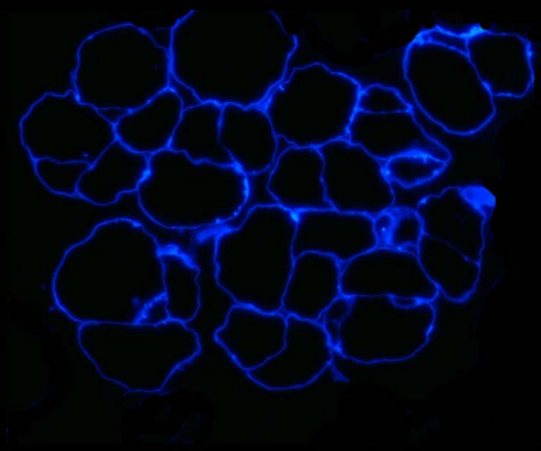
UNIVERSIDAD DE LEÓN

ÁREA DE FISIOLOGÍA VEGETAL

Plasticidad estructural de la pared celular tipo II en cultivos celulares de maíz (*Zea mays* L.) habituados a diclobenil



Hugo Mélida Martínez

León, 2010

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Structural plasticity of type II cell walls in dichlobenil habituated maize (*Zea mays* L.) cell cultures

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TESIS DOCTORAL
2010

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Memoria presentada para
optar al grado de Doctor por
Hugo Mélida Martínez

Fdo. Hugo Mélida Martínez

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Los resultados de esta Tesis Doctoral han sido presentados en los siguientes congresos y reuniones científicas:

- "XI Cell Wall Meeting" (Copenhague, Dinamarca, agosto de 2007).
- "X Congreso Hispano-Luso de Fisiología Vegetal" (Alcalá de Henares, septiembre de 2007).
- "VI Jornada de Fisiología Vegetal de la Sociedad Catalana de Biología" (Barcelona, noviembre de 2008).
- "II EMBO Conference on Plant Molecular Biology; Frontiers of Plant Research" (Cádiz, mayo de 2009).
- "XI Congreso Hispano-Luso de Fisiología Vegetal" (Zaragoza, septiembre de 2009).
- "XVII Congress of the Federation of European Societies of Plant Biology (FESPB)" (Valencia, julio de 2010).
- " XII Cell Wall Meeting" (Oporto, Portugal, julio de 2010).

Parte de los resultados de la siguiente tesis doctoral han dado lugar a las siguientes publicaciones:

- Novel type II cell wall architecture in dichlobenil-habituated maize calluses. H. Mélida, P. García-Angulo, A. Alonso-Simón, A. Encina, J. Álvarez, J.L. Acebes. Planta, 2009, 229: 617–631.

- Unravelling the biochemical and molecular networks involved in maize cells habituation to the cellulose biosynthesis inhibitor dichlobenil. H. Mélida, A. Encina, J. Álvarez, J.L. Acebes, D. Caparrós-Ruiz. Molecular Plant, 2010. En prensa.

- Cellulose Biosynthesis Inhibitors: Their Uses as Potential Herbicides and as Tools in Cellulose and Cell Wall Structural Plasticity Research. En "Cellulose: Structure and Properties, Derivatives and Industrial Uses" (A. Lejeune y T. Deprez, Eds.). J.L. Acebes, A. Encina, P. García-Angulo, A. Alonso-Simón, H. Mélida, J.M. Álvarez. Nova Publishers (New York), 2010. En prensa.

Además, el autor de esta memoria ha participado en las investigaciones que han conducido a las siguientes publicaciones:

- High peroxidase activity and stable changes in the cell wall are related to dichlobenil tolerance. P. García-Angulo, A. Alonso-Simón, H. Mélida, A. Encina, J.L. Acebes, J. Álvarez. Journal of Plant Physiology, 2009, 166: 1229-1240.

- Habituation and dehabituation to dichlobenil: Simply the equivalent of Penelope's weaving and unweaving process? P. García-Angulo, A. Alonso-Simón, H. Mélida, A. Encina, J. Álvarez, J.L. Acebes. Plant Signaling and Behaviour, 2009, 4: 1069-1071.

- FTIR spectroscopy as a tool to monitor changes in plant cell wall architecture associated to cellulose biosynthesis inhibitors. En "Fourier Transform Infrared Spectroscopy: developments, techniques and applications" (OJ Ress, Ed.). A. Alonso-Simón, P. García-Angulo, H. Mélida, A. Encina, JM. Álvarez, JL. Acebes. Nova Publishers (New York), 2010. En prensa.

Agradecimientos

En primer lugar he de agradecer al Ministerio de Ciencia e Innovación por financiar el contrato que me ha "mantenido" durante este periodo (2006-01553) y por costear las estancias en Barcelona y Edimburgo, que tanto han enriquecido la presente Tesis Doctoral. Asimismo, agradezco a la Universidad de León (ULE-2006-2), a la Junta de Castilla y León (LE 048A07) y al Ministerio de Ciencia e Innovación (CGL2008-02470/BOS) por los proyectos de investigación que han apoyado la realización de este trabajo.

Agradezco a mis directores de tesis, los Dres. Antonio Encina García, Jesús Álvarez Fernández y José Luis Acebes Arranz, de la Universidad de León por guiarme durante estos años y darme la oportunidad de trabajar en su laboratorio. Cada uno ha enriquecido diferentes aspectos de este trabajo y a mí personalmente, lo cual les agradezco enormemente.

Agradezco a las Dras. Ana Alonso Simón y Penélope García Angulo por su implicación en mi trabajo, y por su colaboración en la presente Tesis Doctoral, enseñándome a manejar gran cantidad de técnicas en el laboratorio, y dándome sabios consejos para el día a día de un doctorando. A la Dra. Mª Luz Centeno por sus consejos y su colaboración con los experimentos de HPLC.

Al Dr. David Caparrós Ruíz, por acogerme en su laboratorio del Centre de Recerca en AgriGenòmica (CRAG) de Barcelona. A él le debo el capítulo de biología molecular de ésta Tesis Doctoral (a pesar de sus amenazas tijera en mano). Además la estancia en dicho centro me enseñó a ver la ciencia desde otra perspectiva, y fue enriquecedora en muchos sentidos. A la Dra. Silvia Fornalé por su colaboración y consejos durante dicha estancia, y a Sami Irar, responsable del servicio de Proteómica del CRAG por su colaboración en dichos experimentos.

Al Dr. Stephen C. Fry del "Institute of Molecular Plant Sciences" de la Universidad de Edimburgo, todo un modelo a seguir por cualquier científico. Le agradezco todos sus sabios consejos, y sus aportaciones a mi trabajo y a mi formación. Además agradezco a Janice, Kyle, Lenka y Bert entre otros por colaborar en dicha estancia de una u otra manera.

Al Laboratorio de Técnicas Instrumentales de la Universidad de León, y a los responsables de sus servicios de microscopía, cromatografía y radioactividad por permitirme realizar multitud de experimentos en sus instalaciones.

A mis compañeros de carrera Álvaro, Serafín y David, entre otros, que han seguido por la Universidad y que con sus charlas, cafés o partidas de frontón ha hecho más llevadero este trabajo. A las distintas personas que han pasado por el laboratorio en estos años. A María y Asier, que aunque llegaron al laboratorio en mi fase final han traído mucha alegría y ganas de trabajar, y les deseo mucha suerte en sus respectivas tesis, con las cuales espero poder colaborar en mayor o menor manera.

Agradecer de manera especial a mis padres y a mi hermano, que siempre me han apoyado y ayudado en todo lo que han podido, y algún día espero ser tan buen ejemplo como ellos han sido para mí. A mis amigos de Palencia por estar siempre ahí, y ayudarme a desconectar del laboratorio. Y a ti Virginia, que llegaste a mi vida como un rayo de luz, y desde entonces no has dejado de alumbrar, gracias por darme tanto.

Abreviaturas

2,4-D: ácido 2,4-diclorofenoxiacético
2-DE: electroforesis bidemensional
4CL: 4-cumarato CoA ligasa
ADN (DNA): ácido desoxirribonucleico
AIR: residuo insoluble en alcohol
AGP: arabinogalactano proteína
APOX: ascorbato peroxidasa
Ara: arabinosa
ARN (RNA): ácido ribonucleico
ASF: fracción soluble en alcohol
AX: arabinoxilano
B-DFA: dehidrodiferulato en forma benzofurano
BzA: benceno/ácido acético
C3H: cumarato 3-hidroxilasa
C4H: cinamato 4-hidroxilasa
CA (Cou): ácido *p*-cumárico
CAT: catalasa
CBI: inhibidor de biosíntesis de celulosa
CCoAOMT: cafeoil-CoA *O*-metiltransferasa
CesA (CESA): celulosa sintasa
cDNA: ácido desoxirribonucleico codificante
CDTA: ácido *trans*-1,2-diaminociclohexano-*N,N,N´,N´*-tetraacético
CFM: medio libre de células
CHAPS: 1-propanosulfonato-3-[(3-cloroaminopropil)dimetilamonio]
COMT: ácido cafeico *O*-metiltransferasa
CSC: complejo celulosa sintasa
CSL: similar a celulosa sintasa
DCB: 2,6-diclorobenzonitrilo ó diclobenil
DFA: dehidrodiferulato
DH: deshabituado
DMSO: dimetil sulfóxido
DTT: ditiotreitol
DW: peso seco
EDTA: ácido etilendiaminotetraacético
FA (Fer): ácido ferúlico
FTIR: espectroscopía infrarroja por transformada de Fourier
Fuc: fucosa
FW: peso fresco
GalA: ácido galacturónico
GAX: glucuronoarabinoxilano
GC: cromatografía de gases
GFP: proteína fluorescente verde
Glc: glucosa
GPI: glicosilfosfatidilinositol
GPOX: guaiacol peroxidasa
GR: glutatión reductasa
GST: glutatión S-transferasa
Grw: fase de crecimiento activo
GT: glicosiltransferasa
HCT: hidroxicinamoil-CoA sikimato/quinato hidroxicinamoil transferasa
HPLC-PAD: cromatografía líquida de alta eficacia acoplada a detector de fotodiodo
HRAC: "Herbicide Resistance Action Commitee"
Hx: células habituadas a diclobenil creciendo en DCB "x" µM
Hx(y): células habituadas a diclobenil creciendo en DCB "x" µM durante "y" subcultivos
IDA: "immunodot assay"
IPG: gradiente de pH inmovilizado

mAb: anticuerpo monoclonal
MALDI-TOF: “matrix assisted laser desorption ionization coupled to time of flight”
Man: manosa
MAP: proteína asociada a microtúbulos
MASC: compartimento celulosa sintasa asociado a microtúbulos
MLG: glucano mixto
MPBS: tampón fosfato salino conteniendo leche en polvo
MS: espectrometría de masas
M_w: masa molecular media
nc-DFA: dehidrodiferulato no cíclico
NH: no habituado ó control
NH/DCB: no habituado tratado durante tiempos cortos con diclobenil
NIR: espectroscopía en el infrarrojo cercano
PAGE: electroforesis en gel de acrilamida
PAL: fenilalanina amonio liasa
PBS: tampón fosfato salino
PC: componente principal
PCA: análisis de componentes principales
PCR: reacción en cadena de la polimerasa
Rha: ramnosa
rpm: revoluciones por minuto
RT-PCR: reacción en cadena de la polimerasa acoplada a retrotranscripción
SD: desviación estándar
SDS: dodecil sulfato sódico
SmaCC: pequeño compartimento celulosa sintasa
snCR: sobrenadante del residuo de celulosa
Sta: fase estacionaria
Susy: sacarosa sintasa
TFA: ácido trifluoroacético
TLC: cromatografía en capa fina
Ubi: ubiquitina
Uro: ácidos urónicos
UV: ultravioleta
WAK: quinasa asociada a pared
XET: xiloglucano endotransglucosilasa
Xil (Xyl): xilosa
XTH: xiloglucano endotransglucosilasa/hidrolasa
XyG: xiloglucano
YFP: proteína fluorescente amarilla

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Introducción

1. La pared celular de las plantas

Los protoplastos de las células de las plantas terrestres están rodeados por una capa semirrígida compuesta de polisacáridos, proteínas y compuestos fenólicos: la pared celular. Esta estructura actúa ejerciendo una gran influencia en la morfología y el desarrollo de la planta y contribuyendo a la especialización funcional de los tipos celulares. La pared celular probablemente apareció ya en especies acuáticas como las algas verdes carófitas, pero claramente fue un elemento determinante para que las plantas se desarrollasen fuera del agua (Sarkar y col., 2009).

La pared celular proporciona forma y soporte a las plantas, permitiéndolas mantenerse erguidas. Algunas plantas alcanzan alturas superiores a los 100 metros, así que las paredes celulares de dichas plantas deben ser capaces de soportar enormes tensiones, y deben ser muy resistentes. También actúa como barrera frente a agentes ambientales y organismos potencialmente patógenos. Sin embargo, a pesar de la rigidez y aparente impenetrabilidad de las paredes celulares, éstas tienen capacidad de expandirse, y son metabólicamente activas, permitiendo intercambios de materiales y señales entre células.

La formación de la pared celular comienza en la telofase con la aparición de la placa celular, que se origina por acumulación y fusión en el fragmoplasto de vesículas del aparato de Golgi cargadas de polisacáridos no celulósicos, que progresan hasta alcanzar las membranas plasmáticas laterales (Smith, 2001). El desarrollo de la placa celular origina la lámina media, estructura amorfa rica en polisacáridos pécticos que mantiene unidas las células adyacentes, mientras que las membranas de las vesículas dan lugar a las membranas plasmáticas de las células hijas (Aspinall, 1980). Entre la membrana plasmática y la lámina media se deposita la pared celular primaria, que presenta estructura fibrilar debido a la acumulación de microfibrillas de celulosa cristalina embebidas en una matriz de polisacáridos complejos y glicoproteínas, cuya composición varía dependiendo de la especie que consideremos, del tipo de tejido e incluso del grado de diferenciación del tipo celular en cuestión (Knox, 2008).

La pared celular primaria y la lámina media tienen gran importancia en el proceso de extensión o expansión celular, ya que constituyen la única estructura extracitoplasmática de las células que conservan la capacidad de dividirse y/o elongarse. Por el contrario, las células que han perdido esta capacidad suelen depositar en la cara interna de la pared nuevas capas de material, cuya composición depende del tipo concreto de elemento celular. En esas capas suelen aparecer nuevos componentes, como lignina o suberina, modificándose la proporción de los polímeros de pared celular primaria y lámina media. Estas modificaciones originan una estructura multicapa de mayor espesor y ordenación más orientada de las microfibrillas de celulosa, denominada pared celular secundaria.

Se pueden diferenciar dos tipos principales de pared celular primaria (Carpita y Gibeaut, 1993). La mayoría de las especies vegetales (todas las dicotiledóneas y algunas monocotiledóneas) poseen pared celular tipo I, que contiene proporciones semejantes de celulosa y xiloglucano, polisacárido que se une a las microfibrillas de celulosa, fijando su posición y determinando la distancia entre ellas. Las gramíneas y otras monocotiledóneas commelinoides poseen pared celular primaria tipo II, cuya estructura y composición difiere de

aquella característica del resto de angiospermas. En las paredes tipo II los (glucurono)arabinoxilanos (Carpita, 1984) son los principales polisacáridos que se unen a las microfibrillas de celulosa. Además, las paredes tipo II, a diferencia de las tipo I, son pobres en pectinas y proteínas estructurales, pero contienen mayores cantidades de fenoles (hidroxicinamatos). En este capítulo nos centraremos en la estructura de la pared celular primaria tipo II, que es la que presentan los cultivos celulares de la especie (*Zea mays* L.) utilizada en este trabajo.

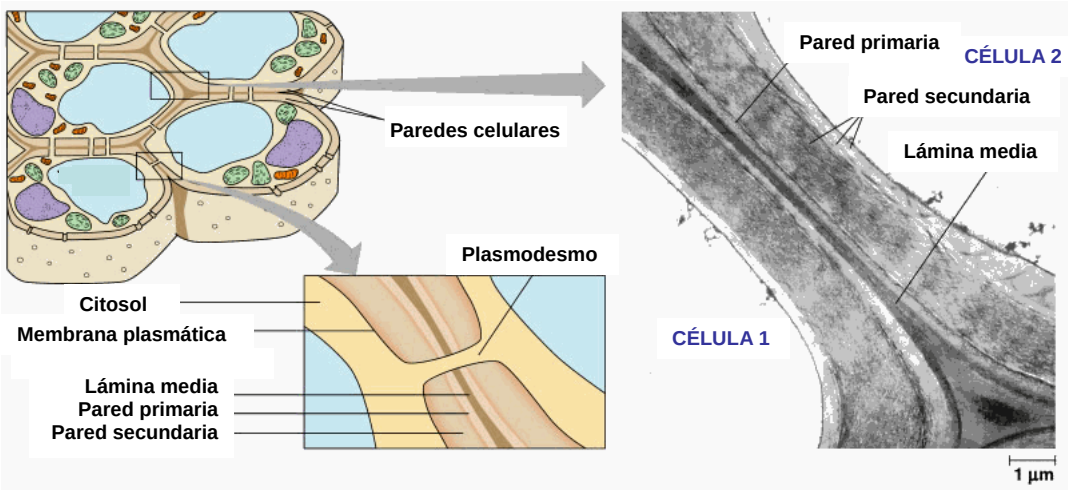

Figura 1. Representación de la pared celular de las plantas. Modificada de Campbell y Reece, 2005.

1.1. Composición química de la pared celular tipo II

Los principales componentes que integran la estructura de la pared celular tipo II se describen a continuación.

1.1.a.- Celulosa

La celulosa constituye aproximadamente un tercio del peso seco total de las plantas. En las paredes celulares primarias, el porcentaje de celulosa varía entre el 15 y el 30% de su peso seco, pudiendo ser aún mayor en paredes celulares secundarias (hasta 50%) (Carpita y McCann, 2000). En células cultivadas *in vitro*, ese porcentaje alcanza aproximadamente el 20% (Blaschek y col., 1981). La celulosa sirve de armazón para la unión del resto de componentes de la pared. No se han descrito diferencias generales entre la celulosa de las paredes celulares tipo I y tipo II.

Las microfibrillas de celulosa están formadas por 36 cadenas de β-1,4-glucano. El enlace β-1,4- hace que cada resto de glucosa esté girado 180° con respecto a las glucosas adyacentes, siendo en realidad el disacárido celobiosa la unidad que se repite en la celulosa. Este hecho, unido a la ausencia de ramificaciones laterales, es responsable de que las cadenas de β-1,4-glucano adquieran una estructura espacial plana y permite la interacción estrecha de unas con otras mediante la formación de puentes de hidrógeno intra e intercatenarios, que dan lugar a una estructura microfibrilar cristalina muy estable (Somerville, 2006).

Las microfibrillas de celulosa ofrecen gran resistencia mecánica en sentido longitudinal y, por tanto, la orientación con que se depositan en la

pared celular primaria determina la dirección con la que se expande la célula, que será perpendicular a la dirección mayoritaria con la que se ha depositado la microfibrilla de celulosa (Emons y Mulder, 2000; Anderson y col., 2010). De esta forma la disposición de las microfibrillas determina la morfología de la célula y en último término la del órgano (Martin y col., 2001) y es esencial para la diferenciación celular (Green y Selker, 1991).

1.1.b. Hemicelulosas

Las hemicelulosas son un grupo de polisacáridos neutros de pared, que se caracterizan por tener cadenas de xilosa, glucosa o manosa unidas mediante enlaces β-1,4- con configuración ecuatorial en C1 y C4 (**Figura 2**) (Scheller y Ulvskov, 2010). Los polisacáridos hemicelulósicos más abundantes en paredes celulares tipo II son los xilanos y el glucano mixto. El xiloglucano, aunque en menor proporción que en paredes celulares primarias tipo I, también está presente.

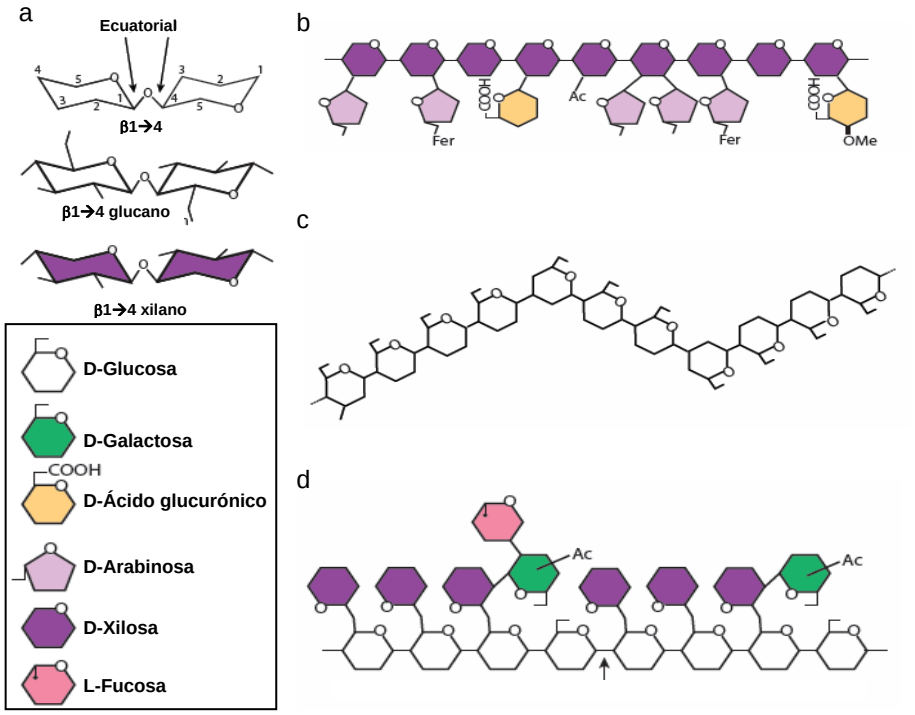

Figura 2. Estructura esquemática de hemicelulosas. **a**: disacáridos constituyentes de las hemicelulosas. **b**: arabinoxilano típico de pared celular tipo II. "Fer" representa ácido ferúlico esterificado y "Ac" grupos acetilo **c**: glucano mixto típico de Poáceas, $[\beta\text{-Glc-}(1\rightarrow4)]_n\text{-}\beta\text{-Glc-}(1\rightarrow3)\text{-}[\beta\text{-Glc-}(1\rightarrow4)]_m$, donde n y m son 3 ó 4. **d**: cadena de xiloglucano $[\beta\text{-Glc-}(1\rightarrow4)]_n$, con ramificaciones laterales. "Ac" representa grupos acetilo. La flecha indica el punto de acción de β-glucanasa. Modificado de Scheller y Ulvskov, 2010.

Los **xilanos** son un grupo de polisacáridos constituidos por una cadena principal de restos β-1,4-xilano. En paredes celulares tipo II los xilanos son los principales polisacáridos no celulósicos, representando aproximadamente entre el 20 y el 40% del peso seco de la pared (Vogel, 2008). Pueden contener restos de arabinosa unidos a la cadena principal de xilosas, denominándose entonces arabinoxilanos. Las sustituciones de arabinosa en las paredes celulares tipo II son mayoritariamente sobre posiciones *O*-3 de las xilosas,

aunque en algunos tejidos son comunes las dobles sustituciones *O*-2/*O*-3. Una característica de los arabinoxilanos de paredes celulares tipo II es la presencia de restos de ácido ferúlico esterificados sobre la posición *O*-5 de arabinosa (Wende y Fry, 1997). Los ésteres de ácido *p*-cumárico también son frecuentes en las paredes celulares de diferentes especies vegetales (Jung y Himmelsbach, 1989).

El **glucano mixto** consiste en una cadena de restos β-1,4-glucano con enlaces β-1,3 intercalados, y es característico de pared celular primaria de poáceas, donde juega un papel importante en la expansión celular, siendo sus niveles muy dependientes de la fase de crecimiento (Obel y col., 2002; Gibeaut y col., 2005). Recientemente se ha demostrado que no son exclusivos de poáceas, sino que aparecen también en hepáticas y *Equisetum* (Popper y Fry, 2003; Fry y col., 2008).

El **xiloglucano** está presente en todas las especies vegetales terrestres. Es la hemicelulosa más abundante de la pared celular primaria tipo I, en la que llega a representar más del 20% del peso seco, pero es considerablemente más escasa en paredes celulares tipo II, en las que puede aportar entre el 1 y 5 % al peso seco de la pared (O´Neil y York, 2003). Consta de una cadena lineal de restos β-1,4-glucano con numerosas ramificaciones α-1,6-xilosa. Algunos de estos restos de xilosa tienen ramificaciones de arabinosa o galactosa, según la especie, y algunas veces la galactosa es sustituida con fucosa (Hayashi, 1989). Pero en el caso del xiloglucano de paredes celulares tipo II la fucosa no está presente, y la galactosa aparece sólo ocasionalmente (Carpita, 1996).

1.1.c. Fenoles

Los ácidos ferúlico y *p*-cumárico, derivados del ácido cinámico, son los principales fenilpropanoides o hidroxicinamatos de la pared celular (Wallace y Fry, 1994). Estos hidroxicinamatos se pueden esterificar entre sí al formar la lignina (Higuchi y col., 1967), con restos α-arabinosil de arabinoxilanos (Wende y Fry, 1997), con restos α-xilosil de xiloglucano (Ishii e Hiroi, 1990), y probablemente con glicoproteínas (Obel y col., 2003). Aun siendo componentes cuantitativamente minoritarios de la pared celular (el ácido ferúlico puede alcanzar un 4% del peso seco de la misma; Saulnier y col., 1999), su papel en esta estructura es muy importante.

Mediante experimentos *in vitro* se ha demostrado que el ácido ferúlico puede experimentar un acoplamiento oxidativo mediado por peroxidasas y peróxido de hidrógeno (Geissman y Neukom, 1971). Las estructuras originadas tras este acoplamiento, conocidas como dehidrodímeros (dehidrodiferulatos, diferulatos, DFAs) permiten la unión de los correspondientes polisacáridos a los que estos fenoles están esterificados (Ishii, 1997; Saulnier y col., 1999), tal y como se representa en la **Figura 3**, en la que se aprecia la unión de dos cadenas de arabinoxilanos mediante este acoplamiento oxidativo. Más tarde se demostró la existencia de este acoplamiento oxidativo *in vivo* (Fry y col., 2000; Fry, 2004; Parker y col., 2005). Esta capacidad de entrecruzamiento de polisacáridos mediante acoplamiento fenólico influye en muchas propiedades de la pared: contribuye al aumento de rigidez celular y consiguiente cese del crecimiento, da cohesión tisular, y refuerza la pared en respuesta a estreses bióticos o abióticos limitando su degradabilidad (Buanafina, 2009).

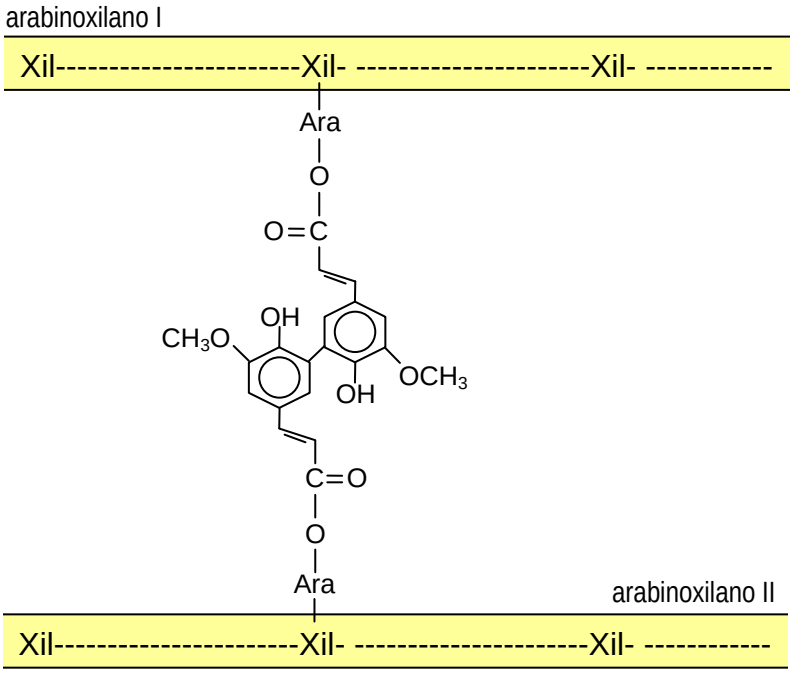

Figura 3. Estructura simplificada del entrecruzamiento de dos cadenas de arabinoxilanos (I y II) mediante el acoplamiento oxidativo del ácido ferúlico. Xil: xilosa, Ara: arabinosa. Modificado de Buanafina, 2009.

1.1.d. Pectinas

Las pectinas son polisacáridos matriciales ricos en ácido galacturónico que se extraen mediante procedimientos relativamente suaves: agua caliente, soluciones acuosas de agentes quelantes o ácidos diluidos. Constituyen hasta el 35% de las paredes tipo I (Willats y col., 2001; Bacic, 2006), pero son componentes minoritarios en las tipo II (5%; Ishii, 1997). Participan en el transporte de iones, en la retención de agua y en la adhesión intercelular, determinan el tamaño de poro de la pared celular y están implicadas en mecanismos de defensa frente a patógenos, heridas y estreses abióticos (Ridley y col., 2001; Jarvis y col., 2003; Verhertbruggen y Knox, 2007).

Las pectinas se pueden dividir en cuatro dominios mayoritarios en función de su composición (Scheller y col., 2007): homogalacturonano, ramnogalacturonano I, ramnogalacturonano II y xilogalacturonano. De nuevo van a existir diferencias en la composición de las pectinas que aparecen en paredes tipo II, debido a que, al igual que el xiloglucano, éstas no contienen fucosa (Carpita, 1996).

1.1.e. Proteínas

Las proteínas son constituyentes esenciales de las paredes celulares, que intervienen en la modificación de sus componentes e interaccionan con proteínas de la membrana plasmática a nivel de la superficie celular (Jamet y col., 2006). Se suelen dividir en dos categorías básicas: proteínas estructurales, usualmente inmovilizadas en la pared celular, y proteínas solubles en el apoplasto, grupo en el cual se incluyen numerosas enzimas (Lee y col., 2004).

Con algunas excepciones, las **proteínas estructurales** son glicoproteínas con secuencias muy repetitivas y ricas en uno o dos

aminoácidos. Hay cuatro clases principales: glicoproteínas ricas en hidroxiprolina, proteínas ricas en prolina, proteínas ricas en glicina y arabinogalactano proteínas. Las proteínas estructurales son mucho menos abundantes en paredes celulares tipo II que en las tipo I (1% frente a 10%; Vogel, 2008).

El análisis de las **proteínas solubles** o débilmente unidas de la pared celular de maíz (Zhu y col., 2006), reveló que la mayoría estaban presentes en dicotiledóneas, aunque una importante proporción (18%) fueron únicas de la gramínea (Ej. endo-1,3;1,4-β-glucanasa). Dentro de este grupo están incluidas las proteínas enzimáticas, la mayor parte de ellas relacionadas con el mecanismo de extensión de la pared, transporte de moléculas, reconocimiento celular y resistencia a patógenos (Rose y col., 2004).

Un buen número de enzimas de la pared celular son hidrolasas y transglicosilasas. Dentro del primer grupo tienen especial importancia las glicanasas, que catalizan la hidrólisis de enlaces glicosídicos, bien en los extremos no reductores de los polisacáridos, liberando uno a uno los restos que los componen (glicosidasas o exoglicanasas), o bien en el interior de la cadena del polímero (endoglicanasas). Además de las glicanasas, hay otros tipos de enzimas hidrolíticas que catalizan la ruptura de enlaces éster-carboxílicos (pectinmetilesterasas), éster-fosfato (fosfatasas) y peptídicos (proteasas).

Las transglicosilasas catalizan reacciones de transglicosilación en las que se rompen enlaces glicosídicos y se transfieren los monosacáridos (exotransglicosilasas) u oligosacáridos (endotransglicosilasas) liberados al extremo no reductor de otro polímero del mismo tipo (Fry, 2004).

Las peroxidasas son enzimas que oxidan el sustrato utilizando peróxido de hidrógeno o hidroperóxidos orgánicos. Actúan generalmente sobre compuestos fenólicos en procesos de lignificación, así como en la formación de puentes isoditirosina entre proteínas ricas en hidroxiprolina y diferulato en arabinoxilanos (Fry, 2004; Lindsay y Fry, 2008). También pueden participar en la destoxificación de xenobióticos (Passardi y col., 2005).

Las expansinas son una familia de proteínas que intervienen en el crecimiento ácido (McQueen-Mason y col., 1992). Estas proteínas regulan la elongación de la pared en células en crecimiento rompiendo las interacciones no covalentes existentes entre las microfibrillas de celulosa y las hemicelulosas (McQueen-Mason y col., 2007). Yennawar y col. (2006) han propuesto el mecanismo de acción de una expansina de maíz (EXPB1), la cual actuaría permitiendo la relajación entre redes de arabinoxilanos y celulosa a través de la ruptura de puentes de hidrógeno.

Las quinasas asociadas a la pared (WAKs), son proteínas clásicamente implicadas en la elongación celular y la modulación del metabolismo de los azúcares (Kohorn y col., 2006). Datos recientes (Seifert y Blaukopf, 2010 y referencias incluidas) atribuyen a este tipo de proteínas un papel clave en la señalización posterior en respuesta a alteraciones en la integridad de la pared celular.

1.2. Arquitectura de la pared celular primaria

Las paredes celulares primarias están constituidas por dos, o tres, redes estructurales independientes, pero capaces de interaccionar entre sí (Carpita y McCann, 2000). La primera red está formada por el entramado celulosa-

hemicelulosa, la segunda de las redes estructurales es la matriz péctica, y la tercera red es la formada por proteínas estructurales o por fenilpropanoides.

Las diferencias en composición de los dos tipos de pared celular determinan diferencias en las arquitecturas que generan. Mientras que en las especies que poseen pared celular tipo I la primera red estructural va a ser de tipo celulosa-xiloglucano, en paredes tipo II (**Figura 4**), los polisacáridos que se unen a las microfibrillas de celulosa fijando su posición van a ser los arabinoxilanos; por tanto la primera red en ellas será de tipo celulosa-arabinoxilano (con trazas de xiloglucano unido débilmente a la celulosa).

En las paredes tipo I la red celulosa-xiloglucano está embebida en la red de pectinas, que va a formar una matriz que entre otras propiedades determina la porosidad de la pared. El hecho de que las paredes celulares tipo II tengan muy pocas pectinas queda contrarrestado con la adición de moléculas de ácido glucurónico a los arabinoxilanos (formando glucuronoarabinoxilanos).

La pared tipo II tiene pocas proteínas estructurales comparada con la tipo I, pero en cambio acumula fenilpropanoides, que van a actuar como elementos de conexión entre arabinoxilanos, permitiendo un reforzamiento extra de esta estructura, particularmente cuando las células terminan de expandirse.

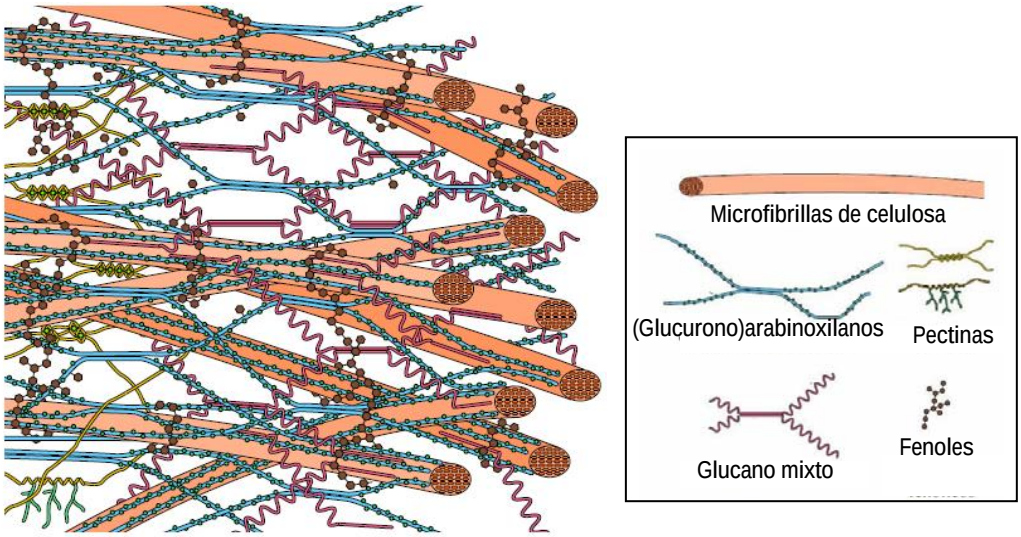

Figura 4. Arquitectura de la pared celular tipo II (Carpita y McCann, 2000).

No se conocen con detalle las uniones que se establecen entre las diferentes redes en paredes celulares tipo II. Sin embargo dichas uniones podrían jugar un papel importante en la estabilización y en el reforzamiento de la estructura de la pared, especialmente en presencia de factores de estrés que afectan a algunos de sus componentes. En este sentido, el estudio de la plasticidad estructural de la pared tipo II tiene gran interés, ya que ayudaría a comprender los mecanismos por los cuales ésta aumenta su fuerza de tensión, especialmente en presencia de diferentes condiciones de estrés. Un aumento en la cantidad de una serie de puentes intra y/o interpoliméricos aparecen como candidatos a contribuir a esa mayor rigidez: arabinoxilano-arabinoxilano,

xiloglucano-xiloglucano, xiloglucano-pectinas, arabinogalactano proteínas-pectinas, celulosa-pectinas.

1.3. Plasticidad estructural de la pared celular primaria

La pared celular primaria posee una elevada capacidad para acomodarse a nuevas condiciones mediante variaciones de su estructura. En los últimos años se han caracterizado paredes celulares con composición alterada debido a modificaciones genéticas, adaptaciones o habituaciones a diferentes tipos de estrés. Gracias a estos estudios se ha avanzado de forma importante en el conocimiento de la composición, estructura y dinámica de la pared celular.

1.3.a. Mutantes de la pared celular

La selección de mutantes con alteraciones en la composición y estructura de la pared celular ha servido para detectar numerosos genes que participan en rutas biosintéticas, en modificaciones de los polímeros *in muro* y en el ensamblaje de los componentes de la pared celular. Tradicionalmente, la identificación de mutantes de la pared celular, ya fueran espontáneos o inducidos, consistía en la búsqueda de alteraciones morfológicas, como el hinchamiento radial de raíces (Baskin y col., 1992), la reducción en la elongación del hipocótilo (Desnos y col., 1996; Nicol y col., 1998), la birrefringencia reducida de tricomas (Potikha y Delmer, 1995; Bischoff y col., 2010), la presencia de xilema colapsado (Turner y Somerville, 1997), o en la obtención de resistencia a inhibidores de la biosíntesis de celulosa, como el isoxabén (Heim y col., 1990) o la taxtomina (Scheible y col., 2003).

El análisis mutacional clásico, que comienza por la observación de cambios fenotípicos y trata de identificar el gen responsable (genética directa) presenta algunos problemas potenciales, ya que a menudo las mutaciones no dan lugar a fenotipos visiblemente anormales y, en otros casos, la mutación suele resultar letal cuando los genes afectados son claves en el proceso implicado. Por ello, se ha recurrido a otros procedimientos de escrutinio como el análisis de la composición en azúcares neutros mediante cromatografía de gases y espectrometría de masas (Reiter y col., 1997), o el rastreo mediante espectroscopías FTIR y/o NIR de alteraciones en la pared celular (Chen y col., 1998; Mouille y col., 2003; McCann y col., 2007; Penning y col., 2009).

En los últimos años el empleo de la genética reversa ha permitido identificar gran cantidad de enzimas, entre ellas glicosiltranferasas implicadas en la síntesis de polisacáridos de pared celular (Richmond y Somerville, 2000; Sarria y col., 2001). Siguiendo esta estrategia se han descrito numerosos mutantes que presentan alteraciones en la estructura y/o síntesis de polisácaridos de pared celular (Arioli y col., 1998; Fagard y col., 2000; Lane y col., 2001).

1.3.b. Tolerancia a diferentes tipos de estrés

La tolerancia a distintos tipos de estrés está asociada frecuentemente a mecanismos que implican la modificación de la pared celular. A través del estudio de dichas modificaciones se puede llegar a comprender, no sólo la contribución global de la pared celular en la tolerancia, sino también el papel de cada uno de sus componentes en particular. Desde hace años se vienen utilizando técnicas de cultivo *in vitro* de células, tejidos y órganos vegetales

para obtener líneas celulares habituadas a diversos tipos de estrés. Las variantes tolerantes se obtienen con o sin el empleo previo de agentes mutagénicos. En este último caso se espera a que la capacidad de respuesta de las células en condiciones de desarrollo restringidas provoque la aparición de células tolerantes, que a la larga, acapararán todo el cultivo. En otros casos la resistencia se transfiere desde otras especies mediante fusión de protoplastos o técnicas de ingeniería genética.

Se ha conseguido habituar suspensiones celulares de tabaco a elevadas concentraciones salinas y a déficit hídrico (Binzel y col., 1988). Las células habituadas presentaban una disminución del volumen celular, en una clara tendencia a restringir la necesidad de agua, y tenían paredes celulares más débiles que contenían menos celulosa y proteína rica en hidroxiprolina (Iraki y col., 1989a, 1989b). Estas células mantenían el contenido total en pectinas pero variaban la organización y composición de los polímeros pécticos, formándose una red laxa de homogalacturonano y ramnogalacturonano que sustituía parcialmente a la red celulosa-xiloglucano.

En una línea de investigación paralela, Sancho y col. (1996) demostraron un aumento de la actividad peroxidasa en el medio de cultivo de células de tomate adaptadas a estrés salino, coincidente con un incremento en lignina en la pared celular, confirmando una correlación inversa entre actividad peroxidasa y capacidad de crecimiento.

Las paredes celulares también participan en la adaptación a las bajas temperaturas, aunque se desconoce el mecanismo exacto por el que se altera la pared celular (Yamada y col., 2002). Algunos autores han propuesto que, al menos en parte, la adaptación ocurre a través del efecto de las oligosacarinas y el ácido abscísico sobre el metabolismo de la pared celular (Zabotin y col., 2009).

Por otra parte, la pared celular interviene en la adaptación de las plantas a concentraciones elevadas de aluminio (Vázquez y col., 1999), de plomo (Wusheng y Donghua, 2010) y de otros metales pesados (Carpena y col., 2000), a través de un aumento en la capacidad de inmovilización del metal pesado en la pared celular. También se ha descrito un aumento de la resistencia mecánica de la pared celular en respuesta a choque hipoosmótico (Cazalé y col., 1998) y estrés mecánico (Yahraus y col., 1995).

1.4. Biosíntesis de los componentes de la pared celular

1.4.a. Celulosa

La celulosa se sintetiza en la membrana plasmática por complejos proteicos, y se deposita directamente en la pared de modo direccional (Somerville, 2006; Mutwil y col., 2008; Taylor, 2008), experimentando una reorientación dinámica posterior a su deposición, que permite una expansión anisotrópica (Anderson y col., 2010). La maquinaria biosintética de la celulosa está localizada en la membrana plasmática formando estructuras conocidas como complejos celulosa sintasa (CSC) o "rosetas" por su aspecto al visualizarlas microscopio electrónico. En las plantas, los CSC se organizan en hexámeros, presumiblemente compuestos por 36 proteínas Celulosa sintasA (CesA) (6 en cada monómero). Cada CSC (en colaboración con otras proteínas) es capaz de catalizar la formación de una microfibrilla completa (36 cadenas de

β-1,4-glucano dispuestas de forma paralela) (Delmer, 1999; Saxena y Brown, 2000; Lerouxel y col., 2006) (**Figura 5**).

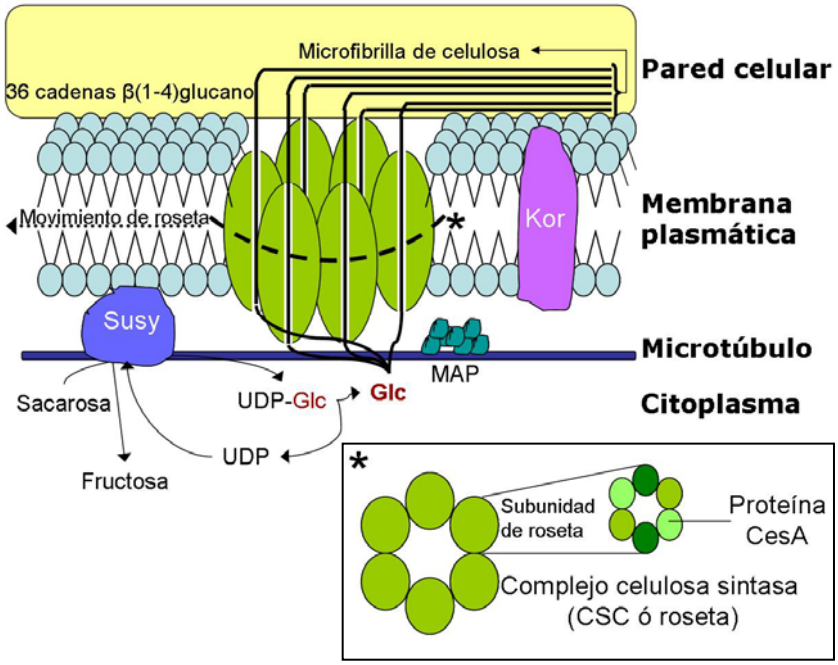

Figura 5. Representación esquemática de la síntesis de celulosa. Se incluyen otras proteínas que parecen participar en el proceso, como sacarosa sintasa (Susy), KORRIGAN (Kor), y proteínas asociadas a microtúbulos (MAP).

Los CSC se constituyen en el aparato de Golgi para posteriormente ser exportados a la membrana plasmática vía exocitosis (Somerville, 2006). Avances recientes en la metodología para la visualización de células vivas ("live cell imaging") han permitido la identificación de varios elementos necesarios para el transporte, reparto y almacenamiento de los CSC (Crowell y col., 2009; Gutiérrez y col., 2009; Wightman y col., 2009; Wightman y Turner, 2010). En concreto, los SmaCCs (pequeños compartimentos celulosa sintasa; Gutiérrez y col., 2009) y los MASCs (compartimentos celulosa sintasa asociados a los microtúbulos; Crowell y col., 2009), son compartimentos muy dinámicos que parecen desempeñar papeles clave tanto en el almacenamiento intracelular de los CSC como en su transferencia a la membrana plasmática (Wightman y Turner, 2010).

Las proteínas CesA forman familias génicas con un número de genes que varía entre especies. *Arabidopsis* (pared tipo I) y maíz (pared tipo II) poseen 10 y 12 genes *CesA*, respectivamente (Holland y col., 2000; Richmond, 2000; Appenzeller y col., 2004). En una misma célula cada complejo requiere tres tipos distintos de subunidades CesA para funcionar correctamente (Taylor y col., 2000). Cada una de estas proteínas posee ocho dominios transmembrana, con el centro activo y los extremos N- y C-terminal en el citosol (Lerouxel y col., 2006). Mediante el uso de marcadores *in vivo* se ha confirmado que la orientación de las microfibrillas de celulosa es controlada por los microtúbulos corticales, los cuales guían las trayectorias de los CSCs activos a través de la membrana plasmática (Paredez y col., 2006).

Otras proteínas implicadas en la síntesis de celulosa son: sacarosa sintasa (Susy), KORRIGAN (Kor), y proteínas asociadas a microtúbulos (MAP).

El sustrato utilizado por los complejos CSC para la síntesis de celulosa es la UDP-glucosa, que es sintetizada mediante la enzima Susy (Haigler y col., 2001). Durante mucho tiempo se ha especulado con que Susy actuaría regulando la síntesis de celulosa controlando los niveles de UDP-glucosa disponibles para los CSC (revisado por Haigler y col., 2001), hecho que no se ha podido demostrar hasta el momento. En cambio, la sobreexpresión en plantas transgénicas de tabaco de varias isoformas de Susy no incrementó los niveles de celulosa en las paredes celulares, hecho que sugiere que el nivel de UDP-glucosa no es el factor limitante para la síntesis de celulosa (Coleman y col., 2006).

La enzima endo-β-1,4-glucanasa, conocida como enzima Kor es capaz de hidrolizar celulosa no cristalina, pero no es activa sobre celulosa microfibrilar, xiloglucano o xilanos (Mølhøj y col., 2001). Esto sugiere que la endoglucanasa Kor está implicada en la terminación de las cadenas durante la biosíntesis de celulosa, en la degradación de cadenas de β-1,4-glucano que no se incorporan correctamente a las microfibrillas (Peng y col., 2002) o en la reducción de estrés por la tensión generada al ensamblarse las cadenas de glucano que forman la microfibrilla (Somerville, 2006).

Las proteínas asociadas a microtúbulos (MAP) son proteínas citosólicas, con capacidad de unión a los microtúbulos. Se cree que las MAPs participan en la síntesis de celulosa acoplando las proteínas CesA a los microtúbulos (Rajangam y col., 2008).

La mayoría de los genes implicados en la síntesis de celulosa se han identificado gracias al estudio de mutantes que muestran alteraciones en la composición y estructura de la pared celular. Algunos se han seleccionado por presentar menor contenido en celulosa (Richmond, 2000), aunque otros mutantes se han detectado al caracterizar otros fenotipos que podrían parecer poco relacionados en la síntesis de este polisacárido. En la **Tabla 1** se expone una relación de los principales mutantes deficientes en celulosa identificados en *Arabidopsis*.

A pesar de que se presupone la existencia de proteínas que participan en la organización macromolecular de los CSC y en la salida de las cadenas de glucano a través de la membrana (Saxena y Brown, 2000, 2005), no se han encontrado propiamente enzimas implicadas directamente en el proceso de cristalización de la celulosa, sino únicamente resultados que indirectamente apuntan a su existencia. En el mutante *rsw* se desorganizan los CSC, además de reducirse la cantidad de celulosa cristalina y aumentar la proporción de un β-1,4-glucano no cristalino fácilmente extraíble de la pared celular (Arioli y col., 1998). Por ello, se deduce que para la correcta cristalización de las microfibrillas se requiere un correcto ensamblaje de las subunidades de celulosa sintasa. El herbicida CGA 325'615 también provoca la liberación de un β-1,4-glucano no cristalino, pero en este caso parece ser que el glucano permanece soluble por estar unido a la proteína CesA (Peng y col., 2001) o a sitosterol-glucósido, que podría funcionar como cebador de la síntesis de celulosa (Peng y col., 2002).

En resumen, la formación de microfibrillas de celulosa se puede dividir en tres pasos: (i) iniciación, usando UDP-glucosa como sustrato donador; (ii)

polimerización de glucosa en cadenas de β-1,4-glucano, y (iii) cristalización de estas cadenas en una microfibrilla (Peng y col., 2002).

Locus	Alelo	Función	Referencia
CesA1	*rsw1-1* *rsw1-2* *rsw1-20* *rsw1-10*	Subunidad catalítica CesA1	Arioli y col., 1998 Gillmor y col., 2002 Beeckman y col., 2002 Fagard y col., 2000
CesA3	*eli1-1; 1-2* *cev1* *ixr1-1; 1-2* *than*	Subunidad catalítica CesA3	Caño-Delgado y col., 2003 Ellis y col., 2002 Scheible y col., 2001 Daras y col., 2009
CesA4	*irx5*	Subunidad catalítica CesA4	Taylor y col., 2003
CesA6	*prc1-1* a*1-12* *ixr2-1*	Subunidad catalítica CesA6	Fagard y col., 2000 Desprez y col., 2002
CesA7	*irx3*	Subunidad catalítica CesA7	Taylor y col., 1999, 2000
CesA8	*irx1*	Subunidad catalítica CesA8	Turner y Somerville, 1997 Taylor y col., 2000
Kob1	*kob1-1; 1-2*	Proteína de membrana tipo II	Pagant y col., 2002
Cob	*cob1-1*	Proteína unida a GPI	Schindelman y col., 2001
Kor1	*kor1-1* *kor1-2* *rsw2-1* a *4* *irx2-1; 2-2*	Endo-β-1,4-glucanasa unida a membrana	Nicol y col., 1998 Zuo y col., 2000 Lane y col., 2001 Mølhøj y col., 2002
Pom1	*pom1-1* a *11* *elp1*	Proteína similar a quitinasa (AtCTL1)	Hauser y col., 1995 Zhong y col., 2002
Rsw3	*rsw3-1*	Glucosidasa II	Burn y col., 2002

Tabla 1. Algunos mutantes de *Arabidopsis* deficientes en celulosa.

1.4.b. Polisacáridos no celulósicos

A diferencia de la celulosa, la síntesis de los polisacáridos no celulósicos de la pared celular ocurre en el aparato de Golgi, y no a nivel de la membrana plasmática. Comienza con la formación de los azúcares precursores y continúa con la polimerización y secreción de los polisacáridos. Una vez exportados a la pared celular vía exocitosis, se intercalan entre las microfibrillas de celulosa (Sandhu y col., 2009). La polimerización de los polisacáridos conlleva la transferencia de un monosacárido desde el nucleótido de monosacárido al extremo no reductor de un polisacárido incipiente, y la liberación de la molécula activadora (Brett y Waldron, 1996). Estas reacciones son catalizadas por glicosiltransferasas (GTs) específicas para cada tipo de enlace y asociadas a la cara interna del aparato de Golgi (Reid, 2000; Scheible y Pauly, 2004; Lairson y col., 2008). Las GTs están clasificadas en 92 familias en la base de datos CAZy (carbohydrate active enzymes). A pesar de los avances durante los últimos años en el conocimiento de la síntesis de estos compuestos todavía existen muchas lagunas. En la **Tabla 2** se han incluido las GTs que hasta el momento se han asociado a la biosíntesis de varios polisacáridos hemicelulósicos.

Actividad	Nombre GT	Familia CAZy	Referencia
Xilanos			
β-1,4-xilano sintasa	IRX9	GT43	Brown y col., 2007; Peña y col., 2007; Lee y col., 2007
	IRX14	GT43	Brown y col., 2007
	IRX10/GUT2	GT47	Brown y col., 2009; Wu y col., 2009
	IRX10-LIKE/GUT1	GT47	Brown y col., 2009; Wu y col., 2009
Síntesis del oligosacárido del extremo terminal reductor	IRX7/FRA8	GT47	Brown y col., 2007
	F8H	GT47	Lee y col., 2009
	IRX8/GAUT12	GT8	Lee y col., 2007; Peña y col., 2007
	PARVUS/GLZ1	GT8	Lao y col., 2003; Lee y col., 2009
α-arabinosil transferasa	-	GT61?	Mitchell y col., 2007
Glucano mixto			
β-1,3-1,4-glucano sintasa	OsCSLF2	GT2	Burton y col., 2006
	OsCSLF4	GT2	Burton y col., 2006
	HvCSLH1	GT2	Doblin y col., 2009
Xiloglucano			
β-1,4-glucano sintasa	CSLC4	GT2	Cocuron y col., 2007
α-1,6-xilosil transferasa	XXT1	GT34	Faik y col., 2002
	XXT2	GT34	Faik y col., 2002
β-1,2-galactosil transferasa	MUR3	GT47	Levy y col., 1991
		GT47	Madson y col., 2003
α-1,6-fucosil transferasa	MUR2/FUT1	GT37	Perrin y col., 1999

Tabla 2. Glicosiltransferasas involucradas en la síntesis de hemicelulosas.

1.4.b.1. Xilanos

La cadena principal de los xilanos puede estar sustituida por diferentes cadenas laterales, y acoplada a un oligosacárido único en su extremo reductor. Debido a esta complejidad y variabilidad en la estructura existe gran incertidumbre acerca de cómo se lleva a cabo la síntesis. Debido a la elevada similitud entre los xilanos y las cadenas β-1,4 de otras hemicelulosas, en un principio se asumió que la síntesis podría estar llevada a cabo por proteínas de las familias CSL (similares a celulosa sintasa). Sin embargo, la caracterización de los mutantes deficientes en xilanos *irx9*, *irx14*, *irx10* e *irx10-like*, indicó que GTs de las familias GT43 y GT47 son responsables de la elongación de la cadena de xilano. La no redundancia entre las proteínas identificadas hasta el momento hace suponer que quizás sean complejos multiproteicos los responsables de la síntesis (Scheller y Ulvskov, 2010).

Además, una característica común de los xilanos, es que presentan en su extremo reductor un oligosacárido del tipo β-Xil-(1→4)-β-Xil-(1→3)-α-Rha-(1→2)-α-GalA-(1→4)-Xil. Los mutantes deficientes en xilanos *irx7*, *irx8* y *parvus* (Lee y col., 2007; Peña y col., 2007) que carecen de este oligosacárido, pero sí fueron capaces de sintetizar xilanos *in vitro* (tras la adición exógena del oligosacáridos) permitieron identificar las proteínas encargadas de su síntesis.

Las sustituciones más comunes de los xilanos deberían ser incorporadas por α-arabinofuranosiltransferasas y α-glucuronosiltransferasas. Ambas actividades han sido detectadas *in vitro* (Baydoun y col., 1989; Porchia y col., 2002; Zeng y col., 2008), pero las GTs responsables de la transferencia no han sido identificadas hasta el momento, aunque se especula con que podrían ser miembros de la familia GT61 (Mitchell y col., 2007).

1.4.b.2. Glucano mixto

La biosíntesis de glucano mixto es llevada a cabo por proteínas de las familias CSLF y CSLH (Burton y col., 2006; Doblin y col., 2009). Estas proteínas

no están presentes en *Arabidopsis*, de modo que la expresión heteróloga de proteínas de arroz de este tipo en *Arabidopsis* y la detección de glucano mixto en las correspondientes plantas transgénicas fue la clave para su identificación.

1.4.b.3. Xiloglucano

Varias de las GTs involucradas en la síntesis de este polisacárido han sido identificadas hasta el momento. La primera en ser identificada fue una fucosiltransferasa (Perrin y col., 1999). Los restos de xilosa son adicionados por α-1,6-xilosiltransferasas pertenecientes a la familia GT34 (Faik y col., 2002; Cavalier y Keegstra, 2006), mientras que la cadena principal de glucano es sintetizada por proteínas de la familia CSLC (Cocuron y col., 2007). En este caso además de GTs, también son necesarias hidrolasas, las cuales realizan un importante procesamiento de las moléculas de xiloglucano después de su síntesis inicial en Golgi (Pauly y col., 2009). Otra modificación importante del xiloglucano es llevada a cabo por las enzimas xiloglucano endotransglucosilasa/hidrolasa (XTH) que actúan sobre una molécula de xiloglucano, cortándola y transfiriendo otra molécula al extremo reductor de la misma.

1.4.c. Fenoles

Los ácidos ferúlico y *p*-cumárico son sintetizados en los plastos a través de la primera parte la ruta de los fenilpropanoides (Vogt, 2010) (**Figura 6**). El primer paso de esta ruta es la desaminación de la fenilalanina mediante la enzima fenilalanina amonio liasa (PAL) para generar ácido cinámico. Existen varias copias de los genes *PAL* en las especies estudiadas (cinco en maíz; Guillaumie y col., 2006), de modo que distintos genes parecen responder a distintos estímulos, y su expresión está muy regulada espacial y temporalmente (Vogt, 2010). Los tres primeros pasos de la ruta, catalizados por PAL, cinamato 4-hidroxilasa (C4H) y 4-cumarato CoA ligasa (4CL), son claves y proporcionan la base para numerosas ramificaciones de la misma que darán lugar a gran cantidad de metabolitos. Así, a través de una serie hidroxilaciones y metilaciones, a partir de fenilalanina se produce feruloil-CoA, que probablemente es el sustrato que se esterifica con los arabinoxilanos (Fry y col., 2000; Lindsay y Fry, 2008), aunque otros autores han sugerido que el sustrato es un feruloil-glucósido (Obel y col., 2003). En cualquier caso estos autores coinciden en que la feruloilación tiene lugar en Golgi. Mediante un análisis bioinformático se predijo que diferentes miembros de la familia génica *pfam* podrían actuar como transferasas feruloil-arabinoxilano (Mitchell y col., 2007), hecho que se ha demostrado muy recientemente en arroz a través de la obtención de mutantes RNAi para cuatro de estos genes (Piston y col., 2010).

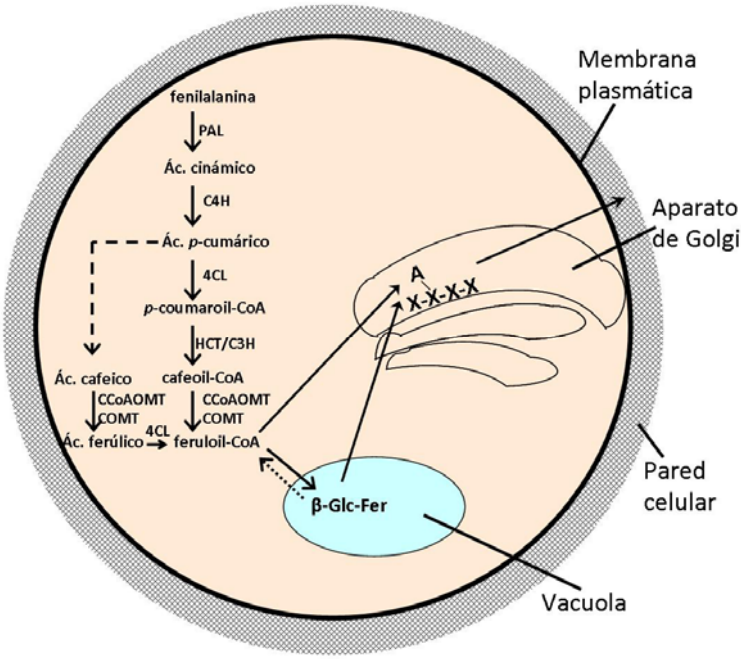

Figura 6. Ilustración de la ruta potencial involucrada en la biosíntesis de fenoles de pared celular. PAL: Fenilalanina Amonio-Liasa, C4H: Cinamato 4-Hidroxilasa, 4CL: 4-Cumarato CoA Ligasa, C3H: Cumarato 3-Hidroxilasa, HCT: Hidroxicinamoil-CoA sikimato/quinato hidroxicinamoil Transferasa, CCoAOMT: Cafeoil-CoA O-MetilTransferasa, COMT: Ácido Cafeico O-MetilTransferasa, β-Glc-Fer: feruloil glucósido. Los pasos no confirmados están indicados con líneas discontinuas. Modificado de Lindsay y Fry, 2008.

2. Uso de inhibidores de la biosíntesis de pared celular

Se han descrito diferentes compuestos que afectan a la biosíntesis de distintos componentes de la pared celular. Estos inhibidores alteran la proporción final de los componentes de la pared celular y la unión entre ellos, y han sido de gran ayuda en el estudio de la formación, organización y estructura de la pared celular primaria y secundaria (Satiat-Jeunemaitre y Darzens, 1986; Suzuki y col., 1992; Taylor y col., 1992; Vaughn y Turley, 1999, 2001); el mecanismo de pérdida de rigidez y la extensión de la pared celular (Hoson y Masuda, 1991; Edelmann y Fry, 1992; Edelmann y Köhler, 1995; Montague, 1995); la formación de la placa celular y el proceso de citocinesis (Vaughn y col., 1996; Nickle y Meinke, 1998); la relación entre citoesqueleto y celulosa (Fisher y Cyr, 1998; DeBolt y col., 2007) y la organización de los complejos celulosa sintasa (Mizuta y Brown, 1992). Asimismo, el uso de estos inhibidores ha permitido abordar con éxito algunos aspectos de la biosíntesis de la celulosa, como es su relación con la biosíntesis de la calosa (Delmer, 1987; Delmer y Amor, 1995).

2.1. Uso de inhibidores de la biosíntesis de celulosa: diclobenil

Los inhibidores de la biosíntesis de celulosa (CBIs) constituyen un grupo variado de compuestos estructuralmente heterogéneos que afectan específicamente al acoplamiento y/o a la deposición de celulosa en plantas superiores. Varios CBIs han sido comercializados como herbicidas, y han sido

clasificados como el grupo L por el "Herbicide Resistance Action Committee" (HRAC). Este grupo incluye diclobenil, clortiamida, isoxabén, taxtomina A, flupoxam y quinclorac (Menne y Köcher, 2007). A continuación se describirá el diclobenil (2,6-diclorobenzonitrilo o DCB), y su utilidad en el estudio de la pared celular, ya que ha sido el inhibidor empleado durante la presente tesis doctoral, y en un capítulo posterior se describirán ampliamente tanto este como otros CBIs.

El DCB es uno de los CBIs más utilizados y mejor caracterizados. Se emplea en agricultura como herbicida de preemergencia de amplio espectro y en investigación como inhibidor de la síntesis de celulosa. Este compuesto inhibe específicamente la síntesis de celulosa en plantas (Brummell y Hall, 1985; Hoson y Masuda, 1991; Corio-Costet y col., 1991; Edelmann y Fry, 1992; Shedletzky y col., 1992; Encina y col., 2002; Alonso-Simón y col., 2004), sin afectar ni a la síntesis de otros polisacáridos de la pared celular (Montezinos y Delmer, 1980; Blaschek y col., 1985; Francey y col., 1989) ni a otros procesos metabólicos, como síntesis de ADN y proteínas, respiración, fosforilación oxidativa, metabolismo lipídico, nucleotídico y de glucosa (Meyer y Herth, 1978; Montezinos y Delmer, 1980; Galbraith y Shields, 1982; Delmer, 1987).

A lo largo de la última década se han propuesto diferentes modos de acción del DCB. En primer lugar se propuso que bloqueaba la iniciación de la biosíntesis de celulosa a través de la inhibición de la síntesis del sitosterol-β-glucósido (Peng y col., 2002). Este modo de acción se basaba en los siguientes resultados obtenidos con DCB: i) inhibición de la síntesis *in vivo* de sitosterol-β-glucósido, ii) reducción de la incorporación de glucosa radioactiva en esteroles de fibras aisladas de algodón a través de la inhibición de la formación de UDP-glucosa o mediante otro efecto indirecto y iii) reversión de las propiedades inhibitorias del DCB por aplicación de sitosterol-β-glucósido. Este modo de acción resulta cuestionable debido tanto a que las condiciones experimentales utilizadas eran forzadas y a que aún no ha sido probado de forma categórica que la síntesis de celulosa se inicie a partir de sitosterol-β-glucósido.

Un segundo modo de acción propuesto consistiría en la alteración de la cristalización de la celulosa más que en la polimerización de la glucosa. La propuesta de este modo de acción parte de la observación de que una severa reducción de la síntesis de celulosa se acompaña de una alteración en la orientación de las microfibrillas restantes de celulosa, tal y como sucede en el mutante deficitario de celulosa *rsw1*, así como en células de *Arabidopsis* tratadas con DCB 1 μM. Es probable que la alteración de la cristalización de la celulosa ocurra a través de una desorganización de los microtúbulos tal y como se observó en células epidérmicas de *Arabidopsis* (Himmelspach y col., 2003). De acuerdo con este modo de acción está el hecho de que la reducción del contenido celulósico en células de alubia habituadas a DCB se acompaña de la acumulación de un β-1,4-glucano no cristalino (Encina et al., 2002; García-Angulo et al., 2006).

Se han propuesto tres posibles dianas para el DCB:

(a) Utilizando un análogo estructural del DCB se identificó un polipéptido de 12-18 kDa como posible diana para este inhibidor (Delmer y col., 1987). Este polipéptido no se corresponde con la celulosa sintasa, ya que se disocia fácilmente de la membrana; más bien se trataría de una proteína moduladora de la especificidad de la glicosiltransferasa.

(b) Otros autores (Nakagawa y Sakurai, 1998), utilizando anticuerpos frente a CesA1 de tabaco, concluyeron que el DCB se uniría específicamente a esta proteína, de modo que alteraría su conformación haciéndola resistente a la degradación proteolítica. Además, el mutante *rsw1*, cuyo locus RSW1 codifica para la subunidad CesA1, presenta dos características semejantes a las provocadas por DCB: contenido reducido de celulosa y acumulación de un β-1,4-glucano no cristalino (Arioli y col., 1998). Pero en ningún caso se demostró una unión directa DCB-CesA1, hecho que hace suponer que el enriquecimiento en CesA1 fue un efecto colateral de la aplicación del DCB.

(c) La tercera diana propuesta es la proteína asociada a microtúbulos MAP20 (Rajangam y col., 2008). Se trata de una proteína citosólica, altamente expresada durante la formación de pared secundaria, con capacidad de unión *in vitro* e *in vivo* a los microtúbulos. Estos autores demostraron que el DCB se une a esta durante la síntesis de celulosa. MAP20 participa en la síntesis de celulosa acoplando las proteínas CesA a los microtúbulos y la idea de que es la diana del DCB se basa en que la unión específica a DCB bloquea el acoplamiento de las proteínas CesA a los microtúbulos.

2.2. Habituación de cultivos celulares a CBIs

A pesar de que los CBIs son herbicidas altamente específicos y activos en concentraciones relativamente bajas, es posible obtener cultivos celulares habituados a crecer en presencia de CBIs, mediante la exposición prolongada a concentraciones crecientes del inhibidor. La utilización de cultivos celulares frente a la utilización de plantas completas, tiene varias ventajas: (i) la posibilidad de tener gran cantidad de células en un espacio reducido al mismo tiempo, donde es fácil controlar y manipular diferentes condiciones, y (ii) la capacidad de seleccionar líneas celulares con determinadas características.

La habituación de cultivos celulares a CBIs refleja la capacidad de las células de sobrevivir con una pared celular modificada, siendo por tanto una técnica muy útil para profundizar en el conocimiento de la plasticidad estructural y composicional de la pared celular. Hasta el momento se han habituado con éxito cultivos celulares de distintas especies a CBIs como DCB (Shedletzky y col., 1990, 1992; Wells y col., 1994; Nakagawa y Sakurai, 1998; 2001; Sabba y col., 1999; Encina y col., 2001, 2002; Alonso-Simón y col., 2004; García-Angulo y col., 2006, 2009a), isoxabén (Corio-Costet y col., 1991; Díaz-Cacho y col., 1999; Sabba y Vaughn, 1999; Manfield y col., 2004), quinclorac (Alonso-Simón y col., 2008) y taxtomina-A (Girard-Martel y col., 2008), mostrando los cultivos habituados una serie de características comunes: menores tasas de crecimiento, células con morfologías irregulares, tendencia a crecer en agregados, y paredes celulares con contenidos en celulosa reducidos compensados por otros componentes de la pared.

2.2.a. Habituación a DCB

Aunque básicamente el mecanismo de habituación es común (un reemplazamiento de la celulosa por otros componentes de la pared celular), los detalles del proceso dependen del tipo de pared (tipo I y tipo II). La mayoría de los cultivos celulares habituados a DCB presentan paredes tipo I: tomate (Shedletzky y col., 1990), tabaco (Shedletzky y col., 1992; Wells y col., 1994; Nakagawa y Sakurai, 1998, 2001; Sabba y col., 1999) y alubia (Encina y col.,

2001, 2002; Alonso-Simón y col., 2004; García-Angulo y col., 2006). En general en especies con pared tipo I la reducción en celulosa estuvo acompañada por la reducción en hemicelulosas y un incremento en pectinas. Además se han descrito otras modificaciones asociadas a la habituación a DCB, como la presencia de un β-1,4-glucano débilmente unido a celulosa, el incremento en las uniones pectina-xiloglucano y en actividad XET, reducciones en arabinogalactano proteínas, cambios en niveles de extensinas y modificaciones en la composición del xiloglucano (Shedletzky y col., 1992; Encina y col., 2002; García-Angulo y col., 2006; Alonso-Simón et al., 2007, 2010a y b). En cambio en una especie con pared tipo II como cebada (Shedletzky y col., 1992) la reducción en celulosa, fue compensada en líneas generales con incrementos en glucano mixto.

2.2.b. Deshabituación

La mayoría de los cambios originados por la habituación a DCB revirtieron cuando los cultivos celulares habituados se cultivan durante largos periodos de tiempo en medio sin DCB (Shedletzky y col., 1990; Encina y col., 2002; García-Angulo y col., 2006). Sin embargo, estos cultivos, denominados deshabituados, retuvieron algunas de las modificaciones de la pared celular asociadas a la habituación, como la presencia de un β-1,4-glucano no cristalino. Cabe destacar que los cultivos deshabituados de alubia resultaron ser mucho más tolerantes al DCB que los no habituados (Encina y col., 2002), hecho que se explicó en parte por la elevada capacidad antioxidante mostrada por los cultivos deshabituados (García-Angulo y col., 2009a). Mediante análisis FTIR se comprobó que la deshabituación sigue una ruta diferente a la de la habituación (García-Angulo y col., 2009b).

3. Objetivos

El objetivo general de este trabajo es estudiar la plasticidad estructural de la pared celular tipo II utilizando cultivos celulares (callos y suspensiones) de maíz habituados al inhibidor de la biosíntesis de celulosa diclobenil (DCB). Este objetivo general se ha abordado a través de los siguientes objetivos parciales:

1. Revisar los usos de los inhibidores de la biosíntesis de celulosa como herramientas en el estudio de la pared celular. Como punto de partida se llevará a cabo una exploración amplia de las investigaciones que han utilizado compuestos capaces de inhibir la síntesis de celulosa orientados al análisis de la pared celular de plantas. Esta revisión proporcionará el fundamento actualizado sobre el que se cimentará el desarrollo posterior del trabajo.

2. Seleccionar y caracterizar líneas celulares de maíz capaces de crecer en presencia de concentraciones letales de DCB. La habituación de cultivos celulares a inhibidores de la biosíntesis de celulosa conlleva modificaciones en la composición y estructura de las paredes celulares tipo I. Se estudiará el efecto inhibitorio del herbicida DCB sobre el crecimiento de cultivos celulares de maíz, mediante la obtención de curvas dosis-respuesta. Seguidamente se habituarán los cultivos celulares a concentraciones letales de DCB mediante subcultivo continuado en concentraciones crecientes del herbicida, partiendo de una concentración equivalente a la I_{50}. Se analizarán los hábitos de crecimiento y morfología (macroscópica y microscópica) de los cultivos habituados, y se hará una caracterización general de las modificaciones de la pared celular originadas durante la habituación a través del uso de una amplia batería de técnicas espectrofotométricas, cromatográficas e inmunocitoquímicas.

3. Analizar mediante técnicas de genómica y proteómica cambios involucrados en la habituación de células de maíz a DCB. Análisis genómicos de líneas celulares de especies con pared tipo I tolerantes a inhibidores de la biosíntesis de celulosa mostraron gran cantidad de genes diferencialmente expresados, entre los que destacan aquellos relacionados con la biosíntesis de celulosa y hemicelulosas. Se utilizarán técnicas de RT-PCR para analizar la expresión de genes supuestamente asociados a la habituación a DCB, principalmente aquellos implicados en la biosíntesis de celulosa y fenoles. Además se llevará a cabo un análisis del proteoma de células habituadas y no habituadas para detectar cambios originados por la habituación en cualquier proceso metabólico. Con el fin de estudiar la posible estabilidad de los cambios asociados a la habituación se analizará también la expresión génica de cultivos deshabituados.

4. Analizar el perfil fenólico de la pared celular primaria de células de maíz con niveles reducidos de celulosa. Las principales modificaciones en las paredes de las células habituadas de otras especies consisten en la compensación de la disminución de celulosa con un aumento en otros componentes de la pared. Se estudiará mediante técnicas cromatográficas

(HPLC) posibles variaciones cuantitativas y cualitativas en el contenido fenólico de las paredes celulares durante el proceso de habituación a DCB, y durante el ciclo de cultivo.

5. Investigar los cambios que experimenta el metabolismo del ácido cinámico en el protoplasma y en la pared celular de células habituadas a DCB. Tratando de profundizar en el conocimiento del posible papel del componente fenólico en la habituación de células de maíz a DCB, se realizarán experimentos de incorporación de ácido cinámico (precursor de los fenoles de la pared celular) radiomarcado. Mediante técnicas de análisis de radioactividad se analizarán los cambios cualitativos y cuantitativos del componente fenólico en distintos compartimentos celulares.

Capítulo I:

Cellulose biosynthesis inhibitors: Their uses as potential herbicides and as tools in cellulose and cell wall structural plasticity research

Capítulo correspondiente a la publicación: Acebes, J.L., Encina, A., García-Angulo, P., Alonso-Simón, A., Mélida, H., Álvarez, J.M. (2010). Cellulose biosynthesis inhibitors: their uses as potential herbicides and as tools in cellulose and cell wall structural plasticity research. En "Cellulose: Structure and Properties, Derivatives and Industrial Uses" (Lejeune A. y Deprez T., Eds,). Nova Publishers (New York). En prensa. ISBN 978-1-60876-388-7.

Cellulose Biosynthesis Inhibitors: Their Uses as Potential Herbicides and as Tools in Cellulose and Cell Wall Structural Plasticity Research

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Abstract

Cellulose biosynthesis inhibitors (CBIs) form a heterogeneous group of structurally unrelated compounds that specifically affect the assembly or the deposition of cellulose. With the exception of thaxtomin A, the only naturally occurring CBI, all other CBIs are synthetic compounds. A number of them (dichlobenil, isoxaben and flupoxam) are used as herbicides and, as such, are listed as group L in the Herbicide Resistance Action Committee classification of herbicides. Recently, targets for isoxaben and dichlobenil have been provided. Other compounds, such as triazofenamide, CGA 325'615, the aminotriazine AE F150944 or the thiazolidinone named compound 1 have proven to be CBIs, but they have not yet been commercialized. Finally, some drugs seems to display a dual effect, acting as CBIs in some cases (i.e., depending on the species or their concentration), or as auxin herbicide (quinclorac) or plant growth retardant (ancymidol) in other circumstances.

CBIs have been used to elucidate cellulose biosynthesis—unraveling the organization of cellulose synthase (CESA) complex including the assembly between CESA subunits—and the relation between cortical microtubules and cellulose deposition, as some CBIs interfere with the microtubule-guided deposition of cellulose microfibrils.

In recent years, several *Arabidopsis* CBI-resistant mutants have been reported. Frequently, but not always, their mutations were related to genes directly involved in cellulose biosynthesis. In other cases, the effects of CBIs on plant growth have been used to associate the phenotype of a set of mutants to the impairment in their cellulose biosynthesis machinery.

The habituation of cell cultures to grow in the presence of high concentrations of different CBIs has been proven to be a powerful tool for insight into the mechanisms underlying the plasticity of plant cell wall structure and composition. CBI-habituated cell cultures reflect the ability of cells to adapt their metabolism and modify their cell walls in order to cope with these new stressful conditions.

New perspectives in CBI uses imply the selection of habituated cells having walls with a reduction in their cellulose content; the manipulation of levels and/or modification of matrix polysaccharides, rendering cell walls with new physicochemical properties; the study of the relationship between cellulose synthesis and other C-sink processes such as phenylpropanoid synthesis; or the elucidation of putative new targets implied in cellulose biosynthesis.

INTRODUCTION

Cellulose biosynthesis inhibitors (CBIs) constitute a varied group of structurally unrelated compounds that specifically affect the assembly or the deposition of cellulose in higher plants (Figure 1, Table 1). Two remarkable reviews focused on CBIs were afforded in 1999 (Sabba and Vaughn) and 2002 (Vaughn), but a set of important data has been raised since then, and the global view of CBIs is now more panoramic.

Several CBIs have been commercialized as herbicides, and therefore appear as group L in the Herbicide Resistance Action Committee (HRAC) classification of herbicides. This classification includes dichlobenil, chlorthiamid, isoxaben, flupoxam and quinclorac (but in this case indicated only for monocots, and it is considered an auxinic herbicide in group O) (Menne and Köcher, 2007).

Some other compounds have also been cited as promising herbicides, such as triazofenamide and triaziflam (Wakabayashi and Böger, 2004), but until now they have not been commercialized or included in this group. Finally, some drugs have been described to display a dual effect (i.e., quinclorac or ancymidol), acting as CBIs in some cases (i.e., depending on the species, or their concentration) and showing an additional mode of action in other circumstances.

The amount of information about each of these compounds is unequal. The objective of this chapter is to show a current view of this kind of compound, attending also to the description of CBI-related mutants and the use of CBIs to elucidate cellulose biosynthesis, and to give insight into the mechanisms underlying the plasticity of plant cell wall structure and composition by means of the habituation of cell cultures to CBIs.

Cellulose Biosynthesis

In recent years, a considerable amount of information regarding cellulose biosynthesis in higher plants has been provided, and some reviews have been reported (Somerville, 2006; Joshi and Mansfield, 2007; Mutwil et al., 2008; Taylor, 2008; Bessueille and Bulone, 2008), so that only a brief presentation of cellulose biosynthesis will be introduced now. Cellulose biosynthesis machinery is located in the plasma membrane, forming 'rosettes', which constitute a transmembrane system (Figure 2). In plants, these rosettes, or cellulose synthase (CESA) complexes, are viewed as hexamers, using freeze-fracture electron microscopy. Each monomer comprises six cellulose synthase proteins, resulting then in 36 individual CESA proteins by rosette, able to synthesize 36 β-(1,4)-glucan chains that will form a microfibril (Doblin et al., 2002). The CESA complexes seem to be assembled in the Golgi apparatus and then exported via exocytosis to the plasma membrane (Somerville, 2006). CESA interacts with the cytoskeleton so that microtubules seem to guide the movement of CESA complexes (Paredez et al., 2006).

In *Arabidopsis thaliana,* primary cell wall CESA complexes are integrated by three unique types of CESA subunits named CESA1, CESA3 and CESA6-related (CESA2, 5 and 9) (Persson et al., 2007), in such a way that CESA3 and CESA6 physically interact (Desprez et al., 2007). Other proteins are claimed to form part of the complex, such as a cytoskeletal-anchored sucrose synthase, which could channel UDP-glucose to CESA (Amor et al., 1995); a membrane anchored endo-β-(1-4)-glucanase named KORRIGAN (Nicol et al., 1998), which seems to act decreasing cellulose crystallinity (Takahashi et al., 2009); a plasma membrane protein denominated KOBITO (Pagant et al., 2002); COBRA, an GPI-anchored protein (Schindelman et al., 2001); etc. However, an exact role for these additional proteins has not yet been provided. In secondary cell walls, CESA complexes are composed of three unique types of CESA subunits, too:

CESA4, CESA7 and CESA8 (Atanassov et al., 2009; Timmers et al., 2009), but in this case purified CESA complexes did not contain any further protein (Atanassov et al., 2009). Analyses of cellulose biosynthesis in other species reflect similar results to that obtained in *Arabidopsis*.

Cellulose microfibril formation can be divided into three steps: (i) initiation, using UDP-glucose as the donor substrate; (ii) polymerization of glucose into β-(1,4)-glucan chains, and (iii) crystallization of β-(1,4)-glucan chains into a microfibril (Peng et al., 2002). Based on *in vitro* experiments, it has been proposed that cellulose biosynthesis is initiated from a sitosterol-β-glucoside as primer molecule (Peng et al., 2002).

CELLULOSE BIOSYNTHESIS INHIBITORS

Dichlobenil

Dichlobenil or 2,6-dichlorobenzonitrile, is the simplest and one of the most studied CBIs. A related herbicide, chlorthiamid (2,6-dichlorothio-benzamide), is converted to dichlobenil in soil as a consequence of microorganism metabolism (Beynon and Wright, 1968). Dichlobenil has been marketed since the 1960s under different trade names (Casoron, Barrier, Silbenil, H 133). Although less effective on monocots (Sabba and Vaughn, 1999), dichlobenil has been extensively used as a broad spectrum preemergence herbicide (Verloop and Nimmo, 1969). The preemergence action of dichlobenil is based on the impairment of seedling growth more than on the inhibition of seed germination. In this way, in the French bean, root growth is 30 times more sensible to dichlobenil than seed germination (Encina, unpubl.). I_{50} values in the micromolar range have been measured for the effect of dichlobenil on root growth: 1 μM on *Lepidium sativum* (Günther and Pestemer, 1990); 0.4 μM on *Arabidopsis* (Heim et al., 1998); 4 μM on the French bean (Encina, unpubl.), and 2 μM on maize (Mélida, unpubl.).

Rapidly expanding cells such as suspension or callus-cultured cells (Shedletzky et al., 1990, 1992; Corio-Costet et al., 1991a, b; Encina et al., 2001, 2002; Mélida et al., 2009); seedling roots and hypocotyls (Himmelspach et al., 2003; DeBolt et al., 2007b); and pollen tubes (Anderson et al., 2002) are sensible to dichlobenil in the nano-micromolar range [I_{50}: 50 nM for soybean suspension-cultured cells (Corio-Costet et al., 1991b); 0.5 μM and 0.3 μM for French bean callus and suspension-cultured cells, respectively (Encina et al., 2001, 2002); and 1.5 μM for maize callus (Mélida et al., 2009)]. The ability of this herbicide to arrest cell plate formation (but not nuclear division [Galbraith and Shields, 1982]) and cell elongation (Vaughn et al., 1996; Sabba et al., 1999; Encina et al., 2001, 2002; Vaughn, 2002) is under the typical dichlobenil-growth retarding and dichlobenil-dwarfing effects (Sabba and Vaugh, 1999 and refs. therein).

Dichlobenil symptomatology also includes radial root or hypocotyl swelling (Umetsu et al., 1976; Eisinger et al., 1983; Montague, 1995; Himmelspach et al., 2003; DeBolt et al., 2007b), inhibition of root-hairs and secondary-root development, and induction of necrotic lesions (Barreiro, unpubl.). Many later effects are also associated with mitotic disrupter

herbicides (Vaughn, 2002), and curiously, to many abiotic stresses (Potters et al., 2007).

Very early studies related the phytotoxic effect of dichlobenil to the inhibition of ATP production by its phenolic degradation products, 2,6-dichlo-3-hydroxybenzonitrile and 2,6-dichloro-4-hydroxybenzonitrile (Moreland et al., 1974). However, in the same year it was demonstrated that dichlobenil itself impaired the incorporation of radiolabelled glucose into cellulose (Hogetsu et al., 1974). Since then, dichlobenil has been demonstrated to inhibit the biosynthesis of cellulose in a wide range of systems by using the same experimental procedure (e.g., Montezinos and Delmer, 1980; Brummell and Hall, 1985; Hoson and Masuda, 1991; Corio-Costet et al., 1991b; Edelmann and Fry, 1992; Shedletzky et al., 1992; García-Angulo et al., 2009), and nowadays no doubts about the specific effect of dichlobenil on cell wall biosynthesis exist.

In plants, the effect of dichlobenil on cellulose biosynthesis seems to be specific, since matrix polysaccharides synthesis is not inhibited upon a short term dichlobenil treatment (Montezinos and Delmer, 1980; Blaschek et al., 1985; Francey et al., 1989). However dichlobenil has also been reported to inhibit non-cellulosic polysaccharides synthesis in algae (Arad et al., 1994; Wang et al., 1997). Other cellular processes such as DNA synthesis, protein synthesis, respiration, oxidative phosphorylation, phospholipid metabolism, nucleoside metabolism and glucose metabolism (including glucose uptake) are not affected by dichlobenil (Meyer and Herth, 1978; Montezinos and Delmer, 1980; Galbraith and Shields, 1982; Delmer et al., 1987).

Besides this specific effect of dichlobenil on cellulose synthesis, several papers address the side effect of dichlobenil on cellulose microfibril orientation (Sugimoto et al., 2001), cellulose-synthesizing complexes organization/motility (Herth, 1987; Mizuta and Brown, 1992; DeBolt et al., 2007b; Wightman et al., 2009) and microtubule organization (Himmelspach et al., 2003).

Mode of action

Despite dichlobenil being recognized and used as a specific CBI since decades (Hogetsu et al., 1974), its mode of action is still unclear. Dichobenil inhibited the *in vivo* synthesis of sitosterol-β-glucosides, and exogenous addition of sitosterol-β-glucosides reverted dichlobenil effects (Peng et al., 2002). Based on these findings, an effect for dichlobenil on blocking the initiation steps of cellulose biosynthesis could be hypothesized. However, the need of a primer for *in vivo* cellulose biosynthesis is now under controversial as experimental conditions used by Peng et al. (2002) seemed to be highly forced and it has been demonstrated that *in vitro* biosynthesis of cellulose did not require any primer (Somerville, 2006).

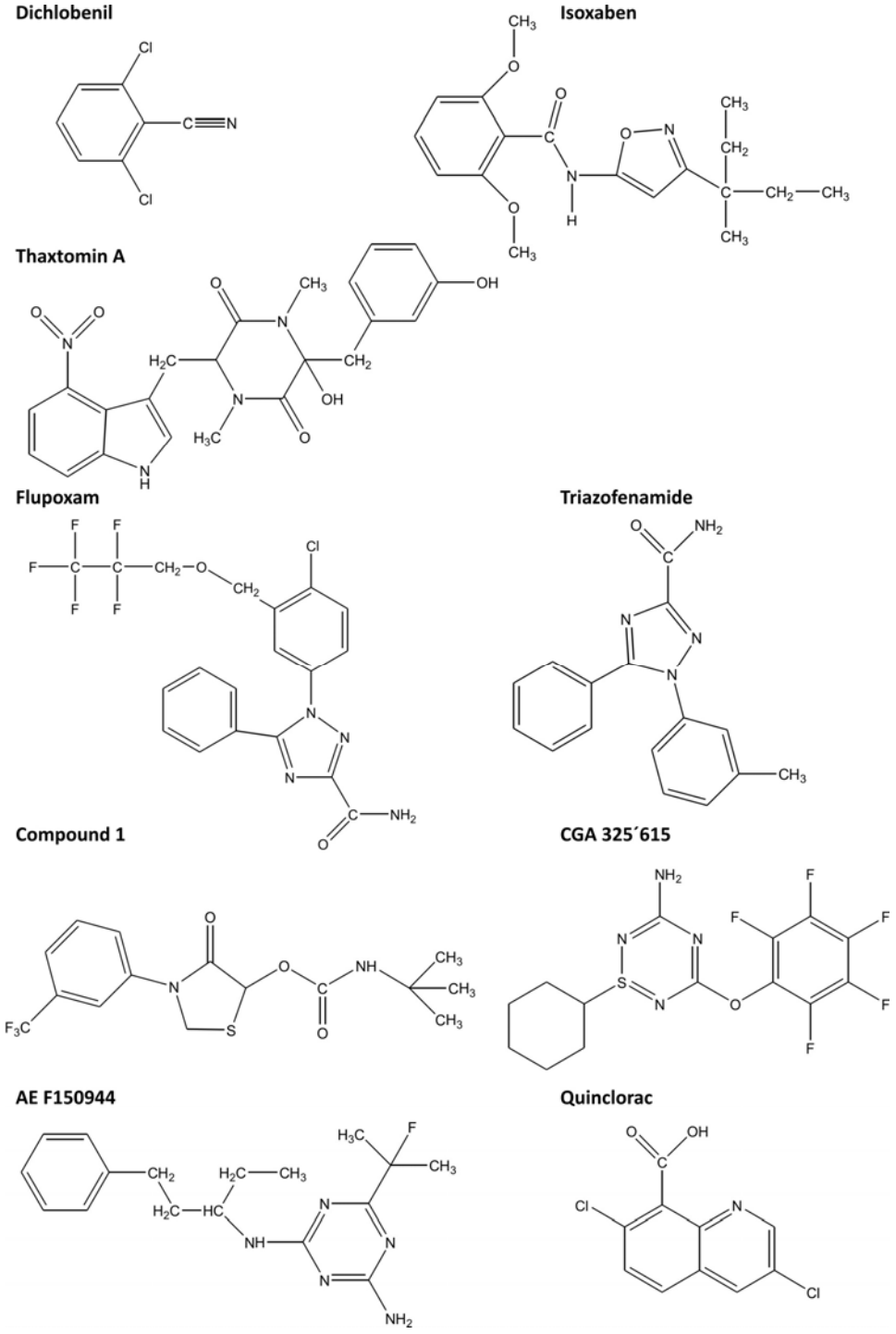

Figure 1. Structures of selected CBIs.

Table 1. Some data about selected CBIs. Accepted chemical names, I_{50} on fresh weight gain of bean calluses, I_{50} on *Arabidopsis* root growth, and selected references about CBIs. (a) García-Angulo, unpubl.; (b) Heim et al., 1998; (c) Heim et al., 1989; (d) Heim et al., 1998; (e) Desprez et al., 2002; (f) Scheible et al., 2003; (g) Hoffmann and Vaughn, 1996 (on *Lepidium sativum* root); (h) Heim et al., 1998; (i) Sharples et al., 1998; (j) Walsh et al., 2006.

