Departamento de Ingeniería y Ciencias Agrarias

Área de Fisiología Vegetal

Structural and metabolic cell wall modifications related to the short-term habituation of maize cell suspensions to dichlobenil

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Modificaciones metabólicas y estructurales de la pared celular asociadas a la habituación incipiente de suspensiones celulares de maíz a diclobenil

Memoria presentada por la Licenciada María de Castro Rodríguez para optar al grado de Doctor por la Universidad de León

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A mis padres, a mis
directores y a Dani

Duda siempre de ti mismo, hasta que los datos no dejen lugar a dudas

Louis Pasteur

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Cell wall modifications during short-term habituation of maize cell suspensions to dichlobenil. M. de Castro, A. Largo, H. Mélida, A. Alonso-Simón, A.E. Encina, J.M. Álvarez, J.L. Acebes, P. García-Angulo. XII Cell Wall Meeting. Oporto, Portugal. Julio 2010.

Metabolismo de polisacáridos hemicelulósicos de suspensiones celulares de maíz habituadas a bajos niveles de DCB. M. de Castro, A. Largo**,** H. Mélida, A. Alonso-simón, A.E. Encina, J.M. Álvarez, J.L. Acebes, S. Fry, P. García-Angulo. XIX Congreso Sociedad Española Fisiología Vegetal. Castellón de la Plana, España. Junio 2011.

The biosynthesis and molecular weight distribution of hemicelluloses in cellulose-deficient maize cells: an example of metabolic plasticity. M. de Castro, A.E. Encina, J.L. Acebes, P. García-Angulo, S. Fry. XIII Cell Wall Meeting. Nantes, Francia. Julio 2013.

Deciphering the Golgi proteome in cellulose-deficient cells. M. de Castro, S. Irar, L. García, A. Largo, D. Caparrós, J.L. Acebes, P. García-Angulo. XIII Cell Wall Meeting. Nantes, Francia. Julio 2013.

Cell wall modifications during short-term habituation of maize cell suspensions to dichlobenil. M. de Castro and A. Largo, A.E. Encina, J.M. Álvarez, J.L. Acebes, P. García-Angulo. XX Congreso Sociedad Española de Fisiología Vegetal. Lisboa (Portugal). Julio 2013.

Además, parte de los resultados de la presente Tesis Doctoral han dado lugar a la siguiente publicación:

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Abbreviations

2,4-D: 2,4-dichloro-phenoxy-acetic acid

2-D: two-dimensional

ACC: 1-aminocyclopropane-1-carboxylic acid

ACS: adenosylmethionine synthetase

ACO 1: 1-aminocyclopropane-1-carboxylate oxidase 1

AIR: alcohol insoluble residue

ANOVA: analysis of variance

Ara: arabinose

CAZy: carbohydrate active enzymes

CBB: coomassie brilliant blue

CBI: cellulose biosynthesis inhibitor

CCoAOMT 1: caffeoyl-CoA *O*-methyltransferase 1

cDNA: complementary deoxyribonucleic acid

CDTA: cyclohexane-trans-1,2-diamine-N,N,N´,N´-tetraacetic sodium salt

CESA: cellulose synthase protein

CFM: cell-free medium fraction

CHAPS: 3-3-(3-cholamidopropyl) diethyl-ammonio-1- propanesulfonate

Cinn: cinnamic acid

COMT: caffeic acid 3-*O*-methyltransferase

CSC: cellulose synthase complex

CSLC: cellulose synthase-like C

DCB: dichlobenil

DMSO: dimethyl sulfoxide

DNA: deoxyribonucleic acid

Dt: doubling time

DTT: dithiothreitol

DW: dry weight

EDTA: ethylene-di-amine-tetra-acetic acid

ESI-MS/MS: electrospray ionization with tandem mass spectrometry

Fer: ferulic acid

FTIR: Fourier transform infrared

Fuc: fucose

Gal: galactose

GalA: galacturonic acid

GC: gas chromatography

Glc: glucose

GlcA: glucuronic acid

GPC: gel-permeation chromatography

GT: glycosyltransferase

HEPES: 2-[4-(2-hydroxyethyl)-l-piperazinyl]-ethanesulfonic acid

I_{50} value: dichlobenil concentration capable of inhibiting dry weight increase by 50% respecting to the control

IDA: immunodot assay

IDP: inosine 5-diphosphate

IDPase: triton-dependent ionidine di-phosphatase

IPG: immobilized pH gradient

Irx: irregular xylem

Kav: partition coefficient for a given gel-permeation chromatography column

LiDS: lithium dodecyl sulfate

Man: mannose

MALDI-TOF/MS: matrix-assisted laser desorption ionization-time of flight mass spectrometry

MAP: microtubule-associated protein

MeGlcA: methyl-glucuronic acid

MM: molecular mass

MPBS: phosphate-buffered saline with 4% fat-free milk powder

M_r: relative molecular mass

M_w: weight-average relative molecular mass

NIR: near infrared

P41: commercial pectin with 41% degree of methyl esterification

PAGE: polyacrylamide gel electrophoresis

PBS: phosphate-buffered saline

PC: paper chromatography

PCA: principal component analysis

PCR: polymerase chain reaction

PCs: principal components

***p*-Cou:** *p*-coumaric acid

pI: isoelectric point

PMF: peptide mass fingerprint

RGP: reversible glycosylation polypeptide

Rha: rhamnose

RNA: ribonucleic acid

RT-PCR: reverse transcription polymerase chain reaction

SEPs: soluble extracellular polymers

SDS: sodium dodecyl sulphate

Shx (n): habituated suspension-cultured cells to x µM DCB during (n) number of culture cycles

Snh: non-habituated suspension-cultured cells

TFA: trifluoroacetic acid

TIFF: tagged image file format

TLC: thin layer chromatography

UDP: uridine 5-diphosphate

V0: void volume

Vi: included volume

Wo: suspension cell culture dry weight at the beginning of the culture cycle

Wt: suspension cell culture dry time at time ´t´ of the culture cycle

Xyl: xylose

Contents

Introduction

THE PLANT CELL WALL

Plant cell walls are partially-rigid structures between 0.1 and 10 μm thick which surround the protoplast. In spite of their apparently impenetrability and stasis, they are dynamic cell compartments with important structural and physiological functions. Cell walls guide, restrict and determine cell growth and have a crucial role in the functional specialisation of the different cell types. As the external cell barrier, the cell wall controls the metabolite exchange between protoplast and external medium (Baron-Epel et al., 1988) and takes part in cell-cell and cell-pathogen recognition processes (Vorwerk et al., 2004; Seifert and Blaukopf, 2010; Wolf et al., 2012). Moreover, cell walls are involved in the modulation of stress signaling responses (Roberts, 2001; Hückelhoven, 2007; Driouich et al., 2012), and also regulate growth activity by being a source of oligosaccharines, biologically active oligosaccharides (Fry et al., 1993, Franková et al., 2012). Furthermore, they show a remarkable compositional and structural plasticity which allows cells to respond to abiotic and biotic stresses (Hamann et al., 2009). In addition to their important physiological functions, cell walls have significant economic implications: they influence the texture and nutritional value of most plant-based products, are a key factor in the processing properties of plant-based foods for human and animal consumption and are considered the source of energy from plant materials in biofuel production (Burton and Fincher, 2012).

In higher plants, the genesis of the cell wall takes place during the cell division process, when the polymers composing the cell wall are deposited starting in the telophase. Golgi vesicles transporting non-cellulosic polysaccharides are responsible for the formation of the cell plate, the structure that will give rise to a totally functional cell wall (Staehelin and Hepler, 1996; Cutler and Ehrhardt, 2002; Segui-Simarro et al., 2004), whereas Golgi vesicle membranes give rise to the plasma membrane of the daughter cells (Aspinall, 1980). As a consequence of the development of the cell plate, the middle lamella appears, which is a structure enriched in pectic polysaccharides shared by adjacent cells, and therefore, responsible for cell adhesion (Aspinall, 1980). Immediately, cells start to synthesise the

primary cell wall, located between the plasma membrane and the middle lamella. By definition, a primary wall layer is the one in which cellulosic microfibrils were deposited while the cell was growing. A primary wall layer will not acquire more cellulose once growth has stopped, although in some cell types other compounds, such as lignin or cutin, are deposited; nevertheless, it remains a primary cell wall (Fry, 2011). In other cell types, a thicker and more regularly arranged second layer of cellulose microfibrils, often impregnated with lignin and suberin, is deposited on the inner face of the wall once cell growth has ceased, and this is called secondary cell wall.

Two types of primary cell walls can be distinguished (Carpita and Gibeaut, 1993), which differ in chemical composition as well as in the taxa to which they are related: type I is characteristic of dicots and non-commelinoids monocots whereas type II is exclusive to commelinoid monocots (Popper, 2008). In this chapter we will focus on the type II structure, which is the cell wall type corresponding to the specie analysed in the present study (*Zea mays*).

I. TYPE II PLANT CELL WALL COMPOSITION AND BIOSYNTHESIS

I.a. Cellulose

Cellulose is considered the scaffolding of the cell wall, to which the remaining cell wall constituents are attached. The cellulose content in both types of primary wall, types I and II, varies from 15% to 30%, whereas in secondary cell walls it may be up to 50% (Carpita and McCann, 2000). In the case of *in vitro* cultured cells, cellulose accounts for is around 20% (Blaschek et al., 1981).

In primary walls, cellulose is deposited in microfibrils, traditionally defined as a crystalline structure consisting of 36 β-1,4-glucan chains arranged in parallel (Delmer, 1999; Saxena and Brown, 2000; Lerouxel et al., 2006). This linked conformation means that each glucose (Glc) residue is positioned at 180° with respect to the contiguous one, with

cellobiose being the disaccharide repeated in cellulose (Saxena et al., 1995). This circumstance, in addition to the absence of lateral chain substitutions, is responsible for the plain spatial structure of the β-1,4-glucan chains, which allows the formation of inter- and intra-strand hydrogen bonds, leading to the highly stable crystalline microfibrillar structure characteristic of cellulose (Somerville, 2006).

BIOSYNTHESIS

Cellulose is known to be synthesised at the plasma membrane (Figure 1; for more details see Guerriero et al., 2010). Subsequently, it is directionally deposited on the cell wall (Somerville, 2006; Taylor, 2008), undergoing dynamic spatial re-arrangement after deposition to allow for anisotropic expansion (Anderson et al., 2010). A protein complex named rosettes or cellulose synthase complex (CSC) is responsible for cellulose biosynthesis (Brown, 1996; Kimura et al., 1999). In plants, CSCs are organised in hexameric structures, with each hexamer containing six cellulose synthase (CESA) proteins. The complete structure consists of 36 CESA units, with each CSC being capable of synthesising the 36 β-1,4-glucan chains of a complete microfibril. This assumption is based on the six-fold symmetry of the rosette structures as well as the estimated lateral size of microfibrils from primary walls. However, since the number of active CESA proteins per rosette has never been experimentally confirmed, the possibility that different rosettes may contain a lower number of catalytically active subunits, leading to a number lower than 36 β-1,4-glucan chains, cannot be ruled out. Therefore, a more correct definition of a microfibril would be a morphologic entity that corresponds to the minimum number of β-1,4-glucan chains required to form a crystalline structure, or equally, the elementary structure produced by an individual rosette (Guerriero et al., 2010).

CSCs are presumably synthesised within the endoplasmic reticulum and then delivered to the Golgi apparatus for assembly before being finally transported to the plasma membrane (Li et al., 2012). Since they are only thought to be functional in the plasma membrane, it is here alone that cellulose biosynthesis is believed to occur. Nevertheless, the

possibility that cellulose synthesis or initiation may take place in another intracellular compartment, for example in the Golgi apparatus, cannot be excluded. The assumption that cellulose synthesis takes place exclusively in the plasma membrane is based on the fact that crystalline microfibrillar structures of β-1,4-glucan have never been observed in the intracellular compartments of higher plants. However, non-crystalline β-1,4-glucan may be synthesised in the Golgi by independent cellulose synthase catalytic subunits which are not part of a rosette; thus, the assembly of this β-1,4-glucan into microfibrillar structures would not be produced (Guerriero et al., 2010). Several hypotheses have emerged to clarify this: a) recently synthesised chains are produced by spatially separate catalytic subunits which are randomly distributed in the Golgi and therefore, the spatial proximity essential for the spontaneous formation of interchain hydrogen bonds is not present, b) a completely assembled rosette or the presence of accessory proteins would be required, which is not possible in the Golgi, c) the interaction of chains in the organelle with other compounds, such as carbohydrates or aglycones, would prevent microfibril formation (Guerriero et al., 2010).

CESA proteins are multigene families with a number of genes that varies among species. Most of them have been identified through the study of mutants showing an altered cell wall structure or composition (Arioli et al., 1998; Taylor et al., 1999; 2000; 2003; Desprez et al., 2002; Daras et al., 2009). Ten and twelve *CESA* genes have been identified in arabidopsis and maize, respectively (Holland et al., 2000; Appenzeller et al., 2004). In an individual cell, each CESA complex requires three different types of CESA subunits to function properly (Taylor et al., 2000). For example, in arabidopsis, *AtCESA1, 3* and *CESA6*-related proteins are essential for cellulose synthesis in primary walls (Desprez et al., 2007; Persson et al., 2007), while *AtCESA4, 7* and *8* are essential in the secondary cell wall (Taylor et al., 2003). Likewise, in maize, *ZmCESA1* to *9* are involved in building the primary cell wall whereas *ZmCESA10* to *12* are involved in the secondary wall. *ZmCESA6, 7* and *8* were thought to be involved in secondary wall synthesis (Holland et al., 2010); However, other authors have suggested that these genes form transitional sequences between the primary and

secondary wall synthesis, and are involved in building the primary wall later in development before the onset of secondary wall formation, due to the closer proximity of these genes to *ZmCESA12* rather than to *ZmCESA10* and *11* (Appenzeller et al., 2004). The structure of a CESA protein consists of eight trans-membrane domains, with the active site and the N and C terminal ends located at the cytosol (Lerouxel et al., 2006). Cortical microtubules control CSC movement and trajectory through the plasma membrane, and are therefore responsible for the orientation of cellulose microfibrils (Paredez et al., 2006).

In addition to CESA, other proteins are involved in cellulose biosynthesis, such as sucrose synthase (Amor et al., 1995), a membrane bound endo-β-1,4-glucanase (KORRIGAN; Nicol et al., 1998) and microtubule-associated proteins (MAP; Sedbrook, 2004).

It has been suggested that sucrose synthase is directly involved in cellulose biosynthesis by channelling uridine 5-diphosphate- (UDP) Glc to the CESA complex, since it is capable of synthesising UDP-Glc from sucrose and UDP (Baroja-Fernández et al., 2012; Li et al., 2012). It has also been reported that sucrose synthase is located close to the plasma membrane or cell walls where cellulose synthesis is very active (Salnikov et al., 2001; 2003; Albrecht and Mustroph, 2003; Persia et al., 2008). In addition, a high expression of several of its isoforms has been detected in tissues active in the production of cellulose (Geisler-Lee et al., 2006). Nevertheless, its physical association with the cellulose synthase machinery has never been experimentally demonstrated (Guerriero et al., 2010).

It has been suggested that KORRIGAN in arabidopsis, and its orthologues in other species, is involved in several possible roles, such as the release of newly synthesised cellulose microfibrils in the cell wall, post-synthetic trimming of imperfections along the newly-synthesised microfibrils or control of the degree of β-1,4-glucan chain polymerisation (Mølhøj et al., 2002). It would only be required at a specific stage of the cellulose biosynthesis process, having a transitory association to cellulose synthase complex (Guerriero et al., 2010).

MAPs are small cytosolic proteins that are also thought to be involved in cellulose synthesis since they participate in the correct assembly and polymerisation of microtubules (Sedbrook, 2004; Wasteneys and Yang, 2004) and in coupling CESA proteins to these (Rajangam et al., 2008).

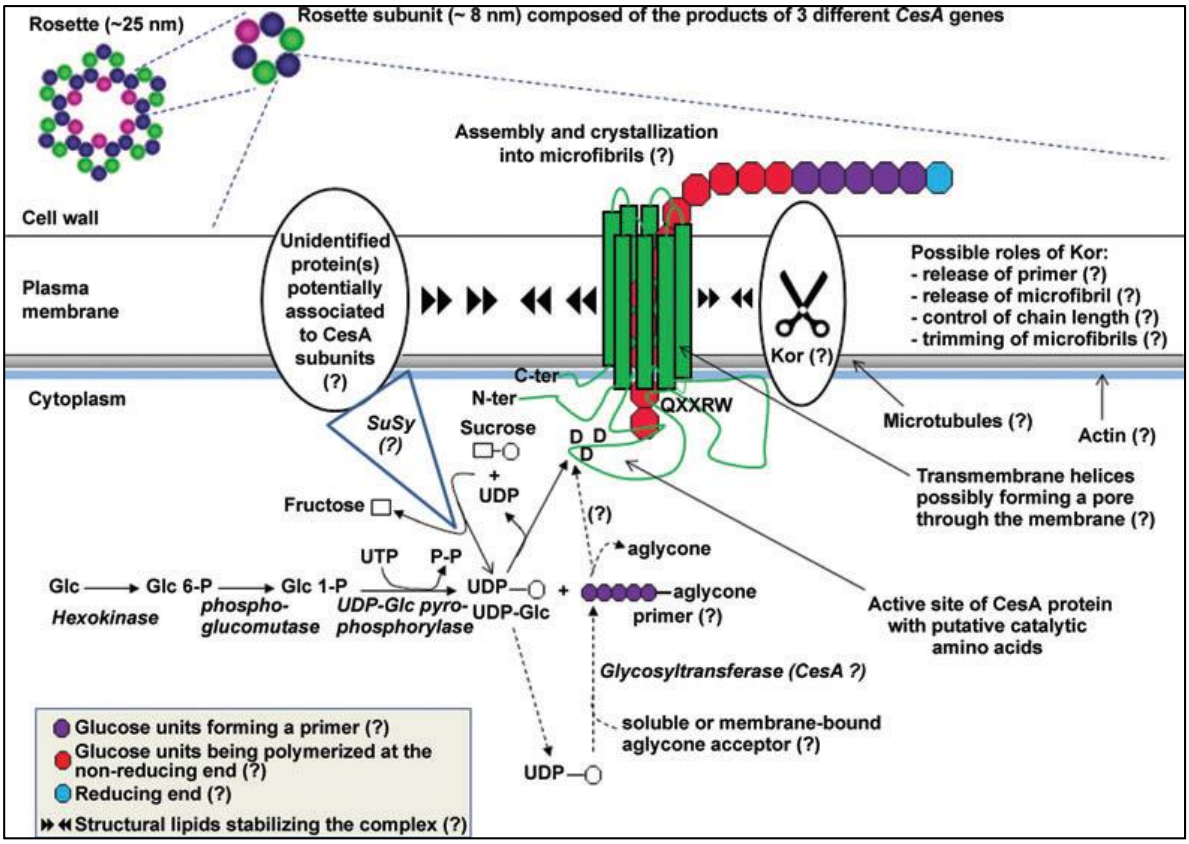

Figure 1. Hypothetical model for the cellulose biosynthesis in higher plants, Kor: endo-β-1,4-glucanase KORRIGAN; SuSy: sucrose synthase (Guerriero et al., 2010)

I.b. Non-cellulosic cell wall polysaccharides

Pectins are matrix polysaccharides enriched in galacturonic acid (GalA). Their content in the Poales is lower than in dicot and gymnosperm walls, approximately 10% by weight (Carpita, 1996). In spite of their significantly lower content in type II cell walls, they perform important physiological functions such as water retention, ion transport, cell adhesion and pore size determination, as well as acting as a defence mechanism against pathogens, wounds and abiotic stresses (Ridley et al., 2001; Jarvis et al., 2003; Verhertbruggen and Knox, 2007; Peaucelle et al., 2012; Wolf and Greiner, 2012). They can be

classified into 4 different domains depending on their composition (Scheller et al., 2007): homogalacturonan, rhamnogalacturonan types I and II and xylogalacturonan. These domains are thought to be conjoined domains (Fry, 2011) supporting the idea that *in muro*, each different domain is not an independent, single polysaccharide (Ishii and Matsunaga, 2001; Coenen et al., 2007).

Homogalacturonan is a polymer of α-1,4-linked GalA which can be methyl-esterified or acetylated (Figure 2), accounting for more than 60% of pectins in the plant cell wall (Ridley et al., 2001). The degree of methyl-esterification has an important role in the cell wall, because homogalacturonan can form a stable gel structure with other pectic molecules by means of Ca^{2+} bridges through the negatively charged unmethylated GalA residues (Liners et al., 1989). The backbone of homogalacturonan is covalently linked to rhamnogalacturonan types I and II, and is also thought to be crosslinked to xyloglucan *in muro* (Popper and Fry, 2008).

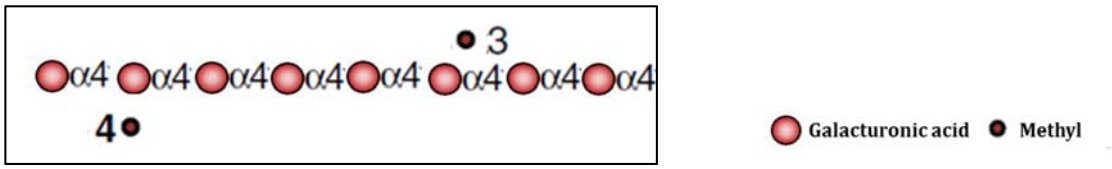

Figure 2. Schematic representation of homogalacturonan structure

Rhamnogalacturonan I is a heteropolymer of rhamnose (Rha) and GalA formed by repeating units of the disaccharide 1,2-α-Rha-1,4-α-GalA (Figure 3) (Carpita and McCann, 2000). Three types of galactan polysaccharides are associated with rhamnogalacturonan I: galactan, and types I and II arabinogalactan. Type I arabinogalactan is only found in association with pectins and is composed of β-1,4-galactose (Gal) chains substituted with mostly arabinose (Ara) units. Type II arabinogalactan is composed of a backbone of β-1,3-Gal with branch points of β-1,6-Gal and is associated with specific proteins, called arabinogalactan proteins (Caffall and Mohnen, 2009) (see section proteins).

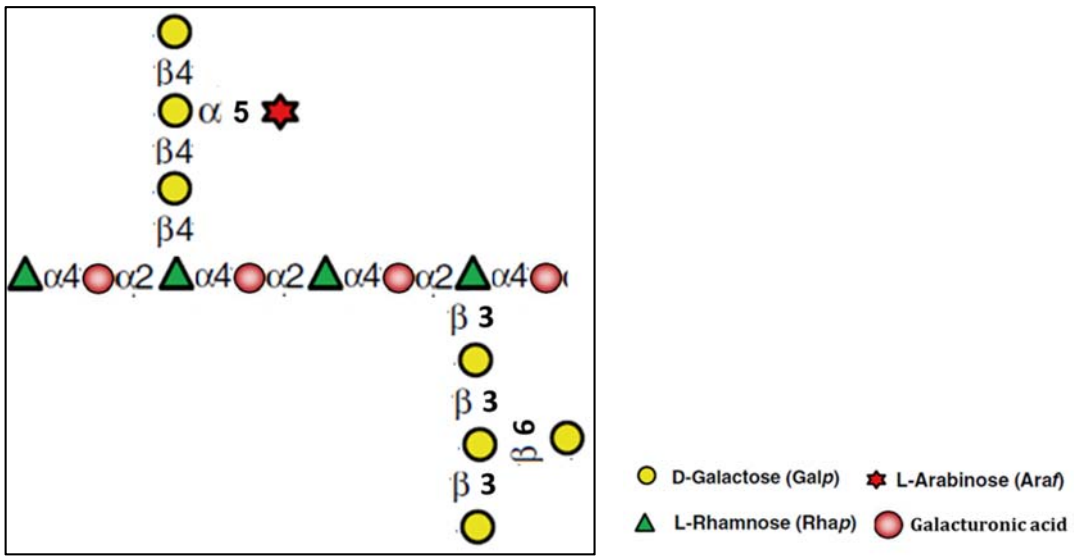

Figure 3. Schematic representation of rhamnogalacturonan I structure

Rhamnogalacturonan II is a substituted galacturonan that is a ubiquitous component of plant walls (Caffall and Mohnen, 2009). It has the richest diversity of sugars and linkage structures known, including apiose, aceric acid, methyl-fucose, methyl-xylose, 3-deoxy-D-manno-2-octulosonic acid, and 3-deoxy-D-lyxo-2-heptulosaric acid. Rhamnogalacturonan II molecules are known to self-associate, forming dimers via a boron bond (Albersheim et al., 2011). Although highly complex, the structure of rhamnogalacturonan II is highly conserved, suggesting that it plays an important role in wall function (Carpita and McCann, 2000).

Hemicelluloses are a group of neutral polysaccharides with a non-crystalline structure, composed of a lineal chain of monosaccharides, mainly Xyl (xylose), Glc or Man (mannose), and generally have short branches (for details see Pauly et al., 2013). The main hemicellulose polysaccharides in type II cell walls are xylans and mixed-linked glucan, but a small proportion of xyloglucan is also found. Through the establishment of hydrogen bonds, xylans and mixed-linked glucan bind tightly to adjacent cellulose microfibrils and therefore, maintain them in the correct spatial conformation.

Xylans are composed of a backbone of 1,4 linked β-Xyl residues, often substituted with GlcA (glucuronic acid) and MeGlcA (methyl-glucuronic acid; glucuronoxylan) (Figure 4A), Ara (arabinoxylan) or a combination of acidic and neutral sugars (glucuronoarabinoxylan) (Figure 4B). Glucuronoxylan is a major component of the secondary walls of dicots, whereas arabinoxylans and, to a lesser extent, glucuronoarabinoxylan, are major components of grasses (York and O'Neill, 2008).