CBI	Chemical name	I_{50} bean calluses[a]	I_{50} *Arabidopsis* root growth	References
Dichlobenil	2,6-dichlorobenzonitrile	0.5 µM	0.4 µM[b]	Delmer, 1987 Delmer et al., 1987
Isoxaben	*N*-[3-(1-ethyl-1-methylpropyl)]-5-isoxazolyl]-2,6-dimethoxybenzamide	3 nM	4.5 nM[c]; 1nM[d]; 1.5 nM[e]	Huggenberger et al., 1982 Heim et al., 1990
Thaxtomin A	(A 4-nitroindol-3-yl containing 2,5-dioxopiperazine)	0.6 nM	25-50 nM[f]	King et al., 1992 Fry and Loria, 2002
Flupoxam	1-[4-chloro-3-[(2,2,3,3,3-pentafluoropropoxymethyl) phenyl]-5-phenyl-1H-1,2,4-triazole-3-carboximide	2 nM	(6 nM[g])	O'Keeffe and Klevorn, 1991 Hoffman and Vaughn, 1996
Triazofenamide	1-(3-methyl phenyl)-5-phenyl-1H-1,2,4-3 triazole-3-carboximide	15 nM	39 nM[h]	Heim et al., 1998
Compound 1	5-tert-butyl-carbamoyloxyl-3-(3-trifluoromethyl) phenyl-4-thiazolidinone	20 µM	<3 µM[i]	Sharples et al., 1998.
CGA 325´615	1-cyclohexyl-5-(2,3,4,5,6-pentafluorophenoxy)-1 λ4,2,4,6-thiatriazin-3-amine	0.5 nM	—	Peng et al., 2001
AE F150944	N2-(1-ethyl-3-phenylpropyl)-6-(1-fluoro-1-methylethyl)-1,3,5-triazine-2,4-diamine	1 nM	—	Kiedaisch et al., 2003
Quinclorac	3,7-dichloro-8-quinoline carboxylic acid	10 µM	8 µM[j]	Koo et al., 1996 Koo et al., 1997 Tresch and Grossmann, 2003

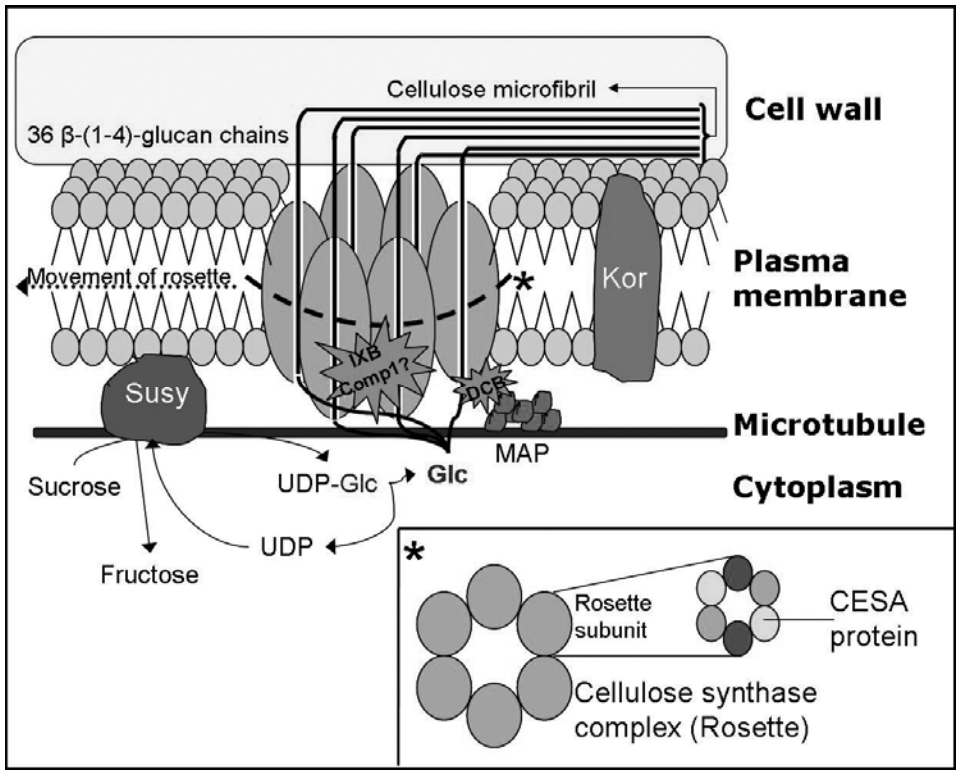

Figure 2. Model of a cellulose synthase complex (rosette). Each subunit (represented as a lobe) contains six CESA proteins, corresponding to three different CESA types (highlighted in the insert by different grey saturation). Other proteins seem to act in the cellulose biosynthesis process, such as sucrose synthase (Susy), KORRIGAN (Kor), a microtubule associated protein (MAP), and other ones, not represented here (see the text). Putative targets for some CBIs have been highlighted in boxes (IXB: isoxaben; Comp1: compound 1; DCB: dichlobenil).

A remarkable characteristic of cell walls from dichlobenil-habituated cells (as it will be detailed further) is the accumulation of a non-crystalline β-1,4)-glucan (Encina et al., 2002; García-Angulo et al., 2006). Moreover, a number of reports point to dichlobenil as a disruptor of the organization, motility or dynamics of the CESA complexes (Herth, 1987; Mizuta and Brown, 1992; DeBolt et al., 2007b). Taken together these results it has been proposed that dichlobenil effect on cellulose biosynthesis would result in an alteration of cellulose crystallization rather than of an inhibition of glucose polymerization. At this respect it is interesting to note that the *rsw1 Arabidopsis* mutants on CESA1 protein, with disassembled CESA complexes, have reduced cellulose contents and accumulate a non-crystalline β-(1,4)-glucan (Arioli et al., 1998).

Three putative targets for dichlobenil have been reported until now. By using a photoreactive analogue of dichlobenil, 2-6-dichlorophenylazide, Delmer et al. (1987) reported a small protein of 12-18 kDa as the dichlobenil target. This small protein resulted easily dissociated from membrane preparations and, therefore, it is unlikely to be a CESA protein. The authors hypothesize for dichlorophenylazide-tagged protein a regulatory role, probably modulating the glycosyl-transferase specificity (Delmer et al., 1987).

Table 2. Selected cellulose-deficient mutants in Arabidopsis. Some of them have a mutation in *CesA* loci, whereas others present a mutation in different genes. Isoxaben-resistant mutants are indicated in bold.

Locus	Mutant alleles	Phenotype	Gene product	Reference
CesA1	*rsw1-1*	Temperature-sensitive, radially expanded cells, dwarf, normal division planes	Cellulose synthase catalytic subunit, CESA1	Arioli et al., 1998
	rsw1-2	Late embryonic, radially expanded cells, dwarf, normal division plane		Gillmor et al., 2002
	rsw1-20			Beeckman et al., 2002
	rsw1-10	Post-embryonic, radially expanded cells, dwarf		Fagard et al., 2000
CesA3	*eli1-1; 1-2*	Post-embryonic, dwarf, accumulation ectopic lignin	Cellulose synthase catalytic subunit, CESA3	Caño-Delgado et al., 2003
	cev1	Increased production of ethylene and jasmonate, stunted root		Ellis et al., 2002
	ixr1-1; 1-2	Semi-dominant, isoxaben-resistant		Scheible et al., 2001
CesA4	*irx5*	Collapsed irregular xylem walls, thinner cell wall, weak stem, adult plants slightly smaller than wild type	Cellulose synthase catalytic subunit, CESA4	Taylor et al., 2003
CesA6	*prc1-1* to *1-12*	Post-embryonic, stunted root and dark-grown hypocotyls, radially expanded cells, gapped cell walls, normal microfibril orientation	Cellulose synthase catalytic subunit, CESA6	Fagard et al., 2000
	ixr2-1	Semi-dominant, isoxaben-resistant		Desprez et al., 2002
CesA7	*irx3*	Collapsed irregular xylem walls, thinner cell walls, weak stem, adult plants slightly smaller than wild type	Cellulose synthase catalytic subunit, CESA7	Taylor et al., 1999, 2000
CesA8	*irx1*	Collapsed irregular xylem walls, weak stem, adult plants slightly smaller than wild type	Cellulose synthase catalytic subunit, CESA8	Turner and Somerville, 1997; Taylor et al., 2000
Kob1	*kob1-1; 1-2*	Dwarf, radially expanded cells, less and disorganized microfibrils in cell elongation zone in root	Type II intrinsic plasma membrane protein	Pagant et al., 2002
Cob	*cob1-1*	Conditional root expansion, T-sensitive, radially expanded cells and stunted root, reduced cell expansion in root	GPI-anchored protein	Schindelman et al., 2001

Table 2. (Continued)

Locus	Mutant alleles	Phenotype	Gene product	Reference
Kor1	*kor1-1*	Post-embryonic, dwarf, radially expanded cells	Membrane-bound endo-β-1,4-glucananse	Nicol et al., 1998
	kor1-2	Late embryonic, dwarf, randomized division planes, aborted cell plates		Zuo et al., 2000
	rsw2-1 to *4*	Temperature-sensitive, radially expanded cells, dwarf, randomized division planes, aborted cell plates		Lane et al., 2001
	irx2-1; 2-2	Collapsed xylem cells, no growth phenotype		Molhoj et al., 2002
Pom1	*pom1-1* to *11*	Conditional root expansion, stunted root, dark-grown hypocotyls, radially expanded cells, normal microfibril orientation	Chitinase-like protein (AtCTL1)	Hauser et al., 1995
	elp1	Ectopic deposition of lignin, incomplete cell walls in some pith cells		Zhong et al., 2002
Cyt1	*cyt1-1; cyt2*	Late embryonic, increased radial expansion , incomplete cell walls, excessive callus accumulation	Mannose-1-phosphatase guanylyl-transferase	Lukowitz et al., 2001
Rsw3	*rsw3-1*	Temperature-sensitive, radially expanded cells, dwarf, longer lag-time before appearance phenotype then rsw1 and 2	Glucosidase II	Burn et al., 2002

Some years later, Nakagawa and Sakurai (1998) published the specific binding of dichlobenil to the catalytic subunit of cellulose synthase. By using a specific antibody against CESA1, it was possible to detect this protein in microsomal fractions of dichlobenil-habituated and control tobacco BY-2 cells. Dichlobenil-habituated cells had reduced contents of cellulose but more CESA1 protein than controls. Following these authors, CESA1 would result stabilized upon dichlobenil binding, rendering it more stable against proteolytic degradation (Nakagawa and Sakurai, 1998). In any case it was demonstrated a direct binding between dichlobenil and CESA1, and therefore the CESA1 enrichment in dichlobenil-habituated cells is likely to be a side effect of dichlobenil action.

Long ago, it was demonstrated that dichlobenil stimulates the accumulation of CESA complexes in the plasma membrane of wheat (Herth, 1987). Recently, live-cell imaging of transgenic plants carrying a yellow fluorescent protein (YFP)-CESA6 fusion showed that a short-term treatment with dichlobenil inhibited the motility of these complexes in *Arabidopsis* cells and promoted their hyperaccumulation at sites in the plasma membrane that may coincide with loading areas of CESA complexes from Golgi (DeBolt et al., 2007b). Further studies confirmed that dichlobenil also slowed the movement

of CESA complexes beneath the zones of formation of secondary wall (Wightman et al., 2009). These findings may reveal the interference of dichlobenil with the circulation of CESA complexes between Golgi and plasma membrane.

In recent times, a microtubule associated protein MAP20 in secondary cell walls of hybrid aspen has been reported as a target for dichlobenil (Rajangam et al., 2008). MAP20 is a small cytosolic protein strongly upregulated during the formation of secondary cell wall. It shares a conserved domain with a classical microtubule associated protein, TPX2, demonstrated to bind microtubules and proteins that use microtubules as guiding templates. In fact, MAP20 binds to microtubules both *in vitro* and *in vivo*. Rajangam et al. (2008) have demonstrated that dichlobenil specifically binds to MAP20 during cellulose synthesis but it does not prevent the binding of the protein to microtubules. Linking up MAP20 function with dichlobenil effects, these authors propose that MAP20 has a specific role in cellulose biosynthesis by coupling CESA proteins with microtubules and that dichlobenil inhibits cellulose biosynthesis by decoupling cellulose synthesis and microtubules through MAP20 inactivation. Finally it is suggested that the small polypeptide described by Delmer et al. (1987) might be a putative ortholog of MAP20 (Rajangam et al., 2008).

Isoxaben

Isoxaben (N-[3-(ethyl-1-methyl propyl)]-5-isoxazolyl-2,6-dimethoxy-benzamide) marketed as Flexidor, Gallery or EL-107 is a selective pre-emergence herbicide widely used for season-long control of dicot weeds in winter cereals (Huggenberger et al., 1982) and for weed control in turf, ornamentals and nursery stocks (Sabba and Vaughn, 1999). The pre-emergence action of isoxaben is based on the ability to impair seedling growth instead of preventing germination (Desprez et al., 2002). Isoxaben is very active in the nanomolar range; I_{50} values of 1.5 nM (Desprez et al., 2002), 4.5 nM (Heim et al., 1989) and 20 nM (Lefebvre et al., 1987) have been reported for the inhibition of *Arabidopsis* (Desprez et al., 2002; Heim et al., 1989) and *Brassica napus* (Lefebvre et al., 1987) seedlings growth. Isoxaben symptomatology evokes that of dichlobenil. *Arabidopsis* seedlings treated with isoxaben show a typical dwarf phenotype caused by the inhibition of hypocotyl and root elongation. Moreover, isoxaben-treated organs typically expand radially, have reduced elongation rates, accumulate callose and show ectopic lignification (Desprez et al., 2002; Caño-Delgado et al., 2003). As dichlobenil, isoxaben also arrests cell plate formation (Samuels et al., 1995; Vaughn et al., 1996; Durso and Vaughn, 1997). At this respect, isoxaben seems to act in a different manner as it has been reported that cell plates of isoxaben-treated BY-2 tobacco cells are reduced in both cellulose and callose, whereas dichlobenil treatment did only affect cellulose biosynthesis. In accordance with this, isoxaben effect is more pronounced due to the inhibition of cell plate formation at an early stage (Durso and Vaughn, 1997; Sabba and Vaughn, 1999). Later, DeBolt et al. (2007b) demonstrate that both dichlobenil and isoxaben promoted the formation of callose on *Arabidopsis* seedlings by inducing the expression of

Pmr4, a pathogen- or wound-induced callose synthase gene (Nishimura et al., 2003).

Isoxaben is also very active at inhibiting the growth of *in vitro* cultured-cells. I_{50} values in the nanomolar range have been reported for different systems: 170 nM for *Arabidopsis* calluses (Heim et al., 1989); 80 nM for soybean cell suspensions (Corio-Costet et al., 1991a, b), and 10 nM for French bean calluses (Díaz-Cacho et al., 1999).

It has been conclusively demonstrated that isoxaben specifically inhibits the glucose incorporation into cellulose in plants, without affecting other metabolic processes such as photosynthesis, respiration, carotenoid or tetrapyrrole biosynthesis (Lefebvre et al., 1987; Heim et al., 1989, 1990; Corio-Costet et al., 1991b; Caño-Delgado et al., 2003). The reported I_{50} values for the inhibition of [^{14}C]glucose incorporation into cellulose are again in the nanomolar range: 10 nM for *Arabidopsis* (Heim et al., 1990) and 40 nM for soybean (Corio-Costet et al., 1991b). Although preliminary results would indicate an effect of isoxaben on protein synthesis (Lefebvre et al., 1987), more accurate experiments demonstrated that the inhibition of protein synthesis, if produced, was not a direct effect of isoxaben (Heim et al., 1990).

Mode of action

The knowledge about the mode of action of isoxaben greatly advanced after the discovery of two loci in *Arabidopsis*, *Ixr1* and *Ixr2*, that conferred resistance to isoxaben (Heim et al., 1989, 1990). Early studies demonstrated that the natural isoxaben-tolerance of some weeds was directly related with a decreased sensitivity at the target site more than with an enhanced herbicide detoxification or reduced herbicide uptake (Heim et al., 1991; Scheneegurt et al., 1994). Therefore the idea of *ixr* mutations affecting the herbicide target was very attractive.

Nowadays it is known that *ixr1* and *ixr2* carry mutations in CESA3 and CESA6 proteins respectively (Scheible et al., 2001; Desprez et al., 2002). No isoxaben-resistant mutants affecting CESA1 (the third "Musketeer" required for cellulose biosynthesis during primary cell wall formation) have been identified, suggesting that CESA1 is not a target for isoxaben (Robert et al., 2004). The *ixr* mutations affect to the C terminus of CESA, far from its active site, making difficult for isoxaben to directly interfere with the catalytic site of the protein (Desprez et al., 2002). Most probably, *ixr* mutants would become isoxaben-insensitive by a conformational change of the target protein.

It is not clear how isoxaben inhibits the cellulose biosynthesis. It has been hypothesized that isoxaben would recognize, directly or indirectly, an isoxaben–sensitive site in the interaction between CESA3 and CESA6. By this way, isoxaben binding would destabilize the rosette (or each rosette constituting particles) leading to an inhibition in the cellulose biosynthesis. In this sense it has been demonstrated that isoxaben causes the clearing of YFP-CESA6-tagged complexes from the membrane (Paredez et al., 2006; DeBolt et al., 2007b). An alternative model explains that an isoxaben-induced conformational change on CESA proteins would block the putative membrane channel needed for the extrusion of the growing cellulose microfibril (Desprez et al., 2002).

As for others CBIs, the inhibition of cellulose biosynthesis by isoxaben is accompanied by side effects on cytoskeleton organization and cell longevity. As dichlobenil, isoxaben disrupts microtubule stability and orientation (Paredez et al., 2008). Same authors demonstrated that isoxaben effect on cortical microtubules is directly linked to the inhibition of cellulose biosynthesis rather than to an alteration of cell wall integrity (Paredez et al., 2008). Recently, it has been demonstrated that isoxaben (like thaxtomin A) specifically induce programmed cell death in suspension-cultured *Arabidopsis* cells (Duval et al., 2005, 2009).

Thaxtomin A

The modified dipeptide thaxtomin A (a 4-nitroindol-3-yl containing 2,5-dioxopiperazine) is the main phytotoxin produced by *Streptomyces scabiei*, the causative agent of common scab disease (King et al., 1992). Thaxtomin A has been reported to have an I_{50} value of 25 to 50 nM on seedling growth of *Arabidopsis* (Scheible et al., 2003). At nanomolar concentrations thaxtomin A causes dramatic cell swelling and root or shoot thickening due to cell hypertrophy (Scheible et al., 2003). Thaxtomin A (1–3 μM) also inhibited normal cell elongation of tobacco protoplasts in a manner that suggested an effect on primary cell wall development (Fry and Loria, 2002).

The inhibition of cellulose synthesis by thaxtomin A induces a genetically controlled cell death in a wide variety of plant species and tissues and in a concentration-dependent manner (Duval et al., 2005). Programmed cell death involves fragmentation of nuclear DNA and requires active gene expression and *de novo* protein synthesis. It was recently demonstrated that this programmed cell death occurs by the activation of common stress-related pathways that would somehow bypass the classical hormone-dependent defense pathways (Duval and Beaudoin, 2009).

Sublethal concentrations of several auxins reduced severity of scab disease (McIntosh et al., 1985) by enhancing tolerance to thaxtomin A (Tegg et al., 2008). Although the mechanism of auxin inhibition of thaxtomin A toxicity is not understood, it could be related to the reversion of programmed cell death by auxins (Gopalan, 2008), or to direct competition for a putative cellular binding since several auxins share chemical similarities to the thaxtomin A molecule (Tegg et al., 2008).

Recently thaxtomin A has been used to select scab disease-resistant potato plants, by means of a cell culture approach. This inhibitor was used as a selection agent applied to potato cells culture media: the surviving variants were recovered and used to regenerate complete plants (Wilson et al., 2009).

Mode of action

The mode of action of thaxtomin A is not known. It has been reported that the symptoms are similar to those caused by CBIs such as dichlobenil and isoxaben (King and Lawrence, 2001; Scheible et al., 2003). In fact, the reduction of seedling growth was accompanied by a reduction of the incorporation of [^{14}C]glucose into the cellulosic fraction of dark-grown seedlings of *Arabidopsis*, that was parallel to a significant increase in the incorporation into pectins, while the incorporation into hemicelluloses was slightly increased (Scheible et

al., 2003; Bischoff et al., 2009). Additional evidence for the inhibition of cellulose biosynthesis was obtained with Fourier transform infrared (FTIR) microspectroscopy. FTIR spectra of thaxtomin A-treated hypocotyls cluster tightly with those of wild-type hypocotyls treated with CBIs (e.g., isoxaben or dichlobenil) and with mutants known to be defective specifically in cellulose synthesis (e.g., *rsw1-2* and *kor-2*) (Robert et al., 2004).

However, it was reported that thaxtomin A also causes substantial wilting in several species after postemergence applications, a symptom dissimilar to that caused by known CBIs (King and Lawrence, 2001).

The modifications in cell wall composition caused by thaxtomin A were accompanied by changes in the expression of *CesA* genes and additional cell wall related genes both in primary (*Korrigan* and *Kobito1*) and secondary (*CesA7, CesA8, Cobra-like 4, Irx8, and Irx9, Cad9*) cell wall synthesis (Bischoff et al., 2009). The alteration in the expression of *CesA* genes of *Arabidopsis* seedlings results in a depletion of CESA complexes from the plasma membrane, coinciding to their accumulation in a microtubule-associated compartment (Bischoff et al., 2009).

Changes in the expression of genes associated with pectin metabolism and cell wall remodeling were also detected after a treatment with thaxtomin A, as it was the case of a pectin acetylesterase and two pectin methylesterases that were found to be upregulated by thaxtomin A (Bischoff et al., 2009). This result agrees with previous data on compensation of the loss of cellulose by an increased amount of pectin with a lower degree of esterification (Burton et al., 2000).

The modification of cell wall composition caused by a thaxtomin A treatment causes an additional cell wall reinforcement by triggering ectopic lignification by a high up-regulation of several genes involved in lignin biosynthesis (Bischoff et al., 2009). Thaxtomin A treatment also provoked the induction of a set of defense genes (Caño-Delgado et al., 2003, Bischoff et al., 2009).

Other CBIs

Triazol carboximide herbicides (flupoxam and triazofenamide)

Flupoxam (1-[4-chloro-3-(2,2,3,3,3-pentafluoropropoxymethyl) phenyl]-5-phenyl-1H-1,2,4-triazole-3-carboximide), and triazofenamide (1- [3-methyl phenyl]-5-phenyl-1H-1,2,4-triazole-3-carboximide), are triazole-carboximide herbicides. Flupoxam, commercialized as Quatatim or KNW-739, inhibits the root growth of watercress (*Lepidium sativum*) by 50% at a concentration of 6 nM. Because flupoxam induces classic club root morphology it was initially characterized as a mitotic disrupter (O'Keeffe and Klevorn, 1991). However, Hoffman and Vaughn (1996) reported later that the effect of flupoxam on watercress roots was different from that expected of a mitotic disrupter although they did not propose an alternative mode of action. By cluster analysis of FTIR spectra, a close relationship among *Arabidopsis* seedlings treated with flupoxam, dichlobenil, isoxaben and cellulose-deficient mutants was observed (Robert el al., 2004). The treatment of cotton fibers with flupoxam (and also with isoxaben) causes spherical shapes and frequently induces cell division. Fibers grown in the presence of isoxaben or flupoxam replaced the entire cell

wall with a pectin sheath of chiefly deesterified pectins, indicating that both herbicides effectively disrupt cellulose biosynthesis and cause radical changes in cell wall structure and composition (Vaughn and Turley, 2001).

The close analog of flupoxam, triazofenamide, was also initially considered as a microtubule polymerization inhibitor (O'Keeffe and Klevorn, 1991). However, using staining and microscopic techniques, this mode of action was later rejected (Hoffman and Vaughn, 1996). Heim et al. (1998) postulated that triazofenamide was a cellulose biosynthesis inhibitor due to the symptoms elicited in an *Arabidopsis* short-term test. Furthermore, triazofenamide inhibits [^{14}C]glucose incorporation into cellulose in a manner similar to isoxaben (Heim et al., 1998). Nevertheless, the exact modes of action of the triazole-carboximide herbicides are still unknown.

Compound 1

Compound 1 (5-tert-butyl-carbamoyloxyl-3-(3-trifluoromethyl) phenyl-4-thiazolidinone) is a thiazolidinone which induces a potent and rapid inhibition of [^{3}H]glucose incorporation into the acid-insoluble cell wall fraction of roots of dicot plants at nanomolar concentration (Sharples et al., 1998). Although many aspects about the mode of action of compound 1 remained unknown it was suggested that compound 1 and isoxaben should share a common mode of action (Sharples et al., 1998) that should differ from the mode of action of triazofenamide, since isoxaben-resistant mutants of *Arabidopsis* are cross resistant to compound 1 (Sharples et al., 1998) but sensitive to triazofenamide (Heim et al., 1998).

CGA 325´615

CGA 325´615 (1-cyclohexyl-5-(2,3,4,5,6-pentafluorophenoxy)-1 λ4,2,4,6-thiatriazin-3-amine) is a herbicide that inhibits synthesis of crystalline cellulose by interfering with glucan chain crystallization and causes an accumulation of non-crystalline β-(1,4)-glucan associated with CESA proteins (Peng et al., 2001). Since oxidizing agents can reverse CGA 325´615 inhibition (Kurek et al., 2002), it has been suggested that this inhibitor affects the oxidative state of the zinc-finger domain of CESA proteins required for the assembly of rosettes, which synthesize multiple β-(1,4)-glucan chains in close proximity in order to facilitate crystallization.

Recently it has been shown that CGA 325'615 treatment of *Arabidopsis* hypocotyls induced internalization of CESA complexes in a microtubule-associated CESA compartment -MASC- (Crowell et al., 2009). Accordingly to these authors, in conditions of cellulose biosynthesis inhibition, CESA complexes are recruited from the membrane into MASCs as a way to regulate the cellulose synthesis. It is interesting to note that CESA complexes internalization is obtained when cell growth is impaired by osmotic stress and that MASCs abundance is negatively correlated with hypocotyl elongation (Crowell et al., 2009). The door is open to speculate on the relationship between CBIs, cellulose biosynthesis and CESA complexes dynamic.

AE F150944

The aminotriazine AE F150944 (N2-(1-ethyl-3-phenylpropyl)-6-(1-fluoro-1-methylethyl)-1,3,5-triazine-2,4-diamine) is structurally distinct from

other CBIs. AE F150944 effectively inhibited [^{14}C]glucose incorporation into crystalline cellulose with I_{50} values of 16.7·nM, 3.67·nM and 0.37·nM during primary wall synthesis in suspension cultures of the monocot *Sorghum halepense*; and secondary and primary wall synthesis in cultured cells of the dicot *Zinnia elegans*, respectively (Kiedaisch et al., 2003). AE F150944 specifically inhibits crystalline cellulose synthesis only in organisms that synthesize cellulose via rosettes. Although it is believed that the effect of AE F150944 is due to the destabilisation of rosettes (Kiedaisch et al., 2003), its molecular target remains to be identified.

COMPOUNDS INHIBITING CELLULOSE BIOSYNTHESIS IN A SECONDARY EFFECT

Some compounds traditionally classified in other categories, such as growth retardants or auxinic herbicides, have also been shown to inhibit cellulose biosynthesis. That is the case of quinclorac, an auxinic herbicide, or ancymidol, an inhibitor of gibberellin biosynthesis. Also, some antimicrotubule agents affect cellulose biosynthesis as a secondary effect.

Quinclorac

Quinclorac (3,7-dichloro-8-quinoline carboxylic acid) is a quinoline carboxylic acid successfully used as an herbicide in crops of rice, barley, sorghum, etc., in order to control monocot and dicot weeds (Grossmann and Kwiatkowski, 2000). This synthetic substance was proposed to be auxin-like, acting by stimulation of ethylene synthesis, accompanied by cyanide accumulation in susceptible species (Grossmann and Kwiatkowski, 1995). However, some symptoms characteristic of auxinic herbicides were absent in the case of quinclorac, such as cell wall acidification due to stimulated H^+-ATPase activity (Theologis, 1987), stimulated respiration or increased RNA content (Koo et al., 1991).

Subsequently, quinclorac was also proved to inhibit cell wall biosynthesis in a dose-dependent manner in maize roots. As shown by Koo et al. (1996), after only 3 hours of treatment, the herbicide inhibited the incorporation of [^{14}C]glucose into cell wall by 33%. The inhibitory effect increased with longer treatments and higher quinclorac concentrations, and affected not only cellulose, but also glucuronoarabinoxylans and, in a minor extent, mixed-linked glucan. In contrast, dichlobenil only inhibited cellulose biosynthesis in a parallel experiment. In a later work by Koo et al. (1997), quinclorac effect on cell wall was tested in both susceptible and tolerant grasses. Cell wall biosynthesis was repressed by the herbicide by 73 and 60% in susceptible grasses, and in a minor extent (36 and 20%) in tolerant counterparts. In addition, roots of tolerant grasses were sensitive to quinclorac, but shoots were extremely tolerant, suggesting a tissue-specific response. This specific response was attributed to the existence of an additional tolerance mechanism or to a less sensitive cell wall synthesis in shoots than in roots. In any case, based on results from these two works, Koo and coauthors proposed

quinclorac to be a cell-wall biosynthesis inhibitor more than an auxinic herbicide.

Nevertheless, Grossmann and Scheltrup (1997) kept on considering quinclorac as auxin herbicide, since they proved that the compound promoted a selective induction of ACC synthase, an enzyme that catalyzes the rate limiting reaction in ethylene biosynthesis. Although ethylene itself is not the agent that directly promotes plant death, the ethylene synthesis stimulation provokes the accumulation of cyanide at physiologically damaging concentrations (Grossmann, 1996, 2000). In addition, ethylene triggers biosynthesis of abscisic acid, which reduces stomatal aperture and consequently, by the declining of photosynthetic activity, causes overproduction of H_2O_2 which contributes to the induction of cell death (Grossmann et al., 2001a). Subsequent work on barnyard grass and maize showed no influence of quinclorac treatment on cellulose biosynthesis (Tresch and Grossmann, 2003). However, long treatment promoted a decline of mixed-linked glucan, similar to that promoted by dichlobenil and also by potassium cyanide. Thus, the reduction of mixed-linked glucan deposition was interpreted as an indirect effect of quinclorac through the stimulated production of cyanide.

An extensive analysis of quinclorac action on cell walls was made on French bean cultured-cells habituated to grow in lethal concentrations of this herbicide (Alonso-Simón et al., 2008). No reduction of cellulose content was found in habituated cells, even at the highest quinclorac concentration used. In contrast, habituated cells showed a lower amount of pectins in their cell walls and extracellular material positively labelled by antibodies specific for highly methyl esterified pectins. Thus, the lower pectin content of cell wall could be due to a deficient de-esterification, which would partially prevent the correct integration and persistence of some pectins in the cell wall. Consequently, quinclorac was proved not to inhibit cell wall biosynthesis in these cells.

In a recent work by Sunohara and Matsumoto (2008), quinclorac effects were compared with those promoted by the synthetic auxin 2,4-D in maize roots. Both compounds stimulated the synthesis of ethylene and subsequent accumulation of cyanide, in a larger extent in 2,4-D. However, cell death rate induced by quinclorac was much higher. Thus, quinclorac toxicity was attributed to reactive oxygen species production induced by this herbicide. Finally, and considering that quinclorac caused similar symptoms to those promoted by cyanide, and that the capacity of metabolize cyanide seems to be different among plant species, the authors suggest that the difference in cyanide detoxification capacity of the plant is the factor which determines if cyanide or reactive oxygen species are primarily responsible of quinclorac herbicide action. Previously, same authors had demonstrated that the tolerance to quinclorac displayed by plant species as rice is related to an increased antioxidant capacity able to cope with quinclorac-induced oxidative injury (Sunohara and Matsumoto, 2004).

Ancymidol

Ancymidol is a plant growth retardant, that interferes with gibberellin biosynthesis by the inhibition of the enzyme ent-kaurene oxidase, thus reducing gibberellin content and further decreasing growth of ancymidol-

treated plants (Shive and Sisler, 1976). Besides this retardant primary action, some effect on cell wall and its polysaccharides have been described. For instance, ancymidol made cells short and thick with galactose-rich cell walls in pea (Tanimoto, 1987), suppressed cell wall extensibility of dwarf pea (Tanimoto, 1994), and changed average molecular weight of cell wall pectins (Tanimoto and Huber, 1997). Nevertheless, these modifications were reversed by gibberellins treatments, and thus due to the inhibition of gibberellin synthesis promoted by ancymidol. However, a recent work described a cellulose biosynthesis inhibitor effect of this compound on tobacco BY-2 cells, not reverted by gibberellin addition (Hofmannová et al., 2008). Ancymidol application on cells resulted in malformations and cell death similar to those induced by dichlobenil and isoxaben. In addition, ancymidol disoriented microtubules and made the cellulose distribution not continuous, provoking protoplasts to regenerate a sparse net of microfibrils, or not cellulose at all, when treated with ancymidol 10 and 100 μM, respectively. This effect was reversible by washing ancymidol from regenerating medium, but not by addition of gibberellin. The mechanism by which ancymidol inhibited cellulose synthesis remains unknown, but it was showed to be different to that of isoxaben, and may be related with the control of cell expansion (Hofmannová et al., 2008).

Coumarin and Derivatives

Other kinds of chemicals have been shown to inhibit cellulose biosynthesis. An important group is that formed by coumarin and its derivatives. Coumarin inhibited specifically [^{14}C]glucose incorporation in cellulose in cotton fibers, since no significant inhibition on [^{14}C]glucose incorporation in callose or noncellulosic glucans has been appreciated (Montezinos and Delmer, 1980). Coumarin was later observed to bind to tubulin and thus to suppress microtubule dynamics (Madari et al., 2003). The relationship between microtubules and cellulose have been repeatedly analyzed, since the orientation of glucan microfibrils and cortical microtubules were very similar or identical and the alignment hypothesis proposed that the orientation of deposited cellulose is associated with underlying cortical microtubules (Heath, 1974; Baskin, 2001). Paredez et al. (2006) visualized cellulose synthase complexes moving along tracks apparently defined by microtubules, confirming the functional relationship between these elements, after some controversy about this alignment hypothesis.

Therefore, compounds that affect microtubules synthesis or orientation should also affect cellulose deposition. In this sense, morlin (a coumarin derivative) was discovered in a screening for compounds that inhibited cellulose biosynthesis (DeBolt et al., 2007a). Morlin was shown to decrease cellulose synthesis in a dose-dependent manner, promoting a reduction in [^{14}C]glucose incorporation in cellulose and a parallel increase in callose biosynthesis. This compound also caused a change in cytoskeletal dynamics, diminishing the velocity of both microtubule polymerization and CESA complexes in plasma membranes. Nevertheless, the use of oryzalin (that completely depletes all detectable microtubules) did not affect CESA complex velocity, probing that the speed of cellulose synthases depends on

polymerization of cellulose and not on microtubules, as have been previously reported (Robinson and Quader, 1981). Then, DeBolt and co-authors (2007a) propose three different hypotheses to explain morlin effects on both cellulose synthesis and microtubules dynamics and organization: i) morlin could have separated effect on both processes; ii) morlin might target a regulatory protein that coordinated cellulose synthesis and microtubules; and iii) morlin targeted a structural protein which interacted with both microtubules and CESA complexes. In any case, the study of morlin action and its target will be a useful tool to unravel the relation between cortical microtubules and cellulose synthesis machinery.

Later, a chemical genetic screening for compounds that affected the cortical microtubule-cellulose microfibrils was performed (Yoneda et al., 2007). When cortical microtubule organization or cellulose microfibril deposition is inhibited, plant cells lose their anisotropy and show swelling. Thus, compounds that caused spherical swelling phenotype (SS compounds) were analyzed. In addition to dichlobenil, two novel compounds that reduced cellulose deposition were identified: SS14 and SS18. SS14 presents the same substructure as morlin or coumarin, but without affecting the cortical microtubule orientation at all. SS18 is a novel compound that might inhibit cellulose synthesis directly or indirectly by affecting substrate synthesis or transport (Yoneda et al., 2007).

Triaziflam

Some other compounds that affect cellulose biosynthesis as well as other cellular processes have been described, but little is known about their mode of action. One example is triaziflam, which also affects photosynthetic electron transport and microtubule formation (Grossmann et al., 2001b). The analysis of this kind of chemicals may be useful to deepen in cellulose synthesis mechanism and its relationships with many other cellular processes.

Oxaziclomefone

Oxaziclomefone is a herbicide that inhibits cell expansion in grass roots. However it did not affect [^{14}C]glucose incorporation into the main sugar residues of their cell walls, including cellulosic glucose, so that it is not considered properly as a CBI (O'Looney and Fry, 2005a, b).

CBI-RELATED MUTANTS

The selection of mutants with alterations in the composition and structure of the cell wall provides a tool to identify new genes involved in biosynthetic pathways, cell wall assembly or modifications of polymers in the cell wall. Traditionally, the identification of cell wall mutants, whether spontaneous or induced, was based on the search for (i) morphological changes such as root radial swelling (Baskin et al., 1992) or reduction in hypocotyl elongation (Nicol et al., 1998; Desnos et al., 1996), (ii) anatomical changes such as low birefringence of the trichomes (Potikha and Delmer, 1995) or collapsed

xylem (Turner and Somerville, 1997), or (iii) resistance to CBIs as isoxaben (Heim et al., 1989, 1990) or thaxtomin A (Scheible et al., 2001).

This classic mutational analysis, from phenotypes to genes (direct genetics), presents two potential problems: often the mutations do not cause visibly abnormal phenotypes due to gene redundancy, and in other cases, the mutations are usually lethal when the affected genes are keys to the concerned process. Therefore, it has resorted to other choices as the analysis of the composition in neutral sugars by gas chromatography and mass spectroscopy (Reiter et al., 1997) or tracking of FTIR spectroscopy alterations in the cell wall (Chen et al., 1998; Mouille et al., 2003; Robert et al., 2004), or the use of reverse genetics. In the last decade a large number of mutants have been involved in the structure and/or synthesis of cell wall polysaccharides, including those affected in the process of cellulose biosynthesis. We will focus on mutants resistant to CBIs, mutants with changed sensitivity to CBIs, and those mutants whose phenotype is similar to CBI-treated plants (Table 2).

Mutants Resistant to CBIs

The screening of *Arabidopsis* mutants resistant to CBIs has allowed clarification, at least in part, of not only the mode of action of these compounds, but also the composition and organization of the cellulose biosynthesis "machinery" of primary cell walls.

Isoxaben resistant mutants (*ixr1-1* and *ixr1-2*) were the first resistant mutants identified for CBIs (Heim et al., 1989, 1990). Semidominant mutations at the *ixr1-1* and *ixr1-2* loci occur in a highly conserved region of the CESA3 near the carboxyl terminus (Scheible et al., 2001). Although the *Ixr1* gene is expressed in the same cells as the structurally related *rsw1* (*CesA1*) cellulose synthase gene, these two *CesA* genes are not functionally redundant (Scheible et al., 2001). The *ixr* mutations appear to directly affect the herbicide target, because resistant cell lines show no alterations in uptake or detoxification of the herbicide (Heim et al., 1991). This mutation confers resistance to the thiazolidinone compound 1, which suggests the same mode of action for both herbicides (Scheible et al., 2001).

Another locus was identified later in the isoxaben resistant mutant (*ixr2-1*) (Desprez et al., 2002). The *ixr2-1* carries a mutation substituting an amino acid close to the C terminus of the CESA6. Initially it was thought that the presence of these two isoxaben-resistant loci (*Ixr1* and *Ixr2*) could be due to CESA3 and CESA6 were redundant. However, mutants with lost function exclusively in CESA6, like *procuste1* (*prc1,* Fagard et al., 2000) did not restore the phenotype with the presence of CESA3. These observations suggested that CESA6 and CESA3 were part of the same protein complex and that the inhibitor directly or indirectly recognizes the place of partnership between the two subunits (see Figure 2).

Isoxaben-resistant mutants CESA3$^{ixr1\text{-}1,\ 1\text{-}2}$ and CESA6$^{ixr\ 2\text{-}1}$ along with other cell wall mutants, have contributed to demonstrate that the complex involved in the cellulose synthesis in primary cell walls contains three CESA catalytic subunits. Through immunolocalization analysis, co-immunoprecipitation and green fluorescent protein (GFP) gene fused expression, it was found that two positions of CESA complexes have being

invariably occupied by CESA1 and CESA3, while in the third position at least three isoforms, CESA2, CESA5 and CESA9 may compete with CESA6 according to the tissue and/or the cell development stage (Robert et al., 2004; Desprez et al., 2007; Persson et al., 2007; Wang et al., 2008). Partial redundancy between CESA2, CESA5, CESA6 and CESA9 could explain the lower isoxaben resistance showed by CESA6^{ixr2} mutants compared withCESA3^{ixr1} (Desprez et al., 2007).

Other CBI mutants appear to have different mechanisms that could be involved in the uptake and/or detoxification of the inhibitor rather than in altered target sites. Such is the case of *txr1*, an *Arabidopsis* thaxtomin A-resistant mutant, which presents a decrease in the rate of inhibitor uptake. The mutated gene has been cloned and encodes a highly conserved and constitutively expressed protein in eukaryotic organisms. This gene could be involved in the regulation of a transport mechanism (Scheible et al., 2003).

By using MES mutagenesis, a putative *Arabidopsis* mutant resistant to dichlobenil has been reported (Heim et al., 1998). This mutant (DH75) was four times more resistant to dichlobenil than the wild type and did not show cross-tolerance to isoxaben or triazofenamide. Although DH75 resistance-mechanism remains unravelled, an alteration in herbicide metabolism has been pointed as its putative cause (Sabba and Vaughn, 1999).

Mutants with Changed Sensitivity to CBIs

At the moment there are no mutants known to be resistant to other CBIs. However, different mutants have been identified with changes in sensitivity to CBIs. A mutant of *Arabidopsis* called *css1* (changed sensitivity to cellulose synthesis inhibitors) grows at rates lower than control but is less sensitive to isoxaben and dichlobenil (Nakagawa and Sakurai, 2006). The *css1* mutation seems to affect a mitochondrial protein (At-nMat1a). Phenotypic analysis of this mutant during the early developmental stages shows changes in several metabolic processes such as amino acid synthesis, triacylglycerides degradation, and in polysaccharides synthesis such as cellulose or starch.

On the other hand it has been observed that plants with loss-of-function mutations in genes required for cell wall synthesis (*CesA2, Cobra, Korrigan* and *CesA6*; see Somerville, 2006) were hypersensitive to CBIs like isoxaben and dichlobenil (DeBolt et al., 2007b).

Some Mutants Show Similar Phenotypes to CBI-Treated Plants

As has been pointed out, plants treated with CBIs as isoxaben, dichlobenil or thaxtomin A showed a decrease in stems growth, and root swelling. When cellulose synthesis is affected, cells expand radially and accumulate callose, lignin and other phenolic compounds (Desprez et al., 2002, Bischoff et al., 2009). This phenotype is shared by many mutants with defects in hormones synthesis or signaling pathways, in mutants with alterations in endocytosis processes (Collings et al., 2008) and with reduced cellulose amount (Lukowitz et al., 2001). Moreover, the application of isoxaben or dichlobenil in control plants results in incomplete cell walls formation (Desprez et al., 2002), an effect also observed in mutants deficient in cellulose (Arioli et al., 1998; Fagard et al., 2000; Lane et al., 2001). Etiolated *Arabidopsis* seedlings treated

with nanomolar concentrations of thaxtomin A display a FTIR spectral phenotype that is most related to those of *Arabidopsis* CESA1^{rsw1} mutant seedlings, or *Arabidopsis* seedlings treated with CBIs like dichlobenil, isoxaben or flupoxam (Scheible et al., 2003; Robert et al., 2004).

Some mutants in catalytic subunits of CESA complex have been isolated and identified on the basis of this phenotype (Table 2), like a set of *radial swelling 1* (*rsw1*) mutants. The *rsw1* have a mutation in the *CesA1* gene, which results in a reduction in cellulose amount, accumulation of non-crystalline β-(1,4)-glucan and radial expansion when plants grow at a restrictive temperature of 31°C (Baskin et al., 1992; Arioli et al., 1998; Sugimoto et al., 2001). Recent evidences suggest that subunits aggregation in the CESA complex changes in extracts depending on the temperature at which CESA1^{rsw1} mutants grow. At a restrictive temperature CESA proteins remain isolated, while at a permissive temperature complexes are formed properly and co-precipitated in a complex of 840 kDa (Wang et al., 2008).

Treatment with isoxaben and other CBIs causes ectopic lignification in roots. A set of mutants also accumulates ectopic lignin. In a selection program to detect mutants of this kind, *eli1* was identified. The *eli1* has a mutation that affects a highly conserved domain of *CesA3* gene (CesA3$^{eli1\text{-}1,\ eli1\text{-}2}$) (Caño-Delgado et al., 2000). Analysis of [^{14}C]glucose incorporation into cellulose in different mutants showed that both *eli1-1* and *eli1-2* have a half reduction in the incorporation of glucose into the acid insoluble fraction of their cell walls (Caño-Delgado et al., 2003). The low level of cellulose in *eli1* is consistent with the cell shape and growth reduction in other mutants as CesA1^{rsw1} at the restrictive temperature, and other mutants that have affected CESA in primary cell wall as *procuste* (CesA6^{prc}) and *korrigan* (*kor*, endo–β-(1,4)-glucanase). CesA3$^{ixr1\text{-}1}$ mutants do not show reduction in cellulose or ectopic lignification after treatment with isoxaben, while control plants showed not only reduction in the synthesis of cellulose, but also showed ectopic lignification in all plant organs, even in the most apical cells of the root that are normally actively dividing (Caño-Delgado et al., 2003). Many other mutants that show a reduction in cellulose amount present an ectopic lignification (Table 2).

To date, no mutants with defects in cellulose synthesis in type II primary cell walls have been reported. However, the *elo* mutants are a class of barley dwarfs initially described as being impaired in cell expansion (Chandler and Robertson, 1999), that have characteristic features similar to cellulose synthesis type I mutants: all *elo* mutants show radial swelling of leaf epidermal and root cortical cells, and lower levels of cellulose in their leaves compared with the wild-type (Lewis et al., 2009). *Elo* genes have not been identified, but the study of these mutants could be an excellent tool to understand the cell expansion in plants with type II cell walls.

CBIS AS TOOLS TO RESEARCH THE STRUCTURAL PLASTICITY OF CELL WALLS

The ability of plant cells to tolerate induced stresses by modifying their cell wall composition and structure has been demonstrated in several works (Iraki et al., 1989; Shedletzky et al., 1992; Encina et al., 2001, 2002; Mélida et

al., 2009). By this meaning, CBIs are valuable tools for the analysis of cell wall structure and biogenesis. These herbicides allow analysis of the connections between the partially independent networks which make up the primary cell wall, and the high plasticity of this structure to accommodate to unfavourable conditions.

Habituation to CBIs

Although CBIs are highly specific and potent herbicides, cell cultures of several species have been habituated to grow in the presence of CBIs by incremental exposure over many culturing cycles. The cell culture utilization is very advantageous with respect to the whole plant for two reasons: (i) the possibility to have lots of cells in a reduced place at the same time, where it is easy to control and manipulate different conditions, and (ii) the availability to select cell lines with specific features. The habituation of cell cultures to CBIs reflects the ability of cells to survive with a modified cell wall and is therefore a valuable experimental technique for gaining an insight into the plasticity of plant cell wall composition and structure. Several cell cultures have been successfully habituated to CBIs such as dichlobenil, isoxaben, quinclorac and thaxtomin A. Habituated cultures usually display some common features: slower growing rates, irregularly shaped cells, a trend to grow in clumps when are suspension cultured, and cell walls with reduced cellulose contents compensated with other cell wall components.

Habituation to Dichlobenil

Two types of primary cell walls having different structure and composition exist in higher plants: type I cell walls are found in dicots, gymnosperms and most monocots, and type II walls are found in graminaceous plants, along with the other commelinoid monocots (Carpita and Gibeaut, 1993; Carpita, 1996). Although the basic mechanism of habituation is common (a replacement of the cellulose network for other cell wall components), the details of the process depend on the type of cell wall, and therefore it has been demonstrated that cells habituate to dichlobenil by using different strategies. Most dichlobenil-habituated cultures belong to type I cell wall species, such as tomato (Shedletzky et al., 1990), tobacco (Shedletzky et al., 1992; Wells et al., 1994; Nakagawa and Sakurai, 1998, 2001; Sabba et al., 1999) and bean (Encina et al., 2001, 2002; Alonso-Simón et al., 2004; García-Angulo et al., 2006). In dichlobenil-habituated type I cell walls there is a marked decrease in the amount of cellulose and hemicelluloses, whereas the quantity of esterified and unesterified pectins is increased. Moreover, in dichlobenil-habituated BY-2 tobacco cells, pectins have been reported to be cross-linked with extensin to form the main cell wall network (Sabba et al., 1999). There are other modifications associated with dichlobenil habituation, such as the presence of a non-crystalline β-(1,4)-glucan tightly bound to cellulose, the accumulation of pectin-enriched cell wall appositions, a putative increase in the extent of pectin–xyloglucan cross-linking and in xyloglucan endotransglucosylase activity, reduced levels of arabinogalactan proteins and changes in the levels of extensin (Shedletzky et al., 1992; Encina et al., 2002; García-Angulo et al., 2006,

Alonso-Simón et al., 2007), and modifications in xyloglucan composition (Alonso-Simón, unpubl.).

To date, barley (Shedletzky et al., 1992) and maize (Mélida et al., 2009) are the only type II cell wall species reported to have been habituated to dichlobenil. These dichlobenil-habituated type II cell walls also displayed a modified architecture: they contained a considerably reduced level of cellulose in the cell wall, effectively compensated for mechanisms (parallel to modifications) quite different to those observed in dicots, and slightly different between both species. Whereas barley cultures habituation implicated a higher proportion of β-glucan and a more extensive cross-linking between arabinoxylans, leading to walls with a reduction in pore size, maize habituated cultures had a more extensive and phenolic cross-linked network of arabinoxylans, without necessitating β-glucan or other polymer enhancement. As a consequence of this modified architecture, walls from dichlobenil-habituated maize cells showed a reduction in their swelling capacity and an increase both in pore size and in resistance to polysaccharide hydrolytic enzymes. From a molecular perspective the application of dichlobenil to maize cell cultures disrupts the "cellulose biosynthesis machinery", affecting to some CESA subunits, but during the habituation, maize cells partially restore this system overexpressing some of these genes (Mélida, unpubl.*). These cultures have also altered the expression of genes involved in the synthesis of phenolic compounds, which is in concordance with the increased arabinoxylans feruloylation observed. The habituation represses a class of proteins usually acting in detoxification of xenobiotics (glutation-S-transferases) and induces other involved in stress and programmed cell death processes (Mélida, unpubl.*).

Habituation to Isoxaben

Isoxaben-habituated cultures seem to have a more heterogeneous habituation mechanism than dichlobenil ones. Former isoxaben-habituated cultures had the same cellulose-xyloglucan proportion than non-habituated ones, and the habituation seemed to be more related to changes in the herbicide target or in the detoxification than in wall modifications (Corio-Costet et al., 1991a). However later results obtained with French bean (Díaz-Cacho et al., 1999), tobacco (Sabba and Vaughn, 1999) and *Arabidopsis* cell cultures (Manfield et al., 2004) showed cell wall changes similar to those described for dichlobenil-habituated cultures. At least in *Arabidopsis*, isoxaben-habituation does not appear to be mediated by stress response processes, nor by functional redundancy within the CESA family (Manfield et al., 2004). Uniquely, amongst the cellulose synthase superfamily, *CslD5* was highly upregulated and might play a role in the biosynthesis of the novel walls of habituated cells (Bernal et al., 2007).

Habituation to Other CBIs

French bean cells have been successfully habituated to grow in the presence of lethal concentrations of quinclorac (Alonso-Simón et al., 2008), such as it was formerly noted. Compared with non-habituated, quinclorac-habituated cells showed irregular shape and accumulated an extracellular material that was more abundant as the level of habituation increased.

* Datos correspondientes al Capítulo III del presente trabajo

Cellulose content was not significantly affected by habituation. In contrast, the distribution and post-depositional modifications of pectins (mainly homogalacturonan and rhamnogalacturonan I) was affected by the habituation process. These results reflect that habituation to quinclorac is not related to cellulose biosynthesis processes.

Habituation to thaxtomin A has also been described in poplar cells (Girard-Martel et al., 2008) and the thaxtomin A-habituated cell walls had less cellulose and were enriched in pectins. The whole genome transcript profiling analysis identified genes involved in many processes, including several genes implicated in glucan and pectin biosynthesis, transcriptional regulation, secondary metabolism and plant defence.

Dehabituation

Most of the cell wall changes induced during the habituation to dichlobenil reverted when cells were dehabituated by culturing them in a medium without the inhibitor (Shedletzky et al., 1990; Encina et al., 2002; García-Angulo et al., 2006). However, dehabituated cell cultures retained some habituation-induced cell wall modifications like reduced levels of arabinogalactan proteins and hydroxyproline-rich glycoproteins epitopes, altered extractability of pectins, and the presence of an amorphous β-(1,4)-glucan (Encina et al., 2002; García-Angulo et al., 2006). Most remarkably, apart from stable changes in cell wall composition and structure, dehabituated cells retained the capacity to cope with lethal concentrations of dichlobenil, as dehabituated cells were 40 times more tolerant to dichlobenil than non-tolerant cells (Encina et al., 2002). In an attempt to explain the dichlobenil resistance of dehabituated cells it was found that these cells had a constitutively increased peroxidase activity indicating a relationship between habituation to dichlobenil and a high antioxidant capacity (García-Angulo et al., 2009).

NEW PERSPECTIVES IN CBI USES

CBIs represent a promising field of experimentation in order to obtain novel herbicides. Active compounds geared towards cell walls are interesting molecules to be commercialized as herbicides, taking into account their assumed lack of toxicity for non-cellulosic organisms. As it has been shown in this chapter, several putative targets for CBIs have been recently hypothesized as a consequence of new data that have been obtained, and more intense research in this field can be predicted.

However, we are a long way from unraveling the exact mechanism of action of most of these CBIs. As the cellulose biosynthesis process is better understood, targets for different CBIs will be ascertained. Reciprocally, the use of CBIs will have an important impact on cellulose biosynthesis studies.

Nowadays, there is growing interest in obtaining cell walls with modified structures directed to change the quality and/or quantity of their components for applications in fields such as food, feed, fibres or fuel. Some of these applications are oriented toward producing dietary fibres with improved properties and other significant polysaccharides in food processing such as

pectins (Willats et al., 2006), feed products with better digestibility (Vogel and Jung, 2001), natural fibres destined for the textile and paper industries (Obembe et al., 2006), or lignocellulosic materials more suitable for biofuels (Pauly and Keegstra, 2008).

Several approaches have been taken in order to obtain these modified cell walls, such as the search for mutants affected in genes related to polysaccharide biosynthesis, the manipulation of genes implied in the modification of cell wall composition (Farrokhi et al., 2006), or the use of enzymes and other proteins able to act on cell wall components (Levy et al., 2002). A promising alternative to these approaches consists of the use of CBIs. In fact, the habituation of cell cultures to diverse CBIs, as it has been mentioned, results in the modification of cell wall composition and structure with quantitative and qualitative changes in their components: CBI-habituated cell walls often have a reduced content in cellulose, compensated by an increment in other polysaccharides, mainly pectins. These cell walls have been demonstrated to have new physicochemical properties such as modifications in pore size, swelling capacity and resistance to polysaccharide hydrolytic enzymes (Shedletzky et al., 1992; Mélida et al., 2009). These materials should be also interesting as a suitable source for studying the relationship between cellulose synthesis and other C-sink processes such as phenylpropanoid synthesis (Mélida et al., 2009), or the elucidation of putative new targets implied in cellulose biosynthesis.

As it has been repeatedly noted in this chapter, CBIs have contributed to the clarification of the mechanism of cellulose biosynthesis and the organization of CESA complexes. In addition to these contributions, in recent years CBIs have been revealed as excellent tools for digging more deeply into the basic processes of plant cell biology, such as the relationship between cellulose biosynthesis and cytoskeleton (Himmelspach et al., 2003; DeBolt, 2007b; Paredez et al., 2008; Wightman et al., 2009), the mechanism of cell wall sensing (Hamman et al., 2009), cell-wall based mechanisms of defence (Caño-Delgado et al., 2003; Bischoff et al., 2009), and the link between cell wall disruption and programmed cell death (Duval, 2005; Bischoff et al., 2009).

CONCLUSION

CBIs are important compounds not only as commercialized herbicides (or molecules with herbicide potential) but also as key tools to unravel the cellulose biosynthesis mechanism. In recent years, a considerable amount of information regarding CBIs has been acquired, and it has been directed as a tool towards new research targets. In the future, it is probable that new CBIs will be discovered, and new research on old and new compounds will open new paths toward the comprehension of cell wall dynamics. However, a long road must be covered to find the mechanism of action of every member of this group of molecules. Surely, new advances in CBIs' characterization will render a better understanding of molecular relations between cellulose and other polysaccharides, such as their interactions, synthesis and potential modifications, and this knowledge would be oriented toward a better understanding of the plasticity in the structure and composition of cell walls.

References

Alonso-Simón, A; Encina, AE; García-Angulo, P; Álvarez, JM; Acebes, JL. FTIR spectroscopy monitoring of cell wall modifications during the habituation of bean (*Phaseolus vulgaris* L.) callus cultures to dichlobenil. *Plant Sci,* 2004, 167, 1273-1281.

Alonso-Simón, A; García-Angulo, P; Encina, A; Acebes, JL; Álvarez, J. Habituation of bean (*Phaseolus vulgaris*) cell cultures to quinclorac and analysis of the subsequent cell wall modifications. *Ann Bot,* 2008, 101, 1329-1339.

Alonso-Simón, A; García-Angulo, P; Encina, A; Álvarez, JM; Acebes, JL; Hayashi, T. Increase in XET activity in bean (*Phaseolus vulgaris* L.) cells habituated to dichlobenil. *Planta*, 2007, 226, 765-771.

Amor, Y; Haigler, CH; Johnson, S; Wainscott, M; Delmer, DP. A membrane-associated form of sucrose synthase and its potential role in synthesis of cellulose and callose in plants. *Proc Natl Acad Sci USA,* 1995, 92, 9353-9357.

Anderson, JR; Barnes, WS; Bedinger, P. 2,6-Dichlorobenzonitrile, a cellulose biosynthesis inhibitor, affects morphology and structural integrity of petunia and lily pollen tubes. *J Plant Physiol,* 2002, 159, 61-67.

Arad, SM; Kolani, R; Simonberkovitch, B; Sivan, A. Inhibition by DCB of cell wall polysaccharide formation in the red microalga *Porphyridium sp.* (Rhodophyta). *Phycologia,* 1994, 33, 158-162.

Arioli, T; Peng, L; Betzner, AS; Burn, J; Wittke, W; Herth, W; Camilleri, C; Plazinski, J; Birch, R; Cork, A; Glover, J; Redmon, J; Williamson, RE. Molecular analysis of cellulose biosynthesis in *Arabidopsis*. *Science,* 1998, 279, 717-719.

Atanassov, II; Pittman, JK; Turner, SR. Elucidating the mechanisms of assembly and subunit interaction of the cellulose synthase complex of *Arabidopsis* secondary cell walls. *J Biol Chem,* 2009, 284, 3833-3841.

Baskin, TI. On the alignment of cellulose microfibrils by cortical microtubules: a review and a model. *Protoplasma*, 2001, 215, 150-171.

Baskin, TI; Betzner, AS; Hoggart, R; Cork, A; Williamson, RE. Root morphology mutants in *Arabidopsis thaliana*. *Aust J Plant Physiol,* 1992, 19, 427-437.

Beeckman, T; Przemeck, GKH; Stamatiou, G; Lau, R; Terryn, N; De Rycke, R; Inzé, D; Berleth, T. Genetic complexity of cellulose synthase A gene function in Arabidopsis embryogenesis. *Plant Physiol*, 2002, 130, 1883-1893.

Bernal, AJ; Jensen, JK; Harholt, J; Sorensen, S; Moller, I; Blaukopf, C; Johansen, B; Lotto, R; Pauly, M; Scheller, HV; Willats, WGT. Disruption of *ATCSLD5* results in reduced growth, reduced xylan and homogalacturonan synthase activity and altered xylan occurrence in Arabidopsis. *Plant J,* 2007, 52, 1-12.

Bessueille, L; Bulone, V. A survey of cellulose biosynthesis in higher plants. *Plant Biotechnol,* 2008, 25, 315-322.

Beynon, KI. & Wright, AN. Persistence, penetration, and breakdown of chlorthiamid and dichlobenil herbicides in field soils of different types. *J Sci Food Agric,* 1968, 19, 718-722.

Bischoff, V; Cookson, SJ; Wu, S; Scheible, W. Thaxtomin A affects CESA-complex density, expression of cell wall genes, cell wall composition, and causes ectopic lignification in *Arabidopsis thaliana* seedlings. *J Exp Bot,* 2009, 60, 955-965.

Blaschek, W; Semler, U; Franz, G. The influence of potential inhibitors on the invivo and invitro cell-wall beta-glucan biosynthesis in tobacco cells. *J Plant Physiol,* 1985, 120, 457-470.

Brummell, DA. & Hall, JL. The role of cell wall synthesis in sustained auxin-induced growth. *Physiol Plant,* 1985, 63, 406-412.

Burn, JE; Hurley, UA; Birch, RJ; Arioli, T; Cork, A; Williamson, RE. The cellulose-deficient *Arabidopsis* mutant *rsw3* is defective in a gene encoding a putative glucosidase II, an enzyme processing N-glycans during ER quality control. *Plant J*, 2002, 32, 949-960.

Burton, RA; Gibeaut, DM; Bacic, A; Findlay, K; Roberts, K; Hamilton, A; Baulcombe, DC; Fincher, GB. Virus-induced silencing of a plant cellulose synthase gene. *Plant Cell,* 2000, 12, 691-705.

Caño-Delgado, A; Metzlaff, K; Bevan, MW. The *eli1* mutation reveals a link between cell expansion and secondary cell wall formation in *Arabidopsis thaliana*. *Development,* 2000, 127, 3395-3405.

Caño-Delgado, A; Penfield, S; Smith, C; Catley, M; Bevan, M. Reduced cellulose synthesis invokes lignification and defense responses in *Arabidopsis thaliana*. *Plant J,* 2003, 34, 351-362.

Carpita, NC. Structure and biogenesis of the cell walls of grasses. *Plant Physiol,* 1996 47, 445-476.

Carpita, NC. & Gilbeaut, DM. Structural models of primary cell walls in flowering plants: consistency of molecular structure with the physical properties of the cell walls during growth. *Plant J,* 1993, 3, 1-30.

Chandler, PM. & Robertson, M. Gibberellin dose-response curves and the characterization of dwarf mutants of barley. *Plant Physiol,* 1999, 120, 623-632.

Chen, L; Carpita, NC; Reiter, WD; Wilson, RH; Jeffries, C; McCann, MC. A rapid method to screen for cell-wall mutants using discriminant analysis of Fourier transform infrared spectra. *Plant J,* 1998, 16, 385-392.

Collings, DA; Gebbie, LK; Howles, PA; Hurley, UA; Birch, RJ; Cork, AH; Hocart, CH; Arioli, T; Williamson, RE. Arabidopsis dynamin-like protein DRP1A: a null mutant with widespread defects in endocytosis, cellulose synthesis, cytokinesis, and cell expansion. *J Exp Bot,* 2008, 59, 361-376.

Corio-Costet., MF; Dall'Agnese, M; Scalla, R (a). Effects of isoxaben on sensitive and tolerant plant cell cultures. I. Metabolic fate of Isoxaben. *Pestic Biochem Physiol,* 1991, 40, 246-254.

Corio-Costet., MF; Dall'Agnese, M; Scalla, R (b). Effects of isoxaben on sensitive and tolerant plant cell cultures. II. Cellular alterations and inhibition of the synthesis of acid insoluble cell wall material. *Pestic Biochem Physiol,* 1991, 40, 255-265.

Crowell, EF; Bischoff, V; Desprez, T; Rolland, A; Stierhof, Y; Schumacher, K; Gonneau, M; Höfte, H; Vernhettes, S. Pausing of golgi bodies on microtubules regulates secretion of cellulose synthase complexes in *Arabidopsis*. *Plant Cell,* 2009, 21, 1141-1154.

DeBolt, S; Gutiérrez, R; Ehrhardt, DW; Melo, CV; Ross, L; Cutler, SR; Somerville, C; Bonetta, D (a). Morlin, an inhibitor of cortical microtubule dynamics and cellulose synthase movement. *Proc Natl Acad Sci USA,* 2007, 104, 5854-5859.

DeBolt, S; Gutiérrez, R; Ehrhardt, DW; Somerville, C (b). Nonmotile cellulose synthase subunits repeatedly accumulate within localized regions at the plasma membrane in Arabidopsis hypocotyl cells following 2,6-dichlorobenzonitrile treatment. *Plant Physiol,* 2007, 145, 334-338.

Delmer, DP. Cellulose biosynthesis. *Annu Rev Plant Physiol,* 1987, 38, 259-290.

Delmer, DP; Read, SM; Cooper, G. Identification of a receptor protein in cotton fibers for the herbicide 2,6-dichlorobenzonitrile. *Plant Physiol,* 1987, 84, 415-420.

Desnos, T; Orbovic, V; Bellini, C; Kronenberger, J; Caboche, M; Traas, J; Höfte, H. *Procuste1* mutants identify two distinct genetic pathways controlling hypocotyl cell elongation, respectively in dark- and light-grown *Arabidopsis* seedlings. *Development,* 1996, 122, 683-693.

Desprez, T; Juraniec, M; Crowell, EF; Jouy, H; Pochylova, Z; Parcy, F; Höfte, H; Gonneau, M; Vernhettes, S. Organization of cellulose synthase complexes involved in primary cell wall synthesis in *Arabidopsis thaliana*. *Proc Natl Acad Sci USA,* 2007, 104, 15572-15577.

Desprez, T; Vernhettes, S; Fagard, M; Refrégier, G; Desnos, T; Aletti, E; Py, N; Pelletier, S; Höfte, H. Resistance against herbicide isoxaben and cellulose deficiency caused by distinct mutations in same cellulose synthase isoform CESA6. *Plant Physiol,* 2002, 128, 482-490.

Díaz-Cacho, P; Moral, R; Encina, A; Acebes, JL; Álvarez, J. Cell wall modifications in bean (*Phaseolus vulgaris*) callus cultures tolerant to isoxaben. *Physiol Plant,* 1999, 107, 54-59.

Doblin, MS; Kurek, I; Jacob-Wilk, D; Delmer, DP. Cellulose biosynthesis in plants: from genes to rosettes. *Plant Cell Physiol,* 2002, 43, 1407-1420.

Durso, NA. & Vaughn, KC. The herbicidal manipulation of callose levels in cell plates radically affects cell plate structure. *Plant Physiol,* 1997, 114, 87(c 351).

Duval, I. & Beaudoin, N. Transcriptional profiling in response to inhibition of cellulose synthesis by thaxtomin A and isoxaben in *Arabidopsis thaliana* suspension cells. *Plant Cell Rep,* 2009, 28, 811-830.

Duval, I; Brochu, V; Simard, M; Beaulieu, C; Beaudoin, N. Thaxtomin A induces programmed cell death in *Arabidopsis thaliana* suspension-cultured cells. *Planta,* 2005, 222, 820-831.

Edelmann, HG. & Fry, SC. Kinetics of integration of xyloglucan into the walls of suspension-cultured rose cells. *J Exp Bot,* 1992, 43, 463-470.

Eisinger, W; Croner, LJ; Taiz, L. Ethylene- induced lateral expansion in etiolated pea stems. Kinetics, cell wall synthesis, and osmotic potential. *Plant Physiol,* 1983 73, 407-412.

Ellis, C; Karafyllidis, I; Wasternack, C; Turner, JG. The Arabidopsis mutant *cev1* links cell wall signaling to jasmonate and ethylene responses. *Plant Cell*, 2002, 14, 1557–1566.

Encina, A; Moral, RM; Acebes, JL; Álvarez, JM. Characterization of cell walls in bean (*Phaseolus vulgaris* L.) callus cultures tolerant to dichlobenil. *Plant Sci,* 2001, 160, 331-339.

Encina, A; Sevillano, JM; Acebes, JL; Álvarez, J. Cell wall modifications of bean (*Phaseolus vulgaris*) cell suspensions during habituation and dehabituation to dichlobenil. *Physiol Plant,* 2002, 114, 182-191.

Fagard, M; Höfte, H; Vernhettes, S. Cell wall mutants. *Plant Physiol Biochem,* 2000, 38, 15-25.

Farrokhi, N; Burton, R; Brownfield, L; Hrmova, M; Wilson, SM; Bacic, A; Fincher, GB. Plant cell wall biosynthesis: genetic, biochemical and functional genomic approaches to the identification of key genes. *Plant Biotech J*, 2006, 4, 145-167.

Francey, Y; Jaquet, JP; Cairoli, S; Buchala, AJ; Meier, H. The biosynthesis of β-glucans in cotton (*Gossypium hirsutum* L.) fibres of ovules cultured in vitro. *J Plant Physiol,* 1989, 134, 485-491.

Fry, BA. & Loria, R. Thaxtomin A: evidence for a plant cell wall target. *Physiol Mol Plant Pathol,* 2002, 60, 1-8.

Galbraith, DW. & Shields, BA. The effects of inhibitors of cell wall synthesis on tobacco protoplast development. *Physiol Plant,* 1982, 55, 25-30.

García-Angulo, P; Alonso-Simón, A; Mélida, H; Encina, A; Acebes, JL; Álvarez, JM. High peroxidase activity and stable changes in the cell wall are related to dichlobenil tolerance. *J Plant Physiol,* 2009, 166, 1229-1240.

García-Angulo, P; Willats, WGT; Encina, AE; Alonso-Simón, A; Álvarez, JM; Acebes, JL. Immunocytochemical characterization of the cell walls of bean cell suspensions during habituation and dehabituation to dichlobenil. *Physiol Plant,* 2006, 127, 87-99.

Gillmor, CS; Poindexter, P; Lorieau, J; Palcic, MM; Somerville, C. α-Glucosidase I is required for cellulose biosynthesis and morphogenesis in *Arabidopsis. J Cell Biol*, 2002, 156, 1003-1013.

Girard-Martel, M; Brochu, V; Duval, I; Beaudoin, N. Characterization and transcription profiles of poplar cells habituated to *Streptomyces scabiei* phytotoxin thaxtomin A. 6th Canadian Plant Genomics Workshop, Toronto 2008, 74.

Gopalan, S. Reversal of an immunity associated plant cell death program by the growth regulator auxin. *BMC Res Notes,* 2008, 1, 126.

Grossmann, K. A role for cyanide, derived from ethylene biosynthesis, in the development of stress symptoms. *Physiol Plant,* 1996, 97, 772-775.

Grossmann, K. Mode of action of auxin herbicides: a new ending to a long, drawn out story. *Trends Plant Sci,* 2000, 5, 506-508.

Grossmann, K. & Kwiatkowski, J. Evidence for a causative role of cyanide, derived from ethylene biosynthesis, in the herbicidal mode of action of quinclorac in barnyard grass. *Pestic Biochem Physiol,* 1995, 51, 150-160.

Grossmann, K. & Kwiatkowski, J. The mechanism of quinclorac selectivity in grasses. *Pestic Biochem Physiol,* 2000, 66, 83-91.

Grossmann, K; Kwiatkowski, J; Tresch, S (a). Auxin herbicides induce H_2O_2 overproduction and tissue damage in cleavers (*Galium aparine* L.). *J Exp Bot,* 2001, 52, 1811-1816.

Grossmann, K. & Scheltrup, F. Selective induction of 1-aminocyclopropane-1-carboxylic acid (ACC) synthase activity is involved in the selectivity of the auxin herbicide quinclorac between barnyard grass and rice. *Pestic Biochem Physiol,* 1997, 58, 145-153.

Grossmann, K; Tresch, S; Plath, P (b). Triaziflam and diaminotriazine derivatives affect enantioselectively multiple herbicide target sites. *Z Naturforsch [C],* 2001, 56, 559-569.

Günther, P. & Pestemer, W. Risk assessment for selected xenobiotics by bioassay methods with higher plants. *Environ Manag,* 1990, 14, 381-388.

Hamman, T; Bennett, M; Mansfield, J; Somerville, C. Identification of cell-wall stress as a hexose-dependent and osmosensitive regulator of plant responses. *Plant J*, 2009, 57, 1015-1026.

Hauser, MT; Morikami, A; Benfey, PN. Conditional root expansion mutants of *Arabidopsis*. *Development* 121, 1995, 1237-1252.

Heath, IB. Unified hypothesis for role of membrane-bound enzyme complexes and microtubules in plant cell wall synthesis. *J Theor Biol,* 1974, 48, 445-449.

Heim, DR; Larrinua, IM; Murdoch, MG; Roberts, JL. Triazofenamide is a cellulose biosynthesis inhibitor. *Pestic Biochem Physiol,* 1998, 59, 163-168.

Heim, DR; Roberts, JL; Phillip, DR; Larrinua, IM. A second locus, *Ixr* B1 in *Arabidopsis thaliana* that confers resistance to the herbicide Isoxaben. *Plant Physiol,* 1990, 92, 695-700.

Heim, DR; Roberts, JL; Pike, PD; Larrinua, IM. Mutation of a locus of *Arabidopsis thaliana* confers resistance to the herbicide Isoxaben. *Plant Physiol,* 1989, 90, 146-150.

Heim, RD; Skomp, JR; Waldron, C; Larrinua, IM. Differential response to isoxaben of cellulose biosynthesis by wild-type and resistant strains of *Arabidopsis thaliana*. *Pestic Biochem Physiol,* 1991, 39, 93-99.

Herth, W. Effects of 2,6-DCB on plasma-membrane rosettes of wheat root cells. *Naturweiss,* 1987, 74, 556-557.

Himmelspach, R; Williamson, RE; Wasteneys, GO. Cellulose microfibril alignment recovers from DCB-induced disruption despite microtubule disorganization. *Plant J,* 2003, 36, 565-575.

Hoffman, JC. & Vaughn, KC. Flupoxam induces classic club root morphology but is not a mitotic disrupter herbicide. *Pestic Biochem Physiol,* 1996, 55, 49-53.

Hofmannová, J; Schwarzerová, K; Havelková, L; Boriková, P; Petrásek, J; Opatrny, Z. A novel, cellulose synthesis inhibitory action of ancymidol impairs plant cell expansion. *J Exp Bot,* 2008, 59, 3963-3974.

Hogetsu, T; Shibaoka, H; Shimokoriyama, M. Involvement of cellulose biosynthesis in actions of gibberellin and kinetin on cell expansion, 2,6-dichlorobenzonitrile as a new cellulose-synthesis inhibitor. *Plant Cell Physiol,* 1974, 15, 389-393.

Hoson, T. & Masuda, Y. Role of polysaccharide synthesis in elongation growth and cell wall loosening in intact rice coleoptiles. *Planta,* 1991 155, 467-472.

Huggenberger, F; Jennings, EA; Ryan, PJ; Burow, KW. EL-107 a new selective herbicide for use in cereals. *Weeds,* 1982, 1, 47-52.

Iraki, NM; Bressan, RA; Hasegawa, PM; Carpita, NC. Alteration of the physical and chemical structure of the primary cell wall of growth-limited plant cells adapted to osmotic stress. *Plant Physiol,* 1989, 91, 39-47.

Joshi, CP. & Mansfield, SD. The cellulose paradox -- simple molecule, complex biosynthesis. *Curr Opin Plant Biol,* 2007, 10, 220-226.

Kiedaisch, BM; Blanton, RL; Haigler, CH. Characterization of a novel cellulose synthesis inhibitor. *Planta,* 2003, 217, 922-930.

King, RR. & Lawrence, CH. Herbicidal properties of the Thaxtomin group of phytotoxins. *J Agric Food Chem,* 2001, 49, 2298-2301.

King, RR; Lawrence, CH; Calhoun, LA. Chemistry of phytotoxins associated with *Streptomyces scabies*, the causal organism of potato common scab. *J Agric Food Chem,* 1992, 40, 834-837.

Koo, SJ; Kwon, YW; Cho, KY. Differences in selectivity and physiological effects of quinclorac between rice and barnyardgrass compared with 2,4-D. *Proc 13th Asian-P WSSC,* 1991, 103-111.

Koo, SJ; Neal, JC; DiTomaso, JM. 3,7-dichloroquinolinecarboxylic acid inhibits cell-wall biosynthesis in maize roots. *Plant Physiol,* 1996, 112, 1383-1389.

Koo, SJ; Neal, JC; DiTomaso, JM. Mechanism of action and selectivity of quinclorac in grass roots. *Pestic Biochem Physiol,* 1997, 57, 44-53.

Kurek, I; Kawagoe, Y; Jacob-Wilk, D; Doblin, M; Delmer, D. Dimerization of cotton fiber cellulose synthase catalytic subunits occurs via oxidation of the zinc-binding domains. *Proc Natl Acad Sci USA,* 2002, 99, 11109-11114.

Lane, D; Wiedemeier, A; Peng, L; Höfte, H; Vernhettes, S; Desprez, T; Hocart, CH; Birch, RJ; Baskin, TI; Burn, JE; Arioli, T; Williamson, RE. Temperature-sensitive alleles of *RSW2* link the KORRIGAN endo-1,4-β-glucanase to cellulose synthesis and cytokinesis in Arabidopsis. *Plant Physiol,* 2001, 126, 278-288.

Lefebvre, A; Maizonnier, D; Gaudry, JC; Clair, D; Scalla, R. Some effects of the herbicide EL-107 on cellular growth and metabolism. *Weed Res,* 1987, 27, 125-134.

Levy, I; Shani, Z; Shoseyor, O. Modification of polysaccharides and plant cell wall by endo-1,4-β-glucanase and cellulose-binding domains. *Biomol Engineer*, 2002, 19, 17-30.

Lewis, D; Bacic, A; Chandler, PM; Newbigin, EJ. Aberrant cell expansion in the elongation mutants of barley. *Plant Cell Physiol,* 2009, 50, 554-571.

Lukowitz, W; Nickle, TC; Meinke, DW; Last, RL; Conklin, PL; Somerville, CR. *Arabidopsis cyt1* mutants are deficient in a mannose-1-phosphate guanylyltransferase and point to a requirement of N-linked glycosylation for cellulose biosynthesis. *Proc Natl Acad Sci USA,* 2001, 98, 2262-2267.

Madari, H; Panda, D; Wilson, L; Jacobs, RS. Dicoumarol: a unique microtubule stabilizing natural product that is synergistic with taxol. *Cancer Res,* 2003, 63, 1214-1220.

Manfield, LW; Orfila, C; McCartney, L; Harholt, J; Bernal, AJ; Scheller, HV; Gilmartin, PM; Mikkelsen, JD; Knox, JP; Willats, WGT. Novel cell wall architecture of isoxaben-habituated *Arabidopsis* suspension-cultured cells: global transcript profiling and cellular analysis. *Plant J,* 2004, 40, 260-275.

McIntosh, AH; Chamberlain, K; Dawson, GW. Foliar sprays against potato common scab-compounds related to 3,5-dichlorophenoxyacetic acid. *Crop Prot,* 1985, 4, 473-480.

Mélida, H; García-Angulo, P; Alonso-Simón, A; Encina, A; Álvarez, J; Acebes, JL. Novel type II cell wall architecture in dichlobenil-habituated maize calluses. *Planta,* 2009, 229, 617-631.

Menne, H. & Köcher, H. HRAC classification of herbicides and resistance development. In: Krämer W, Shirmer U, editors. *Modern crop protection compounds.* Wiley-Vch; 2007, 5-27.

Meyer, Y. & Herth, W. Chemical inhibition of cell wall formation and cytokinesis, but not of nuclear division, in protoplasts of *Nicotiana tabacum* L. cultivated in vitro. *Planta,* 1978, 142, 253-262.

Mizuta, S. & Brown, RM. Effects of 2,6-dichlorobenzonitrile and Tinopal LPW on the structure of the cellulose synthesizing complexes of *Vaucheria hamata*. *Protoplasma,* 1992, 166, 200-207.

Molhøj, M; Pagant, S; Höfte, H. Towards understanding the role of membrane-bound endo-β-1,4-glucanases in cellulose biosynthesis. *Plant Cell Physiol*, 2002, 43, 1399-1406.

Montague, MJ. Gibberellic acid promotes growth and cell wall synthesis in Avena internodes regardless of the orientation of cell expansion. *Physiol Plant,* 1995, 94, 7-18.

Montezinos, D. & Delmer, DP. Characterization of inhibitors of cellulose synthesis in cotton fibers. *Planta,* 1980, 148, 305-311.

Moreland, DE; Hussey, GG; Farmer, FS; Shmuel, M; Trainin, T; Kalman, S; Delmer, D. Comparative effects of dichlobenil and its phenolic alteration products on photophosphorylation and oxidative phosphorylation. *Pestic Biochem Physiol,* 1974, 4, 356-364.

Mouille, G; Robin, S; Lecomte, M; Pagant, S; Höfte, H. Classification and identification of *Arabidopsis* cell wall mutants using Fourier-Transform Infrared (FT-IR) microspectroscopy. *Plant J,* 2003, 35, 393-404.

Mutwil, M; DeBolt, S; Persson, S. Cellulose synthesis: a complex complex. *Curr Opin Plant Biol,* 2008, 11, 252-257.

Nakagawa, N. & Sakurai, N. Increase in the amount of celA1 protein in tobacco BY-2 cells by a cellulose biosynthesis inhibitor, 2,6-dichlorobenzonitrile. *Plant Cell Physiol,* 1998, 39, 779-785.

Nakagawa, N. & Sakurai, N. Cell wall integrity controls expression of endoxyloglucan transferase in tobacco BY2 cells. *Plant Cell Physiol*, 2001, 42, 240-244.

Nakagawa, N. & Sakurai, N. A mutation in At-nMat1a, which encodes a nuclear gene having high similarity to group II intron maturase, causes impaired splicing of mitochondrial NAD4 transcript and altered carbon metabolism in *Arabidopsis thaliana*. *Plant Cell Physiol,* 2006, 47, 772-783.

Nicol, F; His, I; Jauneau, A; Vernhettes, S; Canut, H; Höfte, H. A plasma membrane-bound putative endo-1,4-β-D-glucanase is required for normal wall assembly and cell elongation in *Arabidopsis*. *EMBO J,* 1998, 17, 5563-5576.

Nishimura, MT; Stein M; Hou, BH; Vogel, JP; Edwards, H; Somerville, SC. Loss of a callose synthase results in salicylic acid-dependent disease resistance. *Science*, 2003, 301, 969-972.

Obembe, OO; Jacobsen, E; Visser, RGF, Vincken, JP. Cellulose-hemicellulose networks as target for *in planta* modification of the properties of natural fibres. *Biotechnol Mol Biol Rev*, 2006, 1, 76-86.

O'Keeffe, MG. & Klevorn, TB. Flupoxam: a new preemergence and postemergence herbicide for broad-leaved weed-control in winter cereals. *Brighton Crop Prot Conf Weeds*, 1991, 63-68.

O'Looney, N. & Fry, SC (a). The novel herbicide oxaziclomefone inhibits cell expansion in maize cell cultures without affecting turgor pressure or wall acidification. *New Phytol,* 2005, 168, 1-7.

O'Looney, N. & Fry, SC (b). Oxaziclomefone, a new herbicide, inhibits wall expansion in maize cell-cultures without affecting polysaccharide biosynthesis, xyloglucan transglycosylation, peroxidase action or apoplastic ascorbate oxidation. *Ann Bot,* 2005, 96, 1-11.

Pagant, S; Bichet, A; Sugimoto, K; Lerouxel, O; Desprez, T; McCann, M; Lerouge, P; Vernhettes, S; Hofte, H. *KOBITO1* encodes a novel plasma membrane protein necessary for normal synthesis of cellulose during cell expansion in *Arabidopsis*. *Plant Cell,* 2002, 14, 2001-2013.

Paredez, AR; Persson, S; Ehrhardt, DW; Somerville, CR. Genetic evidence that cellulose synthase activity influences microtubule cortical array organization. *Plant Physiol,* 2008, 147, 1723-1734.

Paredez, AR; Somerville, CR; Ehrhardt, DW. Visualization of cellulose synthase demonstrates functional association with microtubules. *Science,* 2006 312, 1491-1495.

Pauly, M. & Keegstra, K. Cell-wall carbohydrates and their modification as a resource for biofuels. *Plant J*, 2008, 54, 569-568.

Peng, L; Kawagoe, Y; Hoan, P; Delmer, D. Sitosterol-β-glucosidase as primer for cellulose synthesis in plants. *Science,* 2002, 295, 147-150.

Peng, L; Xiang, F; Roberts, E; Kawagoe, Y; Greve, LC; Kreuz, K; Delmer, DP. The experimental herbicide CGA 325´615 inhibits synthesis of crystalline cellulose and causes accumulation of non-crystalline normal β-1,4-glucan associated with CesA protein. *Plant Physiol,* 2001, 126, 981-992.

Persson, S; Paredez, A; Carroll, A; Palsdottir, H; Doblin, M; Poindexter, P; Khitrov, N; Auer, M; Somerville, CR. Genetic evidence for three unique components in primary cell-wall cellulose synthase complexes in *Arabidopsis*. *Proc Natl Acad Sci USA,* 2007, 104, 15566-15571.

Potikha, T. & Delmer, DP. A mutant of *Arabidopsis thaliana* displaying altered patterns of cellulose deposition. *Plant J,* 1995, 7, 453-460.

Potters, G; Pasternak, TP; Guisez, Y; Palme, KJ; Jansen, MAK. Stress-induced morphogenic responses: growing out of trouble? *Trends Plant Sci,* 2007, 12, 98-105.

Rajangam, AS; Kumar, M; Aspeborg, H; Guerriero, G; Arvestad, L; Pansri, P; Brown, CJL; Hober, S; Blomqvist, K; Divne, C; Ezcurra, I; Mellerowicz, E; Sundberg, B; Bulone, V; Teeri, TT. MAP20, a microtubule-associated protein in the secondary cell walls of hybrid aspen, is a target of the cellulose synthesis inhibitor 2,6-dichlorobenzonitrile. *Plant Physiol,* 2008, 148, 1283-1294.

Reiter, W-; Chapple, C; Somerville, CR. Mutants of *Arabidopsis thaliana* with altered cell wall polysaccharide composition. *Plant J,* 1997, 12, 335-345.

Robert, S; Mouille, G; Höfte, H. The mechanism and regulation of cellulose synthesis in primary walls: lessons from cellulose-deficient Arabidopsis mutants. *Cellulose,* 2004, 11, 351-364.

Robinson, DG. & Quader, H. Structure, synthesis, and orientation of microfibrils IX: a freeze-fracture investigation of the *Oocystis* plasma membrane after inhibitor treatment. *Eur J Cell Biol,* 198 125, 278-288.

Sabba, RP; Durso, NA; Vaughn, KC. Structural and immunocytochemical characterization of the walls of dichlobenil-habituated BY-2 tobacco cells. *Int J Plant Sci,* 1999, 160, 275-290.

Sabba, RP. & Vaughn, KC. Herbicides that inhibit cellulose biosynthesis. *Weed Sci,* 1999, 47, 757-763.

Samuels, AL; Giddings, TH; Staehelin, LA. Cytokinesis in tobacco BY-2 and root tip cells: a new model of cell plate formation in higher plants. *J Cell Biol,* 1995, 130, 1345-1357.

Scheible, W-; Eshed, R; Richmond, T; Delmer, D; Somerville, C. Modifications of cellulose synthase confer resistance to isoxaben and thiazolidinone herbicides in *Arabidopsis Ixr1* mutants. *Proc Natl Acad Sci USA,* 2001, 98, 10079-10084.

Scheible, W-; Fry, B; Kochevenko, A; Schindelasch, D; Zimmerli, L; Somerville, S; Loria, R; Somerville, CR. An Arabidopsis mutant resistant to thaxtomin A, a cellulose synthesis inhibitor from *Streptomyces* species. *Plant Cell,* 2003, 15, 1781-1794.

Scheneegurt, MA; Heim, DR; Larrinua, IM. Investigation into the mechanism of Isoxaben tolerance in dicot weeds. *Weed Sci,* 1994, 42, 163-167.

Schindelman, G; Morikami, A; Jung, J; Baskin, TI; Carpita, NC; Derbyshire, P; McCann, MC; Benfey, PN. COBRA encodes a putative GPI-anchored protein, which is polarly localized and necessary for oriented cell expansion in *Arabidopsis*. *Genes Develop,* 2001, 15, 1115-1127.

Sharples, K; Hawkes, TR; Mitchell, G; Edwards, LS; Langford, MP; Langton, DW; Rogers, KM; Townson, JK; Wang, Y. A novel thiazolidinone herbicide is a potent inhibitor of glucose incorporation into cell wall material. *Pestic Sci,* 1998, 54, 368-376.

Shedletzky, E; Shmuel, M; Delmer, DP; Lamport, DTA. Adaptation and growth of tomato cells on the herbicide 2,6-Dichlorobenzonitrile leads to production of unique cell-walls virtually lacking a cellulose-xyloglucan network. *Plant Physiol,* 1990, 94, 980-987.

Shedletzky, E; Shmuel, M; Trainin, T; Kalman, S; Delmer, D. Cell-wall structure in cells adapted to growth on the cellulose-synthesis inhibitor 2,6-Dichlorobenzonitrile - A comparison between two dicotyledonous plants and a graminaceous monocot. *Plant Physiol,* 1992, 100, 120-130.

Shive, JB. & Sisler, HD. Effects of Ancymidol (a growth retardant) and Triarimol (a fungicide) on growth, sterols, and gibberellins of *Phaseolus vulgaris* L. *Plant Physiol,* 1976, 57, 640-644.

Somerville, C. Cellulose synthesis in higher plants. *Annu Rev Cell Dev Biol,* 2006 22, 53-78.

Sugimoto, K; Williamson, R; Wasteneys, GO. Wall architecture in the cellulose-deficient *rsw1* mutant of *Arabidopsis thaliana*: microfibrils but not microtubules lose their transverse alignment before microfibrils become unrecognizable in the mitotic and elongation zones of roots. *Protoplasma,* 2001, 215, 172-183.

Sunohara, Y. & Matsumoto, H. Oxidative injury induced by the herbicide quinclorac on *Echinochloa oryzicola* Vasing. and the involvement of antioxidative ability in its highly selective action in grass species. *Plant Sci*, 2004, 167, 597-606.

Sunohara, Y. & Matsumoto, H. Quinclorac-induced cell death is accompanied by generation of reactive oxygen species in maize root tissue. *Phytochem,* 2008, 69, 2312-2319.

Takahashi, J; Rudsander, UJ; Hedenström, M; Banasiak, A; Harholt, J; Amelot, N; Immerzeel P; Ryden, P; Endo, S; Ibatullin, FM; Brumer, H; del Campillo, E; Master, ER; Scheller, HV; Sundberg, B; Teeri, TT; Mellerowicz, EJ. *KORRIGAN1* and its aspen homologue *PttCel9A1* decrease cellulose crystallinity in Arabidopsis stems. *Plant Cell Physiol,* 2009, 50, 1099-1115.

Tanimoto, E. Gibberellin-dependent root elongation in *Lactuca sativa:* Recovery from growth retardant-suppressed elongation with thickening by low concentration of GA_3. *Plant Cell Physiol,* 1987, 28, 963-973.

Tanimoto, E. Interaction of gibberellin A_3 and ancymidol in the growth and cell-wall extensibility of dwarf pea roots. *Plant Cell Physiol,* 1994, 35, 1019-1028.

Tanimoto, E. & Huber, DJ. Effect of GA_3 on the molecular mass of polyuronides in the cell walls of Alaska pea roots. *Plant Cell Physiol,* 1997, 38, 25-35.

Taylor, NG. Cellulose biosynthesis and deposition in higher plants *New Phytol,* 2008, 178, 239-252.

Taylor, NG; Howells, RM; Huttly, AK; Vickers, K; Turner, SR. Interactions among three distinct CesA proteins essential for cellulose synthesis. *Proc Natl Acad Sci USA*, 2003, 100, 1450-1455.

Taylor, NG; Laurie, S; Turner, SR. Multiple cellulose synthase catalytic subunits are required for cellulose synthesis in Arabidopsis. *Plant Cell*, 2000, 12, 2529-2540.

Taylor, NG; Scheible, WR; Cutler, S; Somerville, CR; Turner, SR. The *irregular xylem3* locus of Arabidopsis encodes a cellulose synthase required for secondary cell wall synthesis. *Plant Cell*, 1999, 11, 769-780.

Tegg, RS; Gill, WM; Thompson, HK; Davies, NW; Ross, JJ; Wilson, CR. Auxin-induced resistance to common scab disease of potato linked to inhibition of thaxtomin A toxicity. *Plant Dis,* 2008, 92, 1321-1328.

Theologis, A. Possible linkage between auxin regulated gene expression, H^+ secretion, and cell elongation: a hypothesis. In Cosgrove DJ, Knievel DP editors. Physiology of Cell Expansion during Plant Growth. American Society of Plant Physiologists, Rockville, MD, 1987, 133-144.

Timmers, J; Vernhettes, S; Desprez, T; Vincken, J; Visser, RGF; Trindade, LM. Interactions between membrane-bound cellulose synthases involved in the synthesis of the secondary cell wall. *FEBS Lett,* 2009, 583, 978-982.

Tresch, S. & Grossmann, K. Quinclorac does not inhibit cellulose (cell wall) biosynthesis in sensitive barnyard grass and maize roots. *Pestic Biochem Physiol,* 2003, 75, 73-78.

Turner, SR. & Somerville, CR. Collapsed xylem phenotype of Arabidopsis identifies mutants deficient in cellulose deposition in the secondary cell wall. *Plant Cell,* 1997, 9, 689-701.

Umetsu, N; Satoh, S; Matsuda, K. Effects of 2,6-dichlorobenzonitrile on suspension-cultured soybean cells. *Plant Cell Physiol,* 1976, 17, 1071-1073.

Vaughn, KC. Cellulose biosynthesis inhibitor herbicides. In: Böger P, Wakabayashi K, Hirai K, editors. *Herbicide classes in development.* Springer, Berlin, 2002, 139-150.

Vaughn, KC; Hoffman, JC; Hahn, MG; Staehelin, LA. The herbicide dichlobenil disrupts cell plate formation: immunogold characterization. *Protoplasma,* 1996, 194, 117-132.

Vaughn, KC. & Turley, RB. Ultrastructural effects of cellulose biosynthesis inhibitor herbicides on developing cotton fibers. *Protoplasma,* 2001, 216, 80-93.

Verloop, A. & Nimmo, WB. Absorption, translocation and metabolism of dichlobenil in bean seedlings. *Weed Res,* 1969, 9, 357-370.

Vogel, KP. & Jung, HJG. Genetic modification of herbaceous plants for feed and fuel. *Crit Rev Plant Sci*, 2001, 20, 15-49.

Wakabayashi, K. & Böger, P. Phytotoxic sites of action for molecular design of modern herbicides (Part 2): Amino acid, lipid and cell wall biosynthesis, and other targets for future herbicides. *Weed Biol Manag,* 2004, 4, 59-70.

Walsh, TA; Neal, R; Merlo, AO; Honma, M; Hicks, GR; Wolff, K; Matsumura, W; Davies, JP. Mutations in an auxin receptor homolog AFB5 and in SGT1b confer resistance to synthetic picolinate auxins and not to 2,4-dichlorophenoxyacetic acid or indole-3-acetic acid in Arabidopsis. *Plant Physiol,* 2006, 142, 542-552.

Wang, J; Elliott, JE; Williamson, RE. Features of the primary wall CESA complex in wild type and cellulose-deficient mutants of *Arabidopsis thaliana*. *J Exp Bot,* 2008, 59, 2627-2637.

Wang, Y; Lu, J; Mollet, J-; Gretz, MR; Hoagland, KD. Extracellular matrix assembly in diatoms (Bacillariophyceae). II. 2,6-dichlorobenzonitrile inhibition of motility and stalk production in the marine diatom *Achnanthes longipes*. *Plant Physiol,* 1997, 113, 1071-1080.

Wells, B; McCann, MC; Shedletzky, E; Delmer, D; Roberts, K. Structural features of cell walls from tomato cells adapted to grow on the herbicide 2,6-dichlorobenzonitrile. *J Microsc,* 1994, 173, 155-164.

Wightman, R; Marshall, R; Turner, SR. A cellulose synthase-containing compartment moves rapidly beneath sites of secondary wall synthesis. *Plant Cell Physiol,* 2009 50, 584–594.

Willats, WGT; Knox, P; Mikkelsen, D. Pectin: new insights into an old polymer are starting to gel. *Trends Food Sci Technol*, 2006, 17, 97–104.

Wilson, CR; Luckman, GA; Tegg, RS; Yuan, ZQ; Wilson AJ; Eyles, A; Conner, AJ. Enhanced resistance to common scab of potato through somatic cell selection in cv. Iwa with the phytotoxin thaxtomin A. *Plant Pathol*, 2009, 58, 137-144.

Yoneda, A; Higaki, T; Kutsuna, N; Kondo, Y; Osada, H; Hasezawa, S; Matsui, M. Chemical genetic screening indentifies a novel inhibitor of parallel alignment of cortical microtubules and cellulose microfibrils. *Plant Cell Physiol*, 2007, 48, 1393-1403.

Zhong, R; Kays, SJ; Schroeder, BP; Ye, ZH. Mutation of a chitinase-like gene causes ectopic deposition of lignin, aberrant cell shapes, and overproduction of ethylene. *Plant Cell*, 2002, 14, 165-179.

Zuo, J; Niu, QW; Nishizawa, N; Wu, Y; Kost, B; Chua, NH. KORRIGAN, an *Arabidopsis* endo-1,4-beta-glucanase, localizes to the cell plate by polarized targeting and is essential for cytokinesis. *Plant Cell*, 2000, 12, 1137-1152.

Capítulo II:

Novel type II cell wall architecture in dichlobenil-habituated maize calluses

Capítulo correspondiente a la publicación: Mélida, H., García-Angulo, P., Alonso-Simón, A., Encina, A., Álvarez, J., Acebes, J.L. (2009). Novel type II cell wall architecture in dichlobenil-habituated maize calluses. Planta. 229, 617-631. DOI 10.1007/s00425-008-0860-8.

Novel type II cell wall architecture in dichlobenil-habituated maize calluses

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Abstract

Growth of maize (*Zea mays* L.) callus-culture cells was inhibited using dichlobenil (2,6 dichlorobenzonitrile, DCB) concentrations ≥ 1 μM; I_{50} value for the effect on inhibited fresh weight gain was 1.5 μM. By increasing the DCB concentration in the culture medium, DCB-habituated cells became thirteen times more tolerant of the inhibitor (I_{50}: 20 μM).

In comparison with non-habituated calluses, DCB-habituated calluses grew slower, were less friable and were formed by irregularly shaped cells surrounded by a thicker cell wall. By using an extensive array of techniques, changes in Type II cell wall composition and structure associated with DCB habituation were studied. Walls from DCB-habituated cells showed a reduction of up to 75% in cellulose content, which was compensated for by a net increase in arabinoxylan content. Arabinoxylans also showed a reduction in their extractability and a marked increase in their relative molecular mass. DCB habituation also involved a shift from ferulate to coumarate-rich cells walls, and enrichment in cell wall esterified hydroxicynnamates and dehydroferulates. The content of polymers such as mixed-glucan, xyloglucan, mannans, pectins or proteins did not vary or was reduced.

These results prove that the architecture of type II cell walls is able to compensate for deficiencies in cellulose content with a more extensive and phenolic cross-linked network of arabinoxylans, without necessitating β-glucan or other polymer enhancement.

As a consequence of this modified architecture, walls from DCB-habituated cells showed a reduction in their swelling capacity and an increase both in pore size and in resistance to polysaccharide hydrolytic enzymes.

Keywords: arabinoxylan; callus culture; cellulose; dichlobenil; FTIR; maize.

Abbreviations: AIR, alcohol insoluble residue; AGPs, arabinogalactan proteins; AX, arabinoxylan; CDTA, 50 mM cyclohexane-*trans*-1,2-diamine-*N,N,N´,N´,*-tetraacetic acid sodium salt; DCB, 2,6-dichlorobenzonitrile or dichlobenil; FTIR, Fourier Transformed Infrared Spectroscopy; GAX, glucuronoarabinoxylan; Hx; dichlobenil-habituated calluses growing in x μM DCB; IDA, immunodot assay; mAb, monoclonal antibody; MPBS, PBS containing 4% fat-free milk powder; M_w, average molecular weight; NH, non-habituated calluses; PBS, 0.1 M phosphate buffer saline; PCA, Principal Component Analysis; Rha, Rhamnose; snCR, supernatant-Cellulose Residue; TFA, trifluoroacetic acid.

Introduction

Previous works have demonstrated the remarkable ability of plant cells to tolerate induced stresses (Iraki et al. 1989; Shedletzky et al. 1992; Encina et al. 2001, 2002) by changing their cell wall composition and structure. The herbicide DCB inhibits the polymerization of Glc into β-1,4-linked glucan and may also affect β-1,4-glucan crystallization at the plasma membrane, but has

little or no short-term effect on other physiological processes (Delmer 1987). The habituation of cell cultures to cellulose biosynthesis inhibitors such as DCB, reflects the ability of cells to survive with a modified wall and is therefore a valuable experimental technique for gaining an insight into the plasticity of plant cell wall composition and structure (Vaughn 2002).

There are two types of cell walls in higher plants. Type I cell walls are found in dicots, gymnosperms and most monocots, and type II walls are found in graminaceous plants, along with the other commelinoid monocots (Carpita and Gibeaut 1993; Carpita 1996). Cellulose, a linear (1,4)-β-D-glucan, is the main load-bearing polysaccharide in both types of walls. In type I cell walls, xyloglucan interlaces the cellulose microfibrils, forming the main load bearing network in the wall. This cellulose-xyloglucan framework is embedded in a matrix of pectic polysaccharides, homogalacturonann and rhamno-galacturonans I and II. Type I cell walls also contain minor amounts of protein, including basic proteins that can interact with the pectin network and with other proteins through intermolecular bridges. Type II cell walls are characterized by a reduction in xyloglucan, pectins and structural proteins, and by a higher content of other noncellulosic-polysaccharides, such as acidic xylans and 'mixed-linkage' (1,3)-(1,4)-β-D-glucan (Carpita et al. 2001). In the case of graminaceous cell walls, matrix pectins are mainly substituted by arabinoxylans and glucuronoarabinoxylan (GAX), which tether adjacent cellulose microfibrils. Also frequent in graminaceous cell walls is the presence of hydroxycinnamic acids (mainly ferulic and *p*-coumaric acid) ester linked to α-L-Ara residues of arabinoxylans (Ishii 1997). Hydroxycinnamic acids contribute to wall assembly by cross linking polysaccharides through the oxidative coupling of feruloyl residues (Fry et al. 2000).

Most DCB-habituated cultures belong to type I cell wall species, such as tomato (Shedletzky et al. 1990), tobacco (Shedletzky et al. 1992; Wells et al. 1994; Nakagawa and Sakurai 1998, 2001) and bean (Encina et al. 2001, 2002; Alonso-Simon et al. 2004). In type I cell walls habituated to DCB, there is a marked reduction in the amount of cellulose and hemicelluloses, whereas the quantity of esterified and unesterified pectins is increased. Moreover, in DCB-habituated BY-2 tobacco cells, pectins have been reported to be cross-linked with extensin to form the main cell wall network (Sabba et al. 1999). Other modifications have been associated with DCB habituation, such as the presence of a non-crystalline β-1,4-glucan tightly bound to cellulose, the accumulation of pectin-enriched cell wall appositions, a putative increase in the extent of pectin-xyloglucan cross-linking, reduced levels of arabinogalactan proteins (AGPs) and changes in the levels of extensin (Shedletzky et al. 1992; Encina et al. 2002; Garcia-Angulo et al. 2006).

To date, barley cultures are the only cells with type II cell walls that have been habituated to DCB (Shedletzky et al. 1992). DCB-habituated barley cells showed a modified architecture: they contained a considerably reduced level of cellulose in the cell wall and this reduction was effectively compensated for by mechanisms (parallel to modifications) quite different to those observed in dicots, which implicated a higher proportion of β-glucan and a more extensive cross-linking between arabinoxylans, leading to walls with a reduction in pore size. The present work addresses the selection and characterisation of a maize cell line able to grow in the presence of lethal concentrations of DCB. The

results show a novel type II cell wall architecture accompanied by unique cell wall properties.

Materials and methods

Cell cultures

Maize callus cultures (*Zea mays* L., Black Mexican sweetcorn, donated by Dr. S.C. Fry, Institute of Molecular Plant Sciences, University of Edinburgh, UK) from immature embryos were grown in Murashige and Skoog media (Murashige and Skoog 1962) supplemented with 9 μM 2,4-D at 25°C under light (Lorences and Fry 1991), and subcultured monthly.

Habituation to dichlobenil

Calluses weighing 1.0 ± 0.1 g were cultured in dichlobenil (supplied by Fluka), in concentrations ranging from 0.01 to 100 μM. DCB was dissolved in dimethyl sulfoxide (DMSO), which did not affect cell growth at this range of concentrations. The cultures were incubated for 30 days, weighed (FW) and heated at 60°C until constant weight was achieved (DW). Growth was expressed as relative increase in FW and the I_{50} was calculated as the concentration of DCB able to inhibit weight increase by 50% with respect to the control.

Calluses were habituated to growth in different DCB concentrations by stepwise transfers with gradual increments of DCB, beginning at 2 μM. At least three subcultures of approximately 30 days were performed between each increase in the DCB concentration. Growth curves were obtained for habituated calluses growing in DCB and for non habituated calluses, by measuring the relative increase in FW every 4-6 days.

Electron microscopy

Callus pieces were fixed in 2.5% (w/v) paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4, and post-fixed with OsO_4 in the same buffer. The samples were dehydrated in an increasing series of ethanol concentrations and embedded in LR White Resin (London Resin Co. Ltd, Basingstoke, UK). This was done by sequentially placing the segments in ethanol and resin (2:1, v/v) for 8 h, ethanol and resin (1:2, v/v) for 8 h, and in pure resin. The samples were transferred to a gelatin capsule, fresh resin added, and the resin polymerized at 60°C for 48 h. Blocks were sectioned (1.5-2 μm thick) with a LKB 2088 ultramicrotome. Ultrathin sections were mounted on copper grids and post-stained with uranyl acetate and lead citrate before observation with a JEOL (JEM-1010) electron microscope. Cell wall thickness was determined by random measurement of cell walls from 30 cells.

Immunolocation

For the immunolocation of cell wall components, the gelatine capsules were polymerized at 37°C for 5 days. Sections were obtained (1.5-2 μm thick) and applied to multi-well slides (ICN Biomedicals, Cleveland, OH, USA) coated with Vectabond reagent (Vector Laboratories, Burlingame, CA, USA). Sections were incubated for 2 h with 0.1 M phosphate buffer saline (PBS) (0.14 M NaCl, 2.7 mM KCl, 7.8 mM $Na_2HPO_4 \cdot 12\ H_2O$, 1.5 mM KH_2PO_4, pH 7.2) containing 4% fat-free milk powder (MPBS) with the primary antibody at a 1/10 dilution. After washing exhaustively with PBS, the sections were incubated in darkness for 2 h with a 1/100 dilution of an antirat immunoglobulin G linked to fluorescein isothiocyanate (Sigma) in MPBS at room temperature. Finally, sections were washed with PBS and mounted in a glycerol/PBS-based antifade solution (Citifluor AF1; Agar Scientific, London, UK) and observed using a Nikon Eclipse-TS100 microscope with epifluorescence irradiation. Cellulose was localized in sections using calcofluor white (fluorescent brightener 28, Sigma). Xylans were probed by using mAb LM10 (specific for 1,4-β-xylans) (McCartney et al. 2005) and LM11 (for xylans and arabinoxylans) (McCartney et al. 2005). Cell wall esterified feruloyl groups were probed with LM12, a new antibody developed at the Paul Knox laboratory, Leeds University. AGPs were probed with mAbs LM2 (Smallwood et al. 1996), MAC207 (Pennell et al. 1989) and JIM8 (Pennell et al. 1991).

Preparation and fractionation of cell walls

Calluses collected during growth in the early stationary phase were frozen and homogenized with liquid nitrogen and treated with 70% ethanol for 5 days at room temperature. The suspension was then centrifuged and the pellet washed with 70% ethanol (x6), acetone (x6), and air dried, in order to obtain the alcohol insoluble residue (AIR). The AIR was treated with 90% DMSO for 8 h at room temperature (x3) and then washed with 0.01 M phosphate buffer pH 7.0 (x2). The washed AIR was then treated with 2.5 U ml^{-1} α-amylase obtained from porcine pancreas (Sigma type VI-A) in 0.01 M phosphate buffer pH 7.0 for 24 h at 37°C (x3). The suspension was filtered through a glass fibre, and the residue washed with 70% ethanol (x6), acetone (x6), air dried and then treated with phenol-acetic-water (2:1:1 by vol.) for 8h at room temperature (x2). This was finally washed with 70% ethanol (x6), acetone (x6) and air dried in order to obtain the cell walls.

For cell wall fractionation, dry cell walls were extracted at room temperature with 50 mM cyclohexane-*trans*-1,2-diamine-*N,N,N´,N´,*-tetraacetic acid sodium salt (CDTA) at pH 6.5 for 8 h and washed with distilled water. The residue was retreated with 0.1 M KOH for 2 h (x2) and washed with distilled water. Then 4 M KOH was added to the residue for 4 h (x2), and washed again with distilled water. The extracts were neutralized with acetic acid, dialysed and lyophilized, representing CDTA, KOH-0.1M and KOH-4M fractions, respectively. The residue after 4 M KOH extraction was suspended in water, adjusted to pH 5 with acetic acid, and dialysed. After centrifugation, the supernatant was filtered and lyophilized; this was referred to as the supernatant-Cellulose Residue (snCR) fraction. The residue was hydrolysed for

2.5 h at 120°C with 2 M trifluoroacetic acid (TFA), and after centrifugation, the supernatant was lyophilized and referred to as the TFA fraction.

Cell wall analyses

Cellulose was quantified in crude cell walls with the Updegraff method (Updegraff 1969), using the hydrolytic conditions described by Saeman (Saeman et al. 1963) and quantifying the glucose released by the anthrone method (Dische 1962).

Tablets for Fourier transform infrared (FTIR) spectroscopy were prepared in a Graseby-Specac press from small samples (2 mg) of cell walls mixed with KBr (1:100, w/w). Spectra were obtained on a Perkin-Elmer instrument at a resolution of 1 cm^{-1}. A window of between 800 and 1800 cm^{-1}, containing information about characteristic polysaccharides, was selected in order to monitor cell wall structure modifications. All spectra were normalized and baseline-corrected with Spectrum v 5.3.1 (2005) software, by Perkin-Elmer. Data were then exported to Microsoft Excel 2003 and all spectra were area-normalized. Cluster analysis was performed using the Ward method, and the Pearson coefficient was selected as distance measurement. Principal component analysis (PCA) was performed using a maximum of five principal components. All analyses were carried out using the Statistica 6.0 software package.

The total sugar content of each fraction was determined by the phenol-sulfuric acid method (Dubois et al. 1956) and expressed as the glucose equivalent. Uronic acid content was determined by the *m*-hydroxydiphenyl method (Blumenkrantz and Asboe-Hansen 1973) using galacturonic acid as a standard. Xyloglucan content was obtained by the iodine-staining method (Kooiman 1960). Neutral sugar analysis was performed as described by Albersheim (Albersheim et al. 1967). Lyophilized samples of each fraction were hydrolysed with 2 M TFA at 121°C for 1 h and the resulting sugars were derivatized to alditol acetates and analysed by gas chromatography (GC) using a Supelco SP-2330 column.

The quantification of (1→3,1→4)-β-glucans was carried out using a direct and specific enzymatic assay with the (1→3,1→4)-β-glucan endo-hydrolase from *Bacillus subtilis* (Mccleary and Codd 1991).

To measure cell wall degradability, cell walls were hydrolyzed (5 mg ml^{-1}) in a mixture of Cellulase R-10 (0.1%), Mazerozyme R-10 (0.1%) and purified Driselase (0.01%) dissolved in sodium acetate 20 mM (pH 4.8) (Grabber et al. 1998). Aliquots were taken at 2, 6, 24, 48 and 72 h, clarified by centrifugation and assayed for total sugars following the method described by Dubois et al. (1956).

In vitro measurement of cell wall swelling examined the relative volume increase that took place after re-hydrating dry walls in 8 mm diameter tubes (Encina et al. 2002).

For amino acid analysis, cell walls were hydrolyzed in 6 N HCl at 110°C for 24 h, filtered through Whatman paper and dried by speed-vac. Soluble amino acids were derivatized by using phenyl isothiocyanate, and then separated, identified and quantified following the method described by Alonso

et al. (1994). Total protein content was estimated by summation of the amounts of amino acids measured.

Assay of esterified phenolic acids

Cell walls (10 mg) were treated in the dark with 1 M NaOH, at room temperature for 12 h to saponify phenolic esters. The solution was acidified by addition of TFA and partitioned against ethyl acetate (x2). The ethyl acetate phases were pooled and mixed with a solution containing 1% 8,5-diferulic acid, 5,5-diferulic acid, 8-*O*-4 diferulic acid, *p*-coumaric acid and ferulic acid as internal markers, then vacuum-dried and re-dissolved in propan-1-ol. Portions of the propanol solution were subjected to TLC on silica-gel in benzene/acetic acid (9:1, v/v).

Gel-permeation chromatography

Hemicellulosic fractions were size-fractionated by gel-permeation chromatography on Sepharose CL-4B (120-125 ml bed-volume in a 1.5 cm diameter column) in pyridin:acetic acid:water (1:1:98, by vol.) at 0.3 ml min^{-1}. The column was calibrated with commercial dextrans of known weight-average relative molecular mass, and hemicellulose average molecular weight (M_w) was obtained using the $K_{av(1/2)}$ method (Kerr and Fry 2003), with the calibration curve [log M_w=-4.999 $K_{av(1/2)}$ + 7.849] obtained for this column. The M_w estimates are nominal rather than absolute because of conformational differences between dextran and hemicelluloses.

Porosity measurements

Porosity was determined using the technique developed by Baron-Epel et al. (1988). Cells at the logarithmic stage of growth were resuspended in 10 mM 2-amino-2(hydroxymethyl)-1.3-propanediol (Tris) (pH 5.5), 10 mM $CaCl_2$, and 0.5 M mannitol in order to induce plasmolysis. They were then suspended in a medium with fluorescein isothiocyanate-dextrans (Sigma) at a final concentration of 500 µg ml^{-1}. Fluorescence was observed using a Nikon Eclipse-TS100 epifluorescent microscope equipped with a Nikon B-2 A filter (450-490 nm excitation, 510 nm dichroic mirror and 520 nm barrier filter).

Immunodot assays

For immunodot assays (IDAs), aliquots of 1 µl from KOH-0.1M and KOH-4M fractions containing 5 µg of total sugars were spotted onto nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany) as a replicated dilution series and probed as described by Willats et al. (1999) and García-Angulo et al. (2006). All primary antibodies (JIM8, LM11 and LM12) were used at 1/5 dilutions.

Results

Effect of DCB on callus cultures and habituation

In order to determine the effect of DCB on maize callus cultures we tested its inhibitory effect on fresh weight gain (Fig. 1). The weight gain of non-habituated calluses (NH) was slightly stimulated at low concentrations of DCB but was diminished by DCB concentrations equal to or higher than 1 µM. The I_{50} was 1.5 µM for FW.

Maize calluses tolerance to lethal concentrations of DCB was achieved by gradually increasing the concentration of the inhibitor in the culture medium, beginning at 2 µM. After 12 subcultures of an average of 30 d each, maize cells were capable of growth in 12 µM DCB (H12). As for non-habituated cell cultures, a stimulation of FW gain was observed for the lowest DCB concentration used. In the case of H12 cells, the I_{50} value for FW inhibition gain was approximately thirteen times higher (20 µM).

Characterization of callus cultures and cells

Habituated cells (H12) growing on DCB had longer lag phases and less FW was accumulated (Fig. 2). Moreover, H12 cells had a higher DW/FW ratio and cell wall yield per DW than non-habituated ones (Table 1).

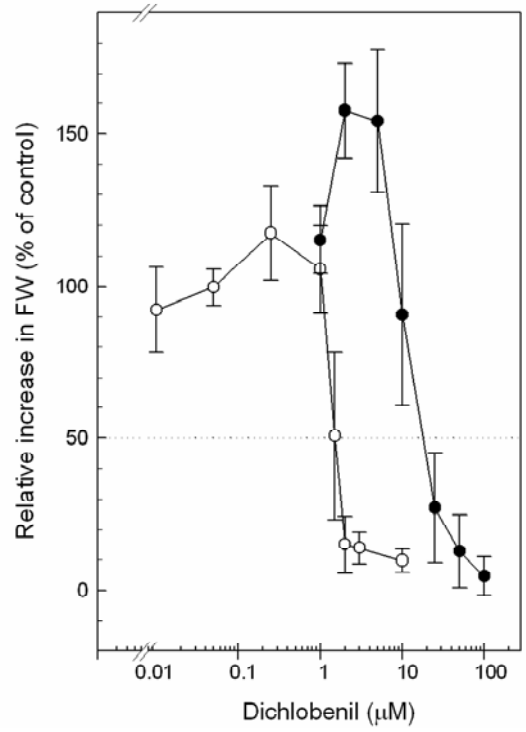

Figure 1. Growth inhibition curves of maize calluses by increasing concentrations of DCB after 30 days of culture. *Open circle* non-habituated calluses, *filled circle* habituated to 12 µM DCB (H12) calluses. Values are means ± SD of six measurements.

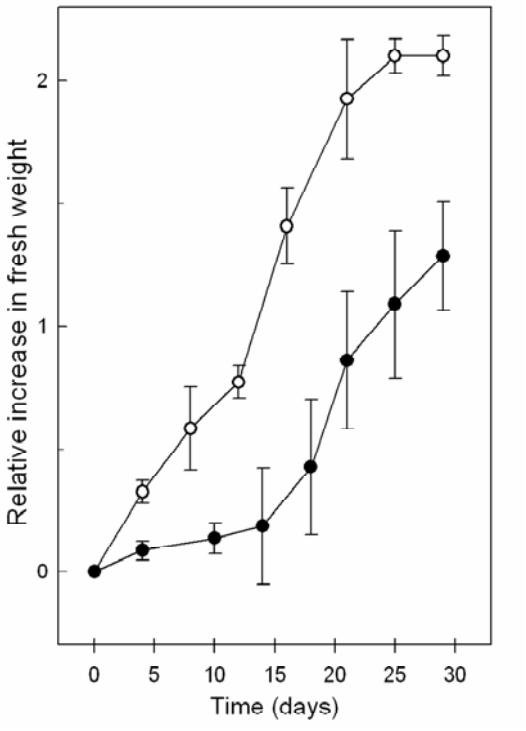

Figure 2. Relative increase in FW of maize callus cultures during the culture. *Open circle* non-habituated calluses, *filled circle* habituated to 12 µM DCB (H12) calluses. Values are means ± SD of six measurements.

H12 calluses were darker and less friable, and formed hard protuberances during growth. H12 cells were rather irregular (Fig. 3b) in comparison with NH cells, which were more or less isodiametrically shaped (Fig. 3a). Groups of cells with thicker walls, apparently arising from the same cell, were frequent in habituated cell preparations (Fig. 3b).

Table 1. Variation of some characteristics of cell lines during habituation.

Cell type	DW/FW[a]	Wall DW/callus DW[a]	Cell wall thickness (μm)[b]	Cell wall swelling[c]
NH	0.0399 ± 0.0074	0.2409 ± 0.0052	0.1344 ± 0.0343	1.72 ± 0.19
H12	0.0623 ± 0.0100	0.5451 ± 0.0029	0.2137 ± 0.1113	0.80 ± 0.13

Cell wall swelling is represented as relative increase in volume.
Values are means ± SD of [a]10, [b]30 or [c]6 measurements.

Cell wall analysis

Ultrastructure and porosity

NH cells were surrounded by a uniform cell wall (Table 1), in comparison with H12 cell walls, which were less uniform (Fig. 3d) and 1.6 times thicker than those from NH ones (Fig. 3c). H12 cell walls (Fig. 3b) were less calcofluor-stained than NH cell walls (Fig. 3a), probably due to the reduction in cellulose.

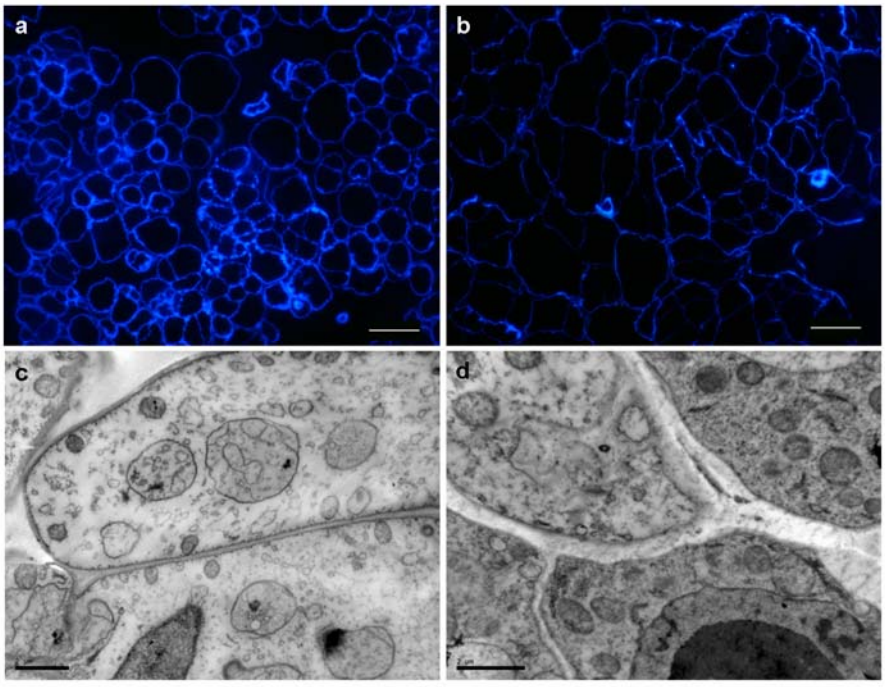

Figure 3. Sections of NH (**a**) and H12 (**b**) callus calcofluor stained. Ultrastructural appearance of cell walls of NH (**c**) and H12 (**d**) callus. *Bars* = 50 μm (**a**, **b**), 2 μm (**c**, **d**).

H12 cell walls half-swelled compared with NH ones (Table 1), but porosity increased (Table 2); limiting diameter of dextrans to be excluded for cell walls increased from 6.6 to 12 nm when NH and H12 cells were compared,

although long incubation times were required by 9 nm of diameter dextrans to go through H12 cell walls.

Table 2. Comparison of porosities of NH and H12 cell walls.

Molecular Mass of FTIC-Dextrans (kDa)	Stokes Diameters (nm)	NH	H12
10	4.6	+[a],+[b],+[c]	+ + +
20	6.6	– – –	± + +
40	9	– – –	– – +
70	12	– – –	– – –

+, Dye penetrates >80% of cell walls; –, dye penetrates <20% of cell walls.

[a] Measurement at 30 min; [b] measurement at 60 min; [c] measurement at 120 min.

FTIR spectroscopy

Changes in the cell wall during the habituation process were monitored by FTIR spectroscopy. Twelve representative FTIR cell wall spectra from non-habituated and habituated to different DCB concentrations calluses were analyzed. FTIR spectra from habituated cell walls showed differences in wavenumbers corresponding to cellulose, phenolic components, arabinose and proteins. They also showed variations in the main phenolic linkage: ester bonds (data not shown). To elucidate differences among these spectra, a multivariate analysis was performed (Fig. 4a).

Principal components 1 and 3 (PC1 and PC3) were used to discriminate between groups of spectra. Cell walls from non-habituated cells were located on the negative side of PC1 and PC3; when the habituation process began, the points corresponding to the cell walls of habituated calluses were displaced to the positive side of PC1 and PC3. Generally, the more habituated the calluses, the more positive the PC1 and PC3 placement. Non-habituated calluses treated for short periods with 6 µM DCB displaced to the positive side of PC1 but to the negative side of PC3, suggesting that the changes in cell walls promoted by DCB were different in habituated and non-habituated cells.

PC3 was more discriminative than PC1. PC3 loading factor plot (Fig. 4b) showed a negative area at the fingerprint region 950-1,175 cm^{-1}, indicative of polysaccharides. In this area, correlated negative peaks could be identified, mainly at 1,160, 1,105, 1,060 and 1,040 cm^{-1}, indicative of cellulose (Carpita et al. 2001). Also, two major protein negative absorbances at 1,550 and 1,650 cm^{-1} were detected. Thus, spectra located at the negative side of PC3, i.e. NH-seemed to have a relatively higher amount of polysaccharides –mainly cellulose- and proteins. In contrast, PC3 showed positive peaks at several wavenumbers indicative of aromatic rings: 1,630 (ring conjugated C=C), 1,600 (aryl ring stretching symmetric), about 1,500 (phenolic ring), and 1,425, 1,180 and 843 (aromatic C-H out of bending) cm^{-1}. The peak at 856 cm^{-1} corresponds to furanoid ring (i.e. Ara*f*); and 1,381, 1,376 and 1,312 cm^{-1} correspond to polysaccharides (Kacurakova et al. 1999).

Summing up, the expected changes in cellulose were accompanied by changes in other components, like arabinose, phenolics and proteins, and multivariate analyses demonstrated that during habituation, cell walls underwent gradual modifications.

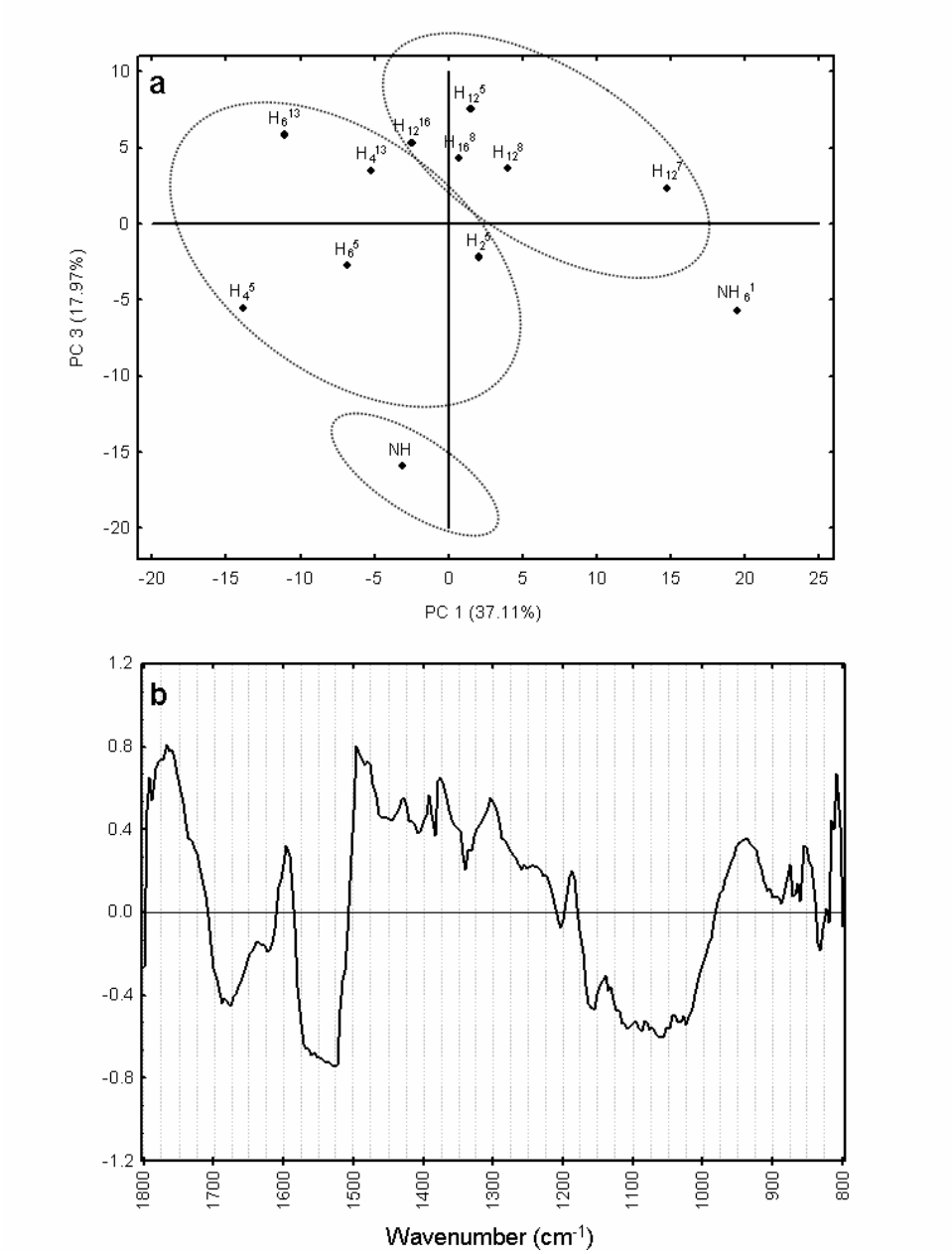

Figure 4. (**a**) Principal component analysis of callus spectra. A plot of the first and third PCs is represented based on the FTIR spectra of non-habituated (NH) and habituated calluses (H_x^n, *x* DCB concentration (µM), *n* number of subcultures in that concentration). NH_6^1, non-habituated calluses treated for 5 days with 6 µM DCB. (**b**) Factor loadings for PC3.

Cellulose content

The increase in DCB concentration during the habituation process caused a reduction in cellulose content (Fig. 5). This reduction correlated with the habituation level until H6. H12 walls had about 25% of the amount of cellulose found in their NH counterpart, with no further change as the level of tolerance increased.

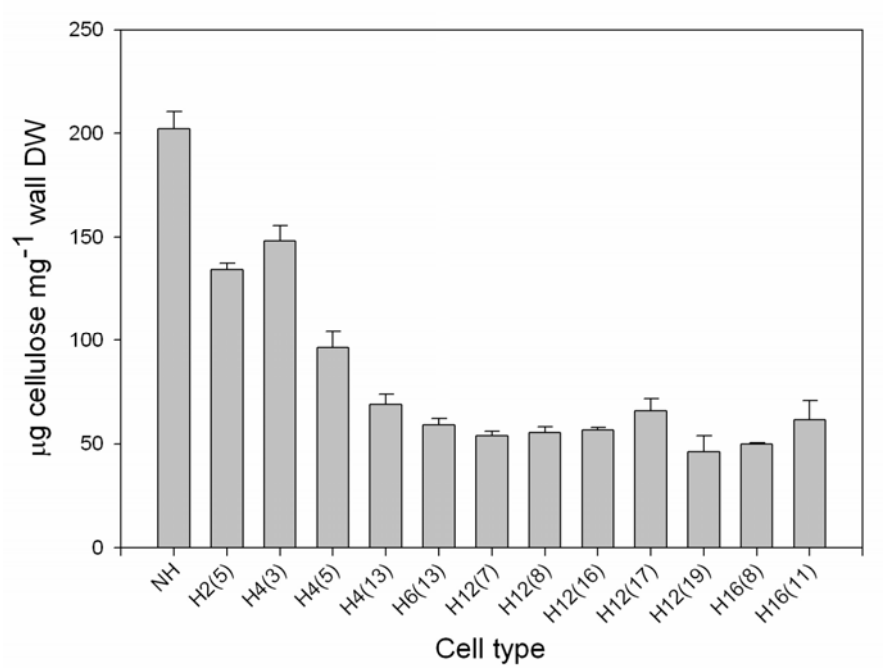

Figure 5. Cellulose content of cell walls isolated from calluses at different DCB tolerance levels. NH, non-habituated callus; $H_x(n)$, habituated callus, where *x* DCB concentration (µM), and *n* number of subcultures in that concentration. Values are means ± S.D. of three measurements.

Cell wall fractionation and sugar analysis

Cell wall fractionation (Fig. 6) showed that the bulk of polysaccharides were extracted from the cell walls by alkali treatment: KOH-0.1M and KOH-4M fractions. These two fractions, plus the TFA fraction, accounted for about 90% of the total cell-wall sugar content. A net increase in the yield of sugars per dry cell wall weight was observed during the habituation to DCB (0.38 in NH vs. 0.48 in H12). This effect was mainly due to a notable increase in polysaccharides extracted with strong alkali (KOH-4M fraction). The total amount of CDTA-extracted polysaccharides was reduced during habituation to DCB, while a slight increase in polysaccharides tightly bound to cellulose (TFA and snCR fractions) was detected. As regards the polysaccharides extracted with diluted alkali (KOH-0.1M fraction), an increase in intermediate levels of tolerance was measured.

Table 3. Xyloglucan (XyG) and mixed-linked glucan (MLG) content of KOH fractions and crude cell walls respectively

	XyG		MLG
Cell type	KOH-0.1M	KOH-4M	
NH	2.84 ± 0.19	19.93 ± 0.42	2.75 ± 0.12
H12	1.63 ± 0.07	12.01 ± 0.63	1.92 ± 0.09

Data units: µg·mg^{-1} wall DW. Values are means ± SD of three measurements.

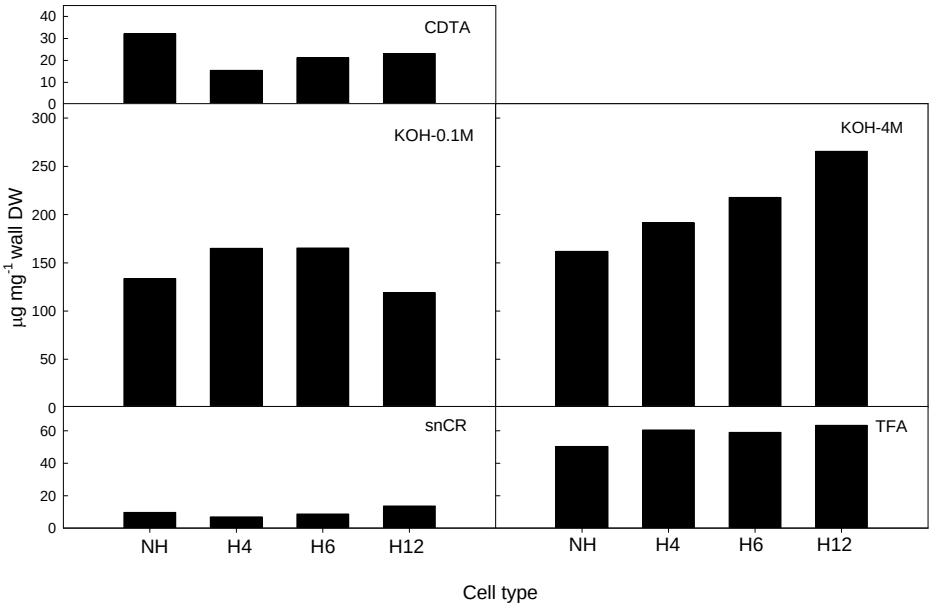

Figure 6. Total sugars in cell wall fractions at different DCB habituation levels. NH, non-habituated calluses; H*x*, habituated calluses where *x* DCB concentration (µM). Calluses with at least eight subcultures in medium containing the indicated concentration of DCB were used. Results came from a representative experiment.

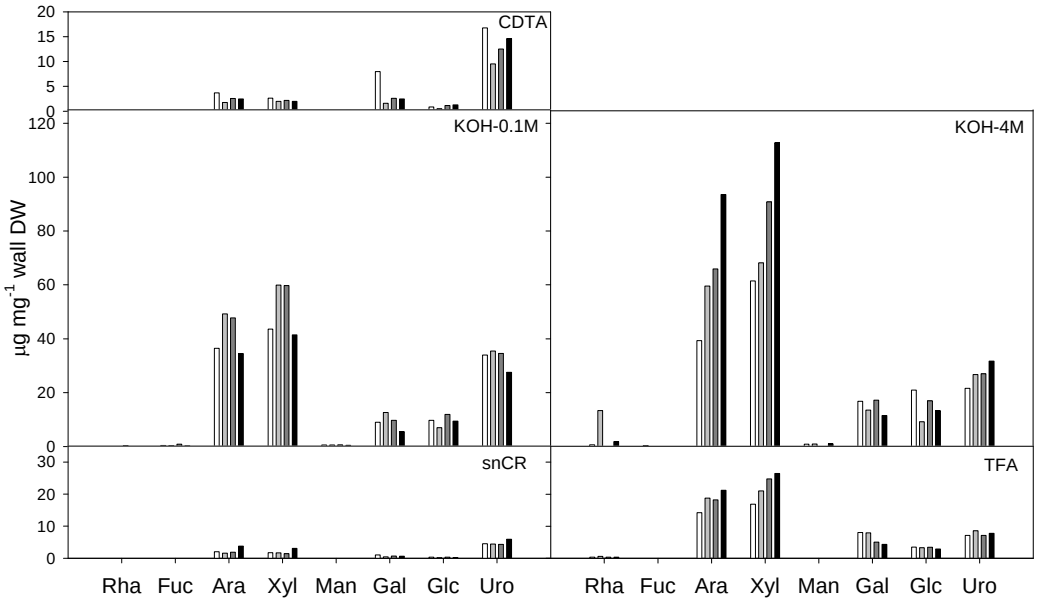

Figure 7. Sugar composition of fractions from cell walls of NH (*open square*), H4 (*light grey square*), H6 (*dark grey square*) and H12 (*black square*) calluses. *Rha* rhamnose, *Fuc* fucose, *Ara* arabinose, *Xyl* xylose, *Man* manose, *Gal* galactose, *Glc* glucose, *Uro* uronic acids.

GC analysis of cell wall fractions (Fig. 7) showed that KOH-0.1M and KOH-4M fractions were composed mainly of Ara and Xyl followed by uronic acids, Gal and Glc, whereas minor fractions such as CDTA and snCR were composed of the same monosaccharides and enriched in uronic acids. The detected variations in the amount of sugar extracted in KOH-0.1M and KOH-4M and TFA fractions were paralleled by variations in Ara and Xyl. Especially important is the gradual enrichment in Ara and Xyl found in KOH-4M and TFA

fractions throughout the habituation process. The reduced levels of Glc reflect the poor levels of xyloglucan and mixed-linked glucan in these cell walls. These two polysaccharides are even further reduced after habituation (Table 3). Therefore, a net enrichment in arabinoxylans, concomitant with a reduction in cellulose, was the main change observed in cell wall composition involved in DCB habituation.

Gel-permeation chromatography

Gel-permeation chromatography showed differences in the molecular mass of hemicellulosic polysaccharides (Table 4). Interesting changes took place in the 4M KOH extractable polysaccharides (Fig. 8). At low and medium habituation levels the M_w of 4M KOH extracted polysaccharides was reduced, but in the H12 fraction a significant increase took place; there was a net shift towards higher molecular masses and a new peak appeared close to the void volume. This peak was isolated and GC-analysed; Ara and Xyl accounted for about 78% (data not shown). In the KOH-0.1M fractions, the opposite tendency was observed: H4 and H6 increased, whilst H12 increased only slightly (Table 4).

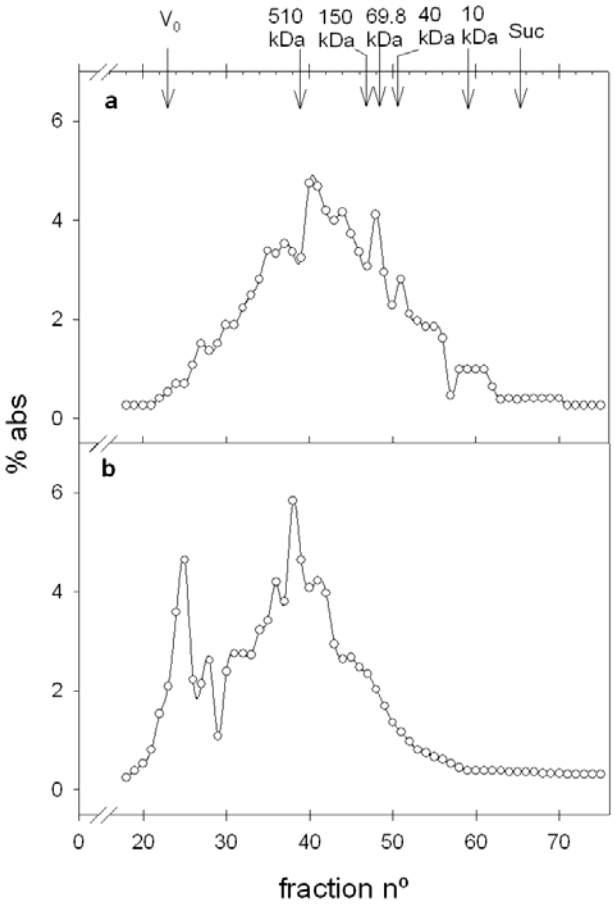

Figure 8. Elution profiles of 4M-KOH-extractable polysaccharides from NH (**a**) and H12 (**b**) calluses. *Markers* blue dextran (V_0), dextrans of 510, 150, 69.8, 40, 10 kDa, and sucrose.

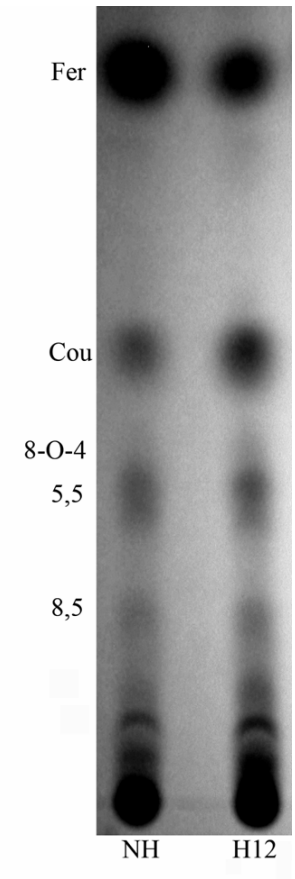

Figure 9. TLC of phenolics alkali extracted from NH and H12 maize walls. Abreviations in the TLC panel represent the position of migration of the following standards: (8,5), 8,5-di-ferulic acid; (5,5), 5,5-diferulic acid; (8-*O*-4), 8-*O*-4 di-ferulic acid; (Cou), *p*-coumaric acid; (Fer), ferulic acid.

Table 4. Average molecular weight (M_w) of the hemicellulosic fractions

Cell type	KOH-0.1M (kDa)	KOH-4M (kDa)
NH	80.28	748.85
H4	446.11	298.18
H6	467.34	467.16
H12	134.75	2612.35

The M_w was obtained using the $K_{av(1/2)}$ method, with the calibration curve [log M_w=-4.999 $K_{av(1/2)}$ + 7.849] obtained for this column.

Protein content and amino acid composition of the cell wall

DCB habituation changed protein content and amino acid composition of cell walls (Table 5). The insoluble protein content of cell walls was reduced in H12 cells by 30%. Expressed as a percentage of total amino acid, an increase in Glu and a reduction in Asp, Pro, Tyr, and Phe was detected in H12 cell walls.

Table 5. Amino acid composition (mol%) of insoluble protein from cell walls.

Amino Acid	Cell type	
	NH	H12
Asp	11.4	2.9
Glu	11.4	36.9
Hyp	2.3	1.7
Asn	4.2	1.1
Ser	0.9	0.6
Thr	3.0	2.6
Ala	10.0	7.6
Pro	3.7	1.7
Tyr	3.1	1.7
Val	18.3	15.4
Ile	3.7	3.4
Leu	4.5	5.5
Phe	6.5	3.7
Trp	9.1	6.8
Lys	3.0	4.5
Arg	4.8	3.6

Values represent the mean of two independent assays.

Cell wall immunoanalysis

Due to increased levels of arabinoxylans in habituated cells, cell walls were probed with LM10 (McCartney et al. 2005), specific for 1,4-β-xylans, and LM11 (McCartney et al. 2005) specific for xylans and arabinoxylans. LM11 epitope was only found in habituated cell walls (Fig. 10b), although the IDA showed that NH cell walls can also bind it, but clearly to a lesser degree (Fig. 11b and e). According to fractionation results, most LM11 labelling appears in the 4M-KOH fractions. No epitopes for LM10 were found in any of the cell types probed (data not shown). Cell wall esterified feruloyl groups were probed with LM12 (Fig. 10c and d). LM12 epitope was found in both cell types but the immunofluorescent labelling in H12 cells (Fig. 10d) seemed weaker than that of NH (Fig. 10c). IDA for LM12 (Fig. 11c and f) showed that most labelling was found in 0.1M-KOH fractions, and was reduced in habituated ones.

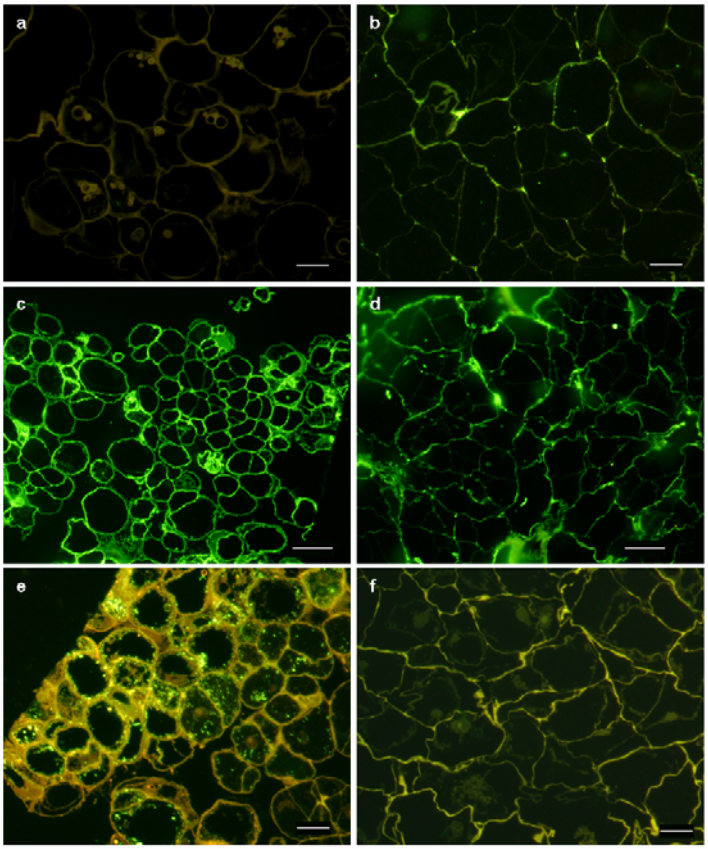

Figure 10. Inmunofluorescent localization of (1→4)-β-D-xylan/arabinoxylan (LM11: **a**, **b**), feruloylates (LM12: **c**, **d**) and arabinogalactan-proteins (JIM8: **e**, **f**) of NH (**a**, **c**, **e**) and H12 (**b**, **d**, **f**) maize cells. *Bars* 20 µm (a, b, e, f), 50 µm (**c**, **d**).

In the case of AGPs, different results were found depending on the antibody used. Whereas no type of cell bound MAC207, both control and habituated cells bound LM2 (data not shown). The difference was in JIM8, where only NH cells bound it (Fig. 10e). IDAs for this antibody (Fig. 11a and d) showed that habituated cells had progressively lesser labelling as habituation level increased.

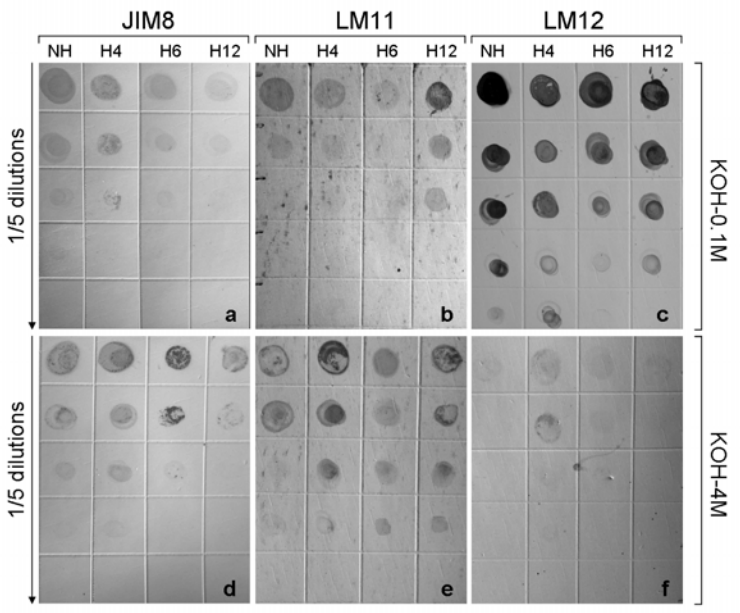

Figure 11. Immunodot assays of KOH cell wall fractions from NH, H4, H6 and H12 calluses, probed with monoclonal antibodies with specifity for arabino-galactan proteins (JIM8: **a**, **d**), xylan/arabinoxylan (LM11: **b**, **e**) and feruloylates (LM12: **c**, **f**).

Cell wall degradability

NH cell walls were quickly and completely degraded by cell wall digesting enzymes, with a total sugar yield of 821 and 1000 μg mg^{-1} being released after 6 h and 72 h of incubation, respectively (Fig. 12). In the hydrolytic conditions assayed, H12 cell walls were significantly more resistant to enzymatic degradation than NH cell walls, and a reduction of 40% in the yield of sugars released was measured after 72 h of incubation. Thus, the modifications that take place during habituation seem to build a strengthened cell wall in response to DCB.

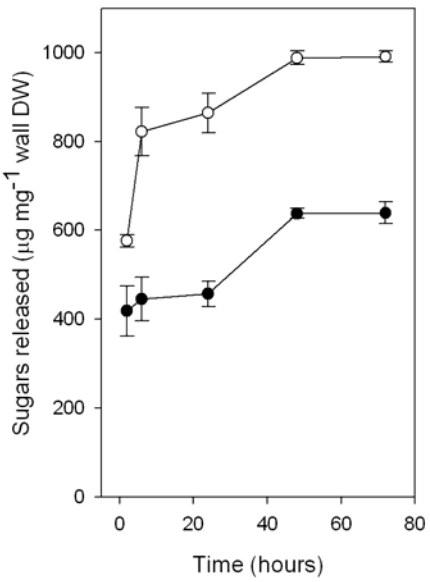

Fig. 12. Cell wall degradability of (*open circle*) NH and (*filled circle*) H12 calluses. Values are means ± SD of nine measurements.

Discussion

Maize calluses have been successfully habituated to lethal DCB concentrations, by gradually increasing the concentration of the inhibitor in the culture medium. This habituation procedure led to progressive widespread changes in callus growth and morphology: habituated calluses grew more slowly, formed hard protuberances, and were darker and harder. Their cells were more irregularly shaped, with a thicker and more irregular cell wall, which contributed to a higher dry weight in proportion to total dry weight. All these characteristics resembled those of other previously described cellulose-inhibitor-habituated cell cultures, such as DCB habituated tomato cell suspensions (Shedletzky et al. 1990), and DCB- or isoxaben-habituated bean calluses (Díaz-Cacho et al. 1999; Encina et al. 2001).

As far as we know, only one other example of a Type II cell wall species (barley) habituated to DCB has been reported to date (Shedletzky et al. 1992). DCB-habituated barley cells also showed a reduced growth rate. However they showed net differences when compared to DCB-habituated maize cells: their cells were not larger than controls, were more isodiametrically-shaped and did not possess thicker cell walls. These differences could be explained -at least partially- by taking into account the different callogenic origin of both cultures. Barley cell cultures were generated from endosperm tissue (Shedletzky et al. 1992) whereas our maize cells were generated from immature embryos.

It has been suggested previously that the mechanism of habituation to DCB and other cellulose biosynthesis inhibitors relies on the ability of the

habituated cells to divide and expand under conditions where cellulose synthesis is inhibited. In fact, maize and barley habituated cultures showed cellulose reductions of up to 70-75%. Both DCB-habituated barley and maize cell cultures compensated for the reduction in cellulose with a higher quantity of hemicellulosic polysaccharides, while uronic acids hardly varied. However, the increment in hemicellulosic polysaccharides had different origins in the cultures: in DCB-habituated barley cells, the only polysaccharide where the proportion rose was mixed-linked glucan, which increased its content four-fold, to reach more than 100 μg mg^{-1} cell wall. However in DCB-habituated maize cells, the reduction in cellulose was paralleled by a net increase in arabinoxylans, whereas other hemicelluloses such as β-glucan and xyloglucan were reduced. Thus, we have proved for the first time that the architecture of type II cell walls is able to compensate for deficiencies in cellulose content without requiring a mixed glucan increment. In this modified architecture, the reduction in cellulose content is compensated for mainly by an increment in arabinoxylan content, whose characteristics appear modified in comparison with those of non-habituated cells.

The enhanced arabinoxylan content of habituated cells was appreciable by probing with LM11, an antibody that binds to xylan or arabinoxylan with a low degree of substitution (McCartney et al. 2005). LM11 labeling of habituated cell walls was much more intense than in non-habituated cell walls. Additionally, the LM11-binding pattern of habituated cell walls shows that LM11 epitopes are mainly localized in cell junctions, in thicker cell walls and in dispersed cell wall areas, pointing to particular cell wall strengthening in some areas.

Arabinoxylans in DCB-habituated maize cells also showed differences in extractability, mean molecular mass and in formation of phenolic bridges, when compared with non-habituated cells.

Cell wall fractionation, GC and IDA for LM11-epitopes of KOH-extracted polysaccharides confirmed the reported net increase of AX in DCB-habituated cells, and showed a shift of AX from mild-alkali-extracted fractions (0.1M-KOH) to strong-alkali-extracted fractions (4M-KOH) and cellulose tightly bound fraction (TFA), pointing to a more extensive cross-linked hemicellulosic network.

Strong-alkali-extracted AX from habituated cell walls also showed a significant increase in M_w. This result can be explained by an increase in the polymerization and/or substitution degree of AX. This is an interesting result taking into account the fact that a major factor controlling AX increase in M_w is their cross-linking through the formation of dehydroferulates, and that alkali treatment has been reported to prevent this by releasing ester bonded feruloyl groups (Kerr and Fry 2004). Therefore, an alternative explanation for this result could be the increment in alkali resistant phenolic bridges (ether-linked phenolic groups), which would render highly cross-linked AX even after the alkali treatment.

Phenolics have an important function in Type II cell walls, as hydroxycinnamic acid derivatives contribute to wall assembly by cross linking polysaccharides through oxidative coupling. Therefore, we tested whether phenolics could contribute to the global tightening in our “stressed” cell walls. FTIR data pointed to an enhanced contribution of phenolics in DCB-habituated

cells, as peaks assigned to phenolic ester (1,725 cm^{-1}) and aromatic rings (1,515, 1,600, 1,630 cm^{-1}) were more pronounced. Furthermore TLC showed interesting changes in phenolic profiles: as described for DCB-habituated barley cultures, there was a shift from ferulic acid-rich walls to *p*-coumaric acid-rich walls (Shedletzky et al. 1992). In addition, in our DCB-habituated maize cells, an enrichment in 5,5 and 8,5 dehydroferulates, and in other compounds with low R_f, which could correspond to trimers or tetramers, was noticed. Therefore, these results indicated a general increase in hydroxycinnamic acid derivatives and in particular, in oxidative coupled derivatives. LM12 immunolocation showed that feruloyl groups were distributed mainly in cell wall areas next to plasmalemma. In conclusion, DCB-habituated maize cells not only showed enrichment in arabinoxylans: they seemed to have a different GAX structure, together with an enrichment in phenolic compounds, which contributes to its cross-linking.

Other polymers do not seem to make a relevant contribution to modifications in the cell wall architecture of DCB-habituated maize cells. Mannose content was very low in both non-habituated and in habituated cells, so that mannan contribution to DCB-habituation was negligible. Xyloglucan levels were very low too, less than 20 μg mg^{-1} cell wall, and they were even lower in habituated cells. CDTA-extracted pectins were present in a low proportion (lower than 20 μg mg^{-1} cell wall) and did not undergo changes throughout the habituation process. Lastly, protein content was even lower than in their non-habituated counterparts, as was shown by FTIR spectroscopy, total Kjeldhal nitrogen determination, and immunochemical approaches. It is interesting to note that this reduction affected some groups of proteins but not others: i.e. LM2 probed AGPs apparently did not vary, whereas JIM8 probed AGPs reduced, as was ascertained using immunolocation and IDAs.

According to Carpita's model (Carpita et al. 2001), type II cell walls are mainly constituted by two domains: a framework of cellulose microfibrils interlaced with tightly adherent β-glucans, GAX of low degrees of arabinosyl substitution and glucomannans; this is then embedded in a matrix which provides an interstitial domain interconnecting the β-glucan-coated microfibrils, constituted by GAX, which is more highly substituted by arabinosyl residues, additional glucomannan and some pectins. According this model, cell wall porosity would be controlled by the content of GAX with a higher degree of arabinosyl substitution, taking into account that this polysaccharide constitutes the major pore-determining interstitial material between the microfibrils (Carpita et al. 2001). In an interesting experiment conducted in order to study xylanase penetration in wheat endosperm, Beaugrand et al. (2005) found complementary evidence that AXs acts to control pore size, as they observed that this penetration was intrinsically linked to AX degradation, and was facilitated by progressive cell wall disassembly. In DCB-habituated maize cells, cellulose microfibrils would be more interspersed by a larger interstitial AX, which determined the notable increment observed in their pore size, in comparison to non-habituated cells. In contrast, reported cell wall porosity of DCB-habituated barley cells was notably minor, this fact being consistent with a higher content in β-glucan, which would be interspersed between microfibrils, consequently reducing pore size between them.

The observed reduction in swelling capacity of DCB-habituated maize cell walls could be related both to the enrichment in their phenolic component –which would increase cell wall hydrophobicity- and to a hydrogen bonding increase due to AX enhancement, both in concentration and in molecular mass, in an opposite way as that described for expansin action: expansins would lead to an enhancement in cell wall swelling capacity breaking hydrogen bonds to release steric constraint of microfibril movement (Thompson 2005; Yennawar et al. 2006).

Another consequence of the modification in cell wall architecture associated with DCB habituation is the change in cell wall degradability. In this respect, the expectation would be that the reduction in crystalline cellulose and the increase in matrix polysaccharides would contribute to enhanced cell wall degradability. However cell walls from DCB-habituated maize cells proved to be less susceptible to enzymatic hydrolysis than non-habituated cell walls. The most probable explanation for this result is related to a phenolic enriched cell wall. Cell wall feruloylation, and particularly increased dimerization of ferulate, has been shown to reduce cell wall degradability by reducing matrix polysaccharide accessibility to hydrolytic enzymes (Grabber 2005 and references therein). Additionally the enrichment in *p*-coumaric acid of our DCB-habituated cell walls would also contribute to a reduction in their degradability as has been previously reported for ruminal digestibility of some grasses (Burritt et al. 1984).

In summary, maize cell cultures from immature embryos have been successfully habituated to DCB. Habituated cells showed a modified cell wall architecture in which the significant reduction in cellulose content was compensated for by an increment in GAXs, which were of a higher molecular mass, more strongly cell wall bound, and more interlaced by means of phenolic bonds. Other cell wall components do not seem to play a significant role in the habituation process. In contrast to previously described type II cell wall architecture, in DCB-habituated maize cells induced to have reduced cellulose content, β-glucan does not fulfill an important role in the acquisition of functional type II cell wall architecture. As a consequence and in contrast to that previously described, the swelling capacity and wall digestibility of maize habituated cell walls was diminished, whereas pore size became bigger. Future studies need to be conducted in order to examine in greater depth the interaction between GAX and phenolics, and to ascertain the genetic control of these modifications in type II cell wall architecture.

Acknowledgements

This work was supported by grants from Junta de Castilla y León (LE 048A07), University of León (ULE-2006-2) and Spanish Science and Innovation Ministry (CGL2008-02470/BOS), and a PhD grant from the FPU program of the Spanish Science and Innovation Ministry to H.M.M. We are grateful to Dr. Paul Knox for his kind gifts of LM10 and LM11 antibodies, to Dr. Stephen C. Fry for his kind provision of maize cell cultures and to Denise Phelps for English language correction.

References

Albersheim P, Nevins PD, English PD, Karr A (1967) A method for the analysis of sugars in plant cell wall polysaccharides by gas liquid chromatography. Carbohydr Res 5:340-345

Alonso ML, Alvarez AI, Zapico J (1994) Rapid analysis of free amino-acids in infant foods. J Liq Chromatogr 17:4019-4030

Alonso-Simón A, Encina AE, García-Angulo P, Alvarez JM, Acebes JL (2004) FTIR spectroscopy monitoring of cell wall modifications during the habituation of bean (*Phaseolus vulgaris* L.) callus cultures to dichlobenil. Plant Sci 167:1273-1281

Baron-Epel O, Gharyal PK, Schindler M (1988) Pectins as mediators of wall porosity in soybean cells. Planta 175:389-395

Beaugrand J, Paes G, Reis D, Takahashi M, Debeire P, O'Donohue M, Chabbert B (2005) Probing the cell wall heterogeneity of micro-dissected wheat caryopsis using both active and inactive forms of a GH11 xylanase. Planta 222:246-257

Blumenkrantz N, Asboe-Hansen G (1973) New method for quantitative determination of uronic acids. Anal Biochem 54:484-489

Burritt EA, Bittner AS, Street JC, Anderson MJ (1984) Correlations of phenolic acids and xylose content of cell-wall with in vitro dry-matter digestibility of 3 maturing grasses. J Dairy Sci 67:1209-1213

Carpita NC (1996) Structure and biogenesis of the cell walls of grasses. Annu Rev Plant Physiol Plant Mol Biol 47:445-476

Carpita NC, Defernez M, Findlay K, Wells B, Shoue DA, Catchpole G, Wilson RH, McCann MC (2001) Cell wall architecture of the elongating maize coleoptile. Plant Physiol 127:551-565

Carpita NC, Gibeaut DM (1993) Structural models of primary cell walls in flowering plants. Consistency of molecular structure with the physical properties of the walls during growth. Plant J 3:1-30

Delmer DP (1987). Cellulose biosynthesis. Annu Rev Plant Physiol Plant Mol Biol 38:259-290

Díaz-Cacho P, Moral R, Encina A, Acebes JL, Alvarez J (1999) Cell wall modifications in bean (*Phaseolus vulgaris*) callus cultures tolerant to isoxaben. Physiol Plant 107:54-59

Dische Z (1962) Color reactions of carbohydrates. In: Whistler RL, Wolfrom ML (eds) Methods in carbohydrate chemistry. Academic Press, New York, pp 475-514

Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F (1956) Colorimetric method for determination of sugars and related substances. Anal Chem 28:350-356

Encina AE, Moral RM, Acebes JL, Alvarez JM (2001) Characterization of cell walls in bean (*Phaseolus vulgaris* L.) callus cultures tolerant to dichlobenil. Plant Sci 160:331-339

Encina A, Sevillano JM, Acebes JL, Alvarez J (2002) Cell wall modifications of bean (*Phaseolus vulgaris*) cell suspensions during habituation and dehabituation to dichlobenil. Physiol Plant 114:182-191

Fry SC, Willis SC, Paterson AEJ (2000) Intraprotoplasmic and wall-localized formation of arabinoxylan-bound diferulates and larger ferulate coupling-products in maize cell-suspension cultures. Planta 211:679-692

García-Angulo P, Willats WGT, Encina A, Alonso-Simón A, Alvarez JM, Acebes JL (2006) Immunocytochemical characterization of the cell walls of bean cell suspensions during habituation and dehabituation to dichlobenil. Physiol Plant 127:87-99

Grabber JH (2005) How do lignin composition, structure, and cross-linking affect degradability? A review of cell wall model studies. Crop Sci 45:820-831

Grabber JH, Ralph J, Hatfield RD (1998) Severe inhibition of maize wall degradation by synthetic lignins formed with coniferaldehyde. J Sci Food Agric 78:81-87

Iraki NM, Bressan RA, Hasegawa PM, Carpita NC (1989) Alteration of the physical and chemical structure of the primary cell wall of growth limited plant cells adapted to osmotic stress. Plant Physiol 91:39-47

Ishii T (1997) Structure and functions of feruloylated polysaccharides. Plant Sci 127:111-127

Kacurakova M, Wellner N, Ebringerova A, Hromadkova Z, Wilson RH, Belton PS (1999) Characterisation of xylan-type polysaccharides and associated cell wall components by FT-IR and FT-Raman spectroscopies. Food Hydrocol 13:35-41

Kerr EM, Fry SC (2004) Extracellular cross-linking of xylan and xyloglucan in maize cell-suspension cultures: the role of oxidative phenolic coupling. Planta 219:73-83

Kerr EM, Fry SC (2003) Pre-formed xyloglucans and xylans increase in molecular weight in three distinct compartments of a maize cell-suspension culture. Planta 217:327-339

Kooiman P (1960). A method for the determination of amyloid in plant seeds. Rec Trav Chim Pays-Bas Belg 79:675-678

Lorences EP, Fry SC (1991) Absolute measurement of cell expansion in plant cell suspension cultures. Plant Cell Tiss Org Cult 24:211-215

McCartney L, Marcus SE, Knox JP (2005) Monoclonal antibodies to plant cell wall xylans and arabinoxylans. J Histochem Cytochem 53:543-546

McCleary BV, Codd R (1991) Measurement of (1→3),(1→4)-β-D-glucan in barley and oats. A streamlined enzymatic procedure. J Sci Food Agric 55:303-312

Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol Plant 15:473-497

Nakagawa N, Sakurai N (1998) Increase in the amount of celA1 protein in tobacco BY-2 cells by a cellulose biosynthesis inhibitor, 2,6-dichlorobenzonitrile. Plant Cell Physiol 39:779-785

Nakagawa N, Sakurai N (2001) Cell wall integrity controls expression of endoxyloglucan transferase in tobacco BY2 cells. Plant Cell Physiol 42:240-244

Pennell RI, Janniche L, Kjellbom P, Scofield GN, Peart JM, Roberts K (1991) Developmental regulation of a plasma membrane arabinogalactan protein epitope in oilseed rape flowers. Plant Cell 3:1317-1326

Pennell R, Knox J, Scofield G, Selvendran R, Roberts K (1989) A family of abundant plasma membrane-associated glycoproteins related to the arabinogalactan proteins is unique to flowering plants. J Cell Biol 108:1967-1977

Sabba RP, Durso NA, Vaughn KC (1999) Structural and immunocytochemical characterization of the walls of dichlobenil-habituated BY-2 tobacco cells. Int J Plant Sci 160:275-290

Saeman JF, Moore WE, Millet MA (1963) Sugar units present. In: Whistler RL (ed) Methods in carbohydrate chemistry. Academic Press, New York, pp 54-69

Shedletzky E, Shmuel M, Delmer DP, Lamport DTA (1990) Adaptation and growth of tomato cells on the herbicide 2,6-dichlorobenzonitrile leads to production of unique cell walls virtually lacking a cellulose-xyloglucan network. Plant Physiol 94:980-987

Shedletzky E, Shmuel M, Trainin T, Kalman S, Delmer D (1992) Cell wall structure in cells adapted to growth on the cellulose synthesis inhibitor 2,6-dichlorobenzonitrile. A comparison between two dicotyledonous plants and a gramineous monocot. Plant Physiol 100:120-130

Smallwood M, Yates EA, Willats WGT, Martin H, Knox JP (1996) Immunochemical comparison of membrane-associated and secreted arabinogalactan-proteins in rice and carrot. Planta 198:452-459

Thompson DS (2005) How do cell walls regulate plant growth? J Exp Bot 56:2275-2285

Updegraff DM. (1969) Semimicro determination of cellulose in biological materials. Anal Biochem 32:420-424

Vaughn KC (2002) Cellulose biosynthesis inhibitor herbicides. In: Böger P, Wakabayashi K, Hirai K (eds.) Herbicide classes in development. Springer, Berlin, pp 139-150

Wells B, Mccann MC, Shedletzky E, Delmer D, Roberts K (1994) Structural features of cell walls from tomato cells adapted to grow on the herbicide 2,6-dichlorobenzonitrile. J Microsc 173:155-164

Willats WGT, Gilmartin PM, Mikkelsen JD, Knox JP (1999) Cell wall antibodies without immunization: generation and use of de-esterified homogalacturonan block-specific antibodies from a naive phage display library. Plant J 18:57-65

Yennawar NH, Li LC, Dudzinski DM, Tabuchi A, Cosgrove DJ (2006) Crystal structure and activities of EXPB1 (Zea m 1), a β-expansin and group-1 pollen allergen from maize. Proc Nat Acad Sci USA 103:14664-14671

Capítulo III:

Unravelling the biochemical and molecular networks involved in maize cell habituation to the cellulose biosynthesis inhibitor dichlobenil

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Capítulo correspondiente a la publicación: Mélida, H., Encina, A., Álvarez, J., Acebes, J.L., Caparrós-Ruiz, D. (2010). Unravelling the biochemical and molecular networks involved in maize cell habituation to the cellulose biosynthesis inhibitor dichlobenil. Molecular Plant. En prensa. DOI: 10.1093/mp/ssq027.

Unravelling the biochemical and molecular networks involved in maize cell habituation to the cellulose biosynthesis inhibitor dichlobenil

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Abstract

The biochemical and molecular processes involved in the habituation of maize cells to growth in the presence of the cellulose biosynthesis inhibitor dichlobenil (DCB) were investigated. DCB affects the synthesis of cellulose both in active and stationary growth phases and alters the expression of several *CesA* genes. Of these, *ZmCesA5* and *ZmCesA7* seem to play a major role in habituating cells to growth in the presence of DCB. As a consequence of the reduction in cellulose, the expression of several genes involved in the synthesis of hydroxycinnamates is increased, resulting in cell walls with higher levels of ferulic and *p*-coumaric acids.

A proteomic analysis revealed that habituation to DCB is linked to modifications in several metabolic pathways. Finally, habituated cells present a reduction in glutathione S-transferase detoxifying activity and antioxidant activities.

Plant cell adaptation to the disturbance of such a crucial process as cellulose biosynthesis requires changes in several metabolic networks, in order to modify cell wall architecture and metabolism, and survive in the presence of the inhibitor. Some of these modifications are described in this paper.

Keywords: abiotic/environmental stress; acclimation - physiological; cell walls; maize; cellulose; dichlobenil; phenylpropanoid.

Introduction

Plant cells are surrounded by an extracellular matrix, the primary cell wall, which is involved in many important processes such as cell elongation, biotic/abiotic stress response, and cell shape maintenance (Ray et al., 1972). It is also believed to be responsible for an elaborate cell wall integrity mechanism (Hamann et al., 2009). The primary plant cell wall consists of cellulose microfibrils embedded in a network of matrix polysaccharides (hemicelluloses and pectins) and structural proteins, the nature and proportions of which differ according to each plant species (Carpita and Gibeaut, 1993), organ, cell type within a tissue, cell development phase and even location within a single cell (Knox, 2008). Most plant species (all dicots and some monocots) have type-I primary cell walls, where xyloglucan, homogalacturonan and rhamno-galacturonan I comprise the principle constituents of matrix polysaccharides (reviewed by Scheller and Ulvskov, 2010). Graminaceous plants (such as maize) and other commelinoid monocots have a cell wall, called type-II, the

architecture and composition of which differ markedly from that characteristic of other angiosperms. In type-II cell walls, the role within the cell wall of the above cited polysaccharides is replaced by (glucurono) arabinoxylans and mixed-linked glucan (Carpita, 1984; Burton and Fincher, 2009).

Compared with type-I, type-II cell walls contain higher amounts of hydroxycinnamates or cell wall phenolics. These phenolics, mainly ferulic acid and *p*-coumaric acid, are found substituting arabinoxylans by ester-linking α-L-arabinosyl residues (Smith and Hartley, 1983; Wende and Fry, 1997). Even in type-II cell walls, phenolics are minor components of the cell wall, however, their contribution to cell wall structure is crucial. It has been demonstrated by *in vivo* experiments, that ester-linked hydroxycinnamates can undergo oxidative-coupling cross-linking adjacent arabinoxylan molecules (Fry et al., 2000; 2004; Parker et al., 2005; Burr and Fry, 2009). By means of its polysaccharide cross-linking activity, phenolic-coupling regulates, mediates or alters a number of cell wall properties, contributing to cell wall assembly, causing cell wall stiffening and growth cessation, promoting tissue cohesion, strengthening cell wall structure in response to biotic and abiotic stresses and limiting cell wall biodegradability (Buanafina 2009). Ferulic and *p*-coumaric acids are synthesized through the phenylpropanoid pathway (Vogt, 2010). The first step of this route is the deamination of L-phenylalanine by phenylalanine ammonia lyase (PAL) to cinnamic acid. Subsequent enzymatic steps catalyzing hydroxylations and methylations produce feruloyl-CoA, which is ester-linked to arabinoxylans (Fry et al., 2000; Lindsay and Fry, 2008). Recently, it has been demonstrated in rice that members of the pfam gene family may act as arabinoxylan feruloyl transferases (Piston et al., 2010).

Cellulose is synthesized at the plasma membrane by an enzymatic complex and is deposited directly into the cell wall in a directional manner (Somerville, 2006; Mutwil et al., 2008; Taylor, 2008), undergoing a dynamic reorientation following deposition which enables its anisotropic expansion (Anderson et al., 2010). Cellulose biosynthesis machinery is located in the plasma membrane, forming 'rosettes' or cellulose synthase (CesA) complexes. In plants, CesA complexes are organized as hexamers, presumably consisting of 36 individual CesA proteins, and some other proteins. It is thought that the CesA complexes are assembled in the Golgi apparatus and then exported to the plasma membrane via exocytosis (Somerville, 2006). *Arabidopsis* (type-I cell wall) and maize (type-II cell wall) have ten and twelve *CesA* genes, respectively (Holland et al., 2000; Richmond, 2000; Appenzeller et al., 2004).

Characterization of mutants affecting CesA1 and CesA3 proteins demonstrated that these two proteins are essential to production of cellulose in *Arabidopsis* primary walls (Desprez et al., 2007; Persson et al., 2007; Daras et al., 2009). Other *Arabidopsis* CesA, such as CesA2, CesA5, CesA6 and CesA9 are also involved in primary cell wall formation but their functions are partially redundant (Desprez et al., 2007; Persson et al., 2007). In contrast, characterization of *Arabidopsis* mutants for *AtCesA4* (*IRX5*: irregular xylem 5), *AtCesA7* (*IRX3*) and *AtCesA8* (*IRX1*) revealed that these three proteins are essential for secondary cell wall formation (Taylor et al., 1999; 2000; 2003; Ha et al., 2002) and do not affect cellulose biosynthesis in primary cell walls (Turner and Somerville, 1997; Ha et al., 2002).

As cellulose represents the main load bearing polysaccharide in cell walls, the habituation of plant cell cultures to lethal concentrations of cellulose biosynthesis inhibitors has emerged as a valuable tool for the study of important mechanisms involved in plant survival, such as cell wall plasticity (both structural and compositional) to cope with cell wall integrity disrupting factors (Acebes et al., 2010).

Although the basic mechanism of habituation is common (a replacement of the cellulose network for other cell wall components), the details of the process depend on the type of cell wall, and thus it has been demonstrated that cells habituate to cellulose biosynthesis inhibitors by using different strategies (Acebes et al., 2010 and refs. therein). In *Arabidopsis*, isoxaben-habituation does not appear to be mediated by stress response processes, nor by functional redundancy within the *CesA* family (Manfield et al., 2004). Amongst the cellulose synthase superfamily, *CslD5 (cellulose synthase-like D5)* is highly upregulated and it might play a role in the biosynthesis of the walls of habituated cells (Bernal et al., 2007). Dehabituation is a feasible strategy to unravel those habituation-related components that are stable (i.e. those changes putatively associated to mutations or epigenetic changes in DNA). Thus, it has been demostrated that most of the cell wall changes induced during habituation reverts when cells are dehabituated by culturing them in a medium without the inhibitor (Shedletzky et al., 1990; Encina et al., 2002; García-Angulo et al., 2006; 2009; Alonso-Simón et al., 2010). However, dehabituated cell cultures retain some habituation-induced modifications. In the case of bean cell cultures the study of DCB dehabituated cells proved that habituation to DCB relied both in reversible (those affecting to cell wall composition and structure) and stable changes (a high guaiacol type peroxidase activity) (García-Angulo et al., 2009).

Maize cell cultures have been habituated to lethal concentrations of DCB (12µM; H12) (Mélida et al. 2009). DCB-habituated cells present modified cell wall architecture with a strong reduction in cellulose which is compensated, at least partially, by a more extensive network of highly feruloylated arabinoxylans in the stationary growth phase.

In this paper, we describe the effect of the herbicide DCB on non-habituated, habituated and dehabituated maize cells at biochemical and molecular levels. In the habituated cells, differences associated with the cell-culture stage were studied. We analyzed the effect of DCB on the expression of genes involved in the biosynthesis of cellulose and hydroxycinnamates. We also applied a proteomic approach to detect changes in the overall metabolism of maize cells habituated to DCB. Based on these results, we also determined detoxifying and antioxidant enzymatic activities associated with DCB habituation.

Results

DCB habituation process implies a reduction in the cellulose content of maize cells

The effect of DCB on cellulose content was assayed in different maize cell cultures (Figure 1). Our results show that cellulose content was not significantly affected when maize cultures were incubated with DCB for a brief period of time (NH/DCB) compared to non-treated cultures (NH). However, in H12 cultures, a strong reduction in cellulose content was observed: 61% in active growth phase and 53% in stationary phase compared with NH cultures. No evidence of cellulose loss during cell wall isolation was found, indicating that the reduction of cellulose content in H12 culture was not due to its loss during the experimental procedure.

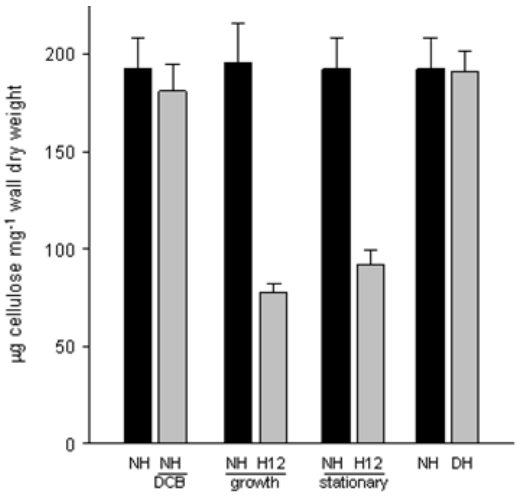


Figure 1. Cellulose content of cell walls isolated from non-habituated (NH), habituated to 12µM DCB (H12; collected in the active growth or in the stationary phase), dehabituated (DH; in the stationary phase) and non-habituated supplemented with DCB for 5-6 days (NH/DCB) maize cell cultures analyzed by the Updegraff method. Values are means ± S.D. of 3 measurements. Growth measured as relative increase in fresh weight of the cells was reduced by 38.9% and 12.4% in H12 and DH cells respectivelly with regard to NH.

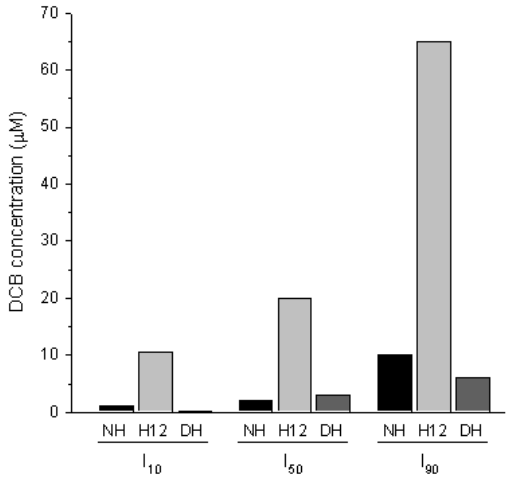


Figure 2. Effect of DCB on the growth of non-habituated (NH), habituated to 12µM DCB (H12) and dehabituated (DH) maize cell cultures expressed as the concentration of DCB (µM) required to inhibit weight increase by 10% (I_{10}), 50% (I_{50}) and 90% (I_{90}) with respect to control.

Our results also show that the reduction of cellulose can be reversed when DCB is subsequently removed for a long period (more than a year) from the culture media (DH cultures).

We also tested the ability of DH cultures to cope with lethal concentrations of DCB, expressed as the concentration of DCB required to inhibit fresh weight gain by 10% (I_{10}), 50% (I_{50}) and 90% (I_{90}) with respect to the control (Figure 2).

The I_{10}, I_{50} and I_{90} values of the DH cultures were comparable to those of the NH cultures, but very dissimilar to those of H12 cultures (about ten times

higher), indicating that DH cultures incubated with DCB display behaviour similar to maize cultures which have never been cultured in the presence of DCB.

DCB affects the expression of some CesA *genes*

As DCB strongly reduces cellulose content in H12 cultures, we further investigated whether the expression of the maize *CesA* genes is affected (Figure 3), using RT-PCR. Our results show that, although the cellulose content was not altered in maize cells treated briefly with DCB (NH/DCB), the presence of this inhibitor downregulated *ZmCesA3, 5, 8* and induced *ZmCesA7*. However, in the habituated H12 cultures, in which a strong decrease in total cellulose content was observed, *ZmCesA1/2, 3, 7*, and *8* were induced in the growth phase, while *ZmCesA7* continued to be induced and *ZmCesA5* repressed, in the stationary phase. Finally, in DH cultures, with cellulose content similar to that of the non treated cells, only the expression of *ZmCesA5* gene was induced. *ZmCesA4, 9, 10, 11* and *12* were also analyzed, but no mRNA was detected in these maize cells.

It has been shown that a mutation in the maize *ZmBk2* gene implies a severe reduction in cellulose content (Ching et al., 2006). We therefore analyzed whether the presence of DCB could also affect the expression of the *ZmBk2L3* gene, as it is the only member of the family expressed during primary cell wall biosynthesis (Brady et al., 2007). In this case, our results indicate that the expression of *ZmBk2L3* gene was not affected by the presence of DCB in the culture media.

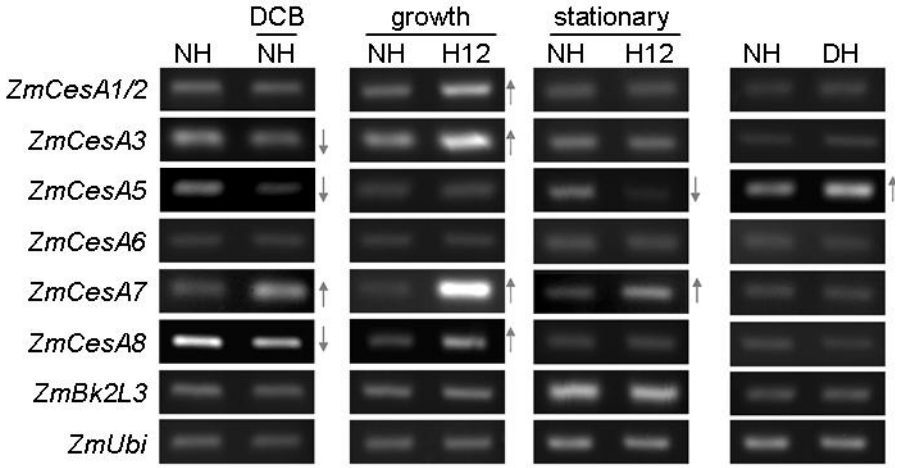


Figure 3. Relative *ZmCesA* gene expression analyzed by RT-PCR of different maize cell cultures and culture phases (key as in Figure 1). ↑, more mRNA accumulation than control (NH); ↓, less mRNA than control. *ZmCesA4, ZmCesA9, ZmCesA10, ZmCesA11, ZmCesA12* and *ZmMAP20* were not detected. *ZmMAP20*: Microtubule associated protein 20, *ZmBk2L3*: Brittle stalk 2 Like 3.

Recently, a microtubule associated protein MAP20 in secondary cell walls of hybrid aspen has been reported as a target for DCB (Rajangam et al., 2008). It was demonstrated that DCB specifically binds to MAP20 during cellulose synthesis in secondary walls. Based on this research, we analyzed the expression of a putative *ZmMAP20* gene but no mRNA could be detected in maize cell cultures, which only have primary cell wall.

DCB affects cell wall phenolics content

In addition to the cellulose content, we have also analyzed the effect of DCB on cell wall phenolic content. Our results show that a short-term DCB treatment (NH/DCB) was sufficient to produce quantitative modifications in the content of cell wall esterified phenolics (Figure 4), resulting in a decrease in ferulic acid and a strong increase in *p*-coumaric acid compared to the NH cultures. In H12 cultures, an increase in both ferulic and *p*-coumaric acids content was measured, showing that H12 cell walls are phenolic-enriched compared to control cells. Finally, our results show that DH cultures had a slightly higher amount of *p*-coumaric acid but showed a reduction in ferulic acid compared to non-treated cells.

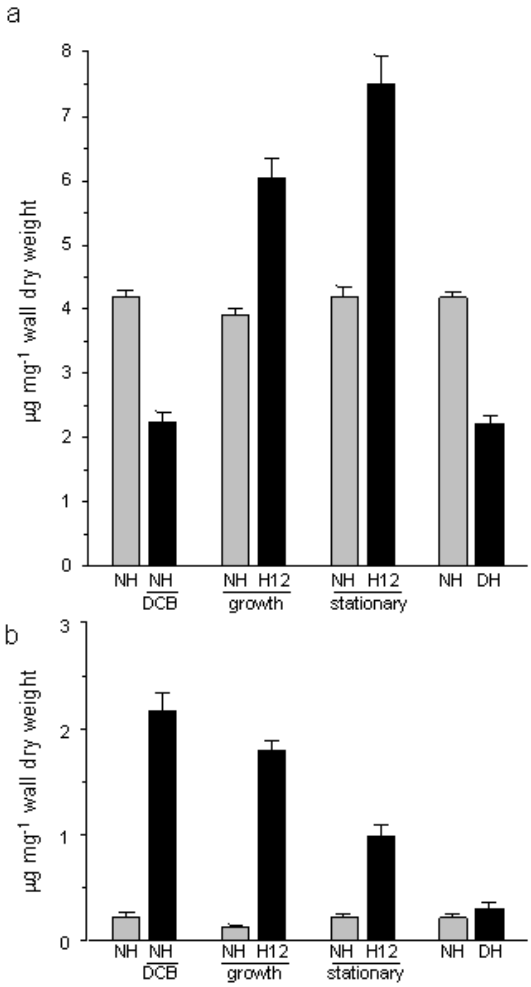


Figure 4. Ferulic (**A**) and *p*-coumaric acid (**B**) content of cell walls isolated from different maize cell cultures (key as in Figure 1) released by treatment with 1M NaOH and measured by HPLC-PAD. Values are mean ± SD of three measurements.

DCB affects the expression of genes involved in the phenylpropanoid pathway

As DCB habituation in H12 maize cultures involves a shift from ferulate to coumarate-rich cells walls and an enrichment in cell wall esterified hydroxycinnamates and dehydroferulates (Melida et al., 2009), we also

analyzed the expression of the phenylpropanoid genes involved in the production of *p*-coumaric and ferulic acid, and feruloyl-CoA (Figure 5A). Results show that the majority of these genes were induced in the H12 cultures during the growing stage, with the exception of *CCoAOMT*, which was repressed (Figure 5B). When these H12 cultures reached the stationary phase, all the genes analyzed were repressed, with the exception of *COMT*, which was induced.

In the short-term DCB-treated cultures (NH/DCB), several genes were also induced. In contrast, in the dehabituated cells, only *4CLc* and both methyl transferases (*COMT* and *CCoAOMT*) were induced.