They are the main hemicellulose polysaccharides in type II cell walls (Fincher, 2009), representing 20% to 40% of the wall dry weight (Vogel, 2008). Poalean xylans show the general structure of all xylans (a backbone of β-1,4-Xyl with Ara, GlcA, or MeGlcA residues attached), but also some unique features. These include the attachment of Ara residues to position 3 (rather than to position 2, characteristic of dicots) in some backbone Xyl residues (Fry, 2011), which often show further substitutions with oligosaccharide side chains containing Xyl, Gal and acetyl groups (Carpita, 1996). Another of their distinctive features is the presence of hydroxycinnamates, mainly Fer (ferulic acid) and *p*-Cou (*p*-coumaric acid), esterified on the Ara residues (Smith and Hartley, 1983; Kato and Nevins, 1985). Through peroxidase action, these hydroxycinnamates are susceptible to oxidative coupling (Geissmann and Neukom, 1971), forming dehydrodiferulates and hence contributing to assembly by cross-linking cell wall polysaccharides (Fry, 2004; Parker et al., 2005).

Xyloglucan consists of a lineal chain of β-1,4-glucan, substituted with α-1,6-Xyl residues which in turn may be branched with Ara or Gal, depending on the species; in addition, Gal residues may also have fucose (Fuc) substitutions attached (Hayashi, 1989) (Figure 5). Although it is the main hemicellulose polysaccharide in type I cell walls, representing around the 20% of wall dry weight, its content is substantially lower in type II cell walls, contributing only 1-5%. Poalean xyloglucan also differs qualitatively from that of dicots, showing a considerably lower Xyl/Glc ratio and a lower Ara, Gal and Fuc substitution content (Fry, 2011). Despite its reduced presence in type II cell walls, xyloglucan is thought

to play an important functional role, since grass tissues often show high xyloglucan-endo-transglucosylase activities (Fry et al., 1992).

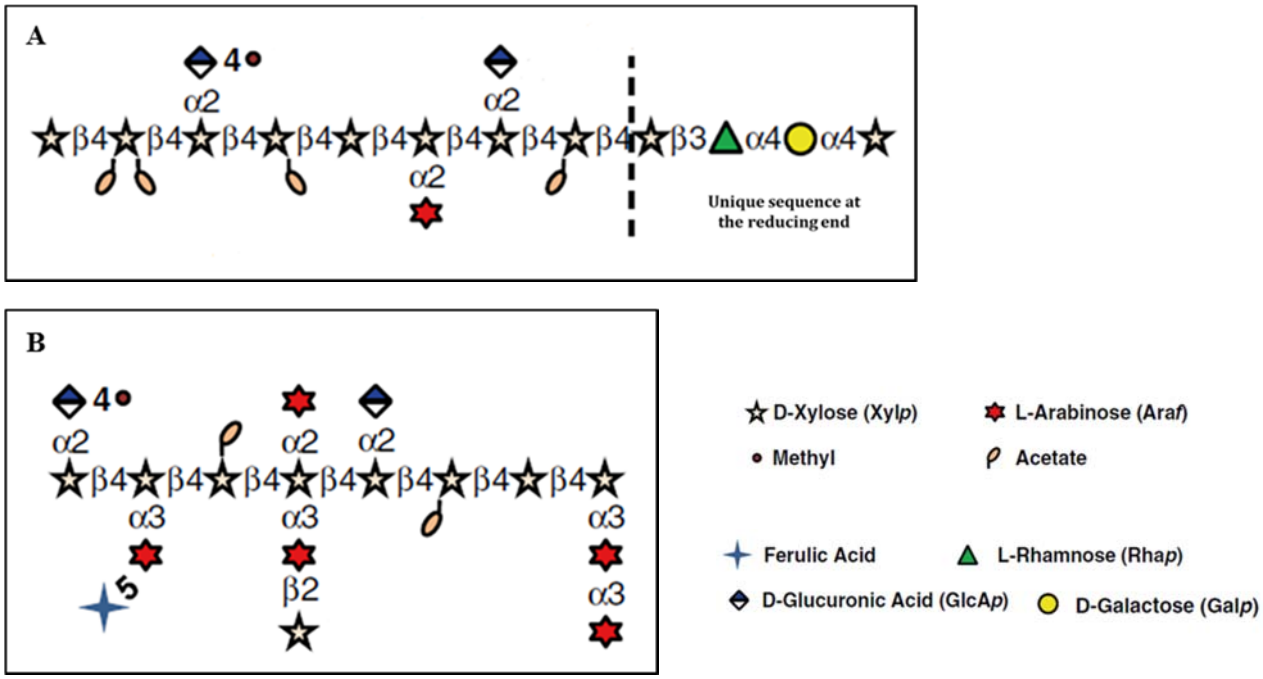

Figure 4. Schematic representation of **A**: glucuronoxylan and **B**: glucuronoarabinoxylan structure (modified from Pauly et al., 2013)

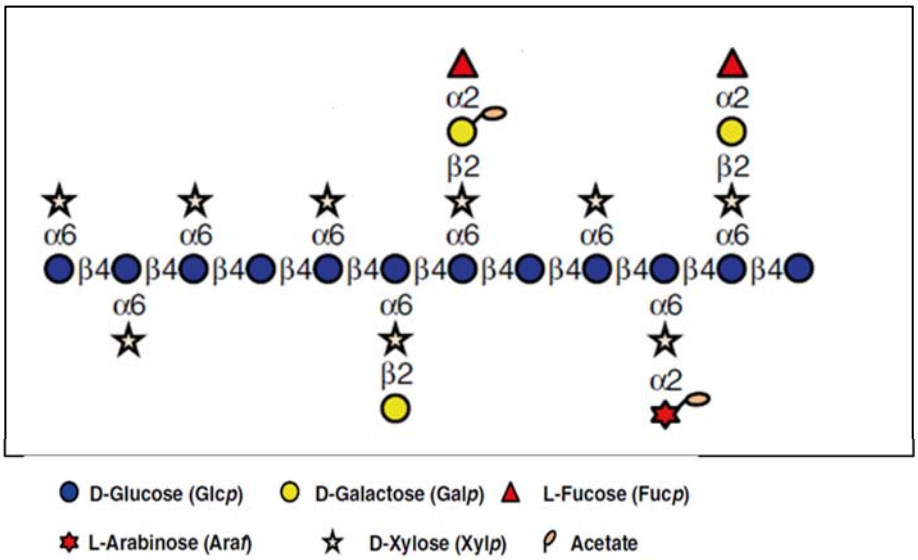

Figure 5. Schematic representation of xyloglucan structure (modified from Pauly et al., 2013)

Mixed-linkage glucan is a hemicellulose of Poalean primary walls, consisting of an un-branched chain of β-Glc residues linked by 1,3 and 1,4 bonds (Figure 6). Playing a crucial role

in primary wall expansion, its levels are highly related to the growth phase (Obel et al., 2003; Gibeaut et al., 2005). It has been reported that it is not exclusive to the Poales, but also appears in liverworts and horsetails (Popper and Fry, 2003; Fry et al., 2008).

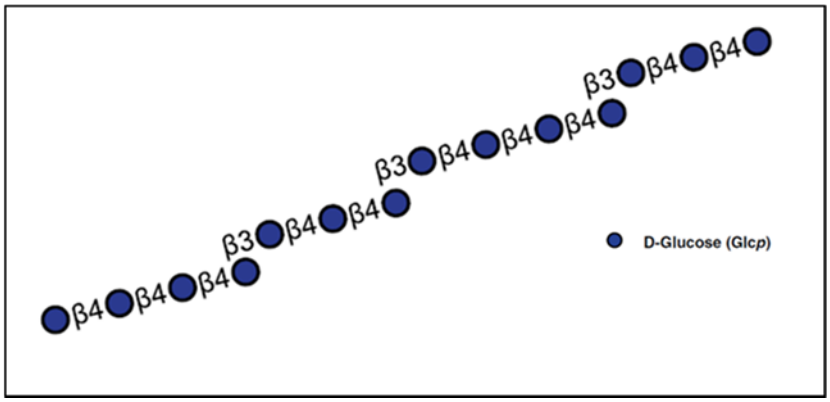

Figure 6. Schematic representation of mixed-linked glucan structure (modified from Pauly et al., 2013)

BIOSYNTHESIS

One of the crucial functions of the Golgi apparatus concerns the synthesis of non-cellulosic cell wall polysaccharides. Unlike cellulose, which is presumably synthesised at the plasma membrane, non-cellulosic polysaccharides (hemicelluloses and pectins) are synthesised and assembled within the Golgi cisternae, and subsequently transported to the cell surface by Golgi-derived vesicles (Driouich et al., 1993; 2012; Lerouxel et al., 2006; Day et al., 2013). Synthesis of non-cellulosic polysaccharides is catalysed by the coordinated action of glycosyltransferases (GTs), enzymes that transfer a sugar residue from an activated nucleotide-sugar onto a specific acceptor. Most plant GTs are type II membrane proteins with a single-pass trans-membrane topology and a catalytic domain in the Golgi lumen, but some of them are multi-pass trans-membrane proteins, with a topological structure similar to the GTs of other organisms (Varki et al., 2009; Rini et al., 2009). As cell wall matrix polysaccharides show a high degree of structural complexity, a spatial organisation of their biosynthesis within the different Golgi stacks should occur, not exclusively between the GTs themselves, but also between GTs, nucleotide-sugar transporters and nucleotide-sugar

interconverting enzymes (Seifert, 2004; Reiter, 2008). In addition, interaction among Golgi proteins has been reported in recent studies (Oikawa et al., 2012). Furthermore, when protein members of a synthesising complex vary, the latter's biochemical and catalytic function also differs (Oikawa et al., 2012). GTs are classified into 92 families in the CAZy (carbohydrate active enzymes) data base. A brief summary of some of the genes codifying for GTs involved in pectin, xylan and xyloglucan synthesis is shown in Tables 1, 2 and 3, respectively.

Due to the high diversity of monosaccharide and glycosidic linkages in pectic polysaccharides, it has been suggested that a minimum of 67 GTs is involved in pectin biosynthesis (Mohnen, 2008). In addition, control of the degree of homogalacturonan methyl-esterification during plant development requires specific methyltransferase and esterase activities (Wolf et al., 2009). *GAUT1*, belonging to the GT8 family, has been identified as a candidate for encoding the galacturonosyltransferase required to form the homogalacturonan backbone in arabidopsis (Sterling et al., 2006). Another gene named *GAUT7*, presenting a 77% similarity to *GAUT1*, is required for galacturonosyltransferase activity, and it is related to the Golgi membrane anchor of *GAUT1* (Atmodjo et al., 2011; 2013). *QUA1* (*GAUT8*) also belongs to the GT8 family (Sterling et al., 2006), and since its corresponding mutant shows reduced galacturonosyltransferase activities, it is thought to be related to homogalacturonan synthesis (Orfila et al., 2005). The existence of a protein complex containing galacturonosyl and methyltransferase activities has also been suggested (Mouille et al., 2007). Similarly, *QUA2* and *QUA3* have been proposed to encode methyltransferases (Miao et al., 2011) since both of them are highly co-regulated or co-expressed with *QUA1* (Drouich et al., 2012) and *GAUT1* and *GAUT7* (Atmodjo et al., 2011; 2013) respectively. It has also been proposed that another gene, *CGR3*, encodes a methyltransferase activity (Held et al., 2011).The *XGD1* gene is involved in transferring β-1,3-Xyl residues to the homogalacturonan backbone to form xylogalacturonan (Jensen et al., 2008). Four other genes, *RGXT1–4* (GT77), have been demonstrated to be involved in the

synthesis of rhamnogalacturonan II, encoding α-1,3-xylosyltransferase activities (Egelund et al., 2006; 2008; Fangel et al., 2011; Liu et al., 2011). Little is known about the GTs involved in rhamnogalacturonan type I biosynthesis and only ARAD1, probably having α-1,5-arabinosyltransferase activity, ARAD2 (both of them members of GT47 family) (Harholt et al., 2012) as well as three *GALS* (*1-3*), encoding galactosyltransferase activities (Liwanag et al., 2012), have been characterised. Finally, an UDP-arabinose mutase has been characterised in rice (Konishi et al., 2007).

Gene	CAZy	Activity	Reference
GAUT1	GT8	HGA: Galacturonosyltransferase (Pr)	Sterling et al., 2006 Atmodjo et al., 2011
GAUT7	GT8	Golgi membrane anchor of GAUT1 (Pr)	Atmodjo et al., 2011
GAUT8 / QUA1	GT8	HGA: Galacturonosyltransferase (Pu)	Bouton et al., 2002 Orfila et al., 2005
QUA2 / TSD2	–	HGA:Methyltransferase (Pu)	Mouille et al., 2007
QUA3	–	HGA:Methyltransferase (Pu)	Miao et al., 2011
CGR3	-	HGA:Methyltransferase (Pu)	Held et al., 2011
XGD1	GT47-C	XGA:Xylosyltransferase (Pr)	Jensen et al., 2008
RGXT1-4	GT77	RG-II:Xylosyltransferase (Pr)	Egelund et al., 2006; 2008 Fangel et al., 2011 Liu et al., 2011
ARAD1	GT47-B	RGI:Arabinosyltransferase (Pu)	Harholt et al., 2012
ARAD2	GT47-B	RGI:Arabinosyltransferase (Pu)	Harholt et al., 2012
GALS1-3	GT92	Galactosyltransferase (Pr)	Liwanag et al., 2012

Table 1. Some genes involved in the pectin synthesis (modified from Drouich et al., 2012 and Atmodjo et al., 2013), HGA: homogalacturonan; RGI: rhamnogalacturonan type I; RGII: rhamnogalacturonan type II; XGA: xylogalacturonan; Pr: proven; Pu: putative

Recent years have witnessed great advances in our knowledge about xylan biosynthesis. Several arabidopsis mutants (named *irx* due to the irregular xylem phenotype they present) with low Xyl content in their walls have been described. *Irx9* (Brown et al., 2007; Pena et al., 2007; belonging to GT43 family), *irx10* [Brown et al., 2009; Wu et al., 2009 (GT47)] and *irx14* [Brown et al., 2007 (GT43)] were found to be defective in making the xylan backbone (Dhugga et al., 2012), indicating that their encoded proteins are involved in xylan chain elongation. On the other hand, *irx7/fra8* [Brown et al., 2007 (GT47)], *irx8* [Lee et al., 2007; Pena et al., 2007 (GT8)] and *parvus* [Lao et al., 2003; Lee et al., 2009 (GT8)] were observed to be defective in the synthesis of the unique sequence at the reducing end of the xylan chain. The suggested role for this sequence is either as a primer or terminator of the

synthesis (York and O'Neill, 2008). The results reported in Pena et al. (2007) indicate that it could be acting as a terminator of the synthesis, since a reduction in the number of glucuronoxylan chains but an increase in their length was observed. Regarding the substitution of the xylan chain, GlcA and MeGlcA residues are added to the backbone by GUX enzymes, with five members in arabidopsis (Pauly et al., 2013). The different GUX enzymes lead to different GlcA xylan substitution patterns (Bromley et al., 2013). Other genes, closely related to *IRX* but with a more general expression pattern and lower expression levels, have been named as their redundant homologue but with the suffix "LIKE", i.e. *IRX9L*, *IRX10L*, *IRX14L* (Brown et al., 2009; 2011; Wu et al., 2010).

Focusing on grasses, the coordinated action of members of a protein complex consisting of a β-1,4-xylosyltransferase (GT43), an α-1,3-arabinosyltransferase (GT47) and an α-1,2-glucuronyltransferase (GT43) has been reported in the glucuronoarabinoxylan synthesis in wheat (Zeng et al., 2010). In the case of maize, Bosch et al. (2011) reported the sequences for the *IRX9*, *IRX10* and *IRX10L* arabidopsis orthologues. More recently, three rice genes orthologues of arabidopsis *IRX9*, *IRX9L* and *IRX14* have been identified, encoding for putative β-1,4-xylan backbone-elongating GTs (Chiniquy et al., 2013). In addition, the unique sequence acting as a primer or terminator is not found in grass glucuronoarabinoxylans, leading to the question of whether xylan synthesis in grasses involves a primer/terminator free mechanism, or whether there is another, undiscovered molecule involved (Pauly et al., 2013). As regards xylan substitutions, a member of the GT61 family, *XAX1*, has been described as being responsible for adding β-Xyl units to form the side chain β-Xyl-α-1,2-Ara in rice (Chiniquy et al., 2012). The corresponding rice *osxax1* mutant is also deficient in Fer and *p*-Cou, although the reason for this is unknown (Chiniquy et al., 2012). In wheat, XAT1 and XAT2 act as α-1,3-arabinosyltransferases (Anders et al., 2012). Finally, a member of the TBL family, TBL29, has been reported to be responsible for xylan acetylation (Xiong et al., 2013). However, and although advances have been made in our understanding of xylan synthesis in grasses in recent years, some aspects of this process still remain unknown.

Gene	CAZy	Activity	Reference
IRX9	GT43	Xylan synthase	Brown et al., 2007 Pena et al., 2007
IRX10	GT47	Xylan synthase	Brown et al., 2009 Wu et al., 2009
IRX10L /GUT1	GT47	Xylan synthase	Brown et al., 2009 Wu et al., 2009
IRX7 / FRA8	GT47	Xylan synthase	Brown et al., 2007 Pena et al., 2007
IRX8	GT8	Xylan synthase	Pena et al., 2007
PARVUS	GT8	Xylan synthase	Lao et al., 2003 Lee et al., 2009
GUX1-5	GT8	Glucurounosyltransferase	Mortimer et al., 2010 Lee et al., 2012 Rennie et al., 2012
XAX1	GT61	Xylosyltransferase	Chiniquy et al., 2012
XAT1-2	GT61	Arabinosyltransferase	Anders et al., 2012
GXMT	-	Methyltransferase	Urbanowicz et al., 2012
GXM1-3	-	Methyltransferase	Lee et al., 2012
IRX15, IRX15L	-	Methyltransferase	Brown et al., 2011 Jensen et al., 2011
TBL29	-	Acetyltransferases	Xiong et al., 2013
IRX14 / IRX14L	GT43	Xylan synthase	Wu et al., 2010
IRX9L	GT43	Xylan synthase	Wu et al., 2010

Table 2. Some genes involved in the xylan synthesis (modified from Pauly et al., 2013 and Jensen et al., 2013)

The xyloglucan backbone is thought to be synthesised by one or more members of the cellulose synthase-like C (*CSLC*) gene family (Pauly et al., 2013), in which the *CSLC4* gene has been identified as a glucan synthase (Cocuron et al., 2007). Regarding the patterning of Xyl substitutions, five genes of the GT34 family have been reported in arabidopsis as xylosyltransferases (Faik et al., 2002; Vuttipongchaikij et al., 2012). These Xyl substituents can be further substituted with Gal units, through the action of the corresponding proteins codified by *MUR3* and *XLT2* genes which belong to GT47 family (Madson et al., 2003; Jensen et al., 2012). *XUT1* is a similar gene to *MUR3* and *XLT2* and is included in the same subclade of the GT47 family (Pauly et al., 2013) that was identified in arabidopsis as being necessary for the presence of GalA acid in xyloglucan (Pena et al., 2012).

In addition, a fucosyltransferase, FUT1, has been shown to add terminal Fuc groups to Gal or GalA residues (Perrin et al., 1999; Faik et al., 2000; Pena et al., 2012). Xyloglucan

also contains acetyl substituents at various positions on the polymers depending on the plant species (Gille and Pauly, 2012). The *AXY4* gene has been identified in arabidopsis as being responsible for these substitutions and is included as a member of the TBL protein family (Bischoff et al., 2010a; 2010b).

Gene	CAZy	Activity	Reference
CSLC4	GT2	Glucan synthase	Cocuron et al., 2007
XXT1-5	GT34	Xylosyltransferase	Faik et al., 2002 Cavalier and Keegstra, 2006 Cavalier et al., 2008
MUR3	GT47	Galactosyltransferase	Madson et al., 2003
XLT2	GT47	Galactosyltransferase	Jensen et al., 2012
XUT1	GT47	Galacturonosyltransferase	Pena et al., 2012
FUT1 / MUR2	GT37	Fucosyltransferase	Perrin et al., 1999 Vanzin et al., 2002 Pena et al., 2012
AXY4 / AXY4L	-	Acetyltransferase	Gille et al., 2011

Table 3. Some genes involved in the xyloglucan synthesis (modified from Pauly et al., 2013)

Mixed-linked glucan biosynthesis is carried out by proteins from the CSLF and CSLH families (Burton et al., 2006; Doblin et al., 2010). Both genes differ in structure as well as in the proteins that they encode (Wang et al., 2010). It has been reported that the overexpression of *CSLF* genes in barley significantly increased the amount of mixed-linked glucan in leaves and endosperm (Burton et al., 2011).

I.c. Proteins

Proteins are also essential constituents of cell walls, being involved in the modification of cell wall components and also interacting with plasma membrane proteins at the cell surface (Jamet et al., 2006). Two main groups of proteins can be distinguished: structural proteins, usually found immobilised at the cell wall, and soluble proteins, which are located at the apoplast and normally have enzymatic activities (Lee et al., 2004).

Structural proteins are less abundant in type II than in type I cell walls (1% and 10% respectively) (Vogel, 2008). They are generally glycoproteins with repeated sequences enriched in one or two amino acids, and can be divided in four main classes: hydroxyproline-rich glycoproteins, proline-rich proteins, glycine-rich proteins and arabinogalactan proteins. Of these, this latter group is one of the best-studied. Arabinogalactan proteins consist of a core protein of varying length and domain complexity, one or more arabinogalactan type II side chains, and often a glycosylphosphatidylinositol lipid anchor. They are involved in important biological processes such as cell division, programmed cell death, pattern formation growth, pollen tube guidance, secondary cell wall deposition, abscission and plant microbe interactions (Seifert and Roberts, 2007). Interestingly, an arabinogalactan protein which covalently links xylans to pectic polysaccharides has recently been reported in arabidopsis (Tan et al., 2013). This finding has important implications for polymer biosynthesis, network formation and apoplastic polymer metabolism.

Regarding the *soluble proteins*, most of them are enzymes related to important processes such as cell wall extension, molecular transport, cell recognition and pathogen resistance (Rose et al., 2002), and include hydrolases, transglycosylases, peroxidases, expansins and kinases.

Hydrolases form the main group of soluble cell wall proteins, including important enzymes such as glycanases, which are responsible for breaking glycosidic bonds (Gilbert, 2010). Depending on whether their cleaving action takes place at the non-reducing terminus or inside of the polysaccharide chain, they can be exoglycanases or endoglycanases respectively (Fry, 2000).

Transglycosylation reactions are also essential in cell growth and are catalysed by transglycosylases, enzymes that cleave glycosidic linkages transferring either a monosaccharide (exotransglycosylases) or an oligosaccharide (endotransglycosylases) from the donor polysaccharide to the non-reducing end of the acceptor (Fry, 2000).

Through hydrogen peroxide or organic hydroperoxides, peroxidases are responsible for the oxidative coupling of phenolic residues, and are involved in arabinoxylan cross-linking, the formation of isodityrosine bridges among hydroxyproline-rich glycoproteins and the lignification process (Fry, 2004; Lindsay and Fry, 2008).

Expansins are involved in acid growth (McQueen-Mason et al., 1992), and hence have a crucial role in the regulation of cell wall elongation in growing cells. Basically, their operation mechanism consists of breaking non-covalent interactions among cellulose microfibrils and hemicelluloses (McQueen-Mason et al., 2007). Specifically in maize, it has been proposed that an expansin (EXPB1) is involved in remodelling the arabinoxylan-cellulose network during cell wall relaxation (Yennawar et al., 2006).

Another important group of proteins are the kinases, known to be involved in the signalling pathways of a wide range of cell processes. Those associated with the cell wall are involved in cell elongation and the modulation of sugar metabolism (Kohorn et al., 2006) as well as in signalling responses to impairment of cell wall integrity (Seifert and Blaukopf, 2010), serving as pectin receptors (Kohorn and Kohorn, 2012).

BIOSYNTHESIS

Synthesis and assembly of cell wall proteins is highly controlled: whereas some of them are developmentally regulated or expressed in a tissue-dependent manner, the synthesis of others occurs in response to wounding, infections or environmental stresses (Albersheim et al., 2011). Cell wall proteins are synthesised and assembled in the endoplasmic reticulum. Proteoglycans such as arabinogalactan, hydroxyproline-rich and glycine-rich proteins later undergo glycosylation, usually in the Golgi apparatus (Figure 7) (Carpita and McCann, 2000).