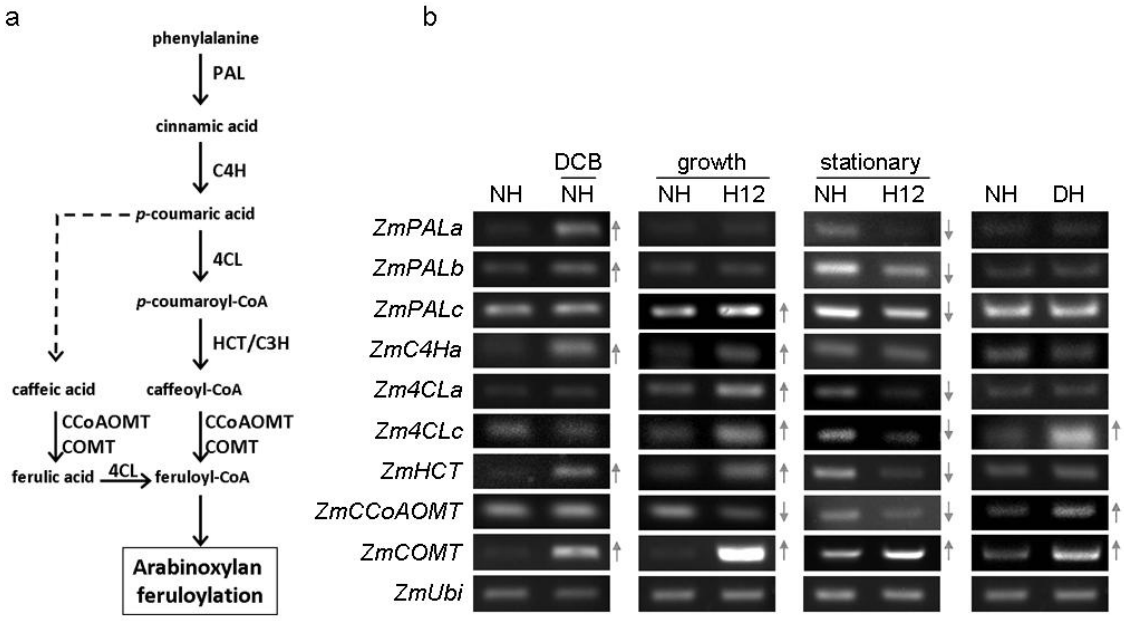


Figure 5. A. Phenolic biosynthetic pathway, from phenylalanine to arabinoxylan feruloylation. Dashed arrows indicate putative pathways. **B**. Relative expression of the genes involved in the synthesis of phenolic compounds of different maize cell cultures (key as in Figure 1). ↑ more mRNA content than control; ↓, less mRNA content than control. PAL: Phenylalanine Ammonia-Lyase, C4H: Cinnamate 4-Hydroxylase, 4CL: 4-Coumarate CoA Ligase, C3H: 4-Coumarate 3-hydroxylase, HCT: Hydroxycinnamoyl-CoA shikimate/quinate hydroxycinnamoyl Transferase, CCoAOMT: Caffeoyl-CoA O-MethylTransferase, COMT: Caffeic acid O-MethylTransferase.

Overall effects of DCB on the metabolism of the habituated cells

In order to attain an overall idea of the effect of DCB on the maize cell habituation process, we compared the proteome of the H12 and NH cell cultures. The 2-DE-based proteomics approach enabled us to compare 906 proteins from the total amount initially isolated (1445 for NH vs. 1217 for H12) (Supplementary Figure 1 online). Twenty seven proteins were identified in only one of the two cell lines (19 absent and 8 present only in H12) and 44 proteins were found to be missregulated (20 downregulated and 24 upregulated in H12). From all the sequenced proteins, we were able to identify 15 proteins (Table 1). Habituation induced carbohydrate metabolism (enolase 1 and glyceraldehyde-3-phosphate dehydrogenase) and some stress-related proteins

(peroxidase and heat shock protein), and repressed some nitrogen and ethylene metabolism proteins (3-isopropylmalate dehydrogenase 2, glutamine synthetase 2, 1-aminocyclopropane-1-carboxylate oxidase 1). Surprisingly, some proteins, such as glutathione S-transferases (GST), which are commonly thought to be involved in herbicide detoxification processes, were repressed in H12 cells compared to the control NH cells.

Table 1. Identification of proteins by MALDI-TOF/MS or by LC-nanoESI-Q-TOF-MS/MS after spot excision of 2-D gels, with an indication of their theoretical and experimental isoelectric point and molecular mass.

Protein group	Protein name (accession no.) (Spot no.)	Theoretical pI /MM	Experimental pI /MM
Present only in H12	Peroxidase 52 precursor (ACG45093) (1)	6.86 / 35350	- / 37055
	Heat shock protein 70 (CAA47948) (2)	5.10 / 71517	- / -
	Translation initiation factor 5A (NP_001105606) (3)	5.61 / 17714	- / 20552
	Elongation factor 1-alpha (P17786) (4)	9.19 / 49599	- / 59693
Induced in H12	Glyceraldehyde-3-phosphate dehydrogenase (CAC80387) (5)	9.01 / 45751	5.70 / 38442
	Enolase1 (NP_001105896) (6)	5.20 / 48262	5.17 / 36871
	Translation initiation factor 5A (NP_001105606) (7)	5.61 / 17714	5.96 / 19911
	Putative xylanase inhibitor (BAB89707) (8)	7.52 / 38873	5.16 / 38408
Repressed in H12	Glutathione S-transferase2 (NP_001105366) (9)	5.77 / 24726	5.81 / 25640
	3-isopropylmalate dehydrogenase 2 (ACG41069) (10)	5.62 / 43142	4.90 / 53129
	Glutamine synthetase1 (NP_001105725) (11)	6.42 / 46300	5.34 / 49092
	1-aminocyclopropane-1-carboxylate oxidase (ACG35410) (12)	4.99 / 34753	5.15 / 41100
Absent in H12	Glutathione S-transferase6 (ACG38008) (13)	5.52 / 25750	5.63 / 25049
	Glutathione S-transferase7 (NP_001105593) (14)	5.29 / 25414	- / 27463

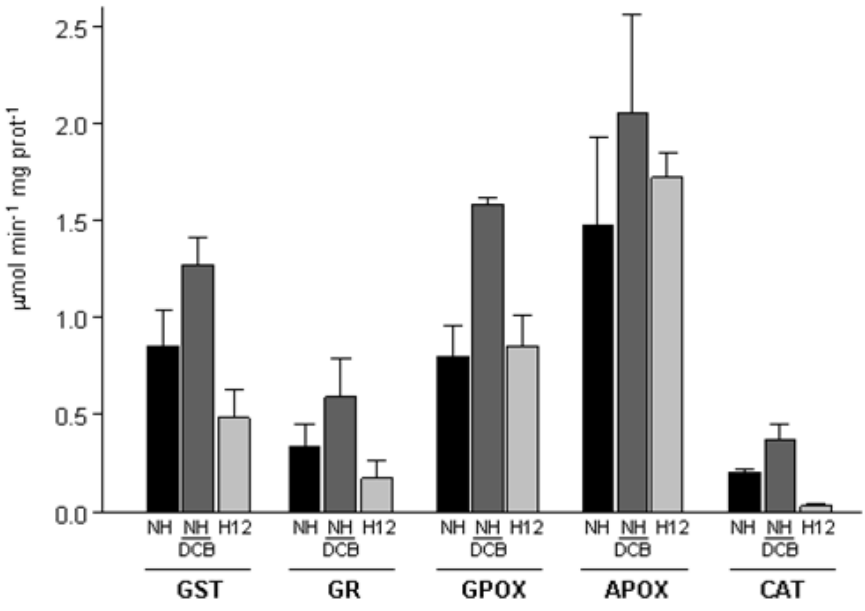


Figure 6. Activity of glutathione S-tranferase (GST), glutathione reductase (GR), guaiacol type peroxidase (GPOX), ascorbate peroxidase (APOX) and catalase (CAT) in non-habituated (NH), habituated to DCB (H12) and non-habituated supplemented with DCB (NH/DCB) maize cultured cells. Values are means ± SD (n= 10) and are expressed as µmol min^{-1} mg $prot^{-1}$.

Glutathione S-transferase, glutathione reductase and catalase activities are reduced in the maize H12 cells

In order to confirm the proteomic results indicating that GST enzymes were repressed in H12, we analyzed the GST activity spectrophotometrically. Our results confirmed that GST activity was reduced in H12 cells compared to the control NH cells (Figure 6). In addition to GST activity, we also analyzed several antioxidant activities (summarised in Figure 6). Results obtained indicate that antioxidant activities such as guaiacol type peroxidase (GPOX) and ascorbate peroxidase (APOX) did not significantly change in H12 cells. Furthermore, these H12 cells presented a reduction in antioxidant activities, such as glutathione reductase (GR) and catalase (CAT) activities. In contrast, a short-term incubation of NH cells in the presence of DCB rendered an increase in all activities tested (Figure 6).

Discussion

Maize calluses were habituated to lethal concentrations of the DCB herbicide by gradually increasing the concentration of the inhibitor in the culture medium. It has previously been suggested that the mechanism of habituation to DCB and other cellulose biosynthesis inhibitors relies on the ability of the habituated cells to divide and expand under conditions where cellulose biosynthesis is inhibited (Vaughn, 2002), but the molecular changes involved in habituation to DCB are poorly understood at present.

In this paper, we show that H12 cell walls present a reduction of more than 50% in cellulose content in the stationary phase, similar to that reported in previous research (Mélida et al., 2009), and that in addition, they also present a strong reduction in cellulose (more than 60%) during the active growing phase. However, when the herbicide was removed from the culture media, maize cells were able to restore normal levels of cellulose. A similar behaviour was observed with DCB-habituated tomato (Shedletzky et al., 1990) and bean cells (Encina et al., 2002; García-Angulo et al., 2006), indicating that plant species having a type-I (tomato and bean) or type-II (maize) wall share some common mechanisms resulting from the removal of the herbicide in the culture media.

It was also reported in a type-I plant specie such as bean that dehabituated cells retain the capacity to cope with lethal concentrations of DCB (Encina et al., 2002; García-Angulo et al., 2009). Interestingly, we show here that this is not the case for type-II, as when the dehabituated cells were newly incubated with DCB they presented a similar behaviour to that of non-habituated cells.

During the active growing phase of the H12 cultures, maize cells induced the expression of several *CesA* genes: *ZmCesA1/2, 3,* phylogenetically grouped with the *Arabidopsis thaliana CesA1* gene involved in the synthesis of cellulose in the primary cell wall. Interestingly, in tobacco cells habituated to 1 μM DCB, a higher amount of the protein celA1 was found, also homologous to AtCesA1 (Nakagawa and Sakurai, 1998). H12 cultures also induced the expression of *ZmCesA7* and *8,* grouped with *Arabidopsis thaliana* CesA proteins involved in

the synthesis of cellulose in the primary to secondary cell wall transition (Appenzeller et al., 2004; Taylor, 2008). In addition to AtCesA1, AtCesA3 is also involved in primary cell wall cellulose biosynthesis (Desprez et al., 2007; Persson et al., 2007). However, expression of maize *CesA* genes most closely related to *AtCesA3* was not detected (*ZmCesA4* and *9*) or was not altered (*ZmCesA5*) in the active growing phase of H12 cells.

Once cells stopped growing (stationary growth phase), the most CesA gene expression was similar to that of control cells, with the exception of *ZmCesA5,* which remained repressed, and *ZmCesA7*, which continued to be induced. Thus, and bearing in mind that caution must be taken when results of gene expression are extrapolated to protein levels, it could be suggested that ZmCesA5 is, at least partially, replaced by ZmCesA7 in the rosettes. As cellulose requirements increase (active growing phase), other CesA proteins (ZmCesA1/2, ZmCesA3 and ZmCesA8) may also replace ZmCesA5 in the rosettes. As a consequence of (partially) removing ZmCesA5 from the rosettes, maize cells would be able to grow in the presence of the herbicide. A similar behaviour occurs in *Arabidopsis*, as when AtCesA3 (the most closely related protein to ZmCesA5) is removed from the rosettes in plants mutated for this gene (*ixr1-1* and *ixr1-2*), these plants become more resistant to the cellulose biosynthesis inhibitor isoxaben (Scheible et al., 2001). In isoxaben-habituated cells, not only AtCesA3, but also AtCesA1, and 6 were downregulated (Manfield et al., 2004).

It is noteworthy that the expression of *ZmCesA5* was induced in dehabituated maize cells, even when maize cells had been grown without DCB for at least one year. However, when dehabituated cells were newly incubated with DCB, they were similarly or slightly more sensitive to the herbicide than control cells. In this case, the increased amount of ZmCesA5 did not affect the total content of cellulose. Thus, this result reinforces the idea that the presence/absence of ZmCesA5 within the rosette could be a critical aspect determining the sensitivity/resistance of the cells to growth in the presence of DCB in the culture media.

Recently, a Microtubule Associated Protein (MAP20) has been reported as a target for DCB in secondary cell walls of hybrid aspen (Rajangam et al., 2008). Linking up MAP20 function with DCB effects, it was proposed that MAP20 has a specific role in cellulose biosynthesis by coupling CesA proteins with microtubules, and that DCB inhibits cellulose biosynthesis by decoupling cellulose synthesis and microtubules through MAP20 inactivation (Rajangam et al., 2008). The connection between microtubules and cellulose synthesis has been proved in recent years (Paredez et al., 2006; Gutierrez et al., 2009). However, the expression of *ZmMAP20* was not detected in maize cultured cells, indicating that ZmMAP20 could be the target of DCB during secondary cell wall synthesis but not during primary cell wall formation.

Mutations in a maize brittle-stalk (*ZmBk2*) gene strongly reduce the total cellulose content in the secondary cell wall (Ching et al., 2006). However, the only member of this family that is expressed in cells producing primary walls (*ZmBk2L3*) (Brady et al., 2007) was not affected in the maize cultures analyzed in this research, suggesting that this gene is not involved in any DCB responses in this case.

Maize cells habituated to DCB presented a significant increase in arabinoxylans (Mélida et al., 2009). Here we show that the phenolic compounds (mainly ferulic acid, but also *p*-coumaric acid) involved in the arabinoxylans cross-link increased in the H12 cell walls. This supports the idea that a more cross-linked network of arabinoxylans is acting as a mechanism to counteract, at least partially, the reduction of cellulose in the H12 cell walls.

Although cells dehabituated to DCB were able to restore the normal levels of cellulose, they presented a significant reduction in ferulic acid in the cell walls, suggesting that some metabolic modifications persist even when DCB has not been present for more than one year in the culture media.

Application of DCB to non habituated cells caused a strong increase in *p*-coumaric acid, suggesting that *p*-coumaric acid enrichment is a response to the toxicity of DCB rather than a mechanism involved in the habituation process. This is in agreement with previous research showing a relationship between *p*-coumaric acid and cellular stresses (Zanardo et al., 2009).

The enrichment in phenolics in H12 cell walls was bound to a global induction of the phenylpropanoid genes involved in the synthesis of these compounds in the active growth phase. However, in the stationary phase, when the cell wall had already been tightened, gene expression levels involved in this metabolic pathway were repressed.

Among the three maize *PAL* genes analyzed, it is interesting to note that *ZmPALa* and *ZmPALb* genes seemed to be involved in short-term response while *ZmPALc* gene may act in the long-term habituation process. Whereas in control cells, ZmCCoAOMT seems to be the main methyl-transferase, our results suggest that ZmCOMT partially replaced ZmCCoAOMT when DCB was present in the culture media.

Plant reaction against DCB stress may involve a wide variety of biochemical and physiological adaptations. In accordance with this idea, habituation to DCB implies an induction of several proteins: a translation initiation factor 5A, that has been shown to be involved in RNA transport and metabolism, regulating cell proliferation, growth and programmed cell death (Feng et al., 2007) and a heat shock protein 70, considered to play a role as a biochemical stress indicator (Tomanek and Sanford, 2003). The fact that H12 cells presented an induction of a putative xylanase inhibitor is in line with the fact that the H12 cells contained higher xylan levels compared to control cells (Mélida et al., 2009). Similarly, an increase in peroxidase activity related to the increased DCB resistance of DCB-dehabituated bean cells has also been reported (García-Angulo et al., 2009). In line with this result, maize H12 cells showed an induction of the peroxidase 52 precursor, indicating that this peroxidase activity could play an important role in maize cell habituation to the herbicide.

Enolase1 and glyceraldehyde-3-phosphate dehydrogenase were induced in H12 cells. These enzymes catalyze the reversal conversion of hexose glucose to pyruvate and it has been reported that hexoses can function as stress indicators when cell wall integrity is impaired (Hamann et al., 2009). Therefore, our results suggest that these two enzymes could play a role as indicators of DCB stress.

Among the proteins that were repressed in H12 cells, two enzymes involved in nitrogen metabolism were identified; 3-isopropylmalate

dehydrogenase 2 and glutamine synthetase 1. A transcriptional relationship between genes involved in carbon and nitrogen metabolism has previously been suggested (Jackson et al., 1993). 1-aminocyclopropane-1-carboxylate oxidase 1 was also repressed, suggesting a reduction of ethylene biosynthesis in habituated cells.

It is noteworthy that several isoforms of GSTs were repressed in H12 cells. GSTs are enzymes involved in detoxification processes to protect plants against xenobiotic damages like DCB (Genter et al., 1998) and are considered as molecular markers of plant stresses (Edwards et al., 2000). A further determination of the GST enzymatic activity confirmed that H12 cells were deficient in this detoxifying activity. Therefore, GST activity does not play a major role in DCB-habituated cells. In contrast, GST activity was enhanced when non habituated cells were incubated short-term with DCB, indicating that, as expected, GST is involved in the process of detoxification.

According to the level of antioxidant activities (GR, GPOX, APOX and CAT) measured in DCB habituated cell cultures, it seems that these cells do not rely on an antioxidant strategy to cope with this herbicide. However, as expected, these activities were induced in maize cells treated short-term with DCB. The behaviour of the H12 maize cells thus contrasted with that observed in bean cells, in which antioxidant capacity is enhanced in habituation to this herbicide (García-Angulo et al., 2009). Results obtained by García-Angulo et al. (2009) did also show that bean dehabituated cells retained an increased GPOX activity what would partially explain that they were more tolerant to DCB than non-habituated cells. In accordance with this explanation, the lack of an antioxidant strategy in the habituation to dichlobenil of maize cells would also explicate that maize dehabituated cells do not differ in DCB sensitivity from non-habituated cells.

The low level of detoxifying/antioxidant activities measured in maize cells habituated to DCB would alternatively be explained as a way to reduce H_2O_2 scavenging, and secondarily, to ensure phenolic dimerization.

In this paper, we show that the habituation of maize cells to the herbicide DCB implies several metabolic modifications: (i) a strong reduction in cellulose and alteration in the expression of several *Cellulose Synthase* genes. Of these, *ZmCesA5* and *ZmCesA7* seem to play a major role in habituating cells to growth in the presence of this herbicide. (ii) Several phenylpropanoid genes involved in the synthesis of hydroxycinnamates are induced, resulting in a strong increase in these compounds in the cell wall. This could be understood as a mechanism to reinforce a cellulose-deficient cell wall. (iii) Several metabolic pathways are altered in habituated cells, such as carbon, nitrogen and ethylene metabolism, as are other proteins typically involved in stress responses. (iv) There is a notable reduction of glutathione S-transferase detoxifying activity, showing that it is not involved in habituation to the herbicide in maize cells. Moreover, this habituation process does not rely on antioxidant strategies. In conclusion, our results show that both the reduction in cellulose content and the increase in phenylpropanoid synthesis observed during habituation of maize cultured cells to a cellulose biosynthesis inhibitor such as dichlobenil provoke changes in gene expression, not only in the genes directly related to cellulose biosynthesis and phenylpropanoid synthesis, but also in others related to such varied aspects as carbon, nitrogen and ethylene

metabolism, detoxification mechanisms, etc. Furthermore, this regulation of gene expression is dependent on the growth phase of habituated cells, and differs considerably from the changes observed in non-habituated cells exposed to the herbicide. Considered together, these changes may be responsible for the capacity displayed by cells to survive exposure to an inhibitor which affects a process as crucial to plant cell as cellulose biosynthesis.

Methods

Cell cultures

Maize callus cultures (*Zea mays* L., Black Mexican sweetcorn) from immature embryos were grown in Murashige and Skoog media (Murashige and Skoog, 1962) supplemented with 9 µM 2,4-D at 25°C under light (Lorences and Fry, 1991), and subcultured monthly. Cell cultures habituated to 12 µM DCB (H12) were obtained from non-habituated (NH) after stepwise transfers with gradual increments of DCB (Mélida et al., 2009), and were subcultured in 12 µM DCB monthly for more than two years. Through long-term (up to 1 year) culturing of H12 cells in a medium lacking DCB, dehabituated cultures (DH) were obtained. For some experiments, NH cells incubated (for 5-6 days) in medium supplemented with 6 µM DCB (NH/DCB) were used. In all cases, (H-DH-NH/DCB) cells were compared with same aged NH cells at the same culture cycle phase.

In order to obtain growth inhibition curves, calluses weighing 1.0 ± 0.1 g were cultured in DCB, in concentrations ranging from 0.01 to 100 µM. DCB was dissolved in dimethyl sulfoxide, which did not affect cell growth at this range of concentrations. The cultures were incubated for 30 days and weighed (FW). Growth was expressed as relative increase in FW and the $I_{10,}$ I_{50} and I_{90} were calculated as the concentration of DCB required to inhibit weight increase by 10%, 50% and 90% respectively compared to non DCB treated cells (control). Six replicates were used in each concentration, and no deviations are shown in Figure 2, due to inhibition percentages are unique values.

Cell wall analyses

Calluses collected in the early stationary phase were frozen and treated as previously described to obtain cell walls (Mélida et al., 2009). In some cases, these were collected in two different growth phases; in the active growth or in the stationary phases. On average, NH cells reached the active growth and stationary phases 16 days and 25 days after subculturing, respectively. Twenty-day-old and 30-day-old H12 cells were considered to be at the active growth and stationary phases respectively (Mélida et al., 2009).

Cellulose was quantified in crude cell walls by the Updegraff method (Updegraff, 1969) using the hydrolytic conditions described by Saeman et al. (1963) and quantifying the glucose released by the anthrone method (Dische, 1962).

Ferulic and *p*-coumaric acids were analyzed by HPLC-PAD. Cell walls (10 mg) were treated in the dark under N_2 with 1 M NaOH, at room temperature for

16 h in order to saponify phenolic esters. The solution was acidified by addition of trifluoroacetic acid and partitioned against ethyl acetate (x2). The ethyl acetate phases were vacuum-dried and re-dissolved in propan-1-ol for HPLC-PAD analysis.

HPLC-PAD analyses were performed using a Waters 2690 chromatograph with a Waters 996 photodiode array detector. Separation was achieved using a Kromasil C18 (Teknokroma) column (250 x 4.6 mm i.d.; 5 µm particle size). The mobile phase consisted of acidified (TFA) 10% acetonitrile (solvent A) and a mix of 40% acetonitrile, 40% methanol and 20% water (solvent B) and followed the binary gradient elution programme: initial conditions 90:10 (A:B), changing to 25:75 after 25 min, then to 0:100 after 5 min and returning to the initial conditions after 10 min. The mobile phase flow was 1 mL/min. The elution profiles were monitored by UV absorbance at 325 and 280 nm. Retention times were compared with freshly prepared standard solutions of ferulic and *p*-coumaric acids. Calibration curves were used to quantify these compounds.

Isolation of total RNA, RT-PCR and PCR

Total RNA was extracted with Trizol Reagent (Invitrogen), and 2 µg of total RNA was reverse-transcribed using M-MLV Reverse Transcriptase (Invitrogen). First-strand cDNA was generated using an oligo(dT)$_{15}$ primer and 1 µl of the first-strand cDNA was used as a template in subsequent PCR reactions. "No-RT" PCR assays were performed to confirm the absence of genomic DNA contamination. For each assay, several numbers of cycles were tested to ensure that amplification was in the exponential range.

The gene-specific primers used for the analysis of *ZmCesA1* (AF200525), *ZmCesA2* (AF200526), *ZmCesA3* (AF200527), *ZmCesA5* (AF200529), *ZmCesA6* (AF200530), *ZmCesA7* (AF200531) were those previously described by Holland et al. (2000). Due to the high sequence similarity between ZmCesA1 and ZmCesA2, the same primer was used for the analysis of both genes (*ZmCesA1/2*). The primers used for the analysis of *ZmCesA4* (AF200528), *ZmCesA8* (AF200532), *ZmCesA9* (AF200533), *ZmCesA10* (AY372244.1), *ZmCesA11* (AY372245.1), *ZmCesA12* (AY372246.1), *ZmBk2L3* (EF078698), *ZmMAP20* (AY110515.1) are shown in the Supplementary Table 1 online.

The sequences of the primers used for the analysis of Caffeic acid O-MethylTransferase *ZmCOMT* (AY323283) and *ZmUbiquitin* (U29159) were those previously described by Fornalé et al. (2006).

The gene-specific primers used for the analysis of Phenylalanine Ammonia-Lyase *ZmPALa* (contig no. 3858636.2.1), *ZmPALb* (contig no. 2161072.2.3), *ZmPALc* (contig no. 2161072.2.1), Cinnamate 4-Hydroxylase *ZmC4Ha* (contig no. 2521589.2.1), 4-Coumarate CoA Ligase *Zm4CLa* (contig no. 3106166.2.1), *Zm4CLc* (contig no. 1716323.2.1), Hydroxycinnamoyl-CoA shikimate/quinate hydroxycinnamoyl Transferase *ZmHCT* (contig no. 2619423.2.1), Caffeoyl-CoA O-MethylTransferase *ZmCCoAOMT* (contig no. 2591258.2.1) and 4-Coumarate 3-hydroxylase *ZmC3H* (contig no. 2643622.2.1) were derived from the "MAIZEWALL" database (Guillaumie et al., 2007).

Protein extraction and 2D-PAGE

NH and H12 maize cells at the stationary phase (1g) were ground in liquid nitrogen. Proteins were solubilized at 4°C in 1.2 mL of lysis buffer (7M urea, 2M thiourea, 40mM Tris-HCl pH 8.0, 50 mM DTT, 4% CHAPS and 0.2% Triton X-100) containing DNase I (53 u/mL^{-1}), RNase (4.9 u/mL^{-1}) and protease inhibitors (1 mM PMSF, 50 mM leupeptin and 10 mM E-64). Protein extracts were clarified twice by centrifugation at 16000 x g for 15 min at 4°C, and the obtained supernatants were saved. Supernatants were precipitated with 15% TCA for 30 min at 4°C and centrifuged at 16000 x g for 15 min. The pellet containing the proteins was mixed with cold acetone (x3) and finally resuspended in lysis buffer. Protein content was quantified by the Bradford assay (Bradford, 1976).

For 2-D analysis, protein extracts were diluted in rehydration solution (7M Urea, 2M thiourea, 18 mM Tris-HCl pH 8.0, 4% CHAPS, 0.5% IPG Buffer in the same range as the IPG strip, and 0.002% Bromophenol Blue) containing 1.6% DeStreak Reagent (Amersham Biosciences). Three hundred µg of total proteins was diluted in a final volume of 300 µl and loaded onto non-linear pH 4-7, 18 cm immobilized pH gradient (IPG) strips (Immobiline DryStrips, Amersham Biosciences) for the first dimension. Isoelectric focusing was performed at 50V for 10 h, 500V in gradient for 1 h 30 min, 1000V in gradient for 1 h 30 min, 2000 V in gradient for 1 h 30 min, 4000 V in gradient for 1 h 30 min, 8000 V in gradient for 2 h and 8000 V holding for 10 h, using EttanTM IPGphor TM Isoelectric Focusing System (Amersham Biosciences). IPG strips were then equilibrated with 50 mM Tris-HCl (pH 8.8), 6 M urea, 30% (v/v) glycerol, 2% SDS, a trace of Bromophenol Blue and 10 mg/mL^{-1} DTT during 15 min, followed by a second equilibration step with the same buffer containing 25 mg/mL^{-1} iodoacetamide instead of DTT, for a further 15 min, with gentle shaking. For the second dimension, the focused strips were loaded and run on SDS-PAGE 12% polyacrylamide gels (26 x 20 x 0.1 cm) using Ettan DALTsix System (Amersham Biosciences), 30 min at 2.5 W/gel, followed by 17 W/gel during 4 h. Gels were stained with CBB G-250 (Bio-Rad). The experiment was repeated with two biological replicates and three experimental replicates per biological sample. The stained gels were scanned with an ImageScanner desktop instrument (Amersham Biosciences) and images were acquired using the LabScan scanning application, in transmission mode, at (16 bits) grey scale level, 300 dpi, zoom factor set at 1:1 (100%), and saved as TIFF (Tagged Image File Format) files. Image analysis was performed using the ImageMasterTM 2D Platinum 5.0 Software (Amersham Biosciences). The optimal parameters for spot detection were: smooth=4, saliency=1.0 and minimum area=5. After automatic spot detection, manual spot editing was carried out. Gel replicates were used to obtain synthetic gels with averaged positions, shapes and optical densities. To evaluate protein expression differences among gels, relative spot volume (% Vol.) was used. This is a normalized value and represents the ratio of a given spot volume to the sum of all spot volumes detected in the gel. Those spots showing a quantitative variation ≥ Ratio 1 and positive GAP were selected as differentially expressed. Statistically significant protein abundance variation was validated by Student's t-Test ($p<0.05$). The selected differential spots were excised from the CBB G-250 stained gels and identified either by PMF using

MALDI-TOF MS or by peptide sequencing at the Proteomics Platform (Barcelona Science Park, Barcelona, Spain). The MALDI-TOF MS analysis was performed using a Voyager DE-PRO (Applied Biosystems) instrument in the reflectron, positive-ion mode. Spectra were mass calibrated externally using a standard peptide mixture. For the analysis, 0.5 mL peptide extract and 0.5 mL matrix (5 mg/mL CHCA) were loaded onto the MALDI plate. When ions corresponding to known trypsin autolytic peptides (m/z 842.5100, 1045.5642, 2111.1046, 2283.1807) were detected at adequate intensities, an automatic internal calibration of the spectra was performed. Data were generated in PKL file format, and were submitted for database searching in MASCOT server. The software packages Protein Prospector v 3.4.1 (UCSF) (Mass Spectrometry Facility, University of California) and MASCOT were used to identify the proteins from the PMF data as reported previously (Carrascal et al., 2002). The SEQUEST software (Thermo-Instruments, Spain) was used for preliminary protein identification from the MS/MS analysis followed by manual sequence data confirmation. Swiss-Prot and non-redundant NCBI databases were used for the search. Searches were performed for the full range of molecular weight and p*I*. No species restriction was applied. When an identity search produced no matches, the homology mode was used.

Antioxidant enzyme assays

Glutathione S-tranferase (GST; EC 2.5.1.18), glutathione reductase (GR; EC 1.8.1.7), guaiacol type peroxidase (GPOX; EC 1.11.1.7), ascorbate peroxidase (APOX; EC 1.11.1.11) and catalase (CAT; EC 1.11.1.6) activities were assayed in NH, H12 and NH/DCB callus-cultured cells. GST activity was determined following the method described by Habig et al. (1974), based on an increase in A_{340} due to reduced reduction in glutathione and chloro-2,4-dinitrobenzene complex formation (ε_{340}= 9.6 mM^{-1} cm^{-1}). GR activity was determined following the method described by Edwards et al. (1990), by measuring the decrease in A_{340} due to NADPH oxidation (ε_{340}= 6.22 mM^{-1} cm^{-1}). GPOX activity was determined following the method described by Adam et al. (1995), based on an increase in A_{470} due to guaiacol oxidation (ε_{470}= 26.6 mM^{-1} cm^{-1}). APOX activity was determined following the method described by Hossain and Asada (1984), by measuring the decrease in A_{290} due to ascorbate oxidation (ε_{290}= 2.8 mM^{-1} cm^{-1}). The method described by Droillard et al. (1987) was followed for CAT activity measurement. This method is based on a decrease in A_{240} due to H_2O_2 decomposition (ε_{240}= 39.58 mM^{-1} cm^{-1}).

Suplementary Data

Supplementary Data are available at Molecular Plant Online.

Funding

This work was supported by the Spanish Ministry of Science and Innovation [CGL2008-02470/BOS and AGL2008-05157] program as well as the CONSOLIDER-INGENIO [CSD2007-00036] program. H-M was financed by a PhD grant from the Spanish Ministry of Science and Innovation's FPU program. This research was carried out within the framework of the "Xarxa de Referència de Biotecnologia" (XarBa) of the Autonomous Government of Catalonia.

Acknowledgements

We are indebted to Dr. S. Fornalé (CRAG), Dr. Alonso-Simón and Dr. García-Angulo for scientific discussions. We would also like to thank S. Irar for his technical support as head of the proteomic service at CRAG, as well as the Biological and Structural Mass Spectrometry Unit of the Barcelona Biomedical Research Institute (IIBB CSIC-IDIBAPS) for the MALDI TOF sequencing, and Denise Phelps for the English revision of the manuscript. No conflict of interest declared.

References

Acebes, J.L., Encina, A., García-Angulo, P., Alonso-Simón, A., Mélida, H., and Álvarez, J.M. (2010). Cellulose biosynthesis inhibitors: their uses as potential herbicides and as tools in cellulose and cell wall structural plasticity research. In Cellulose: Structure and Properties, Derivatives and Industrial Uses, Lejeune A. and Deprez T., eds (New York: Nova Publishers), in press.

Adam, A.L., Bestwick, C.S., Barna, B., and Mansfield, J.W. (1995). Enzymes regulating the accumulation of active oxygen species during the hypersensitive reaction of bean to *Pseudomonas syringae* pv. *phaseolicola* . Planta. **197,** 240-249.

Alonso-Simón, A., Neumetzler, L., García-Angulo, P., Encina, A.E., Acebes, J.L., Álvarez, J.M., and Hayashi, T. (2010). Plasticity of xyloglucan composition in bean (*Phaseolus vulgaris*)-cultured cells during habituation and dehabituation to lethal concentrations of dichlobenil. Mol Plant. In press.

Anderson, C.T., Carroll, A., Akhmetova, L., and Somerville, C. (2010). Real-Time Imaging of cellulose reorientation during cell wall expansion in *Arabidopsis* roots. Plant Physiol. **152,** 787-796.

Appenzeller, L., Doblin, M., Barreiro, R., Wang, H.Y., Niu, X.M., Kollipara, K., Carrigan, L., Tomes, D., Chapman, M., and Dhugga, K.S. (2004). Cellulose synthesis in maize: isolation and expression analysis of the cellulose synthase (*CesA*) gene family. Cellulose. **11,** 287-299.

Bernal, A.J., Jensen, J.K., Harholt, J., Sorensen, S., Moller, I., Blaukopf, C., Johansen, B., de Lotto, R., Pauly, M., Scheller, H.V., and Willats, W.G.T. (2007). Disruption of *ATCSLD5* results in reduced growth, reduced xylan and homogalacturonan synthase activity and altered xylan occurrence in Arabidopsis. Plant J. **52,** 791-802.

Bradford, M.M. (1976). Rapid and sensitive method for quantitation of microgram quantities of protein utilizing principle of protein-dye binding. Anal. Biochem. **72,** 248-254.

Brady, S.M., Song, S., Dhugga, K.S., Rafalski, J.A., and Benfey, P.N. (2007). Combining expression and comparative evolutionary analysis. The *COBRA* gene family. Plant Physiol. **143,** 172-187.

Buanafina, M.M.d.O. (2009). Feruloylation in grasses: current and future perspectives. Mol. Plant. **2,** 861-872.

Burr, S.J., and Fry, S.C. (2009). Feruloylated arabinoxylans are oxidatively cross-linked by extracellular maize peroxidase but not by horseradish peroxidase. Mol Plant. **2,** 883-892.

Burton, R.A., and Fincher, G.B. (2009). (1,3;1,4)-β-D-Glucans in cell walls of the Poaceae, lower plants, and fungi: A tale of two linkages. Mol Plant. **2,** 873-882.

Carpita, N.C. (1984). Cell wall development in maize coleoptiles. Plant Physiol. **76,** 205-212.

Carpita, N.C., and Gibeaut, D.M. (1993). Structural models of primary cell walls in flowering plants. Consistency of molecular structure with the physical properties of the walls during growth. Plant J. **3,** 1-30.

Carrascal, M., Carujo, S., Bachs, O., and Abian, J. (2002). Identification of p21(Cip1) binding proteins by gel electrophoresis and capillary liquid chromatography microelectrospray tandem mass spectrometry. Proteomics. **2,** 455-468.

Ching, A., Dhugga, K.S., Appenzeller, L., Meeley, R., Bourett, T.M., Howard, R.J., and Rafalski, A. (2006). *Brittle stalk 2* encodes a putative glycosylphosphatidylinositol-anchored protein that affects mechanical strength of maize tissues by altering the composition and structure of secondary cell walls. Planta. **224,** 1174-1184.

Daras, G., Rigas, S., Penning, B., Milioni, D., McCann, M.C., Carpita, N.C., Fasseas, C., and Hatzopoulos, P. (2009). The *thanatos* mutation in *Arabidopsis thaliana* cellulose synthase 3 (*AtCesA3*) has a dominant-negative effect on cellulose synthesis and plant growth. New Phytol. **184,** 114-126.

Desprez, T., Juraniec, M., Crowell, E.F., Jouy, H., Pochylova, Z., Parcy, F., Hofte, H., Gonneau, M., and Vernhettes, S. (2007). Organization of cellulose synthase complexes involved in primary cell wall synthesis in *Arabidopsis thaliana*. Proc. Natl. Acad. Sci. U. S. A. **104,** 15572-15577.

Dische, Z. (1962). Color reactions of carbohydrates. In Methods in Carbohydrate Chemistry, Whistler R.L. and Wolfrom R.L., eds (New York: Academic Press), pp. 475-514.

Droillard, M.J., Paulin, A., and Massot, J.C. (1987). Free-radical production, catalase and superoxide-dismutase activities and membrane integrity during senescence of petals of cut carnations (*Dianthus caryophyllus*). Physiol. Plantarum. **71,** 197-202.

Edwards, E.A., Rawsthorne, S., and Mullineaux, P.M. (1990). Subcellular-distribution of multiple forms of glutathione-reductase in leaves of pea (*Pisum sativum* L). Planta. **180,** 278-284.

Edwards, R., Dixon, D.P., and Walbot, V. (2000). Plant glutathione S-transferases: enzymes with multiple functions in sickness and in health. Trends Plant Sci. **5,** 193-198.

Encina, A., Sevillano, J.M., Acebes, J.L., and Álvarez, J. (2002). Cell wall modifications of bean (*Phaseolus vulgaris*) cell suspensions during habituation and dehabituation to dichlobenil. Physiol. Plantarum. **114,** 182-191.

Feng, H., Chen, Q., Feng, J., Zhang, J., Yang, X., and Zuo, J. (2007). Functional characterization of the *Arabidopsis* eukaryotic translation initiation factor 5A-2 that plays a crucial role in plant growth and development by regulating cell division, cell growth, and cell death. Plant Physiol. **144,** 1531-1545.

Fornalé, S., Sonbol, F., Maes, T., Capellades, M., Puigdomenech, P., Rigau, J., and Caparrós-Ruiz, D. (2006). Down-regulation of the maize and *Arabidopsis thaliana* caffeic acid *O*-methyl-transferase genes by two new maize R2R3-MYB transcription factors. Plant Mol. Biol. **62,** 809-823.

Fry, S.C. (2004). Primary cell wall metabolism: tracking the careers of wall polymers in living plant cells. New Phytol. **161,** 641-675.

Fry, S.C., Willis, S.C., and Paterson, A.E.J. (2000). Intraprotoplasmic and wall-localised formation of arabinoxylan-bound diferulates and larger ferulate coupling-products in maize cell-suspension cultures. Planta. **211,** 679-692.

García-Angulo, P., Alonso-Simón, A., Mélida, H., Encina, A., Acebes, J.L., and Álvarez, J.M. (2009). High peroxidase activity and stable changes in the cell wall are related to dichlobenil tolerance. J. Plant Physiol. **166,** 1229-1240.

García-Angulo, P., Willats, W.G.T., Encina, A., Alonso-Simón, A., Álvarez, J.M., and Acebes, J.L. (2006). Immunocytochemical characterization of the cell walls of bean cell suspensions during habituation and dehabituation to dichlobenil. Physiol. Plantarum. **127,** 87-99.

Genter, M.B., Deamer-Melia, N.J., Wetmore, B.A., Morgan, K.T., and Meyer, S.A. (1998). Herbicides and olfactory/neurotoxicity responses. Rev. Toxicol. **2,** 93-112.

Guillaumie, S., San-Clemente, H., Deswarte, C., Martinez, Y., Lapierre, C., Murigneux, A., Barriere, Y., Pichon, M., and Goffner, D. (2007). MAIZEWALL. Database and developmental gene expression profiling of cell wall biosynthesis and assembly in maize. Plant Physiol. **143,** 339-363.

Gutierrez, R., Lindeboom, J.J., Paredez, A.R., Emons, A.M.C., and Ehrhardt, D.W. (2009). *Arabidopsis* cortical microtubules position cellulose synthase delivery to the plasma membrane and interact with cellulose synthase trafficking compartments. Nat. Cell Biol. **11,** 797-806.

Ha, M.A., MacKinnon, I.M., Sturcova, A., Apperley, D.C., McCann, M.C., Turner, S.R., and Jarvis, M.C. (2002). Structure of cellulose-deficient secondary cell walls from the *irx3* mutant of *Arabidopsis thaliana* . Phytochemistry. **61,** 7-14.

Habig, W.H., Pabst, M.J., and Jakoby, W.B. (1974). Glutathione S-Transferases - first enzymatic step in mercapturic acid formation. J. Biol. Chem. **249,** 7130-7139.

Hamann, T., Bennett, M., Mansfield, J., and Somerville, C. (2009). Identification of cell-wall stress as a hexose-dependent and osmosensitive regulator of plant responses. Plant J. **57,** 1015-1026.

Holland, N., Holland, D., Helentjaris, T., Dhugga, K.S., Xoconostle-Cazares, B., and Delmer, D.P. (2000). A comparative analysis of the plant cellulose synthase (*CesA*) gene family. Plant Physiol. **123,** 1313-1323.

Hossain, M.A., and Asada, K. (1984). Inactivation of ascorbate peroxidase in spinach-chloroplasts on dark addition of hydrogen-peroxide - its protection by ascorbate. Plant Cell Physiol. **25,** 1285-1295.

Jackson, S.D., Sonnewald, U., and Willmitzer, L. (1993). Cloning and expression analysis of β-isopropylmalate dehydrogenase from potato. Mol. Gen. Genet. **236,** 309-314.

Knox, J.P. (2008). Revealing the structural and functional diversity of plant cell walls. Curr. Opin. Plant Biol. **11,** 308-313.

Lindsay, S.E., and Fry, S.C. (2008). Control of diferulate formation in dicotyledonous and gramineous cell-suspension cultures. Planta. **227,** 439-452.

Lorences, E.P., and Fry, S.C. (1991). Absolute measurement of cell expansion in plant cell suspension cultures. **24,** 211-215.

Manfield, I.W., Orfila, C., McCartney, L., Harholt, J., Bernal, A.J., Scheller, H.V., Gilmartin, P.M., Mikkelsen, J.D., Knox, J.P., and Willats, W.G.T. (2004). Novel cell wall architecture of isoxaben habituated *Arabidopsis* suspension cultured cells: global transcript profiling and cellular analysis. Plant J. **40,** 260-275.

Mélida, H., García-Angulo, P., Alonso-Simón, A., Encina, A., Álvarez, J., and Acebes, J.L. (2009). Novel type II cell wall architecture in dichlobenil-habituated maize calluses. Planta. **229,** 617-631.

Murashige, T., and Skoog, F. (1962). A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol. Plantarum. **15,** 473-497.

Mutwil, M., Debolt, S., and Persson, S. (2008). Cellulose synthesis: a complex complex. Curr. Opin. Plant Biol. **11,** 252-257.

Nakagawa, N., and Sakurai, N. (1998). Increase in the amount of celA1 protein in tobacco BY-2 cells by a cellulose biosynthesis inhibitor, 2,6-dichlorobenzonitrile. Plant Cell Physiol. **39,** 779-785.

Paredez, A.R., Somerville, C.R., and Ehrhardt, D.W. (2006). Visualization of cellulose synthase demonstrates functional association with microtubules. Science. **312,** 1491-1495.

Parker, M.L., Ng, A., and Waldron, K.W. (2005). The phenolic acid and polysaccharide composition of cell walls of bran layers of mature wheat (*Triticum aestivum* L. cv. Avalon) grains. J. Sci. Food Agric. **85,** 2539-2547.

Persson, S., Paredez, A., Carroll, A., Palsdottir, H., Doblin, M., Poindexter, P., Khitrov, N., Auer, M., and Somerville, C.R. (2007). Genetic evidence for three unique components in primary cell-wall cellulose synthase complexes in *Arabidopsis* . Proc. Natl. Acad. Sci. U. S. A. **104,** 15566-15571.

Piston, F., Uauy, C., Fu, L., Langston, J., Labavitch, J., and Dubcovsky, J. (2010). Down-regulation of four putative arabinoxylan feruloyl transferase genes from family PF02458 reduces ester-linked ferulate content in rice cell walls. Planta. **231,** 677-691.

Rajangam, et al. (2008). MAP20, a Microtubule-Associated Protein in the secondary cell walls of hybrid aspen, is a target of the cellulose synthesis inhibitor 2,6-Dichlorobenzonitrile. Plant Physiol. **148,** 1283-1294.

Ray, P.M., Green, P.B., and Cleland, R. (1972). Role of turgor in plant-cell growth. Nature. **239,** 163-164.

Richmond, T. (2000). Higher plant cellulose synthases. Genome Biol. **1,** reviews3001.1-reviews3001.6.

Saeman, J.F., Moore, W.E., and Millet, M.A. (1963). Sugar units present. In Methods in Carbohydrate Chemistry, Whistler R.L., ed (New York: Academic Press), pp. 54-69.

Scheible, W.R., Eshed, R., Richmond, T., Delmer, D., and Somerville, C. (2001). Modifications of cellulose synthase confer resistance to isoxaben and thiazolidinone herbicides in *Arabidopsis Ixr1* mutants. Proc. Natl. Acad. Sci. U. S. A. **98,** 10079-10084.

Scheller, H.V., and Ulvskov, P. (2010). Hemicelluloses. Annu. Rev. Plant Biol. **61,** 10.1-10.27.

Shedletzky, E., Shmuel, M., Delmer, D.P., and Lamport, D.T.A. (1990). Adaptation and growth of tomato cells on the herbicide 2,6-Dichlorobenzonitrile leads to production of unique cell walls virtually lacking a cellulose-xyloglucan network. Plant Physiol. **94,** 980-987.

Smith, M.M., and Hartley, R.D. (1983). Occurrence and nature of ferulic acid substitution of cell-wall polysaccharides in graminaceous plants. Carbohydr. Res. **118,** 65-80.

Somerville, C. (2006). Cellulose synthesis in higher plants. Annu. Rev. Cell Dev. Biol. **22,** 53-78.

Taylor, N.G. (2008). Cellulose biosynthesis and deposition in higher plants. New Phytol. **178,** 239-252.

Taylor, N.G., Howells, R.M., Huttly, A.K., Vickers, K., and Turner, S.R. (2003). Interactions among three distinct CesA proteins essential for cellulose synthesis. Proc. Natl. Acad. Sci. U. S. A. **100,** 1450-1455.

Taylor, N.G., Laurie, S., and Turner, S.R. (2000). Multiple cellulose synthase catalytic subunits are required for cellulose synthesis in Arabidopsis. Plant Cell. **12,** 2529-2539.

Taylor, N.G., Scheible, W.R., Cutler, S., Somerville, C.R., and Turner, S.R. (1999). The *irregular xylem3* locus of Arabidopsis encodes a cellulose synthase required for secondary cell wall synthesis. Plant Cell. **11,** 769-779.

Tomanek, L., and Sanford, E. (2003). Heat-shock protein 70 (Hsp70) as a biochemical stress indicator: An experimental field test in two congeneric intertidal gastropods (Genus: *Tegula*). Biol. Bull. **205,** 276-284.

Turner, S.R., and Somerville, C.R. (1997). Collapsed xylem phenotype of *Arabidopsis* identifies mutants deficient in cellulose deposition in the secondary cell wall. Plant Cell. **9,** 689-701.

Updegraff, D.M. (1969). Semimicro determination of cellulose in biological materials. Anal. Biochem. **32,** 420-424.

Vaughn, K.C. (2002). Cellulose biosynthesis inhibitor herbicides. In Herbicide Classes in Development, Böger P. Wakabayashi K. and Hirai K., eds (Berlin: Springer-Verlag), pp. 139-150.

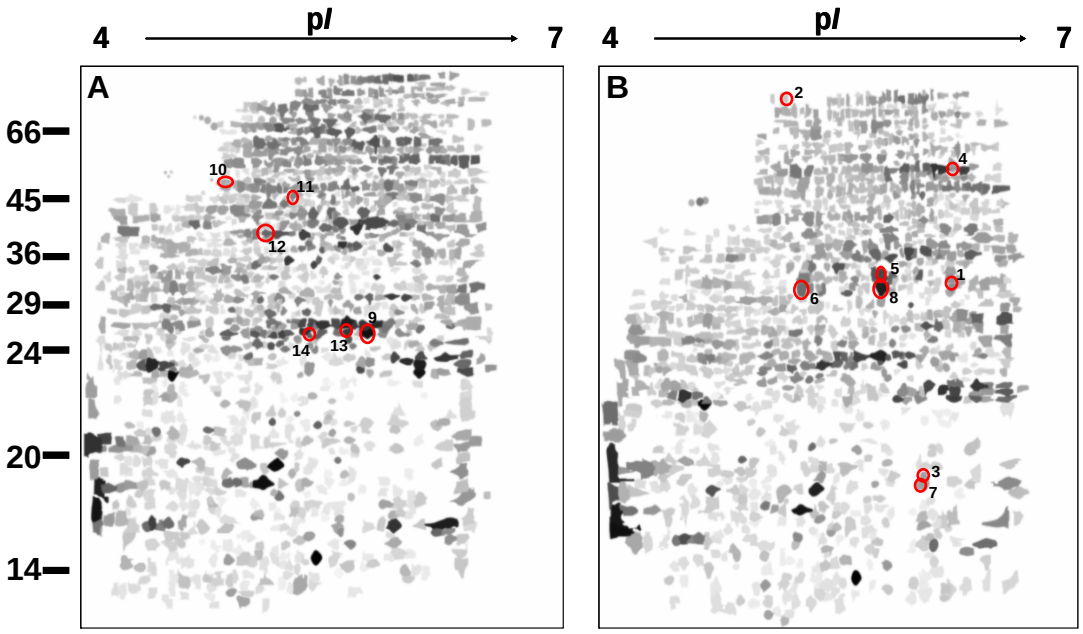
Vogt, T. (2010). Phenylpropanoid biosynthesis. Mol Plant. **3,** 2-20.

Wende, G., and Fry, S.C. (1997). *O*-feruloylated, *O*-acetylated oligosaccharides as side-chains of grass xylans. Phytochemistry. **44,** 1011-1018.

Zanardo, D.I.L., Lima, R.B., Lucio Ferrarese, M.d.L., Bubna, G.A., and Ferrarese-Filho, O. (2009). Soybean root growth inhibition and lignification induced by *p*-coumaric acid. Environ. Exp. Bot. **66,** 25-30.

Supplementary Data

Supplementary Figure 1: 2-D resolution of the maize callus proteins. Comparison of the total proteins of NH (A) and H12 (B) cultures. Numbers on left indicate the positions of the relative molecular mass standards (x 1000) and the p*I* is given at the top. Gels were loaded with 200 µg proteins, visualized by silver staining. Average of 6 gels is shown. The open red circles correspond to the selected spots analyzed by MALDI-TOF MS, which are listed in Table 1.



Supplementary Table 1: primers sequences of *ZmCesA* and *ZmMAP20* genes:

Gene (accession number)	Forward primer sequence	Reverse primer sequence
ZmCesA4 (AF200528)	5´-ggaagtggaaggtttgtactttg-3´	5´-tcaacaaaagaatgcatattaacaca-3´
ZmCesA8 (AF200532)	5´-cccctgtcactcgaagttct-3´	5´-tacactgggcactggaatgt-3´
ZmCesA9 (AF200533)	5´-caactgctagggaggtggaa-3´	5´-ctgtcagccactctccacaa-3´
ZmCesA10 (AY372244.1)	5´-gtttatcccgaaggc-3´	5´-actctgtctcactcag-3´
ZmCesA11 (AY372245.1)	5´-gtcaagatcgacccattcgt-3´	5´-acctacaccacccgcttcag-3´
ZmCesA12 (AY372246.1)	5´-ctttcatcgtcaggac-3´	5´-gacaattctgggtacc-3´
ZmBk2L3 (EF078698)	5´-ttccatggttgcacagaaaa-3´	5´-atcagggaagccttgcatt-3´
ZmMAP20 (AY110515.1)	5´-tcgatcagcattctttttgc-3´	5´-aagaagtgggggaacggtag-3´

Capítulo IV:

The phenolic profile of maize primary cell wall changes in cellulose-deficient cell cultures

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Capítulo correspondiente al manuscrito: Mélida, H., García-Angulo, P., Alonso-Simón, A., Álvarez, J., Acebes, J.L., Encina, A. (2010). The phenolic profile of maize primary cell wall changes in cellulose-deficient cell cultures. Actualmente en revision en "Phytochemistry" (ref. PHYTOCHEM-D-10-00274).

The phenolic profile of maize primary cell wall changes in cellulose-deficient cell cultures

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Abstract

Maize cultured cells habituated to the herbicide dichlobenil (DCB) are distinguished by having a modified cell wall in which cellulose is partially replaced by a more extensive arabinoxylan network. This paper examines the contribution of cell wall esterified hydroxycinnamates to the DCB-mechanism of habituation. For this purpose, differences in the phenolic composition of DCB-habituated and non-habituated cell walls, throughout the cell culture cycle and the habituation process were characterized by HPLC. DCB habituation was accompanied by a net enrichment in cell wall phenolics irrespective of the cell culture phase. The amount of monomeric phenolics was two-fold higher in habituated cell walls. Moreover, habituated cell walls were notably enriched in *p*-coumaric acid. Dehydrodimers were 5 to 6 fold enhanced as a result of DCB habituation and the steep increase in 8,5´-diferulic acid in habituated cell walls would suggest that this dehydrodimer plays a role in DCB habituation. The variety of links between monomeric phenolics was amplified during habituation to DCB, as new putative dehydrodimers were detected in habituated cell walls. In summary, the results obtained show that phenolics play a major role in maintaining the functionality of a cellulose impoverished cell wall.

Keywords: Zea mays; maize; cell wall; DCB; dichlobenil; hydroxycinnamate; ferulic acid; *p*-coumaric acid; HPLC.

Introduction

As with the other grass species and related commelinoid monocots, maize cells have a characteristic primary cell wall (called type II cell wall) in which the main structural net consists of cellulose microfibrils, embedded in a matrix of arabinoxylans (AXs) substituted by cell wall phenolics (hydroxycinnamates) (Carpita, 1984). These hydroxycinnamates are mainly ferulic acid (FA) and *p*-coumaric acid (CA), which can ester-bond to lignin (Higuchi et al., 1967), to the α-L-arabinosyl residues of AXs (Kato and Nevins, 1985; Smith and Hartley, 1983; Wende and Fry, 1997), to the α-D-xylosyl residues of xyloglucan (Ishii and Hiroi, 1990), and probably to glycoproteins (Obel et al., 2003).

Although hydroxycinnamates are minor components of the cell wall (FA can account for up to 3% of graminaceous cell wall -Saulnier et al., 1999-), their contribution to this structure is indispensable. *In vitro* experiments have long demonstrated that FA can undergo oxidative-coupling mediated by peroxidases and H_2O_2 (Geissmann and Neukom, 1971). The identification and structural elucidation of diferulates (also known as dehydrodiferulates = DFA) attached to sugar residues [ie. Xyl-Ara-FA-(5-5)-FA-Ara-Xyl] has provided evidence that FA

dimerization could cross-link cell wall polysaccharides (Grabber et al., 2000; Ishii, 1997; Saulnier et al., 1999). Later, the results of *in vivo* experiments indicated that ester-linked hydroxycinnamates can undergo oxidative-coupling to cross-link adjacent AX molecules (Fry et al., 2000; Fry, 2004; Parker et al., 2005). The polysaccharide cross-linking activity by phenolic-coupling regulates, mediates or alters a number of cell wall properties: contributing to cell wall assembly, causing cell wall stiffening and growth cessation, promoting tissue cohesion, strengthening cell wall structure in response to biotic and abiotic stresses, and limiting cell wall biodegradability (Buanafina, 2009; and refs. therein).

The most frequent coupling products are DFAs. By alkaline hydrolysis of cell wall polysaccharides several naturally-occurring diferuloyl esters have been identified, ie. 5,5´-DFA, 8,8´-DFA, 8,5´-DFA and 8-O-4´-DFA (some of them having different forms) (Lindsay and Fry, 2008; Obel et al., 2003; Ralph et al., 1994; Waldron et al., 1996). In addition to dehydrodimers, trimers and even tetramers have been also described as products of *in vivo* ferulate cross-linking (Bunzel et al., 2003; 2006; Funk, 2005; Rouau et al., 2003). The presence and relevance of oligoferulates is now well accepted and certain evidence would indicate that esterified oligoferulates are more widespread and crucial than dimers in cross-linking type II primary cell walls (Fry et al., 2000; Lindsay and Fry, 2008). In a recent paper, Burr and Fry (2009) demonstrated that after oxidative coupling, strong benzyl-sugar ether bonds are formed via quinone-methide compounds. The same authors proposed that these alkali-resistant bonds play a major role in polysaccharide cross-linking (Burr and Fry, 2009). Polysaccharide linking can occur (1) intracellularly before or during Golgi vesicle transit to cell membrane and/or (2) wall-localized (*in muro*) immediately following polysaccharide secretion or after wall integration (Fry et al., 2000; Mastrangelo et al., 2009; Obel et al., 2003).

We obtained maize (*Zea mays* L.) cell lines habituated to DCB, a well known cellulose biosynthesis inhibitor which specifically inhibits the polymerization of glucose into β-1,4-linked glucan (Delmer, 1987). The habituation of cell cultures to cellulose biosynthesis inhibitors reflects the ability of plant cells to modify cell wall structure and composition in order to cope with abiotic stresses, and therefore represents a valuable tool for improving our knowledge of the mechanisms involved in plant cell wall plasticity and ability to maintain cell wall integrity (Vaughn, 2002). In spite of showing a 75% reduction in cellulose content, DCB-habituated maize cells are able to grow through the acquisition of a modified cell wall in which cellulose is partially replaced by a more extensive network of AXs (Mélida et al., 2009).

The aim of this research was to investigate the phenolic component of cellulose deficient cell walls by focusing on a study of the contribution of cell wall esterified hydroxycinnamates to the DCB-habituation mechanism. To achieve this objective, the phenolic profile of non-habituated (NH) and habituated (H) maize cell walls was characterized by using HPLC-PAD. Attention was paid to variations in the cell wall phenolic profile throughout both a culture cycle of NH vs. H cells (attending to three different growth stages) and DCB process of habituation (H cells able to grow in increasing concentrations of DCB). Our initial hypotheses were that; i) DCB-habituated cell walls should be enriched in cell wall esterified phenolics; ii) DCB-habituated

cell walls would have a higher amount of FA dehydrodimers as a consequence of a more cross-linked cell wall, and iii) that a qualitative variation in the phenolic components should be observed between NH and H cells, among cells with different levels of habituation to DCB, and also throughout the culture cycle.

Results and Discussion

Differences between alkali-extracted phenolics from DCB-habituated and non-habituated cell walls

Cell walls from callus-cultured maize cells at the early stationary (sta) phase were alkali-extracted at room temperature in order to release the ester-linked hydroxycinnamates (Fry, 1983). Compositional analysis of alkali-extracted compounds (Fig. 1) indicated that this fraction was mainly composed of monomeric *trans*- and *cis*- FA **(4,6)**, *trans*- and *cis*- CA **(3,5)** and a set of identified and putative DFAs **(7-14).** In the case of NH maize cells, two DFAs were detected in measurable amounts: 5,5´-DFA **(11)** and 8-O-4´-DFA **(14)**. The 8,5´-DFA **(10)** was also detected, but in trace amounts (Fig. 1, solid line).

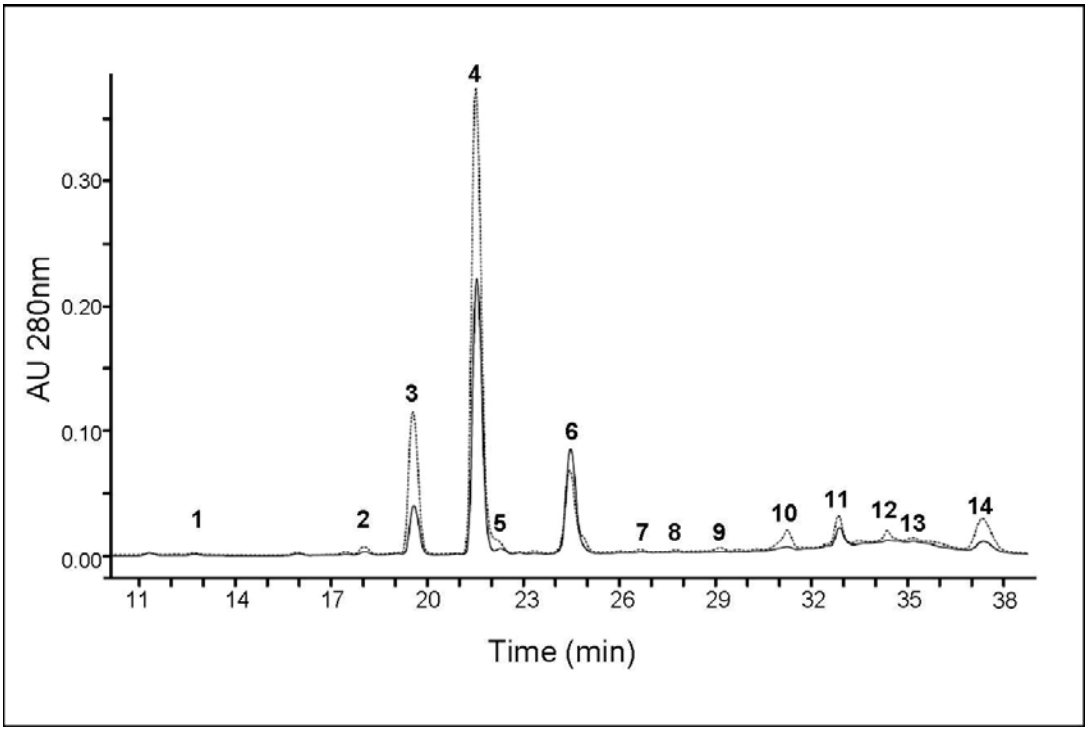


Fig. 1. HPLC-PAD elution profile of phenolic compounds after alkali hydrolysis of cell walls from non-habituated (NH; solid line) and DCB-habituated [H12(24); dotted line] maize callus-cultured cells at the early stationary phase. Peaks were detected at 280 nm. Key to peak identity: **1**, vanillic acid; **2**, vanillin; **3**, *trans*-*p*-coumaric acid; **4**, *trans*-ferulic acid; **5**, *cis*-*p*-coumaric acid; **6**, *cis*-ferulic acid; **7**, 8,8´-DFA aryltetralin; **8**, D1; **9**, D2; **10**, 8,5´-DFA; **11**, 5,5´-DFA; **12**, D3; **13**, D4; **14**, 8-*O*-4´-DFA.

Composition of alkali extracted phenolics from H12 cell walls differed both quantitatively and qualitatively from those of NH cell walls. As a consequence, proportions among major phenolics, and proportions between monomers/dehydromers, underwent important changes in H12 cells.

Four putative dehydrodimers with elution times of 27.77 **(8; D1)**, 29.17 **(9; D2)**, 34.42 **(12, D3)** and 35.22 min **(13, D4)** were exclusively detected in H cell lines (Fig. 1, dotted line). The absorption spectra of D1-4 compounds are shown in Fig. 2. Together with these, 5,5´-DFA **(11),** 8-O-4´-DFA **(14),** 8,5´-DFA **(10)** were detected in measurable quantities and 8,8´-DFA aryltetralin **(7)** was detected in trace amounts.

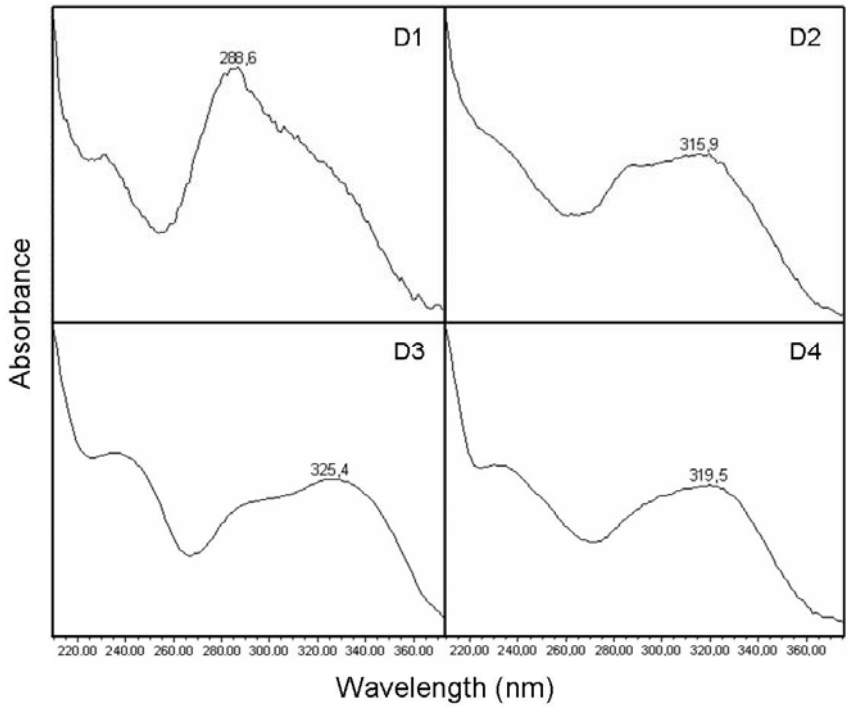


Fig. 2. Absorption spectra of the putative new dehydrodimers detected in cell walls from DCB-habituated [H12(24)] maize cell cultures using HPLC-PAD (see Fig.1).

Changes in alkali-extracted phenolics throughout cell culture cycle

NH cells grew forming homogeneous and friable calluses with a short (2 days) accommodation (lag) phase. On average, NH cells reached the active growth (grw) and sta phases 16 days and 25 days after subculturing, respectively. At the end of the culture cycle, NH cells increased their fresh weight 2.2-fold. H12 calluses did not differ greatly in appearance from NH ones. However, H12 cell cultures had longer lag phases (10 days), reduced growth rates and accumulated less FW (almost 30% less) at the end of the culture cycle. Twenty day-old and 30 day-old H12 cells were considered at the grw and sta phase respectively.

In order to gain further insight into changes in cell wall phenolics throughout the cell culture cycle, NH and long-term DCB-habituated maize cells [H12(24)] were sampled at their corresponding lag (2-5 days), grw (16-20

days) and sta phase (25-30 days), respectively. Cell walls were prepared and then alkali-extracted (Fig. 3). FA was the major monomeric phenolic in the alkali extracted fraction of NH cell walls (Fig. 3a). Cell wall ester-linked FA content did not vary greatly throughout the cell culture cycle, ranging from 3.9 to 4.4 μg mg^{-1} cell wall. CA was also consistently detected in NH cells (about 0.2 μg mg^{-1} cell wall). Contrary to FA, NH cell walls were 1.67-fold more enriched in CA when cells were collected later in the culture cycle (Fig. 3a). Consequently the FA:CA mol ratio was reduced from 31.0 at the lag phase to 17.0 at the sta phase. The amount of ester-wall bound FA and CA found in our experiment was within the range of values already reported for maize callus-cultured cells (Lozovaya et al., 2000). However, Grabber et al. (1995; 1998) found a significantly higher content of FA (14.5 μg mg^{-1} cell wall) and CA (0.4 μg mg^{-1} cell wall) when maize suspension-cultured cells were analysed. These differences would point to variations in cell-wall bound phenolics which depended on the *in vitro* culture system.

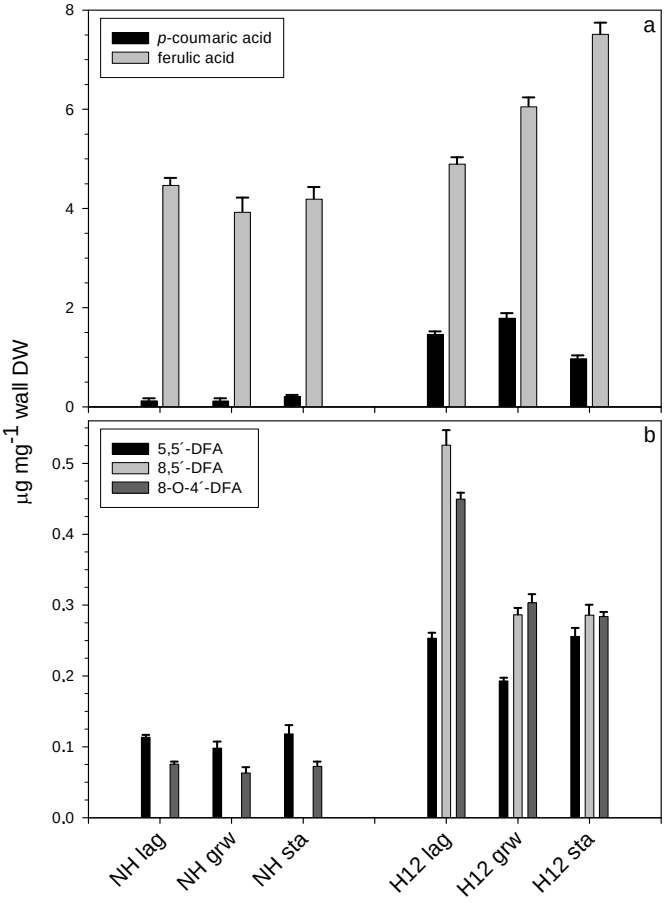


Fig. 3. Variation throughout the culture cycle of the cell wall-esterified phenolics from non-habituated (NH) and DCB-habituated [H12(24)] maize callus-cultured cells. Walls from cells at the accommodation (lag), active growth (grw) and stationary (sta) phase were purified, alkali extracted and HPLC-PAD analysed as described in the experimental section. The content in main (a) monomeric phenolic and (b) dehydrodiferulates is shown. Results shown are representative of three independent experiments giving similar data.

No changes in the total amount of DFAs were noted throughout the cell culture cycle of NH cells, with 5,5´-DFA and 8-O-4´ being the main DFAs (Fig. 1 and Fig. 3b). Therefore FA:DFA ratio did not vary greatly throughout the cell culture cycle of NH cells.

Several studies have indicated a positive correlation between FA cross-linking and growth cessation in various monocot species (Azuma et al., 2005; Kamisaka et al., 1990; MacAdam and Grabber, 2002). However, in relation to DFA variation throughout the cell culture cycle, no correlation between those two variables was established for our callus-cultured maize cells.

Total phenolic content of H12 cell walls was higher than that of NH cell walls, independently of the cell culture phase (9.3 vs 4.6 μg mg^{-1} cell wall at the sta phase; Fig. 3). As H12 cells aged, a steep increase in FA content was observed (Fig. 3a). Consequently, FA content of H12 cell walls was almost double (x 1.9) that of NH ones at the sta phase. This result mirrored the finding that xylose (Xyl) content of H12 was 1.5 times higher than that of NH cell walls at the sta phase (125 vs. 184 μg mg^{-1} cell wall; Mélida et al., 2009). Assuming that 90% of xylosyl residues were derived from AX (Carpita, 1984) a Xyl:FA ratios of 35 and 29 have been calculated for NH and H12 respectively. In other words, although DCB-habituated cell walls were more feruloylated, this did not imply a noticeable increase in the AX feruloylation degree.

H12 cell walls were notably enriched in CA (Fig. 3a) to the point that in certain DCB-habituated cell lines, CA was the major phenolic (see H4, H6 and H12(8) in Fig. 4a). The high CA content of H12 cell walls reduced FA:CA molar ratio, which ranged from 2.8 to 6.5 throughout the H12(24) cell culture cycle (compare with values of 31.0 to 17.0 calculated for NH cell walls). The presence of a CA-rich cell wall associated with DCB-habituation has already been described in barley (Shedletzky et al., 1992) cultured cells. This cell wall CA enrichment is probably an effect of DCB exposure rather than a consequence of DCB-habituation as: (i) a short-term treatment of NH cells with 6 μM DCB causes the cell wall to accumulate CA, and (ii) H12 cells cultured in a medium lacking DCB reduced CA to NH levels (Table 1).

Table 1. Composition of main monomeric phenolics of NH and H12 maize cell walls in the presence or absence of DCB at the early stationary phase. FA: ferulic acid; CA: *p*-coumaric acid.

	[DCB] μM	FA	CA
		μg mg^{-1} cell wall	
NH	0	4.18	0.21
NH	6	2.23	2.15
H12	12	7.50	0.97
H12	0	2.23	0.29

Two of the most prominent features of H12 cell walls are the reduction in AX extractability and the increase in AX average molecular weight (Mélida et al., 2009). Both are probably caused by a more extensive phenolic crosslinking (Burr and Fry, 2009; Kerr and Fry, 2004; Lindsay and Fry, 2008), and therefore, it would be expected for DCB-habituated cells to have an increased level of DFAs in their cell wall. This assumption was confirmed in so far as DFA content of H12 cell walls was on average 4 to 6-fold higher than that of NH cell walls. In

base to DFA content, a 3-fold increase of Xyl:DFA ratio was estimated for H12 cell walls at the stationary phase.

The 8-5´and 8-O-4´ DFAs were the predominant coupling products of FA in H12 cell walls (Fig. 1 and Fig. 3b). Moreover, 8-5´-DFA emerged as a distinctive characteristic of DCB-habituated cell walls, as it was detected in a measurable quantity in H12 cell walls alone.

Total amount of DFAs fell during the cell culture cycle of H12 cells, which could indicate a reduction in FA dimerization as DCB-habituated cells stopped growing (Fig. 3b). Assuming that a reduction in ester-linked DFAs due to a putative AX sloughing is not plausible in callus-cultured cells, we suggest that DFA reduction is the consequence of a DFA population becoming alkali-resistant as a cell wall reinforcement mechanism in H12 cell walls. In this respect, Burr and Fry (2009) demonstrated that oligoferulates attached to sugar residues via alkali-stable bonds (ether-like) predominated in *in vivo* AX crosslinking.

Changes in alkali-extracted phenolics during the habituation to DCB

DCB-habituation is a dynamic process. It has been demonstrated that during the habituation of bean and maize callus-cultured cells to this herbicide, the extent and type of cell wall modifications depended on two factors: i) the concentration of the inhibitor in the culture medium, and ii) length of time cells had been present in a given concentration of the inhibitor (Alonso-Simón et al., 2004). We were interested in discovering whether the cell wall phenolic profile of habituated cells varied depending on the habituation level (i.e. concentration of DCB in the culture medium and number of subcultures in 12 µM DCB; see Supplementary material).

The habituation process was accompanied by both quantitative and qualitative changes in the cell wall phenolic profile. The total amount of major monomeric phenolics (FA+CA) fell as the habituation to DCB proceeded (Fig. 4a). At the same time, the amount of DFAs increased [H4 vs H12(24); Fig. 4 a]. Accordingly, it could be expected that a more extensive cross-linking of the AX network would be observed, as length of habituation to DCB increased.

It is noteworthy that a shift from CA-rich to FA-rich cell walls was observed as the habituation level increased. The relative proportion of each DFA also changed, and a relative increase in 8,5´-DFA was observed during DCB habituation [Fig 4b: H12(8) vs H12(24)]. This latter result, together with the fact that 8,5´-DFA is a minor dehydroferulate in NH cell walls, would seem to indicate that 8,5´-DFA plays a specific role in DCB habituation.

By using an *in vivo* radiolabelling strategy, Obel et al. (2003) suggested that intra-protoplasmic FA dimerization is restricted to the 8,5´ coupling product. Considering 8,5´-DFA as a DCB habituation-related compound, it could be hypothesized that DCB-habituation is linked to an enhanced intra-protoplasmic coupling of FA. However, in a more recent paper, Lindsay and Fry (2008) demonstrated that other DFAs (8,8´; 8-O-4´; 5,5´, 8,5´B) can be formed intra-protoplasmically, at least in maize suspension-cultured cells, which makes this explanation unlikely.

It has been shown that the addition of H_2O_2 to maize cells is followed by an extensive *in-muro* FA cross-linking and that 8,5´-DFA (together with 5,5´ and

8-O-4´-DFA) is especially responsive to this effect (Grabber et al., 1995, Lindsay and Fry, 2008). This could indicate that 8,5´-DFA is the predominant dehydrodimer when an extra cross-linking occurs, as would happen in H12 cells.

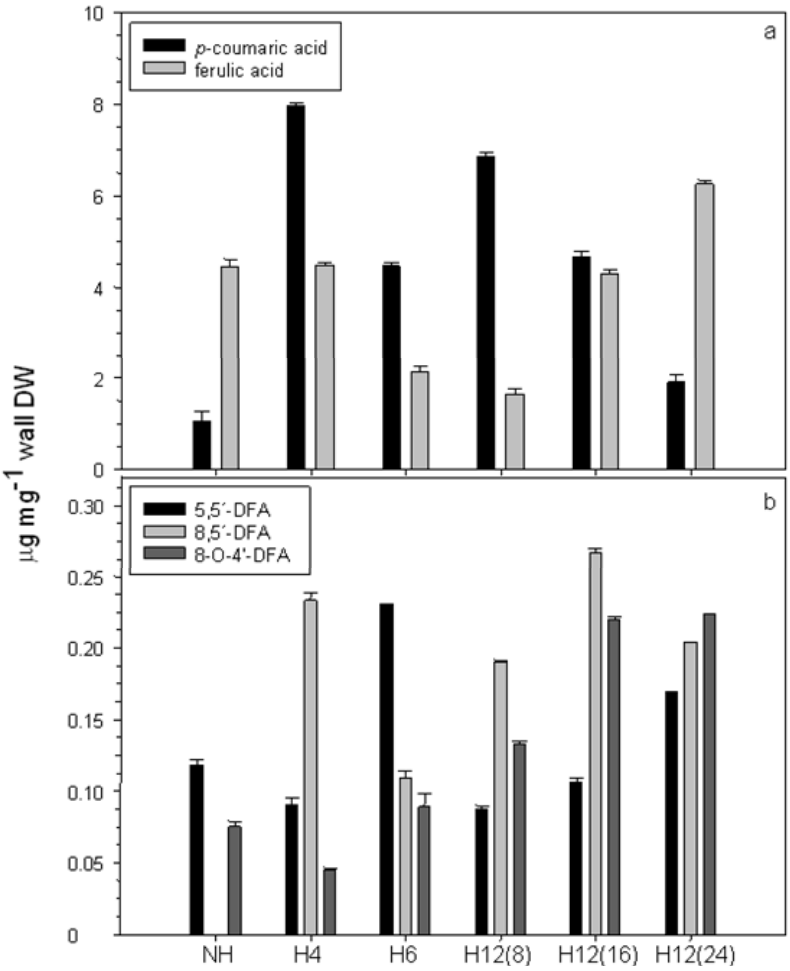


Fig. 4. Effect of exposure time to DCB on the cell wall phenolic composition of DCB-habituated maize callus-cultured cells. Walls from cells at the early stationary phase subcultured in 4 or 6 μM DCB, or 12 μM DCB for 240 (8), 480 (16) and 720 (24) days were prepared, alkali extracted and HPLC-PAD analysed as in Fig. 1. Numbers in brackets indicate the number of subcultures in the presence of 12 μM DCB. The content in main (a) monomeric phenolic and (b) dehydrodiferulates is presented. Values are mean ± SD of three replicates.

Conclusions

Through compositional analysis and structural characterization of DCB habituated cell walls it was possible to prove that these cells compensate for the lack of cellulose without major consequences to their cell wall functionality. The mechanism for such an accommodation consisted in producing a more extensive network of AXs (Mélida et al., 2009).

In this paper it has been demonstrated that:

1- DCB-habituated cell walls increase the total amount of monomeric phenolics and dehydrodimers by a factor of 2 and 5-6, respectively. The enhancement in DFAs is in agreement with a more extensive AX cross-linking, contributing to the reinforcement of a cellulose impoverished cell wall.

2- In spite of having a net enrichment in FA and DFA, the AX feruloylation degree in DCB-habituated cells did not change substantially.

3-A progressive increase in phenolics content was observed during the culture cycle of H12, but not during that of NH.

4- Length of exposure to DCB in the habituation process is associated with changes to the phenolic profile of DCB-habituated cell walls.

5- Although levels of 5,5 and 8-O-4´-DFA are elevated in H12 cell walls, the steep increase in 8,5 in H12 cell walls regarding NH ones suggests that this DFA plays a role in DCB habituation.

6- The variety of links between monomeric phenolics was increased by the habituation to DCB, as four new putative dehydrodimers were detected in H12 cell walls.

According to our results, we conclude that habituation to the cellulose biosynthesis inhibitor DCB, produces a modified cell wall in which phenolics, the units linking AXs, play a highly significant role in maintaining the functionality of a cellulose impoverished cell wall. Based on the quantitative and qualitative changes of cell wall phenolics, this probably indicates an altered phenolic metabolism in DCB-habituated cells. Future work is needed to clarify this particular issue together with others which have emerged from the results presented in this paper.

Experimental

Cell cultures

Maize callus cultures (*Zea mays* L., Black Mexican sweetcorn) from immature embryos were grown in Murashige and Skoog media (Murashige and Skoog 1962) supplemented with 9 µM 2,4-Dichlorophenoxyacetic acid at 25°C under continuous light (Lorences and Fry, 1991), and subcultured monthly. Calluses were habituated to growth in different DCB (supplied by Fluka) concentrations by stepwise transfers with gradual increments, beginning at 2 µM. At least three subcultures of approximately 30 days were performed between each increase in the DCB concentration. To help understanding the habituation process a scheme has been included as Supplementary material. Habituated cultures were named as Hx(y), where x is DCB concentration (µM), and y is number of subcultures in that concentration. Growth curves were obtained for H and for NH calluses, by measuring the relative increase in fresh weight at various culture times.

Phenolic acid analysis

Calluses were frozen and homogenised with liquid nitrogen and treated with 70% ethanol for 5 days at room temperature. The suspension was then

centrifuged and the pellet washed with 70% ethanol (x6), acetone (x6), and air dried, in order to obtain the alcohol insoluble residue (AIR). The AIR was treated with 90% DMSO for 8 h at room temperature (x3) and then washed with 0.01 M phosphate buffer pH 7.0 (x2). The washed AIR was then treated with 2.5 u ml^{-1} α-amylase obtained from porcine pancreas (Sigma type VI-A) in 0.01 M phosphate buffer pH 7.0 for 24 h at 37°C (x3). The suspension was filtered through a glass fibre, and the residue washed with 70% ethanol (x6), acetone (x6), air dried and then treated with phenol-acetic-water (2:1:1 v/v) for 8h at room temperature (x2). This was finally washed with 70% ethanol (x6), acetone (x6) and air dried in order to obtain the cell walls.

Cell walls (10 mg) were treated in the dark under N_2 with 1 M NaOH, at room temperature for 16 h in order to saponify phenolic esters. The solution was acidified by addition of trifluoroacetic acid (TFA) and partitioned against ethyl acetate (x2). The ethyl acetate phases were vacuum-dried and re-dissolved in propan-1-ol for HPLC-PAD analysis.

HPLC-PAD analyses were performed using a Waters 2690 chromatograph with a Waters 996 photodiode array detector. Separation was achieved using a Kromasil C18 (Teknokroma) column (250 x 4.6 mm i.d.; 5 μm particle size). The mobile phase consisted of acidified (TFA) 10% acetonitrile (solvent A) and a mix of 40% acetonitrile and 40% methanol (solvent B) and followed the binary gradient elution programme: initial conditions 90:10 (A:B), changing to 25:75 after 25 min, then to 0:100 after 5 min and returning to the initial conditions after 10 min. The mobile phase flow was 1 ml/min. The elution profiles were monitored by UV absorbance at 325 and 280 nm. Retention times were compared with freshly prepared standard solutions of vanillic acid, vanillin, CA, FA, 5,5´-DFA, 8,8´nc-DFA, 8,5´nc-DFA (namely 8,5´-DFA) and 8,5´B-DFA. Calibration curves and published response factors (Waldron et al., 1996) were used to quantify these compounds.

Acknowledgements

This work was supported by grants from the Junta de Castilla y León (LE 048A07), the Universidad de León (ULE-2006-2) and the Spanish Ministry of Science and Innovation (CGL2008-02470/BOS), in addition to a PhD grant to H.M.M from the Spanish Ministry of Science and Innovation's FPU program. We are grateful to John Ralph, Fachuang Lu, and Hoon Kim of the US Dairy Forage Research Center, USDA-Agricultural Research Service, for their kind gifts of DFAs standards, to Mª Luz Centeno for providing HPLC technical support and to Denise Phelps for the English revision of the manuscript.

References

Alonso-Simón, A., Encina, A.E., García-Angulo, P., Álvarez, J.M., Acebes, J.L., 2004. FTIR spectroscopy monitoring of cell wall modifications during the habituation of bean (*Phaseolus vulgaris* L.) callus cultures to dichlobenil. Plant Sci. 167, 1273-1281.

Azuma, T., Okita, N., Nanmori, T., Yasuda, T., 2005. Relationship between the deposition of phenolic acids in the cell walls and the cessation of rapid growth in internodes of floating rice. Plant. Prod. Sci. 8, 447-453.

Buanafina, M.M.d.O., 2009. Feruloylation in grasses: current and future perspectives. Mol. Plant. 2, 861-872.

Bunzel, M., Ralph, J., Funk, C., Steinhart, H., 2003. Isolation and identification of a ferulic acid dehydrotrimer from saponified maize bran insoluble fiber. Eur. Food Res. Technol. 217, 128-133.

Bunzel, M., Ralph, J., Bruening, P., Steinhart, H., 2006. Structural identification of dehydrotriferulic and dehydrotetraferulic acids isolated from insoluble maize bran fiber. J. Agric. Food Chem. 54, 6409-6418.

Burr, S.J., Fry, S.C., 2009. Extracellular cross-linking of maize arabinoxylans by oxidation of feruloyl esters to form oligoferuloyl esters and ether-like bonds. Plant J. 58, 554-567.

Carpita, N.C., 1984. Cell wall development in maize coleoptiles. Plant Physiol. 76, 205-212.

Delmer, D.P., 1987. Cellulose biosynthesis. Annu. Rev. Plant Physiol. Plant Mol. Biol. 38, 259-290.

Fry, S.C., 2004. Primary cell wall metabolism: tracking the careers of wall polymers in living plant cells. New Phytol. 161, 641-675.

Fry, S.C., Willis, S.C., Paterson, A.E.J., 2000. Intraprotoplasmic and wall-localised formation of arabinoxylan-bound diferulates and larger ferulate coupling-products in maize cell-suspension cultures. Planta 211, 679-692.

Fry, S.C., 1983. Feruloylated pectins from the primary-pell wall - their structure and possible functions. Planta 157, 111-123.

Funk, C., Ralph, J., Steinhart, H., Bunzel, M., 2005. Isolation and structural characterisation of 8-*O*-4/8-*O*-4- and 8-8/8-*O*-4-coupled dehydrotriferulic acids from maize bran. Phytochemistry 66, 363-371.

Geissman, T., Neukom, H., 1971. Cross-linking of phenolcarboxylates of polysaccharides by oxidative phenolic coupling. Helv. Chim. Acta 54, 1108-1112.

Grabber, J.H., Ralph, J., Hatfield, R.D., 2000. Cross-linking of maize walls by ferulate dimerization and incorporation into lignin. J. Agric. Food Chem. 48, 6106-6113.

Grabber, J.H., Hatfield, R.D., Ralph, J., 1998. Diferulate cross-links impede the enzymatic degradation of non-lignified maize walls. J. Sci. Food Agric. 77, 193-200.

Grabber, J.H., Hatfield, R.D., Ralph, J., Zon, J., Amrhein, N., 1995. Ferulate cross-linking in cell-walls isolated from maize cell-suspensions. Phytochemistry 40, 1077-1082.

Higuchi, T., Ito, Y., Shimada, M., Kawamura, I., 1967. Chemical properties of milled wood lignin of grasses. Phytochemistry 6, 1551-1556.

Ishii, T., 1997. Structure and functions of feruloylated polysaccharides. Plant Sci. 127, 111-127.

Ishii, T., Hiroi, T., 1990. Linkage of phenolic-acids to cell-wall polysaccharides of bamboo shoot. Carbohydr. Res. 206, 297-310.

Kamisaka, S., Takeda, S., Takahashi, K., Shibata, K., 1990. Diferulic and ferulic acid in the cell-wall of *Avena* coleoptiles - their relationships to mechanical-properties of the cell-wall. Physiol. Plant. 78, 1-7.

Kato, Y., Nevins, D.J., 1985. Isolation and identification of *O*-(5-*O*-Feruloyl-α-L-Arabinofuranosyl)-(1-3)-*O*-β-D-Xylopyranosyl-(1-4)-D-Xylopyranose as a component of *Zea* shoot cell-walls. Carbohydr. Res. 137, 139-150.

Kerr, E.M., Fry, S.C., 2004. Extracellular cross-linking of xylan and xyloglucan in maize cell-suspension cultures: the role of oxidative phenolic coupling. Planta 219, 73-83.

Lindsay, S.E., Fry, S.C., 2008. Control of diferulate formation in dicotyledonous and gramineous cell-suspension cultures. Planta 227, 439-452.

Lorences, E.P., Fry, S.C., 1991. Absolute measurement of cell expansion in plant cell suspension cultures. Plant Cell Tiss. Organ Cult. 24, 211-215.

Lozovaya, V.V., Gorshkova, T.A., Rumyantseva, N.I., Ulanov, A.V., Valieva, A.I., Yablokova, E.V., Mei, C.S., Widholm, J.M., 2000. Cell wall-bound phenolics in cells of maize (*Zea mays*, Gramineae) and buckwheat (*Fagopyrum tataricum*, Polygonaceae) with different plant regeneration abilities. Plant Sci. 152, 79-85.

MacAdam, J.W., Grabber, J.H., 2002. Relationship of growth cessation with the formation of diferulate cross-links and p-coumaroylated lignins in tall fescue leaf blades. Planta 215, 785-793.

Mastrangelo, L.I., Lenucci, M.S., Piro, G., Dalessandro, G., 2009. Evidence for intra- and extra-protoplasmic feruloylation and cross-linking in wheat seedling roots. Planta 229, 343-355.

Mélida, H., García-Angulo, P., Alonso-Simón, A., Encina, A., Álvarez, J., Acebes, J.L., 2009. Novel type II cell wall architecture in dichlobenil-habituated maize calluses. Planta 229, 617-631.

Murashige, T., Skoog, F., 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol. Plant. 15, 473-497.

Obel, N., Porchia, A.C., Scheller, H.V., 2003. Intracellular feruloylation of arabinoxylan in wheat: evidence for feruloyl-glucose as precursor. Planta 216, 620-629.

Parker, M.L., Ng, A., Waldron, K.W., 2005. The phenolic acid and polysaccharide composition of cell walls of bran layers of mature wheat (*Triticum aestivum* L. cv. Avalon) grains. J. Sci. Food Agric. 85, 2539-2547.

Ralph, J., Quideau, S., Grabber, J.H., Hatfield, R.D., 1994. Identification and synthesis of new ferulic acid dehydrodimers present in grass cell-walls. J. Chem. Soc. -Perkin Trans. 1 , 3485-3498.

Rouau, X., Cheynier, V., Surget, A., Gloux, D., Barron, C., Meudec, E., Louis-Montero, J., Criton, M., 2003. A dehydrotrimer of ferulic acid from maize bran. Phytochemistry 63, 899-903.

Saulnier, L., Crepeau, M.J., Lahaye, M., Thibault, J.F., Garcia-Conesa, M.T., Kroon, P.A., Williamson, G., 1999. Isolation and structural determination of two 5,5'-diferuloyl oligosaccharides indicate that maize heteroxylans are covalently cross-linked by oxidatively coupled ferulates. Carbohydr. Res. 320, 82-92.

Shedletzky, E., Shmuel, M., Trainin, T., Kalman, S., Delmer, D., 1992. Cell wall structure in cells adapted to growth on the cellulose synthesis inhibitor 2,6-Dichlorobenzonitrile. A comparison between 2 dicotyledonous plants and a graminaceous monocot. Plant Physiol. 100, 120-130.

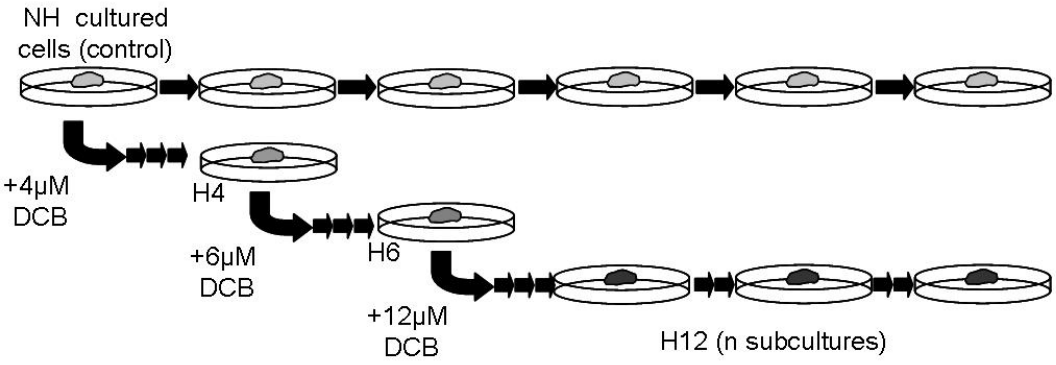
Smith, M.M., Hartley, R.D., 1983. Occurrence and nature of ferulic acid substitution of cell-wall polysaccharides in graminaceous plants. Carbohydr. Res. 118, 65-80.