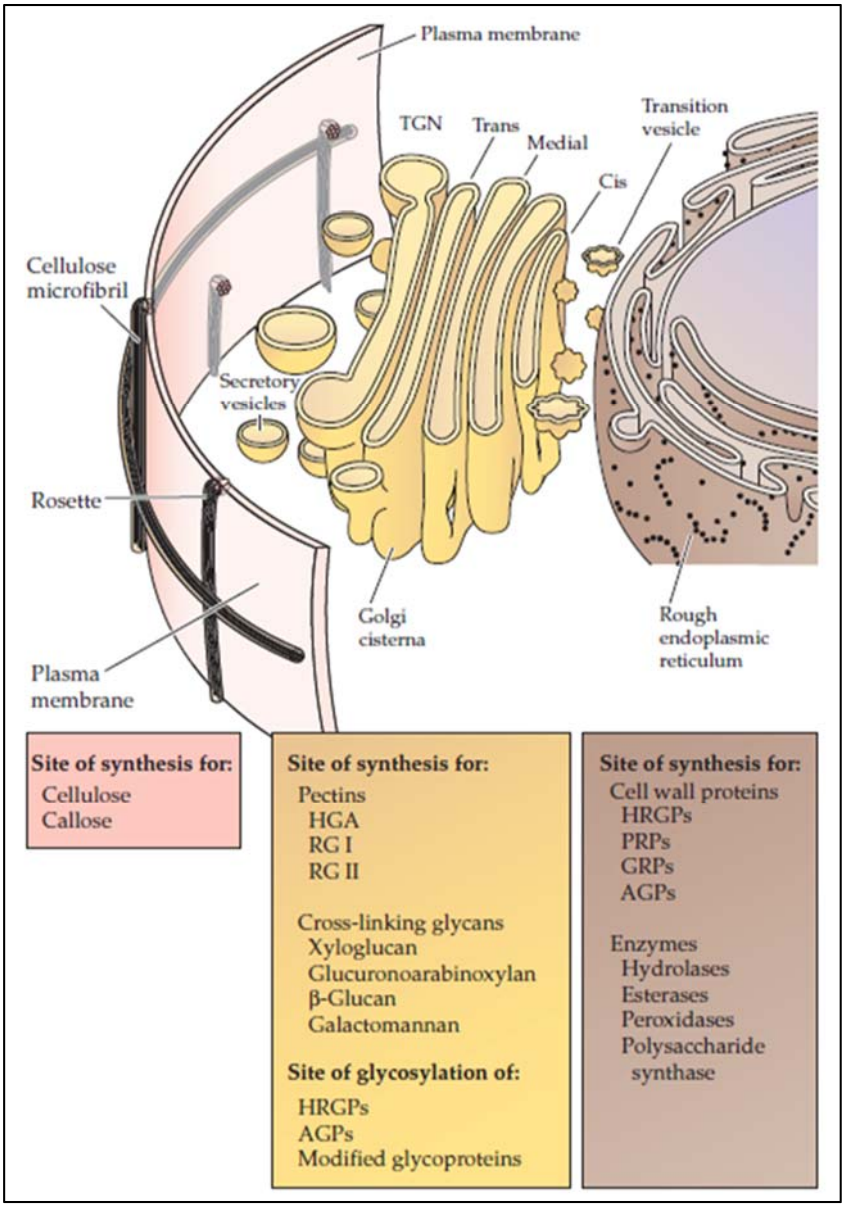

Figure 7. Site of biosynthesis of some cell wall components. HGA: homogalacturonan; RGI: rhamnogalacturonan type I; RGII: rhamnogalacturonan type II; HRGPs: hydroxyproline-rich glycoproteins; PRPs: prolyne-rich proteins; GRPs: glycine-rich proteins and AGPs: arabinogalactan proteins (Carpita and McCann, 2000)

I.d. Phenolic compounds

Phenols are also important compounds in the cell wall of grasses, specifically the hydroxycinnamic acids, the principal of which in cell walls are Fer and *p*-Cou (Wallace and Fry, 1994). They may both be esterified each other when lignin is formed (Higuchi et al., 1967), with Ara and Xyl residues of arabinoxylans (Wende and Fry, 1997) and xyloglucan (Ishii et al., 1990) respectively, and probably with glycoproteins (Obel et al., 2003). Although

a minority component in quantitative terms, they play crucial roles in the cell wall, as they are susceptible to oxidative coupling by peroxidases (Geissmann and Neukom, 1971) to form ester-linked dehydrodiferulates (Fry, 2004; Parker et al., 2005), contributing to cell wall polysaccharide assembly, growth cessation and cell wall reinforcement against abiotic or biotic stresses (Buanafina, 2009).

BIOSYNTHESIS

Fer and *p*-Cou are synthesised in the cytosol following the first part of the phenylpropanoids pathway (Figure 8) (Vogt, 2010), and are esterified on the Ara residues of the arabinoxylan chains, a process that takes place in the Golgi apparatus (Lindsay and Fry, 2008). Regarding the proteins responsible for this incorporation, members of the PFAM family have been described as feruloyltransferases (Piston et al., 2010).

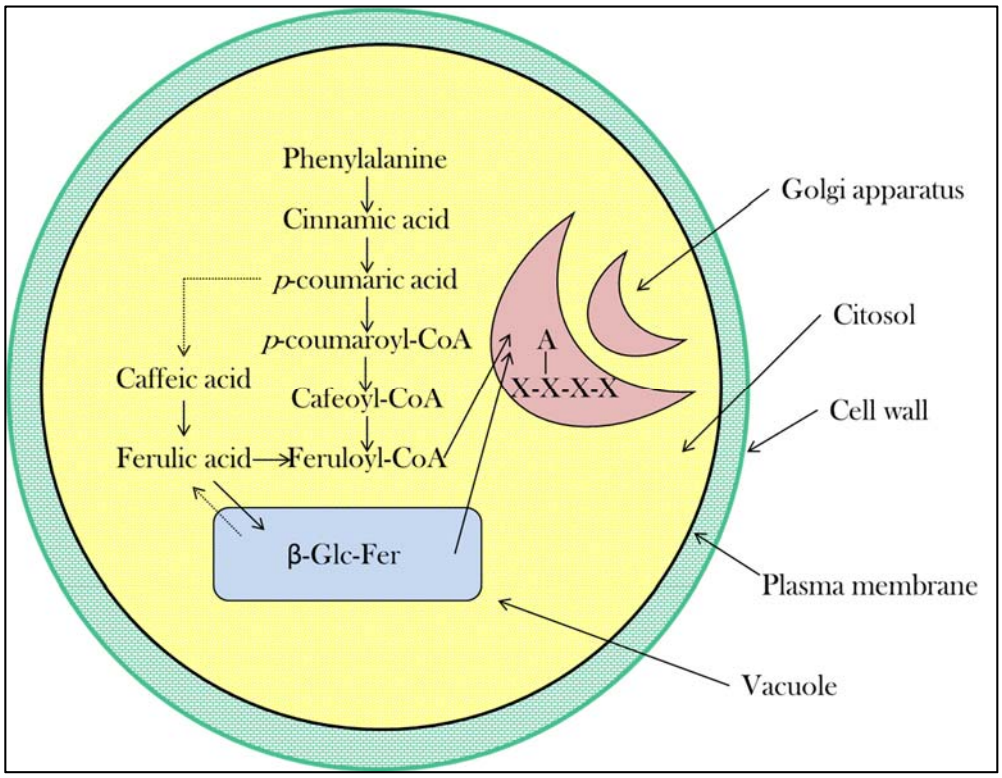

Figure 8. Schematic cellular localization of hydroxycinammates synthesis pathway in plant cells. Glc: glucose; Fer: ferulic acid. Not confirmed steps of the pathway are indicated in dashed lines (Modified from Lindsay and Fry, 2008)

II. THE ARCHITECTURE OF PRIMARY TYPE II CELL WALLS

Primary walls are composed of three independent but interconnected structural networks, consisting of cellulose microfibrils acting as scaffolding and tethered by hemicelluloses embedded in a matrix of pectin polysaccharides (Figure 9) (Carpita and McCann, 2000). Other cell wall elements, such as glycoproteins, phenolic residues, polymers and polyesters such as cutin or suberin, are highly variable between tissues, developmental stages and taxa (Fry, 2011).

Two types of primary cell walls can be distinguished (Carpita and Gibeaut, 1993), which differ in chemical composition as well as in the taxa to which they are related. As mentioned earlier, the *type I primary cell wall* is characteristic of dicots and the non-commelinoid monocots. In both cases, the main hemicellulosic polysaccharide is xyloglucan which is responsible for tethering the cellulose microfibrils, and both groups have similar contents. The xyloglucan-cellulose framework is embedded in a matrix of homogalacturonan and types I and II of rhamnogalacturonan. In addition, some type I cell walls contain large amount of proteins, which can interact with the pectic polysaccharides (Carpita and McCann, 2000).

Type II primary cell wall composition essentially differs from type I in its non-cellulosic polysaccharide content, with lower amounts of xyloglucans and pectins, which are predominantly replaced by glucuronoarabinoxylans. Type II cell walls also show lower protein contents but in contrast, the amount of hydroxycinnamates (mainly Fer and *p*-Cou) is higher. Un-branched arabinoxylans can hydrogen-bond to cellulose or to each other (Carpita and McCann, 2000). This latter possibility can also take place through the Fer residues from different arabinoxylan molecules by covalent cross-linking mediated by oxidative coupling (Fry, 2000). Little is known about how type II cell wall networks interact with each other. Nevertheless, such information would be very valuable since these interactions could be involved in the reinforcing mechanism of the wall structure, especially when walls are subjected to stress conditions affecting one or more of their components.

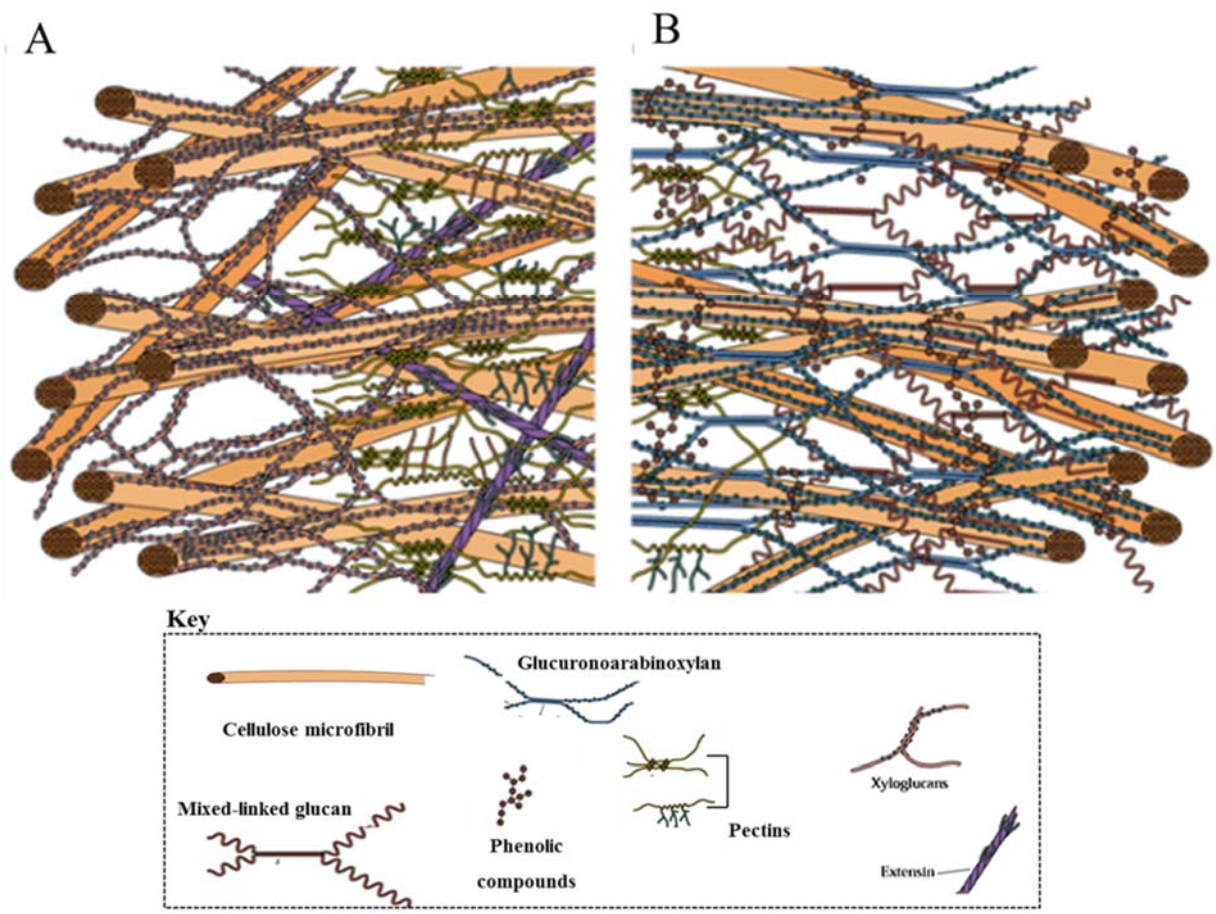

Figure 9. Structural models of **A**: type I and **B**: type II primary cell wall (Modified from Carpita and McCann, 2000)

III. STRUCTURAL PLASTICITY OF THE PRIMARY CELL WALL

Part of the dynamism of cell walls is due to their structure and composition, which endow them with the capacity to adapt to new environmental conditions, as well as to biotic and abiotic stresses. Significant advances in the characterisation of altered cell walls have been achieved in recent years through the study of *in vitro* plant cultured cells, tissues and/or organs. These modified walls are the result of genetic modifications as well as adaptation to biotic and abiotic stresses, such as habituation to cellulose biosynthesis inhibitors. The information gained from the study of these cell wall modifications is extremely valuable, not only because it sheds light on the contribution of the cell wall to adaptation to stress conditions, but also because it improves our knowledge about the precise role of the cell wall component in these coping mechanisms.

The study of cell wall plasticity also has significant economic implications, since an improvement in our knowledge about cell wall synthesis and assembly would have an impact on several important industries, such as textiles, wood, paper and biofuels.

III.a. Mutants

The use of mutants presenting a modified cell wall structure or composition has led to the detection of genes involved in cell wall constituent synthesis, *in muro* processing or assembly. For instance, it has recently been demonstrated that arabidopsis and brachypodium plants expressing hemicellulose- and pectin-specific fungal acetylesterases are resistant to fungal pathogens, but not to bacterial ones, indicating that the degree of cell wall polysaccharide acetylation plays an important role in fungal defence mechanisms (Pogorelko et al., 2013). However, this methodological approach has important limitations, since the mutation may affect essential genes, may be lethal for the mutant or could render the altered phenotype imperceptible, or multigene families may complicate the analysis due to redundant functions. Modifications in the cell wall of these mutants have been screened using gas chromatography and mass spectrometry (Reiter et al., 1997) or Fourier transform infrared (FTIR) and near infrared (NIR) spectroscopy (Chen et al., 1998; Mouille et al., 2003; McCann et al., 2007; Penning et al., 2009), and the study of mutants using these approaches has led in recent years to the identification of several of the enzymes, including GTs (see section non-cellulosic polysaccharides biosynthesis), involved in polysaccharide synthesis (Brown et al., 2007; 2011; Drouich et al., 2012).

III.b. Tolerance to several stresses

Cell wall modifications are related to plant cell strategies to cope with several kind of stress. As mentioned before, several cultured plant cell lines showing tolerance to diverse types of stress have been obtained in recent decades with *in vitro* culture techniques whether using mutagenic agents or not. In this latter type of study, it is expected though that

those cells capable of growing and dividing under such restricted growth conditions become tolerant, which would eventually be accumulated in the culture. Binzel et al. (1988) obtained cultured tobacco cells habituated to high salinity and water deficiency. These cells showed a diminished cell volume and weaker cell walls with lower cellulose and higher hydroxyproline-rich protein content (Iraki et al., 1989a; 1989b). Although no quantitative differences were detected in the total pectin proportion, qualitative modifications in the organisation and composition of the pectic networks were observed, showing a relaxed homogalacturonan-rhamnogalacturonan network which partially substituted the cellulose-xyloglucan one. Another study on cell wall modifications and stress conditions showed an increase in peroxidase activity in the culture medium concomitant with greater lignin contents in the cell wall of tomato cells adapted to saline stress (Sancho et al., 1996).

Through immobilisation of cell walls, plants are capable of adapting to and surviving in higher concentrations of aluminium (Vázquez et al., 1999), lead (Jiang and Liu, 2010) and other heavy metals (Carpena et al., 2000). Furthermore, cell walls are also involved in the coping response to other stresses such as low temperatures (Yamada et al., 2002), hypo-osmotic shock (Cazalé et al., 1998) and mechanical stress (Yahraus et al., 1995). In this regard, it has been reported that cell wall arabinose-enriched polymers (such as pectin-arabinans, arabinogalactan proteins and arabinoxylans) make a major contribution to the general mechanism against desiccation in a wide variety of resurrection plants (Moore et al., 2013).

III.c. Cellulose biosynthesis inhibitors and habituation

Several compounds affecting the biosynthesis of cell wall components have been described (for a review see Acebes et al., 2010; García-Angulo et al., 2012). Their use is considered a valuable tool in the study of the genesis and architecture of cell wall as well as of the processes in which they are involved. Likewise, advances have been achieved in our knowledge of the biosynthesis, organisation and structure of primary and secondary cell

walls through the use of these inhibitors (Satiat-Jeunemaitre and Darzens, 1986; Suzuki et al., 1992; Taylor et al., 1992; Vaughn and Turley, 1999; 2001), in addition to obtaining important information about cell plate formation and cytokinesis (Vaughn et al., 1996; DeBolt et al., 2007) and cell wall extension and the relaxation mechanism (Hoson and Masuda, 1991; Edelmann and Fry, 1992; Montague, 1995). Other aspects related to cellulose biosynthesis have also been studied using these inhibitors, such as the connection between cytoskeleton and cellulose (Fisher and Cyr, 1998; DeBolt et al., 2007; Brabham and DeBolt, 2013), CESA complex organisation (Mizuta and Brown, 1992) and the relationship between cellulose and callose synthesis (Delmer, 1987; Delmer and Amor, 1995).

Cellulose biosynthesis inhibitors (CBIs) are a structurally heterogeneous group of compounds that affects cellulose synthesis, acting specifically on cellulose coupling or deposition in higher plants. They can induce aberrant trajectories in CESA proteins, reduce their velocity or even clear them at the plasma membrane (Acebes et al., 2010; Brabham and DeBolt, 2013). Several of them, including dichlobenil (DCB), the inhibitor used in the present study, have been commercialised as herbicides and are classified as group I by the "Herbicide Resistance Action Committee".

In agriculture DCB is employed as a wide spectrum pre-emergent herbicide. However, it has been used in plant research as means to induce cellulose inhibition (Brummell and Hall, 1985; Hoson and Masuda, 1991; Corio-Costet et al., 1991; Edelmann and Fry, 1992; Shedletzky et al., 1992; García-Angulo et al., 2006; Mélida et al., 2009) since it acts specifically on this process without affecting cell wall polysaccharide synthesis (Montezinos and Delmer, 1980; Blaschek et al., 1985; Francey et al., 1989) or other metabolic processes such as deoxyribonucleic acid (DNA) and protein synthesis, oxidative phosphorylation, cellular respiration or lipid, Glc and nucleotide metabolism (Meyer and Herth, 1978; Montezinos and Delmer, 1980; Galbraith and Shields, 1982; Delmer, 1987).

Several modes of action have been proposed for DCB in the last decade. First, Peng et al. (2002) suggested that DCB inhibited the synthesis of sitosterol-β-glycoside, thereby

blocking cellulose biosynthesis. However, since the experimental conditions used in that study were forced and it has not been proven that cellulose biosynthesis begins from sitosterol-β-glycoside, this hypothesis was debatable. Another DCB action mechanism which has been proposed concerns the alteration of cellulose crystallisation, probably due to disruption of the spatial arrangement of microtubules rather than to glucan chain polymerisation. A study of the *rsw1* cellulose-deficient-mutant as well as arabidopsis cells treated with 1 μM DCB revealed that when cellulose synthesis is reduced, the orientation of the remaining cellulose microfibrils is altered (Arioli et al., 1998; Himmelspach et al., 2003). Consisting with this, and in support of this hypothesis on the mode of action, is the finding that the reduction in cellulose content detected in bean cells habituated to DCB occurred concomitantly with the accumulation of a non-crystalline β-1,4-glucan (Encina et al., 2002; García-Angulo et al., 2006).

Although the target of DCB still remains unclear, several possibilities have been proposed, the most plausible of which is a MAP20 described by Rajangam et al. (2008). MAP20 is involved in cellulose synthesis since it is responsible for coupling CESA proteins to microtubules. These authors demonstrated that DCB interacted with MAP20, blocking the coupling of CESA proteins to microtubules and therefore causing the inhibition of cellulose biosynthesis.

During the last two decades, it has been demonstrated that it is possible to habituate cultured cells to lethal DCB concentrations by subculturing them in stepwise increasing concentrations of the inhibitor in the culture medium (Shedletzky et al., 1992; Wells et al., 1994; Nakagawa and Sakurai, 1998; Sabba et al., 1999; Encina et al., 2001; 2002; Alonso-Simón et al., 2004; García-Angulo et al., 2006; 2009; Mélida et al., 2009; 2010a; 2010b; 2011).

It could be assumed from the studies on DCB-habituation that the habituation mechanism resides in the ability shown by cultured cells to grow and divide with reduced cellulose contents. As a consequence of this process, a series of changes in their metabolism and cell wall composition takes place which differs depending on the cell wall type, DCB

concentration and length of exposure time (Alonso-Simón et al., 2004; Mélida et al., 2009). Most of the studies involving cultured cells habituated to DCB have been performed on cells with a type I cell wall, 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 French bean (Encina et al., 2001; 2002; Alonso-Simón et al., 2004; García-Angulo et al., 2006), where the reduction in cellulose is, generally compensated for by an increase in pectic content, whereas the hemicellulose is reduced. Other modifications have also been described, such as the apparition of the above-mentioned non-crystalline β-1,4-glucan associated with cellulose (Encina et al., 2002; García-Angulo et al., 2006; Alonso-Simón et al., 2007) and an increase in antioxidant activities (García-Angulo et al., 2009). Only two kinds of cultured cell showing type II cell walls have been habituated to DCB: barley (Shedletzky et al., 1992) and maize (Mélida et al., 2009). In the case of maize cells habituated to lethal DCB concentrations, the coping strategy for counteracting reduced cellulose levels is mainly based on a more extensive network of a greater number of cross-linked arabinoxylans with a higher relative molecular mass (M_r).

IV. OBJECTIVES

The main aim of the present study is to shed light on the mechanisms underlying the structural plasticity of type II primary cell walls, paying special attention to metabolic strategies. To this end, cultured maize cells habituated to growing in DCB and showing a moderate reduction in their cellulose content will be used. This central goal has been further divided into four objectives which are detailed below:

Objective I

Monitor and characterise modifications that occur during the first steps of the DCB-habituation process

DCB-habituated cultured cells have developed counteracting mechanisms that may vary depending on the type of primary wall exhibited in question, DCB concentration and the length of time that cells are grown in its presence (Alonso-Simón et al., 2004). Modifications produced in the cell walls of DCB-habituated maize cultured cells have been analysed previously using high DCB concentrations and long-term habituation periods (Mélida et al., 2009), but little attention has been paid to the early modifications which occur during the habituation process. Therefore, the first objective of the present study is a) to monitor the modifications generated throughout the early steps of the DCB-habituation process, b) select a cell line which shows the cell wall features of an initial DCB-habituation with a moderate reduction in cellulose content, c) subsequently characterise its growth pattern and cell wall composition. To this end, a series of spectrophotometric, chromatographic and immunocytochemical techniques will be employed.

Objective II

Obtain further information about the metabolic capacity of cellulose-deficient maize cells

Since maize cell suspensions habituated to DCB compensate for their cellulose-impoverished walls by acquiring a quantitatively and qualitatively modified arabinoxylan network (Mélida et al., 2009; 2010a; 2010b; 2011), it is highly probable that their hemicellulose metabolism is altered. Hence, the second objective is to gain further insight into the metabolism of maize cells with a moderate reduction in their cellulose content throughout the culture cycle. To this end, an *in vivo* pulse-chase experimental approach will be employed, feeding DCB-habituated cell suspension cultures with [^{3}H]Ara as the hemicellulose precursor at lag, early logarithmic and late logarithmic phases of growth. The synthesis and cell fate of ^{3}H-hemicelluloses and ^{3}H-polymers will be subsequently tracked in several cell compartments using a 5 h time-window.

Objective III

Analysis of the molecular weight distribution of polysaccharides and the phenolic metabolism in maize cells with a moderate reduction in their cellulose content

Previous studies on maize cells habituated to DCB have described a reinforced wall acquired through more cross-linked arabinoxylans with higher molecular mass and lower extractability (Mélida et al., 2009; 2011). However, the reported changes were observed in hemicelluloses extracted solely from the cell wall and in the final stage of the culture cycle. Therefore, the third objective will be the study of molecular mass distribution of polysaccharides in several cell compartments throughout the culture cycle in DCB-habituated cells with a mild reduction in their cellulose content. In addition, an analysis of phenolic metabolism will be performed since it is known to be highly related to changes in the molecular weight of hemicelluloses in maize cells. To this end, *in vivo* feeding

experiments will be performed with the radiolabeled precursors, [^{3}H]Ara and [^{14}C]Cinnamic acid (Cinn) for hemicelluloses and hydroxycinnamates, respectively.

Objective IV

Study of the Golgi proteome in DCB-habituated cells

Plasticity of the walls involving modified and remodeled hemicelluloses networks undoubtedly relies on cell metabolism, and therefore on the proteins responsible for such responses. Since crucial metabolic enzymes are known to be Golgi-resident proteins, Golgi-enriched fractions will be obtained from non-habituated and several DCB-habituated maize cells lines. First, a comparative analysis of the Golgi-proteome in different cell lines will be conducted by using two-dimensional (2-D) polyacrylamide gel electrophoresis (PAGE), and subsequently, proteins exhibiting mis-regulation will be sequenced by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF/MS) or electrospray ionization with tandem mass spectrometry (ESI-MS/MS).

Materials and Methods

I. CELL CULTURES

I.a. *In vitro* culture conditions

Maize cell-suspension cultures (*Zea mays* L., Black Mexican sweet corn) from immature embryos were grown in Murashige and Skoog media (Murashige and Skoog, 1962) supplemented with 9 µM 2,4-dichloro-phenoxyacetic acid (2,4-D), 20 g L^{-1} sucrose and adjusted to pH 5.6, at 25°C under light and rotary shaken, and routinely subcultured every 15 days.

I.b. Habituation to DCB

In order to obtain habituated cell cultures to DCB, non-habituated cells (Snh) were stepwise subcultured in a medium supplied with increasing concentrations of DCB dissolved in dimethyl sulfoxide (DMSO) which does not affect cell growth at this range of concentrations. Likewise, in the case of cells suspensions habituated to low DCB levels Snh cells were cultured in a medium containing three DCB concentrations: 0.3 µM, 0.5 µM (I_{50} value: DCB concentration capable of inhibiting dry weight (DW) increase by 50% respecting to the control) and 1 µM. After several subcultures, some of the cells habituated to grow in 1 µM DCB were transferred to medium in which the DCB concentration was increased up to 1.5 µM. Habituated suspension cultures (Sh) are referred to as Sh x (n), where ´x´ indicates DCB concentration (µM) and (n) number of subcultures in that DCB concentration.

The procedure to obtain habituated maize cells suspensions to high DCB levels (6 µM) was slightly different from that described above for the obtaining of maize cultures habituated to low DCB concentrations. In this case, maize callus cultures habituated to grow in 12 µM DCB were the starting cellular material for the habituation process, which were then transferred into liquid medium supplemented with 6 µM DCB (Sh6).

I.c. Cultures characterization

Growth curves of maize cell suspensions were obtained by measuring the increase in DW at different culture times and several growth kinetics parameters were estimated: doubling time (dt), relative growth rate and maximum growth rate.

Cell cluster size was determined after vacuum filtration of cell suspensions using different pore-size meshes, and the relative abundance of the clusters was expressed as the percentage of DW retained in each filter.

II. OBTAINING OF DISCRETE CELL COMPARTMENTS AND FRACTIONS FROM MAIZE CELL SUSPENSIONS

II.a. Cell-free medium collection (CFM) and protoplasmic content extraction

Aliquots of maize cell suspensions were passed through a Poly-Prep column. The filtrate was then collected and labelled as CFM, whereas cells retained in the Poly-Prep column filter were quickly resuspended in homogenisation buffer and subsequently transferred to the glass mortar for being homogenised with a motor-driven Teflon pestle. Resultant homogenate was again passed through a second Poly-Prep column and the filtrate collected. Solid KCl was added to the filtrate, and after 30 min at 4°C, the products were centrifuged several times until the supernatant turned clear. Then, the resultant supernatant was collected and labelled as the protoplasmic fraction.

II.b. Obtaining Golgi-enriched fractions

The entire procedure was carried out at 4°C. Maize cultured cells were first pulverised in liquid and then ground with an omni-mixer in extraction buffer (Zeng et al., 2008). The homogenate was centrifuged and the resultant supernatant was loaded on top of 2 M sucrose buffer and subjected to ultracentrifugation. The upper phase was then discarded and a discontinuous sucrose gradient was developed by overlaying 1.3 M, 1.1 M and 0.25 M

sucrose buffers. The discontinuous sucrose gradient was again ultracentrifuged and the 0.25 M/1.1 M sucrose interface was collected as a Golgi-enriched fraction.

Golgi-enriched fractions were then subjected to routine analysis which included total protein content by using Bradford reagent and a Golgi-membrane marker activity assay: the triton-dependent ionidine di-phosphatase activity (IDPase; Morré et al., 1977).

II.c. Cell wall extraction and fractionation

Two methodological procedures were conducted to obtain cell walls and its fractions, depending on the experimental approach. Likewise, when non-radiolabelling experiments were performed, a more exhaustive protocol for the preparation and obtaining of cell wall fractions was carried out (protocol A). However, experiments in which radioactivity was involved, a simplified version was used for safety reasons, in order to minimize the handling of radiolabelled products (protocols B1 and B2).

Protocol A

Cells collected from suspension cultures were frozen and homogenized with liquid nitrogen using a pestle and mortar, and treated with ethanol for 5 days. The suspension obtained was centrifuged and the pellet was washed several times with ethanol and acetone, and subsequently air dried to obtain the alcohol insoluble residue (AIR). The AIR was treated with DMSO and incubated with α-amylase from porcine pancreas in order to de-starch it. The suspension was filtered and the residue washed again several times with ethanol and acetone, air dried and then treated with phenol/acetic acid/water. This resistant material was finally washed with ethanol and acetone for several times and air dried to obtain the cell walls.

Dried cell walls were subjected to several treatments in order to sequentially solubilize different polysaccharide types leading to a cell wall fractionation. Likewise, they were firstly extracted at room temperature with 50 mM cyclohexane-*trans*-1,2-

diamine-*N,N,N´,N´*-tetraacetic sodium salt (CDTA) and the resulting pellet was treated with 0.1 M KOH. The remaining residue was then treated with 4 M KOH and, afterwards the 4 M KOH-resistant material was subjected to a subsequent extraction with 6 M KOH.

Protocol B1: Studies involving [^{3}H]Ara as metabolic precursor

Maize cell suspensions were homogenised with a motor-driven Teflon pestle. The resultant homogenate was filtered through a Poly-Prep column and the filtrate was then discarded. Cell fragments retained in the filter of the Poly-Prep column were treated with 0.1 M NaOH containing $NaBH_4$. The filtrate was then collected and labelled as 0.1 M NaOH fraction. The remaining residue was then treated with 6 M NaOH containing $NaBH_4$. The filtrate was again collected and labelled as the 6 M NaOH fraction. The 6 M NaOH-resistant material was considered as polymers firmly bound to cellulose residue.

Protocol B2: Studies involving [^{14}C]Cinn as metabolic precursor

Cells were resuspended in 80% ethanol and incubated on a rotary wheel, the samples were filtered and the resultant filtrate was again discarded. The remaining cell fragments were considered the AIR. In some cases, that AIR was de-esterified by saponification with 0.5 M NaOH. Soluble fraction from this treatment was collected as the 0.5 M NaOH fraction. This fraction was acidified by addition of acetic acid and partitioned against ethyl acetate, vacuum-dried and re-dissolved in acidified water. Samples were again subjected to a second ethyl acetate partition and the organic phases were collected, vacuum-dried and re-dissolved in propan-1-ol.

Alkali fractions from the cell wall compartments were acidified by the addition of acetic acid. All the fractions obtained from discrete cell wall compartments were dialysed and stored at -20°C until used.

III. CELL WALL ANALYSES

III.a. Cellulose content determination

Cellulose content 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 Glc released by the anthrone method (Dische, 1962).

III.b. Sugar analyses

Total sugar content of each fraction was determined by the phenol-sulfuric acid method (Dubois et al., 1956) and was expressed as the Glc equivalent. Uronic acid content was determined by the *m*-hydroxydiphenyl method (Blumenkrantz and Asboe-Hansen, 1973) using GalA as standard. Neutral sugar content analysis was performed as described by Albersheim et al. (1967).

III.c. FTIR spectroscopy

Monitoring of cell wall changes in maize cell lines were performed by FTIR. Tablets were prepared in a Graseby-Specac press using cell wall samples mixed with KBr. Spectra were obtained, area normalized and baseline-corrected. Difference spectra were obtained by digital subtraction of non-habituated and habituated cell wall FTIR spectra.

III.d. Immunoanalysis

An extensive analysis of polysaccharide distribution and composition of the cell wall fractions was carried out by immunodot assays (IDAs). Briefly, aliquots from each fraction were spotted onto nitrocellulose membrane as a replicated dilution series of the preceding

one. Then, membranes were incubated with an endo-polygalacturonanase, a β-xylanase and an endo-arabinanase (for details see enzymatic digestions section) following the experimental approach described by Øbro et al. (2007) with minor modifications. After enzymatic digestion, nitrocellulose membranes were blocked with phosphate-buffered saline (PBS) containing 4% fat-free milk powder (MPBS) prior to incubation in monoclonal primary antibodies, with specificity for different pectic and hemicellulosic polysaccharides. After washing extensively under running tap water and PBS, membranes were incubated in secondary antibody (anti-rat horseradish peroxidase conjugate). Membranes were washed as described above and subjected to color development in substrate solution.

Immunolabelling quantification for each fraction was performed by scoring the number of dilutions labelled. These values were then entered into the statistical software (see Statistical analyses section for details) in order to obtain heatmaps.

IV. GENE EXPRESSION ANALYSES

IV.a. Isolation of total ribonucleic acid (RNA), reverse transcription-polymerase chain reaction (RT–PCR) and polymerase chain reaction (PCR)

Total RNA was extracted with Trizol Reagent, and was reverse-transcribed using the Superscript III first strand synthesis system for RT-PCR. First-strand complementary DNA (cDNA) was generated using an oligo(dT)20 primer and used as a template in subsequent PCR reactions. For each assay, several numbers of cycles were tested to ensure that amplification was within the exponential range. Ubiquitin was used as housekeeping.

IV.b. *ZmCESA* gene expression analysis

The gene-specific primers used for the analysis of *ZmCESA1* (*AF200525*), *ZmCESA2* (*AF200526*), *ZmCESA3* (*AF200527*), *ZmCESA5* (*AF200529*), *ZmCESA6* (*AF200530*) and *ZmCESA7* (*AF200531*) genes 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*) and *ZmCESA8* (*AF200532*) were those described in Mélida et al. (2010a).

IV.c. *IRX* arabidopsis homologous gene expression analysis

Primers were used for the analysis of maize genes *GRMZM2G100143*, *GRMZM2G059825* and *gbIBT036881.1,* homologous of arabidopsis *IRX10* (*AT1G27440*), *IRX10-L* (*AT5G61840*) and *IRX9* (*AT2G37090*) respectively (Bosch et al., 2011). Sequences for *GRMZM2G100143* and *GRMZM2G059825* were derived from maize genome browser and that for *gbIBT036881.1* from Phytozome.

V. *IN VIVO* RADIOLABELLING EXPERIMENTS

V.a. Studies involving [^{3}H]Ara as metabolic precursor

Aliquots of maize cell cultures were collected at several growth phases of the culture cycle and independently fed with L-[1-^{3}H]Ara. Due to the disparity that the different cell lines exhibited in the length of time of the culture phases, the days in which the aliquot of the culture was sampled and [^{3}H]Ara added were adjusted depending on the cell line. The incorporation of [^{3}H]Ara into discrete cell compartments was measured at different labelling-time points (30, 60, 120 and 300 min after [^{3}H]Ara was fed) for 5 h in each different culture phase.

V.b. Studies involving [^{14}C]Cinn as metabolic precursor

[^{14}C]Cinn was prepared from L-[U-^{14}C]phenylalanine following the method described by Lindsay and Fry. (2008) with minor modifications. Aliquots of Snh and Sh1.5 cell-suspensions at different phases of the culture cycle were transferred into vials, and shaken to allow them to acclimatise to their new environmental conditions. Then, cells were fed with

[^{14}C]Cinn, and its incorporation and consumption was measured at selected time-points. Where indicated, H_2O_2 was added to some of the samples 120 min after [^{14}C]Cinn, and then shaken for 60 min.

V.c. Assay of radioactivity

V.c.1.Studies involving [^{3}H]Ara as metabolic precursor

Origin of the radioactivity samples imply disparity in the "format" in which it is presented (i.e dissolved, on/within paper chromatogram, polypropylene filter pad) and therefore would determine some radioactivity assay parameters as the type of liquid scintillation used as well as correction factor for counting efficiency in order to perform the quantification.

For the analysis of liquid samples, aliquots of them were assayed for radioactivity by liquid scintillation counting mixing the samples with OptiPhase HiSafe in a 1/10 dilution.
Paper chromatogram spots corresponding to monosaccharide and polymeric material at RF 0 were cut and separately assayed by scintillation counting in 2 ml of OptiScint HiSafe.

When radioactivity of the spots was low, a protocol for the improving of counting sensibility was applied. Likewise, spots were soaked overnight in water and then, OptiPhase HiSafe scintillation liquid was added in a 1/10 dilution, and vials were shaken for 24 h.
In a similar way, the quantification of radioactivity presented in polypropylene filter pad, the entire filter was excised and mixed with OptiPhase HiSafe scintillation liquid. After soaking for 24 h radioactivity was then assayed by counting.

V.c.2. Studies involving [^{14}C]Cinn as metabolic precursor

Liquid samples were mixed with EcoscintA and radioactivity was then assayed by liquid scintillation counting.

In the case of the radioactivity incorporated into the AIR, cell fragments were firstly resuspended in distilled water, EcoscintA was subsequently added and radioactivity was then assayed by counting.

Tracks corresponding to samples from thin layer chromatography (TLC) plates, were cut off into pieces and analysed by scintillation counting through the addition of EcoscintA.

VI. ENZYMATIC DIGESTIONS

Driselase enzymatic digestion was performed as follows: prior to it samples were dried in vacuo, subjected to a mild-hydrolysis in trifluoroacetic acid (TFA) and re-dried to remove TFA. The dried material was re-dissolved in 0.5% (w/v) Driselase in pyridine/acetic acid/water and incubated at 37°C for 96 h. The reaction was stopped by the addition of 15% formic acid.

Treatments with endo-polygalacturonanase (M2) from *Aspergillus aculeatus* (E-PGALUSP) (EC 3.2.1.15), a recombinant β-xylanase from *Neocallimastix patriciarum* (E-XYLNP) (EC 3.2.1.8) and an endo-arabinanase from *Aspergillus niger* (E-EARAB) (EC 3.2.1.99) were carried out at 37°C for 24 h. The enzymes were dissolved to 1 U/ml in 350 mM acetate buffer adjusted to pH 4.7 by the addition of pyridine.

VII. CHROMATOGRAPHY

VII.a. Paper chromatography (PC)

Internal markers were added within samples previous the chromatography analysis, which was developed in 3 MM Whatman paper. PC conditions slightly differed depending on the sample components requested to be resolved. Likewise, when separation of monosaccharides from polymeric material at RF 0 was required, samples were subjected to PC in butan-1-ol/acetic acid/water for 16 h. In the case of samples in which the resolution of

monosaccharides, disaccharides and polymeric RF 0 material would be desirable, PC was conducted by using ethyl acetate/pyridine/water for 18 h (Thompson and Fry, 1997).

The internal markers were stained slightly by dipping the paper in 10% of the standard concentration of aniline hydrogen phthalate (Fry, 2000), dried, and then heated at 105°C until the required intensity of color development was obtained.

VII.b. Gel-permeation chromatography (GPC)

Polymers were size-fractionated on Sepharose CL-4B in pyridine/acetic acid/water at 12.5 ml/h. The Sepharose CL-4B column was calibrated with commercial dextrans of known weight-average relative molecular mass (M_w). M_w was obtained using the $Kav_{(1/2)}$ (Kav: partition coefficient for a given GPC column) method (Kerr and Fry, 2003) with the calibration curve [$\log M_w = -3.547\ Kav_{(1/2)} + 7.2048$] obtained for this column.

VII.c. Gas chromatography (GC)

Lyophilized samples were hydrolyzed with 2 M TFA at 121°C for 1 h and the resulting sugars were derivatized to alditol acetates and analyzed using a Supelco SP-2330 column.

VII.d. TLC

TLC was carried out on plastic-backed silica-gel with a fluorescent indicator in benzene/acetic acid and, during development, plates were exposed to 312 nm.

VIII. ELECTROPHORESIS

VIII.a. Sodium-dodecyl-sulphate (SDS)-PAGE analysis

For SDS-PAGE separation, proteins were treated under reducing conditions (boiled samples) or partial non-reducing conditions (not boiled samples) and separated by standard

SDS-PAGE on a 7.5%, 10% or 12.5% gel. The resultant gels were then stained with coomassie brilliant blue (CBB) R-250.

VIII.b. 2-D PAGE analysis

Previously to 2-D electrophoresis, preparation of samples was required. Briefly, aliquots of each sample were freeze-dried and then dissolved in the lysis buffer, incubated in an ultrasound bath, centrifuged and the resultant supernatant was collected and dialysed. Afterwards, samples were concentrated by Vivaspin columns until the required amount of protein for the 2-D electrophoresis was obtained.

Protein samples were loaded onto non-linear pH 4–7 immobilized pH gradient (IPG) strips to carry out the first dimension, performed in Ettan™ IPGphor Isoelectric Focusing System. Afterwards, IPG strips were collected and equilibrated with a dithiothreitol (DTT) treatment followed by a second treatment with iodoacetamide, loaded on top of a SDS–PAGE 10% polyacrylamide gel and subjected to the second dimension using Ettan DALTsix System. Gels were finally resolved with corresponding stain, CBB G-250 or silver stain (Shevchenko et al., 1996; Irar et al., 2006), leading to two independent but complementary analyses. The experiment was carried out with three technical replicates per biological sample. The stained gels were scanned and analysed (Farinha et al., 2011) and after automatic spot detection, manual spot editing was carried out. To evaluate protein expression differences among gels, relative spot volume (%Vol) was used. Those spots showing a quantitative variation > Ratio 1 and positive GAP were selected as differentially accumulated. Statistically significant protein abundance variation was validated by Student's t-test (p, 0.05) (Farinha et al., 2011). The selected differential spots were excised from the gels and identified either by peptide mass fingerprint (PMF) using MALDI–TOF/MS or by ESI-MS/MS. The software packages Protein Prospector v 3.4.1 from the University of California San Francisco and MASCOT were used to identify the proteins from the PMF data as previously reported (Carrascal et al., 2002). The SEQUEST software 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 isoelectric point (pI). No species restriction was applied. When an identity search produced no matches, the homology mode was used.

IX. STATISTICAL ANALYSES

Principal component analysis (PCA) was performed using a maximum of five principal components (PCs). All these analyses were carried out using the Statistica 6.0 software package.

Significant differences were tested applying a one-factor analysis of variance (ANOVA) (performing Tukey´s test as post-hoc analysis), using the PASW Statistics 18 software package or by Student's t-test (p, 0.05).

Heatmaps were performed by using RKWard statistical software.

Chapter I

Monitoring and characterisation of modifications that occur during the first steps of the DCB-habituation process

ABSTRACT

Studies involving the habituation of plant cell cultures to cellulose biosynthesis inhibitors have achieved significant progress as regards understanding the structural plasticity of cell walls. However, since habituation studies have typically used high concentrations of inhibitors and long-term habituation periods, information on initial changes associated with habituation has usually been missed. This study focuses on monitoring and characterising the earlier cell wall changes occurring during the habituation process of maize cell suspensions to DCB. Cellulose quantification and FTIR spectroscopy of cell walls from 20 cell lines obtained during the first steps of a DCB-habituation process showed transitory decreases in cellulose levels which tended to revert depending on the inhibitor concentration and the length of time that cells were in contact with it. Variations in the cellulose content were concomitant with changes in the expression of several *ZmCESA* genes, mainly involving overexpression of *ZmCESA7* and *ZmCESA8*. In order to explore subsequent changes, a cell line habituated to 1.5 μM DCB was identified as representative of incipient DCB habituation and selected for further analysis. Their cells grew with lower relative and maximum growth rates, determined by longer lag phases and dt, and formed clusters with larger average volume and greater variety of sizes. Their cell walls showed a 33% reduction in cellulose content, which was mainly counteracted by an increase in arabinoxylans, which presented increased extractability. This result was confirmed by IDAs graphically plotted by heatmaps, since habituated cell walls had a more extensive presence of epitopes for arabinoxylans and xylans, but also for homogalacturonan with a low degree of esterification and for galactan side chains of rhamnogalacturonan I. In addition, a partial shift of xyloglucan epitopes towards more easily-extractable fractions was found. However, other epitopes, such as for arabinan side chains of rhamnogalacturonan I or for homogalacturonan with a higher degree of esterification, seemed to be not affected. In conclusion, initial modifications occurring in maize cell walls as a consequence of DCB-habituation includes quantitative and qualitative changes of arabinoxylans and different polysaccharides. Thereby some of the changes that occurred in the cell walls in order to compensate for the lack of cellulose differed according to the DCB-habituation level, and illustrate the ability of plant cells to adopt appropriate coping strategies depending on the herbicide concentration and length of exposure time.

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I. INTRODUCTION

Plant cell walls are dynamic structures whose importance resides principally in the key role they play in plant growth and development, enabling cells to adapt to abiotic or biotic stresses (Wolf et al., 2012). Cell walls influence the properties of most plant-based products, including their texture and nutritional value, and condition the processing properties of plant-based foods for human and animal consumption (Doblin et al., 2010). Cell walls are mainly composed of polysaccharides, which are classified as cellulose, hemicelluloses and pectins, and have lower amounts of proteins, phenolics and other minor components. Cell wall composition varies according to the plant type and species (Sarkar et al., 2009), cell type and position within the plant, developmental stage and history of responses to stresses (Doblin et al., 2010). This variability in the composition of cell walls reflects a certain degree of plasticity as regards cell wall structure and composition. One suitable method to study the mechanisms underlying the plasticity of plant cell wall structure and composition consists in habituating cell cultures to grow in the presence of high concentrations of different CBIs (for a review see Acebes et al., 2010).

CBIs are a group of compounds that specifically affect the assembly and/or deposition of cellulose in the cell wall, by inducing aberrant trajectories, a velocity reduction or even the clearance of CESA subunits at the plasma membrane (Acebes et al., 2010; Brabham and DeBolt, 2013). In the last two decades it has been shown that it is possible to habituate undifferentiated cell cultures (calluses and cell suspensions) of various dicotyledonous plants, such as arabidopsis, tobacco, tomato, bean or poplar, to lethal concentrations of diverse CBIs and related compounds, such as isoxaben, thaxtomin A, DCB or quinclorac, by gradually increasing the concentration in the culture medium. In general terms, in order to cope with the stressful conditions, the cell cultures habituated to these inhibitors modify the characteristic architecture of the type I cell wall (typical of gymnosperms, dicots and most monocots), having reduced levels of cellulose accompanied by a decrease in the hemicellulosic content and a significant increase in pectins (Shedletzky

et al., 1992; Díaz-Cacho et al., 1999; Encina et al., 2001; 2002; García-Angulo et al., 2006; 2009). The modifications produced in cells habituated to these inhibitors not only depend on the inhibitor concentration but also on the period of time that cells grow in contact with the inhibitor (Alonso-Simón et al., 2004).

There are only two reported studies in which cell cultures from plants with type II cell walls (characteristic of graminaceous plants, together with the other commelinoid monocots) have been habituated to a CBI. These were barley cell suspensions (Shedletzky et al., 1992) and maize callus cultures (Mélida et al., 2009; 2010a; 2010b), habituated to DCB in both cases. The modifications found in these type II cell walls differed considerably from those described for type I cell walls, since the drastic reduction in cellulose content was compensated for an increase in the content of heteroxylans, which had lower extractability and higher M_r and M_w. Furthermore, in the case of maize cell cultures obtained in our laboratory, DCB habituation has implied an enrichment of hydroxycinnamates and dehydroferulates esterified on arabinoxylans. The content of other polymers, such as mixed glucan, xyloglucan, mannan, pectins and proteins, was unchanged or reduced (Mélida et al., 2009). As these characteristics differed from those described for DCB-habituated barley cell cultures, and did not look like any other structure previously described for type II cell walls, we proposed that our DCB-habituated maize cells had a unique structure, which was of particular interest in order to gain a deeper understanding of the compositional and structural plasticity of the type II cell wall (Mélida et al., 2009).

Changes associated with the habituation of cell cultures to CBIs have commonly been analysed using high concentrations of herbicide and long-term habituation periods. These kinds of study take advantage of the fact that the cell culture characteristics have been "fixed". However, information about the initial changes that take place during the process of habituation is lost, although cells at these stages probably present a huge variability in relation to their cell wall composition, properties and ability to habituate. A previous work conducted in bean calluses in order to monitor cell wall changes during DCB-habituation,

showed that when calluses were cultured in 0.5 μM DCB for less than 7 subcultures no appreciable changes in cell wall FTIR spectra were detected, but after 13 subcultures the spectra of the cell walls underwent several changes, including an attenuation of the peaks related to cellulose (Alonso-Simón et al., 2004). So, this study established that a) an initial period would be necessary until cell wall modifications arise and b) that a set of cell wall modifications, not only a reduction in cellulose content, would be implied.

Accordingly to these results, the aim of this study was to monitor and characterise the early changes that occur during the DCB-habituation of cells with type II walls, using as a model maize cell suspensions. First of all, we monitored the changes produced throughout the DCB-habituation process, in order to select a cell line that showed an initial contrastable modification in some cell wall features. Next, we compared the cell growth pattern and cell wall composition of Sh1.5 and Snh cells, paying special attention to the variation in composition of polysaccharides, using a set of techniques such as cell wall fractionation, GC, IDAs, enzymatic digestions and heatmaps.

II. MATERIALS AND METHODS

II.a. Cell cultures

Maize cell suspension cultures (*Zea mays* L., Black Mexican sweet corn, donated by Dr. S. C. Fry, Institute of Molecular Plant Sciences, University of Edinburgh, UK) from calluses obtained from immature embryo explants were grown in Murashige and Skoog media (Murashige and Skoog, 1962) supplemented with 9 μM 2,4-D and 20 g L^{-1} sucrose, at 25°C under light and rotary shaken, and routinely subcultured every 15 days.

II.b. Habituation to DCB

Maize cell suspensions were cultured in DCB (supplied by Fluka) concentrations ranging from 0.3 to 1.5 μM. DCB was dissolved in DMSO, which did not affect cell growth at this range of concentrations.