Vaughn, K.C., 2002. Cellulose biosynthesis inhibitor herbicides, in: Böger, P., Wakabayashi, K., Hirai, K. (Eds.), Herbicide Classes in Development. Springer-Verlag, Berlin, pp. 139-150.

Waldron, K.W., Parr, A.J., Ng, A., Ralph, J., 1996. Cell wall esterified phenolic dimers: identification and quantification by reverse phase high performance liquid chromatography and diode array detection. Phytochem. Anal. 7, 305-312.

Supplementary Figure 1 online

Scheme of the habituation process of maize callus cultured cells to DCB. Calluses were habituated to growth in different DCB concentrations by stepwise transfers with gradual increments. At least three subcultures of approximately 30 days were performed between each increase in the DCB concentration.



Capítulo V:

Changes in cinnamic acid derivatives associated to the habituation of maize cells to dichlobenil

Capítulo correspondiente al manuscrito: Mélida, H., Álvarez, J., Acebes, J.L., Encina, A., Fry, SC., (2010). Changes in cinnamic acid derivatives associated to the habituation of maize cells to dichlobenil. Actualmente en revision.

Changes in cinnamic acid derivatives associated to the habituation of maize cells to dichlobenil

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Abstract

The habituation of cell cultures to cellulose biosynthesis inhibitors such as dichlobenil (DCB) reflects the ability of plant cells to modify the structure and composition of cell walls for surviving in new environmental conditions, and represents a valuable tool to improve our knowledge on the mechanisms involved in plant cell wall structural plasticity.

Maize cell lines habituated to lethal concentrations of DCB were able to grow through the acquisition of a modified cell wall in which cellulose was partially replaced by a more extensive network of arabinoxylans. A preliminary characterization of cell-wall phenolics in DCB-habituated cells showed an overall enrichment in these compounds.

The advantages of radiolabelling and using cell-cultures to tracking and studying polysaccaride feruloylation have been widely demonstrated in recent years. The aim of this work was to investigate the phenolic metabolism of non-habituated and DCB-habituated maize cells, by studying the [^{14}C]cinnamate fate in different intraprotoplasmic and wall-localised fractions throughout the cell culture cycle.

Non-habituated and habituated maize cells did not markedly differ in their ability to uptake [^{14}C]cinnamic acid from the medium. However, tracking the radiolabelling of low- and high-M_r [^{14}C]cellular metabolites, interesting differences were found. Habituated cells displayed a higher number and amount of [^{14}C]low-M_r compounds which could act as reserves of [^{14}C]hydroxycinnamoil units, later used for polysaccharide feruloylation.

DCB-habituated cells were highly enriched in esterified [^{14}C]dehydrodiferulates and larger coupling products, supporting the idea that a more cross-linked network of arabinoxylans was acting as a mechanism to counteract the reduction of cellulose in habituated cells. In sum, an extensive and premature cross-linking of hydroxycinnamates was observed in DCB habituated cells as it was expected for a cellulose-deficient cell wall.

Keywords: cell wall; maize; dichlobenil; dehydrodiferulate; ferulate.

Abbreviations: AIR, alcohol insoluble residue; ASF, alcohol soluble fraction; BzA, Benzene/acetic acid; CBI, cellulose biosynthesis inhibitor; CFM, cell free medium; DCB, 2,6-dichlorobenzonitrile or dichlobenil; DFA, dehydrodiferulate; H, dichlobenil-habituated cells; NH, non-habituated cells; TLC, thin-layer chromatography.

Introduction

Primary cell wall of the Poales (grasses, cereals and related plants) is composed of a frame of cellulose microfibrils embedded in a hemicellulosic matrix (arabinoxylans, xyloglucan and in some cases, mixed-linked glucans) and smaller amounts of pectins and glycoproteins (Carpita and Gibeaut 1993).

Poales cell walls are also characterized by the presence of hydroxycinnamates or cell wall phenolics, mainly ferulic and *p*-coumaric acids, which are found substituting arabinoxylans by ester-linking α-L-arabinosyl residues (Smith and Hartley 1983; Kato and Nevins 1985). Hydroxycinnamates are susceptible to oxidative coupling when exposed to hydrogen peroxide plus peroxidase (Geissman and Neukom 1971) and it has been demonstrated that ester-linked dehydrodiferulates formed can cross-link cell wall polysaccharides, contributing to wall assembly (Fry 2004; Parker et al. 2005). 5-5´-dehydro-diferulate was the first discovered dimer, and after this several other dimers have been obtained by alkaline hydrolysis of plant cell wall polysaccharides (Ralph et al. 1994; Waldron et al. 1996). Not only dimers, but also trimers (Bunzel et al. 2003; Rouau et al. 2003; Funk et al. 2005) and tetramers (Bunzel et al. 2006) have been characterised. The variety and complex structures observed suggest that these oligomers of hydroxycinnamates may play an important role in the architecture of the cell wall. Recently Burr and Fry (2009) demonstrated that besides ester-linked diferulates, some alkali-stable (ether-like) bonds contribute to polysaccharide cross-linking. Polysaccharide linking can occur intracellularly, before or during Golgi vesicular transit to cell membrane, and/or wall-localized, just following polysaccharide secretion or after wall integration (Fry et al. 2000; Obel et al. 2003; Mastrangelo et al. 2009). Once feruloylated polysaccharides are cross-linked and incorporated into the cell wall, this structure undergoes a significant alteration in a number of properties such as growth cessation, resistance to pathogens and insects, and cell wall degradability (Buanafina 2009).

Due to the main role of the cellulose in the cell wall structure, cellulose biosynthesis inhibitors (CBIs) have become valuable tools for the analysis of cell wall structure and biogenesis (Sabba and Vaughn 1999; Vaughn 2002; Acebes et al. 2010). Although CBIs are highly specific and potent herbicides, cell cultures of several species have been habituated to grow in the presence of several CBIs by incremental exposure over many culturing cycles, such as dichlobenil (2,6-dichlorobenzonitrile, DCB), which specifically inhibits the polymerization of glucose into β-1,4-linked glucan (Delmer 1987). The habituation of different cell cultures to DCB (Shedletzky et al. 1990; Encina et al. 2001, 2002) reflects the ability of plant cells to modify cell wall structure and composition in order to cope with abiotic stresses, and therefore represents a valuable tool for improving our knowledge of the mechanisms involved in plant cell wall structural plasticity and ability to maintain cell wall integrity. We obtained maize cell lines habituated to lethal concentrations of DCB (Mélida et al. 2009), which in spite of showing a 75% reduction in cellulose content, were able to grow through the acquisition of a modified cell wall in which cellulose was partially replaced by a more extensive network of arabinoxylans. A preliminary TLC characterization of cell-wall esterified phenolics in DCB-habituated cells showed an overall enrichment in cell wall esterified hydroxycinnamates and a shift from ferulic acid to *p*-coumaric acid enriched cell walls.

The advantages of radiolabelling and using cell-cultures to tracking and studying polysaccharide feruloylation and related items have been widely demonstrated in recent years (Fry et al. 2000; Kerr and Fry 2003, 2004; Obel et al. 2003; Encina and Fry 2005; Lindsay and Fry 2008; Burr and Fry 2009).

Experiments involving [^{14}C]ferulate-feeding (Obel et al. 2003) do not necessarily trace the major natural pathway to polysaccharide feruloylation, but [^{14}C]cinnamate, which is metabolically inert in the apoplast, and just begins to be metabolised when is taken up by the cells, does it (Lindsay and Fry 2008).

The aim of this work was to investigate the phenolic metabolism in DCB-habituated maize cells, studying the [^{14}C]cinnamate fate in different intraprotoplasmic and wall-localised fractions throughout the culture cycle of non-habituated (NH) and DCB-habituated (H) maize cells.

Materials and methods

Cell cultures

Maize callus cultures (*Zea mays* L., Black Mexican sweetcorn) from immature embryos were grown in Murashige and Skoog media (Murashige and Skoog 1962) supplemented with 9 μM 2,4-Dichlorophenoxyacetic acid and 8% agar at 25°C under light (Lorences and Fry 1991), and subcultured fortnightly. Calluses were habituated to growth in lethal DCB (Fluka) concentrations (12 μM) by stepwise transfers with gradual increments, beginning at 2 μM (Mélida et al. 2009) and were transferred to medium supplemented with 6 μM DCB, but without agar, to obtain maize DCB-habituated liquid cultured cells.

Chemicals

[^{14}C]Cinnamic acid was prepared from L-[U-^{14}C]phenylalanine (460 Ci/mol; Amersham) as described by Lindsay and Fry (2008), and stored at -20°C.

Chromatography

Thin-layer chromatography (TLC) was carried out on plastic-backed silica-gel plates with a fluorescent indicator (Merck) in benzene/acetic acid (9:1, v/v; BzA). During development, TLC plates were exposed to 366-nm UV to keep hydroxycinnamates as rapidly inter-converting single spot of *cis/trans* isomers. Authentic markers were located under 254-nm UV radiation. Based on HPLC retention times (Waldron et al. 1996), TLC R_F-values (Encina personal communication), and previous published data (Lindsay and Fry 2008), six putative dehydrodiferulates (DFAs) were suggested to appear. D3 co-migrated with authentic 5-5´-DFA.

Radiolabelling of liquid cultures with [^{14}C]cinnamate and assay of radioactivity

Aliquots (500 μl) of NH and H maize cell-suspension cultures (1, 3, 5, 7, 9, 11, 13 and 15 days after sub-culturing) were transferred into flat-bottomed vials (i.d. 11 mm) loosely capped with aluminium foil, where were then left for 1 h to "acclimatise" to their new environment (25°C and shaking, 150 rpm). [^{14}C]Cinnamic acid (2.7 kBq), in 10 μl H_2O, was then added to each vial. Samples

of medium (10 µl) were removed at each time point (up to 2 h) and assayed by scintillation counting. After 2 h the cultures were filtered obtaining cell-free medium (CFM) and cells. Cells were washed and incubated (18 h) with 500 µl of 75% ethanol on a rotating wheel. The alcohol-soluble fraction (ASF) and dry alcohol-insoluble residue (AIR) were collected. AIR was saponified with 0.5 M NaOH for 18 h, to release esterified hydroxycinnamates, acidified by addition of trifluoroacetic acid (TFA) and partitioned against ethyl acetate (x2). The ethyl acetate phases were vacuum-dried and re-dissolved in propan-1-ol (named as 0.5M-AIR). The residue after 0.5 M NaOH saponification was then treated with 6 M NaOH 18h at 37°C in order to break alkali-stable (ether-like) bonds, acidified, partitioned and vacuum-dried (named as 6M-AIR). Portions of all the fractions (including the final residue after 6 M NaOH treatment) were assayed by scintillation counting. Aliquots (15 µl) of CFM, ASF, 0.5M-AIR and 6M-AIR were TLC-subjected. The TLC plates were autoradiographed on Kodak film, and in some cases were cut into 1-cm strips, which were assayed for radioactivity. Aqueous or ethanolic solutions were mixed with ten volumes of OptiPhase (Wallac Oy), and dry samples were moistened in 2 ml of OptiScint (Wallac Oy).

Further experiments were carried out using 300 µl-aliquots of NH and H maize cell cultures (7 and 15 days after sub-culturing), which were transferred into flat-bottomed vials (i.d. 7.5 mm) and developed as indicated previously. [^{14}C]Cinnamic acid (1.6 kBq), in 10 µl H_2O, was then added to each vial, and after 120 min H_2O_2 was added to some vials to give a final concentration of 1 mM. At selected time-points (up to 3 h) 750 µl of 100% ethanol containing 7% formic acid were added to each culture vial, capped and left on a rotating wheel for 18 h. ASF and AIR were obtained and assayed as before.

Results

Uptake of [^{14}C]cinnamic acid from the medium

NH and H maize cell-suspension cultures did not markedly differ in their ability to uptake [^{14}C]cinnamic from the culture medium (Fig. 1). In most cases, and independently of the cell line, the net uptake of ^{14}C ceased after 30 min, reaching 60 to 80% of total radioactivity added. Calculated on the base of the radioactivity remaining in the medium after a 15 min incubation, [^{14}C]cinnamic acid consumption rates among 2.9 and 4.7% min^{-1} were estimated for both cell lines, depending on the cell age.

The TLC analysis of the ^{14}C-labelled compounds present in the cell culture medium (Fig. 2) showed that both, NH and H maize cells, completely removed the [^{14}C]cinnamic acid after 120 min incubation. Accordingly to the peak of radioactivity detected at R_F0, the chemical nature of the ^{14}C-labelled compounds found in the culture medium would correspond to polymeric and/or highly hydrophilic material.

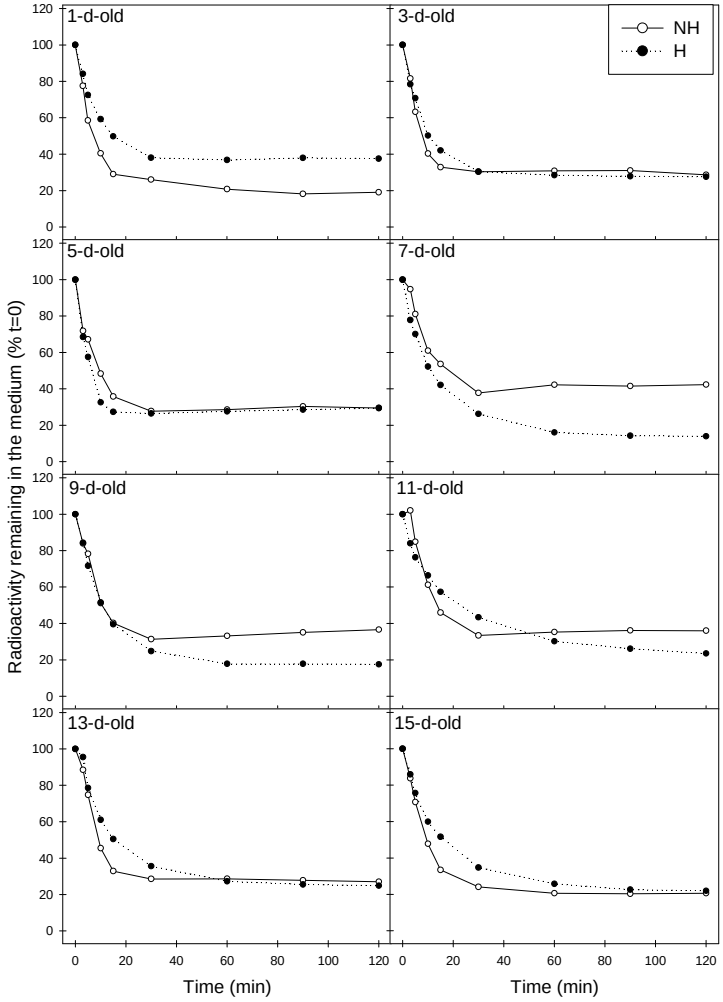

Fig. 1 Time-course of consumption of [^{14}C]-cinnamic acid by non-habituated (NH; *open circle*) and DCB habituated (H; *filled circle*) maize cell cultures (from 1 to 15 days after subculturing). [^{14}C]Cinnamic acid (2.7 kBq) was fed to 500-µl cultures. Portions of cell-free medium were assayed for radioactivity at each time-point

[^{14}C]radiolabelling of cellular metabolites along the culture cycle of maize cells

Low-M_r (ASF) and high-M_r (0.5M-AIR/6M-AIR) cellular metabolites were assayed throughout the culture cycle of NH and H maize cell cultures (Fig. 3). Samples were taken after 120 min [^{14}C]cinnamic acid feeding. ASF of NH (Fig. 3a) did not contain many different compounds, just some low-R_F ones, and the amount of these diminished along the cycle. The opposite fashion was observed for H cells (Fig. 3b), where the number and the amount of compounds increased when the culture cycle advanced, especially after day 9. Even free *p*-[^{14}C]coumarate was present in these cultures after day 7.

The cellular polymers (AIR fraction) were subjected to a sequential fractionation in order to distinguish between alkali labile (ester linked units) and alkali resistant (ether linked units) material.

After 0.5M-NaOH saponification of AIR (Fig. 3c and d) ester-bonded [^{14}C]metabolites were expected to be released. Based on TLC results the total amount and diversity of [^{14}C]ester-linked compounds were higher in H cell cultures than in NH ones. Ester-linked [^{14}C]ferulate and *p*-[^{14}C]coumarate were detected in both cell lines one day after subculturing. Quantitative and qualitative differences between H and NH cells were found in the region where DFAs (named as D1-D6) putatively migrate.

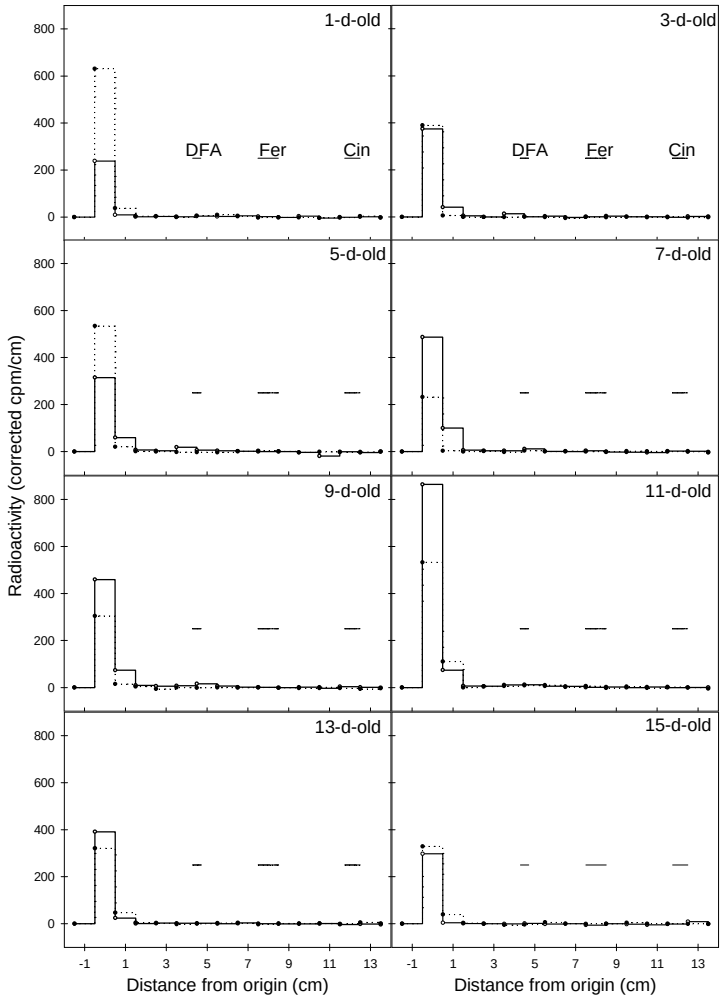

Fig. 2 TLC of cell-free medium (CFM) from non-habituated (NH; *open circle-continuous line*) and habituated (H; *filled circle-dotted line*) to DCB maize cell cultures (from 1 to 15 days after subculturing). CFM obtained as in Fig. 1, was TLC chromatographed in BzA. Strips (1 cm) were assayed for ^{14}C. External markers (DFA, 5-5´-dehydrodiferulic acid; Fer, ferulic acid and Cin, cinnamic acid) were run and located under UV prior to scintillation-counting

On the base of co-migration with authentic 5-5´-DFA and R_F-values on TLC using this and other solvent systems (Baydoun et al. 2004; Lindsay and Fry 2008) the putative assignation of spots to DFA was as follow:

D1: 8-5B´DFA
D2: 8-O-4´DFA
D3: 5-5´DFA
D4: 8-5´DFA
D5: 8-8´DFA
D6: 8-8A´DFA

In NH cells D1 (followed by D5) was the major [^{14}C]DFA. A strong accumulation of putative [^{14}C]8-5B´DFA was observed in 15-day-old cells. D1-D6 were consistently detected throughout the culture cycle of H cells, and from day 7 after subculturing, a substantial increase of radiolabelled DFAs was detected. In any case the radiolabelling intensity of D1-D6 compounds was higher in H than in NH throughout the cell culture cycle.

6M-NaOH saponification (Fig. 3e and f) released relative alkali-stable (ether-like) bonds. While only *p*-[^{14}C]coumarate was detected after this treatment in NH cells (from day 7), [^{14}C]ferulate, *p*-[^{14}C]coumarate and traces of low-R_F compounds were detected in H cultures from day 3 after subculturing.

Interestingly, radioactive material at R_F0 that was detected only in aged

NH cells (7 to 15 day-old) become radioactive shortly in H ones (1 day after subculturing).

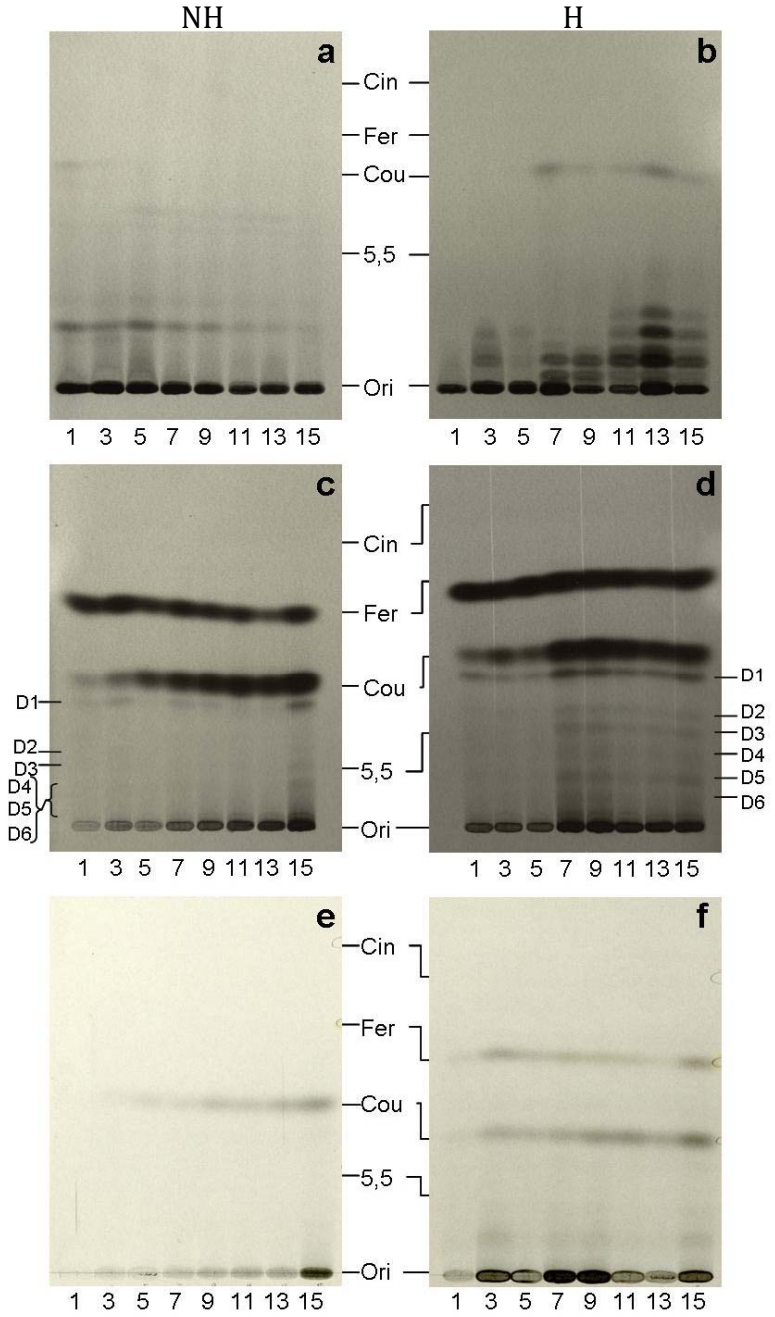

Fig. 3 Autoradiography of TLCs of alcohol-soluble fractions (ASF; low-Mr ^{14}C-compounds) (**a** and **b**), polymer-esterified [^{14}C]cinnamate-derivatives (after 0.5M NaOH treatment) (**c** and **d**) and polymer-etherified (alkali-stable) [^{14}C]cinnamate-derivatives (after 6M NaOH at 37°C treatment) (**e** and **f**) from non-habituated (NH; **a**, **c** and **e**) and DCB-habituated (H; **b**, **d** and **f**) maize cell cultures. [^{14}C]Cinnamic acid (2.7 kBq) was fed to 500-µl cultures aged 1, 3, 5,...15 days after subculturing. Samples were taken 120 min after feeding. Fractions were chromatographed in BzA and then autoradiographed. The positions of the origin (Ori) and external standards are indicated (*Cin*, cinnamic acid; *Fer*, ferulic acid; *Cou*, *p*-coumaric acid; *5,5*, 5-5´-diferulic acid). "D1"-"D6" in **c** and **d** indicate putative [^{14}C]dehydro-diferulates. Spot D3 is identified as 5-5´-dehydrodiferulic acid

Kinetics of radiolabelling between different pools along the culture cycle

To achieve an overall picture of the distribution of radioactivity between major pools throughout the culture cycle of NH and H maize cells, obtained fractions (CFM, ASF, 0.5M-AIR, 6M-AIR and the final residue) were assayed for ^{14}C (Fig. 4).

In the case of NH cultures a gradual increase in CFM fraction was observed throughout the cell culture cycle up to 11-day old cells, but a decrease was observed in 13- and 15-d-old cells. However, CFM fraction kept steady or even decreased as H cell cultures aged. In average, CFM fraction accounted for less radioactivity percentage in H cells than in NH ones throughout the cell culture cycle.

The kinetics of [^{14}C] partitioning into ASF fraction varied between cell lines. In NH cell line, a gradual reduction of this pool was observed as cells aged. In the case of H cells the level of ASF [^{14}C] labelled compounds kept steady throughout the cell culture cycle.

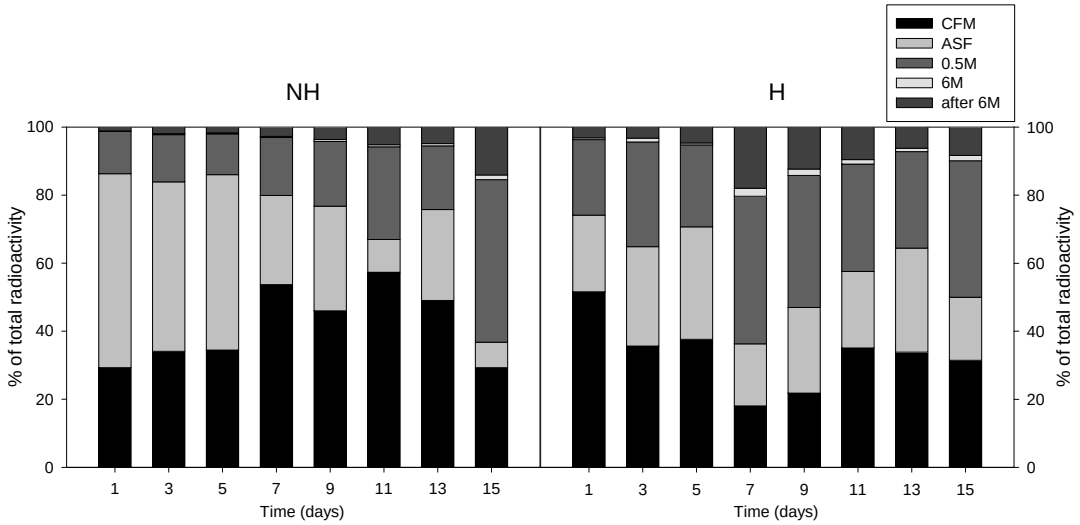

Fig. 4 Distribution of radioactivity between major pools. Fractions from the experiment reported in Fig. 1 and 3 were assayed for ^{14}C. Cell free medium (CFM) and cellular material from non-habituated (NH) and DCB-habituated (H) maize cell cultures were analysed. Cellular material was fractionated into alcohol-soluble fraction (ASF) and alcohol-insoluble residue (AIR). The AIR was sequentially fractionated into 0.5M-NaOH and 6M-NaOH extractable material. All these fractions and the final residue after extractions were assayed for ^{14}C

In NH maize cells, the proportion of radioactivity incorporated into AIR by 120 min increased as cells aged. Further, a sharp increase of radioactivity recovered in this fraction (up to ≈ 64%) was detected 15 days after subculturing. When the kinetics of radiolabelling of the AIR fraction for H cells was assayed a peak of incorporation was observed 7 to 9 days after subculturing. With the exception of very aged cells, the proportion of radioactivity recovered in AIR fraction was higher in H cell cultures than in NH ones when cells with the same age were compared.

Most of the radioactivity incorporated into AIR, was fractionated into an alkali labile fraction (0.5M-NaOH extractable material). Further, a variable

proportion of AIR labelling needed strong alkali (6M-NaOH extractable material) to be extracted or pooled in the final residue (6M-NaOH residue) being regarded as alkali resistant [^{14}C]-compounds. In NH cells a gradual increment was observed in the proportion of this residue as cells aged. Both alkali-labile and alkali-resistant [^{14}C]-compounds were more abundant in H cells that in NH.

Radioactivity incorporation to low-molecular-weight fraction of 7- and 15-d-old maize cultures

Seven- and 15-d-old maize cultures were deeper analysed as the radioactivity was tracked in different pools during 180 min (adding H_2O_2 after 120 min). After approximately 10 (NH) or 30 (H) min, [^{14}C]cinnamate was largely replaced by other [^{14}C]metabolites (Figs. 5, 7 and 8). Up to 26% of the ^{14}C added remained as ethanol soluble [^{14}C]metabolites (ASF) in both 7- and 15-d-old cultures (Fig. 4) 120 min after feeding.

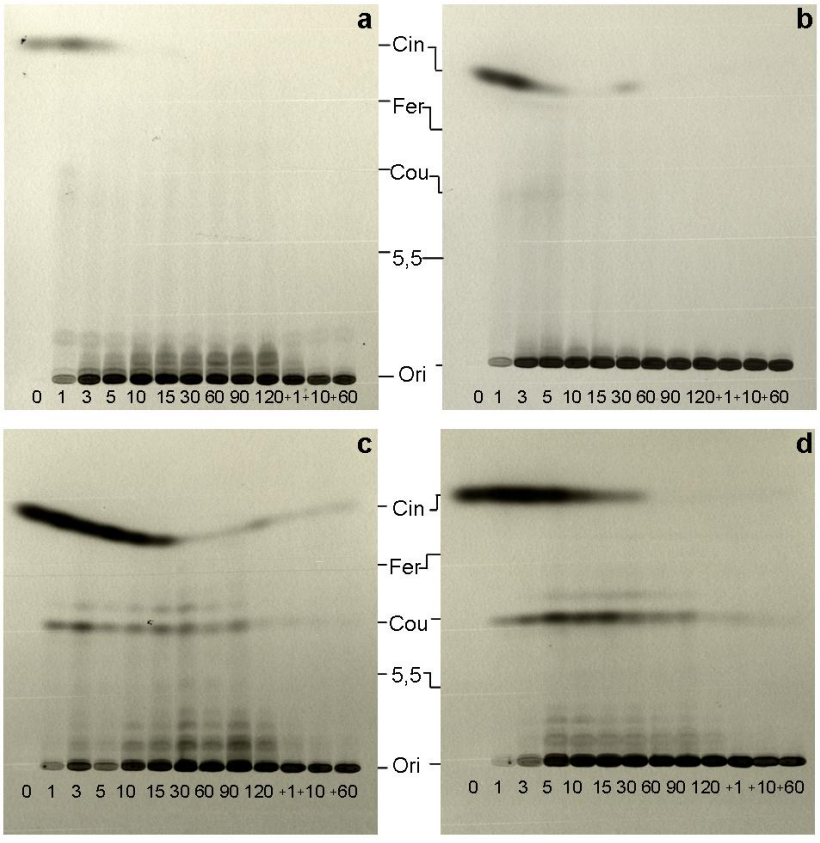

Fig. 5 Autoradiography of TLCs of ASFs (low-Mr ^{14}C-compounds) from 7 (**a** and **c**) and 15-d-old (**b** and **d**) maize cultures; non-habituated (NH; **a** and **b**) and DCB-habituated (H; **c** and **d**). [^{14}C]Cinnamate was fed (1.6 kBq) to 300 µl cultures, which were harvested at 0, 1, 2,...120 min, as indicated. After 120 min H_2O_2 (final concentration 1mM) was added to further cultures, which were harvested after an additional period of incubation (+1, +10 and +60 min). ASFs were chromatographed in BzA and then autoradiographed. The positions of the origin and external standards are indicated (key as in Fig. 3)

In contrast with NH cells in which free *p*-[^{14}C]coumarate was detected in traces (see 15-day-old NH cells; Fig. 5b), free *p*-[^{14}C]coumarate was clearly

detected in 7 and 15-day-old H cells up to 90 min after ^{14}C feeding. Habituated cells were characterized for the rapid labelling (1 min after [^{14}C]cinnamic acid feeding) of several polar [^{14}C]compounds (R_F0 + low-R_F material). These highly hydrophilic compounds were stable for 120 min and partially disappeared from ASF after H_2O_2 addition. Polar [^{14}C]compounds were less abundant and diverse in NH cells, especially in 15-day-old cells, where low-R_F material turned over 30 min after [^{14}C]cinnamic acid feeding.

Radioactivity incorporation to high-M_r compounds; different requirements of H_2O_2

Following the kinetics of incorporation (Fig. 6) of [^{14}C]compounds into the AIR of NH and H cells during 180 min (adding H_2O_2 at 120 min) interesting differences were observed.

Seven-d-old NH cells slowly (during first 120 min) incorporated to the AIR ≈ 18% of the total radioactivity added, and the addition of H_2O_2 stimulated this incorporation up to 44%. Although H cells (7- and 15-d-old) incorporated higher amounts of radioactivity (≈ 30%) during first 60 min in contrast to 7-d-old NH cells, similar trends between both cell lines were observed as the addition of H_2O_2 stimulated again the incorporation up to high levels (more than 50%). In the other hand 15-d-old NH cells continuously incorporated radioactivity in higher levels than the other cells, and clearly in this case the requirement of H_2O_2 was not a limiting factor and its addition did not change substantially the radioactivity incorporation to the AIR.

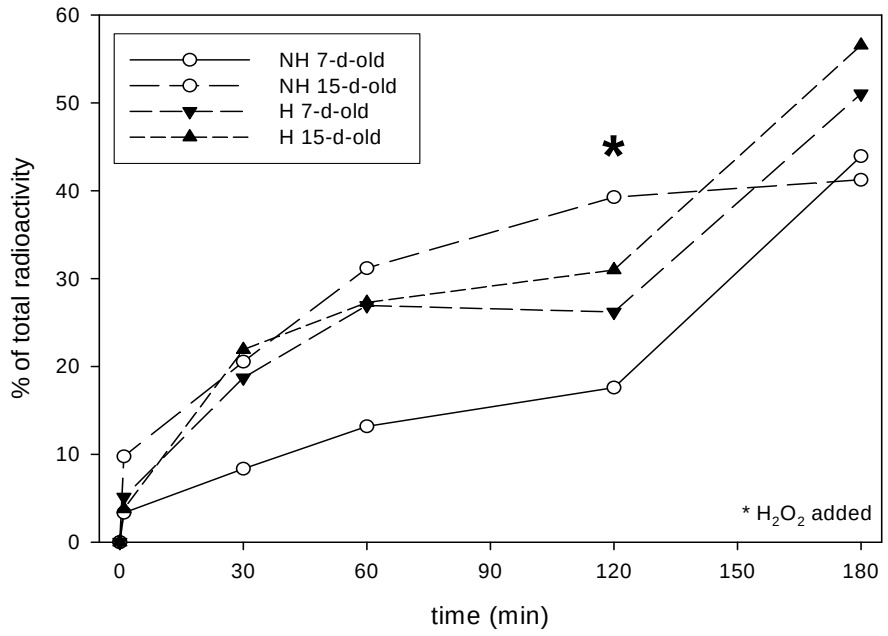

Fig. 6 Kinetics of incorporation of [^{14}C]compounds into the alcohol-insoluble residue (AIR) of non-habituated (NH) and DCB-habituated (H) maize cell cultures. [^{14}C]Cinnamate was fed (1.6 kBq) to replicate cultures, which were harvested at 0, 30, 60 and 120 min, as indicated. After 120 min H_2O_2 (final concentration 1mM) was added to further cultures, which were harvested after an additional period of incubation (60 min). AIR was assayed for radioactivity

Polymer-esterified derivatives

Esterified [^{14}C]ferulate and [^{14}C]*p*-coumarate were found in both NH and H cells since initials time-points (Fig. 7). As expected, radiolabelling kinetic indicated that [^{14}C]*p*-coumaroyl preceded [^{14}C]feruloyl groups. Radiolabelled material at R_F0 was increasingly accumulated from 30-min after [^{14}C] feeding. In the case of 7-day-old NH cells, the addition of H_2O_2 highly stimulated the radiolabelling of esterified monomers and DFAs. In fact, no putative DFAs were detected before H_2O_2 addition.

In 15-day-old NH cells, the levels of ^{14}C incorporation into AIR-esterified [^{14}C]*p*-coumarate and [^{14}C]ferulate were higher than in younger NH cells (Fig. 7). Moreover, [^{14}C]*p*-coumarate levels reached a plateau 3 min after [^{14}C]cinnamic acid feeding, and kept steady for 120 min. In aged NH cells putative 8-5B´DFA (spot D1) appeared 15 min after [^{14}C]cinnamic feeding. In addition, slow migrating compounds became radiolabelled earlier than in 7-day-old NH cells. In the case of aged NH cells, the requirement of H_2O_2 was not a limiting factor and its addition did not substantially change changed either the amount of radiolabelling of AIR (see Fig. 6 also) or the type of radiolabelled compound (Fig. 7).

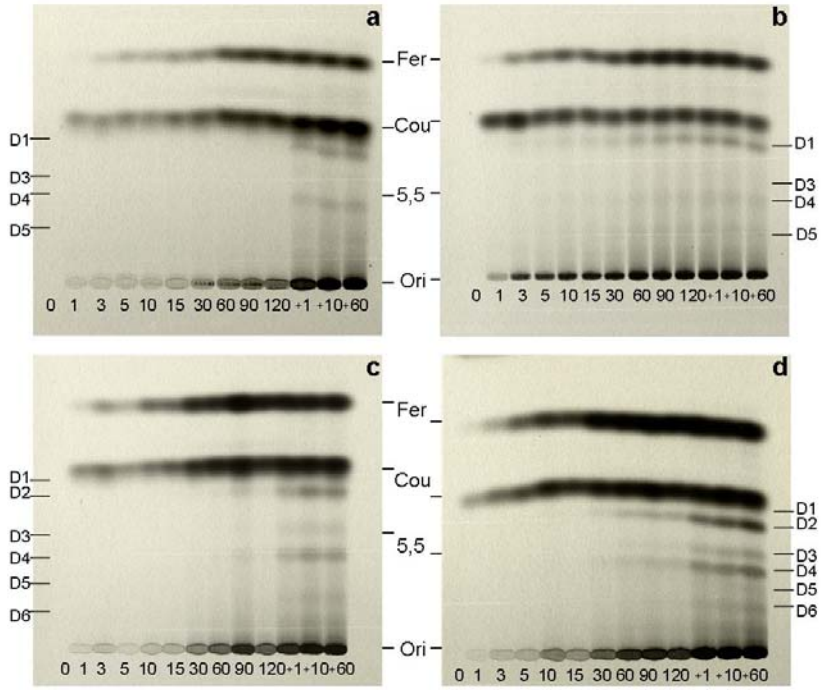

Fig. 7 Autoradiography of TLCs of polymer-esterified [^{14}C]cinnamate-derivatives (after 0.5M NaOH treatment) synthesised by 7 (**a** and **c**) and 15-d-old (**b** and **d**) maize cultures; non-habituated (NH; **a** and **b**) and DCB-habituated (H; **c** and **d**). The cultures were incubated with [^{14}C]cinnamate for 0-120 min, as indicated, followed by an additional incubation period after treatment with H_2O_2 as in Fig. 5. ^{14}C-compounds were chromatographed in BzA and then autoradiographed. The positions of the origin and external standards are indicated (key as in Fig. 3)

A similar pattern of AIR-radiolabelling was observed for H cells, although these ones (both 7- and 15-d-old) incorporated higher amounts or

radioactivity (see Fig. 6 also) than same aged NH cells. In accordance with the level of radiolabelling of putative DFAs, the addition of H_2O_2 had a marked effect on the increase of dimerisation in aged H cells.

Polymer-etherified derivatives

TLC of 6M-NaOH released [^{14}C]compounds (Fig. 8) by 7-d-old NH just displayed the [^{14}C]*p*-coumarate spot, which was more intense after about 30 min, but in 7-d-old H cells this spot was slightly more intense and appeared sooner. Moreover the [^{14}C]ferulate spot was also detectable after about 30 min. This trend was similar in 15-d-old NH cells, but H cells same aged displayed after H_2O_2 addition besides low-R_F spots putatively corresponding to radiolabelled dimers, trimers or oligomers.

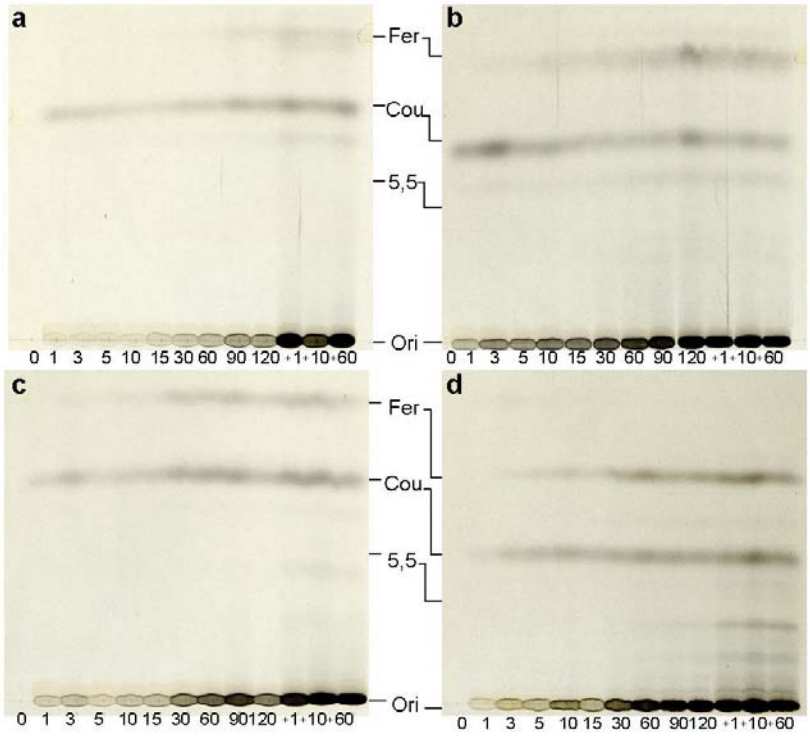

Fig. 8 Autoradiography of TLCs of polymer-etherified (alkali-stable) [^{14}C]cinnamate-derivatives (after 6M NaOH at 37°C treatment) synthesised in 7 (**a** and **c**) and 15-d-old (**b** and **d**) maize cultures; non-habituated (NH; **a** and **b**) and DCB-habituated (H; **c** and **d**). The cultures were incubated with [^{14}C]cinnamate for 0-120 min, as indicated, followed by an additional incubation period after treatment with H_2O_2 as in Fig. 5. ^{14}C-compounds were chromatographed in BzA and then autoradiographed. The positions of the origin and external standards are indicated (key as in Fig. 3)

TLC of 6M-NaOH released [^{14}C]compounds (Fig. 8) by 7 and 15 day-old cells did not much differ between NH and H cells. Major polymer etherified [^{14}C]hydroxyccinamates were [^{14}C]*p*-coumarate followed by [^{14}C]ferulate. No ether-linked [^{14}C]ferulate was detected in young NH cell walls.

As in the case of polymer esterified phenolics (fig 7) [^{14}C]*p*-coumaroyl cell wall incorporation preceded [^{14}C]feruloyl groups.

Radiolabelled material at R_F0 was increasingly detected from 1-min after [^{14}C] feeding. The R_F0 spot was more intense in H12 cells when compared with NH ones, specially in 30 to 120 min radiolabelled samples.

In all the cases, H_2O_2 addition increased the amount of R_F0 cell wall etherified compounds. Moreover, in the case of aged H cells, H_2O_2 addition rendered low R_F radiolabelled compounds (dimers, trimers or oligomers).

Discussion

It has previously been suggested that feruloyl-arabinoxylan cross-linking has consequences in the control of cell expansion and in the response of plant cells to stresses as a defence mechanism (Bolwell et al. 1998; Fry et al. 2000; Buanafina 2009). Maize cells habituated to lethal concentrations of DCB presented a modified cell wall composition and architecture (Mélida et al. 2009), that allowed cells to cope with this toxic compound. Based on preliminary works, we hypothesized for the phenolic component of the cell wall an outstanding role in the habituation process to the cellulose biosynthesis inhibitor DCB. In this work, by using pulse-chase experiments with [^{14}C]cinnamic acid, and tracking radiolabelled compounds through different pools, an attempt to describe changes on phenolic metabolism of a cellulose impoverished cell wall has been conducted.

The fate of [^{14}C]cinnamic acid uptake by maize cultured cells was comparable to that shown by Lindsay and Fry (2008). As cinnamic acid is a lipophilic weak acid, it penetrates the plasma membrane easily and was completely removed from the medium in few minutes. NH and H maize liquid cultured cells did not markedly differ in their ability to uptake [^{14}C]cinnamic acid from the culture medium. However, important differences in the [^{14}C]radiolabelled products present in different cellular pools from NH and H cells were found. TLCs of CFM after the radiolabelling showed a single peak of radioactivity at R_F0, putatively corresponding to polymeric and/or highly hydrophilic material. It is likely to consider these compounds as cellular material, probably cell wall [^{14}C]feruloyl-arabinoxylans, sloughed into the cell culture medium (Fry et al. 2000; Kerr and Fry 2003, 2004; Lindsay and Fry 2008). Therefore, the rapid uptake was followed by a reappearance of radioactivity in the medium, indicating a release of those [^{14}C]hemicelluloses which missed their chance for the integration in the cell wall immediately after secretion and were therefore not retained within the wall. In the case of NH cultures, after a gradual increase in CFM fraction throughout the cell culture cycle, a decrease took place in aged NH cell cultures. This would be explained by phenolic cross-linking of extracellular [^{14}C]feruloyl-arabinoxylans into the cell wall. Therefore a second chance for integration within the cell wall seemed to take place in old cells. In average, CFM fraction accounted for fewer radioactivity in H cell cultures throughout the cell culture cycle. This may indicate a more efficient integration of newly synthesized [^{14}C]hemicelluloses into the wall of DCB habituated cells, which may thus play an important role in wall assembly (Kerr and Fry 2003).

Tracking the radiolabelling of low- and high-M_r cellular metabolites during the culture cycle, considerable differences between non-habituated and

DCB-habituated maize cultured cells were found. Some polar low-M_r compounds, probably including [^{14}C]hydroxycinnamoyl conjugates of sugars or other hydrophilic substances, e.g. β-glucosyl esters (Harborne and Corner 1961; Fry et al. 2000; Obel et al. 2003) were detected in both cell lines. However habituated cells displayed a higher variety and amount of these compounds and an increasing accumulation along the culture cycle could also be observed. These compounds could act as reserves of [^{14}C]hydroxycinnamoil units, later used for polysaccharide feruloylation directly or indirectly from feruloyl-CoA (Fry 1984; Fry et al. 2000; Obel et al. 2003; Lindsay and Fry 2008). Habituated cells required higher levels of these reserves. Short-time feeding experiments of 7- and 15-d-old maize cultures reported results in line with these. Interestingly these compounds disappeared from this fraction when H_2O_2 was added.

The AIR fraction (cellular polymers) includes mature polysaccharides already deposited in the cell wall, but also newly synthesised polysaccharides in the Golgi bodies or being transported in vesicles (Lindsay and Fry 2008). NH cells increased the proportion of radioactivity incorporated into AIR as cells aged. In other words; the older the NH cells, the higher amount of [^{14}C]hydroxycinnamates were incorporated into the cell wall, and therefore, more substrates would be available to cross-link adjacent polysaccharides. This result is in line with several studies that have indicated a positive correlation between phenolic cross-linking and growth cessation in various monocot species (Kamisaka et al. 1990; MacAdam and Grabber 2002; Azuma et al. 2005). Habituated cells incorporated a high proportion of radioactivity into AIR since early growth phases. Moreover, with the exception of very aged cells, the proportion of radioactivity recovered in AIR fraction was higher in H cell cultures than in NH ones when cells with the same age were compared. In base to these results, a higher amount of cell wall hydroxycinnamates and a higher and earlier phenolic cross-linking degree would be expected for H cells.

Most of the radioactivity incorporated into AIR was fractionated into an alkali labile fraction. With regard to cell wall esterified hydroxycinnamates, [^{14}C]ferulate and *p*-[^{14}C]coumarate were present in both cell lines along the culture cycle and were attached to a polysaccharide chain very quickly, as these compounds became detectable radioactive within 1 min, as was previously observed by Fry et al. (2000). Regarding to esterified [^{14}C]DFAs, qualitative and quantitative differences were achieved between cell lines. Habituated cells were highly enriched in [^{14}C]DFAs, supporting the idea that a more cross-linked network of arabinoxylans was acting as a mechanism to counteract, at least partially, the reduction of cellulose in H cells. According to the appearance of [^{14}C]DFAs at short-time, ferulic acid dimerization began before in H cells, although aged NH cells also showed high levels of DFAs. While cross-linking seems related with growth cessation in NH cells, in the case of H cells this mechanism seems to be a tightening strategy since early cell growth phases.

The possibility of benzyl ether bonds contributing to hemicelluloses cross-linking in primary cell walls had been suggested some time ago (Kerr and Fry, 2004). Very recently it was demonstrated that after oxidative coupling, strong benzyl-sugar ether bonds are formed via quinone-methide compounds (Burr and Fry 2009). In this paper we provide results pointing to the presence of these alkali-resistant-bonded polysaccharides both in NH and H cell walls.

[^{14}C]Ferulate, *p*-[^{14}C]coumarate and R_F0 compounds were detected in NH and H cultures. As expected, timing of the incorporation indicated that *p*-[^{14}C]coumarate was incorporated before than [^{14}C]ferulate. [^{14}C]Ferulate and *p*-[^{14}C]coumarate released after a strong alkaly treatment could represent a population of ether linked hydroxycinnamates forming brigdes between polysaccharides and lignin-like compounds (Lam et al, 2001). The presence of this sort of linkages has been proposed to occur during the primary cell wall formation of grasses (Iiyama et al. 1990; Lam et al. 1994). Considering that not much differences in the amount and diversity of ether-linked hydroxi-cinnamates were found between NH and H cells, a minor role in DCB habituation is proposed for this sort of very strong phenolic-mediated linkages between polysaccharides.

Ferulate cross-linking varies with the physiological condition of cells along the cell culture cycle (Burr and Fry 2009). Same authors proposed that main factor controlling ferulate cross-linking is peroxidase action instead of peroxidase activity being the rate of H_2O_2 production and the presence/absence of low-M_r inhibitors as described by Encina and Fry (2005), main factors controlling the peroxidase action. Here we report that throughout the cell culture cycle, cross-linking activity started earlier in DCB-habituated cells as a strong accumulation of [^{14}C]DFAs and larger coupling products were obtained from cellular polymers of very young H cells (1-day-old). Moreover the sudden increase in this sort of [^{14}C]compounds observed for 7-day-old H cells (same effect is observed in very aged NH cells) suggest a burst in H_2O_2 production and/or the disappearance of a putative phenolic cross-linking inhibitor early in the cell culture cycle. In line with this we show that the exogenous H_2O_2 addition stimulated dimerization in H cells in all cases, but not in the oldest NH cells where dimerization seems to have already taken place, and no substrates for peroxidase would be available. So, although habituated cells showed higher dimerization levels, the H_2O_2 was still a limiting factor for even a more extensive feruloylation. Further experiments involving low-M_r inhibitors as those described by Encina and Fry (2005) in habituated cells will help to elucidate the role of these compounds controlling peroxidase action.

In sum, by using pulse-chase experiments with [^{14}C]cinnamate it has been shown that DCB habituation relates with a modification of citosolic phenolic metabolism that render an increased accumulation of precursor for polysaccharide feruloylation. Moreover a more efficient integration of newly synthesized hemicelluloses together with an extensive and premature cross-linking of hydroxycinnamates was observed in dichlobenil habituated cells as it was expected for a cellulose-deficient cell wall, to be reinforced.

Acknowledgements

This work was supported by the Spanish Ministry of Science and Innovation program [CGL2008-02470]. H-M was financed by a PhD grant from the Spanish Ministry of Science and Innovation's FPU program. We are indebted to Dr. Alonso-Simón and Dr. García-Angulo for scientific discussions and to Mrs Janice Miller for excellent technical assistance at Dr. Fry´s lab.

References

Acebes JL, Encina A, García-Angulo P, Alonso-Simón A, Mélida H, Álvarez JM (2010) Cellulose biosynthesis inhibitors: their uses as potential herbicides and as tools in cellulose and cell wall structural plasticity research. In: A. Lejeune and T. Deprez (eds) Cellulose: Structure and Properties, Derivatives and Industrial Uses. Nova Publishers, New York. In press

Azuma T, Okita N, Nanmori T, Yasuda T (2005) Relationship between the deposition of phenolic acids in the cell walls and the cessation of rapid growth in internodes of floating rice. Plant Prod Sci 8:447-453

Baydoun EA, Pavlencheva N, Cumming CM, Waldron KW, Brett CT (2004) Control of dehydrodiferulate cross-linking in pectins from sugar-beet tissues. Phytochemistry 65:1107-1115

Bolwell GP, Davies DR, Gerrish C, Auh CK, Murphy TM (1998) Comparative biochemistry of the oxidative burst produced by rose and french bean cells reveals two distinct mechanisms. Plant Physiol 116:1379-1385

Buanafina MMdO (2009) Feruloylation in grasses: current and future perspectives. Mol Plant 2:861-872

Bunzel M, Ralph J, Bruning P, Steinhart H (2006) Structural identification of dehydrotriferulic and dehydrotetraferulic acids isolated from insoluble maize bran fiber. J Agric Food Chem 54:6409-6418

Bunzel M, Ralph J, Funk C, Steinhart H (2003) Isolation and identification of a ferulic acid dehydrotrimer from saponified maize bran insoluble fiber. Eur Food Res Technol 217:128-133

Burr SJ, Fry SC (2009) Extracellular cross-linking of maize arabinoxylans by oxidation of feruloyl esters to form oligoferuloyl esters and ether-like bonds. Plant J 58:554-567

Carpita NC, Gibeaut DM (1993) Structural models of primary cell walls in flowering plants. Consistency of molecular structure with the physical properties of the walls during growth. Plant J 3:1-30

Delmer DP (1987) Cellulose biosynthesis. Annu Rev Plant Physiol Plant Mol Biol 38:259-290

Encina A, Fry SC (2005) Oxidative coupling of a feruloyl-arabinoxylan trisaccharide (FAXX) in the walls of living maize cells requires endogenous hydrogen peroxide and is controlled by a low-Mr apoplastic inhibitor. Planta 223:77-89

Encina A, Moral RM, Acebes JL, Álvarez JM (2001) Characterization of cell walls in bean (*Phaseolus vulgaris* L.) callus cultures tolerant to dichlobenil. Plant Sci 160:331-339

Encina A, Sevillano JM, Acebes JL, Álvarez J (2002) Cell wall modifications of bean (*Phaseolus vulgaris*) cell suspensions during habituation and dehabituation to dichlobenil. Physiol Plantarum 114:182-191

Fry SC (1984) Incorporation of [^{14}C]Cinnamate into hydrolase-resistant components of the primary cell wall of spinach. Phytochemistry 23:59-64

Fry SC (2004) Oxidative coupling of tyrosine and ferulic acid residues: Intra- and extra-protoplasmatic occurrence, predominance of trimers and larger products, and possible role in inter-polymeric cross-linking. Phytochem Rev 3:97-111

Fry SC, Willis SC, Paterson AEJ (2000) Intraprotoplasmic and wall-localised formation of arabinoxylan-bound diferulates and larger ferulate coupling-products in maize cell-suspension cultures. Planta 211:679-692

Funk C, Ralph J, Steinhart H, Bunzel M (2005) Isolation and structural characterisation of 8-*O*-4/8-*O*-4- and 8-8/8-*O*-4-coupled dehydrotriferulic acids from maize bran. Phytochemistry 66:363-371

Geissman T, Neukom H (1971) Cross-linking of phenolcarboxylates of polysaccharides by oxidative phenolic coupling. Helv Chim Acta 54:1108-1112

Grabber JH, Hatfield RD, Ralph J, Zon J, Amrhein N (1995) Ferulate cross-linking in cell-walls isolated from maize cell-suspensions. Phytochemistry 40:1077-1082

Harborne JB, Corner JJ (1961) Plant polyphenols. 4. Hydroxycinnamic acid-sugar derivatives. Biochem J 81:242-250

Iiyama K, Lam TBT, Stone BA (1990) Phenolic-acid bridges between polysaccharides and lignin in wheat internodes. Phytochemistry 29:733-737

Kamisaka S, Takeda S, Takahashi K, Shibata K (1990) Diferulic and ferulic acid in the cell-wall of *Avena* coleoptiles - their relationships to mechanical-properties of the cell-wall. Physiol Plant 78:1-7

Kato Y, Nevins DJ (1985) Isolation and identification of *O*-(5-*O*-Feruloyl-α-L-Arabinofuranosyl)-(1-3)-*O*-β-D-Xylopyranosyl-(1-4)-D-Xylopyranose as a component of *Zea* shoot cell-walls. Carbohydr Res 137:139-150

Kerr EM, Fry SC (2003) Pre-formed xyloglucans and xylans increase in molecular weight in three distinct compartments of a maize cell-suspension culture. Planta 217:327-339

Kerr EM, Fry SC (2004) Extracellular cross-linking of xylan and xyloglucan in maize cell-suspension cultures: the role of oxidative phenolic coupling. Planta 219:73-83

Lam TBT, Iiyama K, Stone BA (1994) An approach to the estimation of ferulic acid bridges in unfractionated cell-walls of wheat internodes. Phytochemistry 37:327-333

Lam TBT, Kadoya K, Iiyama K (2001) Bonding of hydroxycinnamic acids to lignin: ferulic and *p*-coumaric acids are predominantly linked at the benzyl position of lignin, not the beta-position, in grass cell walls. Phytochemistry 57:987-992

Lindsay SE, Fry SC (2008) Control of diferulate formation in dicotyledonous and gramineous cell-suspension cultures. Planta 227:439-452

Lorences EP, Fry SC (1991) Absolute measurement of cell expansion in plant cell suspension cultures. Plant Cell Tiss Organ Cult 24:211-215

MacAdam JW, Grabber JH (2002) Relationship of growth cessation with the formation of diferulate cross-links and p-coumaroylated lignins in tall fescue leaf blades. Planta 215:785-793

Mastrangelo LI, Lenucci MS, Piro G, Dalessandro G (2009) Evidence for intra- and extra-protoplasmic feruloylation and cross-linking in wheat seedling roots. Planta 229:343-355

Mélida H, García-Angulo P, Alonso-Simón A, Encina A, Álvarez J, Acebes JL (2009) Novel type II cell wall architecture in dichlobenil-habituated maize calluses. Planta 229:617-631

Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol Plant 15:473-497

Obel N, Porchia AC, Scheller HV (2003) Intracellular feruloylation of arabinoxylan in wheat: evidence for feruloyl-glucose as precursor. Planta 216:620-629

Parker ML, Ng A, Waldron KW (2005) The phenolic acid and polysaccharide composition of cell walls of bran layers of mature wheat (*Triticum aestivum* L. cv. Avalon) grains. J Sci Food Agric 85:2539-2547

Ralph J, Quideau S, Grabber JH, Hatfield RD (1994) Identification and synthesis of new ferulic acid dehydrodimers present in grass cell-walls. J Chem Soc -Perkin Trans 1 3485-3498

Rouau X, Cheynier V, Surget A, Gloux D, Barron C, Meudec E, Louis-Montero J, Criton M (2003) A dehydrotrimer of ferulic acid from maize bran. Phytochemistry 63:899-903

Sabba RP, Vaughn KC (1999) Herbicides that inhibit cellulose biosynthesis. Weed Sci 47:757-763

Shedletzky E, Shmuel M, Delmer DP, Lamport DTA (1990) Adaptation and growth of tomato cells on the herbicide 2,6-dichlorobenzonitrile leads to production of unique cell walls virtually lacking a cellulose-xyloglucan network. Plant Physiol 94:980-987

Smith MM, Hartley RD (1983) Occurrence and nature of ferulic acid substitution of cell-wall polysaccharides in graminaceous plants. Carbohydr Res 118:65-80

Vaughn KC (2002) Cellulose biosynthesis inhibitor herbicides. In: P. Böger, K. Wakabayashi and K. Hirai (eds) Herbicide Classes in Development. Springer-Verlag, Berlin pp 139-150

Waldron KW, Parr AJ, Ng A, Ralph J (1996) Cell wall esterified phenolic dimers: identification and quantification by reverse phase high performance liquid chromatography and diode array detection. Phytochem Anal 7:305-312

Discusión general

El progreso en el desarrollo de diferentes industrias, como la textil, del papel, alimentaria, energética y de la construcción está vinculado en gran medida al avance en el conocimiento de aspectos fundamentales de la pared celular, como son su estructura, formación y posible modificación (Cosgrove y col., 2009). El hecho de que las paredes celulares pueden modificar su composición y estructura para adaptar su metabolismo a diferentes condiciones de estrés ha abierto un panorama insospechado en las aplicaciones de las paredes celulares con arquitectura modificada. Actualmente el maíz se ha convertido en especie estratégica para las industrias agroalimentaria y bioenergética, por tanto el estudio de su pared celular y su potencial modificación ha cobrado un interés creciente. Una de las aproximaciones para estudiar esta plasticidad estructural de la pared celular ha sido la habituación de cultivos celulares a concentraciones elevadas de inhibidores de la biosíntesis de celulosa.

En los cultivos celulares de todas las especies que se han habituado a concentraciones elevadas de al menos tres de estos inhibidores -diclobenil (DCB), isoxabén y taxtomina A-, el contenido de celulosa experimenta un descenso notable, aunque este descenso no impide que las células mantengan su capacidad para dividirse y expandirse. Este descenso de celulosa se acompaña de otros cambios en la pared de células habituadas que varían en función del inhibidor y de la especie.

La gran mayoría de los estudios de habituación a este tipo de inhibidores se han centrado en especies que tienen una pared celular tipo I: tomate (Shedletzky y col., 1990; Wells y col., 1994), tabaco (Nakagawa y Sakurai, 1998; 2001; Sabba y col., 1999), arabidopsis (Manfield y col., 2004) y alubia (Díaz-Cacho y col., 1999; Encina y col., 2001; 2002; Alonso-Simón y col., 2004; 2008; García-Angulo y col., 2006). En la mayoría de estos cultivos el descenso en celulosa, que provoca la habituación a DCB, se acompaña de un aumento en la cantidad de pectinas y de una reducción de la de hemicelulosas. Los cultivos celulares con paredes celulares tipo I habituados a isoxabén son bastante más heterogéneos que los habituados a DCB, pero mantienen la pauta descrita para la habituación a DCB, en alubia (Díaz-Cacho y col., 1999), tabaco (Sabba y Vaughn, 1999) y arabidopsis (Manfield y col., 2004).

Hasta el inicio de esta tesis doctoral, sólo se había descrito un caso de habituación a un inhibidor de la biosíntesis de celulosa en una especie con pared celular tipo II. Se trataba de cultivos celulares de cebada habituados a DCB (Shedletzky y col., 1992). Además, los cambios que acompañaban a la habituación de estas células habían sido caracterizados solamente de forma preliminar, ya que ese no era el objetivo principal de dicho trabajo. En esta tesis doctoral se han habituado a DCB cultivos celulares de maíz, y se ha caracterizado de forma exhaustiva la pared celular de los cultivos habituados.

La habituación de maíz a DCB se logró mediante incrementos graduales de su concentración en el medio de cultivo. El proceso de habituación implicó menores tasas de crecimiento de los cultivos y alteraciones morfológicas, a nivel estructural y ultraestructural (Capítulo II, **Figuras 2 y 3**), comparables a las descritas en cultivos de otras especies con paredes celulares tipo I habituados a DCB, como suspensiones de tomate (Shedletzky y col., 1990), y suspensiones y callos de alubia (Encina y col., 2001, 2002).

Tal y como ya se había observado anteriormente en cebada (Shedletzky y col., 1992), la habituación de los cultivos celulares de maíz a DCB se acompañaba de un descenso en el contenido de celulosa y de un aumento en polisacáridos hemicelulósicos. En el caso de callos de maíz la reducción en el contenido de celulosa fue gradual a lo largo del proceso de habituación (menor cantidad de celulosa al incrementar la concentración del inhibidor y el número de subcultivos) y alcanzó reducciones de hasta el 75% (Capítulo II, **Figura 5**; Capítulo III, **Figura 1**). La diferencia en la habituación de ambas especies con pared del tipo II fue el tipo concreto de hemicelulosas que aumentó, ya que en los cultivos de cebada se incrementó el contenido de glucano mixto mientras que en los de maíz fue el de arabinoxilanos. Además, el contenido de otros componentes de la pared celular, como glucano mixto, xiloglucano, pectinas o proteínas, no aumentó o se vio reducido. Esta era la primera vez que se observaba una compensación por arabinoxilanos de una deficiencia en celulosa (Capítulo II, **Figuras 5, 7, 10 y 11**).

Los arabinoxilanos de células habituadas fueron más difícilmente extraíbles y tuvieron mayores masas moleculares medias (Capítulo II, **Figuras 6 y 8**), respecto a los de las células control, apuntando a la presencia de una mayor y más entrecruzada red de arabinoxilanos en las células habituadas. Una primera caracterización del contenido fenólico de las paredes de células habituadas reveló un notable incremento en estos compuestos (Capítulo II, **Figura 9**), hecho que sugirió que el incremento en la masa molecular media de los arabinoxilanos estaría controlado en gran medida mediante el entrecruzamiento por la formación de dehidrodímeros, tal y como se había propuesto en ocasiones anteriores (Fry y col., 2000; Kerr y Fry, 2003, 2004; Lindsay y Fry, 2008). Además, este mayor entrecruzamiento de la red de arabinoxilanos por compuestos fenólicos podría ser responsable de la mayor resistencia a la degradación enzimática de las paredes de células habituadas (Capítulo II, **Figura 12**), resultado que en principio pudiera sorprender por tratarse de paredes con niveles muy reducidos de celulosa y enriquecidas en componentes matriciales, pero que se podría explicar por una menor accesibilidad de las enzimas hidrolíticas a los polisacáridos matriciales debido a su vez a un enriquecimiento en fenoles y/o a una mayor dimerización fenólica (Grabber, 2005; Buanafina, 2009).

Tanto la reducción del contenido en celulosa, como el aumento en fenilpropanoides, estuvieron acompañados por cambios en la expresión génica, no sólo de aquellos genes directamente relacionados con estos componentes, sino también de otros relacionados con el metabolismo del carbono, nitrógeno y etileno, y con mecanismos de detoxificación.

La expresión de varios genes *ZmCesA* resultó alterada (Capítulo III, **Figura 3**), y en concreto *ZmCesA5* y *ZmCesA7* parecieron tener un papel destacado en la habituación al inhibidor. *ZmCesA5* fue el gen *CesA* cuya expresión se vio más afectada durante la habituación, deshabituación y tratamientos cortos con DCB. La sobreexpresión de *ZmCesA7* en todas las condiciones estudiadas apunta a ZmCesA7 como la subunidad que podría reemplazar a ZmCesA5 en las rosetas de células habituadas. Los resultados obtenidos indicaron que la presencia/ausencia de ZmCesA5 en la roseta podría ser un aspecto crítico determinando la sensibilidad/resistencia de las células a crecer en presencia de DCB. Durante la habituación se podrían originar rosetas

con configuraciones diferentes a las originales, y que posiblemente tendrían una funcionalidad alterada, pero que permitirían a las células habituadas crecer en presencia de concentraciones inicialmente letales de DCB. En línea con estos resultados, los mutantes de arabidopsis, *ixr1-1* y *ixr1-2,* que carecieron de la proteína AtCesA3 (proteína con una elevada homología con ZmCesA5) fueron resistentes al inhibidor de la biosíntesis de celulosa isoxabén (Scheible y col., 2001).

La mayoría de los genes relacionados con la síntesis de hidroxicinamatos resultaron inducidos en células habituadas (Capítulo III, **Figura 5**). La sobreexpresión de estos genes concuerda con el enriquecimiento generalizado de fenoles en la pared de células habituadas.

Además, la regulación de la expresión génica fue dependiente de la fase de cultivo, de modo que en general, en fases iniciales de cultivo una mayor cantidad de genes relacionados con estas dos redes metabólicas estuvieron sobreexpresados, y en cambio en fases finales de crecimiento hubo una mayor represión, indicando un control preciso en el uso de los recursos celulares para lograr la supervivencia en presencia del agente inhibidor.

Varias isoformas de la enzima glutatión S-transferasa (GST) resultaron reprimidas en células habituadas (Capítulo III, **Tabla 1**). Estas enzimas están relacionadas con procesos de detoxificación, protegiendo a las plantas frente al efecto de xenobióticos, como el DCB, y son consideradas como marcadores moleculares de estrés en plantas (Genter y col., 1998; Edwards y col., 2000). La actividad GST, que si bien resultaba incrementada tras tratamientos cortos con elevadas concentraciones de DCB en células no habituadas, resultó reducida en células habituadas (Capítulo III, **Figura 6**). El análisis de varias actividades antioxidantes (guaiacol peroxidasa, ascorbato peroxidasa, catalasa, glutatión reductasa, y otros datos que no han sido incluidos, como la actividad superóxido dismutasa y el grado de peroxidación lipídica) mostró que estas apenas estuvieron incrementadas, o incluso aparecieron reducidas, en células habituadas (Capítulo III, **Figura 6**). Estos resultados parecen indicar que en el caso del maíz, la habituación al DCB residiría exclusivamente en modificaciones de la pared celular. En la habituación de cultivos celulares de alubia al mismo inhibidor, se demostró que en el proceso de habituación a DCB, además de las modificaciones en la estructura y composición de la pared celular sí tiene importancia una estrategia antioxidante (García-Angulo y col., 2009a).

Aunque los fenoles son componentes cuantitativamente minoritarios en la pared celular, su papel en ella tiene gran importancia desde un punto de vista funcional. Se estudió en detalle la contribución del componente fenólico al proceso de habituación utilizando HPLC (Capítulo IV) y radiomarcaje (Capítulo V). Mediante HPLC se demostró un incremento (dos veces más) en fenoles monoméricos (ácidos ferúlico y *p*-cumárico). A este respecto cabe destacar que el proceso de habituación al DCB se caracterizó por un enriquecimiento significativo de las paredes celulares en ácido *p*-cumárico, muy probablemente como consecuencia de un efecto de exposición al DCB más que como efecto directo de la habituación.

Es de esperar que la modificación en la estructura y función de una pared celular tipo II deficiente en celulosa dependa en mayor medida de la riqueza en dehidrodímeros (anticipando un mayor grado de entrecruzamiento de los polisacáridos matriciales) que del contenido en monofenoles. Por esta

razón, y desde un punto de vista de la funcionalidad de la pared celular habituada al DCB, fue más relevante el cambio cuantitativo en dehidrodímeros (Capítulo IV, **Figuras 1, 3 y 4**). En promedio, el contenido en dehidrodímeros de las paredes celulares habituadas al DCB fue de 4 a 6 veces mayor que el de las paredes celulares no habituadas. Este resultado explicaría la reducción en la extractabilidad y el incremento en la masa molecular relativa de los arabinoxilanos de paredes celulares habituadas y avalaría la hipótesis según la cual, en cultivos celulares de maíz habituados al DCB, el déficit de celulosa es compensado por una red de arabinoxilanos más entrecruzados.

La variación en dehidrodímeros no fue sólo cuantitativa sino también cualitativa. La habituación originó nuevos tipos de enlaces entre fenoles monoméricos, ya que se detectaron hasta cuatro posibles nuevos dehidrodímeros en las paredes de estas células (Capítulo IV, **Figuras 1 y 2**). La estructura de estos nuevos compuestos se caracterizará en futuros experimentos, mediante técnicas de espectrometría de masas y resonancia magnética nuclear. Además, el seguimiento de estos compuestos a lo largo del proceso de habituación, demostró que dicho proceso es dinámico, ya que el perfil fenólico se va modificando a lo largo de los subcultivos (Capítulo II, **Figura 4**). Aunque los niveles de los dehidrodímeros 5,5 y 8-*O*-4 resultan elevados en paredes celulares de cultivos habituados, el importante incremento del dehidrodímero 8,5 sugirió un papel destacado de dicho compuesto en la habituación a DCB.

El estudio del componente fenólico se completó con experimentos de radiomarcaje, usando [^{14}C]ácido cinámico. Cultivos habituados y no habituados no difirieron en su capacidad de incorporación del compuesto (Capítulo V, **Figura 1**), sin embargo, el análisis de metabolitos radiomarcados presentes en distintos compartimentos celulares mostró importantes diferencias entre ambas líneas (Capítulo V, **Figuras 3 y 4**). Los cultivos habituados presentaron mayor variedad y cantidad de compuestos citosólicos de bajo peso molecular (Capítulo V, **Figuras 3 y 5**), los cuales posiblemente sirvan de reservas de unidades hidroxicinamoil que posteriormente pueden ser usadas en la feruloilación de polisacáridos (Fry, 1984; Fry y col., 2000; Obel y col., 2003; Lindsay y Fry, 2008). Además, los cultivos habituados resultaron muy enriquecidos en dehidrodímeros esterificados y eterificados sobre polisacáridos de la pared celular, así como en compuestos de mayores masas moleculares, posiblemente correspondiendo a trímeros y/o oligómeros (Capítulo V, **Figuras 7 y 8**). Todos estos resultados indicaron que la dimerización de ácido ferúlico comienza antes en células habituadas y podría ser responsable del probable mayor entrecruzamiento de arabinoxilanos que acompaña a la habituación a DCB.

El proceso denominado deshabituación consiste en retirar el DCB del medio de cultivo de células habituadas, es decir en cultivar células habituadas en ausencia del inhibidor, y permite estudiar la estabilidad de los cambios asociados a la habituación y determinar su naturaleza (mutaciones, cambios epigenéticos, etc). Trabajos previos demostraron que la mayoría de los cambios inducidos durante la habituación revierten al cultivar las células en medio sin inhibidor (Shedletzky y col., 1990; Encina y col., 2002; García-Angulo y col., 2006, 2009a). Cabe destacar el hecho de que cultivos celulares deshabituados de especies vegetales con pared tipo I, son capaces de tolerar concentraciones

del inhibidor letales para células control, y mantienen la elevada capacidad antioxidante propia de las células habituadas (García-Angulo y col., 2009a y b); sin embargo en el caso del maíz, los cultivos deshabituados siguieron una pauta semejante, en líneas generales, a la de los cultivos no habituados.

Globalmente, los resultados obtenidos con este trabajo permiten concluir que la habituación de cultivos de células de maíz al inhibidor de la biosíntesis de celulosa DCB modifica la composición y estructura de la pared celular tipo II. En esta modificación los fenoles, las moléculas que unen a los arabinoxilanos entre sí, juegan un papel clave en el mantenimiento de la funcionalidad de una pared celular deficitaria en celulosa. Este y otros cambios en la pared celular durante la habituación a DCB ponen de manifiesto que los mecanismos que participan en la plasticidad estructural son diferentes en paredes celulares tipo I y tipo II, y son cruciales en la supervivencia ante agentes externos tóxicos para una célula vegetal.

Conclusiones

1. Se han habituado células de maíz a concentraciones de diclobenil (DCB) hasta ocho veces superiores a su correspondiente I_{50}. Las células habituadas crecieron más lentamente, presentaron un aspecto más irregular y paredes celulares más engrosadas. Durante la habituación a DCB las células modificaron la composición y arquitectura de la pared celular con una reducción de hasta el 75% en el contenido en celulosa, que fue compensada, al menos parcialmente, por un aumento en el de arabinoxilanos, que además experimentaron un incremento en masa molecular y aparecieron más fuertemente unidos a la pared celular. El contenido de otros componentes de la pared celular, como glucano mixto, xiloglucano, pectinas o proteínas, no aumentó o se vio reducido. Como consecuencia de esta nueva arquitectura las paredes de las células habituadas tuvieron mayores tamaños de poro y una menor degradabilidad y capacidad de hidratación.

2. La habituación a DCB se acompañó de cambios cuantitativos y cualitativos en el componente fenólico de la pared celular, entre los que destacan el aumento en el contenido de fenoles monoméricos (ácidos ferúlico y *p*-cumárico), en la proporción de dímeros, y la formación de nuevos enlaces entre los monómeros que los constituían. La evolución del perfil fenólico durante la habituación a DCB reflejó un dinamismo en el proceso de dimerización fenólica, de modo que, a lo largo del proceso de habituación se apreciaron diferencias en la proporción relativa de los dehidrodímeros 5,5 y 8-*0*-4, pero sobre todo del 8,5, que pareció jugar un papel clave en el proceso de habituación.

3. Mediante radiomarcaje con ácido [^{14}C]cinámico se comprobó que la habituación al DCB se acompañaba de modificaciones en el metabolismo citosólico de los fenoles cuya principal consecuencia fue la acumulación de intermediarios de bajo peso molecular, que servirían de precursores para la feruloilación de los polisacáridos de la pared celular. Asimismo, el análisis *in muro* de los metabolitos de [^{14}C]cinámico indicó que las células habituadas estaban enriquecidas en dehidrodiferulatos y compuestos de mayor masa molecular esterificados y eterificados sobre la pared celular. La cinética de aparición de [^{14}C]dehidrodiferulatos indicó que la dimerización del ácido ferúlico se producía antes en células habituadas. Estos resultados avalan la hipótesis de que un prematuro y mayor entrecruzamiento de la red de arabinoxilanos constituye un mecanismo compensatorio de la deficiencia de celulosa.

4. Se han detectado cambios en la expresión génica durante la habituación de células de maíz a DCB. Las células habituadas mostraron una mayor expresión de la mayoría de los genes implicados en la ruta de biosíntesis de fenilpropanoides. Estas células experimentaron también cambios en el nivel de expresión de diferentes genes de celulosa sintasas, entre los que destacan *ZmCesA5* y *ZmCesA7*. La regulación de la expresión génica dependió de la fase de crecimiento de las células, y difirió a su vez de la de los cultivos no habituados expuestos a altas concentraciones del herbicida. Además, el análisis proteómico reveló cambios en las células habituadas en procesos tan variados como el metabolismo del carbono, nitrógeno y etileno. La habituación a DCB en

células de maíz, a diferencia de lo descrito en células de especies con pared celular tipo I, no estuvo acompañada por un aumento en actividades enzimáticas antioxidantes (guaiacol peroxidasa, ascorbato peroxidasa, catalasa y glutatión reductasa) o detoxificadoras (glutatión S-transferasa).

5. Las células habituadas a DCB y cultivadas en ausencia del inhibidor, denominadas células deshabituadas, mantuvieron una sobreexpresión constitutiva de varios genes relacionados con la biosíntesis de la celulosa y la de los fenoles. Sin embargo, no presentaron una mayor tolerancia a DCB y mostraron características semejantes, en líneas generales, a las de los cultivos no habituados.

Bibliografía general

Alonso-Simón A, Encina AE, García-Angulo P, Álvarez JM, Acebes JL (2004) FTIR spectroscopy monitoring of cell wall modifications during the habituation of bean (*Phaseolus vulgaris* L.) callus cultures to dichlobenil. Plant Sci 167: 1273-1281

Alonso-Simón A, García-Angulo P, Encina A, Acebes JL, Álvarez JM (2008) Habituation of bean (*Phaseolus vulgaris*) cell cultures to quinclorac and analysis of the subsequent cell wall modifications. Ann Bot 101: 1329-1339

Alonso-Simón A, García-Angulo P, Encina A, Álvarez JM, Acebes JL, Hayashi T (2007) Increase in XET activity in bean (*Phaseolus vulgaris* L.) cells habituated to dichlobenil. Planta 226: 765-771

Alonso-Simón A, García-Angulo P, Mélida H, Encina A, Álvarez JM, Acebes JL (2010b) FTIR spectroscopy as a tool to monitor changes in plant cell wall architecture associated to cellulose biosynthesis inhibitors. En "Fourier Transform Infrared Spectroscopy: developments, techniques and applications" (OJ Ress, Ed.). Nova Publishers, New York. En prensa

Alonso-Simón A, Neumetzler L, García-Angulo P, Encina A, Acebes JL, Álvarez JM, Hayashi T (2010a). Plasticity of xyloglucan composition in bean (*Phaseolus vulgaris*) cultured cells during habituation and dehabituation to lethal concentrations of dichlobenil. Mol Plant. En prensa

Anderson CT, Carroll A, Akhmetova L, Somerville C (2010) Real-Time Imaging of cellulose reorientation during cell wall expansion in *Arabidopsis* roots. Plant Physiol 152: 787-796

Appenzeller L, Doblin M, Barreiro R, Wang H, Niu X, Kollipara K, Carrigan L, Tomes D, Chapman M, Dhugga KS (2004) Cellulose synthesis in maize: isolation and expression analysis of cellulose synthase (*CesA*) gene family. Cellulose 11: 287-299

Arioli T, Peng L, Betzner AS, Burn J, Wittke W, Herth W, Camilleri C, Höfte H, Plazinski J, Birch R, Cork A, Glover J, Redmond J, Williamson RE (1998) Molecular analysis of cellulose biosynthesis in *Arabidopsis*. Science 279: 717-720

Aspinall GO (1980) Chemistry of cell wall polysaccharides. En "The Biochemistry of Plants, a Comprehensive Treatise" Vol 3 (J Preiss, Ed.). Academic Press, New York, pp 473-500

Bacic A (2006) Breaking an impasse in pectin biosynthesis. Proc Natl Acad Sci U S A 103: 5639-5640

Baskin TI, Betzner AS, Hoggart R, Cork A, Williamson RE (1992) Root morphology mutants in *Arabidopsis thaliana*. Aust J Plant Physiol 19: 427-437

Baydoun EAH, Waldron KW, Brett CT (1989) The interaction of Xylosyltransferase and Glucuronyltransferase involved in glucuronoxylan synthesis in pea (*Pisum sativum*) epicotyls. Biochem J 257: 853-858

Beeckman T, Przemeck GKH, Stamatiou G, Lau R, Terryn N, De Rycke R, Inze D, Berleth T (2002) Genetic complexity of cellulose synthase A gene function in *Arabidopsis* embryogenesis. Plant Physiol 130: 1883-1893

Binzel ML, Hess FD, Bressan RA, Hasegawa PM (1988) Intracellular compartmentation of ions in salt adapted tobacco cells. Plant Physiol 86: 607-614

Bischoff V, Nita S, Neumetzler L, Schindelasch D, Urbain A, Eshed R, Persson S, Delmer D, Scheible WR (2010) *TRICHOME BIREFRINGENCE* and its homolog *At5g01360* encode novel plant-specific DUF231 proteins required for cellulose biosynthesis in *Arabidopsis thaliana*. Plant Physiol 153. En prensa

Blaschek W, Semler U, Franz G (1985) The influence of potential inhibitors on the *in vivo* and *in vitro* cell-wall β-glucan biosynthesis in tobacco cells. J Plant Physiol 120: 457-470

Brett C, Waldron K (1996) Physiology and biochemistry of plant cell wall. Chapman and Hall, London

Brown DM, Goubet F, Vicky WWA, Goodacre R, Stephens E, Dupree P, Turner SR (2007) Comparison of five xylan synthesis mutants reveals new insight into the mechanisms of xylan synthesis. Plant J 52: 1154-1168

Brown DM, Zhang Z, Stephens E, Dupree P, Turner SR (2009) Characterization of IRX10 and IRX10-like reveals an essential role in glucuronoxylan biosynthesis in *Arabidopsis*. Plant J 57: 732-746

Brummell DA, Hall JL (1985) The role of cell wall synthesis in sustained auxin-induced growth. Physiol Plant 63: 406-412

Buanafina MMdO (2009) Feruloylation in grasses: current and future perspectives. Mol Plant 2: 861-872

Burn JE, Hurley UA, Birch RJ, Arioli T, Cork A, Williamson RE (2002) The cellulose-deficient *Arabidopsis* mutant *rsw3* is defective in a gene encoding a putative glucosidase II, an enzyme processing N-glycans during ER quality control. Plant J 32: 949-960

Burton RA, Wilson SM, Hrmova M, Harvey AJ, Shirley NJ, Stone BA, Newbigin EJ, Bacic A, Fincher GB (2006) Cellulose synthase-like *CslF* genes mediate the synthesis of cell wall (1,3;1,4)-beta-D-glucans. Science 311: 1940-1942

Campbell NA, Reece JB (2005) Biology. Pearson Benjamin Cummings, San Francisco

Caño-Delgado A, Penfield S, Smith C, Catley M, Bevan M (2003) Reduced cellulose synthesis invokes lignification and defense responses in *Arabidopsis thaliana*. Plant J 34: 351-362

Carpena RO, Esteban E, Sarro MJ, Penalosa J, Garate A, Lucena JJ, Zornoza P (2000) Boron and calcium distribution in nitrogen-fixing pea plants. Plant Sci 151: 163-170

Carpita NC (1984) Cell wall development in maize coleoptiles. Plant Physiol 76: 205-212

Carpita NC (1996) Structure and biogenesis of the cell walls of grasses. Annu Rev Plant Physiol Plant Mol Biol 47: 445-476

Carpita NC, Gibeaut DM (1993) Structural models of primary cell walls in flowering plants. Consistency of molecular structure with the physical properties of the walls during growth. Plant J 3: 1-30

Carpita NC, McCann MC (2000) The cell wall. En "Biochemistry and Molecular Biology of Plants" (B Buchanan, W Gruissem, R Jones, Eds.). American Society of Plant Biologists, Rockville, pp 52-108

Cavalier DM, Keegstra K (2006) Two xyloglucan xylosyltransferases catalyze the addition of multiple xylosyl residues to cellohexaose. J Biol Chem 281: 34197-34207

Cazalé AC, Rouet-Mayer MA, Barbier-Brygoo H, Mathieu Y, Laurière C (1998) Oxidative burst and hypoosmotic stress in tobacco cell suspensions. Plant Physiol 116: 659-669

Chen L, Carpita NC, Reiter W-, Wilson RH, Jeffries C, McCann MC (1998) A rapid method to screen for cell-wall mutants using discriminant analysis of Fourier transform infrared spectra. Plant J 16: 385-392

Cocuron J, Lerouxel O, Drakakaki G, Alonso AP, Liepman AH, Keegstra K, Raikhel N, Wilkerson CG (2007) A gene from the cellulose synthase-like C family encodes a β-1,4 glucan synthase. Proc Natl Acad Sci U S A 104: 8550-8555

Coleman HD, Ellis DD, Gilbert M, Mansfield SD (2006) Up-regulation of sucrose synthase and UDP-glucose pyrophosphorylase impacts plant growth and metabolism. Plant Biotechnol J 4: 87-101

Corio-Costet. MF, Dall`aquese M, Scalla R (1991) Effects of isoxaben on sensitive and tolerant plant cell cultures. II. Cellular alterations and inhibition of the synthesis of acid insoluble cell wall material. Pestic Biochem Physiol 40: 255-265

Cosgrove DJ, Fincher G, Höfte H (2009) Editorial. Mol Plant 2: 839

Crowell EF, Bischoff V, Desprez T, Rolland A, Stierhof Y, Schumacher K, Gonneau M, Hoefte H, Vernhettes S (2009) Pausing of golgi bodies on microtubules regulates secretion of cellulose synthase complexes in *Arabidopsis*. Plant Cell 21: 1141-1154

Daras G, Rigas S, Penning B, Milioni D, McCann MC, Carpita NC, Fasseas C, Hatzopoulos P (2009) The *thanatos* mutation in *Arabidopsis thaliana* cellulose synthase 3 (*AtCesA3*) has a dominant-negative effect on cellulose synthesis and plant growth. New Phytol 184: 114-126

Debolt S, Gutierrez R, Ehrhardt DW, Somerville C (2007) Nonmotile cellulose synthase subunits repeatedly accumulate within localized regions at the plasma membrane in Arabidopsis hypocotyl cells following 2,6-dichlorobenzonitrile treatment. Plant Physiol 145: 334-338

Delmer DP (1987) Cellulose biosynthesis. Annu Rev Plant Physiol Plant Mol Biol 38: 259-290

Delmer DP (1999) Cellulose biosynthesis: exciting times for a difficult field of study. Annu Rev Plant Physiol Plant Mol Biol 50: 245-276

Delmer DP, Amor Y (1995) Cellulose biosynthesis. Plant Cell 7: 987-1000

Desnos T, Orbovic V, Bellini C, Kronenberger J, Caboche M, Traas J, Höfte H (1996) *Procuste1* mutants identify two distinct genetic pathways controlling hypocotyl cell elongation, respectively in dark- and light-grown *Arabidopsis* seedlings. Development 122: 683-693

Desprez T, Vernhettes S, Fagard M, Refrégier G, Desnos T, Aletti E, Py N, Pelletier S, Höfte H (2002) Resistance against herbicide isoxaben and cellulose deficiency caused by distinct mutations in same cellulose synthase isoform CESA6. Plant Physiol 128: 482-490

Díaz-Cacho P, Moral R, Encina A, Acebes JL, Álvarez J (1999) Cell wall modifications in bean (*Phaseolus vulgaris*) callus cultures tolerant to isoxaben. Physiol Plant 107: 54-59

Doblin MS, Pettolino FA, Wilson SM, Campbell R, Burton RA, Fincher GB, Newbigin E, Bacic A (2009) A barley cellulose synthase-like *CSLH* gene mediates (1,3;1,4)-beta-D-glucan synthesis in transgenic *Arabidopsis*. Proc Natl Acad Sci U S A 106: 5996-6001

Edelmann HG, Fry SC (1992) Effect of cellulose synthesis inhibition on growth and the integration of xyloglucan into pea internode cell-walls. Plant Physiol 100: 993-997

Edelmann HG, Köhler K (1995) Auxin increases elastic wall-properties in rye coleoptiles - Implications for the mechanism of wall loosening. Physiol Plant 93: 85-92

Edwards R, Dixon DP, Walbot V (2000) Plant glutathione S-transferases: enzymes with multiple functions in sickness and in health. Trends Plant Sci 5: 193-198

Ellis C, Karafyllidis I, Wasternack C, Turner JG (2002) The *Arabidopsis* mutant *cev1* links cell wall signaling to jasmonate and ethylene responses. Plant Cell 14: 1557-1566

Emons AMC, Mulder BM (2000) How the deposition of cellulose microfibrils builds cell wall architecture. Trends Plant Sci 5: 35-40

Encina A, Moral RM, Acebes JL, Álvarez J (2001) Characterization of cell walls in bean (*Phaseolus vulgaris* L.) callus cultures tolerant to dichlobenil. Plant Sci 160: 331-339

Encina A, Sevillano JM, Acebes JL, Álvarez J (2002) Cell wall modifications of bean (*Phaseolus vulgaris*) cell suspensions during habituation and dehabituation to dichlobenil. Physiol Plant 114: 182-191

Fagard M, Höfte H, Vernhettes S (2000) Cell wall mutants. Plant Physiol Biochem 38 (1/2): 15-25

Faik A, Price NJ, Raikhel NV, Keegstra K (2002) An *Arabidopsis* gene encoding an alpha-xylosyltransferase involved in xyloglucan biosynthesis. Proc Natl Acad Sci U S A 99: 7797-7802

Fisher DD, Cyr RJ (1998) Extending the microtubule/microfibril paradigm - Cellulose synthesis is required for normal cortical microtubule alignment in elongating cells. Plant Physiol 116: 1043-1051

Francey Y, Jaquet JP, Cairoli S, Buchala AJ, Meier H (1989) The biosynthesis of β-glucans in cotton (*Gossypium hirsutum* L.) fibres of ovules cultured in vitro. J Plant Physiol 134: 485-491

Fry SC (1984) Incorporation of [^{14}C]Cinnamate into hydrolase-resistant components of the primary cell wall of spinach. Phytochemistry 23: 59-64

Fry SC (2004) Primary cell wall metabolism: tracking the careers of wall polymers in living plant cells. New Phytol 161: 641-675

Fry SC, Mohler KE, Nesselrode BHWA, Frankova L (2008) Mixed-linkage beta-D-glucan: xyloglucan endotransglucosylase, a novel wall-remodelling enzyme from *Equisetum* (horsetails) and charophytic algae. Plant J 55: 240-252

Fry SC, Willis SC, Paterson AEJ (2000) Intraprotoplasmic and wall-localised formation of arabinoxylan-bound diferulates and larger ferulate coupling-products in maize cell-suspension cultures. Planta 211: 679-692

Galbraith DW, Shields BA (1982) The effects of inhibitors of cell wall synthesis on tobacco protoplast development. Physiol Plant 55: 25-30

García-Angulo P, Alonso-Simón A, Mélida H, Encina A, Acebes JL, Álvarez JM (2009a) High peroxidase activity and stable changes in the cell wall are related to dichlobenil tolerance. J Plant Physiol 166: 1229-1240

García-Angulo P, Alonso-Simón A, Mélida H, Encina A, Álvarez JM, Acebes JL (2009b) Habituation and dehabituation to dichlobenil: simply the equivalent of Penelope's weaving and unweaving process? Plant Sign Behav 4: 1069-1071

García-Angulo P, Willats WGT, Encina AE, Alonso-Simón A, Álvarez JM, Acebes JL (2006) Immunocytochemical characterization of the cell walls of bean cell suspensions during habituation and dehabituation to dichlobenil. Physiol Plant 127: 87-99