Initially, Snh maize cells were cultured in media containing three DCB concentrations: 0.3 μM (lower than the I_{50}), 0.5 μM (the I_{50} value) and 1 μM (higher than the I_{50} value). After seven subcultures, some of the cells habituated to growth in 1 μM DCB were transferred into media containing 1.5 μM DCB.

II.c. Growth measurements

Growth curves of Snh and Sh1.5 cell lines were obtained by measuring the increase in DW at different culture times. Dt was defined as the time required for a cell to divide or a cell population to double in size in the logarithmic phase of growth, and was estimated by using the equation lnWt = lnWo + 0.693t/dt. Thus, Wt or Wo logarithmic plotting versus culture time is a straight line where the ordinate at the origin corresponds to the Wt or Wo natural logarithm. Relative growth rate was determined by the slope of the straight line (0.693t/dt). Maximum growth rate was the maximum difference in DW per time unit between two consecutive measurements in growth kinetics.

Cell cluster size was determined after vacuum filtration of cell suspensions that were sequentially passed through different pore-size meshes, and the relative abundance of the clusters was expressed as the percentage of DW retained in each filter.

II.d. *ZmCESA* gene expression analysis: 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 the Superscript III first strand synthesis system for RT-PCR (Invitrogen). First-strand cDNA was generated using an oligo(dT)20 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 within 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*) genes 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*) and *ZmCESA8* (*AF200532*) were those described in Mélida et al. (2010a).

II.e. Preparation and fractionation of cell walls

Cells collected from suspension cultures in the stationary phase of growth were frozen and homogenised with liquid nitrogen using a pestle and mortar, and treated with 70% ethanol for 5 days at room temperature. The suspension obtained was centrifuged and the pellet was washed with 70% ethanol (x6) and acetone (x6), and subsequently air dried to obtain the 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 incubated with 2.5 U ml^{-1} α-amylase 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 fiber filter, and the residue washed with 70% ethanol (x6) and acetone (x6), air dried and then treated with phenol/acetic acid/water (2/1/1 by vol.) for 8 h at room temperature (x2). This residue was finally washed with 70% ethanol (x6) and acetone (x6) and then air dried to obtain the cell walls.

In order to carry out a cell wall fractionation, dried cell walls were extracted at room temperature with 50 mM CDTA (10 mg ml^{-1}) and then washed with distilled water. The resulting pellet was treated with 0.1 M KOH (10 mg ml^{-1}) for 2 h (x2) and washed again with distilled water. The remaining residue was then treated with 4 M KOH (10 mg ml^{-1}) for 4 h

(x2) and subsequently washed with distilled water. Then 6 M KOH (10 mg ml^{-1}) was finally added to the pellet and treated at 37°C for 24 h. KOH extracts were neutralised with acetic acid to pH 5.

Extracts of each treatment were dialysed (48 h) and lyophilised, and referred to CDTA, 0.1 M KOH, 4 M KOH and 6 M KOH fractions, respectively.

II.f. Cell wall analyses

II.f.1. Cellulose content determination

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

II.f.2. FTIR spectroscopy

Tablets for FTIR spectroscopy were prepared in a Graseby-Specac press using cell wall samples (2 mg) mixed with KBr (1:100, w/w). Spectra were obtained on a Perkin-Elmer instrument at a resolution of 1 cm^{-1}. A window between 800 and 1800 cm^{-1}, containing information about characteristic polysaccharides, was selected in order to monitor cell wall structure modifications. All the spectra were normalized and baseline-corrected with Spectrum v 5.3.1 software, by Perkin-Elmer. Data were then exported to Microsoft Excel 2003 and all spectra were area-normalized. Difference spectra were obtained by digital subtraction of Snh and Sh cell wall FTIR spectra.

II.f.3. Sugar analyses

Total sugar content of each fraction was determined by the phenol-sulfuric acid method (Dubois et al., 1956) and was expressed as the Glc equivalent. Uronic acid content was determined by the *m*-hydroxydiphenyl method (Blumenkrantz and Asboe-Hansen, 1973) using GalA as standard. Neutral sugar content analysis was performed as described by

Albersheim et al. (1967). Briefly, a lyophilized sample of each fraction was hydrolyzed with 2 M TFA at 121°C for 1 h and the resulting sugars were derivatized to alditol acetates and analyzed by GC using a Supelco SP-2330 column.

II.f.4. Analysis of polysaccharides by enzymatic epitope deletion

An extensive analysis of polysaccharide distribution and composition of the cell wall fractions was carried out by IDAs. Briefly, aliquots of 1 µl from each fraction were spotted onto nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany) as a replicated 1/5 dilution series of the precedent one, being 5 different dilutions for each fraction in all. Then, these membranes were incubated with either an endo-polygalacturonanase (M2) from *Aspergillus aculeatus* (E-PGALUSP) (EC 3.2.1.15), a recombinant β-xylanase from *Neocallimastix patriciarum* (E-XYLNP) (EC 3.2.1.8) and an endo-arabinanase from *Aspergillus niger* (E-EARAB) (EC 3.2.1.99) (all of them purchased from Megazyme) at 37°C for 24 h, following the experimental approach described by Øbro et al. (2007) with minor modifications. The enzymes were dissolved to 1 U/ml in 350 mM acetate buffer adjusted to pH 4.7 by the addition of pyridine. After enzymatic digestion, nitrocellulose membranes were blocked with PBS (0.14 M NaCl, 2.7 mM KCl, 7.8 mM $Na_2HPO_4 \cdot 12H_2O$, 1.5 mM KH_2PO_4, pH 7.2) containing 4% fat-free milk powder (MPBS) prior to incubation in primary antibody (hybridoma supernatants diluted 1/10 in MPBS) for 1.5 h at room temperature. After washing extensively under running tap water and PBS, membranes were incubated in secondary antibody (antirat horseradish peroxidase conjugate, Sigma) diluted 1/1000 in MPBS for 1.5 h at room temperature. Membranes were washed as described above prior to colour development in substrate solution [25 ml de-ionized water, 5 ml methanol containing 10 mg ml^{-1} 4-chloro-1-naphtol, 30 ml 6% (v/v) H_2O_2]. Colour development was stopped by washing the membranes. Samples of commercial pectin (P41, Danisco), xyloglucan (kindly donated by Dr. T Hayashi) and an arabinoxylan-enriched fraction (AX; obtained from maize calluses habituated to 12 µM DCB, kindly donated by Dr. H. Mélida) were used as reference

compounds. Monoclonal antibodies assayed and their corresponding epitope recognized were: JIM5 (J5; homogalacturonan with low degree of methylesterification; Clausen et al., 2003), JIM7 (J7; homogalacturonan with high degree of methylesterification; Knox et al., 1990), LM5 (L5; β-1,4-galactan side chain of rhamnogalacturonan type I; Jones et al., 1997), LM6 (L6; α-1,5-arabinan side chain of rhamnogalacturonan type I and arabinogalactan proteins; Willats et al., 1998), LM10 (L10; unsubstituted and relative low substituted β-1,4-D-xylan). No cross activity with LM11 (L11; unsubstituted and relative low substituted β-1,4-D-xylan; McCartney et al., 2005) and LM15 (L15; non-fucosylated xyloglucan; Marcus et al., 2008) were observed.

II.f.5. IDAs related heatmaps

Immunolabeling semi-quantification for each fraction was performed by scoring the number of dilutions labeled. Thus, a value ranging from 1 to 5 was assigned depending on the number of colored spots which appeared after incubation with the secondary antibody. These values were then entered into the statistical software (see Statistical analyses section for details) in order to obtain heatmaps.

II.g. Statistical analyses

PCA was performed using a maximum of five PCs. All these analyses were carried out using the Statistica 6.0 software package.

Significant differences in the cellulose content were tested applying a one-factor ANOVA (performing Tukey´s test as post-hoc analysis), using the PASW Statistics 18 software package.

Heatmaps were performed by using RKWard statistical software.

III. RESULTS

III.a. Monitoring of early cell wall modifications during DCB-habituation

III.a.1. FTIR spectroscopy and multivariate analysis

FTIR spectra from cell walls corresponding to Snh and habituated (Sh) to 0.3 (Sh0.3), 0.5 (Sh0.5), 1 (Sh1) and 1.5 (Sh1.5) µM DCB maize cell suspensions which had been for different number of culture cycles growing in the presence of DCB were obtained and analyzed (Figure 1). The main differences among cell lines were detected in the fingerprint area (900-1200 cm^{-1}). In this area of the spectra, many polysaccharides absorb IR radiation, including cellulose.

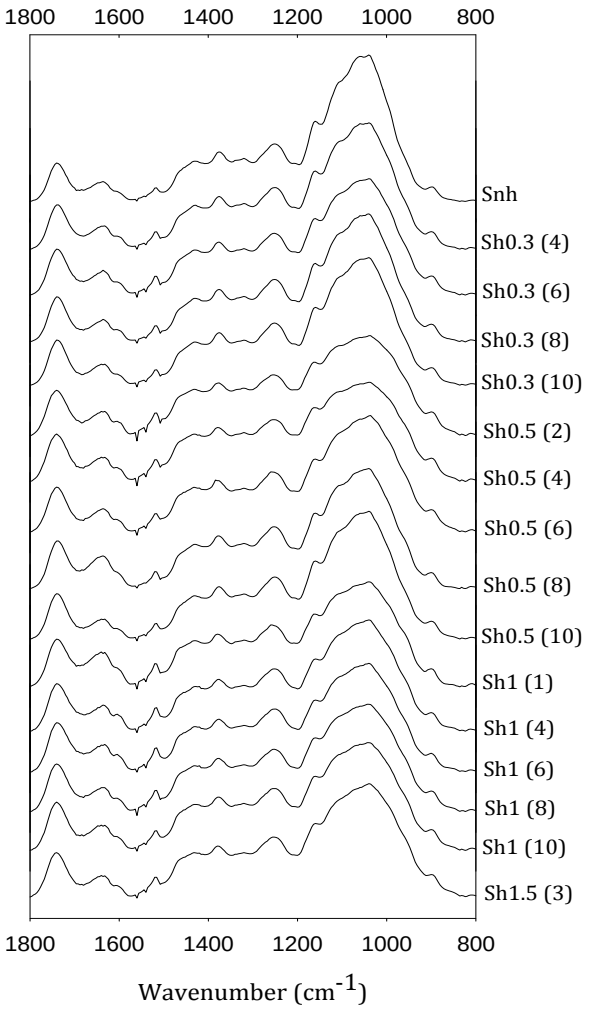


Figure 1. Fourier Transform Infrared (FTIR) spectra obtained from cell walls from non-habituated (Snh) and different dichlobenil- (DCB) habituated (Sh) maize cell suspensions (Shx (n); x indicates DCB concentration (µM) and (n) number of subcultures in that DCB concentration)

In order to better appreciate the variations between Sh and Snh cell wall spectra, difference spectra for each cell line were obtained (Figure 2). These spectra showed noticeable variation among Snh and most of the Sh cell lines subjected to short-term DCB habituation [i.e. see Snh-Sh0.3(2); Snh-Sh0.5(2) and Snh-Sh1(1)]. Spectra obtained from Sh cells showed decreased peaks in wavenumbers associated with cellulose (1039, 1056, 1060, 1105 and 1160 cm^{-1}) (Alonso-Simón et al., 2004; 2011). However, these variations were gradually attenuated in the difference spectra corresponding to cell walls of Sh0.3 and Sh0.5 cell lines as the number of culture cycles in presence of DCB was increased [see Snh-Sh0.3(4-10); Snh-Sh0.5(4-10)]. In contrast, the differences observed in the Sh1 cell line were more marked and they did not revert as the number of subcultures was increased [see Snh-Sh1(4-10)]. In addition other wavenumbers not associated to cellulose, such as 950 cm^{-1} -ascribed to pectins- or 1520 cm^{-1} -attributed to phenolic rings- (Alonso-Simón et al., 2011), and others unattributed such as 1190 and 1480 cm^{-1} were also affected, indicating that other cell wall components have been modified.

PCA of FTIR spectra showed two groups (Figure 3) and the spectra were mainly discriminated by PC1 (approx. 71 % of total variance explained). Spectra obtained from cell walls corresponding to Snh and Sh suspensions habituated to lower DCB concentrations (Sh0.3 and Sh0.5) which had been growing in presence of the inhibitor for a greater number of culture cycles were located on the positive side of PC1. In contrast, spectra corresponding to cells habituated to higher DCB concentrations (Sh1 and Sh1.5) and those corresponding to cells which had been growing for a lesser number of culture cycles in contact with lower concentrations of the inhibitor were grouped on the negative side of PC1.

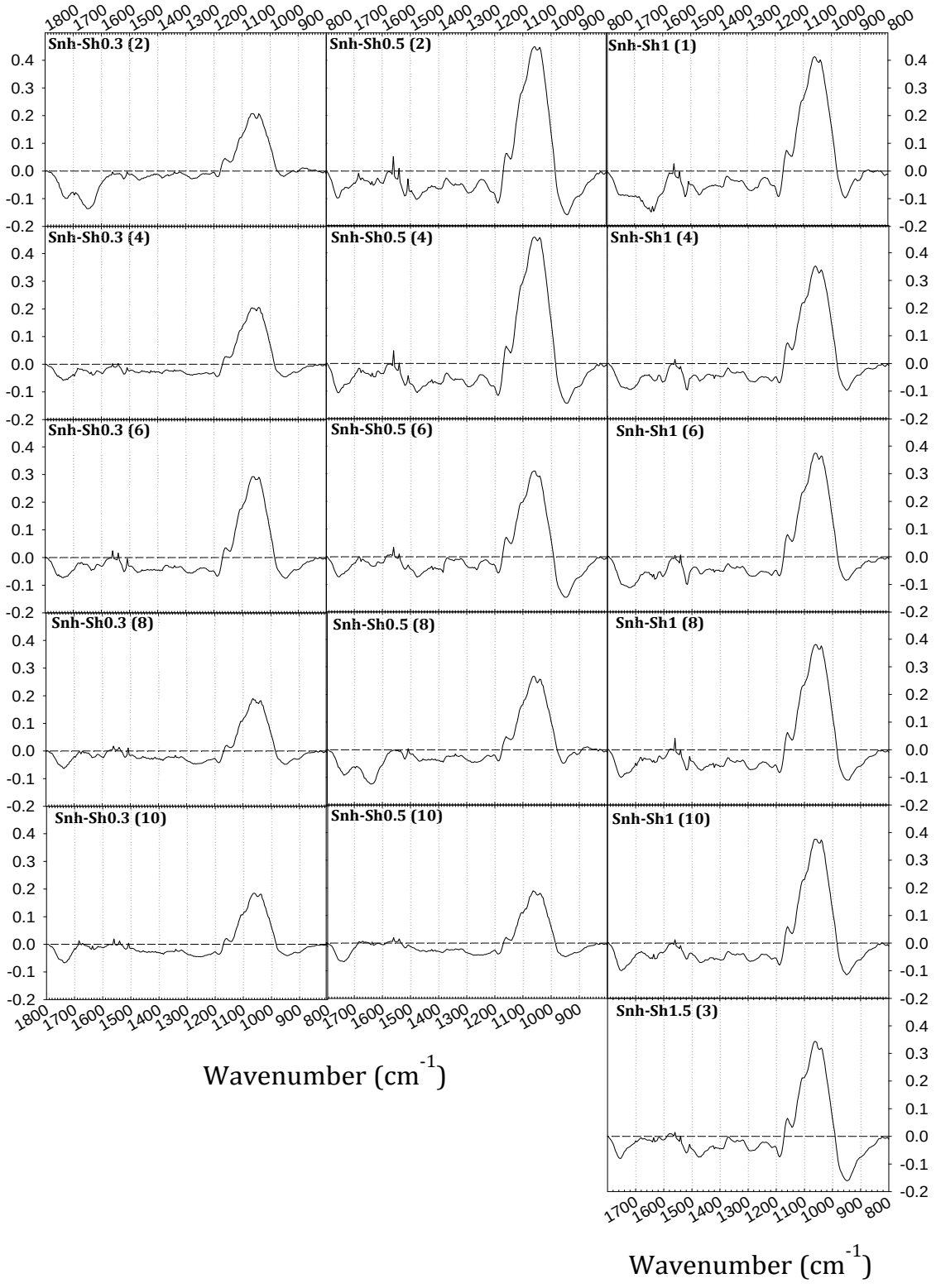


Figure 2. Difference spectra obtained from the digital subtraction of the FTIR spectra of cell walls from Snh and different DCB-habituated Sh maize cell suspensions (legend as Figure 1)

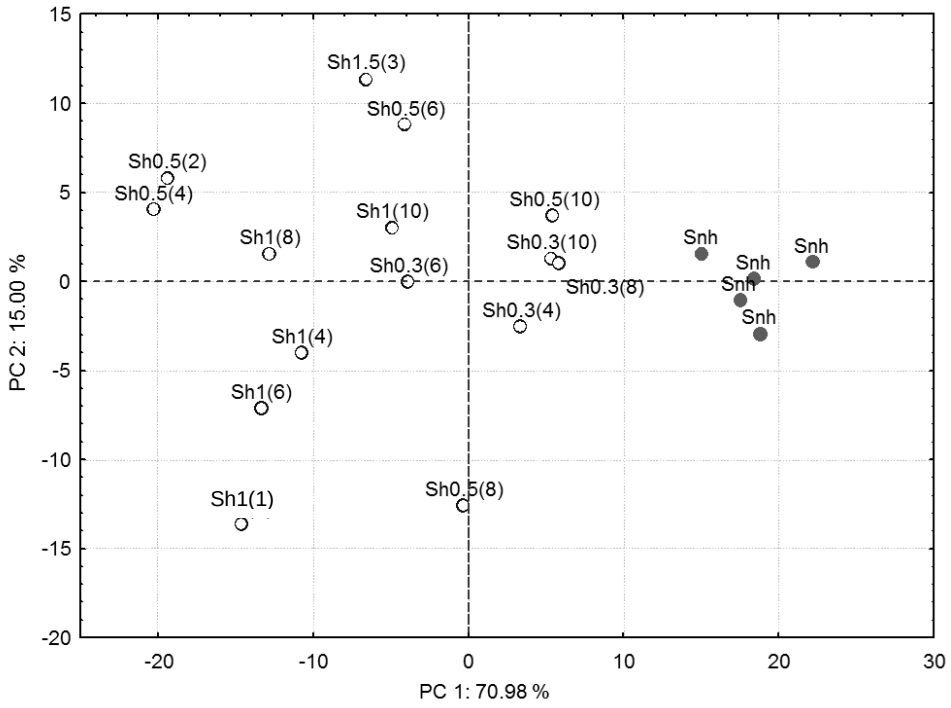


Figure 3. Principal components analysis (PCA) of maize cell suspensions FTIR spectra. A plot of the first and second Principal Components (PCs) is represented based on the FTIR spectra of cell walls from Snh and Sh maize cell suspensions along several culture cycles (legend as Figure 1)

III.a.2. Cellulose content

Cellulose content was influenced by DCB concentration as well as by the time that maize cells were growing in its presence (Figure 4). In Snh cell walls, cellulose represented roughly 27% of the cell wall weight. However, a reduction in the cellulose level occurred when maize cells were exposed to DCB for one or two culture cycles. As the number of subcultures in presence of DCB was increased, the cellulose content of cell walls from Sh suspensions habituated to lower DCB concentrations (Sh0.3 and Sh0.5) gradually reverted to that of the control level. In fact, after growing in contact with the inhibitor for ten culture cycles, their cellulose content reached values that did not differ significantly from those of the Snh. In contrast, this complete reversion did not occur in the Sh1 cell line, at least not in the same number of culture cycles as Sh0.3 and Sh0.5 cell lines did.

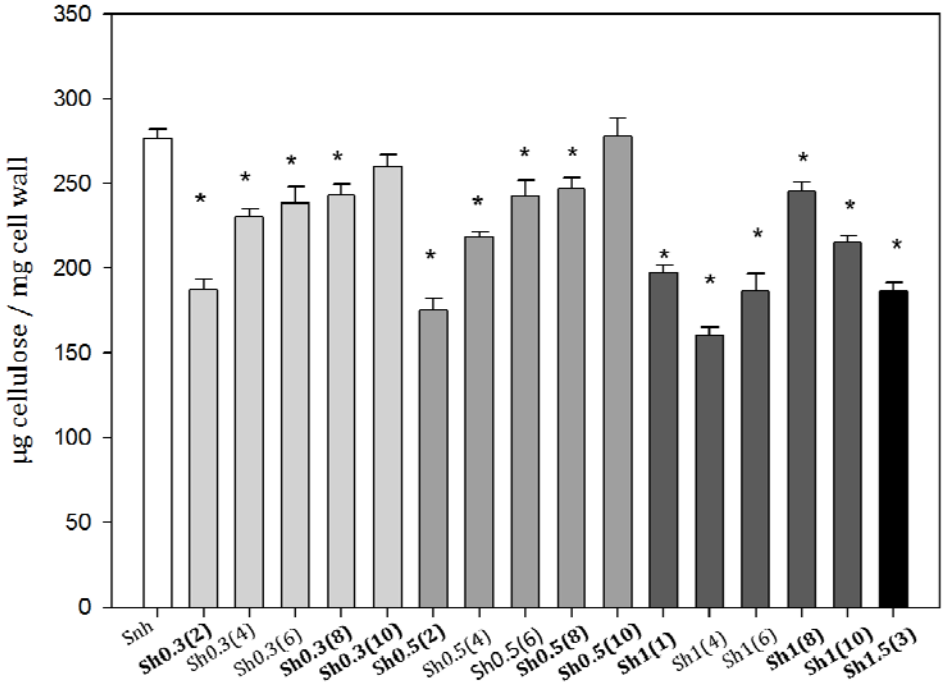


Figure 4. Cellulose content of cell walls from Snh and Sh maize cell suspensions (legend as Figure 1). The asterisk reflects significant differences among non-habituated and DCB-habituated cell lines according to Tukey test ($p<0.05$). Values represented are mean ± standard error (SE) of nine measurements (n=9). References in bold refer to the cell lines which were selected for further *ZmCESA* genes expression analysis (Figure 5)

III.a.3. *ZmCESA* genes expression

ZmCESA8 and *ZmCESA7* gene expression was induced in those cell cultures which were grown for a high number of culture cycles in the presence of DCB -Sh0.3(10) and Sh0.5(10)- respectively (Figure 5). An interesting trend was observed in cells habituated to 1 µM DCB: an induction of *ZmCESA7* expression was detected after 8 and 10 culture cycles -Sh1(8) and Sh1(10)-, and in the former line, *ZmCESA8* expression was also enhanced. However, none of these genes was induced in DCB habituated cells in their first culture cycle -Sh1(1)-. Lastly, the expression of *ZmCESA7* was also induced in Sh1.5(3). No expression of *ZmCESA3* was observed in any of the cell lines analyzed, and no differences in mRNA accumulation in *ZmCESA1/2*, *ZmCESA4*, *ZmCESA5* and *ZmCESA6* with respect to the Snh were detected.

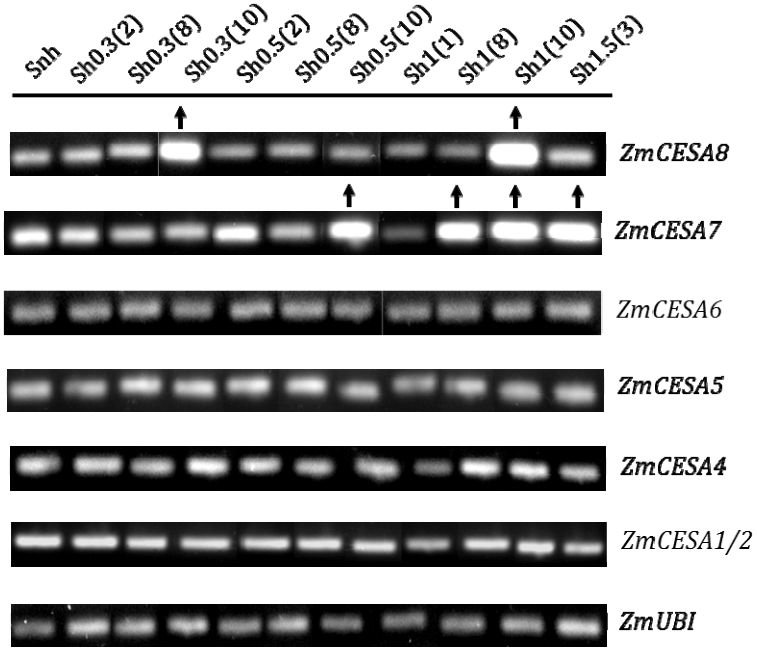


Figure 5. Relative *ZmCESA* gene expression analyzed by RT–PCR of different maize cell suspensions (legend as Figure 1). ↑; more mRNA accumulation than control (Snh); *ZmCESA3* was not included because no expression was detected. *ZmUBI*: Ubiquitine gene expression. Ubiquitine was used as the housekeeping gene due to its constitutive expression

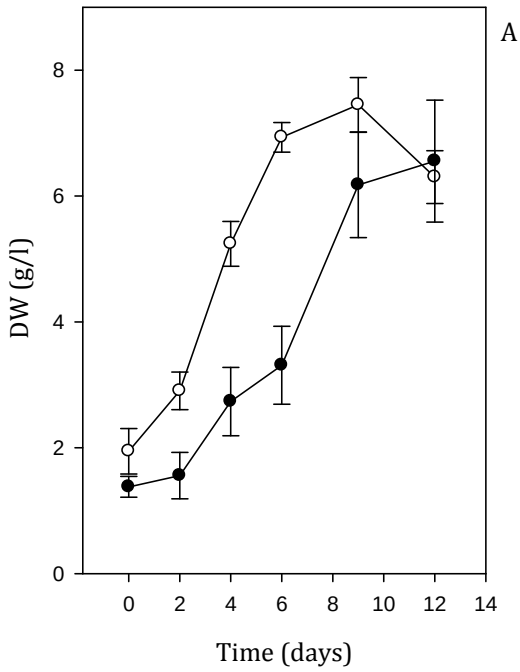
The Sh1.5 cell line was selected for further analysis since it was representative of an early DCB-habituation based on the following criteria: a) a 33% reduction of cellulose content, later demonstrated to be irreversible (data not shown) was observed, b) FTIR spectrum indicated that cellulose, as well as other polysaccharides, were affected, and c) PCA results pointed to differences with Snh cells but also with the remaining Sh cells, indicating features of an incipient DCB habituation.

III.b. Culture characteristics

Growth of Snh and Sh1.5 maize cell suspensions throughout the culture cycle was analyzed (Figure 6A). Sh1.5 cells showed a longer lag phase and a less pronounced logarithmic phase of growth when compared with the Snh cells, which determined longer dt and lower relative and maximum growth rates (Figure 6B).

Snh cells showed homogeneous cluster-size since 88% of their DW was found to have diameters ranging between 710 and 400 μm (Figure 7). In contrast, a larger average size and greater variety of sizes was observed in the Sh1.5 cells, and 73% of their DW was retained in filters with pore sizes ranging from 3000 to 710 μm.

A



B

Cell line	Dt	Relative growth rate	Maximum growth rate (g / l day)
Snh	2.3	0.3	1.17
Sh1.5	3.6	0.19	0.96

Figure 6. **A:** Growth of maize cell suspensions during the culture cycle. *Open circle*; Snh. *Filled circle;* Sh1.5. Values represented are mean ± SE of nine measurements (n=9); **B:** Growth parameters obtained from the growth curves of Snh and Sh1.5 maize cell suspensions. Dt: doubling time

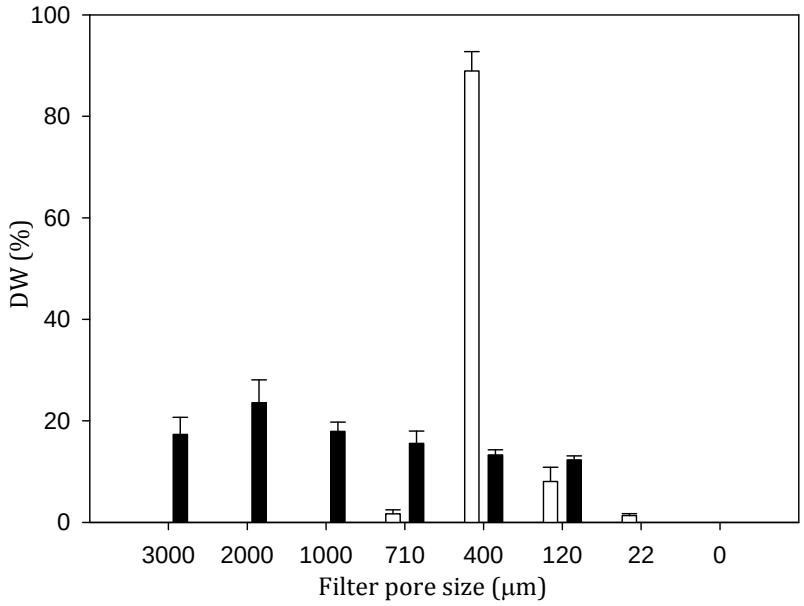


Figure 7. Size of cell clusters of maize cell suspensions at the stationary phase. *White bars*; Snh. *Black bars;* Sh1.5. Values represented are mean ± SE of three measurements (n=3)

III.c. Cell wall features

III.c.1. Cell wall fractionation and sugar composition

The results showed that most polysaccharides were extracted by alkali treatments, especially in 4 M KOH and 6 M KOH fractions. Sh1.5 cell walls were enriched in total sugars, mainly extracted with 4 M KOH followed by 0.1 M KOH (Figure 8A).

GC and uronic acid analysis of cell wall fractions (Figure 8B) revealed that alkali fractions were composed mainly of Ara and Xyl, followed by Glc, uronic acids and Gal, whereas the CDTA fraction was enriched in uronic acids and Ara. Ara and Xyl were responsible for the increase in neutral sugars detected in the 0.1 M KOH and 4 M KOH fractions in Sh1.5 cell walls. A decrease in Glc, especially in fractions extracted with 4 M KOH and 6 M KOH, was observed in Sh1.5 cells. Finally, Sh1.5 cells showed a slight increase in uronic acids, mainly those extracted in alkali fractions.

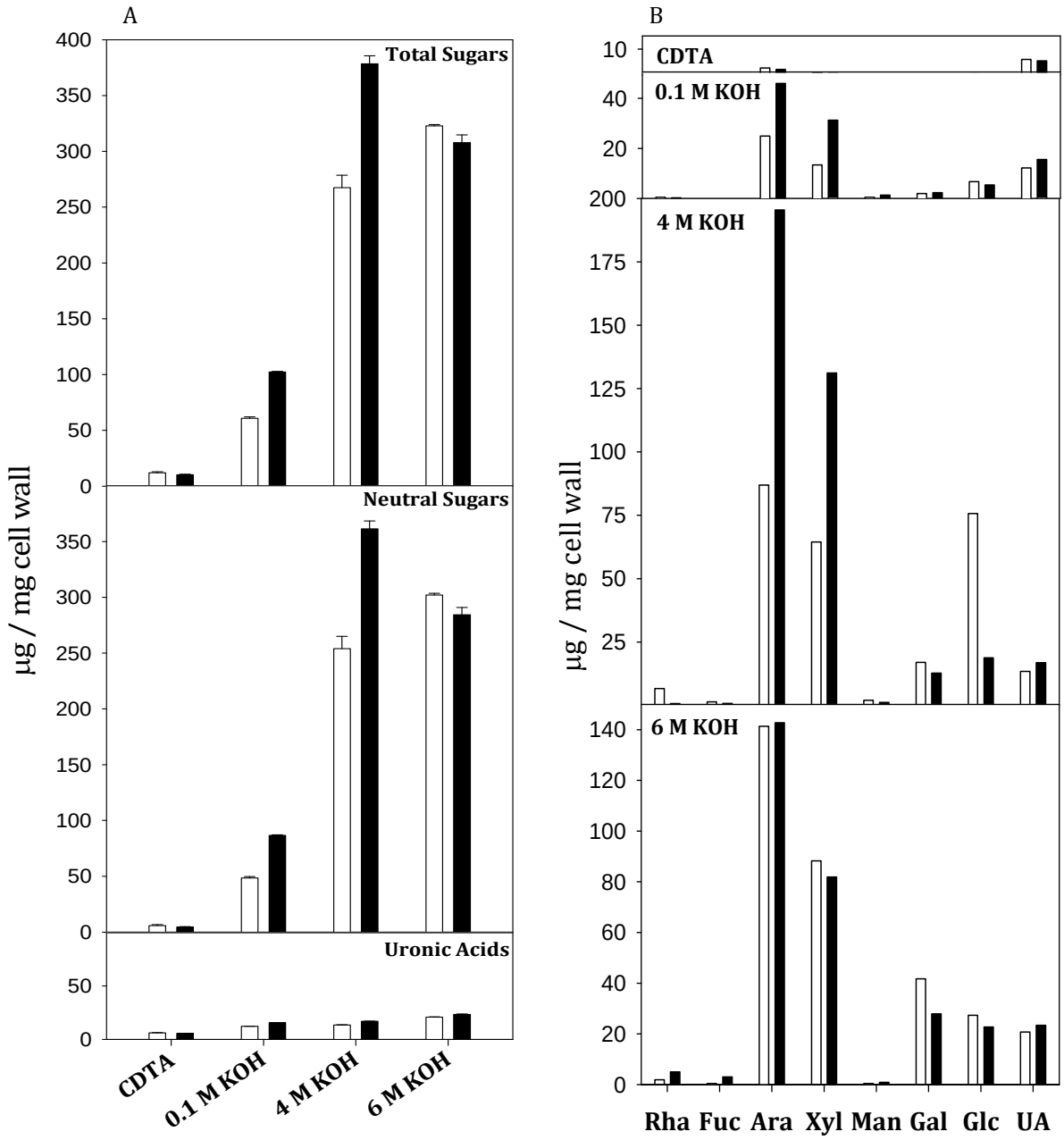


Figure 8. A: Total sugars, neutral sugars and uronic acids contents of fractions from cell walls of *white bars*; Snh and *black bars* ; Sh1.5 maize cell suspensions. Values represented are mean ± SE of three measurements (n=3); **B:** Monosaccharide composition of fractions from cell walls of *white bars*; Snh and *black bars*; Sh1.5 maize cell suspensions. Rha: rhamnose, Fuc: fucose, Ara: arabinose, Xyl: xylose, Man: mannose, Gal: galactose, Glc: glucose, UA: uronic acids

III.c.2. Polysaccharide epitope screening after enzymatic digestion

In order to detect cell wall changes in the distribution of epitopes in short-term DCB-habituated cells, aliquots of cell wall fractions of Snh and Sh1.5 cell suspensions were loaded onto nitrocellulose membranes as 1/5 dilutions, subjected to enzymatic digestions by using

an endo-polygalacturonanase, a β-xylanase and an endo-arabinanase and subsequently probed with monoclonal antibodies for pectic and hemicellulosic polysaccharides.

Heatmaps were generated with the resulting pool of data (Figures 9B and 10). As the total number of dilutions assayed was 5, a value ranging from 0 to 5 was assigned. This value depended on the sum of colored spots (corresponding to labeled epitopes in each dilution) found in the heatmap (see methods). In order to illustrate how heatmap were performed, a representative IDAs for LM10 is shown in Figure 9A. Likewise, when LM10 was assayed, the scoring values assigned were 0 for all the Snh cell wall fractions, and 0, 1, 1 and 2 for Sh1.5 CDTA, 0.1 M KOH, 4 M KOH and 6 M KOH fractions, respectively. In addition, it should be taken into consideration that in the case of Snh heatmap the maximum value assigned was 4 due to the fact that in any case 5 coloured spots were found.

Two different types of groupings appeared in the heatmap: a dendrogram with cell wall fractions and a dendrogram with the different antibodies probed. These groupings depended on the distribution pattern of labeled epitopes: proximity in grouping is directly related with similarity of epitopes distribution across the cell wall fractions.

In figure 9B it is shown a heatmap performed only with the results obtained from the non-enzymatic digested nitrocellulose membranes (controls). Closest cell wall fractions in the Snh and Sh1.5 cell lines, and consequently the most similar in terms of the distribution of labeled epitopes, were 6 M KOH and 4 M KOH, followed by 0.1 M KOH and CDTA. Epitopes for β-1,4-xylans, recognized by LM10, were exclusively found in all the alkali-extracted cell wall fractions from Sh1.5. The pattern of LM11 labeling which detected specifically epitopes for xylans and arabinoxylans was similar in both cell lines, with the exception that the Sh1.5 6 M KOH cell wall fraction showed an increased intensity of labelling.

Respect to pectic polysaccharides, cell wall fractions from both cell lines were probed with antibodies recognizing homogalacturonan and rhamnogalacturonan I. Results obtained for JIM5 and JIM7, antibodies for homogalacturonan with low and high degree of methylesterification respectively, showed that no differences in the pattern of labeling were

found for JIM7 among cell line fractions, whereas a greater intensity of labeling for JIM5 was found in CDTA and 0.1 M KOH Sh1.5 cell line fractions when compared to Snh.

Detection of epitopes for β-1,4-galactan side chains of rhamnogalacturonan I (LM5) and non-fucosylated xyloglucan (LM15) revealed a greater degree of labeling in all the alkali-extracted fractions from Sh1.5 cells, especially in the 4 M KOH fraction. Moreover, these two epitopes were always related to Sh1.5 cells. In contrast, in Snh alkali-extracted fractions, an association was observed between the distribution of LM5 and LM6 epitopes. No differences between cell lines were found in these fractions when LM6 was probed. Nevertheless, α-1,5-arabinan side chains of rhamnogalacturonan I epitopes were only detected in the CDTA fraction of Snh cells.

In order to better appreciate the differences induced after enzymatic digestions, individual heatmaps were generated for Snh and Sh1.5 cells (Figure 10). In the heatmap for the Snh cell line (Figure 10A), the closest cell wall fractions were 0.1 M KOH and 4 M KOH, followed by 6 M KOH and finally CDTA, whereas in the Sh1.5 heatmap (Figure 10B), 0.1 M KOH was firstly grouped with 6 M KOH, followed by 4 M KOH and lastly with CDTA. As could be deduced from the greater intensity of colour, it was in the 6 M KOH and 4 M KOH cell wall fractions that the greatest amount of labelled epitopes was detected in both cell lines.

The dendrogram illustrating the enzymatic treatments, controls and antibodies probed shows two principal branches for both cell lines: 1 and 2. Each of these subsequently divided into another two sub-branches (Figure 10). However, groupings differed depending on the cell line, indicating variations between cell lines in epitope distribution in the different polysaccharides. In Snh cells, the distribution of epitopes for JIM7, LM15, LM10 and LM11 was not substantially affected by any enzymatic treatment; however, endo-polygalacturonanase treatment produced modifications in JIM5, LM5 and LM6 epitope distribution. Epitopes for JIM5 disappeared from the CDTA and in the 0.1 M and 4 M alkali-extracted fractions. Disappearance of epitopes for LM6 was detected in the CDTA and 0.1 M alkali-extracted fractions, whereas those for LM5 appeared in the 6 M KOH fraction when

subjected to endo-polygalacturonanase enzymatic digestion. Endo-arabinanase treatment also induced changes in LM5 and LM6 epitopes. Similarly to the endo-polygalacturonanase treatment, the intensity of LM5 epitope labelling was greater in the 6 M KOH fraction after endo-arabinanase treatment; however in the LM6 epitopes this intensity was reduced in all the cell wall fractions.

The influence of enzymatic digestions on the pattern of labelling of epitopes extracted in the Sh1.5 cell wall fractions showed differences when compared with Snh (Figure 10B). A greater proportion of epitopes were affected in the Sh1.5 fractions than in Snh cells after enzymatic digestions. As was expected, endo-polygalacturonanase treatment led to the disappearance of epitopes for JIM5 (in the 0.1 M KOH fraction) and JIM7 (in the CDTA fraction). In Sh1.5 cell wall fractions, both endo-polygalacturonanase and endo-arabinanase treatments affected epitope distribution for xylans (LM10) as well as those specific for rhamnogalacturonan I side chains (LM5 and LM6). Finally, LM10 and LM11 epitopes disappeared from all cell wall fractions except the CDTA fraction after xylanase digestion.

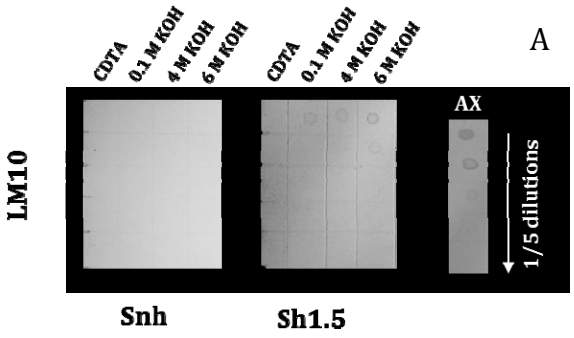
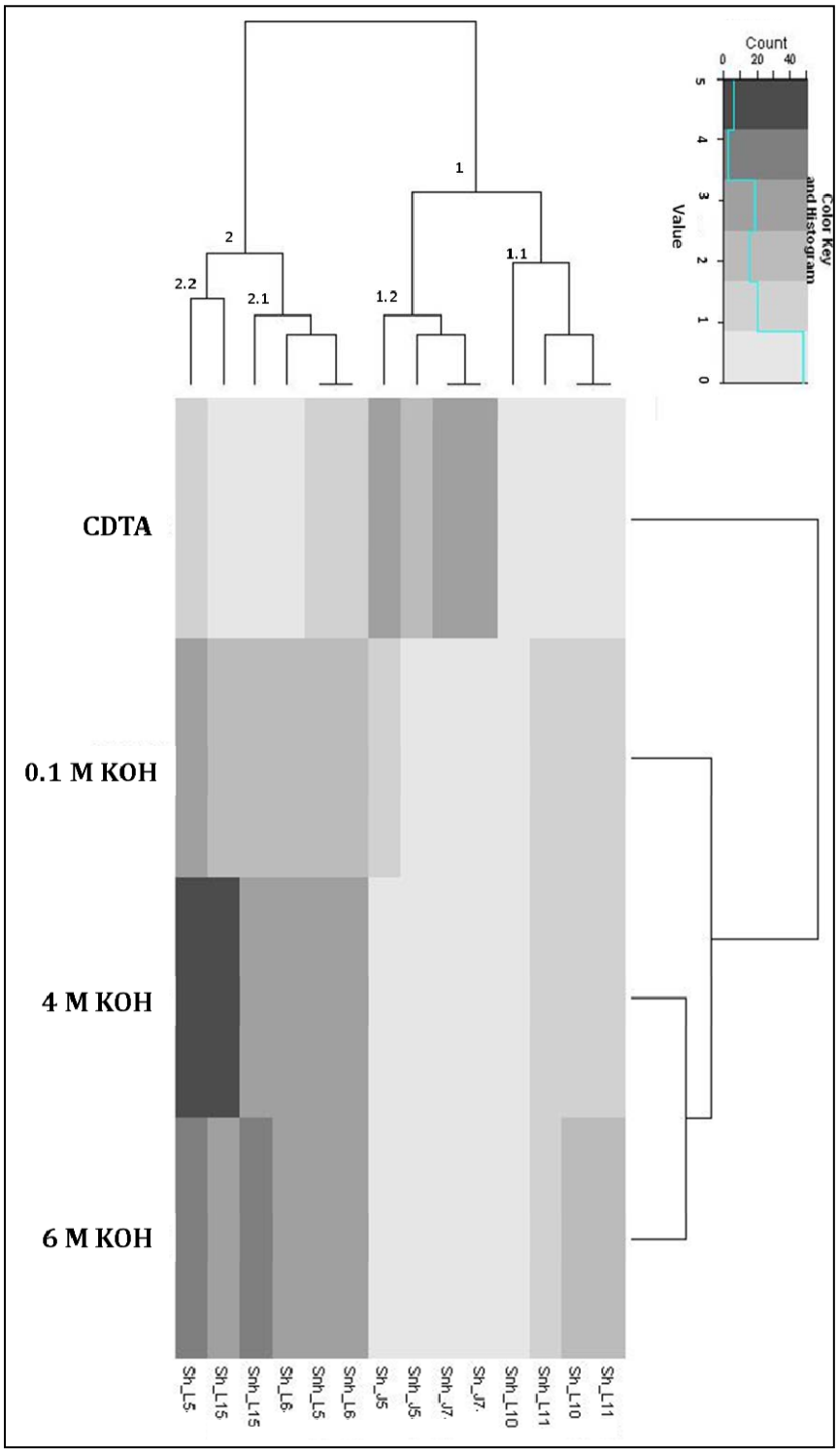


Figure 9. **A:** Representative immunodot assay (IDA). Cell wall fractions obtained from Snh and Sh1.5 were probed with LM10, monoclonal antibody specific for xylans. Any labelling was observed for Snh fractions, whereas 0, 1, 1 and 2 colored spots were detected respectively in CDTA, 0.1 M KOH, 4 M KOH and 6 M KOH fractions from Sh1.5 cells. AX: arabinoxylan-enriched fraction used as standard. **B:** Heatmap of data obtained from IDAs of Snh and Sh cell wall fractions (see next page). A value ranging from 0 (no colored spot detected) to 5 (5 colored spots detected) was assigned to each antibody (J5: JIM5, J7: JIM7, L5: LM5, L6; LM5, L10:LM10, L11:LM11, L15:LM15) and in each cell wall fraction (CDTA, 0.1 M KOH, 4 M KOH, 6 M KOH), depending on the amount of colored spots (corresponding to dilutions) that were shown after revealing

B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6 M KOH
Sh_L5
Sh_L15
Smh_L15
Sh_L6
Smh_L5
Smh_L6
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Smh_J7
Sh_J7
Smh_L10
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A

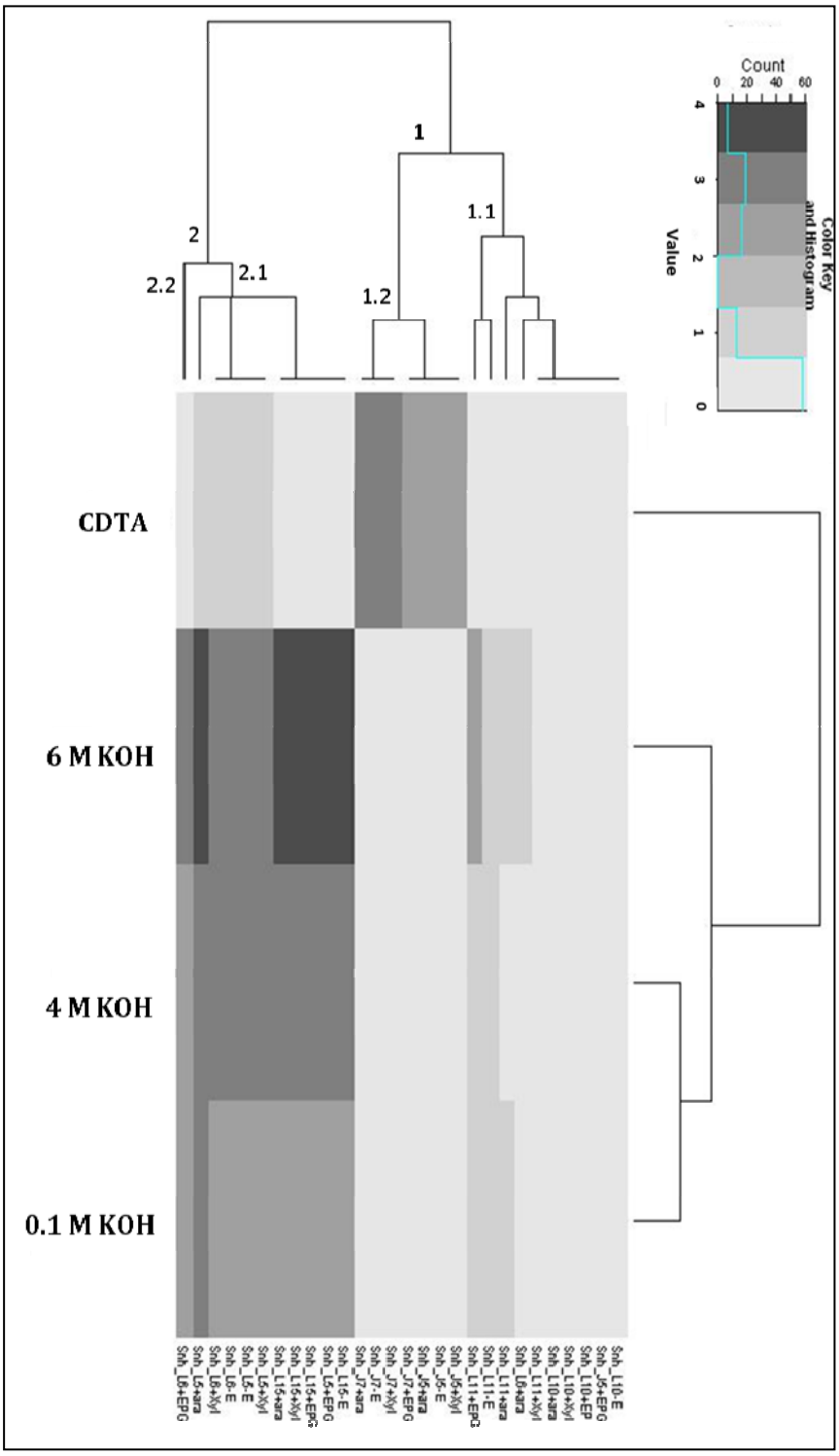
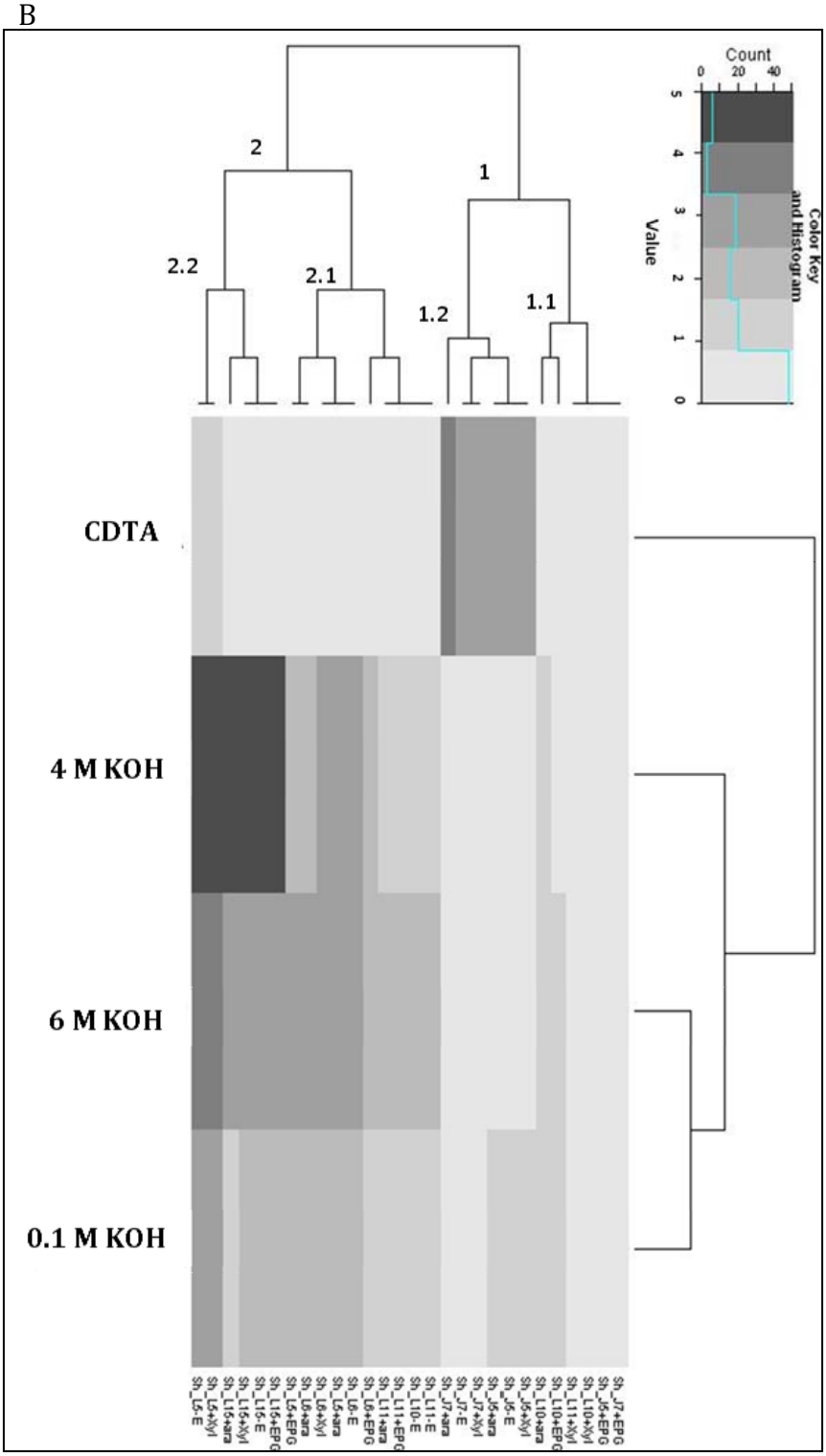