Geissman T, Neukom H (1971) Cross-linking of phenolcarboxylates of polysaccharides by oxidative phenolic coupling. Helv Chim Acta 54: 1108-1112

Genter MB, Deamer-Melia NJ, Wetmore BA, Morgan KT, Meyer SA (1998) Herbicides and olfactory/neurotoxicity responses. Rev Toxicol 2: 93-112

Gibeaut DM, Pauly M, Bacic A, Fincher GB (2005) Changes in cell wall polysaccharides in developing barley (*Hordeum vulgare*) coleoptiles. Planta 221: 729-738

Gillmor CS, Poindexter P, Lorieau J, Palcic MM, Somerville C (2002) α-glucosidase I is required for cellulose biosynthesis and morphogenesis in *Arabidopsis*. J Cell Biol 156: 1003-1013

Girard-Martel M, Brochu V, Duval I, Beaudoin N (2008) Characterization and transcription profiles of poplar cells habituated to *Streptomyces scabiei* phytotoxin thaxtomin A. 6th Canadian Plant Genomics Workshop, pp 74

Grabber JH (2005) How do lignin composition, structure, and cross-linking affect degradability? A review of cell wall model studies. Crop Sci 45: 820-831

Green PB, Selker JML (1991) Mutual alignments of cell walls, cellulose and cytoskeletons: their role in meristems. En "The Cytoskeletal Basis of Plant Growth and Form" (Lloyd, Ed.). Academic Press, San Diego, pp 303-322

Guillaumie S, San-Clemente H, Deswarte C, Martinez Y, Lapierre C, Murigneux A, Barriere Y, Pichon M, Goffner D (2007) MAIZEWALL. Database and developmental gene expression profiling of cell wall biosynthesis and assembly in maize. Plant Physiol 143: 339-363

Gutierrez R, Lindeboom JJ, Paredez AR, Emons AMC, Ehrhardt DW (2009) *Arabidopsis* cortical microtubules position cellulose synthase delivery to the plasma membrane and interact with cellulose synthase trafficking compartments. Nat Cell Biol 11: 797-806

Haigler CH, Ivanova-Datcheva M, Hogan PS, Salnikov VV, Hwang S, Martin K, Delmer DP (2001) Carbon partitioning to cellulose synthesis. Plant Mol Biol 47: 29-51

Hauser MT, Morikami A, Benfey PN (1995) Conditional root expansion mutants of *Arabidopsis*. Development 121: 1237-1252

Hayashi T (1989) Xyloglucans in the primary cell wall. Annu Rev Plant Physiol Plant Mol Biol 40: 139-168

Heim DR, Skomp JR, Tschabold EE, Larrinua IM (1990) Isoxaben inhibits the synthesis of acid insoluble cell wall materials in *Arabidopsis thaliana*. Plant Physiol 93: 695-700

Higuchi T, Ito Y, Shimada M, Kawamura I (1967) Chemical properties of milled wood lignin of grasses. Phytochemistry 6: 1551-1556

Himmelspach R, Williamson RE, Wasteneys GO (2003) Cellulose microfibril alignment recovers from DCB-induced disruption despite microtubule disorganization. Plant J 36: 565-575

Holland N, Holland D, Helentjaris T, Dhugga KS, Xoconostle-Cazares B, Delmer DP (2000) A comparative analysis of the plant cellulose synthase (*CesA*) gene family. Plant Physiol 123: 1313-1324

Hoson T, Masuda Y (1991) Role of polysaccharide synthesis in elongation growth and cell-wall loosening in intact rice coleoptiles. Plant Cell Physiol 32: 763-769

Iraki NM, Bressan RA, Hasegawa PM, Carpita NC (1989a) Alteration of the physical and chemical structure of the primary cell wall of growth limited plant cells adapted to osmotic stress. Plant Physiol 91: 39-47

Iraki NM, Singh N, Bressan RA, Carpita NC (1989b) Cell walls of tobacco cells and changes in composition associated with reduced growth upon adaptation to water and saline stress. Plant Physiol 91: 48-53

Ishii T (1997) Structure and functions of feruloylated polysaccharides. Plant Sci 127: 111-127

Ishii T, Hiroi T (1990) Linkage of phenolic-acids to cell-wall polysaccharides of bamboo shoot. Carbohydr Res 206: 297-310

Jamet E, Canut H, Boudart G, Pont-Lezica RF (2006) Cell wall proteins: a new insight through proteomics. Trends Plant Sci 11: 33-39

Jarvis MC, Briggs SPH, Knox JP (2003) Intercellular adhesion and cell separation in plants. Plant Cell Environ 26: 977-989

Jung HJG, Himmelsbach DS (1989) Isolation and characterization of wheat straw lignin. J Agric Food Chem 37: 81-87

Kerr EM, Fry SC (2003) Pre-formed xyloglucans and xylans increase in molecular weight in three distinct compartments of a maize cell-suspension culture. Planta 217: 327-339

Knox JP (2008) Revealing the structural and functional diversity of plant cell walls. Curr Opin Plant Biol 11: 308-313

Kohorn BD, Kobayashi M, Johansen S, Friedman HP, Fischer A, Byers N (2006) Wall-associated kinase 1 (WAK1) is crosslinked in endomembranes, and transport to the cell surface requires correct cell-wall synthesis. J Cell Sci 119: 2282-2290

Lairson LL, Henrissat B, Davies GJ, Withers SG (2008) Glycosyltransferases: Structures, functions, and mechanisms. Annu Rev Biochem 77: 521-555

Lane D, Wiedemeier A, Peng L, Höfte H, Vernhettes S, Desprez T, Hocart CH, Birch RJ, Baskin TI, Burn JE, Arioli T, Williamson RE (2001) Temperature-sensitive alleles of *RSW2* link the KORRIGAN endo-1,4-β-glucanase to cellulose synthesis and cytokinesis in Arabidopsis. Plant Physiol 126: 278-288

Lee C, Teng Q, Huang W, Zhong R, Ye Z (2009) The F8H glycosyltransferase is a functional paralog of FRA8 involved in glucuronoxylan biosynthesis in Arabidopsis. Plant Cell Physiol 50: 812-827

Lee C, Zhong R, Richardson EA, Himmelsbach DS, McPhail BT, Ye Z (2007) The *PARVUS* gene is expressed in cells undergoing secondary wall thickening and is essential for glucuronoxylan biosynthesis. Plant Cell Physiol 48: 1659-1672

Lee SJ, Saravanan RS, Damasceno CMB, Yamane H, Kim BD, Rose JKC (2004) Digging deeper into the plant cell wall proteome. Plant Physiol Biochem 42: 979-988

Lerouxel O, Cavalier DM, Liepman AH, Keegstra K (2006) Biosynthesis of plant cell wall polysaccharides - a complex process. Curr Opin Plant Biol 9: 621-630

Levy S, York WS, Stuikeprill R, Meyer B, Staehelin LA (1991) Simulations of the static and dynamic molecular-conformations of xyloglucan - the role of the fucosylated side-chain in surface-specific side-chain folding. Plant J 1: 195-215

Lindsay SE, Fry SC (2008) Control of diferulate formation in dicotyledonous and gramineous cell-suspension cultures. Planta 227: 439-452

Madson M, Dunand C, Li XM, Verma R, Vanzin GF, Calplan J, Shoue DA, Carpita NC, Reiter WD (2003) The *MUR3* gene of *Arabidopsis* encodes a xyloglucan galactosyltransferase that is evolutionarily related to animal exostosins. Plant Cell 15: 1662-1670

Manfield IW, Orfila C, McCartney L, Harholt J, Bernal AJ, Scheller HV, Gilmartin PM, Mikkelsen JD, Knox JP, Willats WGT (2004) Novel cell wall architecture of isoxaben habituated *Arabidopsis* suspension cultured cells: global transcript profiling and cellular analysis. Plant J 40: 260-275

Martin C, Bhatt K, Baumann K (2001) Shaping in plant cells. Curr Opin Plant Biol 4: 540-549

McCann MC, Defernez M, Urbanowicz BR, Tewari JC, Langewisch T, Olek A, Wells B, Wilson RH, Carpita NC (2007) Neural network analyses of infrared spectra for classifying cell wall architectures. Plant Physiol 143: 1314-1326

McQueen-Mason S, Durachko DM, Cosgrove DJ (1992) Two endogenous proteins that induce cell-wall extension in plants. Plant Cell 4: 1425-1433

McQueen-Mason S, Le NT, Brocklehurst D (2007) Expansins. En "Plant Cell Monographs" Vol 5 (JP Verbelen, K Vissenberg, Eds.). Springer-Verlag, Berlin, pp 117-138

Menne H, Köcher H (2007) HRAC classification of herbicides and resistance development. En "Modern crop protection compounds" (W Krämer, U Shirmer, Eds.) Wiley, pp 5-27

Meyer Y, Herth W (1978) Chemical inhibition of cell wall formation and cytokinesis, but not of nuclear division, in protoplasts of *Nicotiana tabacum* L. cultivated in vitro. Planta 142: 253-262

Mitchell RAC, Dupree P, Shewry PR (2007) A novel bioinformatics approach identifies candidate genes for the synthesis and feruloylation of arabinoxylan. Plant Physiol 144: 43-53

Mizuta S, Brown RM (1992) Effects of 2,6-dichlorobenzonitrile and Tinopal Lpw on the structure of the cellulose synthesizing complexes of *Vaucheria hamata*. Protoplasma 166: 200-207

Mølhøj M, Ulvskov P, Dal Degan F (2001) Characterization of a functional soluble form of a *Brassica napus* membrane-anchored endo-1,4-β-glucanase heterologously expressed in *Pichia pastoris*. Plant Physiol 127: 674-684

Mølhøj M, Pagant SR, Höfte H (2002) Towards understanding the role of membrane-bound endo-β-1,4-glucanases in cellulose biosynthesis. Plant Cell Physiol 43: 1399-1406

Montague MJ (1995) Gibberellic acid promotes growth and cell-wall synthesis in *Avena* internodes regardless of the orientation of cell expansion. Physiol Plant 94: 7-18

Montezinos D, Delmer DP (1980) Characterization of inhibitors of cellulose synthesis in cotton fibers. Planta 148: 305-311

Mouille G, Robin S, Lecomte M, Pagant S, Höfte H (2003) Classification and identification of *Arabidopsis* cell wall mutants using Fourier-Transform Infrared (FT-IR) microspectroscopy. Plant J 35: 393-404

Mutwil M, Debolt S, Persson S (2008) Cellulose synthesis: a complex complex. Curr Opin Plant Biol 11: 252-257

Nakagawa N, Sakurai N (1998) Increase in the amount of celA1 protein in tobacco BY-2 cells by a cellulose biosynthesis inhibitor, 2,6-dichlorobenzonitrile. Plant Cell Physiol 39: 779-785

Nakagawa N, Sakurai N (2001) Cell wall integrity controls expression of endoxyloglucan transferase in tobacco BY2 cells. Plant Cell Physiol 42: 240-244

Nickle TC, Meinke DW (1998) A cytokinesis-defective mutant of *Arabidopsis* (*cyt1*) characterized by embryonic lethality, incomplete cell walls, and excessive callose accumulation. Plant J 15: 321-332

Nicol F, His I, Jauneau A, Vernhettes S, Canut H, Höfte H (1998) A plasma membrane-bound putative endo-1,4-β-D-glucanase is required for normal wall assembly and cell elongation in *Arabidopsis*. EMBO J 17: 5563-5576

O´Neil MA, York WS (2003) The composition and structure of plant primary cell wall. En "The Plant Cell Wall" (JKC Rose, Ed.). CRC Press, Boca Ratón, pp 1-54

Obel N, Porchia AC, Scheller HV (2002) Dynamic changes in cell wall polysaccharides during wheat seedling development. Phytochemistry 60: 603-610

Obel N, Porchia AC, Scheller HV (2003) Intracellular feruloylation of arabinoxylan in wheat: evidence for feruloyl-glucose as precursor. Planta 216: 620-629

Pagant S, Bichet A, Sugimoto K, Lerouxel O, Desprez T, McCann M, Lerouge P, Vernhettes S, Höfte H (2002) *KOBITO1* encodes a novel plasma membrane protein necessary for normal synthesis of cellulose during cell expansion in Arabidopsis. Plant Cell 14: 2001-2013

Paredez AR, Somerville CR, Ehrhardt DW (2006) Visualization of cellulose synthase demonstrates functional association with microtubules. Science 312: 1491-1495

Parker ML, Ng A, Waldron KW (2005) The phenolic acid and polysaccharide composition of cell walls of bran layers of mature wheat (*Triticum aestivum* L. cv. Avalon) grains. J Sci Food Agric 85: 2539-2547

Passardi F, Cosio C, Penel C, Dunand C (2005) Peroxidases have more functions than a Swiss army knife. Plant Cell Rep 24: 255-265

Pauly M, Gunl M, Souza A, Neumetzler L, Florian K (2009) The substitution pattern of plant cell wall cross-linking glycans is determined by apoplastic glycosidases. Annu Meet Am Soc Plant Biol, Abstr P15004

Peng LC, Kawagoe Y, Hogan P, Delmer D (2002) Sitosterol-β-glucoside as primer for cellulose synthesis in plants. Science 295: 147-150

Peng LC, Xiang F, Roberts E, Kawagoe Y, Greve LC, Kreuz K, Delmer DP (2001) The experimental herbicide CGA 325'615 inhibits synthesis of crystalline cellulose and

causes accumulation of non-crystalline β-1,4-glucan associated with CesA protein. Plant Physiol 126: 981-992

Penning BW, Hunter CT,III, Tayengwa R, Eveland AL, Dugard CK, Olek AT, Vermerris W, Koch KE, McCarty DR, Davis MF, Thomas SR, McCann MC, Carpita NC (2009) Genetic resources for maize cell wall biology. Plant Physiol 151: 1703-1728

Peña MJ, Zhong R, Zhou G, Richardson EA, O'Neill MA, Darvill AG, York WS, Ye Z (2007) *Arabidopsis* irregular xylem8 and irregular xylem9: Implications for the complexity of glucuronoxylan biosynthesis. Plant Cell 19: 549-563

Perrin RM, DeRocher AE, Bar-Peled M, Zeng WQ, Norambuena L, Orellana A, Raikhel NV, Keegstra K (1999) Xyloglucan fucosyltransferase, an enzyme involved in plant cell wall biosynthesis. Science 284: 1976-1979

Piston F, Uauy C, Fu L, Langston J, Labavitch J, Dubcovsky J (2010) Down-regulation of four putative arabinoxylan feruloyl transferase genes from family PF02458 reduces ester-linked ferulate content in rice cell walls. Planta 231: 677-691

Popper ZA, Fry SC (2003) Primary cell wall composition of bryophytes and charophytes. Ann Bot 91: 1-12

Porchia AC, Sorensen SO, Scheller HV (2002) Arabinoxylan biosynthesis in wheat. Characterization of arabinosyltransferase activity in Golgi membranes. Plant Physiol 130: 432-441

Potikha T, Delmer DP (1995) A mutant of *Arabidopsis thaliana* displaying altered patterns of cellulose deposition. Plant J 7: 453-460

Rajangam AS, Kumar M, Aspeborg H, Guerriero G, Arvestad L, Pansri P, Brown CJ, Hober S, Blomqvist K, Divne C, Ezcurra I, Mellerowicz E, Sundberg B, Bulone V, Teeri TT (2008) MAP20, a Microtubule-Associated Protein in the secondary cell walls of hybrid aspen, is a target of the cellulose synthesis inhibitor 2,6-Dichlorobenzonitrile. Plant Physiol 148: 1283-1294

Reid JSG (2000) Cementing the wall: cell wall polysaccharide synthesising enzymes. Curr Opin Plant Biol 3: 512-516

Reiter WD, Chapple C, Somerville CR (1997) Mutants of *Arabidopsis thaliana* with altered cell wall polysaccharide composition. Plant J 12: 335-345

Richmond T (2000) Higher plant cellulose synthases. Genome Biol 1: reviews3001.1-reviews3001.6

Richmond TA, Somerville CR (2000) The cellulose synthase superfamily. Plant Physiol 124: 495-498

Ridley BL, O'Neill MA, Mohnen DA (2001) Pectins: structure, biosynthesis, and oligogalacturonide-related signaling. Phytochemistry 57: 929-967

Rose JKC, Braam J, Fry SC, Nishitani K (2002) The XTH family of enzymes involved in xyloglucan endotransglucosylation and endohydrolysis: Current perspectives and a new unifying nomenclature. Plant Cell Physiol 43: 1421-1435

Sabba RP, Durso NA, Vaughn KC (1999) Structural and immunocytochemical characterization of the walls of dichlobenil-habituated BY-2 tobacco cells. Int J Plant Sci 160: 275-290

Sabba RP, Vaughn KC (1999) Characterization of BY-2 tobacco cells habituated to the cellulose-inhibiting herbicides dichlobenil and isoxaben. Plant Biol 99: 186

Sancho MA, deForchetti SM, Pliego F, Valpuesta V, Quesada MA (1996) Peroxidase activity and isoenzymes in the culture medium of NaCl adapted tomato suspension cells. Plant Cell Tissue Organ Cult 44: 161-167

Sandhu APS, Randhawa GS, Dhugga KS (2009) Plant cell wall matrix polysaccharide biosynthesis. Mol Plant 2: 840-850

Sarkar P, Bosneaga E, Auer M (2009) Plant cell walls throughout evolution: towards a molecular understanding of their design principles. J Exp Bot 60: 3615-3635

Sarria R, Wagner TA, O'Neill MA, Faik A, Wilkerson CG, Keegstra K, Raikhel NV (2001) Characterization of a family of *Arabidopsis* genes related to xyloglucan fucosyltransferase1. Plant Physiol 127: 1595-1606

Satiat-Jeunemaitre B, Darzens D (1986) *In vivo* hemicelluloses-cellulose equilibrium modifications: effects on helicoidal cell wall assembly. Cell Wall´s 86, pp 68-69

Saulnier L, Crepeau MJ, Lahaye M, Thibault JF, Garcia-Conesa MT, Kroon PA, Williamson G (1999) Isolation and structural determination of two 5,5'-diferuloyl oligosaccharides indicate that maize heteroxylans are covalently cross-linked by oxidatively coupled ferulates. Carbohydr Res 320: 82-92

Saxena IM, Brown RM (2000) Cellulose synthases and related enzymes. Curr Opin Plant Biol 3: 523-531

Saxena IM, Brown RM (2005) Cellulose biosynthesis: Current views and evolving concepts. Ann Bot 96: 9-21

Scheible WR, Eshed R, Richmond T, Delmer D, Somerville C (2001) Modifications of cellulose synthase confer resistance to isoxaben and thiazolidinone herbicides in *Arabidopsis Ixr1* mutants. Proc Natl Acad Sci U S A 98: 10079-10084

Scheible WR, Fry B, Kochevenko A, Schindelasch D, Zimmerli L, Somerville S, Loria R, Somerville CR (2003) An *Arabidopsis* mutant resistant to thaxtomin A, a cellulose synthesis inhibitor from *Streptomyces* species. Plant Cell 15: 1781-1794

Scheible WR, Pauly M (2004) Glycosyltransferases and cell wall biosynthesis: novel players and insights. Curr Opin Plant Biol 7: 285-295

Scheller HV, Jensen JK, Sorensen SO, Harholt J, Geshi N (2007) Biosynthesis of pectin. Physiol Plant 129: 283-295

Scheller HV, Ulvskov P (2010) Hemicelluloses. Annu Rev Plant Biol 61: 263-289

Schindelman G, Morikami A, Jung J, Baskin TI, Carpita NC, Derbyshire P, McCann MC, Benfey PN (2001) *COBRA* encodes a putative GPI-anchored protein, which is polarly localized and necessary for oriented cell expansion in *Arabidopsis*. Genes Develop 15: 1115-1127

Seifert GJ, Blaukopf C (2010) Irritable walls: The plant extracellular matrix and signaling. Plant Physiol 153. En prensa

Shedletzky E, Shmuel M, Delmer DP, Lamport DTA (1990) Adaptation and growth of tomato cells on the herbicide 2,6-Dichlorobenzonitrile leads to production of unique cell-walls virtually lacking a cellulose-xyloglucan network. Plant Physiol 94: 980-987

Shedletzky E, Shmuel M, Trainin T, Kalman S, Delmer D (1992) Cell-wall structure in cells adapted to growth on the cellulose-synthesis inhibitor 2,6-Dichlorobenzonitrile - A comparison between two dicotyledonous plants and a graminaceous monocot. Plant Physiol 100: 120-130

Smith LG (2001) Plant cell division: Building walls in the right places. Nat Rev Mol Cell Biol 2: 33-39

Somerville C (2006) Cellulose synthesis in higher plants. Annu Rev Cell Dev Biol 22: 53-78

Suzuki K, Ingold E, Sugiyama M, Fukuda H, Komamine A (1992) Effects of 2,6-dichlorobenzonitrile on differentiation to tracheary elements of isolated mesophyll cells of *Zinnia elegans* and formation of secondary cell walls. Physiol Plant 86: 43-48

Taylor NG (2008) Cellulose biosynthesis and deposition in higher plants. New Phytol 178: 239-252

Taylor NG, Howells RM, Huttly AK, Vickers K, Turner SR (2003) Interactions among three distinct CesA proteins essential for cellulose synthesis. Proc Natl Acad Sci U S A 100: 1450-1455

Taylor NG, Laurie S, Turner SR (2000) Multiple cellulose synthase catalytic subunits are required for cellulose synthesis in Arabidopsis. Plant Cell 12: 2529-2539

Taylor NG, Owen TP, Koonce LT, Haigler CH (1992) Dispersed lignin in tracheary elements treated with cellulose synthesis inhibitors provides evidence that molecules of the secondary cell wall mediate wall patterning. Plant J 2: 959-970

Taylor NG, Scheible WR, Cutler S, Somerville CR, Turner SR (1999) The *irregular xylem3* locus of Arabidopsis encodes a cellulose synthase required for secondary cell wall synthesis. Plant Cell 11: 769-779

Turner SR, Somerville CR (1997) Collapsed xylem phenotype of Arabidopsis identifies mutants deficient in cellulose deposition in the secondary cell wall. Plant Cell 9: 689-701

Vaughn KC (2002) Cellulose biosynthesis inhibitor herbicides. En "Herbicide Classes in Development" (P Böger, K Wakabayashi, K Hirai, Eds.). Springer-Verlag, Berlin, pp 139-150

Vaughn KC, Hoffman JC, Hahn MG, Staehelin LA (1996) The herbicide dichlobenil disrupts cell plate formation: Immunogold characterization. Protoplasma 194: 117-132

Vaughn KC, Turley RB (1999) The primary walls of cotton fibers contain an ensheathing pectin layer. Protoplasma 209: 226-237

Vaughn KC, Turley RB (2001) Ultrastructural effects of cellulose biosynthesis inhibitor herbicides on developing cotton fibers. Protoplasma 216: 80-93

Vázquez MD, Poschenrieder C, Corrales I, Barceló J (1999) Change in apoplastic aluminum during the initial growth response to aluminum by roots of a tolerant maize variety. Plant Physiol 119: 435-444

Verhertbruggen Y, Knox JP (2007) Pectic polysaccharides and expanding cell walls. En "Plant Cell Monographs" Vol 5 (JP Verbelen, K Vissenberg, Eds.). Springer-Verlag, Berlin, pp 139-158

Vogel J (2008) Unique aspects of the grass cell wall. Curr Opin Plant Biol 11: 301-307

Vogt T (2010) Phenylpropanoid biosynthesis. Mol Plant 3: 2-20

Wallace G, Fry SC (1994) Phenolic components of the plant cell wall. Int Rev Cytol 151: 229-267

Wells B, McCann MC, Shedletzky E, Delmer D, Roberts K (1994) Structural features of cell walls from tomato cells adapted to grow on the herbicide 2,6-dichlorobenzonitrile. J Microsc 173: 155-164

Wende G, Fry SC (1997) *O*-feruloylated, *O*-acetylated oligosaccharides as side-chains of grass xylans. Phytochemistry 44: 1011-1018

Wightman R, Marshall R, Turner SR (2009) A cellulose synthase-containing compartment moves rapidly beneath sites of secondary wall synthesis. Plant Cell Physiol 50: 584-594

Wightman R, Turner S (2010) Trafficking of the plant cellulose synthase complex. Plant Physiol 153. En prensa

Willats WGT, Orfila C, Limberg G, Buchholt HC, van Alebeek GJWM, Voragen AGJ, Marcus SE, Christensen TMIE, Mikkelsen JD, Murray BS, Knox JP (2001) Modulation of the degree and pattern of methyl-esterification of pectic homogalacturonan in plant cell walls - Implications for pectin methyl esterase action, matrix properties, and cell adhesion. J Biol Chem 276: 19404-19413

Wu A, Rihouey C, Seveno M, Hornblad E, Singh SK, Matsunaga T, Ishii T, Lerouge P, Marchant A (2009) The *Arabidopsis* IRX10 and IRX10-LIKE glycosyltransferases are critical for glucuronoxylan biosynthesis during secondary cell wall formation. Plant J 57: 718-731

Wusheng J, Donghua L (2010) Pb-induced cellular defense system in the root meristematic cells of *Allium sativum* L. BMC Plant Biol 10: 40

Yahraus T, Chandra S, Legendre L, Low PS (1995) Evidence for a mechanically induced oxidative burst. Plant Physiol 109: 1259-1266

Yamada T, Kuroda K, Jitsuyama Y, Takezawa D, Arakawa K, Fujikawa S (2002) Roles of the plasma membrane and the cell wall in the responses of plant cells to freezing. Planta 215: 770-778

Yennawar NH, Li LC, Dudzinski DM, Tabuchi A, Cosgrove DJ (2006) Crystal structure and activities of EXPB1 (Zea m 1), a beta-expansin and group-1 pollen allergen from maize. Proc Natl Acad Sci U S A 103: 14664-14671

Zabotin AI, Barisheva TS, Trofimova OI, Toroschina TE, Larskaya IA, Zabotina OA (2009) Oligosaccharin and ABA synergistically affect the acquisition of freezing tolerance in winter wheat. Plant Physiol Biochem 47: 854-858

Zeng W, Chatterjee M, Faik A (2008) UDP-xylose-stimulated glucuronyltransferase activity in wheat microsomal membranes: Characterization and role in glucurono (arabino) xylan biosynthesis. Plant Physiol 147: 78-91

Zhong RQ, Kays SJ, Schroeder BP, Ye ZH (2002) Mutation of a chitinase-like gene causes ectopic deposition of lignin, aberrant cell shapes, and overproduction of ethylene. Plant Cell 14: 165-179

Zhu JM, Chen SX, Alvarez S, Asirvatham VS, Schachtman DP, Wu YJ, Sharp RE (2006) Cell wall proteome in the maize primary root elongation zone. I. Extraction and identification of water-soluble and lightly ionically bound proteins. Plant Physiol 140: 311-325

Zuo JR, Niu QW, Nishizawa N, Wu Y, Kost B, Chua NH (2000) KORRIGAN, an *Arabidopsis* endo-1,4-β-glucanase, localizes to the cell plate by polarized targeting and is essential for cytokinesis. Plant Cell 12: 1137-1152

Anexos

ANEXO I

In: Cellulose: Structure and Properties…
Editors: Arnaud Lejeune and Thibaut Deprez
ISBN: 978-1-60876-388-7

Chapter 2

CELLULOSE BIOSYNTHESIS INHIBITORS: THEIR USES AS POTENTIAL HERBICIDES AND AS TOOLS IN CELLULOSE AND CELL WALL STRUCTURAL PLASTICITY RESEARCH

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ABSTRACT

Cellulose biosynthesis inhibitors (CBIs) form a heterogeneous group of structurally unrelated compounds that specifically affect the assembly or the deposition of cellulose. With the exception of thaxtomin A, the only naturally occurring CBI, all other CBIs are synthetic compounds. A number of them (dichlobenil, isoxaben and flupoxam) are used as herbicides and, as such, are listed as group L in the Herbicide Resistance Action Committee classification of herbicides. Recently, targets for isoxaben and dichlobenil have been provided. Other compounds, such as triazofenamide, CGA 325'615, the aminotriazine AE F150944 or the thiazolidinone named compound 1 have proven to be CBIs, but they have not yet been commercialized. Finally, some drugs seems to display a dual effect, acting as CBIs in some cases (i.e., depending on the species or their concentration), or as auxin herbicide (quinclorac) or plant growth retardant (ancymidol) in other circumstances.

CBIs have been used to elucidate cellulose biosynthesis—unraveling the organization of cellulose synthase (CESA) complex including the assembly between CESA subunits—and the relation between cortical microtubules and cellulose deposition, as some CBIs interfere with the microtubule-guided deposition of cellulose microfibrils.

In recent years, several *Arabidopsis* CBI-resistant mutants have been reported. Frequently, but not always, their mutations were related to genes directly involved in cellulose biosynthesis. In other cases, the effects of CBIs on plant growth have been used to associate the phenotype of a set of mutants to the impairment in their cellulose biosynthesis machinery.

The habituation of cell cultures to grow in the presence of high concentrations of different CBIs has been proven to be a powerful tool for insight into the mechanisms underlying the plasticity of plant cell wall structure and composition. CBI-habituated cell cultures reflect the ability of cells to adapt their metabolism and modify their cell walls in order to cope with these new stressful conditions.

New perspectives in CBI uses imply the selection of habituated cells having walls with a reduction in their cellulose content; the manipulation of levels and/or modification of matrix polysaccharides, rendering cell walls with new physicochemical properties; the study of the relationship between cellulose synthesis and other C-sink processes such as phenylpropanoid synthesis; or the elucidation of putative new targets implied in cellulose biosynthesis.

INTRODUCTION

Cellulose biosynthesis inhibitors (CBIs) constitute a varied group of structurally unrelated compounds that specifically affect the assembly or the deposition of cellulose in higher plants (Figure 1, Table 1). Two remarkable reviews focused on CBIs were afforded in 1999 (Sabba and Vaughn) and 2002 (Vaughn), but a set of important data has been raised since then, and the global view of CBIs is now more panoramic.

Several CBIs have been commercialized as herbicides, and therefore appear as group L in the Herbicide Resistance Action Committee (HRAC) classification of herbicides. This classification includes dichlobenil, chlorthiamid, isoxaben, flupoxam and quinclorac (but in this case indicated only for monocots, and it is considered an auxinic herbicide in group O) (Menne and Köcher, 2007).

Some other compounds have also been cited as promising herbicides, such as triazofenamide and triaziflam (Wakabayashi and Böger, 2004), but until now they have not been commercialized or included in this group. Finally, some drugs have been described to display a dual effect (i.e., quinclorac or ancymidol), acting as CBIs in some cases (i.e., depending on the species, or their concentration) and showing an additional mode of action in other circumstances.

The amount of information about each of these compounds is unequal. The objective of this chapter is to show a current view of this kind of compound, attending also to the description of CBI-related mutants and the use of CBIs to elucidate cellulose biosynthesis, and to give insight into the mechanisms underlying the plasticity of plant cell wall structure and composition by means of the habituation of cell cultures to CBIs.

Cellulose Biosynthesis

In recent years, a considerable amount of information regarding cellulose biosynthesis in higher plants has been provided, and some reviews have been reported (Somerville, 2006; Joshi and Mansfield, 2007; Mutwil et al., 2008; Taylor, 2008; Bessueille and Bulone, 2008), so that only a brief presentation of cellulose biosynthesis will be introduced now. Cellulose biosynthesis machinery is located in the plasma membrane, forming ‘rosettes’, which constitute a transmembrane system (Figure 2). In plants, these rosettes, or cellulose synthase (CESA) complexes, are viewed as hexamers, using freeze-fracture electron microscopy. Each

monomer comprises six cellulose synthase proteins, resulting then in 36 individual CESA proteins by rosette, able to synthesize 36 β-(1,4)-glucan chains that will form a microfibril (Doblin et al., 2002). The CESA complexes seem to be assembled in the Golgi apparatus and then exported via exocytosis to the plasma membrane (Somerville, 2006). CESA interacts with the cytoskeleton so that microtubules seem to guide the movement of CESA complexes (Paredez et al., 2006).

In *Arabidopsis thaliana,* primary cell wall CESA complexes are integrated by three unique types of CESA subunits named CESA1, CESA3 and CESA6-related (CESA2, 5 and 9) (Persson et al., 2007), in such a way that CESA3 and CESA6 physically interact (Desprez et al., 2007). Other proteins are claimed to form part of the complex, such as a cytoskeletal-anchored sucrose synthase, which could channel UDP-glucose to CESA (Amor et al., 1995); a membrane anchored endo-β-(1-4)-glucanase named KORRIGAN (Nicol et al., 1998), which seems to act decreasing cellulose crystallinity (Takahashi et al., 2009); a plasma membrane protein denominated KOBITO (Pagant et al., 2002); COBRA, an GPI-anchored protein (Schindelman et al., 2001); etc. However, an exact role for these additional proteins has not yet been provided. In secondary cell walls, CESA complexes are composed of three unique types of CESA subunits, too: CESA4, CESA7 and CESA8 (Atanassov et al., 2009; Timmers et al., 2009), but in this case purified CESA complexes did not contain any further protein (Atanassov et al., 2009). Analyses of cellulose biosynthesis in other species reflect similar results to that obtained in *Arabidopsis*.

Cellulose microfibril formation can be divided into three steps: (i) initiation, using UDP-glucose as the donor substrate; (ii) polymerization of glucose into β-(1,4)-glucan chains, and (iii) crystallization of β-(1,4)-glucan chains into a microfibril (Peng et al., 2002). Based on *in vitro* experiments, it has been proposed that cellulose biosynthesis is initiated from a sitosterol-β-glucoside as primer molecule (Peng et al., 2002).

CELLULOSE BIOSYNTHESIS INHIBITORS

Dichlobenil

Dichlobenil or 2,6-dichlorobenzonitrile, is the simplest and one of the most studied CBIs. A related herbicide, chlorthiamid (2,6-dichlorothiobenzamide), is converted to dichlobenil in soil as a consequence of microorganism metabolism (Beynon and Wright, 1968). Dichlobenil has been marketed since the 1960s under different trade names (Casoron, Barrier, Silbenil, H 133). Although less effective on monocots (Sabba and Vaughn, 1999), dichlobenil has been extensively used as a broad spectrum preemergence herbicide (Verloop and Nimmo, 1969). The preemergence action of dichlobenil is based on the impairment of seedling growth more than on the inhibition of seed germination. In this way, in the French bean, root growth is 30 times more sensible to dichlobenil than seed germination (Encina, unpubl.). I_{50} values in the micromolar range have been measured for the effect of dichlobenil on root growth: 1 μM on *Lepidium sativum* (Günther and Pestemer, 1990); 0.4 μM on *Arabidopsis* (Heim et al., 1998); 4 μM on the French bean (Encina, unpubl.), and 2 μM on maize (Mélida, unpubl.).

Rapidly expanding cells such as suspension or callus-cultured cells (Shedletzky et al., 1990, 1992; Corio-Costet et al., 1991a, b; Encina et al., 2001, 2002; Mélida et al., 2009);

seedling roots and hypocotyls (Himmelspach et al., 2003; DeBolt et al., 2007b); and pollen tubes (Anderson et al., 2002) are sensible to dichlobenil in the nano-micromolar range [I_{50}: 50 nM for soybean suspension-cultured cells (Corio-Costet et al., 1991b); 0.5 μM and 0.3 μM for French bean callus and suspension-cultured cells, respectively (Encina et al., 2001, 2002); and 1.5 μM for maize callus (Mélida et al., 2009)]. The ability of this herbicide to arrest cell plate formation (but not nuclear division [Galbraith and Shields, 1982]) and cell elongation (Vaughn et al., 1996; Sabba et al., 1999; Encina et al., 2001, 2002; Vaughn, 2002) is under the typical dichlobenil-growth retarding and dichlobenil-dwarfing effects (Sabba and Vaugh, 1999 and refs. therein).

Dichlobenil symptomatology also includes radial root or hypocotyl swelling (Umetsu et al., 1976; Eisinger et al., 1983; Montague, 1995; Himmelspach et al., 2003; DeBolt et al., 2007b), inhibition of root-hairs and secondary-root development, and induction of necrotic lesions (Barreiro, unpubl.). Many later effects are also associated with mitotic disrupter herbicides (Vaughn, 2002), and curiously, to many abiotic stresses (Potters et al., 2007).

Very early studies related the phytotoxic effect of dichlobenil to the inhibition of ATP production by its phenolic degradation products, 2,6-dichlo-3-hydroxybenzonitrile and 2,6-dichloro-4-hydroxybenzonitrile (Moreland et al., 1974). However, in the same year it was demonstrated that dichlobenil itself impaired the incorporation of radiolabelled glucose into cellulose (Hogetsu et al., 1974). Since then, dichlobenil has been demonstrated to inhibit the biosynthesis of cellulose in a wide range of systems by using the same experimental procedure (e.g., Montezinos and Delmer, 1980; Brummell and Hall, 1985; Hoson and Masuda, 1991; Corio-Costet et al., 1991b; Edelmann and Fry, 1992; Shedletzky et al., 1992; García-Angulo et al., 2009), and nowadays no doubts about the specific effect of dichlobenil on cell wall biosynthesis exist.

In plants, the effect of dichlobenil on cellulose biosynthesis seems to be specific, since matrix polysaccharides synthesis is not inhibited upon a short term dichlobenil treatment (Montezinos and Delmer, 1980; Blaschek et al., 1985; Francey et al., 1989). However dichlobenil has also been reported to inhibit non-cellulosic polysaccharides synthesis in algae (Arad et al., 1994; Wang et al., 1997). Other cellular processes such as DNA synthesis, protein synthesis, respiration, oxidative phosphorylation, phospholipid metabolism, nucleoside metabolism and glucose metabolism (including glucose uptake) are not affected by dichlobenil (Meyer and Herth, 1978; Montezinos and Delmer, 1980; Galbraith and Shields, 1982; Delmer et al., 1987).

Besides this specific effect of dichlobenil on cellulose synthesis, several papers address the side effect of dichlobenil on cellulose microfibril orientation (Sugimoto et al., 2001), cellulose-synthesizing complexes organization/motility (Herth, 1987; Mizuta and Brown, 1992; DeBolt et al., 2007b; Wightman et al., 2009) and microtubule organization (Himmelspach et al., 2003).

Mode of action

Despite dichlobenil being recognized and used as a specific CBI since decades (Hogetsu et al., 1974), its mode of action is still unclear. Dichobenil inhibited the *in vivo* synthesis of sitosterol-β-glucosides, and exogenous addition of sitosterol-β-glucosides reverted dichlobenil effects (Peng et al., 2002). Based on these findings, an effect for dichlobenil on blocking the initiation steps of cellulose biosynthesis could be hypothesized. However, the need of a primer

for *in vivo* cellulose biosynthesis is now under controversial as experimental conditions used by Peng et al. (2002) seemed to be highly forced and it has been demonstrated that *in vitro* biosynthesis of cellulose did not require any primer (Somerville, 2006).

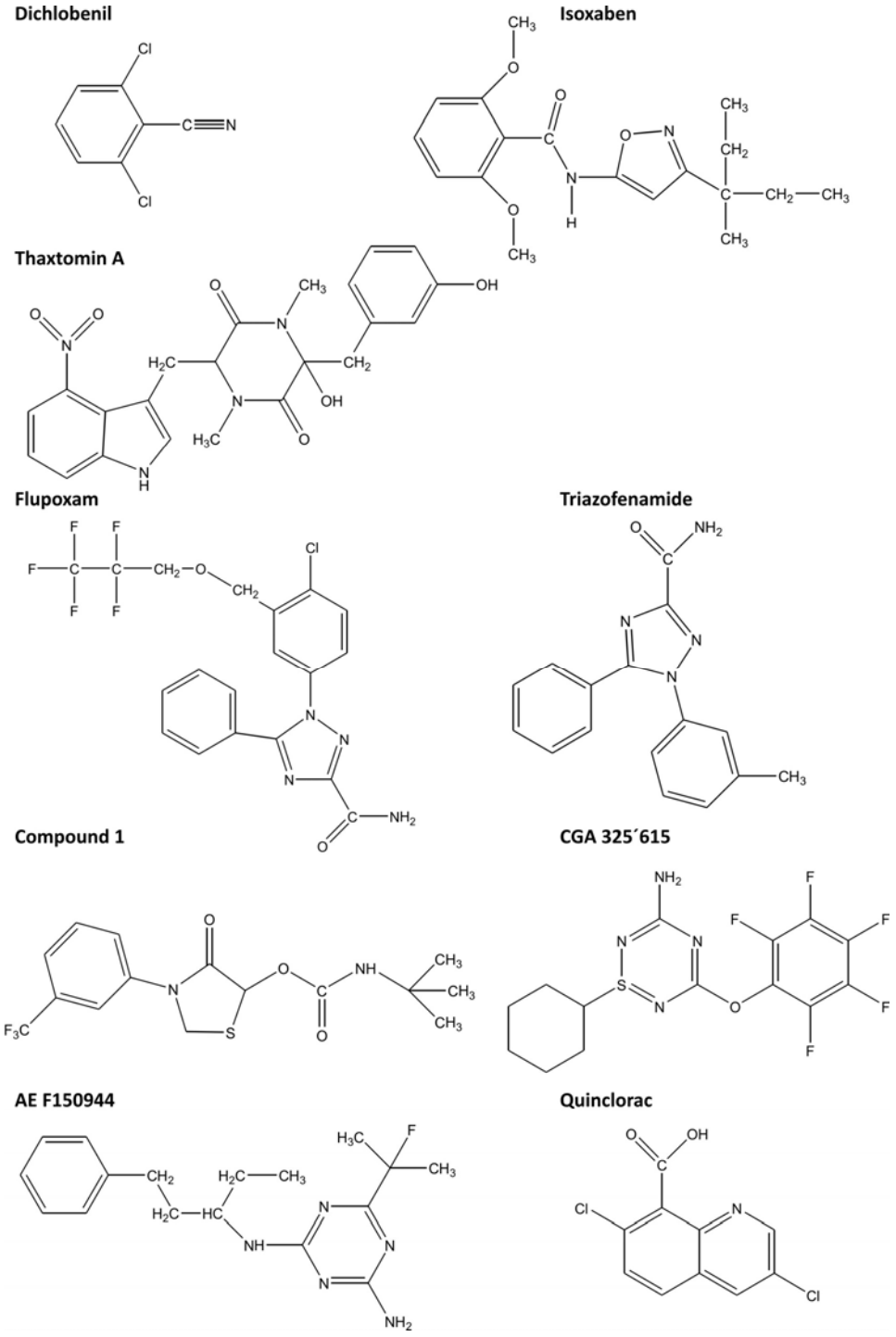

Figure 1. Structures of selected CBIs.

Table 1. Some data about selected CBIs. Accepted chemical names, I_{50} on fresh weight gain of bean calluses, I_{50} on *Arabidopsis* root growth, and selected references about CBIs. (a) García-Angulo, unpubl.; (b) Heim et al., 1998; (c) Heim et al., 1989; (d) Heim et al., 1998; (e) Desprez et al., 2002; (f) Scheible et al., 2003; (g) Hoffmann and Vaughn, 1996 (on *Lepidium sativum* root); (h) Heim et al., 1998; (i) Sharples et al., 1998; (j) Walsh et al., 2006

CBI	Chemical name	I_{50} bean calluses[a]	I_{50} *Arabidopsis* root growth	References
Dichlobenil	2,6-dichlorobenzonitrile	0.5 µM	0.4 µM[b]	Delmer, 1987 Delmer et al., 1987
Isoxaben	*N*-[3-(1-ethyl-1-methylpropyl)]-5-isoxazolyl]-2,6-dimethoxybenzamide	3 nM	4.5 nM[c]; 1nM[d]; 1.5 nM[e]	Huggenberger et al., 1982 Heim et al., 1990
Thaxtomin A	(A 4-nitroindol-3-yl containing 2,5-dioxopiperazine)	0.6 nM	25-50 nM[f]	King et al., 1992 Fry and Loria, 2002
Flupoxam	1-[4-chloro-3-[(2,2,3,3,3-pentafluoropropoxymethyl) phenyl]-5-phenyl-1H-1,2,4-triazole-3-carboximide	2 nM	(6 nM[g])	O'Keeffe and Klevorn, 1991 Hoffman and Vaughn, 1996
Triazofenamide	1-(3-methyl phenyl)-5-phenyl-1H-1,2,4-3 triazole-3-carboximide	15 nM	39 nM[h]	Heim et al., 1998
Compound 1	5-tert-butyl-carbamoyloxyl-3-(3-trifluoromethyl) phenyl-4-thiazolidinone	20 µM	<3 µM[i]	Sharples et al., 1998.
CGA 325´615	1-cyclohexyl-5-(2,3,4,5,6-pentafluorophenoxy)-1 λ4,2,4,6-thiatriazin-3-amine	0.5 nM	—	Peng et al., 2001
AE F150944	N2-(1-ethyl-3-phenylpropyl)-6-(1-fluoro-1-methylethyl)-1,3,5-triazine-2,4-diamine	1 nM	—	Kiedaisch et al., 2003
Quinclorac	3,7-dichloro-8-quinoline carboxylic acid	10 µM	8 µM[j]	Koo et al., 1996 Koo et al., 1997 Tresch and Grossmann, 2003

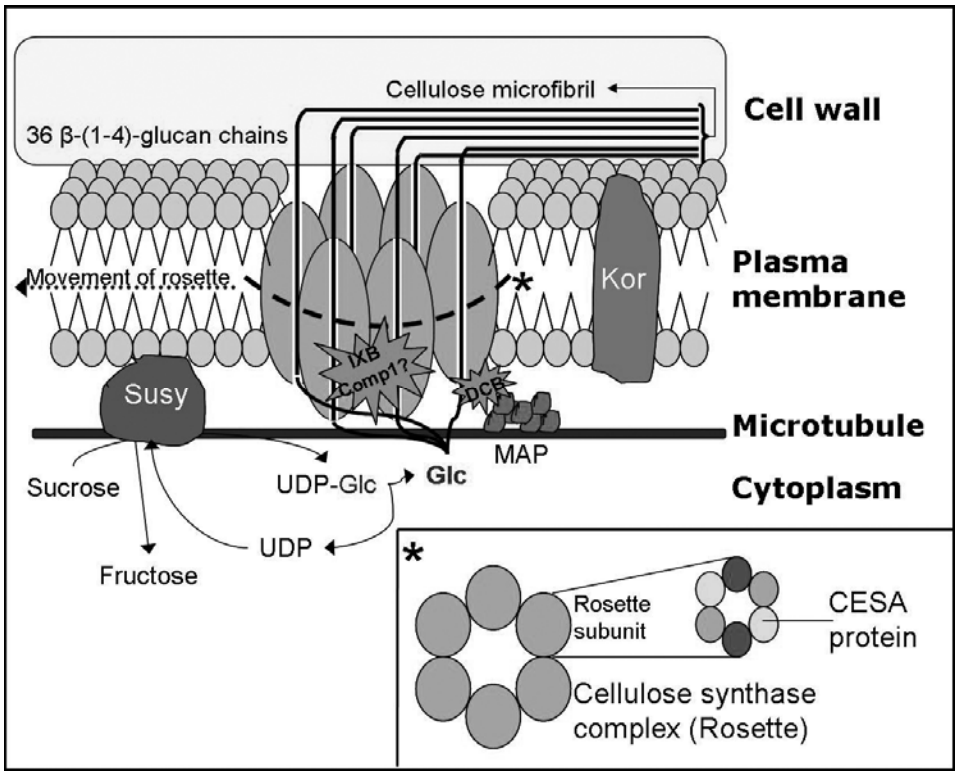

Figure 2. Model of a cellulose synthase complex (rosette). Each subunit (represented as a lobe) contains six CESA proteins, corresponding to three different CESA types (highlighted in the insert by different grey saturation). Other proteins seem to act in the cellulose biosynthesis process, such as sucrose synthase (Susy), KORRIGAN (Kor), a microtubule associated protein (MAP), and other ones, not represented here (see the text). Putative targets for some CBIs have been highlighted in boxes (IXB: isoxaben; Comp1: compound 1; DCB: dichlobenil).

A remarkable characteristic of cell walls from dichlobenil-habituated cells (as it will be detailed further) is the accumulation of a non-crystalline β-(1,4)-glucan (Encina et al., 2002; García-Angulo et al., 2006). Moreover, a number of reports point to dichlobenil as a disruptor of the organization, motility or dynamics of the CESA complexes (Herth, 1987; Mizuta and Brown, 1992; DeBolt et al., 2007b). Taken together these results it has been proposed that dichlobenil effect on cellulose biosynthesis would result in an alteration of cellulose crystallization rather than of an inhibition of glucose polymerization. At this respect it is interesting to note that the *rsw1 Arabidopsis* mutants on CESA1 protein, with disassembled CESA complexes, have reduced cellulose contents and accumulate a non-crystalline β-(1,4)-glucan (Arioli et al., 1998).

Three putative targets for dichlobenil have been reported until now. By using a photoreactive analogue of dichlobenil, 2-6-dichlorophenylazide, Delmer et al. (1987) reported a small protein of 12-18 kDa as the dichlobenil target. This small protein resulted easily dissociated from membrane preparations and, therefore, it is unlikely to be a CESA protein. The authors hypothesize for dichlorophenylazide-tagged protein a regulatory role, probably modulating the glycosyl-transferase specificity (Delmer et al., 1987).

Table 2. Selected cellulose-deficient mutants in Arabidopsis. Some of them have a mutation in *CesA* loci, whereas others present a mutation in different genes. Isoxaben-resistant mutants are indicated in bold.

Locus	Mutant alleles	Phenotype	Gene product	Reference
CesA1	*rsw1-1*	Temperature-sensitive, radially expanded cells, dwarf, normal division planes	Cellulose synthase catalytic subunit, CESA1	Arioli et al., 1998
	rsw1-2	Late embryonic, radially expanded cells, dwarf, normal division plane		Gillmor et al., 2002
	rsw1-20			Beeckman et al., 2002
	rsw1-10	Post-embryonic, radially expanded cells, dwarf		Fagard et al., 2000
CesA3	*eli1-1; 1-2*	Post-embryonic, dwarf, accumulation ectopic lignin	Cellulose synthase catalytic subunit, CESA3	Caño-Delgado et al., 2003
	cev1	Increased production of ethylene and jasmonate, stunted root		Ellis et al., 2002
	ixr1-1; 1-2	Semi-dominant, isoxaben-resistant		Scheible et al., 2001
CesA4	*irx5*	Collapsed irregular xylem walls, thinner cell wall, weak stem, adult plants slightly smaller than wild type	Cellulose synthase catalytic subunit, CESA4	Taylor et al., 2003
CesA6	*prc1-1* to *1-12*	Post-embryonic, stunted root and dark-grown hypocotyls, radially expanded cells, gapped cell walls, normal microfibril orientation	Cellulose synthase catalytic subunit, CESA6	Fagard et al., 2000
	ixr2-1	Semi-dominant, isoxaben-resistant		Desprez et al., 2002
CesA7	*irx3*	Collapsed irregular xylem walls, thinner cell walls, weak stem, adult plants slightly smaller than wild type	Cellulose synthase catalytic subunit, CESA7	Taylor et al.,1999, 2000
CesA8	*irx1*	Collapsed irregular xylem walls, weak stem, adult plants slightly smaller than wild type	Cellulose synthase catalytic subunit, CESA8	Turner and Somerville, 1997; Taylor et al., 2000
Kob1	*kob1-1; 1-2*	Dwarf, radially expanded cells, less and disorganized microfibrils in cell elongation zone in root	Type II intrinsic plasma membrane protein	Pagant et al., 2002
Cob	*cob1-1*	Conditional root expansion, T-sensitive, radially expanded cells and stunted root, reduced cell expansion in root	GPI-anchored protein	Schindelman et al., 2001

Table 2. (Continued)

Locus	Mutant alleles	Phenotype	Gene product	Reference
Kor1	*kor1-1*	Post-embryonic, dwarf, radially expanded cells	Membrane-bound endo-β-1,4-glucananse	Nicol et al., 1998
	kor1-2	Late embryonic, dwarf, randomized division planes, aborted cell plates		Zuo et al., 2000
	rsw2-1 to *4*	Temperature-sensitive, radially expanded cells, dwarf, randomized division planes, aborted cell plates		Lane et al., 2001
	irx2-1; 2-2	Collapsed xylem cells, no growth phenotype		Molhoj et al., 2002
Pom1	*pom1-1* to *11*	Conditional root expansion, stunted root, dark-grown hypocotyls, radially expanded cells, normal microfibril orientation	Chitinase-like protein (AtCTL1)	Hauser et al., 1995
	elp1	Ectopic deposition of lignin, incomplete cell walls in some pith cells		Zhong et al., 2002
Cyt1	*cyt1-1; cyt2*	Late embryonic, increased radial expansion , incomplete cell walls, excessive callus accumulation	Mannose-1-phosphatase guanylyl-transferase	Lukowitz et al., 2001
Rsw3	*rsw3-1*	Temperature-sensitive, radially expanded cells, dwarf, longer lag-time before appearance phenotype then rsw1 and 2	Glucosidase II	Burn et al., 2002

Some years later, Nakagawa and Sakurai (1998) published the specific binding of dichlobenil to the catalytic subunit of cellulose synthase. By using a specific antibody against CESA1, it was possible to detect this protein in microsomal fractions of dichlobenil-habituated and control tobacco BY-2 cells. Dichlobenil-habituated cells had reduced contents of cellulose but more CESA1 protein than controls. Following these authors, CESA1 would result stabilized upon dichlobenil binding, rendering it more stable against proteolytic degradation (Nakagawa and Sakurai, 1998). In any case it was demonstrated a direct binding between dichlobenil and CESA1, and therefore the CESA1 enrichment in dichlobenil-habituated cells is likely to be a side effect of dichlobenil action.

Long ago, it was demonstrated that dichlobenil stimulates the accumulation of CESA complexes in the plasma membrane of wheat (Herth, 1987). Recently, live-cell imaging of transgenic plants carrying a yellow fluorescent protein (YFP)-CESA6 fusion showed that a short-term treatment with dichlobenil inhibited the motility of these complexes in *Arabidopsis*

cells and promoted their hyperaccumulation at sites in the plasma membrane that may coincide with loading areas of CESA complexes from Golgi (DeBolt et al., 2007b). Further studies confirmed that dichlobenil also slowed the movement of CESA complexes beneath the zones of formation of secondary wall (Wightman et al., 2009). These findings may reveal the interference of dichlobenil with the circulation of CESA complexes between Golgi and plasma membrane.

In recent times, a microtubule associated protein MAP20 in secondary cell walls of hybrid aspen has been reported as a target for dichlobenil (Rajangam et al., 2008). MAP20 is a small cytosolic protein strongly upregulated during the formation of secondary cell wall. It shares a conserved domain with a classical microtubule associated protein, TPX2, demonstrated to bind microtubules and proteins that use microtubules as guiding templates. In fact, MAP20 binds to microtubules both *in vitro* and *in vivo*. Rajangam et al. (2008) have demonstrated that dichlobenil specifically binds to MAP20 during cellulose synthesis but it does not prevent the binding of the protein to microtubules. Linking up MAP20 function with dichlobenil effects, these authors propose that MAP20 has a specific role in cellulose biosynthesis by coupling CESA proteins with microtubules and that dichlobenil inhibits cellulose biosynthesis by decoupling cellulose synthesis and microtubules through MAP20 inactivation. Finally it is suggested that the small polypeptide described by Delmer et al. (1987) might be a putative ortholog of MAP20 (Rajangam et al., 2008).

Isoxaben

Isoxaben (N-[3-(ethyl-1-methyl propyl)]-5-isoxazolyl-2,6-dimethoxybenzamide) marketed as Flexidor, Gallery or EL-107 is a selective pre-emergence herbicide widely used for season-long control of dicot weeds in winter cereals (Huggenberger et al., 1982) and for weed control in turf, ornamentals and nursery stocks (Sabba and Vaughn, 1999). The pre-emergence action of isoxaben is based on the ability to impair seedling growth instead of preventing germination (Desprez et al., 2002). Isoxaben is very active in the nanomolar range; I_{50} values of 1.5 nM (Desprez et al., 2002), 4.5 nM (Heim et al., 1989) and 20 nM (Lefebvre et al., 1987) have been reported for the inhibition of *Arabidopsis* (Desprez et al., 2002; Heim et al., 1989) and *Brassica napus* (Lefebvre et al., 1987) seedlings growth. Isoxaben symptomatology evokes that of dichlobenil. *Arabidopsis* seedlings treated with isoxaben show a typical dwarf phenotype caused by the inhibition of hypocotyl and root elongation. Moreover, isoxaben-treated organs typically expand radially, have reduced elongation rates, accumulate callose and show ectopic lignification (Desprez et al., 2002; Caño-Delgado et al., 2003). As dichlobenil, isoxaben also arrests cell plate formation (Samuels et al., 1995; Vaughn et al., 1996; Durso and Vaughn, 1997). At this respect, isoxaben seems to act in a different manner as it has been reported that cell plates of isoxaben-treated BY-2 tobacco cells are reduced in both cellulose and callose, whereas dichlobenil treatment did only affect cellulose biosynthesis. In accordance with this, isoxaben effect is more pronounced due to the inhibition of cell plate formation at an early stage (Durso and Vaughn, 1997; Sabba and Vaughn, 1999). Later, DeBolt et al. (2007b) demonstrate that both dichlobenil and isoxaben promoted the formation of callose on *Arabidopsis* seedlings

by inducing the expression of *Pmr4*, a pathogen- or wound-induced callose synthase gene (Nishimura et al., 2003).

Isoxaben is also very active at inhibiting the growth of *in vitro* cultured-cells. I_{50} values in the nanomolar range have been reported for different systems: 170 nM for *Arabidopsis* calluses (Heim et al., 1989); 80 nM for soybean cell suspensions (Corio-Costet et al., 1991a, b), and 10 nM for French bean calluses (Díaz-Cacho et al., 1999).

It has been conclusively demonstrated that isoxaben specifically inhibits the glucose incorporation into cellulose in plants, without affecting other metabolic processes such as photosynthesis, respiration, carotenoid or tetrapyrrole biosynthesis (Lefebvre et al., 1987; Heim et al., 1989, 1990; Corio-Costet et al., 1991b; Caño-Delgado et al., 2003). The reported I_{50} values for the inhibition of [^{14}C]glucose incorporation into cellulose are again in the nanomolar range: 10 nM for *Arabidopsis* (Heim et al., 1990) and 40 nM for soybean (Corio-Costet et al., 1991b). Although preliminary results would indicate an effect of isoxaben on protein synthesis (Lefebvre et al., 1987), more accurate experiments demonstrated that the inhibition of protein synthesis, if produced, was not a direct effect of isoxaben (Heim et al., 1990).

Mode of action

The knowledge about the mode of action of isoxaben greatly advanced after the discovery of two loci in *Arabidopsis*, *Ixr1* and *Ixr2*, that conferred resistance to isoxaben (Heim et al., 1989, 1990). Early studies demonstrated that the natural isoxaben-tolerance of some weeds was directly related with a decreased sensitivity at the target site more than with an enhanced herbicide detoxification or reduced herbicide uptake (Heim et al., 1991; Scheneegurt et al., 1994). Therefore the idea of *ixr* mutations affecting the herbicide target was very attractive.

Nowadays it is known that *ixr1* and *ixr2* carry mutations in CESA3 and CESA6 proteins respectively (Scheible et al., 2001; Desprez et al., 2002). No isoxaben-resistant mutants affecting CESA1 (the third "Musketeer" required for cellulose biosynthesis during primary cell wall formation) have been identified, suggesting that CESA1 is not a target for isoxaben (Robert et al., 2004). The *ixr* mutations affect to the C terminus of CESA, far from its active site, making difficult for isoxaben to directly interfere with the catalytic site of the protein (Desprez et al., 2002). Most probably, *ixr* mutants would become isoxaben-insensitive by a conformational change of the target protein.

It is not clear how isoxaben inhibits the cellulose biosynthesis. It has been hypothesized that isoxaben would recognize, directly or indirectly, an isoxaben–sensitive site in the interaction between CESA3 and CESA6. By this way, isoxaben binding would destabilize the rosette (or each rosette constituting particles) leading to an inhibition in the cellulose biosynthesis. In this sense it has been demonstrated that isoxaben causes the clearing of YFP-CESA6-tagged complexes from the membrane (Paredez et al., 2006; DeBolt et al., 2007b). An alternative model explains that an isoxaben-induced conformational change on CESA proteins would block the putative membrane channel needed for the extrusion of the growing cellulose microfibril (Desprez et al., 2002).

As for others CBIs, the inhibition of cellulose biosynthesis by isoxaben is accompanied by side effects on cytoskeleton organization and cell longevity. As dichlobenil, isoxaben disrupts microtubule stability and orientation (Paredez et al.,

2008). Same authors demonstrated that isoxaben effect on cortical microtubules is directly linked to the inhibition of cellulose biosynthesis rather than to an alteration of cell wall integrity (Paredez et al., 2008). Recently, it has been demonstrated that isoxaben (like thaxtomin A) specifically induce programmed cell death in suspension-cultured *Arabidopsis* cells (Duval et al., 2005, 2009).

Thaxtomin A

The modified dipeptide thaxtomin A (a 4-nitroindol-3-yl containing 2,5-dioxopiperazine) is the main phytotoxin produced by *Streptomyces scabiei*, the causative agent of common scab disease (King et al., 1992). Thaxtomin A has been reported to have an I_{50} value of 25 to 50 nM on seedling growth of *Arabidopsis* (Scheible et al., 2003). At nanomolar concentrations thaxtomin A causes dramatic cell swelling and root or shoot thickening due to cell hypertrophy (Scheible et al., 2003). Thaxtomin A (1–3 μM) also inhibited normal cell elongation of tobacco protoplasts in a manner that suggested an effect on primary cell wall development (Fry and Loria, 2002).

The inhibition of cellulose synthesis by thaxtomin A induces a genetically controlled cell death in a wide variety of plant species and tissues and in a concentration-dependent manner (Duval et al., 2005). Programmed cell death involves fragmentation of nuclear DNA and requires active gene expression and *de novo* protein synthesis. It was recently demonstrated that this programmed cell death occurs by the activation of common stress-related pathways that would somehow bypass the classical hormone-dependent defense pathways (Duval and Beaudoin, 2009).

Sublethal concentrations of several auxins reduced severity of scab disease (McIntosh et al., 1985) by enhancing tolerance to thaxtomin A (Tegg et al., 2008). Although the mechanism of auxin inhibition of thaxtomin A toxicity is not understood, it could be related to the reversion of programmed cell death by auxins (Gopalan, 2008), or to direct competition for a putative cellular binding since several auxins share chemical similarities to the thaxtomin A molecule (Tegg et al., 2008).

Recently thaxtomin A has been used to select scab disease-resistant potato plants, by means of a cell culture approach. This inhibitor was used as a selection agent applied to potato cells culture media: the surviving variants were recovered and used to regenerate complete plants (Wilson et al., 2009).

Mode of action

The mode of action of thaxtomin A is not known. It has been reported that the symptoms are similar to those caused by CBIs such as dichlobenil and isoxaben (King and Lawrence, 2001; Scheible et al., 2003). In fact, the reduction of seedling growth was accompanied by a reduction of the incorporation of [^{14}C]glucose into the cellulosic fraction of dark-grown seedlings of *Arabidopsis*, that was parallel to a significant increase in the incorporation into pectins, while the incorporation into hemicelluloses was slightly increased (Scheible et al., 2003; Bischoff et al., 2009). Additional evidence for the inhibition of cellulose biosynthesis was obtained with Fourier transform infrared (FTIR) microspectroscopy. FTIR spectra of thaxtomin A-treated hypocotyls cluster tightly with those of wild-type hypocotyls treated with

CBIs (e.g., isoxaben or dichlobenil) and with mutants known to be defective specifically in cellulose synthesis (e.g., *rsw1-2* and *kor-2*) (Robert et al., 2004).

However, it was reported that thaxtomin A also causes substantial wilting in several species after postemergence applications, a symptom dissimilar to that caused by known CBIs (King and Lawrence, 2001).

The modifications in cell wall composition caused by thaxtomin A were accompanied by changes in the expression of *CesA* genes and additional cell wall related genes both in primary (*Korrigan* and *Kobito1*) and secondary (*CesA7, CesA8, Cobra-like 4, Irx8, and Irx9, Cad9*) cell wall synthesis (Bischoff et al., 2009). The alteration in the expression of *CesA* genes of *Arabidopsis* seedlings results in a depletion of CESA complexes from the plasma membrane, coinciding to their accumulation in a microtubule-associated compartment (Bischoff et al., 2009).

Changes in the expression of genes associated with pectin metabolism and cell wall remodeling were also detected after a treatment with thaxtomin A, as it was the case of a pectin acetylesterase and two pectin methylesterases that were found to be upregulated by thaxtomin A (Bischoff et al., 2009). This result agrees with previous data on compensation of the loss of cellulose by an increased amount of pectin with a lower degree of esterification (Burton et al., 2000).

The modification of cell wall composition caused by a thaxtomin A treatment causes an additional cell wall reinforcement by triggering ectopic lignification by a high up-regulation of several genes involved in lignin biosynthesis (Bischoff et al., 2009). Thaxtomin A treatment also provoked the induction of a set of defense genes (Caño-Delgado et al., 2003, Bischoff et al., 2009).

Other CBIs

Triazol carboximide herbicides (flupoxam and triazofenamide)

Flupoxam (1-[4-chloro-3-(2,2,3,3,3-pentafluoropropoxymethyl) phenyl]-5-phenyl-1H-1,2,4-triazole-3-carboximide), and triazofenamide (1- [3-methyl phenyl]-5-phenyl-1H-1,2,4-triazole-3-carboximide), are triazole-carboximide herbicides. Flupoxam, commercialized as Quatatim or KNW-739, inhibits the root growth of watercress (*Lepidium sativum*) by 50% at a concentration of 6 nM. Because flupoxam induces classic club root morphology it was initially characterized as a mitotic disrupter (O'Keeffe and Klevorn, 1991). However, Hoffman and Vaughn (1996) reported later that the effect of flupoxam on watercress roots was different from that expected of a mitotic disrupter although they did not propose an alternative mode of action. By cluster analysis of FTIR spectra, a close relationship among *Arabidopsis* seedlings treated with flupoxam, dichlobenil, isoxaben and cellulose-deficient mutants was observed (Robert el al., 2004). The treatment of cotton fibers with flupoxam (and also with isoxaben) causes spherical shapes and frequently induces cell division. Fibers grown in the presence of isoxaben or flupoxam replaced the entire cell wall with a pectin sheath of chiefly deesterified pectins, indicating that both herbicides effectively disrupt cellulose biosynthesis and cause radical changes in cell wall structure and composition (Vaughn and Turley, 2001).

The close analog of flupoxam, triazofenamide, was also initially considered as a microtubule polymerization inhibitor (O'Keeffe and Klevorn, 1991). However, using staining

and microscopic techniques, this mode of action was later rejected (Hoffman and Vaughn, 1996). Heim et al. (1998) postulated that triazofenamide was a cellulose biosynthesis inhibitor due to the symptoms elicited in an *Arabidopsis* short-term test. Furthermore, triazofenamide inhibits [^{14}C]glucose incorporation into cellulose in a manner similar to isoxaben (Heim et al., 1998). Nevertheless, the exact modes of action of the triazole-carboximide herbicides are still unknown.

Compound 1

Compound 1 (5-tert-butyl-carbamoyloxyl-3-(3-trifluoromethyl) phenyl-4-thiazolidinone) is a thiazolidinone which induces a potent and rapid inhibition of [^{3}H]glucose incorporation into the acid-insoluble cell wall fraction of roots of dicot plants at nanomolar concentration (Sharples et al., 1998). Although many aspects about the mode of action of compound 1 remained unknown it was suggested that compound 1 and isoxaben should share a common mode of action (Sharples et al., 1998) that should differ from the mode of action of triazofenamide, since isoxaben-resistant mutants of *Arabidopsis* are cross resistant to compound 1 (Sharples et al., 1998) but sensitive to triazofenamide (Heim et al., 1998).

CGA 325´615

CGA 325´615 (1-cyclohexyl-5-(2,3,4,5,6-pentafluorophenoxy)-1 λ4,2,4,6-thiatriazin-3-amine) is a herbicide that inhibits synthesis of crystalline cellulose by interfering with glucan chain crystallization and causes an accumulation of non-crystalline β-(1,4)-glucan associated with CESA proteins (Peng et al., 2001). Since oxidizing agents can reverse CGA 325´615 inhibition (Kurek et al., 2002), it has been suggested that this inhibitor affects the oxidative state of the zinc-finger domain of CESA proteins required for the assembly of rosettes, which synthesize multiple β-(1,4)-glucan chains in close proximity in order to facilitate crystallization.

Recently it has been shown that CGA 325'615 treatment of *Arabidopsis* hypocotyls induced internalization of CESA complexes in a microtubule-associated CESA compartment –MASC- (Crowell et al., 2009). Accordingly to these authors, in conditions of cellulose biosynthesis inhibition, CESA complexes are recruited from the membrane into MASCs as a way to regulate the cellulose synthesis. It is interesting to note that CESA complexes internalization is obtained when cell growth is impaired by osmotic stress and that MASCs abundance is negatively correlated with hypocotyl elongation (Crowell et al., 2009). The door is open to speculate on the relationship between CBIs, cellulose biosynthesis and CESA complexes dynamic.

AE F150944

The aminotriazine AE F150944 (N2-(1-ethyl-3-phenylpropyl)-6-(1-fluoro-1-methylethyl)-1,3,5-triazine-2,4-diamine) is structurally distinct from other CBIs. AE F150944 effectively inhibited [^{14}C]glucose incorporation into crystalline cellulose with I_{50} values of 16.7·nM, 3.67·nM and 0.37·nM during primary wall synthesis in suspension cultures of the monocot *Sorghum halepense*; and secondary and primary wall synthesis in cultured cells of the dicot *Zinnia elegans*, respectively (Kiedaisch et al., 2003). AE F150944 specifically inhibits crystalline cellulose synthesis only in

organisms that synthesize cellulose via rosettes. Although it is believed that the effect of AE F150944 is due to the destabilisation of rosettes (Kiedaisch et al., 2003), its molecular target remains to be identified.

COMPOUNDS INHIBITING CELLULOSE BIOSYNTHESIS IN A SECONDARY EFFECT

Some compounds traditionally classified in other categories, such as growth retardants or auxinic herbicides, have also been shown to inhibit cellulose biosynthesis. That is the case of quinclorac, an auxinic herbicide, or ancymidol, an inhibitor of gibberellin biosynthesis. Also, some antimicrotubule agents affect cellulose biosynthesis as a secondary effect.

Quinclorac

Quinclorac (3,7-dichloro-8-quinoline carboxylic acid) is a quinoline carboxylic acid successfully used as an herbicide in crops of rice, barley, sorghum, etc., in order to control monocot and dicot weeds (Grossmann and Kwiatkowski, 2000). This synthetic substance was proposed to be auxin-like, acting by stimulation of ethylene synthesis, accompanied by cyanide accumulation in susceptible species (Grossmann and Kwiatkowski, 1995). However, some symptoms characteristic of auxinic herbicides were absent in the case of quinclorac, such as cell wall acidification due to stimulated H^+-ATPase activity (Theologis, 1987), stimulated respiration or increased RNA content (Koo et al., 1991).

Subsequently, quinclorac was also proved to inhibit cell wall biosynthesis in a dose-dependent manner in maize roots. As shown by Koo et al. (1996), after only 3 hours of treatment, the herbicide inhibited the incorporation of [^{14}C]glucose into cell wall by 33%. The inhibitory effect increased with longer treatments and higher quinclorac concentrations, and affected not only cellulose, but also glucuronoarabinoxylans and, in a minor extent, mixed-linked glucan. In contrast, dichlobenil only inhibited cellulose biosynthesis in a parallel experiment. In a later work by Koo et al. (1997), quinclorac effect on cell wall was tested in both susceptible and tolerant grasses. Cell wall biosynthesis was repressed by the herbicide by 73 and 60% in susceptible grasses, and in a minor extent (36 and 20%) in tolerant counterparts. In addition, roots of tolerant grasses were sensitive to quinclorac, but shoots were extremely tolerant, suggesting a tissue-specific response. This specific response was attributed to the existence of an additional tolerance mechanism or to a less sensitive cell wall synthesis in shoots than in roots. In any case, based on results from these two works, Koo and coauthors proposed quinclorac to be a cell-wall biosynthesis inhibitor more than an auxinic herbicide.

Nevertheless, Grossmann and Scheltrup (1997) kept on considering quinclorac as auxin herbicide, since they proved that the compound promoted a selective induction of ACC synthase, an enzyme that catalyzes the rate limiting reaction in ethylene biosynthesis. Although ethylene itself is not the agent that directly promotes plant death, the ethylene synthesis stimulation provokes the accumulation of cyanide at physiologically damaging concentrations (Grossmann, 1996, 2000). In addition, ethylene triggers biosynthesis of

abscisic acid, which reduces stomatal aperture and consequently, by the declining of photosynthetic activity, causes overproduction of H_2O_2 which contributes to the induction of cell death (Grossmann et al., 2001a). Subsequent work on barnyard grass and maize showed no influence of quinclorac treatment on cellulose biosynthesis (Tresch and Grossmann, 2003). However, long treatment promoted a decline of mixed-linked glucan, similar to that promoted by dichlobenil and also by potassium cyanide. Thus, the reduction of mixed-linked glucan deposition was interpreted as an indirect effect of quinclorac through the stimulated production of cyanide.

An extensive analysis of quinclorac action on cell walls was made on French bean cultured-cells habituated to grow in lethal concentrations of this herbicide (Alonso-Simón et al., 2008). No reduction of cellulose content was found in habituated cells, even at the highest quinclorac concentration used. In contrast, habituated cells showed a lower amount of pectins in their cell walls and extracellular material positively labelled by antibodies specific for highly methyl esterified pectins. Thus, the lower pectin content of cell wall could be due to a deficient de-esterification, which would partially prevent the correct integration and persistence of some pectins in the cell wall. Consequently, quinclorac was proved not to inhibit cell wall biosynthesis in these cells.

In a recent work by Sunohara and Matsumoto (2008), quinclorac effects were compared with those promoted by the synthetic auxin 2,4-D in maize roots. Both compounds stimulated the synthesis of ethylene and subsequent accumulation of cyanide, in a larger extent in 2,4-D. However, cell death rate induced by quinclorac was much higher. Thus, quinclorac toxicity was attributed to reactive oxygen species production induced by this herbicide. Finally, and considering that quinclorac caused similar symptoms to those promoted by cyanide, and that the capacity of metabolize cyanide seems to be different among plant species, the authors suggest that the difference in cyanide detoxification capacity of the plant is the factor which determines if cyanide or reactive oxygen species are primarily responsible of quinclorac herbicide action. Previously, same authors had demonstrated that the tolerance to quinclorac displayed by plant species as rice is related to an increased antioxidant capacity able to cope with quinclorac-induced oxidative injury (Sunohara and Matsumoto, 2004).

Ancymidol

Ancymidol is a plant growth retardant, that interferes with gibberellin biosynthesis by the inhibition of the enzyme ent-kaurene oxidase, thus reducing gibberellin content and further decreasing growth of ancymidol-treated plants (Shive and Sisler, 1976). Besides this retardant primary action, some effect on cell wall and its polysaccharides have been described. For instance, ancymidol made cells short and thick with galactose-rich cell walls in pea (Tanimoto, 1987), suppressed cell wall extensibility of dwarf pea (Tanimoto, 1994), and changed average molecular weight of cell wall pectins (Tanimoto and Huber, 1997). Nevertheless, these modifications were reversed by gibberellins treatments, and thus due to the inhibition of gibberellin synthesis promoted by ancymidol. However, a recent work described a cellulose biosynthesis inhibitor effect of this compound on tobacco BY-2 cells, not reverted by gibberellin addition (Hofmannová et al., 2008). Ancymidol application on cells resulted in malformations and cell death similar to those induced by dichlobenil and isoxaben. In addition, ancymidol disoriented microtubules and made the cellulose distribution

not continuous, provoking protoplasts to regenerate a sparse net of microfibrils, or not cellulose at all, when treated with ancymidol 10 and 100 μM, respectively. This effect was reversible by washing ancymidol from regenerating medium, but not by addition of gibberellin. The mechanism by which ancymidol inhibited cellulose synthesis remains unknown, but it was showed to be different to that of isoxaben, and may be related with the control of cell expansion (Hofmannová et al., 2008).

Coumarin and Derivatives

Other kinds of chemicals have been shown to inhibit cellulose biosynthesis. An important group is that formed by coumarin and its derivatives. Coumarin inhibited specifically [^{14}C]glucose incorporation in cellulose in cotton fibers, since no significant inhibition on [^{14}C]glucose incorporation in callose or noncellulosic glucans has been appreciated (Montezinos and Delmer, 1980). Coumarin was later observed to bind to tubulin and thus to suppress microtubule dynamics (Madari et al., 2003). The relationship between microtubules and cellulose have been repeatedly analyzed, since the orientation of glucan microfibrils and cortical microtubules were very similar or identical and the alignment hypothesis proposed that the orientation of deposited cellulose is associated with underlying cortical microtubules (Heath, 1974; Baskin, 2001). Paredez et al. (2006) visualized cellulose synthase complexes moving along tracks apparently defined by microtubules, confirming the functional relationship between these elements, after some controversy about this alignment hypothesis.

Therefore, compounds that affect microtubules synthesis or orientation should also affect cellulose deposition. In this sense, morlin (a coumarin derivative) was discovered in a screening for compounds that inhibited cellulose biosynthesis (DeBolt et al., 2007a). Morlin was shown to decrease cellulose synthesis in a dose-dependent manner, promoting a reduction in [^{14}C]glucose incorporation in cellulose and a parallel increase in callose biosynthesis. This compound also caused a change in cytoskeletal dynamics, diminishing the velocity of both microtubule polymerization and CESA complexes in plasma membranes. Nevertheless, the use of oryzalin (that completely depletes all detectable microtubules) did not affect CESA complex velocity, probing that the speed of cellulose synthases depends on polymerization of cellulose and not on microtubules, as have been previously reported (Robinson and Quader, 1981). Then, DeBolt and co-authors (2007a) propose three different hypotheses to explain morlin effects on both cellulose synthesis and microtubules dynamics and organization: i) morlin could have separated effect on both processes; ii) morlin might target a regulatory protein that coordinated cellulose synthesis and microtubules; and iii) morlin targeted a structural protein which interacted with both microtubules and CESA complexes. In any case, the study of morlin action and its target will be a useful tool to unravel the relation between cortical microtubules and cellulose synthesis machinery.

Later, a chemical genetic screening for compounds that affected the cortical microtubule-cellulose microfibrils was performed (Yoneda et al., 2007). When cortical microtubule organization or cellulose microfibril deposition is inhibited, plant cells lose their anisotropy and show swelling. Thus, compounds that caused spherical swelling phenotype (SS compounds) were analyzed. In addition to dichlobenil, two novel compounds that reduced cellulose deposition were identified: SS14 and SS18. SS14 presents the same substructure as morlin or coumarin, but without affecting the cortical microtubule orientation at all. SS18 is a

novel compound that might inhibit cellulose synthesis directly or indirectly by affecting substrate synthesis or transport (Yoneda et al., 2007).

Triaziflam

Some other compounds that affect cellulose biosynthesis as well as other cellular processes have been described, but little is known about their mode of action. One example is triaziflam, which also affects photosynthetic electron transport and microtubule formation (Grossmann et al., 2001b). The analysis of this kind of chemicals may be useful to deepen in cellulose synthesis mechanism and its relationships with many other cellular processes.

Oxaziclomefone

Oxaziclomefone is a herbicide that inhibits cell expansion in grass roots. However it did not affect [^{14}C]glucose incorporation into the main sugar residues of their cell walls, including cellulosic glucose, so that it is not considered properly as a CBI (O'Looney and Fry, 2005a, b).

CBI-RELATED MUTANTS

The selection of mutants with alterations in the composition and structure of the cell wall provides a tool to identify new genes involved in biosynthetic pathways, cell wall assembly or modifications of polymers in the cell wall. Traditionally, the identification of cell wall mutants, whether spontaneous or induced, was based on the search for (i) morphological changes such as root radial swelling (Baskin et al., 1992) or reduction in hypocotyl elongation (Nicol et al., 1998; Desnos et al., 1996), (ii) anatomical changes such as low birefringence of the trichomes (Potikha and Delmer, 1995) or collapsed xylem (Turner and Somerville, 1997), or (iii) resistance to CBIs as isoxaben (Heim et al., 1989, 1990) or thaxtomin A (Scheible et al., 2001).

This classic mutational analysis, from phenotypes to genes (direct genetics), presents two potential problems: often the mutations do not cause visibly abnormal phenotypes due to gene redundancy, and in other cases, the mutations are usually lethal when the affected genes are keys to the concerned process. Therefore, it has resorted to other choices as the analysis of the composition in neutral sugars by gas chromatography and mass spectroscopy (Reiter et al., 1997) or tracking of FTIR spectroscopy alterations in the cell wall (Chen et al., 1998; Mouille et al., 2003; Robert et al., 2004), or the use of reverse genetics. In the last decade a large number of mutants have been involved in the structure and/or synthesis of cell wall polysaccharides, including those affected in the process of cellulose biosynthesis. We will focus on mutants resistant to CBIs, mutants with changed sensitivity to CBIs, and those mutants whose phenotype is similar to CBI-treated plants (Table 2).

Mutants Resistant to CBIs

The screening of *Arabidopsis* mutants resistant to CBIs has allowed clarification, at least in part, of not only the mode of action of these compounds, but also the composition and organization of the cellulose biosynthesis "machinery" of primary cell walls.

Isoxaben resistant mutants (*ixr1-1* and *ixr1-2*) were the first resistant mutants identified for CBIs (Heim et al., 1989, 1990). Semidominant mutations at the *ixr1-1* and *ixr1-2* loci occur in a highly conserved region of the CESA3 near the carboxyl terminus (Scheible et al., 2001). Although the *Ixr1* gene is expressed in the same cells as the structurally related *rsw1* (*CesA1*) cellulose synthase gene, these two *CesA* genes are not functionally redundant (Scheible et al., 2001). The *ixr* mutations appear to directly affect the herbicide target, because resistant cell lines show no alterations in uptake or detoxification of the herbicide (Heim et al., 1991). This mutation confers resistance to the thiazolidinone compound 1, which suggests the same mode of action for both herbicides (Scheible et al., 2001).

Another locus was identified later in the isoxaben resistant mutant (*ixr2-1*) (Desprez et al., 2002). The *ixr2-1* carries a mutation substituting an amino acid close to the C terminus of the CESA6. Initially it was thought that the presence of these two isoxaben-resistant loci (*Ixr1* and *Ixr2*) could be due to CESA3 and CESA6 were redundant. However, mutants with lost function exclusively in CESA6, like *procuste1* (*prc1*, Fagard et al., 2000) did not restore the phenotype with the presence of CESA3. These observations suggested that CESA6 and CESA3 were part of the same protein complex and that the inhibitor directly or indirectly recognizes the place of partnership between the two subunits (see Figure 2).

Isoxaben-resistant mutants CESA3$^{ixr1\text{-}1,\ 1\text{-}2}$ and CESA6$^{ixr\ 2\text{-}1}$ along with other cell wall mutants, have contributed to demonstrate that the complex involved in the cellulose synthesis in primary cell walls contains three CESA catalytic subunits. Through immunolocalization analysis, co-immunoprecipitation and green fluorescent protein (GFP) gene fused expression, it was found that two positions of CESA complexes have being invariably occupied by CESA1 and CESA3, while in the third position at least three isoforms, CESA2, CESA5 and CESA9 may compete with CESA6 according to the tissue and/or the cell development stage (Robert et al., 2004; Desprez et al., 2007; Persson et al., 2007; Wang et al., 2008). Partial redundancy between CESA2, CESA5, CESA6 and CESA9 could explain the lower isoxaben resistance showed by CESA6^{ixr2} mutants compared withCESA3^{ixr1} (Desprez et al., 2007).

Other CBI mutants appear to have different mechanisms that could be involved in the uptake and/or detoxification of the inhibitor rather than in altered target sites. Such is the case of *txr1*, an *Arabidopsis* thaxtomin A-resistant mutant, which presents a decrease in the rate of inhibitor uptake. The mutated gene has been cloned and encodes a highly conserved and constitutively expressed protein in eukaryotic organisms. This gene could be involved in the regulation of a transport mechanism (Scheible et al., 2003).

By using MES mutagenesis, a putative *Arabidopsis* mutant resistant to dichlobenil has been reported (Heim et al., 1998). This mutant (DH75) was four times more resistant to dichlobenil than the wild type and did not show cross-tolerance to isoxaben or triazofenamide. Although DH75 resistance-mechanism remains unravelled, an alteration in herbicide metabolism has been pointed as its putative cause (Sabba and Vaughn, 1999).

Mutants with Changed Sensitivity to CBIs

At the moment there are no mutants known to be resistant to other CBIs. However, different mutants have been identified with changes in sensitivity to CBIs. A mutant of *Arabidopsis* called *css1* (changed sensitivity to cellulose synthesis inhibitors) grows at rates lower than control but is less sensitive to isoxaben and dichlobenil (Nakagawa and Sakurai, 2006). The *css1* mutation seems to affect a mitochondrial protein (At-nMat1a). Phenotypic analysis of this mutant during the early developmental stages shows changes in several metabolic processes such as amino acid synthesis, triacylglycerides degradation, and in polysaccharides synthesis such as cellulose or starch.

On the other hand it has been observed that plants with loss-of-function mutations in genes required for cell wall synthesis (*CesA2, Cobra, Korrigan* and *CesA6*; see Somerville, 2006) were hypersensitive to CBIs like isoxaben and dichlobenil (DeBolt et al., 2007b).

Some Mutants Show Similar Phenotypes to CBI-Treated Plants

As has been pointed out, plants treated with CBIs as isoxaben, dichlobenil or thaxtomin A showed a decrease in stems growth, and root swelling. When cellulose synthesis is affected, cells expand radially and accumulate callose, lignin and other phenolic compounds (Desprez et al., 2002, Bischoff et al., 2009). This phenotype is shared by many mutants with defects in hormones synthesis or signaling pathways, in mutants with alterations in endocytosis processes (Collings et al., 2008) and with reduced cellulose amount (Lukowitz et al., 2001). Moreover, the application of isoxaben or dichlobenil in control plants results in incomplete cell walls formation (Desprez et al., 2002), an effect also observed in mutants deficient in cellulose (Arioli et al., 1998; Fagard et al., 2000; Lane et al., 2001). Etiolated *Arabidopsis* seedlings treated with nanomolar concentrations of thaxtomin A display a FTIR spectral phenotype that is most related to those of *Arabidopsis* CESA1^{rsw1} mutant seedlings, or *Arabidopsis* seedlings treated with CBIs like dichlobenil, isoxaben or flupoxam (Scheible et al., 2003; Robert et al., 2004).

Some mutants in catalytic subunits of CESA complex have been isolated and identified on the basis of this phenotype (Table 2), like a set of *radial swelling 1* (*rsw1*) mutants. The *rsw1* have a mutation in the *CesA1* gene, which results in a reduction in cellulose amount, accumulation of non-crystalline β-(1,4)-glucan and radial expansion when plants grow at a restrictive temperature of 31°C (Baskin et al., 1992; Arioli et al., 1998; Sugimoto et al., 2001). Recent evidences suggest that subunits aggregation in the CESA complex changes in extracts depending on the temperature at which CESA1^{rsw1} mutants grow. At a restrictive temperature CESA proteins remain isolated, while at a permissive temperature complexes are formed properly and co-precipitated in a complex of 840 kDa (Wang et al., 2008).

Treatment with isoxaben and other CBIs causes ectopic lignification in roots. A set of mutants also accumulates ectopic lignin. In a selection program to detect mutants of this kind, *eli1* was identified. The *eli1* has a mutation that affects a highly conserved domain of *CesA3* gene (CesA3$^{eli1\text{-}1,\ eli1\text{-}2}$) (Caño-Delgado et al., 2000). Analysis of [^{14}C]glucose incorporation into cellulose in different mutants showed that both *eli1-1* and *eli1-2* have a half reduction in the incorporation of glucose into the acid insoluble fraction of their cell walls (Caño-Delgado

et al., 2003). The low level of cellulose in *eli1* is consistent with the cell shape and growth reduction in other mutants as CesA1^{rsw1} at the restrictive temperature, and other mutants that have affected CESA in primary cell wall as *procuste* (CesA6^{prc}) and *korrigan* (*kor*, endo–β-(1,4)-glucanase). CesA3$^{ixr1\text{-}1}$ mutants do not show reduction in cellulose or ectopic lignification after treatment with isoxaben, while control plants showed not only reduction in the synthesis of cellulose, but also showed ectopic lignification in all plant organs, even in the most apical cells of the root that are normally actively dividing (Caño-Delgado et al., 2003). Many other mutants that show a reduction in cellulose amount present an ectopic lignification (Table 2).

To date, no mutants with defects in cellulose synthesis in type II primary cell walls have been reported. However, the *elo* mutants are a class of barley dwarfs initially described as being impaired in cell expansion (Chandler and Robertson, 1999), that have characteristic features similar to cellulose synthesis type I mutants: all *elo* mutants show radial swelling of leaf epidermal and root cortical cells, and lower levels of cellulose in their leaves compared with the wild-type (Lewis et al., 2009). *Elo* genes have not been identified, but the study of these mutants could be an excellent tool to understand the cell expansion in plants with type II cell walls.

CBIs AS TOOLS TO RESEARCH THE STRUCTURAL PLASTICITY OF CELL WALLS

The ability of plant cells to tolerate induced stresses by modifying their cell wall composition and structure has been demonstrated in several works (Iraki et al., 1989; Shedletzky et al., 1992; Encina et al., 2001, 2002; Mélida et al., 2009). By this meaning, CBIs are valuable tools for the analysis of cell wall structure and biogenesis. These herbicides allow analysis of the connections between the partially independent networks which make up the primary cell wall, and the high plasticity of this structure to accommodate to unfavourable conditions.

Habituation to CBIs

Although CBIs are highly specific and potent herbicides, cell cultures of several species have been habituated to grow in the presence of CBIs by incremental exposure over many culturing cycles. The cell culture utilization is very advantageous with respect to the whole plant for two reasons: (i) the possibility to have lots of cells in a reduced place at the same time, where it is easy to control and manipulate different conditions, and (ii) the availability to select cell lines with specific features. The habituation of cell cultures to CBIs reflects the ability of cells to survive with a modified cell wall and is therefore a valuable experimental technique for gaining an insight into the plasticity of plant cell wall composition and structure. Several cell cultures have been successfully habituated to CBIs such as dichlobenil, isoxaben, quinclorac and thaxtomin A. Habituated cultures usually display some common features: slower growing rates, irregularly shaped cells, a trend to grow in clumps when are suspension cultured, and cell walls with reduced cellulose contents compensated with other cell wall components.

Habituation to Dichlobenil

Two types of primary cell walls having different structure and composition exist in higher plants: type I cell walls are found in dicots, gymnosperms and most monocots, and type II walls are found in graminaceous plants, along with the other commelinoid monocots (Carpita and Gibeaut, 1993; Carpita, 1996). Although the basic mechanism of habituation is common (a replacement of the cellulose network for other cell wall components), the details of the process depend on the type of cell wall, and therefore it has been demonstrated that cells habituate to dichlobenil by using different strategies. Most dichlobenil-habituated cultures belong to type I cell wall species, such as tomato (Shedletzky et al., 1990), tobacco (Shedletzky et al., 1992; Wells et al., 1994; Nakagawa and Sakurai, 1998, 2001; Sabba et al., 1999) and bean (Encina et al., 2001, 2002; Alonso-Simón et al., 2004; García-Angulo et al., 2006). In dichlobenil-habituated type I cell walls there is a marked decrease in the amount of cellulose and hemicelluloses, whereas the quantity of esterified and unesterified pectins is increased. Moreover, in dichlobenil-habituated BY-2 tobacco cells, pectins have been reported to be cross-linked with extensin to form the main cell wall network (Sabba et al., 1999). There are other modifications associated with dichlobenil habituation, such as the presence of a non-crystalline β-(1,4)-glucan tightly bound to cellulose, the accumulation of pectin-enriched cell wall appositions, a putative increase in the extent of pectin–xyloglucan cross-linking and in xyloglucan endotransglucosylase activity, reduced levels of arabinogalactan proteins and changes in the levels of extensin (Shedletzky et al., 1992; Encina et al., 2002; García-Angulo et al., 2006, Alonso-Simón et al., 2007), and modifications in xyloglucan composition (Alonso-Simón, unpubl.).

To date, barley (Shedletzky et al., 1992) and maize (Mélida et al., 2009) are the only type II cell wall species reported to have been habituated to dichlobenil. These dichlobenil-habituated type II cell walls also displayed a modified architecture: they contained a considerably reduced level of cellulose in the cell wall, effectively compensated for mechanisms (parallel to modifications) quite different to those observed in dicots, and slightly different between both species. Whereas barley cultures habituation implicated a higher proportion of β-glucan and a more extensive cross-linking between arabinoxylans, leading to walls with a reduction in pore size, maize habituated cultures had a more extensive and phenolic cross-linked network of arabinoxylans, without necessitating β-glucan or other polymer enhancement. As a consequence of this modified architecture, walls from dichlobenil-habituated maize cells showed a reduction in their swelling capacity and an increase both in pore size and in resistance to polysaccharide hydrolytic enzymes. From a molecular perspective the application of dichlobenil to maize cell cultures disrupts the “cellulose biosynthesis machinery”, affecting to some CESA subunits, but during the habituation, maize cells partially restore this system overexpressing some of these genes (Mélida, unpubl.). These cultures have also altered the expression of genes involved in the synthesis of phenolic compounds, which is in concordance with the increased arabinoxylans feruloylation observed. The habituation represses a class of proteins usually acting in detoxification of xenobiotics (glutation-S-transferases) and induces other involved in stress and programmed cell death processes (Mélida, unpubl.).