Figure 10. Heatmaps of data obtained from IDAs of **A:** Snh and **B:** Sh1.5 (see next page) maize cell suspensions obtained after the enzymatic digestion of the cell wall fractions. A value ranging from 0 (no colored spot detected) to 5 (5 colored spots detected) was assigned to each enzymatic treatment (EPG: endo-polygalacturonanase, Xyl: β-xylanase, Ara: endo-arabinanase and –E: control, where no enzyme was added), antibody (J5: JIM5, J7: JIM7, L5: LM5, L6; LM5, L10:LM10, L11:LM11, L15:LM15) in each cell wall fraction (CDTA, 0.1 M KOH, 4 M KOH, 6 M KOH), depending on the amount of colored spots (corresponding to dilutions) that were shown after revealing

B


Count
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1.1
CDTA
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6 M KOH
0.1 M KOH
Sh_L5-E
Sh_L5+Xyl
Sh_L16+Xyl
Sh_L16-E
Sh_L16+EPG
Sh_L5+EPG
Sh_L6+ara
Sh_L6+Xyl
Sh_L5+ara
Sh_L6-E
Sh_L6+EPG
Sh_L11+ara
Sh_L11+EPG
Sh_L10-E
Sh_L11-E
Sh_J7+ara
Sh_J7-E
Sh_J7+Xyl
Sh_J5+ara
Sh_J5-E
Sh_J5+Xyl
Sh_L10+ara
Sh_L10+EPG
Sh_L11+Xyl
Sh_L10+Xyl
Sh_J5+EPG
Sh_J7+EPG

IV. DISCUSSION

One of the most conspicuous changes detected by FTIR monitoring of the cell walls from the cell lines studied was a reduction in the cellulose content. In fact, the main differences among spectra of Snh and DCB-habituated (Sh) maize cell walls appeared in the fingerprint region (900-1200 cm^{-1}), in which a wide range of polysaccharides absorb IR radiation, including cellulose. These differences became more marked as the DCB concentration in the culture medium was increased. However, differences among the spectra of Snh and Sh exposed to lower DCB concentrations (Sh0.3 and Sh0.5) were attenuated when the number of culture cycles that cells were grown in contact with the inhibitor was increased. These data were confirmed by the results obtained from the analysis of cellulose content. A 23% and 27% reduction in the cellulose content was observed when maize cells were exposed to 0.3 and 0.5 μM DCB, respectively, but after 10 culture cycles growing in presence of the inhibitor, these levels reverted to values close to the Snh value. At higher DCB concentrations (Sh1 and Sh1.5), differences in the fingerprint region among spectra were greater with respect to the Snh spectra and eventual recovery of the cellulose content was not observed. The gradual reversion in cellulose content produced when cells were growing in low DCB concentrations and the number of culture cycles in its presence was increased, had previously been described in bean cell suspensions (García-Angulo et al., 2006) and callus-cultured cells (Alonso-Simón et al., 2004), but interestingly, in these cells with type I walls, an initial period of about seven subcultures was required previously to the detection of any changes in their cell walls FTIR profiles, whereas in our maize cells, which have type II cell walls, this lag period has not been observed.

One possible explanation for this observed transient reversion in cellulose content at the initial steps of the DCB-habituation process could be that increasing concentrations of herbicide may promote some change in the expression level of *ZmCESA* genes. In fact, it has been found that *ZmCESA* gene expression was mis-regulated in maize cultured cells habituated to high (12 μM) DCB concentrations (Mélida et al., 2010a). An induction of

ZmCESA7 and *ZmCESA8*, thought to be involved in producing the primary cell wall before the onset of secondary wall formation (Appenzeller et al., 2004; Bosch et al., 2011), was detected in some of our short-term habituated cell lines. It seems that maize cells start to overexpress these isoforms when the number of culture cycles growing in presence of DCB is increased or when the concentration of the inhibitor exceeds a threshold. Similarly, maize cells subjected to long-term habituation to high DCB concentrations also showed an induction of *ZmCESA7* and *ZmCESA8* genes (Mélida et al., 2010a). This could be indicative of an important role for these two *CESA* isoforms in habituation to all DCB levels. It could be hypothesized that, although less efficient in cellulose synthesis, CESA7 and CESA8 may be more resistant to the effects of DCB.

In addition to cellulose, FTIR monitoring revealed that other polysaccharides seem to be also affected by DCB-habituation. So, in order to unmask other actors playing a role in early DCB-habituation events, Sh1.5 cell line was selected for further studies.

In the Sh1.5 cell line the observed 33% reduction in cellulose content was compensated by a net increase in 0.1 M KOH and 4 M KOH extracted arabinoxylans. IDAs also showed in this cell line an increase in the proportion of epitopes for arabinoxylan/xylan (LM10 and LM11). Interestingly, no LM10 labeling was observed neither in Snh cell walls in our work, nor in maize calluses habituated to high DCB concentrations -4, 6 or 12 μM- (Mélida et al., 2009). The divergence between both studies could be related to the difference of plant material (calluses or suspension cell cultures), but would be also linked to a transient modification in chemical composition in some subsets of arabinoxylans populations at the first steps of the habituation.

Other hemicelluloses, such as xyloglucan, seems also to be involved in the early events of DCB-habituation, since an increase in the detection of LM15 epitopes was observed in the Sh1.5 4 M KOH fraction, pointing to more easily-extractable xyloglucan molecules. Reinforcing this finding, a lower presence of LM15 epitopes was found in the 6 M KOH fraction from Sh1.5 cells, treatment which would extract strongly-linked molecules.

Furthermore, sugar composition analysis points to an increase of uronic acid-rich polysaccharides, especially in alkali-extracted fractions, in the early steps of habituation. Likewise, in such alkali-extracted fractions, IDAs revealed an enhancement of LM5 labeling and, therefore, an increase in galactan side chains of rhamnogalacturonan I in the habituated cell line. Also, an enrichment of epitopes for JIM5 (homogalacturonan with a low degree of esterification) was found in the mild-alkali (0.1 M KOH) extracted fraction, as well as in the CDTA-extracted fraction.

The fact that other related epitopes such as those for homogalacturonan with a higher degree of esterification (JIM7) or for arabinan side chains of rhamnogalacturonan I (LM6) did not change between cell lines, indicates that only some specific subsets of polysaccharides were affected during the early DCB-habituation. Enzymatic treatments affected differently depending on the cell line. Thus, these results may be indicative of structural differences in polysaccharides among cell lines, which could prevent or facilitate the enzyme action. However, further analyses are required in order to clarify it.

It is interesting to note how in the type II cell walls of our cell cultures, characterised by a rather low amount of pectic polysaccharides, an increase in such type of polysaccharides is enhanced in the first steps of DCB-habituation. This fact is comparable with the observed trend in DCB-habituated plant cultured cells having type I walls, as bean, in which the reduction in the cellulose content is mainly counteracted by an increase in pectic polysaccharides (Encina et al., 2001; García-Angulo et al., 2006). Previous work carried out in DCB or isoxaben habituated cultured cells having type I cell walls, are characterised by a substantial increase in JIM5-reactive low-esterified pectins, which would point to more Ca^{+2}-cross-linked pectins (Wells et al., 1994; Sabba et al., 1999; Manfield et al., 2004; García-Angulo et al., 2006). As pectic polysaccharides are involved in cell-cell adhesion (Willats et al., 2001), the higher amount of these polysaccharides observed in our habituated cells could explain the fact that these cultured cells developed larger cell clusters than the Snh cultures, resembling to that described for DCB-habituated bean cell suspensions (Encina et al., 2001;

García-Angulo et al., 2009). Nevertheless, and as a supplementary explanation, such slower and altered growth patterns detected in Sh1.5 cells could be also related with their cellulose-deficient wall, which would, therefore, cause an impaired cell division. On the other hand, and although xyloglucan is known not to be an abundant hemicellulose in type II cell walls (Fry, 2000), our results shown that this hemicellulose may play some role during this early stress situation, as the detection of LM15 epitopes increased (at least in 4 M KOH fraction) during the first steps of DCB habituation.

Interesting differences in cell wall modifications appear when early DCB-habituation events are compared with those described for maize calluses habituated to intermediate and high DCB levels (Mélida et al., 2009; 2010a; 2010b). In long-term DCB habituated cells, a 75% of reduction in cellulose content was detected, and this value remained stable even when DCB concentration in the culture medium increased from 6 to 12 μM. In contrast, in short term DCB-habituation, the reduction in cellulose content was lower (23-33%) and tended to revert when DCB concentration in the culture medium ranged from 0.3 and 0.5 μM. Moreover, in long-term DCB-habituation the reduction in the amount of cellulose was compensated by an increase in arabinoxylans, but the content of other polysaccharides, such as pectins or xyloglucan, was not affected or even was reduced. However, as mentioned above, in Sh1.5 cultures besides the not so drastic increase in the arabinoxylan amount, other polysaccharides such as rhamnogalacturonan I, homogalacturonan with a low degree of esterification, and even xyloglucan seemed to be involved. Finally, although further studies are required, our preliminary results suggest that antioxidant activities play an important role during the first steps of the DCB habituation process (Largo-Gosens, personal communication), in contrast to those described in cultures habituated long-term to high DCB concentrations (Mélida et al., 2010a).

In sum, monitoring of short-term DCB habituation of maize cell suspensions has revealed that the main modification produced during the first steps of this process consisted in the reduction of cellulose content, and it has been confirmed that changes in these

cellulose levels were promoted by two main factors: the inhibitor concentration and the number of culture cycles that cells were in its presence. Wall composition of Sh1.5 cells was modified as a consequence of habituation to low DCB levels, showing a reduction of 33% in the cellulose content. Furthermore, an induction of *ZmCESA7* and *ZmCESA8* took place and this effect was revealed as a constant feature of DCB habituation. Sh1.5 cells counteracted the lack of cellulose with an increase in arabinoxylans. In addition to arabinoxylans, other polysaccharides, such as galactan side chains of rhamnogalacturonan I, homogalacturonan with a low degree of esterification and xyloglucan, seemed to have a role in the first steps of DCB-habituation. Thereby some of the modifications occurred in the cell walls in order to compensate for the lack of cellulose differed according to the DCB-habituation level, and demonstrates the remarkable dynamic plasticity of maize cell cultures, which enables them to cope with different DCB habituation conditions by altering their cell wall composition.

Chapter II

Study of the metabolic capacity of cellulose-deficient maize cells

ABSTRACT

The capacity of DCB-habituated maize cells to survive with reduced cellulose content derives from their ability to counteract the lack in cellulose by acquiring a modified arabinoxylan network. Since it is probable that this fact entails alterations in their metabolism of polysaccharides, the aim of this study was to investigate such alterations in DCB-habituated maize cell cultures showing a mild reduction in their cellulose content at different stages in the culture cycle. Using a pulse-chase radio-labelling experimental approach, the DCB-habituated cell cultures were fed with [^{3}H]Ara as radio-labelled precursor, and the metabolism of ^{3}H-hemicelluloses and ^{3}H-polymers was tracked in several cell compartments for 5 h.

The habituated cells exhibited difficulties in the uptake of the [^{3}H]Ara, showing a slower and less efficient metabolism regardless of the culture stage of growth. Moreover, lower proportions of strongly cell wall bound hemicelluloses ([^{3}H]xylans and [^{3}H]xyloglucan) as well as ^{3}H-polymers ([^{3}H]Ara-containing and Driselase-digestion resistant polymers) were grafted onto the cell wall, while a higher percentage of soluble extracellular polymers were sloughed into the culture medium. These findings could be relate, at least partially, to the cell wall cellulose-deficiency, and therefore to a reduction in "binding points" to cellulose and/or a reduced capacity to incorporate arabinoxylans into the cell wall by extra-protoplasmic phenolic cross-linking.

I. INTRODUCTION

Plant cell walls are dynamic structures essential for cell division, enlargement and differentiation and are also involved in the modulation of stress signalling responses (Roberts, 2001; Huckelhoven, 2007; Driouich et al., 2012). The generic composition of the primary plant cell wall consists of a cellulose framework tethered by hemicelluloses embedded in a matrix of pectins (Demura and Ye, 2010; Pauly and Keegstra, 2010). In grasses and cereals, the cellulose framework is strengthened by heteroxylans, which present the general structure of all xylans (a backbone of Xyl with Ara, GlcA, or MeGlcA residues attached) but also some unique features such as the presence of hydroxycinnamates, mainly Fer and *p*-Cou esterified on Ara residues (Smith and Hartley, 1983; Kato and Nevins, 1985). Hydroxycinnamates are susceptible to oxidative coupling by peroxidases (Geissmann and Neukom, 1971) to form ester-linked dehydrodiferulates which contribute to wall assembly by cross-linking cell wall polysaccharides (Fry, 2004; Parker et al., 2005).

Heteroxylans from grasses are the third most abundant component of plant biomass, and they are the main non-cellulosic polysaccharide in this type of wall (Zhong et al., 2009; Fincher, 2009). Non-cellulosic polysaccharides are potential sugar sources for ethanol fermentation and therefore, for bioethanol production (Cosgrove, 2005; Komatsu and Yanagawa, 2013). In addition to their economic importance, heteroxylans play an essential role in cell expansion. In order for this to take place, primary walls require the integration of these heteroxylans and other newly synthesised polymers, as well as a modification of the pre-existing wall bound ones (Kerr and Fry, 2003). Thus, an improved knowledge of their synthesis and assembly is crucial to understanding and manipulating plant growth and morphogenesis, with the consequent economic implications.

Since mRNA might not be translated and *in vitro* assays may not reproduce natural cell conditions (with the subsequent loss of real information), *in vivo* metabolic studies appear to be the best experimental approach, making it possible to monitor *in vivo* the behaviour of the hemicellulosic substrates themselves (Kerr and Fry, 2003). Such *in vivo*

analyses have typically been carried out in suspension-cultured cells (Edelmann and Fry, 1992; Thompson and Fry, 1997; 2000; Encina and Fry, 2005; Kerr and Fry, 2003; 2004; Burr and Fry, 2009), and although these may differ in some details from plantlets or plants, they are excellent, easy to manipulate model systems. Moreover, some suspension-cultured cells (including maize) release polysaccharides into the culture medium (which can be considered as an extension of the apoplast). Since these sloughed polysaccharides do not require chemical treatment to be extracted from the cell wall, they retain their molecular integrity and have been demonstrated to constitute an excellent model system for studying several aspects of cell wall biology (Kerr and Fry, 2003; 2004; Burr and Fry, 2009). Feeding these cell suspension systems (cells plus culture medium) with radio-labelled precursors has been widely shown to be as a useful, sensitive and precise methodology for tracking the synthesis and cellular traffic (from synthesis in the Golgi apparatus to integration in the cell wall and/or sloughing into the culture medium) of hemicelluloses in living cells: this approach has the advantage that all the substrates are endogenous and therefore the reactions detected are ones that occur naturally (Thompson and Fry, 1997; Kerr and Fry, 2003; 2004; Lindsay and Fry, 2008; Mélida et al., 2011). Specifically, when [^{3}H]Ara was supplied to maize cell-suspension cultures, the *in vivo* careers of endogenous xyloglucan and xylan molecules in several cell compartments were successfully tracked, confirming the effectiveness of this experimental approach for tracking heteroxylan metabolism (Kerr and Fry, 2003).

Habituation of cultured cells to CBIs has proven a valuable tool to study cell wall plasticity and biogenesis (for a review see Acebes et al., 2010). Within CBIs, DCB specifically inhibits the polymerisation of Glc into β-1,4-linked glucan (Delmer, 1987; García-Angulo et al., 2012) thereby causing a reduction in cellulose content. Several plant cell cultures have been habituated to grow in presence of DCB (Shedletzky et al., 1992; Encina et al., 2001; 2002; García-Angulo et al., 2006; 2009; Mélida et al., 2009), and it has been found that the magnitude of the cellulose reduction provoked by the inhibitor depends on its concentration as well as on the length of time that cells are grown in its presence (Alonso-Simón et al.,

2004). The capacity of habituated cells to survive resides in their ability to modify their cell wall composition in order to counteract the lack of cellulose. In this respect, it has been proven that maize cell suspensions habituated to DCB compensate for the reduction in cellulose levels by modifying their cell wall matrix, involving quantitative and qualitative changes in the arabinoxylan network. Based on previous results (Mélida et al., 2009; 2010a; 2010b and 2011), it is highly probable that arabinoxylan metabolism in DCB-habituated cells is both quantitatively and qualitatively altered. Therefore, the aim pursued in the present study was to gain a greater insight in the metabolic capacity of cellulose-deficient maize cells throughout the culture cycle. The elucidation of differences in location, timing and intensity of hemicellulose biosynthesis in these cellulose-deficient cells could help us to 1) shed light on the mechanisms that account for the rearrangement of the hemicellulose network in cellulose deficient conditions and 2) improve our knowledge about the general mechanisms underlying the structural plasticity of primary cell walls. To this end, DCB-habituated maize cell cultures were fed with [^{3}H]Ara as radio-labelled precursor at lag, early logarithmic and late logarithmic growth phase, and the synthesis and cellular traffic of ^{3}H-hemicelluloses and ^{3}H-polymers were tracked in several cell compartments for 5 h.

II. MATERIALS AND METHODS

II.a. Cell-suspension cultures and habituation to DCB

Maize cell-suspension cultures (*Zea mays* L., Black Mexican sweet corn) from immature embryos were grown in Murashige and Skoog media (Murashige and Skoog, 1962) supplemented with 9 μM 2,4-D and 20 g L^{-1} sucrose, at 25°C under light and rotary shaken, and routinely subcultured every 15 days.

In order to obtain habituated cell cultures, Snh cells were cultured in a medium supplied with 1 μM DCB. DCB was dissolved in DMSO, which does not affect cell growth at this range of concentrations. After seven subcultures, some of the cells habituated to grow in

1 µM DCB were transferred to medium containing 1.5 µM DCB (Sh1.5). Growth curves of Snh and Sh1.5 maize cell lines were obtained by measuring the increase in DW at different culture times and it was observed that Sh1.5 cells presented longer culture phases than Snh cells (de Castro et al., 2013; Chapter I, Figure 6A). Therefore, these growth curves were subsequently used to select the most appropriate days for sampling in order to ensure that Snh and Sh1.5 cell lines were at the same stage of the culture cycle.

II.b. Radio-labelling and sample collection of ^{3}H-hemicelluloses and ^{3}H-polymers

Thirteen-ml aliquots from Snh and Sh1.5 maize cell cultures were collected at lag, logarithmic and early stationary growth phases. Each 13-ml aliquot was independently fed with 1 MBq of L-[1-^{3}H]Ara (148 GBq/mmol; Amersham International, Bucks., U.K). The days on which the 13-ml aliquot of culture was sampled and [^{3}H]Ara was added were adjusted depending on the cell line at early and late logarithmic phases. Thus, for lag phase, the first day of culture was selected for both cell lines. In the case of the early-logarithmic phase, 4th and 8th days of culture were chosen in Snh and Sh1.5 cell lines, respectively. Finally, for the late- logarithmic phase, the 8th and 12th days were selected for Snh and Sh1.5 cell lines respectively.

The incorporation of [^{3}H]Ara into ^{3}H-hemicelluloses and ^{3}H-polymers in both cell lines was measured at different labelling-time points for 5 h in each different culture phase. Thus, 1-ml samples were collected 30, 60, 120 and 300 min (labelling-time points) after [^{3}H]Ara was fed from the independent 13-ml aliquots of culture.

II.b.1. CFM collection

Each 1-ml sample collected at the different labelling-time points was filtered through an empty Poly-Prep column (Biorad). Then, cells were rinsed with 5 ml of ice-cold fresh medium. The filtrate was pooled with the rinses and labelled as CFM fraction.

II.b.2. Protoplasmic content extraction

The washed cells retained in the Poly-Prep column filter, were quickly resuspended in 1 ml of homogenisation buffer [50 mM collidine acetate (pH 7.0) containing 2% (w/v) lithium dodecyl sulfate (LiDS), 10% (v/v) glycerol, 5 mM sodium thiosulfate and 10 mM DTT (added freshly)] and stored at -20°C in the Poly-Prep column.

The cell suspension was thawed at 1.5°C and transferred to the 10-ml glass mortar of a Potter–Elvehjem homogeniser by rinsing with 1 ml of chilled homogenisation buffer (x3). The cell suspension was then homogenised with a motor-driven Teflon pestle for 10 min. The homogenate was passed through a second empty Poly-Prep column and the filtrate collected. The cell fragments were rinsed with 1 ml H_2O (x4).

Solid KCl was added to the filtrate and rinses to a final concentration of 0.67 M in order to precipitate the dodecyl sulphate as its insoluble K^+ salt. After 30 min at 4°C, the products were centrifuged at 3000 rpm for 5 min. The supernatant was removed and re-centrifuged. This procedure was repeated until the supernatant turned clear. Then, the supernatant was collected and labelled as the protoplasmic fraction.

II.b.3. Extraction of cell wall fractions

Cell fragments retained in the filter of the Poly-Prep column were treated with 1 ml of 0.1 M NaOH containing 1% (w/v) $NaBH_4$ for 24 h at room temperature. The filtrate was then collected and the residue washed with 1 ml of the same extractant (x3). Filtrate and washes were pooled and labelled as 0.1 M NaOH fraction. The remaining residue was then treated with 1 ml of 6 M NaOH containing 1% (w/v) $NaBH_4$ for 24 h at 37°C. The filtrate was again collected and the residue washed with 1 ml of the same solution (x3). Filtrate and washes were then pooled and labelled as 6 M NaOH fraction. Both the 0.1 M NaOH and 6 M NaOH fractions, were acidified to pH 4.7 by the addition of acetic acid.

The residue in the Poly-Prep column was then washed with 1 ml of distilled water slightly acidified with acetic acid (x3). The remaining residue (6 M NaOH-insoluble material)

still retained in the Poly-Prep column filter was considered to be from ^{3}H-polymers firmly bound to cellulose residue.

II.c. Assay of radioactivity in the uptake of [^{3}H]Ara and incorporation into ^{3}H-polymers

An analysis of the CFM and protoplasmic fractions was performed as described by Kerr and Fry (2003) with minor modifications. Briefly, 5 µl of 10% (w/v) cold Ara was added to an aliquot of 60 µl of each sample as an internal marker. The sample was then subjected to PC in butan-1-ol/acetic acid/water (12/3/5, by vol.) for 16 h. The internal marker was stained slightly by dipping the paper in 10% of the standard concentration of aniline hydrogen phthalate (Fry, 2000), dried, and then heated at 105°C for 2 min. This revealed the position of the Ara without appreciably decreasing the efficiency of scintillation counting (Kerr and Fry, 2003). The Ara spot and the origin zone (RF 0; containing the polymers) were then cut and separately assayed for ^{3}H by liquid scintillation counting in 2 ml of OptiScint HiSafe (Fisher).

For the analysis of cell wall fractions, an aliquot of 500 µl of each sample was assayed for radioactivity by liquid scintillation counting in 5 ml of OptiPhase HiSafe (Fisher).

In the case of the ^{3}H-polymers firmly bound to cellulose, the entire polypropylene filter pad was excised and mixed with 15 ml of OptiPhase HiSafe scintillation liquid. After soaking for 24 h, radioactivity was then assayed by counting.