Habituation to Isoxaben

Isoxaben-habituated cultures seem to have a more heterogeneous habituation mechanism than dichlobenil ones. Former isoxaben-habituated cultures had the same cellulose-xyloglucan proportion than non-habituated ones, and the habituation seemed to be more related to changes in the herbicide target or in the detoxification than in wall modifications (Corio-Costet et al., 1991a). However later results obtained with French bean (Díaz-Cacho et al., 1999), tobacco (Sabba and Vaughn, 1999) and *Arabidopsis* cell cultures (Manfield et al., 2004) showed cell wall changes similar to those described for dichlobenil-habituated cultures. At least in *Arabidopsis*, isoxaben-habituation does not appear to be mediated by stress response processes, nor by functional redundancy within the CESA family (Manfield et al., 2004). Uniquely, amongst the cellulose synthase superfamily, *CslD5* was highly upregulated and might play a role in the biosynthesis of the novel walls of habituated cells (Bernal et al., 2007).

Habituation to Other CBIs

French bean cells have been successfully habituated to grow in the presence of lethal concentrations of quinclorac (Alonso-Simón et al., 2008), such as it was formerly noted. Compared with non-habituated, quinclorac-habituated cells showed irregular shape and accumulated an extracellular material that was more abundant as the level of habituation increased. Cellulose content was not significantly affected by habituation. In contrast, the distribution and post-depositional modifications of pectins (mainly homogalacturonan and rhamnogalacturonan I) was affected by the habituation process. These results reflect that habituation to quinclorac is not related to cellulose biosynthesis processes.

Habituation to thaxtomin A has also been described in poplar cells (Girard-Martel et al., 2008) and the thaxtomin A-habituated cell walls had less cellulose and were enriched in pectins. The whole genome transcript profiling analysis identified genes involved in many processes, including several genes implicated in glucan and pectin biosynthesis, transcriptional regulation, secondary metabolism and plant defence.

Dehabituation

Most of the cell wall changes induced during the habituation to dichlobenil reverted when cells were dehabituated by culturing them in a medium without the inhibitor (Shedletzky et al., 1990; Encina et al., 2002; García-Angulo et al., 2006). However, dehabituated cell cultures retained some habituation-induced cell wall modifications like reduced levels of arabinogalactan proteins and hydroxyproline-rich glycoproteins epitopes, altered extractability of pectins, and the presence of an amorphous β-(1,4)-glucan (Encina et al., 2002; García-Angulo et al., 2006). Most remarkably, apart from stable changes in cell wall composition and structure, dehabituated cells retained the capacity to cope with lethal concentrations of dichlobenil, as dehabituated cells were 40 times more tolerant to dichlobenil than non-tolerant cells (Encina et al., 2002). In an attempt to explain the dichlobenil

resistance of dehabituated cells it was found that these cells had a constitutively increased peroxidase activity indicating a relationship between habituation to dichlobenil and a high antioxidant capacity (García-Angulo et al., 2009).

NEW PERSPECTIVES IN CBI USES

CBIs represent a promising field of experimentation in order to obtain novel herbicides. Active compounds geared towards cell walls are interesting molecules to be commercialized as herbicides, taking into account their assumed lack of toxicity for non-cellulosic organisms. As it has been shown in this chapter, several putative targets for CBIs have been recently hypothesized as a consequence of new data that have been obtained, and more intense research in this field can be predicted.

However, we are a long way from unraveling the exact mechanism of action of most of these CBIs. As the cellulose biosynthesis process is better understood, targets for different CBIs will be ascertained. Reciprocally, the use of CBIs will have an important impact on cellulose biosynthesis studies.

Nowadays, there is growing interest in obtaining cell walls with modified structures directed to change the quality and/or quantity of their components for applications in fields such as food, feed, fibres or fuel. Some of these applications are oriented toward producing dietary fibres with improved properties and other significant polysaccharides in food processing such as pectins (Willats et al., 2006), feed products with better digestibility (Vogel and Jung, 2001), natural fibres destined for the textile and paper industries (Obembe et al., 2006), or lignocellulosic materials more suitable for biofuels (Pauly and Keegstra, 2008).

Several approaches have been taken in order to obtain these modified cell walls, such as the search for mutants affected in genes related to polysaccharide biosynthesis, the manipulation of genes implied in the modification of cell wall composition (Farrokhi et al., 2006), or the use of enzymes and other proteins able to act on cell wall components (Levy et al., 2002). A promising alternative to these approaches consists of the use of CBIs. In fact, the habituation of cell cultures to diverse CBIs, as it has been mentioned, results in the modification of cell wall composition and structure with quantitative and qualitative changes in their components: CBI-habituated cell walls often have a reduced content in cellulose, compensated by an increment in other polysaccharides, mainly pectins. These cell walls have been demonstrated to have new physicochemical properties such as modifications in pore size, swelling capacity and resistance to polysaccharide hydrolytic enzymes (Shedletzky et al., 1992; Mélida et al., 2009). These materials should be also interesting as a suitable source for studying the relationship between cellulose synthesis and other C-sink processes such as phenylpropanoid synthesis (Mélida et al., 2009), or the elucidation of putative new targets implied in cellulose biosynthesis.

As it has been repeatedly noted in this chapter, CBIs have contributed to the clarification of the mechanism of cellulose biosynthesis and the organization of CESA complexes. In addition to these contributions, in recent years CBIs have been revealed as excellent tools for digging more deeply into the basic processes of plant cell biology, such as the relationship between cellulose biosynthesis and cytoskeleton (Himmelspach et al., 2003; DeBolt, 2007b; Paredez et al., 2008; Wightman et al., 2009), the mechanism of cell wall sensing (Hamman et

al., 2009), cell-wall based mechanisms of defence (Caño-Delgado et al., 2003; Bischoff et al., 2009), and the link between cell wall disruption and programmed cell death (Duval, 2005; Bischoff et al., 2009).

CONCLUSION

CBIs are important compounds not only as commercialized herbicides (or molecules with herbicide potential) but also as key tools to unravel the cellulose biosynthesis mechanism. In recent years, a considerable amount of information regarding CBIs has been acquired, and it has been directed as a tool towards new research targets. In the future, it is probable that new CBIs will be discovered, and new research on old and new compounds will open new paths toward the comprehension of cell wall dynamics. However, a long road must be covered to find the mechanism of action of every member of this group of molecules. Surely, new advances in CBIs' characterization will render a better understanding of molecular relations between cellulose and other polysaccharides, such as their interactions, synthesis and potential modifications, and this knowledge would be oriented toward a better understanding of the plasticity in the structure and composition of cell walls.

REFERENCES

Alonso-Simón, A; Encina, AE; García-Angulo, P; Álvarez, JM; Acebes, JL. FTIR spectroscopy monitoring of cell wall modifications during the habituation of bean (*Phaseolus vulgaris* L.) callus cultures to dichlobenil. *Plant Sci,* 2004, 167, 1273-1281.

Alonso-Simón, A; García-Angulo, P; Encina, A; Acebes, JL; Álvarez, J. Habituation of bean (*Phaseolus vulgaris*) cell cultures to quinclorac and analysis of the subsequent cell wall modifications. *Ann Bot,* 2008, 101, 1329-1339.

Alonso-Simón, A; García-Angulo, P; Encina, A; Álvarez, JM; Acebes, JL; Hayashi, T. Increase in XET activity in bean (*Phaseolus vulgaris* L.) cells habituated to dichlobenil. *Planta*, 2007, 226, 765-771.

Amor, Y; Haigler, CH; Johnson, S; Wainscott, M; Delmer, DP. A membrane-associated form of sucrose synthase and its potential role in synthesis of cellulose and callose in plants. *Proc Natl Acad Sci USA,* 1995, 92, 9353-9357.

Anderson, JR; Barnes, WS; Bedinger, P. 2,6-Dichlorobenzonitrile, a cellulose biosynthesis inhibitor, affects morphology and structural integrity of petunia and lily pollen tubes. *J Plant Physiol,* 2002, 159, 61-67.

Arad, SM; Kolani, R; Simonberkovitch, B; Sivan, A. Inhibition by DCB of cell wall polysaccharide formation in the red microalga *Porphyridium sp.* (Rhodophyta). *Phycologia,* 1994, 33, 158-162.

Arioli, T; Peng, L; Betzner, AS; Burn, J; Wittke, W; Herth, W; Camilleri, C; Plazinski, J; Birch, R; Cork, A; Glover, J; Redmon, J; Williamson, RE. Molecular analysis of cellulose biosynthesis in *Arabidopsis*. *Science,* 1998, 279, 717-719.

Atanassov, II; Pittman, JK; Turner, SR. Elucidating the mechanisms of assembly and subunit interaction of the cellulose synthase complex of *Arabidopsis* secondary cell walls. *J Biol Chem,* 2009, 284, 3833-3841.

Baskin, TI. On the alignment of cellulose microfibrils by cortical microtubules: a review and a model. *Protoplasma*, 2001, 215, 150-171.

Baskin, TI; Betzner, AS; Hoggart, R; Cork, A; Williamson, RE. Root morphology mutants in *Arabidopsis thaliana*. *Aust J Plant Physiol,* 1992, 19, 427-437.

Beeckman, T; Przemeck, GKH; Stamatiou, G; Lau, R; Terryn, N; De Rycke, R; Inzé, D; Berleth, T. Genetic complexity of cellulose synthase A gene function in Arabidopsis embryogenesis. *Plant Physiol*, 2002, 130, 1883-1893.

Bernal, AJ; Jensen, JK; Harholt, J; Sorensen, S; Moller, I; Blaukopf, C; Johansen, B; Lotto, R; Pauly, M; Scheller, HV; Willats, WGT. Disruption of *ATCSLD5* results in reduced growth, reduced xylan and homogalacturonan synthase activity and altered xylan occurrence in Arabidopsis. *Plant J,* 2007, 52, 1-12.

Bessueille, L; Bulone, V. A survey of cellulose biosynthesis in higher plants. *Plant Biotechnol,* 2008, 25, 315-322.

Beynon, KI. & Wright, AN. Persistence, penetration, and breakdown of chlorthiamid and dichlobenil herbicides in field soils of different types. *J Sci Food Agric,* 1968, 19, 718-722.

Bischoff, V; Cookson, SJ; Wu, S; Scheible, W. Thaxtomin A affects CESA-complex density, expression of cell wall genes, cell wall composition, and causes ectopic lignification in *Arabidopsis thaliana* seedlings. *J Exp Bot,* 2009, 60, 955-965.

Blaschek, W; Semler, U; Franz, G. The influence of potential inhibitors on the invivo and invitro cell-wall beta-glucan biosynthesis in tobacco cells. *J Plant Physiol,* 1985, 120, 457-470.

Brummell, DA. & Hall, JL. The role of cell wall synthesis in sustained auxin-induced growth. *Physiol Plant,* 1985, 63, 406-412.

Burn, JE; Hurley, UA; Birch, RJ; Arioli, T; Cork, A; Williamson, RE. The cellulose-deficient Arabidopsis mutant rsw3 is defective in a gene encoding a putative glucosidase II, an enzyme processing N-glycans during ER quality control. *Plant J*, 2002, 32, 949-960.

Burton, RA; Gibeaut, DM; Bacic, A; Findlay, K; Roberts, K; Hamilton, A; Baulcombe, DC; Fincher, GB. Virus-induced silencing of a plant cellulose synthase gene. *Plant Cell,* 2000, 12, 691-705.

Caño-Delgado, A; Metzlaff, K; Bevan, MW. The *eli1* mutation reveals a link between cell expansion and secondary cell wall formation in *Arabidopsis thaliana*. *Development,* 2000, 127, 3395-3405.

Caño-Delgado, A; Penfield, S; Smith, C; Catley, M; Bevan, M. Reduced cellulose synthesis invokes lignification and defense responses in *Arabidopsis thaliana*. *Plant J,* 2003, 34, 351-362.

Carpita, NC. Structure and biogenesis of the cell walls of grasses. *Plant Physiol,* 1996 47, 445-476.

Carpita, NC. & Gilbeaut, DM. Structural models of primary cell walls in flowering plants: consistency of molecular structure with the physical properties of the cell walls during growth. *Plant J,* 1993, 3, 1-30.

Chandler, PM. & Robertson, M. Gibberellin dose-response curves and the characterization of dwarf mutants of barley. *Plant Physiol,* 1999, 120, 623-632.

Chen, L; Carpita, NC; Reiter, WD; Wilson, RH; Jeffries, C; McCann, MC. A rapid method to screen for cell-wall mutants using discriminant analysis of Fourier transform infrared spectra. *Plant J,* 1998, 16, 385-392.

Collings, DA; Gebbie, LK; Howles, PA; Hurley, UA; Birch, RJ; Cork, AH; Hocart, CH; Arioli, T; Williamson, RE. Arabidopsis dynamin-like protein DRP1A: a null mutant with widespread defects in endocytosis, cellulose synthesis, cytokinesis, and cell expansion. *J Exp Bot,* 2008, 59, 361-376.

Corio-Costet, MF; Dall'Agnese, M; Scalla, R (a). Effects of isoxaben on sensitive and tolerant plant cell cultures. I. Metabolic fate of Isoxaben. *Pestic Biochem Physiol,* 1991, 40, 246-254.

Corio-Costet, MF; Dall'Agnese, M; Scalla, R (b). Effects of isoxaben on sensitive and tolerant plant cell cultures. II. Cellular alterations and inhibition of the synthesis of acid insoluble cell wall material. *Pestic Biochem Physiol,* 1991, 40, 255-265.

Crowell, EF; Bischoff, V; Desprez, T; Rolland, A; Stierhof, Y; Schumacher, K; Gonneau, M; Höfte, H; Vernhettes, S. Pausing of golgi bodies on microtubules regulates secretion of cellulose synthase complexes in *Arabidopsis*. *Plant Cell,* 2009, 21, 1141-1154.

DeBolt, S; Gutiérrez, R; Ehrhardt, DW; Melo, CV; Ross, L; Cutler, SR; Somerville, C; Bonetta, D (a). Morlin, an inhibitor of cortical microtubule dynamics and cellulose synthase movement. *Proc Natl Acad Sci USA,* 2007, 104, 5854-5859.

DeBolt, S; Gutiérrez, R; Ehrhardt, DW; Somerville, C (b). Nonmotile cellulose synthase subunits repeatedly accumulate within localized regions at the plasma membrane in Arabidopsis hypocotyl cells following 2,6-dichlorobenzonitrile treatment. *Plant Physiol,* 2007, 145, 334-338.

Delmer, DP. Cellulose biosynthesis. *Annu Rev Plant Physiol,* 1987, 38, 259-290.

Delmer, DP; Read, SM; Cooper, G. Identification of a receptor protein in cotton fibers for the herbicide 2,6-dichlorobenzonitrile. *Plant Physiol,* 1987, 84, 415-420.

Desnos, T; Orbovic, V; Bellini, C; Kronenberger, J; Caboche, M; Traas, J; Höfte, H. *Procuste1* mutants identify two distinct genetic pathways controlling hypocotyl cell elongation, respectively in dark- and light-grown *Arabidopsis* seedlings. *Development,* 1996, 122, 683-693.

Desprez, T; Juraniec, M; Crowell, EF; Jouy, H; Pochylova, Z; Parcy, F; Höfte, H; Gonneau, M; Vernhettes, S. Organization of cellulose synthase complexes involved in primary cell wall synthesis in *Arabidopsis thaliana*. *Proc Natl Acad Sci USA,* 2007, 104, 15572-15577.

Desprez, T; Vernhettes, S; Fagard, M; Refrégier, G; Desnos, T; Aletti, E; Py, N; Pelletier, S; Höfte, H. Resistance against herbicide isoxaben and cellulose deficiency caused by distinct mutations in same cellulose synthase isoform CESA6. *Plant Physiol,* 2002, 128, 482-490.

Díaz-Cacho, P; Moral, R; Encina, A; Acebes, JL; Álvarez, J. Cell wall modifications in bean (*Phaseolus vulgaris*) callus cultures tolerant to isoxaben. *Physiol Plant,* 1999, 107, 54-59.

Doblin, MS; Kurek, I; Jacob-Wilk, D; Delmer, DP. Cellulose biosynthesis in plants: from genes to rosettes. *Plant Cell Physiol,* 2002, 43, 1407-1420.

Durso, NA. & Vaughn, KC. The herbicidal manipulation of callose levels in cell plates radically affects cell plate structure. *Plant Physiol,* 1997, 114, 87(c 351).

Duval, I. & Beaudoin, N. Transcriptional profiling in response to inhibition of cellulose synthesis by thaxtomin A and isoxaben in *Arabidopsis thaliana* suspension cells. *Plant Cell Rep,* 2009, 28, 811-830.

Duval, I; Brochu, V; Simard, M; Beaulieu, C; Beaudoin, N. Thaxtomin A induces programmed cell death in *Arabidopsis thaliana* suspension-cultured cells. *Planta,* 2005, 222, 820-831.

Edelmann, HG. & Fry, SC. Kinetics of integration of xyloglucan into the walls of suspension-cultured rose cells. *J Exp Bot,* 1992, 43, 463-470.

Eisinger, W; Croner, LJ; Taiz, L. Ethylene- induced lateral expansion in etiolated pea stems. Kinetics, cell wall synthesis, and osmotic potential. *Plant Physiol,* 1983 73, 407-412.

Ellis, C; Karafyllidis, I; Wasternack, C; Turner, JG. The Arabidopsis mutant *cev1* links cell wall signaling to jasmonate and ethylene responses. *Plant Cell*, 2002, 14, 1557–1566.

Encina, A; Moral, RM; Acebes, JL; Álvarez, JM. Characterization of cell walls in bean (*Phaseolus vulgaris* L.) callus cultures tolerant to dichlobenil. *Plant Sci,* 2001, 160, 331-339.

Encina, A; Sevillano, JM; Acebes, JL; Álvarez, J. Cell wall modifications of bean (*Phaseolus vulgaris*) cell suspensions during habituation and dehabituation to dichlobenil. *Physiol Plant,* 2002, 114, 182-191.

Fagard, M; Höfte, H; Vernhettes, S. Cell wall mutants. *Plant Physiol Biochem,* 2000, 38, 15-25.

Farrokhi, N; Burton, R; Brownfield, L; Hrmova, M; Wilson, SM; Bacic, A; Fincher, GB. Plant cell wall biosynthesis: genetic, biochemical and functional genomic approaches to the identification of key genes. *Plant Biotech J*, 2006, 4, 145-167.

Francey, Y; Jaquet, JP; Cairoli, S; Buchala, AJ; Meier, H. The biosynthesis of β-glucans in cotton (*Gossypium hirsutum* L.) fibres of ovules cultured in vitro. *J Plant Physiol,* 1989, 134, 485-491.

Fry, BA. & Loria, R. Thaxtomin A: evidence for a plant cell wall target. *Physiol Mol Plant Pathol,* 2002, 60, 1-8.

Galbraith, DW. & Shields, BA. The effects of inhibitors of cell wall synthesis on tobacco protoplast development. *Physiol Plant,* 1982, 55, 25-30.

García-Angulo, P; Alonso-Simón, A; Mélida, H; Encina, A; Acebes, JL; Álvarez, JM. High peroxidase activity and stable changes in the cell wall are related to dichlobenil tolerance. *J Plant Physiol,* 2009, 166, 1229-1240.

García-Angulo, P; Willats, WGT; Encina, AE; Alonso-Simón, A; Álvarez, JM; Acebes, JL. Immunocytochemical characterization of the cell walls of bean cell suspensions during habituation and dehabituation to dichlobenil. *Physiol Plant,* 2006, 127, 87-99.

Gillmor, CS; Poindexter, P; Lorieau, J; Palcic, MM; Somerville, C. α-Glucosidase I is required for cellulose biosynthesis and morphogenesis in *Arabidopsis. J Cell Biol*, 2002, 156, 1003-1013.

Girard-Martel, M; Brochu, V; Duval, I; Beaudoin, N**.** Characterization and transcription profiles of poplar cells habituated to *Streptomyces scabiei* phytotoxin thaxtomin A. 6th Canadian Plant Genomics Workshop, Toronto 2008, 74.

Gopalan, S. Reversal of an immunity associated plant cell death program by the growth regulator auxin. *BMC Res Notes,* 2008, 1, 126.

Grossmann, K. A role for cyanide, derived from ethylene biosynthesis, in the development of stress symptoms. *Physiol Plant,* 1996, 97, 772-775.

Grossmann, K. Mode of action of auxin herbicides: a new ending to a long, drawn out story. *Trends Plant Sci,* 2000, 5, 506-508.

Grossmann, K. & Kwiatkowski, J. Evidence for a causative role of cyanide, derived from ethylene biosynthesis, in the herbicidal mode of action of quinclorac in barnyard grass. *Pestic Biochem Physiol,* 1995, 51, 150-160.

Grossmann, K. & Kwiatkowski, J. The mechanism of quinclorac selectivity in grasses. *Pestic Biochem Physiol,* 2000, 66, 83-91.

Grossmann, K; Kwiatkowski, J; Tresch, S (a). Auxin herbicides induce H_2O_2 overproduction and tissue damage in cleavers (*Galium aparine* L.). *J Exp Bot,* 2001, 52, 1811-1816.

Grossmann, K. & Scheltrup, F. Selective induction of 1-aminocyclopropane-1-carboxylic acid (ACC) synthase activity is involved in the selectivity of the auxin herbicide quinclorac between barnyard grass and rice. *Pestic Biochem Physiol,* 1997, 58, 145-153.

Grossmann, K; Tresch, S; Plath, P (b). Triaziflam and diaminotriazine derivatives affect enantioselectively multiple herbicide target sites. *Z Naturforsch [C],* 2001, 56, 559-569.

Günther, P. & Pestemer, W. Risk assessment for selected xenobiotics by bioassay methods with higher plants. *Environ Manag,* 1990, 14, 381-388.

Hamman, T; Bennett, M; Mansfield, J; Somerville, C. Identification of cell-wall stress as a hexose-dependent and osmosensitive regulator of plant responses. *Plant J*, 2009, 57, 1015-1026.

Hauser, MT; Morikami, A; Benfey, PN. Conditional root expansion mutants of *Arabidopsis*. *Development* 121, 1995, 1237-1252.

Heath, IB. Unified hypothesis for role of membrane-bound enzyme complexes and microtubules in plant cell wall synthesis. *J Theor Biol,* 1974, 48, 445-449.

Heim, DR; Larrinua, IM; Murdoch, MG; Roberts, JL. Triazofenamide is a cellulose biosynthesis inhibitor. *Pestic Biochem Physiol,* 1998, 59, 163-168.

Heim, DR; Roberts, JL; Phillip, DR; Larrinua, IM. A second locus, *Ixr* B1 in *Arabidopsis thaliana* that confers resistance to the herbicide Isoxaben. *Plant Physiol,* 1990, 92, 695-700.

Heim, DR; Roberts, JL; Pike, PD; Larrinua, IM. Mutation of a locus of *Arabidopsis thaliana* confers resistance to the herbicide Isoxaben. *Plant Physiol,* 1989, 90, 146-150.

Heim, RD; Skomp, JR; Waldron, C; Larrinua, IM. Differential response to isoxaben of cellulose biosynthesis by wild-type and resistant strains of *Arabidopsis thaliana*. *Pestic Biochem Physiol,* 1991, 39, 93-99.

Herth, W. Effects of 2,6-DCB on plasma-membrane rosettes of wheat root cells. *Naturwiss,* 1987, 74, 556-557.

Himmelspach, R; Williamson, RE; Wasteneys, GO. Cellulose microfibril alignment recovers from DCB-induced disruption despite microtubule disorganization. *Plant J,* 2003, 36, 565-575.

Hoffman, JC. & Vaughn, KC. Flupoxam induces classic club root morphology but is not a mitotic disrupter herbicide. *Pestic Biochem Physiol,* 1996, 55, 49-53.

Hofmannová, J; Schwarzerová, K; Havelková, L; Boriková, P; Petrásek, J; Opatrny, Z. A novel, cellulose synthesis inhibitory action of ancymidol impairs plant cell expansion. *J Exp Bot,* 2008, 59, 3963-3974.

Hogetsu, T; Shibaoka, H; Shimokoriyama, M. Involvement of cellulose biosynthesis in actions of gibberellin and kinetin on cell expansion, 2,6-dichlorobenzonitrile as a new cellulose-synthesis inhibitor. *Plant Cell Physiol,* 1974, 15, 389-393.

Hoson, T. & Masuda, Y. Role of polysaccharide synthesis in elongation growth and cell wall loosening in intact rice coleoptiles. *Planta,* 1991 155, 467-472.

Huggenberger, F; Jennings, EA; Ryan, PJ; Burow, KW. EL-107 a new selective herbicide for use in cereals. *Weeds,* 1982, 1, 47-52.

Iraki, NM; Bressan, RA; Hasegawa, PM; Carpita, NC. Alteration of the physical and chemical structure of the primary cell wall of growth-limited plant cells adapted to osmotic stress. *Plant Physiol,* 1989, 91, 39-47.

Joshi, CP. & Mansfield, SD. The cellulose paradox -- simple molecule, complex biosynthesis. *Curr Opin Plant Biol,* 2007, 10, 220-226.

Kiedaisch, BM; Blanton, RL; Haigler, CH. Characterization of a novel cellulose synthesis inhibitor. *Planta,* 2003, 217, 922-930.

King, RR. & Lawrence, CH. Herbicidal properties of the Thaxtomin group of phytotoxins. *J Agric Food Chem,* 2001, 49, 2298-2301.

King, RR; Lawrence, CH; Calhoun, LA. Chemistry of phytotoxins associated with *Streptomyces scabies*, the causal organism of potato common scab. *J Agric Food Chem,* 1992, 40, 834-837.

Koo, SJ; Kwon, YW; Cho, KY. Differences in selectivity and physiological effects of quinclorac between rice and barnyardgrass compared with 2,4-D. *Proc 13th Asian-P WSSC,* 1991, 103-111.

Koo, SJ; Neal, JC; DiTomaso, JM. 3,7-dichloroquinolinecarboxylic acid inhibits cell-wall biosynthesis in maize roots. *Plant Physiol,* 1996, 112, 1383-1389.

Koo, SJ; Neal, JC; DiTomaso, JM. Mechanism of action and selectivity of quinclorac in grass roots. *Pestic Biochem Physiol,* 1997, 57, 44-53.

Kurek, I; Kawagoe, Y; Jacob-Wilk, D; Doblin, M; Delmer, D. Dimerization of cotton fiber cellulose synthase catalytic subunits occurs via oxidation of the zinc-binding domains. *Proc Natl Acad Sci USA,* 2002, 99, 11109-11114.

Lane, D; Wiedemeier, A; Peng, L; Höfte, H; Vernhettes, S; Desprez, T; Hocart, CH; Birch, RJ; Baskin, TI; Burn, JE; Arioli, T; Williamson, RE. Temperature-sensitive alleles of *RSW2* link the KORRIGAN endo-1,4-β-glucanase to cellulose synthesis and cytokinesis in Arabidopsis. *Plant Physiol,* 2001, 126, 278-288.

Lefebvre, A; Maizonnier, D; Gaudry, JC; Clair, D; Scalla, R. Some effects of the herbicide EL-107 on cellular growth and metabolism. *Weed Res,* 1987, 27, 125-134.

Levy, I; Shani, Z; Shoseyor, O. Modification of polysaccharides and plant cell wall by endo-1,4-β-glucanase and cellulose-binding domains. *Biomol Engineer*, 2002, 19, 17-30.

Lewis, D; Bacic, A; Chandler, PM; Newbigin, EJ. Aberrant cell expansion in the elongation mutants of barley. *Plant Cell Physiol,* 2009, 50, 554-571.

Lukowitz, W; Nickle, TC; Meinke, DW; Last, RL; Conklin, PL; Somerville, CR. *Arabidopsis cyt1* mutants are deficient in a mannose-1-phosphate guanylyltransferase and point to a requirement of N-linked glycosylation for cellulose biosynthesis. *Proc Natl Acad Sci USA,* 2001, 98, 2262-2267.

Madari, H; Panda, D; Wilson, L; Jacobs, RS. Dicoumarol: a unique microtubule stabilizing natural product that is synergistic with taxol. *Cancer Res,* 2003, 63, 1214-1220.

Manfield, LW; Orfila, C; McCartney, L; Harholt, J; Bernal, AJ; Scheller, HV; Gilmartin, PM; Mikkelsen, JD; Knox, JP; Willats, WGT. Novel cell wall architecture of isoxaben-habituated *Arabidopsis* suspension-cultured cells: global transcript profiling and cellular analysis. *Plant J,* 2004, 40, 260-275.

McIntosh, AH; Chamberlain, K; Dawson, GW. Foliar sprays against potato common scab-compounds related to 3,5-dichlorophenoxyacetic acid. *Crop Prot,* 1985, 4, 473-480.

Mélida, H; García-Angulo, P; Alonso-Simón, A; Encina, A; Álvarez, J; Acebes, JL. Novel type II cell wall architecture in dichlobenil-habituated maize calluses. *Planta,* 2009, 229, 617-631.

Menne, H. & Köcher, H. HRAC classification of herbicides and resistance development. In: Krämer W, Shirmer U, editors. *Modern crop protection compounds.* Wiley-Vch; 2007, 5-27.

Meyer, Y. & Herth, W. Chemical inhibition of cell wall formation and cytokinesis, but not of nuclear division, in protoplasts of *Nicotiana tabacum* L. cultivated in vitro. *Planta,* 1978, 142, 253-262.

Mizuta, S. & Brown, RM. Effects of 2,6-dichlorobenzonitrile and Tinopal LPW on the structure of the cellulose synthesizing complexes of *Vaucheria hamata*. *Protoplasma,* 1992, 166, 200-207.

Molhoj, M; Pagant, S; Höfte, H. Towards understanding the role of membrane-bound endo-ß-1,4-glucanases in cellulose biosynthesis. *Plant Cell Physiol*, 2002, 43, 1399-1406.

Montague, MJ. Gibberellic acid promotes growth and cell wall synthesis in Avena internodes regardless of the orientation of cell expansion. *Physiol Plant,* 1995, 94, 7-18.

Montezinos, D. & Delmer, DP. Characterization of inhibitors of cellulose synthesis in cotton fibers. *Planta,* 1980, 148, 305-311.

Moreland, DE; Hussey, GG; Farmer, FS; Shmuel, M; Trainin, T; Kalman, S; Delmer, D. Comparative effects of dichlobenil and its phenolic alteration products on photophosphorylation and oxidative phosphorylation. *Pestic Biochem Physiol,* 1974, 4, 356-364.

Mouille, G; Robin, S; Lecomte, M; Pagant, S; Höfte, H. Classification and identification of *Arabidopsis* cell wall mutants using Fourier-Transform Infrared (FT-IR) microspectroscopy. *Plant J,* 2003, 35, 393-404.

Mutwil, M; DeBolt, S; Persson, S. Cellulose synthesis: a complex complex. *Curr Opin Plant Biol,* 2008, 11, 252-257.

Nakagawa, N. & Sakurai, N. Increase in the amount of celA1 protein in tobacco BY-2 cells by a cellulose biosynthesis inhibitor, 2,6-dichlorobenzonitrile. *Plant Cell Physiol,* 1998, 39, 779-785.

Nakagawa, N. & Sakurai, N. Cell wall integrity controls expression of endoxyloglucan transferase in tobacco BY2 cells. *Plant Cell Physiol*, 2001, 42, 240-244.

Nakagawa, N. & Sakurai, N. A mutation in At-nMat1a, which encodes a nuclear gene having high similarity to group II intron maturase, causes impaired splicing of mitochondrial NAD4 transcript and altered carbon metabolism in *Arabidopsis thaliana*. *Plant Cell Physiol,* 2006, 47, 772-783.

Nicol, F; His, I; Jauneau, A; Vernhettes, S; Canut, H; Höfte, H. A plasma membrane-bound putative endo-1,4-β-D-glucanase is required for normal wall assembly and cell elongation in *Arabidopsis*. *EMBO J,* 1998, 17, 5563-5576.

Nishimura, MT; Stein M; Hou, BH; Vogel, JP; Edwards, H; Somerville, SC. Loss of a callose synthase results in salicylic acid-dependent disease resistance. *Science*, 2003, 301, 969-972.

Obembe, OO; Jacobsen, E; Visser, RGF, Vincken, JP. Cellulose-hemicellulose networks as target for *in planta* modification of the properties of natural fibres. *Biotechnol Mol Biol Rev*, 2006, 1, 76-86.

O'Keeffe, MG. & Klevorn, TB. Flupoxam: a new preemergence and postemergence herbicide for broad-leaved weed-control in winter cereals. *Brighton Crop Prot Conf Weeds*, 1991, 63-68.

O'Looney, N. & Fry, SC (a). The novel herbicide oxaziclomefone inhibits cell expansion in maize cell cultures without affecting turgor pressure or wall acidification. *New Phytol,* 2005, 168, 1-7.

O'Looney, N. & Fry, SC (b). Oxaziclomefone, a new herbicide, inhibits wall expansion in maize cell-cultures without affecting polysaccharide biosynthesis, xyloglucan transglycosylation, peroxidase action or apoplastic ascorbate oxidation. *Ann Bot,* 2005, 96, 1-11.

Pagant, S; Bichet, A; Sugimoto, K; Lerouxel, O; Desprez, T; McCann, M; Lerouge, P; Vernhettes, S; Hofte, H. *KOBITO1* encodes a novel plasma membrane protein necessary for normal synthesis of cellulose during cell expansion in *Arabidopsis*. *Plant Cell,* 2002, 14, 2001-2013.

Paredez, AR; Persson, S; Ehrhardt, DW; Somerville, CR. Genetic evidence that cellulose synthase activity influences microtubule cortical array organization. *Plant Physiol,* 2008, 147, 1723-1734.

Paredez, AR; Somerville, CR; Ehrhardt, DW. Visualization of cellulose synthase demonstrates functional association with microtubules. *Science,* 2006 312, 1491-1495.

Pauly, M. & Keegstra, K. Cell-wall carbohydrates and their modification as a resource for biofuels. *Plant J*, 2008, 54, 569-568.

Peng, L; Kawagoe, Y; Hoan, P; Delmer, D. Sitosterol-β-glucosidase as primer for cellulose synthesis in plants. *Science,* 2002, 295, 147-150.

Peng, L; Xiang, F; Roberts, E; Kawagoe, Y; Greve, LC; Kreuz, K; Delmer, DP. The experimental herbicide CGA 325´615 inhibits synthesis of crystalline cellulose and causes accumulation of non-crystalline normal β-1,4-glucan associated with CesA protein. *Plant Physiol,* 2001, 126, 981-992.

Persson, S; Paredez, A; Carroll, A; Palsdottir, H; Doblin, M; Poindexter, P; Khitrov, N; Auer, M; Somerville, CR. Genetic evidence for three unique components in primary cell-wall cellulose synthase complexes in *Arabidopsis*. *Proc Natl Acad Sci USA,* 2007, 104, 15566-15571.

Potikha, T. & Delmer, DP. A mutant of *Arabidopsis thaliana* displaying altered patterns of cellulose deposition. *Plant J,* 1995, 7, 453-460.

Potters, G; Pasternak, TP; Guisez, Y; Palme, KJ; Jansen, MAK. Stress-induced morphogenic responses: growing out of trouble? *Trends Plant Sci,* 2007, 12, 98-105.

Rajangam, AS; Kumar, M; Aspeborg, H; Guerriero, G; Arvestad, L; Pansri, P; Brown, CJL; Hober, S; Blomqvist, K; Divne, C; Ezcurra, I; Mellerowicz, E; Sundberg, B; Bulone, V; Teeri, TT. MAP20, a microtubule-associated protein in the secondary cell walls of hybrid aspen, is a target of the cellulose synthesis inhibitor 2,6-dichlorobenzonitrile. *Plant Physiol,* 2008, 148, 1283-1294.

Reiter, W-; Chapple, C; Somerville, CR. Mutants of *Arabidopsis thaliana* with altered cell wall polysaccharide composition. *Plant J,* 1997, 12, 335-345.

Robert, S; Mouille, G; Höfte, H. The mechanism and regulation of cellulose synthesis in primary walls: lessons from cellulose-deficient Arabidopsis mutants. *Cellulose,* 2004, 11, 351-364.

Robinson, DG. & Quader, H. Structure, synthesis, and orientation of microfibrils IX: a freeze-fracture investigation of the *Oocystis* plasma membrane after inhibitor treatment. *Eur J Cell Biol,* 198 125, 278-288.

Sabba, RP; Durso, NA; Vaughn, KC. Structural and immunocytochemical characterization of the walls of dichlobenil-habituated BY-2 tobacco cells. *Int J Plant Sci,* 1999, 160, 275-290.

Sabba, RP. & Vaughn, KC. Herbicides that inhibit cellulose biosynthesis. *Weed Sci,* 1999, 47, 757-763.

Samuels, AL; Giddings, TH; Staehelin, LA. Cytokinesis in tobacco BY-2 and root tip cells: a new model of cell plate formation in higher plants. *J Cell Biol,* 1995, 130, 1345-1357.

Scheible, W-; Eshed, R; Richmond, T; Delmer, D; Somerville, C. Modifications of cellulose synthase confer resistance to isoxaben and thiazolidinone herbicides in *Arabidopsis Ixr1* mutants. *Proc Natl Acad Sci USA,* 2001, 98, 10079-10084.

Scheible, W-; Fry, B; Kochevenko, A; Schindelasch, D; Zimmerli, L; Somerville, S; Loria, R; Somerville, CR. An Arabidopsis mutant resistant to thaxtomin A, a cellulose synthesis inhibitor from *Streptomyces* species. *Plant Cell,* 2003, 15, 1781-1794.

Scheneegurt, MA; Heim, DR; Larrinua, IM. Investigation into the mechanism of Isoxaben tolerance in dicot weeds. *Weed Sci,* 1994, 42, 163-167.

Schindelman, G; Morikami, A; Jung, J; Baskin, TI; Carpita, NC; Derbyshire, P; McCann, MC; Benfey, PN. COBRA encodes a putative GPI-anchored protein, which is polarly localized and necessary for oriented cell expansion in *Arabidopsis*. *Genes Develop,* 2001, 15, 1115-1127.

Sharples, K; Hawkes, TR; Mitchell, G; Edwards, LS; Langford, MP; Langton, DW; Rogers, KM; Townson, JK; Wang, Y. A novel thiazolidinone herbicide is a potent inhibitor of glucose incorporation into cell wall material. *Pestic Sci,* 1998, 54, 368-376.

Shedletzky, E; Shmuel, M; Delmer, DP; Lamport, DTA. Adaptation and growth of tomato cells on the herbicide 2,6-Dichlorobenzonitrile leads to production of unique cell-walls virtually lacking a cellulose-xyloglucan network. *Plant Physiol,* 1990, 94, 980-987.

Shedletzky, E; Shmuel, M; Trainin, T; Kalman, S; Delmer, D. Cell-wall structure in cells adapted to growth on the cellulose-synthesis inhibitor 2,6-Dichlorobenzonitrile - A comparison between two dicotyledonous plants and a graminaceous monocot. *Plant Physiol,* 1992, 100, 120-130.

Shive, JB. & Sisler, HD. Effects of Ancymidol (a growth retardant) and Triarimol (a fungicide) on growth, sterols, and gibberellins of *Phaseolus vulgaris* L. *Plant Physiol,* 1976, 57, 640-644.

Somerville, C. Cellulose synthesis in higher plants. *Annu Rev Cell Dev Biol,* 2006 22, 53-78.

Sugimoto, K; Williamson, R; Wasteneys, GO. Wall architecture in the cellulose-deficient *rsw1* mutant of *Arabidopsis thaliana*: microfibrils but not microtubules lose their transverse alignment before microfibrils become unrecognizable in the mitotic and elongation zones of roots. *Protoplasma,* 2001, 215, 172-183.

Sunohara, Y. & Matsumoto, H. Oxidative injury induced by the herbicide quinclorac on *Echinochloa oryzicola* Vasing. and the involvement of antioxidative ability in its highly selective action in grass species. *Plant Sci*, 2004, 167, 597-606.

Sunohara, Y. & Matsumoto, H. Quinclorac-induced cell death is accompanied by generation of reactive oxygen species in maize root tissue. *Phytochem,* 2008, 69, 2312-2319.

Takahashi, J; Rudsander, UJ; Hedenström, M; Banasiak, A; Harholt, J; Amelot, N; Immerzeel P; Ryden, P; Endo, S; Ibatullin, FM; Brumer, H; del Campillo, E; Master, ER; Scheller, HV; Sundberg, B; Teeri, TT; Mellerowicz, EJ. *KORRIGAN1* and its aspen homologue *PttCel9A1* decrease cellulose crystallinity in Arabidopsis stems. *Plant Cell Physiol,* 2009, 50, 1099-1115.

Tanimoto, E. Gibberellin-dependent root elongation in *Lactuca sativa:* Recovery from growth retardant-suppressed elongation with thickening by low concentration of GA_3. *Plant Cell Physiol,* 1987, 28, 963-973.

Tanimoto, E. Interaction of gibberellin A_3 and ancymidol in the growth and cell-wall extensibility of dwarf pea roots. *Plant Cell Physiol,* 1994, 35, 1019-1028.

Tanimoto, E. & Huber, DJ. Effect of GA_3 on the molecular mass of polyuronides in the cell walls of Alaska pea roots. *Plant Cell Physiol,* 1997, 38, 25-35.

Taylor, NG. Cellulose biosynthesis and deposition in higher plants *New Phytol,* 2008, 178, 239-252.

Taylor, NG; Howells, RM; Huttly, AK; Vickers, K; Turner, SR. Interactions among three distinct CesA proteins essential for cellulose synthesis. *Proc Natl Acad Sci USA*, 2003, 100, 1450-1455.

Taylor, NG; Laurie, S; Turner, SR. Multiple cellulose synthase catalytic subunits are required for cellulose synthesis in Arabidopsis. *Plant Cell*, 2000, 12, 2529-2540.

Taylor, NG; Scheible, WR; Cutler, S; Somerville, CR; Turner, SR. The *irregular xylem3* locus of Arabidopsis encodes a cellulose synthase required for secondary cell wall synthesis. *Plant Cell*, 1999, 11, 769-780.

Tegg, RS; Gill, WM; Thompson, HK; Davies, NW; Ross, JJ; Wilson, CR. Auxin-induced resistance to common scab disease of potato linked to inhibition of thaxtomin A toxicity. *Plant Dis,* 2008, 92, 1321-1328.

Theologis, A. Possible linkage between auxin regulated gene expression, H^+ secretion, and cell elongation: a hypothesis. In Cosgrove DJ, Knievel DP editors. Physiology of Cell Expansion during Plant Growth. American Society of Plant Physiologists, Rockville, MD, 1987, 133-144.

Timmers, J; Vernhettes, S; Desprez, T; Vincken, J; Visser, RGF; Trindade, LM. Interactions between membrane-bound cellulose synthases involved in the synthesis of the secondary cell wall. *FEBS Lett,* 2009, 583, 978-982.

Tresch, S. & Grossmann, K. Quinclorac does not inhibit cellulose (cell wall) biosynthesis in sensitive barnyard grass and maize roots. *Pestic Biochem Physiol,* 2003, 75, 73-78.

Turner, SR. & Somerville, CR. Collapsed xylem phenotype of Arabidopsis identifies mutants deficient in cellulose deposition in the secondary cell wall. *Plant Cell,* 1997, 9, 689-701.

Umetsu, N; Satoh, S; Matsuda, K. Effects of 2,6-dichlorobenzonitrile on suspension-cultured soybean cells. *Plant Cell Physiol,* 1976, 17, 1071-1073.

Vaughn, KC. Cellulose biosynthesis inhibitor herbicides. In: Böger P, Wakabayashi K, Hirai K, editors. *Herbicide classes in development.* Springer, Berlin, 2002, 139-150.

Vaughn, KC; Hoffman, JC; Hahn, MG; Staehelin, LA. The herbicide dichlobenil disrupts cell plate formation: immunogold characterization. *Protoplasma,* 1996, 194, 117-132.

Vaughn, KC. & Turley, RB. Ultrastructural effects of cellulose biosynthesis inhibitor herbicides on developing cotton fibers. *Protoplasma,* 2001, 216, 80-93.

Verloop, A. & Nimmo, WB. Absorption, translocation and metabolism of dichlobenil in bean seedlings. *Weed Res,* 1969, 9, 357-370.

Vogel, KP. & Jung, HJG. Genetic modification of herbaceous plants for feed and fuel. *Crit Rev Plant Sci*, 2001, 20, 15-49.

Wakabayashi, K. & Böger, P. Phytotoxic sites of action for molecular design of modern herbicides (Part 2): Amino acid, lipid and cell wall biosynthesis, and other targets for future herbicides. *Weed Biol Manag,* 2004, 4, 59-70.

Walsh, TA; Neal, R; Merlo, AO; Honma, M; Hicks, GR; Wolff, K; Matsumura, W; Davies, JP. Mutations in an auxin receptor homolog AFB5 and in SGT1b confer resistance to synthetic picolinate auxins and not to 2,4-dichlorophenoxyacetic acid or indole-3-acetic acid in Arabidopsis. *Plant Physiol,* 2006, 142, 542-552.

Wang, J; Elliott, JE; Williamson, RE. Features of the primary wall CESA complex in wild type and cellulose-deficient mutants of *Arabidopsis thaliana*. *J Exp Bot,* 2008, 59, 2627-2637.

Wang, Y; Lu, J; Mollet, J-; Gretz, MR; Hoagland, KD. Extracellular matrix assembly in diatoms (Bacillariophyceae). II. 2,6-dichlorobenzonitrile inhibition of motility and stalk production in the marine diatom *Achnanthes longipes*. *Plant Physiol,* 1997, 113, 1071-1080.

Wells, B; McCann, MC; Shedletzky, E; Delmer, D; Roberts, K. Structural features of cell walls from tomato cells adapted to grow on the herbicide 2,6-dichlorobenzonitrile. *J Microsc,* 1994, 173, 155-164.

Wightman, R; Marshall, R; Turner, SR. A cellulose synthase-containing compartment moves rapidly beneath sites of secondary wall synthesis. *Plant Cell Physiol,* 2009 50, 584–594.

Willats, WGT; Knox, P; Mikkelsen, D. Pectin: new insights into an old polymer are starting to gel. *Trends Food Sci Technol*, 2006, 17, 97–104.

Wilson, CR; Luckman, GA; Tegg, RS; Yuan, ZQ; Wilson AJ; Eyles, A; Conner, AJ. Enhanced resistance to common scab of potato through somatic cell selection in cv. Iwa with the phytotoxin thaxtomin A. *Plant Pathol*, 2009, 58, 137-144.

Yoneda, A; Higaki, T; Kutsuna, N; Kondo, Y; Osada, H; Hasezawa, S; Matsui, M. Chemical genetic screening indentifies a novel inhibitor of parallel alignment of cortical microtubules and cellulose microfibrils. *Plant Cell Physiol*, 2007, 48, 1393-1403.

Zhong, R; Kays, SJ; Schroeder, BP; Ye, ZH. Mutation of a chitinase-like gene causes ectopic deposition of lignin, aberrant cell shapes, and overproduction of ethylene. *Plant Cell*, 2002, 14, 165-179.

Zuo, J; Niu, QW; Nishizawa, N; Wu, Y; Kost, B; Chua, NH. KORRIGAN, an *Arabidopsis* endo-1,4-β-glucanase, localizes to the cell plate by polarized targeting and is essential for cytokinesis. *Plant Cell*, 2000, 12, 1137-1152.

Planta (2009) 229:617–631
DOI 10.1007/s00425-008-0860-8

ORIGINAL ARTICLE

Novel type II cell wall architecture in dichlobenil-habituated maize calluses

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Received: 3 October 2008 / Accepted: 10 November 2008 / Published online: 2 December 2008

Abstract Growth of maize (*Zea mays* L.) callus-culture cells was inhibited using dichlobenil (2,6 dichlorobenzonitrile, DCB) concentrations ≥1 μM; I_{50} value for the effect on inhibited fresh weight gain was 1.5 μM. By increasing the DCB concentration in the culture medium, DCB-habituated cells became 13 times more tolerant of the inhibitor (I_{50}: 20 μM). In comparison with non-habituated calluses, DCB-habituated calluses grew slower, were less friable and were formed by irregularly shaped cells surrounded by a thicker cell wall. By using an extensive array of techniques, changes in type II cell wall composition and structure associated with DCB habituation were studied. Walls from DCB-habituated cells showed a reduction of up to 75% in cellulose content, which was compensated for by a net increase in arabinoxylan content. Arabinoxylans also showed a reduction in their extractability and a marked increase in their relative molecular mass. DCB habituation also involved a shift from ferulate to coumarate-rich cells walls, and enrichment in cell wall esterified hydroxycinnamates and dehydroferulates. The content of polymers such as mixed-glucan, xyloglucan, mannans, pectins or proteins did not vary or was reduced. These results prove that the architecture of type II cell walls is able to compensate for deficiencies in cellulose content with a more extensive and phenolic cross-linked network of arabinoxylans, without necessitating β-glucan or other polymer enhancement. As a consequence of this modified architecture, walls from DCB-habituated cells showed a reduction in their swelling capacity and an increase both in pore size and in resistance to polysaccharide hydrolytic enzymes.

Keywords Arabinoxylan · Callus culture · Cellulose · Dichlobenil · FTIR · Maize

Abbreviations

AIR	Alcohol insoluble residue
AGPs	Arabinogalactan proteins
AX	Arabinoxylan
CDTA	50 mM cyclohexane-*trans*-1,2-diamine-*N*,*N*,*N*′,*N*′-tetraacetic acid sodium salt
DCB	2,6-Dichlorobenzonitrile or dichlobenil
FTIR	Fourier transform infrared
GAX	Glucuronoarabinoxylan
H*x*	Dichlobenil-habituated calluses growing in *x* (μM) DCB
IDA	Immunodot assay
mAb	Monoclonal antibody
MPBS	PBS containing 4% fat-free milk powder
M_w	Average molecular weight
NH	Non-habituated calluses
PBS	0.1 M phosphate buffer saline
PCA	Principal component analysis
Rha	Rhamnose
snCR	Supernatant-cellulose residue
TFA	Trifluoroacetic acid

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Introduction

Previous works have demonstrated the remarkable ability of plant cells to tolerate induced stresses (Iraki et al. 1989; Shedletzky et al. 1992; Encina et al. 2001, 2002) by changing

their cell wall composition and structure. The herbicide DCB inhibits the polymerization of Glc into β-1,4-linked glucan and may also affect β-1,4-glucan crystallization at the plasma membrane, but has little or no short-term effect on other physiological processes (Delmer 1987). The habituation of cell cultures to cellulose biosynthesis inhibitors such as DCB, reflects the ability of cells to survive with a modified wall and is therefore a valuable experimental technique for gaining an insight into the plasticity of plant cell wall composition and structure (Vaughn 2002).

There are two types of cell walls in higher plants. Type I cell walls are found in dicots, gymnosperms and most monocots, and type II walls are found in graminaceous plants, along with the other commelinoid monocots (Carpita and Gibeaut 1993; Carpita 1996). Cellulose, a linear (1,4)-β-D-glucan, is the main load-bearing polysaccharide in both types of walls. In type I cell walls, xyloglucan interlaces the cellulose microfibrils, forming the main load-bearing network in the wall. This cellulose-xyloglucan framework is embedded in a matrix of pectic polysaccharides, homogalacturonan and rhamnogalacturonans I and II. Type I cell walls also contain minor amounts of protein, including basic proteins that can interact with the pectin network and with other proteins through intermolecular bridges. Type II cell walls are characterized by a reduction in xyloglucan, pectins and structural proteins, and by a higher content of other noncellulosic-polysaccharides, such as acidic xylans and 'mixed-linkage' (1,3)-(1,4)-β-D-glucan (Carpita et al. 2001). In the case of graminaceous cell walls, matrix pectins are mainly substituted by arabinoxylans and glucuronoarabinoxylan (GAX), which tether adjacent cellulose microfibrils. Also frequent in graminaceous cell walls is the presence of hydroxycinnamic acids (mainly ferulic and *p*-coumaric acid) ester linked to α-L-Ara residues of arabinoxylans (Ishii 1997). Hydroxycinnamic acids contribute to wall assembly by cross linking polysaccharides through the oxidative coupling of feruloyl residues (Fry et al. 2000).

Most DCB-habituated cultures belong to type I cell wall species, such as tomato (Shedletzky et al. 1990), tobacco (Shedletzky et al. 1992; Wells et al. 1994; Nakagawa and Sakurai 1998, 2001) and bean (Encina et al. 2001, 2002; Alonso-Simón et al. 2004). In type I cell walls habituated to DCB, there is a marked reduction in the amount of cellulose and hemicelluloses, whereas the quantity of esterified and unesterified pectins is increased. Moreover, in DCB-habituated BY-2 tobacco cells, pectins have been reported to be cross-linked with extensin to form the main cell wall network (Sabba et al. 1999). Other modifications have been associated with DCB habituation, such as the presence of a non-crystalline β-1,4-glucan tightly bound to cellulose, the accumulation of pectin-enriched cell wall appositions, a putative increase in the extent of pectin–xyloglucan cross-linking, reduced levels of arabinogalactan proteins (AGPs) and changes in the levels of extensin (Shedletzky et al. 1992; Encina et al. 2002; García-Angulo et al. 2006).

To date, barley cultures are the only cells with type II cell walls that have been habituated to DCB (Shedletzky et al. 1992). DCB-habituated barley cells showed a modified architecture: they contained a considerably reduced level of cellulose in the cell wall and this reduction was effectively compensated for by mechanisms (parallel to modifications) quite different to those observed in dicots, which implicated a higher proportion of β-glucan and a more extensive cross-linking between arabinoxylans, leading to walls with a reduction in pore size.

The present work addresses the selection and characterisation of a maize cell line able to grow in the presence of lethal concentrations of DCB. The results show a novel type II cell wall architecture accompanied by unique cell wall properties.

Materials and methods

Cell cultures

Maize callus cultures (*Zea mays* L., Black Mexican sweetcorn, donated by Dr. S. C. Fry, Institute of Molecular Plant Sciences, University of Edinburgh, UK) from immature embryos were grown in Murashige and Skoog media (Murashige and Skoog 1962) supplemented with 9 μM 2,4-D at 25°C under light (Lorences and Fry 1991), and subcultured monthly.

Habituation to dichlobenil

Calluses weighing 1.0 ± 0.1 g were cultured in dichlobenil (supplied by Fluka), in concentrations ranging from 0.01 to 100 μM. DCB was dissolved in dimethyl sulfoxide (DMSO), which did not affect cell growth at this range of concentrations. The cultures were incubated for 30 days, weighed (FW) and heated at 60°C until constant weight was achieved (DW). Growth was expressed as relative increase in FW and the I_{50} was calculated as the concentration of DCB able to inhibit weight increase by 50% with respect to the control.

Calluses were habituated to growth in different DCB concentrations by stepwise transfers with gradual increments of DCB, beginning at 2 μM. At least three subcultures of approximately 30 days were performed between each increase in the DCB concentration. Growth curves were obtained for habituated calluses growing in DCB and for non habituated calluses, by measuring the relative increase in FW every 4–6 days.

Electron microscopy

Callus pieces were fixed in 2.5% (w/v) paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4, and post-fixed with OsO_4 in the same buffer. The samples were dehydrated in an increasing series of ethanol concentrations and embedded in LR White Resin (London Resin Co. Ltd, Basingstoke, UK). This was done by sequentially placing the segments in ethanol and resin (2:1, v/v) for 8 h, ethanol and resin (1:2, v/v) for 8 h, and in pure resin. The samples were transferred to a gelatin capsule, fresh resin added, and the resin polymerized at 60°C for 48 h. Blocks were sectioned (1.5–2 μm thick) with a LKB 2088 ultramicrotome. Ultrathin sections were mounted on copper grids and post-stained with uranyl acetate and lead citrate before observation with a JEOL (JEM-1010) electron microscope. Cell wall thickness was determined by random measurement of cell walls from 30 cells.

Immunolocation

For the immunolocation of cell wall components, the gelatine capsules were polymerized at 37°C for 5 days. Sections were obtained (1.5–2 μm thick) and applied to multi-well slides (ICN Biomedicals, Cleveland, OH, USA) coated with Vectabond reagent (Vector Laboratories, Burlingame, CA, USA). Sections were incubated for 2 h with 0.1 M phosphate buffer saline (PBS) (0.14 M NaCl, 2.7 mM KCl, 7.8 mM Na_2HPO_4 12 H_2O, 1.5 mM KH_2PO_4, pH 7.2) containing 4% fat-free milk powder (MPBS) with the primary antibody at a 1/10 dilution. After washing exhaustively with PBS, the sections were incubated in darkness for 2 h with a 1/100 dilution of an antirat immunoglobulin G linked to fluorescein isothiocyanate (Sigma) in MPBS at room temperature. Finally, sections were washed with PBS and mounted in a glycerol/PBS-based antifade solution (Citifluor AF1; Agar Scientific, London, UK) and observed using a Nikon Eclipse-TS100 microscope with epifluorescence irradiation. Cellulose was localized in sections using calcofluor white (fluorescent brightener 28, Sigma). Xylans were probed by using mAb LM10 (specific for 1,4-β-xylans) (McCartney et al. 2005) and LM11 (for xylans and arabinoxylans) (McCartney et al. 2005). Cell wall esterified feruloyl groups were probed with LM12, a new antibody developed at the Paul Knox laboratory (Leeds University, UK). AGPs were probed with mAbs LM2 (Smallwood et al. 1996), MAC207 (Pennell et al. 1989) and JIM8 (Pennell et al. 1991).

Preparation and fractionation of cell walls

Calluses collected during growth in the early stationary phase were frozen and homogenized with liquid nitrogen and treated with 70% ethanol for 5 days at room temperature. The suspension was then centrifuged and the pellet washed with 70% ethanol (×6), acetone (×6), and air dried, in order to obtain the alcohol insoluble residue (AIR). The AIR was treated with 90% DMSO for 8 h at room temperature (×3) and then washed with 0.01 M phosphate buffer pH 7.0 (×2). The washed AIR was then treated with 2.5 U ml^{-1} α-amylase obtained from porcine pancreas (Sigma type VI-A) in 0.01 M phosphate buffer pH 7.0 for 24 h at 37°C (×3). The suspension was filtered through a glass fibre, and the residue washed with 70% ethanol (×6), acetone (×6), air dried and then treated with phenol–acetic–water (2:1:1, by vol.) for 8 h at room temperature (×2). This was finally washed with 70% ethanol (×6), acetone (×6) and air dried in order to obtain the cell walls.

For cell wall fractionation, dry cell walls were extracted at room temperature with 50 mM cyclohexane-*trans*-1,2-diamine-*N*,*N*,*N*′,*N*′-tetraacetic acid sodium salt (CDTA) at pH 6.5 for 8 h and washed with distilled water. The residue was retreated with 0.1 M KOH for 2 h (×2) and washed with distilled water. Then 4 M KOH was added to the residue for 4 h (×2), and washed again with distilled water. The extracts were neutralized with acetic acid, dialysed and lyophilized, representing CDTA, KOH-0.1 M and KOH-4 M fractions, respectively. The residue after 4 M KOH extraction was suspended in water, adjusted to pH 5 with acetic acid, and dialysed. After centrifugation, the supernatant was filtered and lyophilized; this was referred to as the supernatant-cellulose residue (snCR) fraction. The residue was hydrolysed for 2.5 h at 120°C with 2 M trifluoroacetic acid (TFA), and after centrifugation, the supernatant was lyophilized and referred to as the TFA fraction.

Cell wall analyses

Cellulose was quantified in crude cell walls with the Updegraff method (Updegraff 1969), using the hydrolytic conditions described by Saeman et al. (1963) and quantifying the glucose released by the anthrone method (Dische 1962).

Tablets for Fourier transform infrared (FTIR) spectroscopy were prepared in a Graseby-Specac press from small samples (2 mg) of cell walls mixed with KBr (1:100, w/w). Spectra were obtained on a Perkin–Elmer instrument at a resolution of 1 cm^{-1}. A window of between 800 and 1,800 cm^{-1}, containing information about characteristic polysaccharides, was selected in order to monitor cell wall structure modifications. All spectra were normalized and baseline-corrected with Spectrum v 5.3.1 (2005) software, by Perkin–Elmer. Data were then exported to Microsoft Excel 2003 and all spectra were area-normalized. Cluster analysis was performed using the Ward method, and the Pearson coefficient was selected as distance measurement. Principal component analysis (PCA) was performed using a maximum of five principal components. All analyses were carried out using the Statistica 6.0 software package.

The total sugar content of each fraction was determined by the phenol–sulfuric acid method (Dubois et al. 1956) and expressed as the glucose equivalent. Uronic acid content was determined by the *m*-hydroxydiphenyl method (Blumenkrantz and Asboe-Hansen 1973) using galacturonic acid as a standard. Xyloglucan content was obtained by the iodine-staining method (Kooiman 1960). Neutral sugar analysis was performed as described by Albersheim et al. (1967). Lyophilized samples of each fraction were hydrolysed with 2 M TFA at 121°C for 1 h and the resulting sugars were derivatized to alditol acetates and analysed by gas chromatography (GC) using a Supelco SP-2330 column.

The quantification of (1 → 3,1 → 4)-β-glucans was carried out using a direct and specific enzymatic assay with the (1 → 3,1 → 4)-β-glucan endo-hydrolase from *Bacillus subtilis* (McCleary and Codd 1991).

To measure cell wall degradability, cell walls were hydrolysed (5 mg ml^{-1}) in a mixture of cellulase R-10 (0.1%), macerozyme R-10 (0.1%) and purified driselase (0.01%) dissolved in sodium acetate 20 mM (pH 4.8) (Grabber et al. 1998). Aliquots were taken at 2, 6, 24, 48 and 72 h, clarified by centrifugation and assayed for total sugars following the method described by Dubois et al. (1956).

In vitro measurement of cell wall swelling examined the relative volume increase that took place after re-hydrating dry walls in 8 mm diameter tubes (Encina et al. 2002).

For amino acid analysis, cell walls were hydrolysed in 6 N HCl at 110°C for 24 h, filtered through Whatman paper and dried by speed-vac. Soluble amino acids were derivatized by using phenyl isothiocyanate, and then separated, identified and quantified following the method described by Alonso et al. (1994). Total protein content was estimated by summation of the amounts of amino acids measured.

Assay of esterified phenolic acids

Cell walls (10 mg) were treated in the dark with 1 M NaOH, at room temperature for 12 h to saponify phenolic esters. The solution was acidified by addition of TFA and partitioned against ethyl acetate (×2). The ethyl acetate phases were pooled and mixed with a solution containing 1% 8,5-diferulic acid, 5,5-diferulic acid, 8-*O*-4 diferulic acid, *p*-coumaric acid and ferulic acid as internal markers, then vacuum-dried and re-dissolved in propan-1-ol. Portions of the propanol solution were subjected to TLC on silica-gel in benzene/acetic acid (9:1, v/v).

Gel-permeation chromatography

Hemicellulosic fractions were size-fractionated by gel-permeation chromatography on Sepharose CL-4B (120–125 ml bed-volume in a 1.5 cm diameter column) in pyridin: acetic acid:water (1:1:98, by vol.) at 0.3 ml min^{-1}. The column was calibrated with commercial dextrans of known weight-average relative molecular mass, and hemicellulose average molecular weight (M_w) was obtained using the $K_{av(1/2)}$ method (Kerr and Fry 2003), with the calibration curve [$\log M_w = -4.999K_{av(1/2)} + 7.849$] obtained for this column. The M_w estimates are nominal rather than absolute because of conformational differences between dextran and hemicelluloses.

Porosity measurements

Porosity was determined using the technique developed by Baron-Epel et al. (1988). Cells at the logarithmic stage of growth were resuspended in 10 mM 2-amino-2(hydroxymethyl)-1,3-propanediol (Tris; pH 5.5), 10 mM $CaCl_2$, and 0.5 M mannitol in order to induce plasmolysis. They were then suspended in a medium with fluorescein isothiocyanate-dextrans (Sigma) at a final concentration of 500 μg ml^{-1}. Fluorescence was observed using a Nikon Eclipse-TS100 epifluorescent microscope equipped with a Nikon B-2 A filter (450–490 nm excitation, 510 nm dichroic mirror and 520 nm barrier filter).

Immunodot assays

For immunodot assays (IDAs), aliquots of 1 μl from KOH-0.1 M and KOH-4 M fractions containing 5 μg of total sugars were spotted onto nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany) as a replicated dilution series and probed as described by Willats et al. (1999) and García-Angulo et al. (2006). All primary antibodies (JIM8, LM11 and LM12) were used at 1/5 dilutions.

Results

Effect of DCB on callus cultures and habituation

In order to determine the effect of DCB on maize callus cultures we tested its inhibitory effect on fresh weight gain (Fig. 1). The weight gain of non-habituated calluses (NH) was slightly stimulated at low concentrations of DCB, but was diminished by DCB concentrations equal to or higher than 1 μM. The I_{50} was 1.5 μM for FW.

Maize calluses' tolerance to lethal concentrations of DCB was achieved by gradually increasing the concentration of the inhibitor in the culture medium, beginning at 2 μM. After 12 subcultures of an average of 30 days each, maize cells were capable of growth in 12 μM DCB (H12). As for non-habituated cell cultures, a stimulation of FW gain was observed for the lowest DCB concentration used. In the case of H12 cells, the I_{50} value for FW inhibition gain was approximately 13 times higher (20 μM).

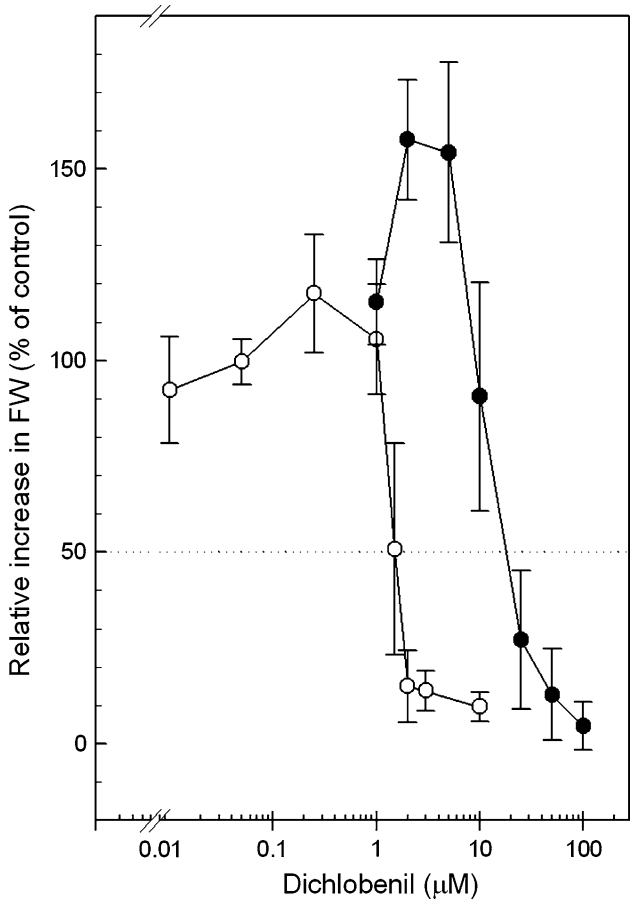

Fig. 1 Growth inhibition curves of maize calluses by increasing concentrations of DCB after 30 days of culture. *Open circle* non-habituated (NH) calluses, *filled circle* habituated to 12 μM DCB (H12) calluses. Values are mean ± SD of six measurements

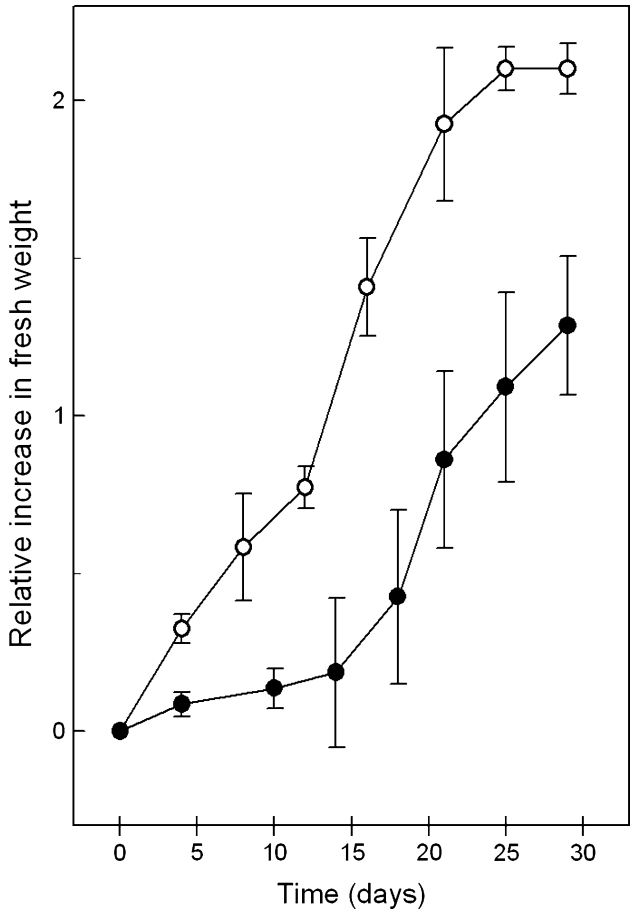

Fig. 2 Relative increase in FW of maize calluses during the culture. *Open circle* NH calluses, *filled circle* H12 calluses. Values are mean ± SD of six measurements

Characterization of callus cultures and cells

Habituated cells (H12) growing on DCB had longer lag phases and less FW was accumulated (Fig. 2). Moreover, H12 cells had a higher DW/FW ratio and cell wall yield per DW than non-habituated ones (Table 1).

H12 calluses were darker and less friable, and formed hard protuberances during growth. H12 cells were rather irregular (Fig. 3b) in comparison with NH cells, which were more or less isodiametrically shaped (Fig. 3a). Groups of cells with thicker walls, apparently arising from the same cell, were frequent in habituated cell preparations (Fig. 3b).

Cell wall analysis

Ultrastructure and porosity

NH cells were surrounded by a uniform cell wall (Table 1), in comparison with H12 cell walls, which were less uniform (Fig. 3d) and 1.6 times thicker than those from NH ones (Fig. 3c). H12 cell walls (Fig. 3b) were less calcofluor-stained than NH cell walls (Fig. 3a), probably due to the reduction in cellulose.

H12 cell walls half-swelled compared with NH ones (Table 1), but porosity increased (Table 2); limiting diameter of dextrans to be excluded for cell walls increased from 6.6 to 12 nm when NH and H12 cells were compared, although long incubation times were required by 9-nm-diameter dextrans to go through H12 cell walls.

FTIR spectroscopy

Changes in the cell wall during the habituation process were monitored by FTIR spectroscopy. Twelve representative FTIR cell wall spectra from non-habituated and habituated to different DCB concentrations calluses were analysed. FTIR spectra from habituated cell walls showed differences in wavenumbers corresponding to cellulose, phenolic components, arabinose and proteins. They also showed variations in the main phenolic linkage: ester bonds (data not shown). To elucidate differences among these spectra, a multivariate analysis was performed (Fig. 4a).

Table 1 Variation of some characteristics of cell lines during habituation

Cell type	DW/FW[a]	Wall DW/callus DW[a]	Cell wall thickness (μm)[b]	Cell wall swelling[c]
NH	0.0399 ± 0.0074	0.2409 ± 0.0052	0.1344 ± 0.0343	1.72 ± 0.19
H12	0.0623 ± 0.0100	0.5451 ± 0.0029	0.2137 ± 0.1113	0.80 ± 0.13

Cell wall swelling is represented as relative increase in volume

Values are mean ± SD of [a]10, [b]30 or [c]6 measurements

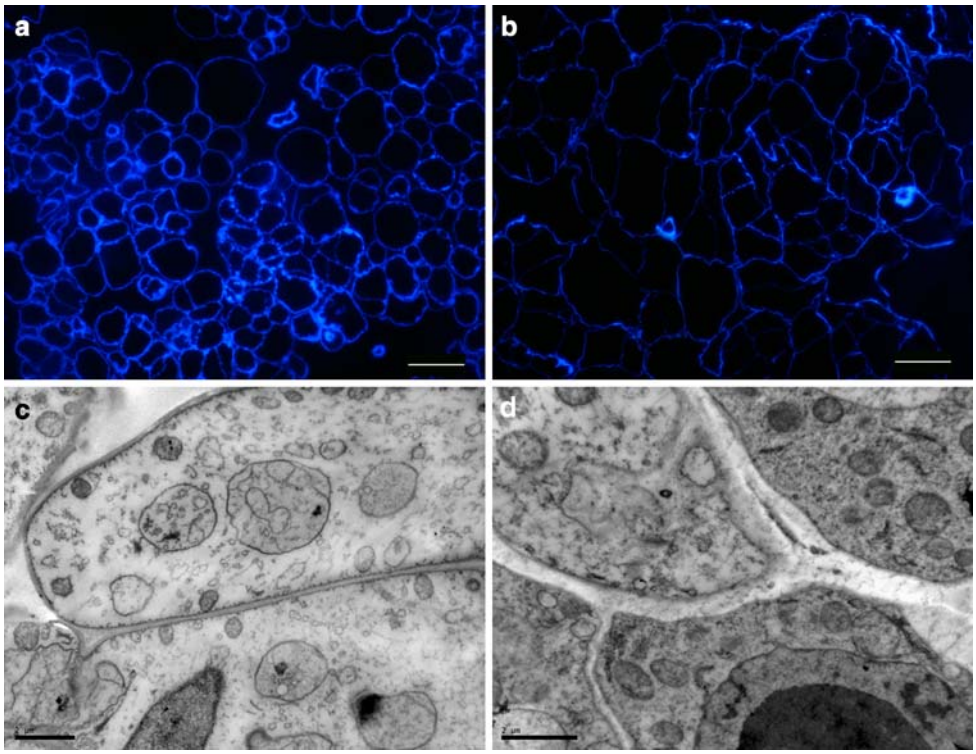

Fig. 3 Sections of NH (**a**) and H12 (**b**) callus calcofluor stained. Ultra-structural appearance of cell walls of NH (**c**) and H12 (**d**) callus. *Bars* 50 μm (**a**, **b**), 2 μm (**c**, **d**)

Table 2 Comparison of porosities of NH and H12 cell walls

Molecular mass of FTIC-dextrans (kDa)	Stokes diameters (nm)	NH	H12
10	4.6	+[a], +[b], +[c]	+, +, +
20	6.6	−, −, −	±, +, +
40	9	−, −, −	−, −, +
70	12	−, −, −	−, −, −

+, Dye penetrates >80% of cell walls; −, dye penetrates <20% of cell walls

[a] Measurement at 30 min

[b] Measurement at 60 min

[c] Measurement at 120 min

Principal components 1 and 3 (PC1 and PC3) were used to discriminate between groups of spectra. Cell walls from non-habituated cells were located on the negative side of PC1 and PC3; when the habituation process began, the points corresponding to the cell walls of habituated calluses were displaced to the positive side of PC1 and PC3. Generally, the more habituated the calluses, the more positive the PC1 and PC3 placement. NH treated for short periods with 6 μM DCB displaced to the positive side of PC1 but to the negative side of PC3, suggesting that the changes in cell walls promoted by DCB were different in habituated and non-habituated cells.

PC3 was more discriminative than PC1. PC3 loading factor plot (Fig. 4b) showed a negative area at the fingerprint region 950–1,175 cm^{-1}, indicative of polysaccharides. In this area, correlated negative peaks could be identified, mainly at 1,160, 1,105, 1,060 and 1,040 cm^{-1}, indicative of cellulose (Carpita et al. 2001). Also, two major protein negative absorbances at 1,550 and 1,650 cm^{-1} were detected. Thus, spectra located at the negative side of PC3, i.e. NH-seemed to have a relatively higher amount of polysaccharides—mainly cellulose—and proteins. In contrast, PC3 showed positive peaks at several wavenumbers indicative of aromatic rings: 1,630 (ring conjugated C=C), 1,600 (aryl ring stretching symmetric), about 1,500 (phenolic ring), and 1,425, 1,180 and 843 (aromatic C–H out of bending) cm^{-1}. The peak at 856 cm^{-1} corre-

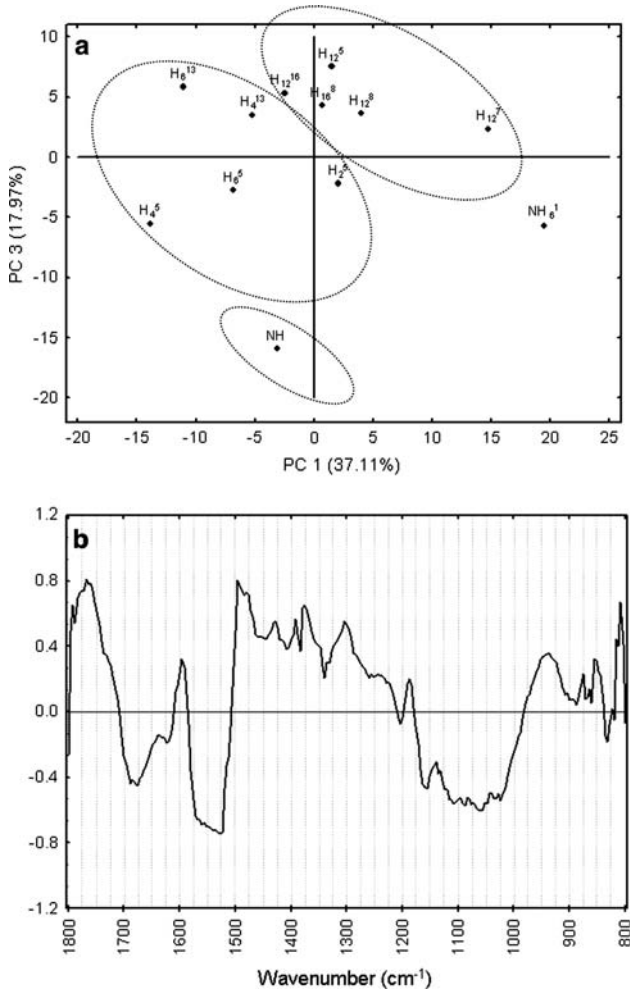

Fig. 4 **a** Principal component analysis of callus spectra. A plot of the first and third PCs is represented based on the FTIR spectra of non-habituated (NH) and habituated calluses (H_x^n, x DCB concentration (μM), n number of subcultures in that concentration). NH_6^1, non-habituated calluses treated for 5 days with 6 μM DCB. **b** Factor loadings for PC3

sponds to furanoid ring (i.e. Ara*f*); and 1,381, 1,376 and 1,312 cm^{-1} correspond to polysaccharides (Kacurakova et al. 1999).

Summing up, the expected changes in cellulose were accompanied by changes in other components, like arabinose, phenolics and proteins, and multivariate analyses demonstrated that during habituation, cell walls underwent gradual modifications.

Cellulose content

The increase in DCB concentration during the habituation process caused a reduction in cellulose content (Fig. 5). This reduction correlated with the habituation level until H6. H12 walls had about 25% of the amount of cellulose found in their NH counterpart, with no further change as the level of tolerance increased.

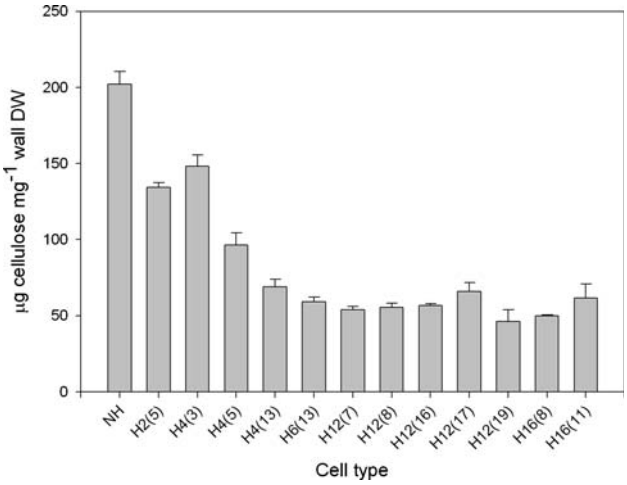

Fig. 5 Cellulose content of cell walls isolated from calluses at different DCB tolerance levels. NH, non-habituated callus; $H_x(n)$, habituated callus, where x DCB concentration (μM), and n number of subcultures in that concentration. Values are mean ± SD of three measurements

Cell wall fractionation and sugar analysis

Cell wall fractionation (Fig. 6) showed that the bulk of polysaccharides were extracted from the cell walls by alkali treatment: KOH-0.1 M and KOH-4 M fractions. These two fractions, plus the TFA fraction, accounted for about 90% of the total cell-wall sugar content. A net increase in the yield of sugars per dry cell wall weight was observed during the habituation to DCB (0.38 in NH vs. 0.48 in H12). This effect was mainly due to a notable increase in polysaccharides extracted with strong alkali (KOH-4 M fraction). The total amount of CDTA-extracted polysaccharides was reduced during habituation to DCB, while a slight increase in polysaccharides tightly bound to cellulose (TFA and snCR fractions) was detected. As regards the polysaccharides extracted with diluted alkali (KOH-0.1 M fraction), an increase in intermediate levels of tolerance was measured.

GC analysis of cell wall fractions (Fig. 7) showed that KOH-0.1 M and KOH-4 M fractions were composed mainly of Ara and Xyl followed by uronic acids, Gal and Glc, whereas minor fractions such as CDTA and snCR were composed of the same monosaccharides and enriched with uronic acids. The detected variations in the amount of sugar extracted in KOH-0.1 M and KOH-4 M and TFA fractions were paralleled by variations in Ara and Xyl. Especially important is the gradual enrichment in Ara and Xyl found in KOH-4 M and TFA fractions throughout the habituation process. The reduced levels of Glc reflect the poor levels of xyloglucan and mixed-linked glucan in these cell walls. These two polysaccharides are even further reduced after habituation (Table 3).

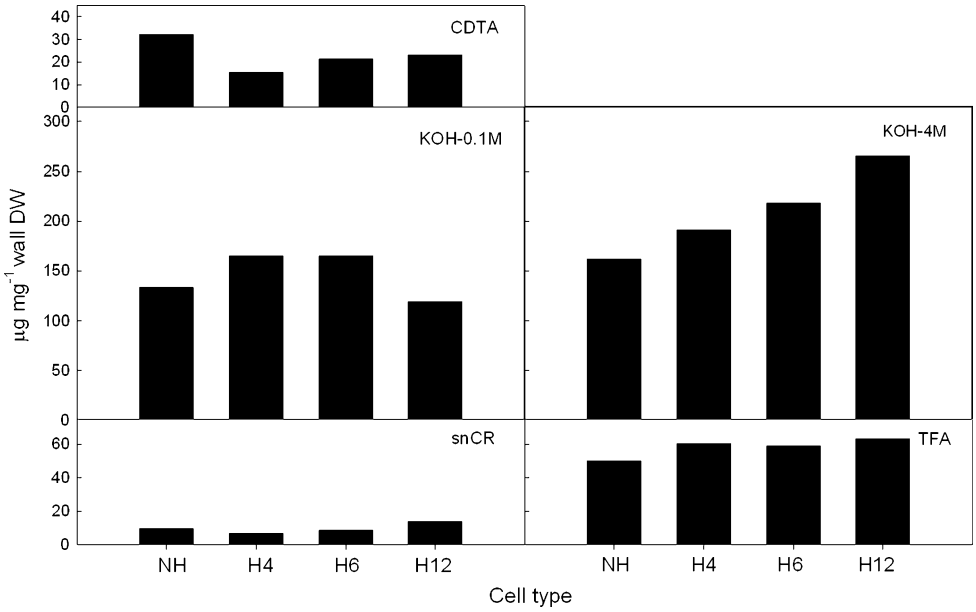

Fig. 6 Total sugars in cell wall fractions at different DCB habituation levels. NH, non-habituated calluses; H*x*, habituated calluses where *x* DCB concentration (μM). Calluses with at least eight subcultures in medium containing the indicated concentration of DCB were used. Results came from a representative experiment

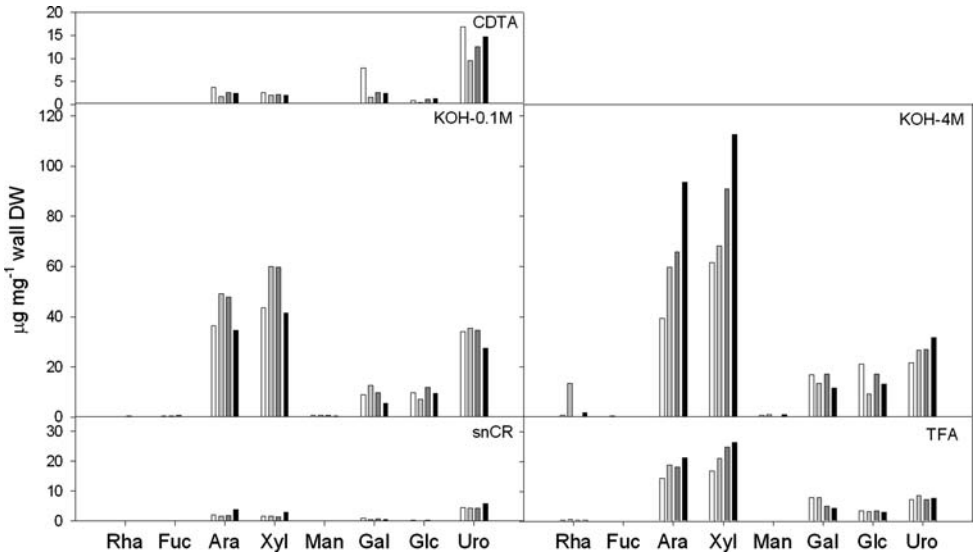

Fig. 7 Sugar composition of fractions from cell walls of NH (*open square*), H4 (*light grey square*), H6 (*dark grey square*) and H12 (*black square*) calluses. *Rha* rhamnose, *Fuc* fucose, *Ara* arabinose, *Xyl* xylose, *Man* mannose, *Gal* galactose, *Glc* glucose, *Uro* uronic acids

Table 3 Xyloglucan (XyG) and mixed-linked glucan (MLG) content of KOH fractions and crude cell walls, respectively

Cell type	XyG		MLG
	KOH-0.1 M	KOH-4 M	
NH	2.84 ± 0.19	19.93 ± 0.42	2.75 ± 0.12
H12	1.63 ± 0.07	12.01 ± 0.63	1.92 ± 0.09

Data units: μg mg^{-1} wall DW. Values are mean ± SD of three measurements

Therefore, a net enrichment in arabinoxylans, concomitant with a reduction in cellulose, was the main change observed in cell wall composition involved in DCB habituation.

Gel-permeation chromatography

Gel-permeation chromatography showed differences in the molecular mass of hemicellulosic polysaccharides (Table 4). Interesting changes took place in the 4 M KOH extractable polysaccharides (Fig. 8). At low and medium habituation levels the M_w of 4 M KOH extracted polysaccharides was reduced, but in the H12 fraction a significant increase took place; there was a net shift towards higher molecular masses and a new peak appeared close to the void volume. This peak was isolated and GC-analysed; Ara and Xyl accounted for about 78% (data not shown). In the KOH-0.1 M fractions, the opposite tendency was observed: H4 and H6 increased, whilst H12 increased only slightly (Table 4).

Table 4 Average molecular weight (M_w) of the hemicellulosic fractions

Cell type	KOH-0.1 M (kDa)	KOH-4 M (kDa)
NH	80.28	748.85
H4	446.11	298.18
H6	467.34	467.16
H12	134.75	2612.35

The M_w was obtained using the $K_{av(1/2)}$ method, with the calibration curve [$\log M_w = -4.999K_{av(1/2)} + 7.849$] obtained for this column

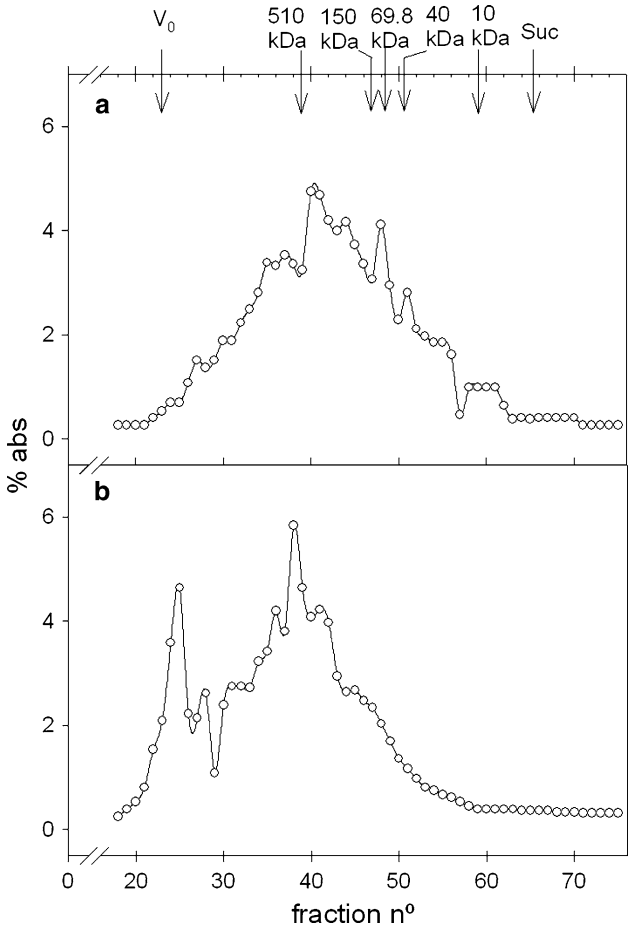

Fig. 8 Elution profiles of 4 M-KOH-extractable polysaccharides from NH (**a**) and H12 (**b**) calluses. *Markers* blue dextran (V_0), dextrans of 510, 150, 69.8, 40, 10 kDa, and sucrose

Phenolics analysis

Saponified phenolics were TLC subjected (Fig. 9). The main phenolic component released from NH cell walls was ferulic acid, followed by *p*-coumaric and 5,5-diferulic acid. Habituation to DCB clearly involved a shift from ferulic acid to *p*-coumaric acid enriched cell walls. Habituated cell walls also showed increased levels of 5,5, 8-*O*-4 and 8,5 diferulic acids and low R_f material probably corresponding to dimers, trimers or larger coupling products. The difference between mild and strong alkali treatment was not qualitative but quantitative both in NH and in H12 (data not shown).

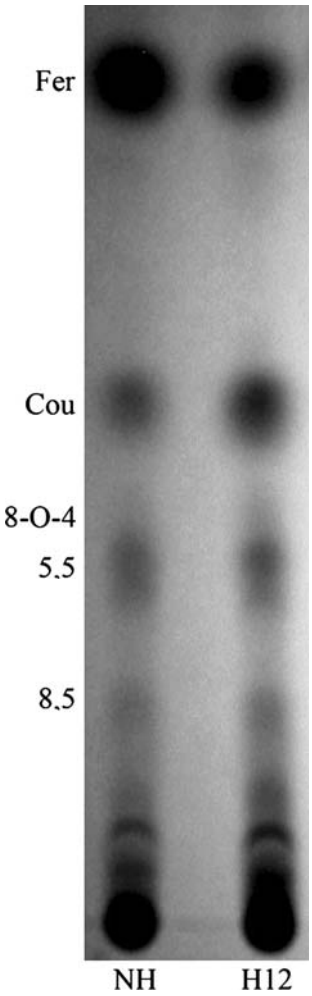

Fig. 9 TLC of phenolics alkali extracted from NH and H12 maize walls. Abbreviations in the TLC panel represent the position of migration of the following standards: (8,5), 8,5-di-ferulic acid; (5,5), 5,5-di-ferulic acid; (8-*O*-4), 8-*O*-4 di-ferulic acid; (Cou), *p*-coumaric acid; (Fer), ferulic acid

Protein content and amino acid composition of the cell wall

DCB habituation changed protein content and amino acid composition of cell walls (Table 5). The insoluble protein content of cell walls was reduced in H12 cells by 30%. Expressed as a percentage of total amino acid, an increase in Glu and a reduction in Asp, Pro, Tyr, and Phe was detected in H12 cell walls.

Cell wall immunoanalysis

Due to increased levels of arabinoxylans in habituated cells, cell walls were probed with LM10 (McCartney et al. 2005),

Table 5 Amino acid composition (mol%) of insoluble protein from cell walls

Amino Acid	Cell type	
	NH	H12
Asp	11.4	2.9
Glu	11.4	36.9
Hyp	2.3	1.7
Asn	4.2	1.1
Ser	0.9	0.6
Thr	3.0	2.6
Ala	10.0	7.6
Pro	3.7	1.7
Tyr	3.1	1.7
Val	18.3	15.4
Ile	3.7	3.4
Leu	4.5	5.5
Phe	6.5	3.7
Trp	9.1	6.8
Lys	3.0	4.5
Arg	4.8	3.6

Values represent the mean of two independent assays

specific for 1,4-β-xylans, and LM11 (McCartney et al. 2005) specific for xylans and arabinoxylans. LM11 epitope was only found in habituated cell walls (Fig. 10b), although the IDA showed that NH cell walls can also bind it, but clearly to a lesser degree (Fig. 11b, e). According to fractionation results, most LM11 labelling appears in the 4 M-KOH fractions. No epitopes for LM10 were found in any of the cell types probed (data not shown). Cell wall esterified feruloyl groups were probed with LM12 (Fig. 10c, d). LM12 epitope was found in both cell types, but the immunofluorescent labelling in H12 cells (Fig. 10d) seemed weaker than that of NH (Fig. 10c). IDA for LM12 (Fig. 11c, f) showed that most labelling was found in 0.1 M-KOH fractions, and was reduced in habituated ones.

In the case of AGPs, different results were found depending on the antibody used. Whereas no type of cell bound MAC207, both control and habituated cells bound LM2 (data not shown). The difference was in JIM8, where only NH cells bound it (Fig. 10e). IDAs for this antibody (Fig. 11a, d) showed that habituated cells had progressively lesser labelling as habituation level increased.

Cell wall degradability

NH cell walls were quickly and completely degraded by cell wall digesting enzymes, with a total sugar yield of 821 and 1,000 μg mg^{-1} being released after 6 and 72 h of incubation, respectively (Fig. 12). In the hydrolytic conditions assayed, H12 cell walls were significantly more resistant to enzymatic degradation than NH cell walls, and a reduction of 40% in the yield of sugars released was measured after 72 h of incubation. Thus, the modifications that take place during habituation seem to build a strengthened cell wall in response to DCB.

Discussion

Maize calluses have been successfully habituated to lethal DCB concentrations, by gradually increasing the concentration of the inhibitor in the culture medium. This habituation procedure led to progressive widespread changes in callus growth and morphology: habituated calluses grew more slowly, formed hard protuberances, and were darker and harder. Their cells were more irregularly shaped, with a thicker and more irregular cell wall, which contributed to a higher dry weight in proportion to total dry weight. All these characteristics resembled those of other previously described cellulose-inhibitor-habituated cell cultures, such as DCB-habituated tomato cell suspensions (Shedletzky et al. 1990), and DCB- or isoxaben-habituated bean calluses (Díaz-Cacho et al. 1999; Encina et al. 2001).

As far as we know, only one other example of a type II cell wall species (barley) habituated to DCB has been reported to date (Shedletzky et al. 1992). DCB-habituated barley cells also showed a reduced growth rate. However, they showed net differences when compared to DCB-habituated maize cells: their cells were not larger than controls, were more isodiametrically-shaped and did not possess thicker cell walls. These differences could be explained—at least partially—by taking into account the different callogenic origin of both cultures. Barley cell cultures were generated from endosperm tissue (Shedletzky et al. 1992) whereas our maize cells were generated from immature embryos.

It has been suggested previously that the mechanism of habituation to DCB and other cellulose biosynthesis inhibitors relies on the ability of the habituated cells to divide and expand under conditions where cellulose synthesis is inhibited. In fact, maize and barley habituated cultures showed cellulose reductions of up to 70–75%. Both DCB-habituated barley and maize cell cultures compensated for the reduction in cellulose with a higher quantity of hemicellulosic polysaccharides, while uronic acids hardly varied. However, the increment in hemicellulosic polysaccharides had different origins in the cultures: in DCB-habituated barley cells, the only polysaccharide where the proportion rose was mixed-linked glucan, which increased its content fourfold, to reach more than 100 μg mg^{-1} cell wall. However in DCB-habituated maize cells, the reduction in cellulose was paralleled by a net increase in arabinoxylans, whereas other hemicelluloses such as β-glucan and xyloglucan were reduced. Thus, we have proven for the first time that the architecture of type II cell walls is able to compensate for

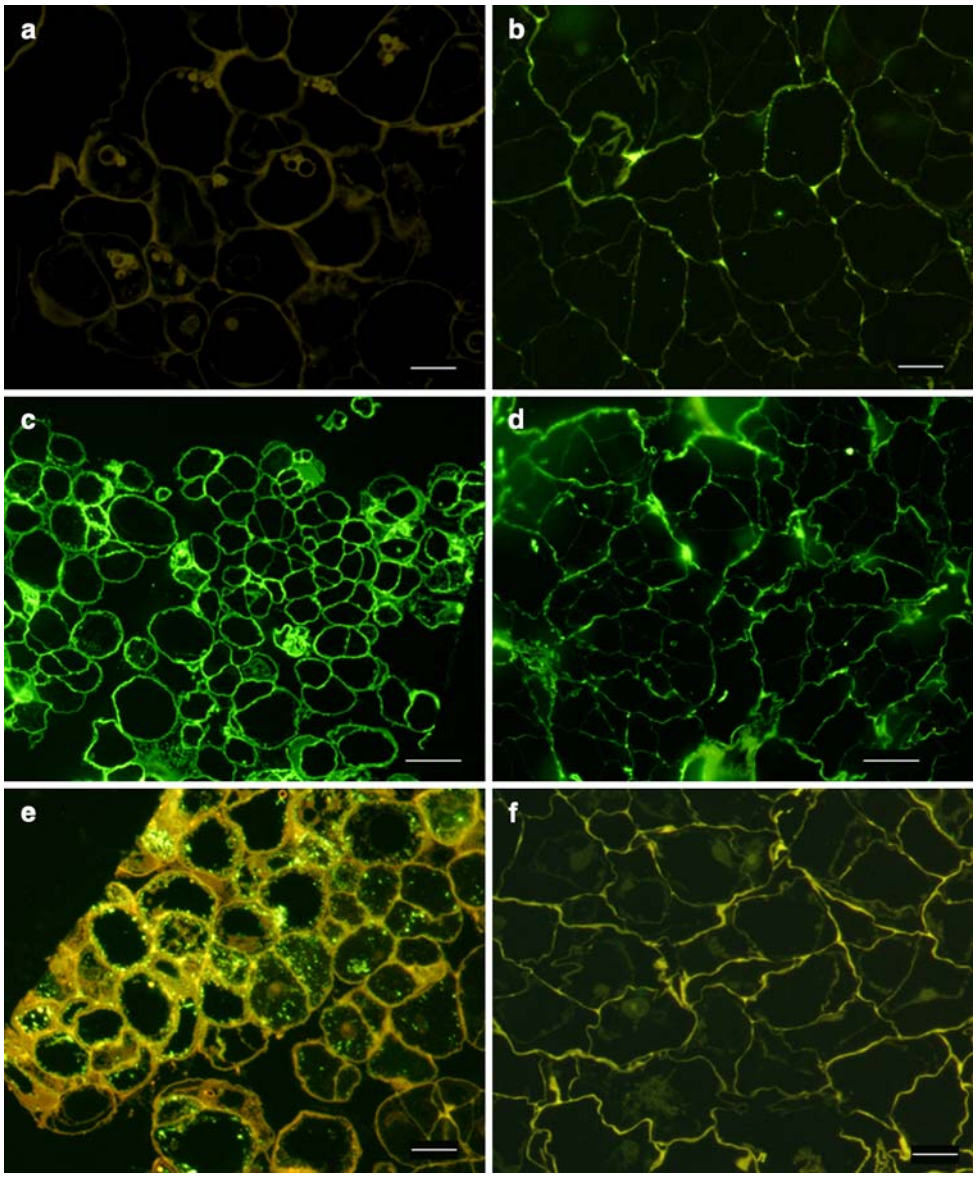

Fig. 10 Immunofluorescent localization of (1 → 4)-β-D-xylan/arabinoxylan (LM11: **a**, **b**), feruloylates (LM12: **c**, **d**) and arabinogalactan-proteins (JIM8: **e**, **f**) of NH (**a**, **c**, **e**) and H12 (**b**, **d**, **f**) maize cells. *Bars* 20 μm (**a**, **b**, **e**, **f**), 50 μm (**c**, **d**)

deficiencies in cellulose content without requiring a mixed glucan increment. In this modified architecture, the reduction in cellulose content is compensated for mainly by an increment in arabinoxylan content, whose characteristics appear modified in comparison with those of non-habituated cells.

The enhanced arabinoxylan content of habituated cells was appreciable by probing with LM11, an antibody that binds to xylan or arabinoxylan with a low degree of substitution (McCartney et al. 2005). LM11 labelling of habituated cell walls was much more intense than in non-habituated cell walls. Additionally, the LM11-binding pattern of habituated cell walls shows that LM11 epitopes are mainly localized in cell junctions, in thicker cell walls and in dispersed cell wall areas, pointing to particular cell wall strengthening in some areas.

Arabinoxylans in DCB-habituated maize cells also showed differences in extractability, mean molecular mass and in the formation of phenolic bridges, when compared with non-habituated cells.

Cell wall fractionation, GC and IDA for LM11-epitopes of KOH-extracted polysaccharides confirmed the reported net increase of AX in DCB-habituated cells, and showed a shift of AX from mild-alkali-extracted fractions (0.1 M-KOH) to strong-alkali-extracted fractions (4 M-KOH) and cellulose tightly bound fraction (TFA), pointing to a more extensive cross-linked hemicellulosic network.

Strong-alkali-extracted AX from habituated cell walls also showed a significant increase in M_w. This result can be explained by an increase in the polymerization and/or substitution degree of AX. This is an interesting result taking into account the fact that a major factor controlling AX

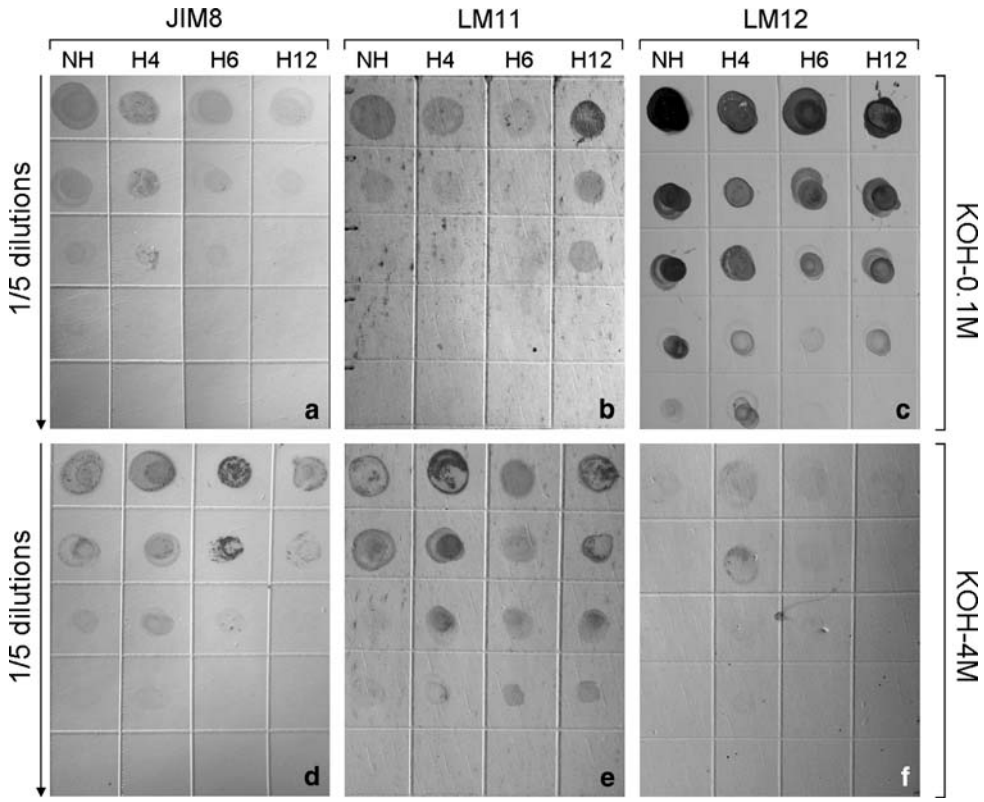

Fig. 11 Immunodot assays of KOH cell wall fractions from NH, H4, H6 and H12 calluses, probed with monoclonal antibodies with specificity for arabinogalactan proteins (JIM8: **a**, **d**), xylan/arabinoxylan (LM11: **b**, **e**) and feruloylates (LM12: **c**, **f**)

increase in M_w is their cross-linking through the formation of dehydroferulates, and that alkali treatment has been reported to prevent this by releasing ester bonded feruloyl groups (Kerr and Fry 2004). Therefore, an alternative explanation for this result could be the increment in alkali resistant phenolic bridges (ether-linked phenolic groups), which would render highly cross-linked AX even after the alkali treatment.

Phenolics have an important function in type II cell walls, as hydroxycinnamic acid derivatives contribute to wall assembly by cross linking polysaccharides through oxidative coupling. Therefore, we tested whether phenolics could contribute to the global tightening in our "stressed" cell walls. FTIR data pointed to an enhanced contribution of phenolics in DCB-habituated cells, as peaks assigned to phenolic ester (1,725 cm^{-1}) and aromatic rings (1,515, 1,600, 1,630 cm^{-1}) were more pronounced. Furthermore, TLC showed interesting changes in phenolic profiles: as described for DCB-habituated barley cultures, there was a shift from ferulic acid-rich walls to *p*-coumaric acid-rich walls (Shedletzky et al. 1992). In addition, in our DCB-habituated maize cells, an enrichment in 5,5 and 8,5 dehydroferulates, and in other compounds with low R_f, which could correspond to trimers or tetramers, was noticed. Therefore, these results indicated a general increase in hydroxycinnamic acid derivatives and in particular, in oxidative coupled derivatives. LM12 immunolocation showed that feruloyl groups were distributed mainly in cell wall areas next to plasmalemma. In conclusion, DCB-habituated maize cells not only showed enrichment in arabinoxylans: they also seemed to have a different GAX structure, together with an enrichment in phenolic compounds, which contributes to its cross-linking.

Other polymers do not seem to make a relevant contribution to modifications in the cell wall architecture of DCB-habituated maize cells. Mannose content was very low in both non-habituated and in habituated cells, so that mannan contribution to DCB-habituation was negligible. Xyloglucan levels were very low too, less than 20 μg mg^{-1} cell wall, and they were even lower in habituated cells. CDTA-extracted pectins were present in a low proportion (lower than 20 μg mg^{-1} cell wall) and did not undergo changes throughout the habituation process. Last, protein content was even lower than in their non-habituated counterparts, as was shown by FTIR spectroscopy, total Kjeldhal nitrogen determination, and immunochemical approaches. It is interesting to note that this reduction affected some groups of proteins, but not others: i.e. LM2 probed AGPs apparently did not vary, whereas JIM8 probed AGPs reduced, as was ascertained using immunolocation and IDAs.

According to Carpita's model (Carpita et al. 2001), type II cell walls are mainly constituted by two domains: a framework of cellulose microfibrils interlaced with tightly adherent β-glucans, GAX of low degrees of arabinosyl substitution and glucomannans; this is then embedded in a

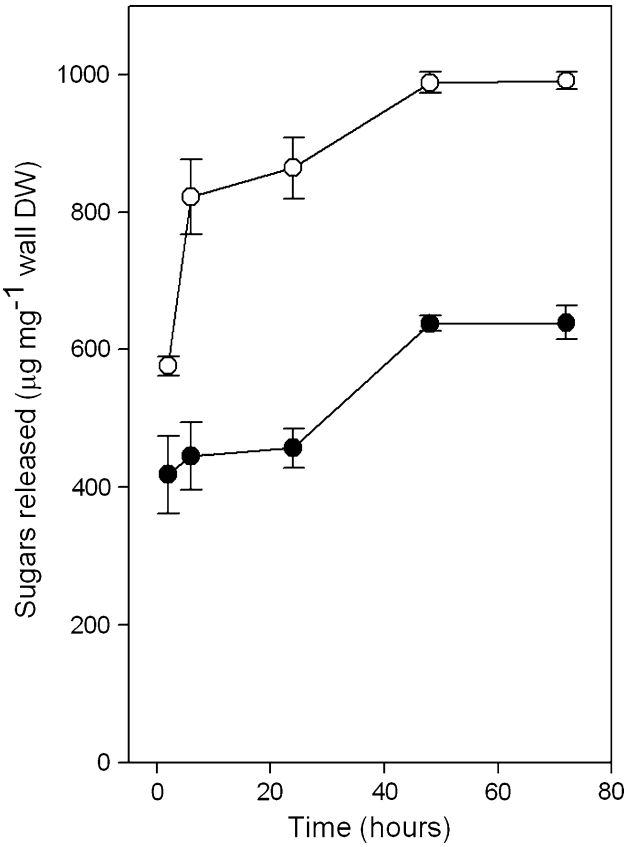

Fig. 12 Cell wall degradability of (*open circle*) NH and (*filled circle*) H12 calluses. Values are mean ± SD of nine measurements

matrix which provides an interstitial domain interconnecting the β-glucan-coated microfibrils, constituted by GAX, which is more highly substituted by arabinosyl residues, additional glucomannan and some pectins. According this model, cell wall porosity would be controlled by the content of GAX with a higher degree of arabinosyl substitution, taking into account that this polysaccharide constitutes the major pore-determining interstitial material between the microfibrils (Carpita et al. 2001). In an interesting experiment conducted in order to study xylanase penetration in wheat endosperm, Beaugrand et al. (2005) found complementary evidence that AXs acts to control pore size, as they observed that this penetration was intrinsically linked to AX degradation, and was facilitated by progressive cell wall disassembly. In DCB-habituated maize cells, cellulose microfibrils would be more interspersed by a larger interstitial AX, which determined the notable increment observed in their pore size, in comparison to non-habituated cells. In contrast, reported cell wall porosity of DCB-habituated barley cells was notably minor, this fact being consistent with a higher content in β-glucan, which would be interspersed between microfibrils, consequently reducing pore size between them.

The observed reduction in swelling capacity of DCB-habituated maize cell walls could be related both to the enrichment in their phenolic component—which would increase cell wall hydrophobicity—and to a hydrogen bonding increase due to AX enhancement, both in concentration and in molecular mass, in an opposite way as that described for expansin action: expansins would lead to an enhancement in cell wall swelling capacity breaking hydrogen bonds to release steric constraint of microfibril movement (Thompson 2005; Yennawar et al. 2006).

Another consequence of the modification in cell wall architecture associated with DCB habituation is the change in cell wall degradability. In this respect, the expectation would be that the reduction in crystalline cellulose and the increase in matrix polysaccharides would contribute to enhanced cell wall degradability. However, cell walls from DCB-habituated maize cells proved to be less susceptible to enzymatic hydrolysis than non-habituated cell walls. The most probable explanation for this result is related to a phenolic-enriched cell wall. Cell wall feruloylation, and particularly increased dimerization of ferulate, has been shown to reduce cell wall degradability by reducing matrix polysaccharide accessibility to hydrolytic enzymes (Grabber 2005 and references therein). Additionally, the enrichment in *p*-coumaric acid of our DCB-habituated cell walls would also contribute to a reduction in their degradability as has been previously reported for ruminal digestibility of some grasses (Burritt et al. 1984).

In summary, maize cell cultures from immature embryos have been successfully habituated to DCB. Habituated cells showed a modified cell wall architecture in which the significant reduction in cellulose content was compensated for by an increment in GAXs, which were of a higher molecular mass, more strongly cell wall bound, and more interlaced by means of phenolic bonds. Other cell wall components do not seem to play a significant role in the habituation process. In contrast to previously described type II cell wall architecture, in DCB-habituated maize cells induced to have reduced cellulose content, β-glucan does not fulfill an important role in the acquisition of functional type II cell wall architecture. As a consequence and in contrast to that previously described, the swelling capacity and wall digestibility of maize habituated cell walls was diminished, whereas pore size became bigger. Future studies need to be conducted in order to examine in greater depth the interaction between GAX and phenolics, and to ascertain the genetic control of these modifications in type II cell wall architecture.

Acknowledgments This work was supported by grants from Junta de Castilla y León (LE 048A07), University of León (ULE-2006-2) and Spanish Science and Innovation Ministry (CGL2008-02470/BOS), and a PhD grant from the FPU program of the Spanish Science and Innovation Ministry to H.M.M. We are grateful to Dr. Paul Knox

for his kind gifts of LM10, LM11 and LM12 antibodies, to Dr. Stephen C. Fry for his kind provision of maize cell cultures and to Denise Phelps for English language correction.

References

Albersheim P, Nevins PD, English PD, Karr A (1967) A method for the analysis of sugars in plant cell wall polysaccharides by gas liquid chromatography. Carbohydr Res 5:340–345

Alonso ML, Alvarez AI, Zapico J (1994) Rapid analysis of free amino-acids in infant foods. J Liq Chromatogr 17:4019–4030

Alonso-Simón A, Encina AE, García-Angulo P, Alvarez JM, Acebes JL (2004) FTIR spectroscopy monitoring of cell wall modifications during the habituation of bean (*Phaseolus vulgaris* L.) callus cultures to dichlobenil. Plant Sci 167:1273–1281

Baron-Epel O, Gharyal PK, Schindler M (1988) Pectins as mediators of wall porosity in soybean cells. Planta 175:389–395

Beaugrand J, Paes G, Reis D, Takahashi M, Debeire P, O'Donohue M, Chabbert B (2005) Probing the cell wall heterogeneity of micro-dissected wheat caryopsis using both active and inactive forms of a GH11 xylanase. Planta 222:246–257

Blumenkrantz N, Asboe-Hansen G (1973) New method for quantitative determination of uronic acids. Anal Biochem 54:484–489

Burritt EA, Bittner AS, Street JC, Anderson MJ (1984) Correlations of phenolic acids and xylose content of cell-wall with in vitro dry-matter digestibility of 3 maturing grasses. J Dairy Sci 67:1209–1213

Carpita NC (1996) Structure and biogenesis of the cell walls of grasses. Annu Rev Plant Physiol Plant Mol Biol 47:445–476

Carpita NC, Gibeaut DM (1993) Structural models of primary cell walls in flowering plants. Consistency of molecular structure with the physical properties of the walls during growth. Plant J 3:1–30

Carpita NC, Defernez M, Findlay K, Wells B, Shoue DA, Catchpole G, Wilson RH, McCann MC (2001) Cell wall architecture of the elongating maize coleoptile. Plant Physiol 127:551–565

Delmer DP (1987) Cellulose biosynthesis. Annu Rev Plant Physiol Plant Mol Biol 38:259–290

Díaz-Cacho P, Moral R, Encina A, Acebes JL, Alvarez J (1999) Cell wall modifications in bean (*Phaseolus vulgaris*) callus cultures tolerant to isoxaben. Physiol Plant 107:54–59

Dische Z (1962) Color reactions of carbohydrates. In: Whistler RL, Wolfrom ML (eds) Methods in carbohydrate chemistry. Academic Press, New York, pp 475–514

Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F (1956) Colorimetric method for determination of sugars and related substances. Anal Chem 28:350–356

Encina AE, Moral RM, Acebes JL, Alvarez JM (2001) Characterization of cell walls in bean (*Phaseolus vulgaris* L.) callus cultures tolerant to dichlobenil. Plant Sci 160:331–339

Encina A, Sevillano JM, Acebes JL, Alvarez J (2002) Cell wall modifications of bean (*Phaseolus vulgaris*) cell suspensions during habituation and dehabituation to dichlobenil. Physiol Plant 114:182–191

Fry SC, Willis SC, Paterson AEJ (2000) Intraprotoplasmic and wall-localized formation of arabinoxylan-bound diferulates and larger ferulate coupling-products in maize cell-suspension cultures. Planta 211:679–692

García-Angulo P, Willats WGT, Encina A, Alonso-Simón A, Alvarez JM, Acebes JL (2006) Immunocytochemical characterization of the cell walls of bean cell suspensions during habituation and dehabituation to dichlobenil. Physiol Plant 127:87–99

Grabber JH (2005) How do lignin composition, structure, and cross-linking affect degradability? A review of cell wall model studies. Crop Sci 45:820–831

Grabber JH, Ralph J, Hatfield RD (1998) Severe inhibition of maize wall degradation by synthetic lignins formed with coniferaldehyde. J Sci Food Agric 78:81–87

Iraki NM, Bressan RA, Hasegawa PM, Carpita NC (1989) Alteration of the physical and chemical structure of the primary cell wall of growth limited plant cells adapted to osmotic stress. Plant Physiol 91:39–47

Ishii T (1997) Structure and functions of feruloylated polysaccharides. Plant Sci 127:111–127

Kacurakova M, Wellner N, Ebringerova A, Hromadkova Z, Wilson RH, Belton PS (1999) Characterisation of xylan-type polysaccharides and associated cell wall components by FT-IR and FT-Raman spectroscopies. Food Hydrocoll 13:35–41

Kerr EM, Fry SC (2003) Pre-formed xyloglucans and xylans increase in molecular weight in three distinct compartments of a maize cell-suspension culture. Planta 217:327–339

Kerr EM, Fry SC (2004) Extracellular cross-linking of xylan and xyloglucan in maize cell-suspension cultures: the role of oxidative phenolic coupling. Planta 219:73–83

Kooiman P (1960) A method for the determination of amyloid in plant seeds. Recl Trav Chim Pays Bas Belg 79:675–678

Lorences EP, Fry SC (1991) Absolute measurement of cell expansion in plant cell suspension cultures. Plant Cell Tissue Organ Cult 24:211–215

McCartney L, Marcus SE, Knox JP (2005) Monoclonal antibodies to plant cell wall xylans and arabinoxylans. J Histochem Cytochem 53:543–546

McCleary BV, Codd R (1991) Measurement of $(1 \rightarrow 3),(1 \rightarrow 4)$-β-d-glucan in barley and oats. A streamlined enzymatic procedure. J Sci Food Agric 55:303–312

Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol Plant 15:473–497

Nakagawa N, Sakurai N (1998) Increase in the amount of celA1 protein in tobacco BY–2 cells by a cellulose biosynthesis inhibitor, 2, 6-dichlorobenzonitrile. Plant Cell Physiol 39:779–785

Nakagawa N, Sakurai N (2001) Cell wall integrity controls expression of endoxyloglucan transferase in tobacco BY2 cells. Plant Cell Physiol 42:240–244

Pennell R, Knox J, Scofield G, Selvendran R, Roberts K (1989) A family of abundant plasma membrane-associated glycoproteins related to the arabinogalactan proteins is unique to flowering plants. J Cell Biol 108:1967–1977

Pennell RI, Janniche L, Kjellbom P, Scofield GN, Peart JM, Roberts K (1991) Developmental regulation of a plasma membrane arabinogalactan protein epitope in oilseed rape flowers. Plant Cell 3:1317–1326

Sabba RP, Durso NA, Vaughn KC (1999) Structural and immunocytochemical characterization of the walls of dichlobenil-habituated BY-2 tobacco cells. Int J Plant Sci 160:275–290

Saeman JF, Moore WE, Millet MA (1963) Sugar units present. In: Whistler RL (ed) Methods in carbohydrate chemistry. Academic Press, New York, pp 54–69

Shedletzky E, Shmuel M, Delmer DP, Lamport DTA (1990) Adaptation and growth of tomato cells on the herbicide 2, 6-dichlorobenzonitrile leads to production of unique cell walls virtually lacking a cellulose-xyloglucan network. Plant Physiol 94:980–987

Shedletzky E, Shmuel M, Trainin T, Kalman S, Delmer D (1992) Cell wall structure in cells adapted to growth on the cellulose synthesis inhibitor 2, 6-dichlorobenzonitrile. A comparison between two dicotyledonous plants and a gramineous monocot. Plant Physiol 100:120–130

Smallwood M, Yates EA, Willats WGT, Martin H, Knox JP (1996) Immunochemical comparison of membrane-associated and secreted arabinogalactan-proteins in rice and carrot. Planta 198:452–459

Thompson DS (2005) How do cell walls regulate plant growth? J Exp Bot 56:2275–2285

Updegraff DM (1969) Semimicro determination of cellulose in biological materials. Anal Biochem 32:420–424

Vaughn KC (2002) Cellulose biosynthesis inhibitor herbicides. In: Böger P, Wakabayashi K, Hirai K (eds) Herbicide classes in development. Springer, Berlin, pp 139–150

Wells B, Mccann MC, Shedletzky E, Delmer D, Roberts K (1994) Structural features of cell walls from tomato cells adapted to grow on the herbicide 2, 6-dichlorobenzonitrile. J Microsc 173:155–164

Willats WGT, Gilmartin PM, Mikkelsen JD, Knox JP (1999) Cell wall antibodies without immunization: generation and use of de-esterified homogalacturonan block-specific antibodies from a naive phage display library. Plant J 18:57–65

Yennawar NH, Li LC, Dudzinski DM, Tabuchi A, Cosgrove DJ (2006) Crystal structure and activities of EXPB1 (Zea m 1), a β-expansin and group-1 pollen allergen from maize. Proc Natl Acad Sci USA 103:14664–14671

Unraveling the Biochemical and Molecular Networks Involved in Maize Cell Habituation to the Cellulose Biosynthesis Inhibitor Dichlobenil

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ABSTRACT The biochemical and molecular processes involved in the habituation of maize cells to growth in the presence of the cellulose biosynthesis inhibitor dichlobenil (DCB) were investigated. DCB affects the synthesis of cellulose both in active and stationary growth phases and alters the expression of several *CesA* genes. Of these, *ZmCesA5* and *ZmCesA7* seem to play a major role in habituating cells to growth in the presence of DCB. As a consequence of the reduction in cellulose, the expression of several genes involved in the synthesis of hydroxycinnamates is increased, resulting in cell walls with higher levels of ferulic and *p*-coumaric acids. A proteomic analysis revealed that habituation to DCB is linked to modifications in several metabolic pathways. Finally, habituated cells present a reduction in glutathione S-transferase detoxifying activity and antioxidant activities. Plant cell adaptation to the disturbance of such a crucial process as cellulose biosynthesis requires changes in several metabolic networks, in order to modify cell wall architecture and metabolism, and survive in the presence of the inhibitor. Some of these modifications are described in this paper.

Key words: Abiotic/environmental stress; acclimation—physiological; cell walls; maize; cellulose; dichlobenil; phenylpropanoid.

INTRODUCTION

Plant cells are surrounded by an extracellular matrix, the primary cell wall, which is involved in many important processes such as cell elongation, biotic/abiotic stress response, and cell shape maintenance (Ray et al., 1972). It is also believed to be responsible for an elaborate cell wall integrity mechanism (Hamann et al., 2009). The primary plant cell wall consists of cellulose microfibrils embedded in a network of matrix polysaccharides (hemicelluloses and pectins) and structural proteins, the nature and proportions of which differ according to each plant species (Carpita and Gibeaut, 1993), organ, cell type within a tissue, cell development phase, and even location within a single cell (Knox, 2008). Most plant species (all dicots and some monocots) have type-I primary cell walls, where xyloglucan, homogalacturonan, and rhamnogalacturonan I comprise the principle constituents of matrix polysaccharides (reviewed by Scheller and Ulvskov, 2010). Graminaceous plants (such as maize) and other commelinoid monocots have a cell wall, called type-II, the architecture and composition of which differ markedly from that characteristic of other angiosperms. In type-II cell walls, the role within the cell wall of the above cited polysaccharides is replaced by (glucurono) arabinoxylans and mixed-linked glucan (Carpita, 1984; Burton and Fincher, 2009).

Compared with type-I, type-II cell walls contain higher amounts of hydroxycinnamates or cell wall phenolics. These phenolics, mainly ferulic acid and *p*-coumaric acid, are found substituting arabinoxylans by ester-linking α-L-arabinosyl residues (Smith and Hartley, 1983; Wende and Fry, 1997). Even in type-II cell walls, phenolics are minor components of the cell wall; however, their contribution to cell wall structure is crucial. It has been demonstrated by *in vivo* experiments that ester-linked hydroxycinnamates can undergo oxidative-coupling cross-linking adjacent arabinoxylan molecules (Fry et al., 2000; Fry, 2004; Parker et al., 2005; Burr and Fry, 2009). By means of its polysaccharide cross-linking activity, phenolic-coupling

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doi: 10.1093/mp/ssq027
Received 23 February 2010; accepted 20 April 2010

regulates, mediates, or alters a number of cell wall properties, contributing to cell wall assembly, causing cell wall stiffening and growth cessation, promoting tissue cohesion, strengthening cell wall structure in response to biotic and abiotic stresses, and limiting cell wall biodegradability (Buanafina, 2009). Ferulic and *p*-coumaric acids are synthesized through the phenylpropanoid pathway (Vogt, 2010). The first step of this route is the deamination of L-phenylalanine by phenylalanine ammonia lyase (PAL) to cinnamic acid. Subsequent enzymatic steps catalyzing hydroxylations and methylations produce feruloyl-CoA, which is ester-linked to arabinoxylans (Fry et al., 2000; Lindsay and Fry, 2008). Recently, it has been demonstrated in rice that members of the pfam gene family may act as arabinoxylan feruloyl transferases (Piston et al., 2010).

Cellulose is synthesized at the plasma membrane by an enzymatic complex and is deposited directly into the cell wall in a directional manner (Somerville, 2006; Mutwil et al., 2008; Taylor, 2008), undergoing a dynamic reorientation following deposition that enables its anisotropic expansion (Anderson et al., 2010). Cellulose biosynthesis machinery is located in the plasma membrane, forming 'rosettes' or cellulose synthase (CesA) complexes. In plants, CesA complexes are organized as hexamers, presumably consisting of 36 individual CesA proteins and some other proteins. It is thought that the CesA complexes are assembled in the Golgi apparatus and then exported to the plasma membrane via exocytosis (Somerville, 2006). *Arabidopsis* (type-I cell wall) and maize (type-II cell wall) have 10 and 12 *CesA* genes, respectively (Holland et al., 2000; Richmond, 2000; Appenzeller et al., 2004).

Characterization of mutants affecting CesA1 and CesA3 proteins demonstrated that these two proteins are essential to production of cellulose in *Arabidopsis* primary walls (Desprez et al., 2007; Persson et al., 2007; Daras et al., 2009). Other *Arabidopsis* CesA, such as CesA2, CesA5, CesA6, and CesA9, are also involved in primary cell wall formation but their functions are partially redundant (Desprez et al., 2007; Persson et al., 2007). In contrast, characterization of *Arabidopsis* mutants for *AtCesA4* (*IRX5*: irregular xylem 5), *AtCesA7* (*IRX3*), and *AtCesA8* (*IRX1*) revealed that these three proteins are essential for secondary cell wall formation (Taylor et al., 1999, 2000, 2003; Ha et al., 2002) and do not affect cellulose biosynthesis in primary cell walls (Turner and Somerville, 1997; Ha et al., 2002).

As cellulose represents the main load-bearing polysaccharide in cell walls, the habituation of plant cell cultures to lethal concentrations of cellulose biosynthesis inhibitors has emerged as a valuable tool for the study of important mechanisms involved in plant survival, such as cell wall plasticity (both structural and compositional) to cope with cell wall integrity disrupting factors (Acebes et al., 2010).

Although the basic mechanism of habituation is common (a replacement of the cellulose network for other cell wall components), the details of the process depend on the type of cell wall and thus it has been demonstrated that cells habituate to cellulose biosynthesis inhibitors by using different strategies (Acebes et al., 2010 and references therein). In *Arabidopsis*, isoxaben habituation does not appear to be mediated by stress response processes, nor by functional redundancy within the *CesA* family (Manfield et al., 2004). Amongst the cellulose synthase superfamily, *CslD5* (*cellulose synthase-like D5*) is highly up-regulated and it might play a role in the biosynthesis of the walls of habituated cells (Bernal et al., 2007). Dehabituation is a feasible strategy to unravel those habituation-related components that are stable (i.e. those changes putatively associated with mutations or epigenetic changes in DNA). Thus, it has been demonstrated that most of the cell wall changes induced during habituation revert when cells are dehabituated by culturing them in a medium without the inhibitor (Shedletzky et al., 1990; Encina et al., 2002; García-Angulo et al., 2006, 2009; Alonso-Simón et al., 2010). However, dehabituated cell cultures retain some habituation-induced modifications. In the case of bean cell cultures, the study of DCB dehabituated cells proved that habituation to DCB relied both in reversible (those affecting to cell wall composition and structure) and stable changes (a high guaiacol-type peroxidase activity) (García-Angulo et al., 2009).

Maize cell cultures have been habituated to lethal concentrations of DCB (12 μM; H12) (Mélida et al., 2009). DCB-habituated cells present modified cell wall architecture with a strong reduction in cellulose that is compensated, at least partially, by a more extensive network of highly feruloylated arabinoxylans in the stationary growth phase.

In this paper, we describe the effect of the herbicide DCB on non-habituated, habituated, and dehabituated maize cells at biochemical and molecular levels. In the habituated cells, differences associated with the cell-culture stage were studied. We analyzed the effect of DCB on the expression of genes involved in the biosynthesis of cellulose and hydroxycinnamates. We also applied a proteomic approach to detect changes in the overall metabolism of maize cells habituated to DCB. Based on these results, we also determined detoxifying and antioxidant enzymatic activities associated with DCB habituation.

RESULTS

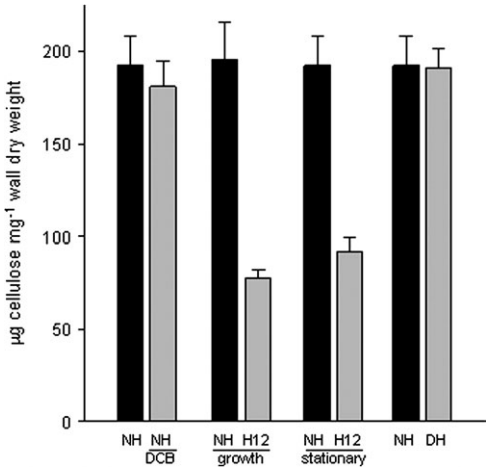
DCB Habituation Process Implies a Reduction in the Cellulose Content of Maize Cells

The effect of DCB on cellulose content was assayed in different maize cell cultures (Figure 1). Our results show that cellulose content was not significantly affected when maize cultures were incubated with DCB for a brief period of time (NH/DCB) compared to non-treated cultures (NH). However, in H12 cultures, a strong reduction in cellulose content was observed: 61% in active growth phase and 53% in stationary phase compared with NH cultures. No evidence of cellulose loss during cell wall isolation was found, indicating that the reduction in cellulose content in H12 culture was not due to its loss during the experimental procedure.

Our results also show that the reduction in cellulose can be reversed when DCB is subsequently removed for a long period (more than a year) from the culture media (DH cultures).

We also tested the ability of DH cultures to cope with lethal concentrations of DCB, expressed as the concentration of DCB required to inhibit fresh weight gain by 10% (I_{10}), 50% (I_{50}), and 90% (I_{90}) with respect to the control (Figure 2).

The I_{10}, I_{50}, and I_{90} values of the DH cultures were comparable to those of the NH cultures, but very dissimilar to those of H12 cultures (about 10 times higher), indicating that DH cultures incubated with DCB display behavior similar to maize cultures that have never been cultured in the presence of DCB.



[AQ2] **Figure 1.** Cellulose Content of Cell Walls Isolated from Non-Habituated (NH), Habituated to 12 µM DCB (H12; Collected in the Active Growth or in the Stationary Phase), Dehabituated (DH; in the Stationary Phase), and Non-Habituated Supplemented with DCB for 5–6 d (NH/DCB) Maize Cell Cultures Analyzed by the Updegraff Method.
Values are means ± S.D. of three measurements. Growth measured as relative increase in fresh weight of the cells was reduced by 38.9 and 12.4% in H12 and DH cells, respectively, with regard to NH.

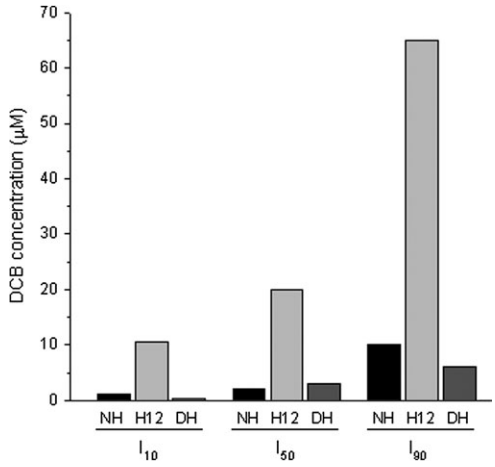


Figure 2. Effect of DCB on the Growth of Non-Habituated (NH), Habituated to 12 µM DCB (H12), and Dehabituated (DH) Maize Cell Cultures Expressed as the Concentration of DCB (µM) Required to Inhibit Weight Increase by 10% (I_{10}), 50% (I_{50}), and 90% (I_{90}) with Respect to Control.

DCB Affects the Expression of Some *CesA* Genes

As DCB strongly reduces cellulose content in H12 cultures, we further investigated whether the expression of the maize *CesA* genes is affected (Figure 3), using RT–PCR. Our results show that although the cellulose content was not altered in maize cells treated briefly with DCB (NH/DCB), the presence of this inhibitor down-regulated *ZmCesA3*, *5*, *8*, and induced *ZmCesA7*. However, in the habituated H12 cultures, in which a strong decrease in total cellulose content was observed, *ZmCesA1/2*, *3*, *7*, and *8* were induced in the growth phase, while *ZmCesA7* continued to be induced and *ZmCesA5* repressed in the stationary phase. Finally, in DH cultures, with cellulose content similar to that of the non treated cells, only the expression of *ZmCesA5* gene was induced. *ZmCesA4*, *9*, *10*, *11*, and *12* were also analyzed, but no mRNA was detected in these maize cells.

It has been shown that a mutation in the maize *ZmBk2* gene implies a severe reduction in cellulose content (Ching et al., 2006). We therefore analyzed whether the presence of DCB could also affect the expression of the *ZmBk2L3* gene, as it is the only member of the family expressed during primary cell wall biosynthesis (Brady et al., 2007). In this case, our results indicate that the expression of *ZmBk2L3* gene was not affected by the presence of DCB in the culture media.

Recently, a microtubule-associated protein MAP20 in secondary cell walls of hybrid aspen has been reported as a target for DCB (Rajangam et al., 2008). It was demonstrated that DCB specifically binds to MAP20 during cellulose synthesis in secondary walls. Based on this research, we analyzed the expression of a putative *ZmMAP20* gene but no mRNA could be detected in maize cell cultures, which only have primary cell wall.

DCB Affects Cell Wall Phenolics Content

In addition to the cellulose content, we have also analyzed the effect of DCB on cell wall phenolic content. Our results show that a short-term DCB treatment (NH/DCB) was sufficient to produce quantitative modifications in the content of cell wall-esterified phenolics (Figure 4), resulting in a decrease in ferulic acid and a strong increase in *p*-coumaric acid compared to the NH cultures. In H12 cultures, an increase in both ferulic and *p*-coumaric acids content was measured, showing that H12 cell walls are phenolic-enriched compared to control cells. Finally, our results show that DH cultures had a slightly higher amount of *p*-coumaric acid but showed a reduction in ferulic acid compared to non-treated cells.

DCB Affects the Expression of Genes Involved in the Phenylpropanoid Pathway

As DCB habituation in H12 maize cultures involves a shift from ferulate to coumarate-rich cell walls and an enrichment in cell wall-esterified hydroxycinnamates and dehydroferulates (Mélida et al., 2009), we also analyzed the expression of the phenylpropanoid genes involved in the production of *p*-coumaric and ferulic acid, and feruloyl-CoA (Figure 5A).

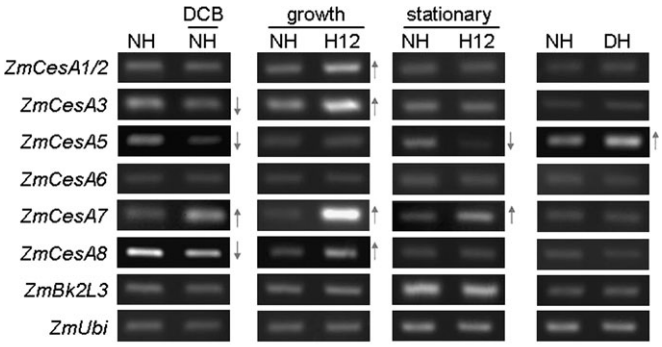


Figure 3. Relative *ZmCesA* Gene Expression Analyzed by RT–PCR of Different Maize Cell Cultures and Culture Phases (Key as in Figure 1). ↑, more mRNA accumulation than control (NH); ↓, less mRNA than control. *ZmCesA4*, *ZmCesA9*, *ZmCesA10*, *ZmCesA11*, *ZmCesA12*, and *ZmMAP20* were not detected. *ZmMAP20*, microtubule-associated protein 20; *ZmBk2L3*, brittle stalk 2 Like 3.

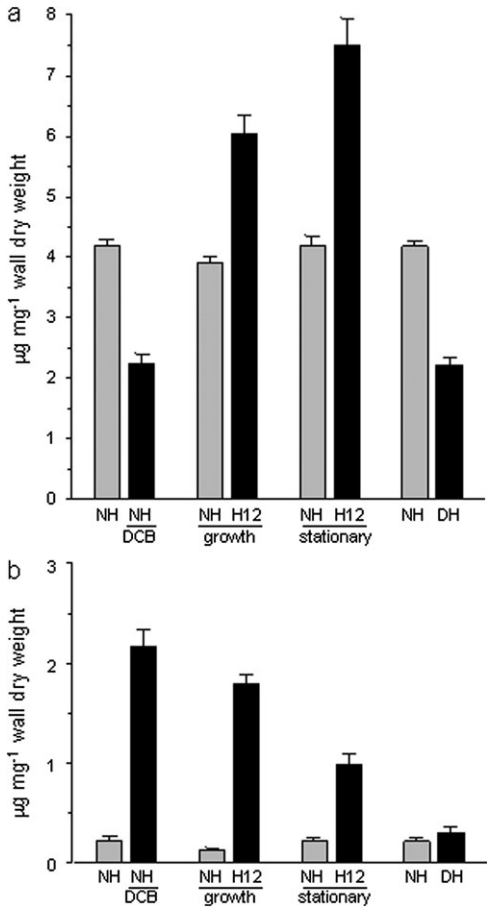


Figure 4. Ferulic **(A)** and *p*-Coumaric Acid **(B)** Content of Cell Walls Isolated from Different Maize Cell Cultures (Key as in Figure 1) Released by Treatment with 1 M NaOH and Measured by HPLC–PAD. Values are mean ± SD of three measurements.

Results show that the majority of these genes were induced in the H12 cultures during the growing stage, with the exception of *CCoAOMT*, which was repressed (Figure 5B). When these H12 cultures reached the stationary phase, all the genes analyzed were repressed, with the exception of *COMT*, which was induced.

In the short-term DCB-treated cultures (NH/DCB), several genes were also induced. In contrast, in the dehabituated cells, only 4CLc and both methyl transferases (COMT and CCoAOMT) were induced.

Overall Effects of DCB on the Metabolism of the Habituated Cells

In order to attain an overall idea of the effect of DCB on the maize cell habituation process, we compared the proteome of the H12 and NH cell cultures. The 2-DE-based proteomics approach enabled us to compare 906 proteins from the total amount initially isolated (1445 for NH versus 1217 for H12) (Supplemental Figure 1). Twenty-seven proteins were identified in only one of the two cell lines (19 absent and eight present only in H12) and 44 proteins were found to be misregulated (20 down-regulated and 24 up-regulated in H12). From all the sequenced proteins, we were able to identify 15 proteins (Table 1). Habituation induced carbohydrate metabolism (enolase 1 and glyceraldehyde-3-phosphate dehydrogenase) and some stress-related proteins (peroxidase and heat shock protein), and repressed some nitrogen and ethylene metabolism proteins (3-isopropylmalate dehydrogenase 2, glutamine synthetase 2, 1-aminocyclopropane-1-carboxylate oxidase 1). Surprisingly, some proteins, such as glutathione S-transferases (GST), which are commonly thought to be involved in herbicide detoxification processes, were repressed in H12 cells compared to the control NH cells.

Glutathione S-Transferase, Glutathione Reductase, and Catalase Activities Are Reduced in the Maize H12 Cells

In order to confirm the proteomic results indicating that GST enzymes were repressed in H12, we analyzed the GST activity spectrophotometrically. Our results confirmed that GST activity was reduced in H12 cells compared to the control NH cells (Figure 6). In addition to GST activity, we also analyzed several antioxidant activities (summarized in Figure 6). Results obtained indicate that antioxidant activities such as guaiacol-type peroxidase (GPOX) and ascorbate peroxidase (APOX) did not significantly change in H12 cells. Furthermore, these H12 cells presented a reduction in antioxidant activities, such as glutathione reductase (GR) and catalase (CAT) activities. In contrast, a short-term incubation of NH cells in the presence of DCB rendered an increase in all activities tested (Figure 6).

DISCUSSION

Maize calluses were habituated to lethal concentrations of the DCB herbicide by gradually increasing the concentration of the inhibitor in the culture medium. It has previously been suggested that the mechanism of habituation to DCB and other cellulose biosynthesis inhibitors relies on the ability of the habituated cells to divide and expand under conditions in which

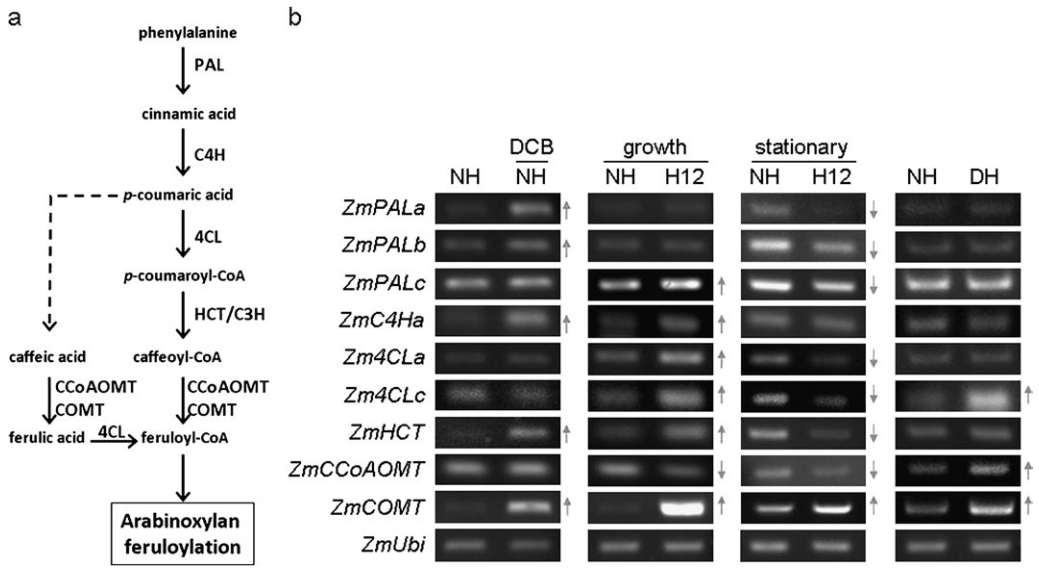


Figure 5. (A) Phenolic biosynthetic pathway, from phenylalanine to arabinoxylan feruloylation. Dashed arrows indicate putative pathways. **(B)** Relative expression of the genes involved in the synthesis of phenolic compounds of different maize cell cultures (key as in Figure 1). ↑ more mRNA content than control; ↓, less mRNA content than control; PAL, Phenylalanine Ammonia-Lyase; C4H, Cinnamate 4-Hydroxylase; 4CL, 4-Coumarate CoA Ligase; C3H, 4-coumarate 3-hydroxylase; HCT, Hydroxycinnamoyl-CoA shikimate/quinate hydroxycinnamoyl Transferase, CCoAOMT, Caffeoyl-CoA O-MethylTransferase; COMT, Caffeic acid O-MethylTransferase.

Table 1. Identification of Proteins by MALDI–TOF/MS or by LC-nanoESI-Q-TOF–MS/MS after Spot Excision of 2-D Gels, with an Indication of their Theoretical and Experimental Isoelectric Point and Molecular Mass.

Protein group	Protein name (accession no.) (spot no.)	Theoretical pI/MM	Experimental pI/MM
Present only in H12	Peroxidase 52 precursor (ACG45093) (1)	6.86/35 350	–/37 055
	Heat shock protein 70 (CAA47948) (2)	5.10/71 517	–/–
	Translation initiation factor 5A (NP_001105606) (3)	5.61/17 714	–/20 552
	Elongation factor 1-alpha (P17786) (4)	9.19/49 599	–/59 693
Induced in H12	Glyceraldehyde-3-phosphate dehydrogenase (CAC80387) (5)	9.01/45 751	5.70/38 442
	Enolase1 (NP_001105896) (6)	5.20/48 262	5.17/36 871
	Translation initiation factor 5A (NP_001105606) (7)	5.61/17 714	5.96/19 911
	Putative xylanase inhibitor (BAB89707) (8)	7.52/38 873	5.16/38 408
Repressed in H12	Glutathione S-transferase2 (NP_001105366) (9)	5.77/24 726	5.81/25 640
	3-isopropylmalate dehydrogenase 2 (ACG41069) (10)	5.62/43 142	4.90/53 129
	Glutamine synthetase1 (NP_001105725) (11)	6.42/46 300	5.34/49 092
	1-aminocyclopropane-1-carboxylate oxidase (ACG35410) (12)	4.99/34 753	5.15/41 100
Absent in H12	Glutathione S-transferase6 (ACG38008) (13)	5.52/25 750	5.63/25 049
	Glutathione S-transferase7 (NP_001105593) (14)	5.29/25 414	–/27 463

cellulose biosynthesis is inhibited (Vaughn, 2002), but the molecular changes involved in habituation to DCB are poorly understood at present.

In this paper, we show that H12 cell walls present a reduction of more than 50% in cellulose content in the stationary phase, similar to that reported in previous research (Mélida et al., 2009), and that in addition, they also present a strong reduction in cellulose (more than 60%) during the active growing phase. However, when the herbicide was removed from the culture media, maize cells were able to restore normal levels of cellulose. A similar behavior was observed with DCB-habituated tomato (Shedletzky et al., 1990) and bean cells (Encina et al., 2002; García-Angulo et al., 2006), indicating that plant species having a type-I (tomato and bean) or type-II (maize) wall share some common mechanisms resulting from the removal of the herbicide in the culture media.

It was also reported in a type-I plant specie such as bean that dehabituated cells retain the capacity to cope with lethal

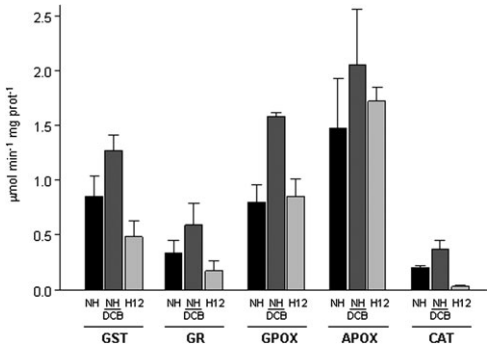


Figure 6. Activity of Glutathione S-Tranferase (GST), Glutathione Reductase (GR), Guaiacol-Type Peroxidase (GPOX), Ascorbate Peroxidase (APOX), and Catalase (CAT) in Non-Habituated (NH), Habituated to DCB (H12), and Non-Habituated Supplemented with DCB (NH/DCB) Maize Cultured Cells.
Values are means ± SD (n = 10) and are expressed as µmol min^{-1} mg $prot^{-1}$.

concentrations of DCB (Encina et al., 2002; García-Angulo et al., 2009). Interestingly, we show here that this is not the case for type-II, as when the dehabituated cells were newly incubated with DCB, they presented a similar behavior to that of non-habituated cells.

During the active growing phase of the H12 cultures, maize cells induced the expression of several *CesA* genes: *ZmCesA1/2*, *3*, phylogenetically grouped with the *Arabidopsis thaliana CesA1* gene involved in the synthesis of cellulose in the primary cell wall. Interestingly, in tobacco cells habituated to 1 µM DCB, a higher amount of the protein celA1 was found, also homologous to AtCesA1 (Nakagawa and Sakurai, 1998). H12 cultures also induced the expression of *ZmCesA7* and *8*, grouped with *Arabidopsis thaliana* CesA proteins involved in the synthesis of cellulose in the primary to secondary cell wall transition (Appenzeller et al., 2004; Taylor et al., 2008). In addition to AtCesA1, AtCesA3 is also involved in primary cell wall cellulose biosynthesis (Desprez et al., 2007; Persson et al., 2007). However, expression of maize *CesA* genes most closely related to *AtCesA3* was not detected (*ZmCesA4* and *9*) or was not altered (*ZmCesA5*) in the active growing phase of H12 cells.

Once cells stopped growing (stationary growth phase), the most CesA gene expression was similar to that of control cells, with the exception of *ZmCesA5*, which remained repressed, and *ZmCesA7*, which continued to be induced. Thus, and bearing in mind that caution must be taken when results of gene expression are extrapolated to protein levels, it could be suggested that ZmCesA5 is, at least partially, replaced by ZmCesA7 in the rosettes. As cellulose requirements increase (active growing phase), other CesA proteins (ZmCesA1/2, ZmCesA3, and ZmCesA8) may also replace ZmCesA5 in the rosettes. As a consequence of (partially) removing ZmCesA5 from the rosettes, maize cells would be able to grow in the presence of the herbicide. A similar behavior occurs in *Arabidopsis*, as when AtCesA3 (the most closely related protein to ZmCesA5) is removed from the rosettes in plants mutated for this gene (*ixr1-1* and *ixr1-2*), these plants become more resistant to the cellulose biosynthesis inhibitor isoxaben (Scheible et al., 2001). In isoxaben-habituated cells, not only AtCesA3, but also AtCesA1, and 6 were down-regulated (Manfield et al., 2004).

It is noteworthy that the expression of *ZmCesA5* was induced in dehabituated maize cells, even when maize cells had been grown without DCB for at least 1 year. However, when dehabituated cells were newly incubated with DCB, they were similarly or slightly more sensitive to the herbicide than control cells. In this case, the increased amount of ZmCesA5 did not affect the total content of cellulose. Thus, this result reinforces the idea that the presence/absence of ZmCesA5 within the rosette could be a critical aspect determining the sensitivity/resistance of the cells to growth in the presence of DCB in the culture media.

Recently, a Microtubule Associated Protein (MAP20) has been reported as a target for DCB in secondary cell walls of hybrid aspen (Rajangam et al., 2008). Linking up MAP20 function with DCB effects, it was proposed that MAP20 has a specific role in cellulose biosynthesis by coupling CesA proteins with microtubules, and that DCB inhibits cellulose biosynthesis by decoupling cellulose synthesis and microtubules through MAP20 inactivation (Rajangam et al., 2008). The connection between microtubules and cellulose synthesis has been proved in recent years (Paredez et al., 2006; Gutierrez et al., 2009). However, the expression of *ZmMAP20* was not detected in maize cultured cells, indicating that ZmMAP20 could be the target of DCB during secondary cell wall synthesis but not during primary cell wall formation.

Mutations in a maize brittle-stalk (*ZmBk2*) gene strongly reduce the total cellulose content in the secondary cell wall (Ching et al., 2006). However, the only member of this family that is expressed in cells producing primary walls (*ZmBk2L3*) (Brady et al., 2007) was not affected in the maize cultures analyzed in this research, suggesting that this gene is not involved in any DCB responses in this case.

Maize cells habituated to DCB presented a significant increase in arabinoxylans (Mélida et al., 2009). Here, we show that the phenolic compounds (mainly ferulic acid, but also *p*-coumaric acid) involved in the arabinoxylans cross-link increased in the H12 cell walls. This supports the idea that a more cross-linked network of arabinoxylans is acting as a mechanism to counteract, at least partially, the reduction of cellulose in the H12 cell walls.

Although cells dehabituated to DCB were able to restore the normal levels of cellulose, they presented a significant reduction in ferulic acid in the cell walls, suggesting that some metabolic modifications persist even when DCB has not been present for more than 1 year in the culture media.

Application of DCB to non-habituated cells caused a strong increase in *p*-coumaric acid, suggesting that *p*-coumaric acid enrichment is a response to the toxicity of DCB rather than a mechanism involved in the habituation process. This is in agreement with previous research showing a relationship between *p*-coumaric acid and cellular stresses (Zanardo et al., 2009).

The enrichment in phenolics in H12 cell walls was bound to a global induction of the phenylpropanoid genes involved in the synthesis of these compounds in the active growth phase. However, in the stationary phase, when the cell wall had already been tightened, gene expression levels involved in this metabolic pathway were repressed.

Among the three maize *PAL* genes analyzed, it is interesting to note that *ZmPALa* and *ZmPALb* genes seemed to be involved in short-term response while *ZmPALc* gene may act in the long-term habituation process. Whereas in control cells, ZmCCoAOMT seems to be the main methyl-transferase, our results suggest that ZmCOMT partially replaced ZmCCoAOMT when DCB was present in the culture media.

Plant reaction against DCB stress may involve a wide variety of biochemical and physiological adaptations. In accordance with this idea, habituation to DCB implies an induction of several proteins: a translation initiation factor 5A that has been shown to be involved in RNA transport and metabolism, regulating cell proliferation, growth and programmed cell death (Feng et al., 2007) and a heat shock protein 70, considered to play a role as a biochemical stress indicator (Tomanek and Sanford, 2003). The fact that H12 cells presented an induction of a putative xylanase inhibitor is in line with the fact that the H12 cells contained higher xylan levels compared to control cells (Mélida et al., 2009). Similarly, an increase in peroxidase activity related to the increased DCB resistance of DCB-dehabituated bean cells has also been reported (García-Angulo et al., 2009). In line with this result, maize H12 cells showed an induction of the peroxidase 52 precursor, indicating that this peroxidase activity could play an important role in maize cell habituation to the herbicide.

Enolase1 and glyceraldehyde-3-phosphate dehydrogenase were induced in H12 cells. These enzymes catalyze the reversal conversion of hexose glucose to pyruvate and it has been reported that hexoses can function as stress indicators when cell wall integrity is impaired (Hamann et al., 2009). Therefore, our results suggest that these two enzymes could play a role as indicators of DCB stress.

Among the proteins that were repressed in H12 cells, two enzymes involved in nitrogen metabolism were identified: 3-isopropylmalate dehydrogenase 2 and glutamine synthetase 1. A transcriptional relationship between genes involved in carbon and nitrogen metabolism has previously been suggested (Jackson et al., 1993). 1-aminocyclopropane-1-carboxylate oxidase 1 was also repressed, suggesting a reduction in ethylene biosynthesis in habituated cells.

It is noteworthy that several isoforms of GSTs were repressed in H12 cells. GSTs are enzymes involved in detoxification processes to protect plants against xenobiotic damage like DCB (Genter et al., 1998) and are considered as molecular markers of plant stresses (Edwards et al., 2000). A further determination of the GST enzymatic activity confirmed that H12 cells were deficient in this detoxifying activity. Therefore, GST activity does not play a major role in DCB-habituated cells. In contrast, GST activity was enhanced when non-habituated cells were incubated short-term with DCB, indicating that, as expected, GST is involved in the process of detoxification.

According to the level of antioxidant activities (GR, GPOX, APOX, and CAT) measured in DCB habituated cell cultures, it seems that these cells do not rely on an antioxidant strategy to cope with this herbicide. However, as expected, these activities were induced in maize cells treated short-term with DCB. The behavior of the H12 maize cells thus contrasted with that observed in bean cells, in which antioxidant capacity is enhanced in habituation to this herbicide (García-Angulo et al., 2009). Results obtained by García-Angulo et al. (2009) did also show that bean dehabituated cells retained an increased GPOX activity that would partially explain that they were more tolerant to DCB than non-habituated cells. In accordance with this explanation, the lack of an antioxidant strategy in the habituation to dichlobenil of maize cells would also explicate that maize dehabituated cells do not differ in DCB sensitivity from non-habituated cells.

The low level of detoxifiyng/antioxidant activities measured in maize cells habituated to DCB would alternatively be explained as a way to reduce H_2O_2 scavenging and, secondarily, to ensure phenolic dimerization.

In this paper, we show that the habituation of maize cells to the herbicide DCB implies several metabolic modifications: (1) a strong reduction in cellulose and alteration in the expression of several *Cellulose Synthase* genes. Of these, *ZmCesA5* and *ZmCesA7* seem to play a major role in habituating cells to growth in the presence of this herbicide. (2) Several phenylpropanoid genes involved in the synthesis of hydroxycinnamates are induced, resulting in a strong increase in these compounds in the cell wall. This could be understood as a mechanism to reinforce a cellulose-deficient cell wall. (3) Several metabolic pathways are altered in habituated cells, such as carbon, nitrogen, and ethylene metabolism, as are other proteins typically involved in stress responses. (4) There is a notable reduction in glutathione S-transferase detoxifying activity, showing that it is not involved in habituation to the herbicide in maize cells. Moreover, this habituation process does not rely on antioxidant strategies. In conclusion, our results show that both the reduction in cellulose content and the increase in phenylpropanoid synthesis observed during habituation of maize cultured cells to a cellulose biosynthesis inhibitor such as dichlobenil provoke changes in gene expression, not only in the genes directly related to cellulose biosynthesis and phenylpropanoid synthesis, but also in others related to such varied aspects as carbon, nitrogen and ethylene metabolism, detoxification mechanisms, etc. Furthermore, this regulation of gene expression is dependent on the growth phase of habituated cells and differs considerably from the changes observed in non-habituated cells exposed to the herbicide. Considered together, these changes may be responsible for the capacity displayed by cells to survive exposure to an inhibitor that affects a process as crucial to plant cell as cellulose biosynthesis.

METHODS

Cell Cultures

Maize callus cultures (*Zea mays* L., Black Mexican sweetcorn) from immature embryos were grown in Murashige and Skoog media (Murashige and Skoog, 1962) supplemented with 9 μM 2,4-D at 25°C under light (Lorences and Fry, 1991) and sub-cultured monthly. Cell cultures habituated to 12 μM DCB (H12) were obtained from non-habituated (NH) after stepwise transfers with gradual increments of DCB (Mélida et al., 2009), and were sub-cultured in 12 μM DCB monthly for more than 2 years. Through long-term (up to 1 year) culturing of H12 cells in a medium lacking DCB, dehabituated cultures (DH) were obtained. For some experiments, NH cells incubated (for 5–6 d) in medium supplemented with 6 μM DCB (NH/DCB) were used. In all cases (H-DH-NH/DCB), cells were compared with same aged NH cells at the same culture cycle phase.

In order to obtain growth inhibition curves, calluses weighing 1.0 ± 0.1 g were cultured in DCB, in concentrations ranging from 0.01 to 100 μM. DCB was dissolved in dimethyl sulfoxide, which did not affect cell growth at this range of concentrations. The cultures were incubated for 30 d and weighed (FW). Growth was expressed as relative increase in FW and the I_{10}, I_{50}, and I_{90} were calculated as the concentration of DCB required to inhibit weight increase by 10, 50, and 90%, respectively, compared to non-DCB-treated cells (control). Six replicates were used in each concentration and no deviations are shown in Figure 2, due to inhibition percentages being unique values.

Cell Wall Analyses

Calluses collected in the early stationary phase were frozen and treated as previously described to obtain cell walls (Mélida et al., 2009). In some cases, these were collected in two different growth phases: in the active growth or in the stationary phases. On average, NH cells reached the active growth and stationary phases 16 and 25 d after sub-culturing, respectively. Twenty-day-old and 30-day-old H12 cells were considered to be at the active growth and stationary phases, respectively (Mélida et al., 2009).

Cellulose was quantified in crude cell walls by the Updegraff method (Updegraff, 1969) using the hydrolytic conditions described by Saeman et al. (1963) and quantifying the glucose released by the anthrone method (Dische, 1962).

Ferulic and *p*-coumaric acids were analyzed by HPLC–PAD. Cell walls (10 mg) were treated in the dark under N_2 with 1 M NaOH, at room temperature for 16 h in order to saponify phenolic esters. The solution was acidified by addition of trifluoroacetic acid and partitioned against ethyl acetate (× 2). The ethyl acetate phases were vacuum-dried and re-dissolved in propan-1-ol for HPLC–PAD analysis.

HPLC–PAD analyses were performed using a Waters 2690 chromatograph with a Waters 996 photodiode array detector. Separation was achieved using a Kromasil C18 (Teknokroma) column (250 × 4.6 mm i.d.; 5 μm particle size). The mobile phase consisted of acidified (TFA) 10% acetonitrile (solvent A) and a mix of 40% acetonitrile, 40% methanol, and 20% water (solvent B), and followed the binary gradient elution program: initial conditions 90:10 (A:B), changing to 25:75 after 25 min, then to 0:100 after 5 min and returning to the initial conditions after 10 min. The mobile phase flow was 1 mL min^{-1}. The elution profiles were monitored by UV absorbance at 325 and 280 nm. Retention times were compared with freshly prepared standard solutions of ferulic and *p*-coumaric acids. Calibration curves were used to quantify these compounds.

Isolation of Total RNA, RT–PCR, and PCR

Total RNA was extracted with Trizol Reagent (Invitrogen), and 2 μg of total RNA was reverse-transcribed using M-MLV Reverse Transcriptase (Invitrogen). First-strand cDNA was generated using an oligo$(dT)_{15}$ primer and 1 μl of the first-strand cDNA was used as a template in subsequent PCR reactions. 'No-RT' PCR assays were performed to confirm the absence of genomic DNA contamination. For each assay, several numbers of cycles were tested to ensure that amplification was in the exponential range.

The gene-specific primers used for the analysis of *ZmCesA1* (AF200525), *ZmCesA2* (AF200526), *ZmCesA3* (AF200527), *ZmCesA5* (AF200529), *ZmCesA6* (AF200530), and *ZmCesA7* (AF200531) were those previously described by Holland et al. (2000). Due to the high-sequence similarity between ZmCesA1 and ZmCesA2, the same primer was used for the analysis of both genes (*ZmCesA1/2*). The primers used for the analysis of *ZmCesA4* (AF200528), *ZmCesA8* (AF200532), *ZmCesA9* (AF200533), *ZmCesA10* (AY372244.1), *ZmCesA11* (AY372245.1), *ZmCesA12* (AY372246.1), *ZmBk2L3* (EF078698), and *ZmMAP20* (AY110515.1) are shown in Supplemental Table 1.

The sequences of the primers used for the analysis of Caffeic acid O-MethylTransferase *ZmCOMT* (AY323283) and *ZmUbiquitin* (U29159) were those previously described by Fornalé et al. (2006).

The gene-specific primers used for the analysis of Phenylalanine Ammonia-Lyase *ZmPALa* (contig no. 3858636.2.1), *ZmPALb* (contig no. 2161072.2.3), *ZmPALc* (contig no. 2161072.2.1), Cinnamate 4-Hydroxylase *ZmC4Ha* (contig no. 2521589.2.1), 4-Coumarate CoA Ligase *Zm4CLa* (contig no. 3106166.2.1), *Zm4CLc* (contig no. 1716323.2.1), Hydroxycinnamoyl-CoA shikimate/quinate hydroxycinnamoyl Transferase *ZmHCT* (contig no. 2619423.2.1), Caffeoyl-CoA O-MethylTransferase *ZmCCoAOMT* (contig no. 2591258.2.1), and 4-coumarate 3-hydroxylase *ZmC3H* (contig no. 2643622.2.1) were derived from the 'MAIZEWALL' database (Guillaumie et al., 2007).

Protein Extraction and 2D–PAGE

NH and H12 maize cells at the stationary phase (1 g) were ground in liquid nitrogen. Proteins were solubilized at 4°C in 1.2 mL of lysis buffer (7 M urea, 2 M thiourea, 40 mM Tris-HCl pH 8.0, 50 mM DTT, 4% CHAPS and 0.2% Triton

X-100) containing DNase I (53 u mL^{-1}), RNase (4.9 u mL^{-1}) and protease inhibitors (1 mM PMSF, 50 mM leupeptin and 10 mM E–64). Protein extracts were clarified twice by centrifugation at 16 000 *g* for 15 min at 4°C, and the obtained supernatants were saved. Supernatants were precipitated with 15% TCA for 30 min at 4°C and centrifuged at 16 000 *g* for 15 min. The pellet containing the proteins was mixed with cold acetone (× 3) and finally re-suspended in lysis buffer. Protein content was quantified by the Bradford assay (Bradford, 1976).

For 2-D analysis, protein extracts were diluted in rehydration solution (7 M Urea, 2 M thiourea, 18 mM Tris-HCl pH 8.0, 4% CHAPS, 0.5% IPG Buffer in the same range as the IPG strip, and 0.002% Bromophenol Blue) containing 1.6% DeStreak Reagent (Amersham Biosciences). Three hundred μg of total proteins was diluted in a final volume of 300 μl and loaded onto non-linear pH 4–7, 18-cm immobilized pH gradient (IPG) strips (Immobiline DryStrips, Amersham Biosciences) for the first dimension. Isoelectric focusing was performed at 50 V for 10 h, 500 V in gradient for 1 h 30 min, 1000 V in gradient for 1 h 30 min, 2000 V in gradient for 1 h 30 min, 4000 V in gradient for 1 h 30 min, 8000 V in gradient for 2 h, and 8000 V holding for 10 h, using EttanTM IPGphor TM Isoelectric Focusing System (Amersham Biosciences). IPG strips were then equilibrated with 50 mM Tris-HCl (pH 8.8), 6 M urea, 30% (v/v) glycerol, 2% SDS, a trace of Bromophenol Blue and 10 mg mL^{-1} DTT during 15 min, followed by a second equilibration step with the same buffer containing 25 mg mL^{-1} iodoacetamide instead of DTT, for a further 15 min, with gentle shaking. For the second dimension, the focused strips were loaded and run on SDS–PAGE 12% polyacrylamide gels (26 × 20 × 0.1 cm) using Ettan DALTsix System (Amersham Biosciences), 30 min at 2.5 W gel^{-1}, followed by 17 W gel^{-1} during 4 h. Gels were stained with CBB G-250 (Bio-Rad). The experiment was repeated with two biological replicates and three experimental replicates per biological sample. The stained gels were scanned with an ImageScanner desktop instrument (Amersham Biosciences) and images were acquired using the LabScan scanning application, in transmission mode, at (16-bits) grayscale level, 300 dpi, zoom factor set at 1:1 (100%), and saved as TIFF (Tagged Image File Format) files. Image analysis was performed using the ImageMasterTM 2D Platinum 5.0 Software (Amersham Biosciences). The optimal parameters for spot detection were: smooth = 4, saliency = 1.0, and minimum area = 5. After automatic spot detection, manual spot editing was carried out. Gel replicates were used to obtain synthetic gels with averaged positions, shapes, and optical densities. To evaluate protein expression differences among gels, relative spot volume (% Vol.) was used. This is a normalized value and represents the ratio of a given spot volume to the sum of all spot volumes detected in the gel. Those spots showing a quantitative variation ⩾ Ratio 1 and positive GAP were selected as differentially expressed. Statistically significant protein abundance variation was validated by Student's *t*-test ($p < 0.05$). The selected differential spots were excised from the CBB G-250 stained gels and identified either by PMF using MALDI–TOF MS or by peptide sequencing at the Proteomics Platform (Barcelona Science Park, Barcelona, Spain). The MALDI–TOF MS analysis was performed using a Voyager DE-PRO (Applied Biosystems) instrument in the reflectron, positive-ion mode. Spectra were mass calibrated externally using a standard peptide mixture. For the analysis, 0.5 mL peptide extract and 0.5 mL matrix (5 mg mL^{-1} CHCA) were loaded onto the MALDI plate. When ions corresponding to known trypsin autolytic peptides (m/z 842.5100, 1045.5642, 2111.1046, 2283.1807) were detected at adequate intensities, an automatic internal calibration of the spectra was performed. Data were generated in PKL file format and were submitted for database searching in MASCOT server. The software packages Protein Prospector v 3.4.1 (UCSF) (Mass Spectrometry Facility, University of California) and MASCOT were used to identify the proteins from the PMF data as reported previously (Carrascal et al., 2002). The SEQUEST software (Thermo-Instruments, Spain) was used for preliminary protein identification from the MS/MS analysis followed by manual sequence data confirmation. Swiss-Prot and non-redundant NCBI databases were used for the search. Searches were performed for the full range of molecular weight and p*I*. No species restriction was applied. When an identity search produced no matches, the homology mode was used.

Antioxidant Enzyme Assays

Glutathione S-tranferase (GST; EC 2.5.1.18), glutathione reductase (GR; EC 1.8.1.7), guaiacol type peroxidase (GPOX; EC 1.11.1.7), ascorbate peroxidase (APOX; EC 1.11.1.11), and catalase (CAT; EC 1.11.1.6) activities were assayed in NH, H12, and NH/DCB callus-cultured cells. GST activity was determined following the method described by Habig et al. (1974), based on an increase in A_{340} due to reduced reduction in glutathione and chloro-2,4-dinitrobenzene complex formation (ε_{340} = 9.6 mM^{-1} cm^{-1}). GR activity was determined following the method described by Edwards et al. (1990), by measuring the decrease in A_{340} due to NADPH oxidation (ε_{340} = 6.22 mM^{-1} cm^{-1}). GPOX activity was determined following the method described by Adam et al. (1995), based on an increase in A_{470} due to guaiacol oxidation (ε_{470} = 26.6 mM^{-1} cm^{-1}). APOX activity was determined following the method described by Hossain and Asada (1984), by measuring the decrease in A_{290} due to ascorbate oxidation (ε_{290}= 2.8 mM^{-1} cm^{-1}). The method described by Droillard et al. (1987) was followed for CAT activity measurement. This method is based on a decrease in A_{240} due to H_2O_2 decomposition (ε_{240} = 39.58 mM^{-1} cm^{-1}).

SUPPLEMENTARY DATA

Supplementary Data are available at *Molecular Plant Online*.

FUNDING

This work was supported by the Spanish Ministry of Science and Innovation (CGL2008-02470/BOS and AGL2008-05157) program as

well as the CONSOLIDER-INGENIO (CSD2007-00036) program. H.-M. was financed by a PhD grant from the Spanish Ministry of Science and Innovation's FPU program. This research was carried out within the framework of the 'Xarxa de Referència de Biotecnologia' (XarBa) of the Autonomous Government of Catalonia.

ACKNOWLEDGMENTS

We are indebted to Dr S. Fornalé (CRAG), Dr Alonso-Simón, and Dr García-Angulo for scientific discussions. We would also like to thank S. Irar for his technical support as head of the proteomic service at CRAG, as well as the Biological and Structural Mass Spectrometry Unit of the Barcelona Biomedical Research Institute (IIBB CSIC-IDIBAPS) for the MALDI–TOF sequencing, and Denise Phelps for the English revision of the manuscript. No conflict of interest declared.

REFERENCES

Acebes, J.L., Encina, A., García-Angulo, P., Alonso-Simón, A., Mélida, H., and Álvarez, J.M. (2010). Cellulose biosynthesis inhibitors: their uses as potential herbicides and as tools in cellulose and cell wall structural plasticity research. In Cellulose: Structure and Properties, Derivatives and Industrial Uses, Lejeune A. and Deprez T., eds (New York: Nova Publishers)) in press.

Adam, A.L., Bestwick, C.S., Barna, B., and Mansfield, J.W. (1995). Enzymes regulating the accumulation of active oxygen species during the hypersensitive reaction of bean to *Pseudomonas syringae* pv. *phaseolicola*. Planta. **197,** 240–249.

Alonso-Simón, A., Neumetzler, L., García-Angulo, P., Encina, A.E., Acebes, J.L., Álvarez, J.M., and Hayashi, T. (2010). Plasticity of xyloglucan composition in bean (*Phaseolus vulgaris*)-cultured cells during habituation and dehabituation to lethal concentrations of dichlobenil. Mol Plant. 10.1093/mp/ssq011.

Anderson, C.T., Carroll, A., Akhmetova, L., and Somerville, C. (2010). Real-time imaging of cellulose reorientation during cell wall expansion in *Arabidopsis* roots. Plant Physiol. **152,** 787–796.

Appenzeller, L., Doblin, M., Barreiro, R., Wang, H.Y., Niu, X.M., Kollipara, K., Carrigan, L., Tomes, D., Chapman, M., and Dhugga, K.S. (2004). Cellulose synthesis in maize: isolation and expression analysis of the cellulose synthase (*CesA*) gene family. Cellulose. **11,** 287–299.

Bernal, A.J., et al. (2007). Disruption of *ATCSLD5* results in reduced growth, reduced xylan and homogalacturonan synthase activity and altered xylan occurrence in *Arabidopsis*. Plant J. **52,** 791–802.

Bradford, M.M. (1976). Rapid and sensitive method for quantitation of microgram quantities of protein utilizing principle of protein-dye binding. Anal. Biochem. **72,** 248–254.

Brady, S.M., Song, S., Dhugga, K.S., Rafalski, J.A., and Benfey, P.N. (2007). Combining expression and comparative evolutionary analysis: the COBRA gene family. Plant Physiol. **143,** 172–187.

Buanafina, M.M.d.O. (2009). Feruloylation in grasses: current and future perspectives. Mol. Plant. **2,** 861–872.

Burr, S.J., and Fry, S.C. (2009). Feruloylated arabinoxylans are oxidatively cross-linked by extracellular maize peroxidase but not by horseradish peroxidase. Mol. Plant. **2,** 883–892.

Burton, R.A., and Fincher, G.B. (2009). (1,3;1,4)-B-D-glucans in cell walls of the Poaceae, lower plants, and fungi: a tale of two linkages. Mol. Plant. **2,** 873–882.

Carpita, N.C. (1984). Cell wall development in maize coleoptiles. Plant Physiol. **76,** 205–212.

Carpita, N.C., and Gibeaut, D.M. (1993). Structural models of primary cell walls in flowering plants: consistency of molecular structure with the physical properties of the walls during growth. Plant J. **3,** 1–30.

Carrascal, M., Carujo, S., Bachs, O., and Abian, J. (2002). Identification of p21(Cip1) binding proteins by gel electrophoresis and capillary liquid chromatography microelectrospray tandem mass spectrometry. Proteomics. **2,** 455–468.

Ching, A., Dhugga, K.S., Appenzeller, L., Meeley, R., Bourett, T.M., Howard, R.J., and Rafalski, A. (2006). *Brittle stalk 2* encodes a putative glycosylphosphatidylinositol-anchored protein that affects mechanical strength of maize tissues by altering the composition and structure of secondary cell walls. Planta. **224,** 1174–1184.

Daras, G., Rigas, S., Penning, B., Milioni, D., McCann, M.C., Carpita, N.C., Fasseas, C., and Hatzopoulos, P. (2009). The *thanatos* mutation in *Arabidopsis thaliana* cellulose synthase 3 (*AtCesA3*) has a dominant-negative effect on cellulose synthesis and plant growth. New Phytol. **184,** 114–126.

Desprez, T., Juraniec, M., Crowell, E.F., Jouy, H., Pochylova, Z., Parcy, F., Hofte, H., Gonneau, M., and Vernhettes, S. (2007). Organization of cellulose synthase complexes involved in primary cell wall synthesis in *Arabidopsis thaliana*. Proc. Natl Acad. Sci. U S A. **104,** 15572–15577.

Dische, Z. (1962). Color reactions of carbohydrates. In Methods in Carbohydrate Chemistry, Whistler R.L. and Wolfrom R.L., eds (New York: Academic Press), pp. 475–514.

Droillard, M.J., Paulin, A., and Massot, J.C. (1987). Free-radical production, catalase and superoxide-dismutase activities and membrane integrity during senescence of petals of cut carnations (*Dianthus caryophyllus*). Physiol. Plantarum. **71,** 197–202.

Edwards, E.A., Rawsthorne, S., and Mullineaux, P.M. (1990). Subcellular-distribution of multiple forms of glutathione-reductase in leaves of pea (*Pisum sativum* L). Planta. **180,** 278–284.

Edwards, R., Dixon, D.P., and Walbot, V. (2000). Plant glutathione S-transferases: enzymes with multiple functions in sickness and in health. Trends Plant Sci. **5,** 193–198.

Encina, A., Sevillano, J.M., Acebes, J.L., and Álvarez, J. (2002). Cell wall modifications of bean (*Phaseolus vulgaris*) cell suspensions during habituation and dehabituation to dichlobenil. Physiol. Plantarum. **114,** 182–191.

Feng, H., Chen, Q., Feng, J., Zhang, J., Yang, X., and Zuo, J. (2007). Functional characterization of the *Arabidopsis* eukaryotic translation initiation factor 5A-2 that plays a crucial role in plant growth and development by regulating cell division, cell growth, and cell death. Plant Physiol. **144,** 1531–1545.

Fornalé, S., Sonbol, F., Maes, T., Capellades, M., Puigdomenech, P., Rigau, J., and Caparrós-Ruiz, D. (2006). Down-regulation of the maize and *Arabidopsis thaliana* caffeic acid *O*-methyltransferase genes by two new maize R2R3-MYB transcription factors. Plant Mol. Biol. **62,** 809–823.

Fry, S.C. (2004). Primary cell wall metabolism: tracking the careers of wall polymers in living plant cells. New Phytol. **161,** 641–675.

Fry, S.C., Willis, S.C., and Paterson, A.E.J. (2000). Intraprotoplasmic and wall-localised formation of arabinoxylan-bound diferulates and larger ferulate coupling-products in maize cell-suspension cultures. Planta. **211,** 679–692.

García-Angulo, P., Alonso-Simón, A., Mélida, H., Encina, A., Acebes, J.L., and Álvarez, J.M. (2009). High peroxidase activity and stable changes in the cell wall are related to dichlobenil tolerance. J. Plant Physiol. **166,** 1229–1240.

García-Angulo, P., Willats, W.G.T., Encina, A., Alonso-Simón, A., Álvarez, J.M., and Acebes, J.L. (2006). Immunocytochemical characterization of the cell walls of bean cell suspensions during habituation and dehabituation to dichlobenil. Physiol. Plantarum. **127,** 87–99.

Genter, M.B., Deamer-Melia, N.J., Wetmore, B.A., Morgan, K.T., and Meyer, S.A. (1998). Herbicides and olfactory/neurotoxicity responses. Rev. Toxicol. **2,** 93–112.

Guillaumie, S., San-Clemente, H., Deswarte, C., Martinez, Y., Lapierre, C., Murigneux, A., Barriere, Y., Pichon, M., and Goffner, D. (2007). MAIZEWALL: database and developmental gene expression profiling of cell wall biosynthesis and assembly in maize. Plant Physiol. **143,** 339–363.

Gutierrez, R., Lindeboom, J.J., Paredez, A.R., Emons, A.M.C., and Ehrhardt, D.W. (2009). *Arabidopsis* cortical microtubules position cellulose synthase delivery to the plasma membrane and interact with cellulose synthase trafficking compartments. Nat. Cell Biol. **11,** 797–806.

Ha, M.A., MacKinnon, I.M., Sturcova, A., Apperley, D.C., McCann, M.C., Turner, S.R., and Jarvis, M.C. (2002). Structure of cellulose-deficient secondary cell walls from the *irx3* mutant of *Arabidopsis thaliana*. Phytochemistry. **61,** 7–14.

Habig, W.H., Pabst, M.J., and Jakoby, W.B. (1974). Glutathione S-transferases: first enzymatic step in mercapturic acid formation. J. Biol. Chem. **249,** 7130–7139.

Hamann, T., Bennett, M., Mansfield, J., and Somerville, C. (2009). Identification of cell-wall stress as a hexose-dependent and osmosensitive regulator of plant responses. Plant J. **57,** 1015–1026.

Holland, N., Holland, D., Helentjaris, T., Dhugga, K.S., Xoconostle-Cazares, B., and Delmer, D.P. (2000). A comparative analysis of the plant cellulose synthase (*CesA*) gene family. Plant Physiol. **123,** 1313–1323.

Hossain, M.A., and Asada, K. (1984). Inactivation of ascorbate peroxidase in spinach-chloroplasts on sark addition of hydrogen-peroxide: its protection by ascorbate. Plant Cell Physiol. **25,** 1285–1295.

Jackson, S.D., Sonnewald, U., and Willmitzer, L. (1993). Cloning and expression analysis of b-isopropylmalate dehydrogenase from potato. Mol. Gen. Genet. **236,** 309–314.

Knox, J.P. (2008). Revealing the structural and functional diversity of plant cell walls. Curr. Opin. Plant Biol. **11,** 308–313.

Lindsay, S.E., and Fry, S.C. (2008). Control of diferulate formation in dicotyledonous and gramineous cell-suspension cultures. Planta. **227,** 439–452.

Lorences, E.P., and Fry, S.C. (1991). Absolute measurement of cell expansion in plant cell suspension cultures. Plant Cell Tiss. Org. **24,** 211–215.

Manfield, I.W., Orfila, C., McCartney, L., Harholt, J., Bernal, A.J., Scheller, H.V., Gilmartin, P.M., Mikkelsen, J.D., Knox, J.P., and Willats, W.G.T. (2004). Novel cell wall architecture of isoxaben habituated *Arabidopsis* suspension cultured cells: global transcript profiling and cellular analysis. Plant J. **40,** 260–275.

Mélida, H., García-Angulo, P., Alonso-Simón, A., Encina, A., Álvarez, J., and Acebes, J.L. (2009). Novel type II cell wall architecture in dichlobenil-habituated maize calluses. Planta. **229,** 617–631.

Murashige, T., and Skoog, F. (1962). A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol. Plantarum. **15,** 473–497.

Mutwil, M., Debolt, S., and Persson, S. (2008). Cellulose synthesis: a complex complex. Curr. Opin. Plant Biol. **11,** 252–257.

Nakagawa, N., and Sakurai, N. (1998). Increase in the amount of celA1 protein in tobacco BY-2 cells by a cellulose biosynthesis inhibitor, 2,6-Dichlorobenzonitrile. Plant Cell Physiol. **39,** 779–785.

Paredez, A.R., Somerville, C.R., and Ehrhardt, D.W. (2006). Visualization of cellulose synthase demonstrates functional association with microtubules. Science. **312,** 1491–1495.

Parker, M.L., Ng, A., and Waldron, K.W. (2005). The phenolic acid and polysaccharide composition of cell walls of bran layers of mature wheat (*Triticum aestivum* L. cv. Avalon) grains. J. Sci. Food Agric. **85,** 2539–2547.

Persson, S., Paredez, A., Carroll, A., Palsdottir, H., Doblin, M., Poindexter, P., Khitrov, N., Auer, M., and Somerville, C.R. (2007). Genetic evidence for three unique components in primary cell-wall cellulose synthase complexes in *Arabidopsis*. Proc. Natl Acad. Sci. U S A. **104,** 15566–15571.

Piston, F., Uauy, C., Fu, L., Langston, J., Labavitch, J., and Dubcovsky, J. (2010). Down-regulation of four putative arabinoxylan feruloyl transferase genes from family PF02458 reduces ester-linked ferulate content in rice cell walls. Planta. **231,** 677–691.

Rajangam, A.S., et al. (2008). MAP20, a microtubule-associated protein in the secondary cell walls of hybrid aspen, is a target of the cellulose synthesis inhibitor 2,6-Dichlorobenzonitrile. Plant Physiol. **148,** 1283–1294.

Ray, P.M., Green, P.B., and Cleland, R. (1972). Role of turgor in plant-cell growth. Nature. **239,** 163–164.

Richmond, T. (2000). Higher plant cellulose synthases. Genome Biol. **1**, reviews3001.1–reviews3001.6.

Saeman, J.F., Moore, W.E., and Millet, M.A. (1963). Sugar units present. In Methods in Carbohydrate Chemistry, Whistler R.L., ed. (New York: Academic Press), pp. 54–69.

Scheible, W.R., Eshed, R., Richmond, T., Delmer, D., and Somerville, C. (2001). Modifications of cellulose synthase confer resistance to isoxaben and thiazolidinone herbicides in *Arabidopsis Ixr1* mutants. Proc. Natl Acad. Sci. U S A. **98,** 10079–10084.

Scheller, H.V., and Ulvskov, P. (2010). Hemicelluloses. Annu. Rev. Plant Biol. **61,** 10. 1–10.27.

Shedletzky, E., Shmuel, M., Delmer, D.P., and Lamport, D.T.A. (1990). Adaptation and growth of tomato cells on the herbicide 2,6-Dichlorobenzonitrile leads to production of unique cell walls

virtually lacking a cellulose-xyloglucan network. Plant Physiol. **94,** 980–987.

Smith, M.M., and Hartley, R.D. (1983). Occurrence and nature of ferulic acid substitution of cell-wall polysaccharides in graminaceous plants. Carbohydr. Res. **118,** 65–80.

Somerville, C. (2006). Cellulose synthesis in higher plants. Annu. Rev. Cell Dev. Biol. **22,** 53–78.

Taylor, N.G. (2008). Cellulose biosynthesis and deposition in higher plants. New Phytol. **178,** 239–252.

Taylor, N.G., Howells, R.M., Huttly, A.K., Vickers, K., and Turner, S.R. (2003). Interactions among three distinct CesA proteins essential for cellulose synthesis. Proc. Natl Acad. Sci. U S A. **100,** 1450–1455.

Taylor, N.G., Laurie, S., and Turner, S.R. (2000). Multiple cellulose synthase catalytic subunits are required for cellulose synthesis in *Arabidopsis*. Plant Cell. **12,** 2529–2539.

Taylor, N.G., Scheible, W.R., Cutler, S., Somerville, C.R., and Turner, S.R. (1999). The *irregular xylem3* locus of *Arabidopsis* encodes a cellulose synthase required for secondary cell wall synthesis. Plant Cell. **11,** 769–779.

Tomanek, L., and Sanford, E. (2003). Heat-shock protein 70 (Hsp70) as a biochemical stress indicator: an experimental field test in two congeneric intertidal gastropods (genus: *Tegula*). Biol. Bull. **205,** 276–284.

Turner, S.R., and Somerville, C.R. (1997). Collapsed xylem phenotype of *Arabidopsis* identifies mutants deficient in cellulose deposition in the secondary cell wall. Plant Cell. **9,** 689–701.

Updegraff, D.M. (1969). Semimicro determination of cellulose in biological materials. Anal. Biochem. **32,** 420–424.

Vaughn, K.C. (2002). Cellulose biosynthesis inhibitor herbicides. In Herbicide Classes in Development, Böger, P. Wakabayashi, K. Hirai, K., eds (Berlin: Springer-Verlag), pp. 139–150.

Vogt, T. (2010). Phenylpropanoid biosynthesis. Mol. Plant. **3,** 2–20.

Wende, G., and Fry, S.C. (1997). *O*-feruloylated, *O*-acetylated oligosaccharides as side-chains of grass xylans. Phytochemistry. **44,** 1011–1018.

Zanardo, D.I.L., Lima, R.B., Lucio Ferrarese, M.d.L., Bubna, G.A., and Ferrarese-Filho, O. (2009). Soybean root growth inhibition and lignification induced by *p*-coumaric acid. Environ. Exp. Bot. **66,** 25–30.