II.d. Fractions composition assay

Samples of CFM, protoplasmic and cell wall fractions were dialysed against distilled water with 0.1% (w/v) chlorobutanol at 4°C for 72 h. Then, the dialysed samples were centrifuged for 15 min at 3000 rpm in order to separate the soluble from the insoluble material in water, and the soluble portion was collected. Samples were dried in vacuo and re-dissolved in 500 µl of 0.1 M TFA, heated at 85°C for 1 h to cleave arabinofuranosyl linkages, and re-dried to remove TFA. The dried material was re-dissolved in 20 µl 0.5% (w/v)

Driselase (Sigma Chemical Co.; partially purified by the method of Fry 2000) in pyridine/acetic acid/water (1/1/23 by vol. pH 4.7 containing 0.5% chlorobutanol), and incubated at 37°C for 96 h. The reaction was stopped by the addition of 15% formic acid. The mild acid pre-treatment greatly increased the yield of Xyl and xylobiose generated during subsequent digestion with Driselase and did not decrease the yield of isoprimeverose (Kerr and Fry, 2003). An internal marker mixture containing Xyl, Ara, Glc, isoprimeverose and xylobiose (≈ 50 μg each) was then added to the digest, which was subjected to PC on Whatman 3MM in ethyl acetate/pyridine/water (9/3/2 by vol.) for 18 h (Thompson and Fry, 1997). The internal markers were slightly stained with aniline hydrogen-phthalate and the identified spots were cut out and assayed for ^{3}H (as described above). Results from the radioactivity counting of the ^{3}H-labelled diagnostic fragments were plotted and named as follows: polymers which had [^{3}H]Ara (radioactivity detected in [^{3}H]Ara spot from the paper chromatogram); [^{3}H]xylans (summation of the amounts of radioactivity from [^{3}H]Xyl and [^{3}H]xylobiose spots), [^{3}H]xyloglucan (radioactivity detected when counting the [^{3}H]isoprimeverose spot) and undigested ^{3}H-polymers (radioactivity on the origin spot of the paper chromatogram).

III. RESULTS

III.a. Uptake of [^{3}H]Ara from the culture medium

Snh and Sh1.5 maize cell suspensions showed differences in [^{3}H]Ara uptake depending on the culture phase, with consumption being more efficient at the late logarithmic phase in both cell lines (0.114 and 0.055 KBq min^{-1} for Snh and Sh1.5 respectively) (Figure 1). However, Sh1.5 cells were less efficient in [^{3}H]Ara uptake than Snh cells, regardless of the culture phase (Figure 1B *vs* 1A), showing much lower estimated consumption rates of [^{3}H]Ara (roughly half) than Snh cells in all the culture stages analysed (data not shown). This was particularly marked at the late logarithmic phase of the culture

cycle, when Snh cells completely depleted the [^{3}H]Ara within the 300 min monitored. This depletion was not accomplished by Sh1.5 cells.

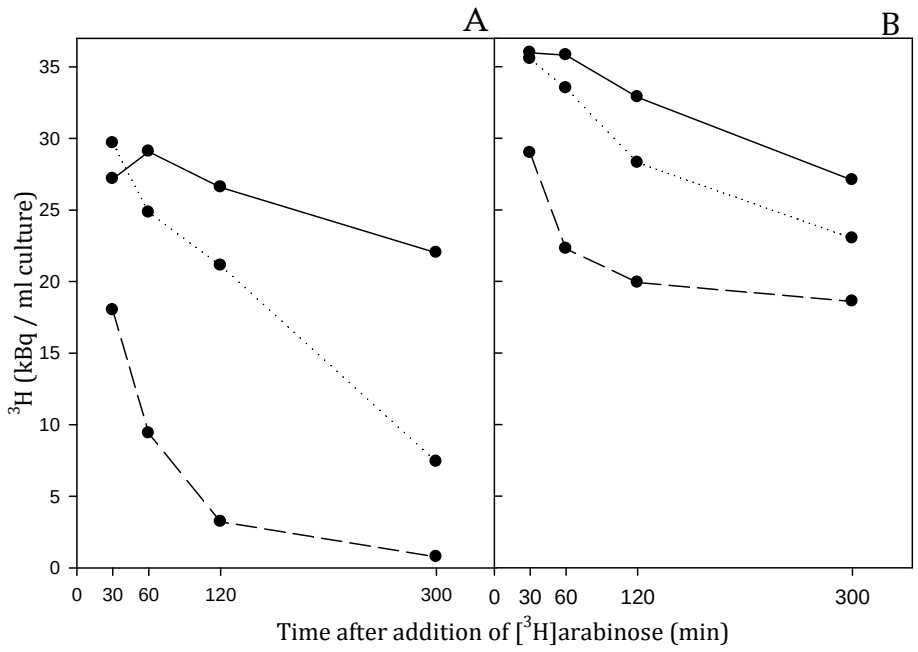


Figure 1. Consumption of [^{3}H]Ara in **A**: Snh and **B**: Sh1.5 DCB maize cell suspensions during *solid line;* lag, *dotted line*; early logarithmic and *dashed line;* late logarithmic phases of culture cycle. Results shown are representative of two independent experiments

III.b. Kinetics of incorporation of [^{3}H]Ara into hemicelluloses and polymers

As previously shown for [^{3}H]Ara uptake, differences in the kinetics of the incorporation of ^{3}H into hemicelluloses and polymers were observed depending on the cell line (Figure 2). In Snh cells, a gradual increase in the capacity to metabolise [^{3}H]Ara was observed throughout the culture cycle (Figure 2A). Thus, as culture cycle progressed, the length of time that unmetabolised [^{3}H]Ara and ^{3}H-polymers which were being synthesised remained in the protoplasm began to decrease. Furthermore, the time required for ^{3}H-polymers to be grafted onto the cell wall or sloughed into the medium decreased over the course of the cell culture cycle. This trend was not observed in the Sh1.5 cell line, which showed delayed kinetics when compared with Snh cells (Figure 2B).

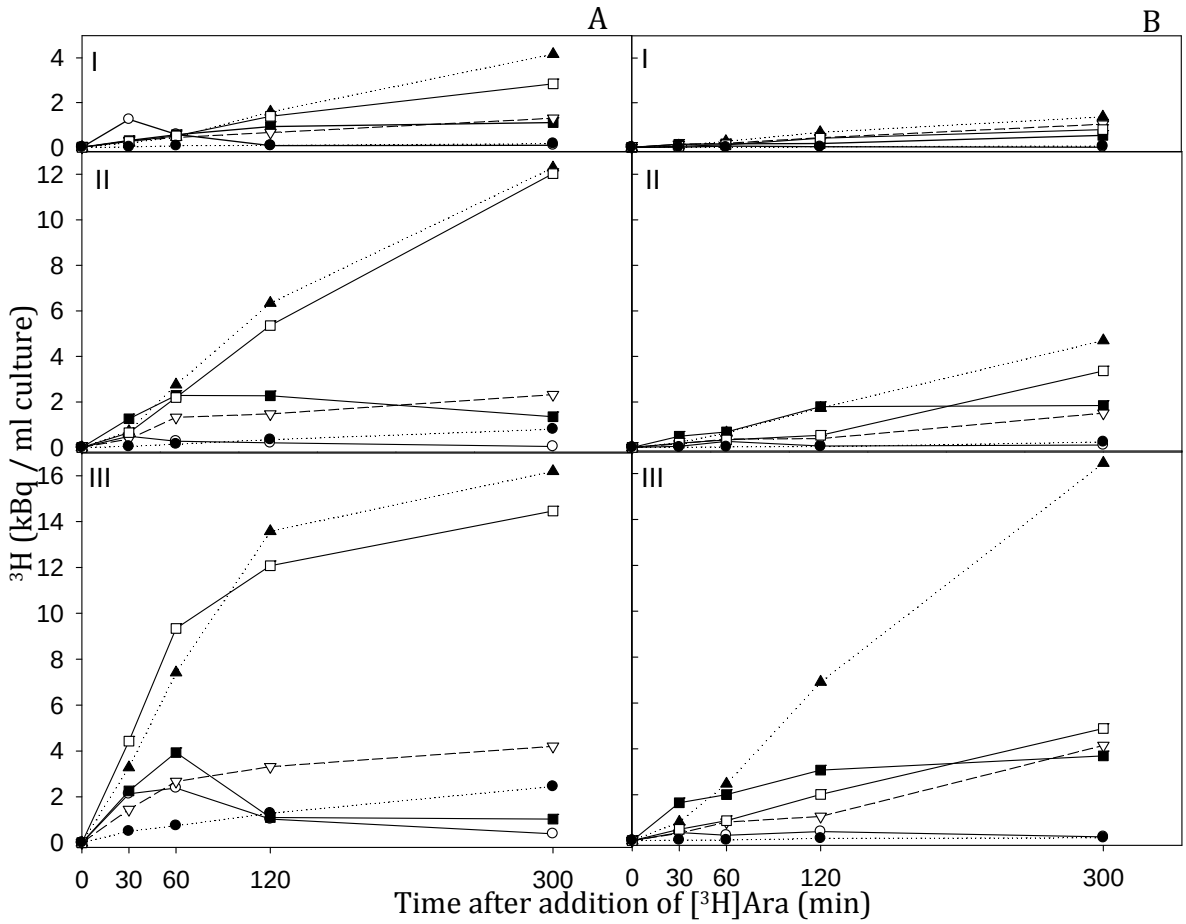


Figure 2. Incorporation of [^{3}H]Ara into the polymers of discrete compartments of **A**: Snh and **B**: Sh1.5 maize cell suspensions during lag (I), early logarithmic (II) and late logarithmic (III) phases of culture cycle. Samples were taken during 5 h at different labeling time points (30, 60, 120 and 300 min). The compartments assayed for total ^{3}H-polymers were protoplasmic (■), cell wall-bound [0.1 M NaOH-extractable (▽) and 6 M NaOH-extractable (□)] and soluble extracellular polymers (SEPs) sloughed to culture medium (▲). ^{3}H-polymers that were firmly bound to the α-cellulose residue (●) and unmetabolised [^{3}H]Ara detected within the protoplasm (○) were also assayed. Results shown are representative of two independent experiments

In both cell lines, the most active phase of the culture cycle as regards [^{3}H]Ara incorporation into hemicelluloses and polymers was the late logarithmic (Figure 2AIII and 2BIII). The kinetics obtained from this phase of the culture cycle clearly illustrated the differences mentioned above regarding to the metabolic capacity of synthesis and incorporation of ^{3}H-hemicelluloses and ^{3}H-polymers. At this phase of the culture cycle, Snh cells rapidly metabolised and incorporated the [^{3}H]Ara into ^{3}H-polymers in the protoplasm reaching a maximum peak of synthesis 60 min after [^{3}H]Ara feeding. Subsequently, the synthesis of ^{3}H-polymers in the protoplasm decreased, showing constant values during the remaining 240 min monitored. This sudden decrease could be related to the removal of these

^{3}H-polymers from the protoplasm and their incorporation into the cell wall as ^{3}H-hemicelluloses, or their sloughing into the culture medium ([^{3}H]soluble extracellular polymers; [^{3}H]SEPs) (Figure 2AIII). In contrast, Sh1.5 cells did not show a marked peak of ^{3}H-polymer synthesis in the protoplasm, and the incorporation of [^{3}H]Ara into any ^{3}H-hemicelluloses or [^{3}H]SEPs did not reach a plateau phase, as happened in Snh cells (Figure 2BIII). This may indicate that Sh1.5 cells had a delayed metabolism and, therefore, lower rates of synthesis of ^{3}H-polymers and ^{3}H-hemicelluloses.

The fate of the ^{3}H-polymers when leaving the protoplasm was also altered in Sh1.5 cells when compared with Snh cells (Figure 2). Snh cells preferentially incorporated [^{3}H]Ara into strong alkali-extracted-hemicelluloses (6 M NaOH) and/or SEPs (Figure 2A) followed by mild alkali-extracted hemicelluloses and ^{3}H-polymers firmly bound to cellulose residue. Sh1.5 cells incorporated lower amounts of [^{3}H]Ara into cell wall polymers (^{3}H-polymers firmly bound to cellulose residue and NaOH-extracted ^{3}H-hemicelluloses, mainly those extracted with 6 M), (Figure 2B) but the amount of [^{3}H]SEPs detected in the culture medium of Sh1.5 cells at late logarithmic phase of the culture cycle was roughly equal to that observed in Snh cells (Figure 2AIII *vs* 2BIII). However, when data from the incorporation of the [^{3}H]Ara into the different types of polymers and hemicelluloses were expressed as a percentage of the total radioactivity incorporated at the end of the 300 min of monitoring, a relative increase in the incorporation of [^{3}H]Ara into 0.1 M NaOH-extracted hemicelluloses was detected in Sh1.5 cells.

III.c. Kinetics of synthesis, cell wall integration and sloughing of ^{3}H-labelled diagnostic fragments

^{3}H-polymers and ^{3}H-hemicelluloses from the different cell compartments were firstly Driselase-digested and then subjected to PC in order to obtain the diagnostic fragments. In each cell line (Snh and Sh1.5), polymers which had [^{3}H]Ara residues, [^{3}H]xylans, [^{3}H]xyloglucan and ^{3}H-polymers resistant to Driselase digestion showed similar kinetics in

each cell compartment and culture phase analysed (Figures 3, 4, 5 and 6). Nevertheless, variations between cell lines and within the same cell line, among cell compartments and phases of culture cycle were found.

III.c.1. Protoplasmic compartment

Synthesis of the different polymers and hemicelluloses occurred exponentially during the lag phase in both cell lines (Figure 3). As with the general kinetics, 60 min after [^{3}H]Ara was fed at early and late logarithmic phases of the culture cycle, Snh cells reached a peak in synthesis of [^{3}H]xylans, [^{3}H]xyloglucan, polymers which had [^{3}H]Ara and undigested ^{3}H-polymers, which subsequently decreased (Figure 3A). In contrast, Sh1.5 cells did not show any peak in synthesis of [^{3}H]xylans, [^{3}H]xyloglucan, polymers which had [^{3}H]Ara and undigested ^{3}H-polymers during the early logarithmic phase of the culture cycle. At late logarithmic phase, maximum synthesis was detected later in Sh1.5 than in Snh cells, 120 min after [^{3}H]Ara was supplied, and the rapid decrease in the peak in synthesis detected in Snh was not observed in the Sh1.5 protoplasm (Figure 3B). This indicates that [^{3}H]xylans, [^{3}H]xyloglucan, polymers which had [^{3}H]Ara and undigested ^{3}H-polymers remained in the protoplasm of the habituated cells for a longer period of time after being wall-integrated or sloughed.

III.c.2. Cell wall compartment

The kinetics of ^{3}H-labelled diagnostic fragments from polysaccharides extracted with mild (0.1 M NaOH) and strong alkali treatment (6 M NaOH) from Snh and Sh1.5 cells are shown in Figures 4 and 5, respectively. Snh and Sh1.5 cells integrated a similar proportion of [^{3}H]xylans, [^{3}H]xyloglucan, polymers which had [^{3}H]Ara and undigested ^{3}H-polymers extracted with mild alkali treatment (Figure 4), whereas the amount of those extracted with strong alkali treatment was much lower in Sh1.5 cells (Figure 5). However, the kinetics of ^{3}H-labelled diagnostic fragments obtained by both (0.1 M and 6 M NaOH) alkali treatments

showed differences depending on the cell line (Figure 4A *vs* Figure 4B and Figure 5A *vs* Figure 5B).

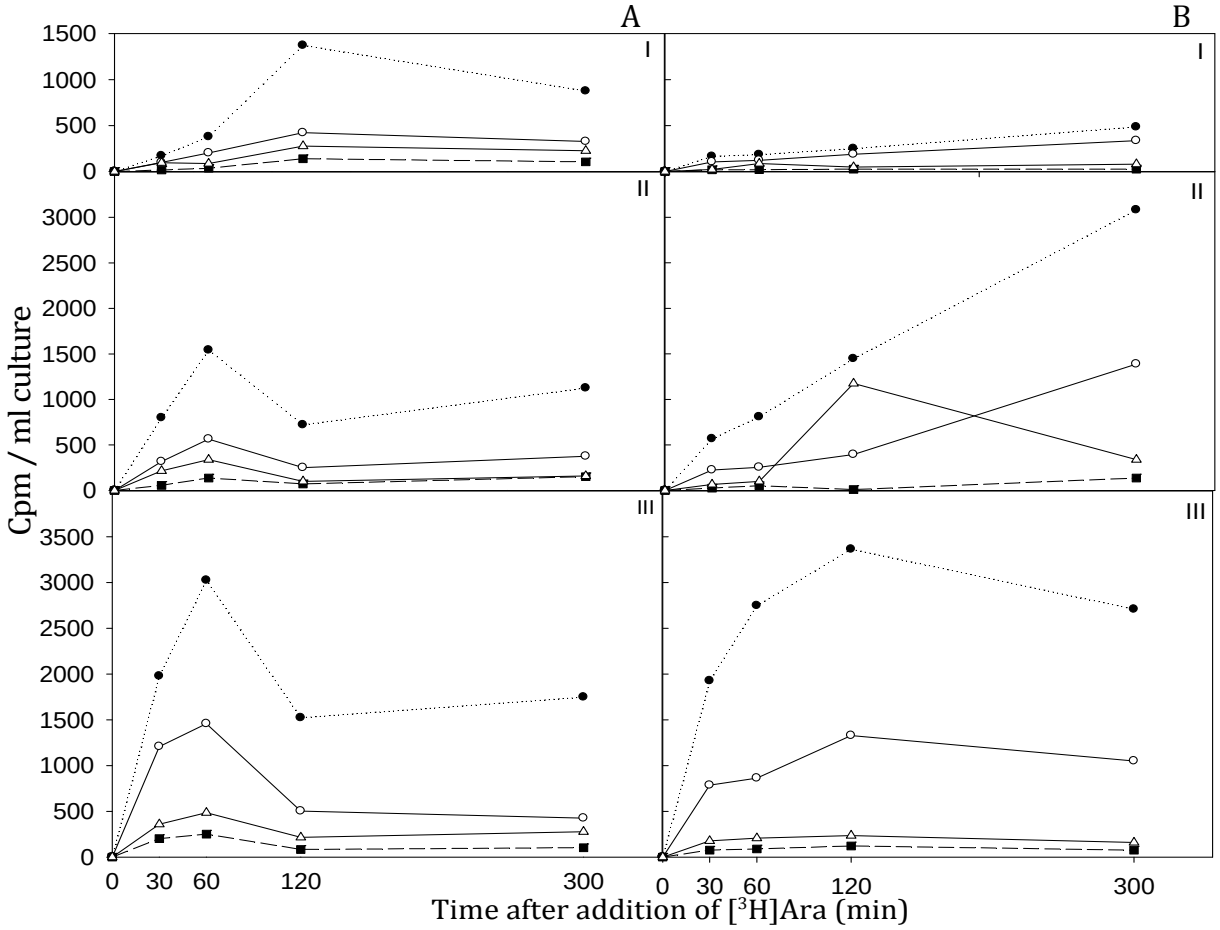


Figure 3. Pulse-chase kinetics of radiolabeling of polymers in the protoplasm after Driselase enzymatic digestion of **A**: Snh and **B**: Sh1.5 maize cell suspensions. Diagnostic ^{3}H-labelled fragments were those for: polymers which have Ara (●); xylans (○); xyloglucan (■) and undigested polymers (△). Samples were taken during 5 h at different labeling time points during lag (I), early logarithmic (II) and late logarithmic (III) phases of culture cycle. Results shown are representative of two independent experiments

During the lag phase, polymers which had [^{3}H]Ara, [^{3}H]xylans, [^{3}H]xyloglucan and the Driselase-resistant ^{3}H-polymers, either extracted with mild or strong alkali treatment, were gradually integrated into the cell walls of Snh and Sh1.5 cells (Figure 4AI, 4BI, 5AI and 5BI). At the early logarithmic phase, the cell lines started to show disparate trends in the incorporation of different types of ^{3}H-polymers and ^{3}H-hemicelluloses (Figure 4AII, 4BII, 5AII and 5BII), and this disparity was more marked at the late logarithmic culture phase

(Figure 4AIII, 4BIII, 5AIII and 5BIII). This incorporation of polymers extracted with either mild or strong alkali treatment occurred more rapidly within the first 120 min in the Snh cell walls. However, although polymers which had [^{3}H]Ara, [^{3}H]xylans, [^{3}H]xyloglucan as well as Driselase-resistant ^{3}H-polymers continued being integrated into the cell wall from 120 min to 300 min, this increase was not so pronounced as that observed during the first 120 min (Figure 4AIII and Figure 5AIII). In contrast, the incorporation of ^{3}H-polymers or ^{3}H-hemicelluloses into habituated cell walls mainly occurred from 120 min to 300 min (Figure 4BIII and Figure 5BIII), indicating a delayed integration when compared with Snh cells.

III.c.3. Extracellular culture medium

The kinetics of the appearance of polymers which had [^{3}H]Ara, [^{3}H]xylans, [^{3}H]xyloglucan and Driselase-resistant ^{3}H-polymers in the culture medium of both Snh and Sh1.5 cell lines were similar, showing gradual sloughing at the lag and early logarithmic phases of the culture cycle (Figures 6AI, 6AII, 6BI and 6II). At the late logarithmic phase, an increase in the sloughing of polymers which had [^{3}H]Ara, [^{3}H]xylans, [^{3}H]xyloglucan and Driselase-resistant ^{3}H-polymers into the culture medium was observed from 60 min to 120 min in both cell lines, indicating that their release into the culture medium took place later than their binding to the cell wall (Figures 6AIII and 6BIII). From 120 min to 300 min, Snh cells did not increase the amounts of polymers which had [^{3}H]Ara, [^{3}H]xylans, [^{3}H]xyloglucan and Driselase-resistant ^{3}H-polymers which were sloughed with respect to the value detected at 120 min, showing a constant value. In contrast, within this period of time, Sh1.5 cells continued releasing them exponentially until the end of the monitoring period.

III.d. Composition of the ^{3}H-polymers and ^{3}H-hemicelluloses

The composition of the ^{3}H-polymers and ^{3}H-hemicelluloses did not differ depending on the culture stage and cellular compartment assayed (Figures 3, 4, 5 and 6).

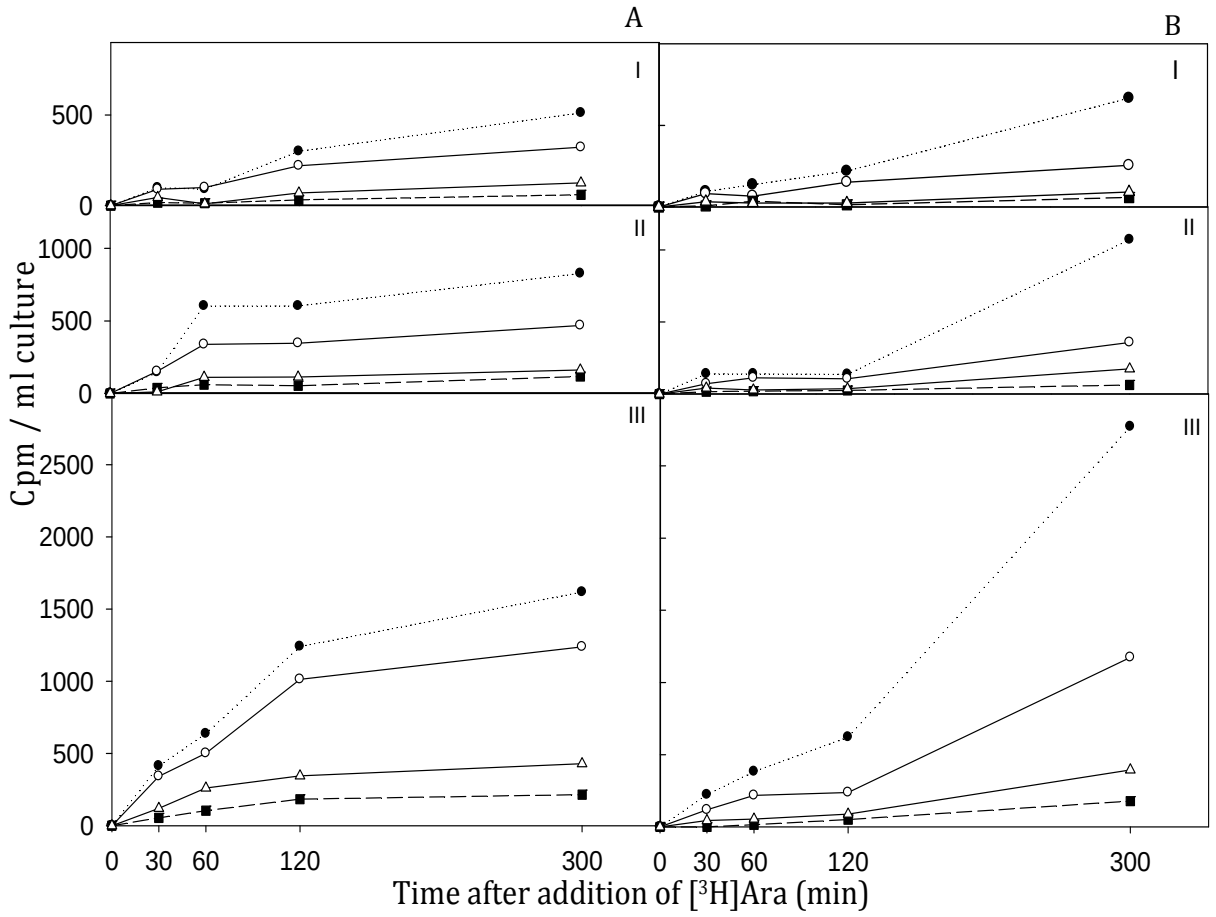
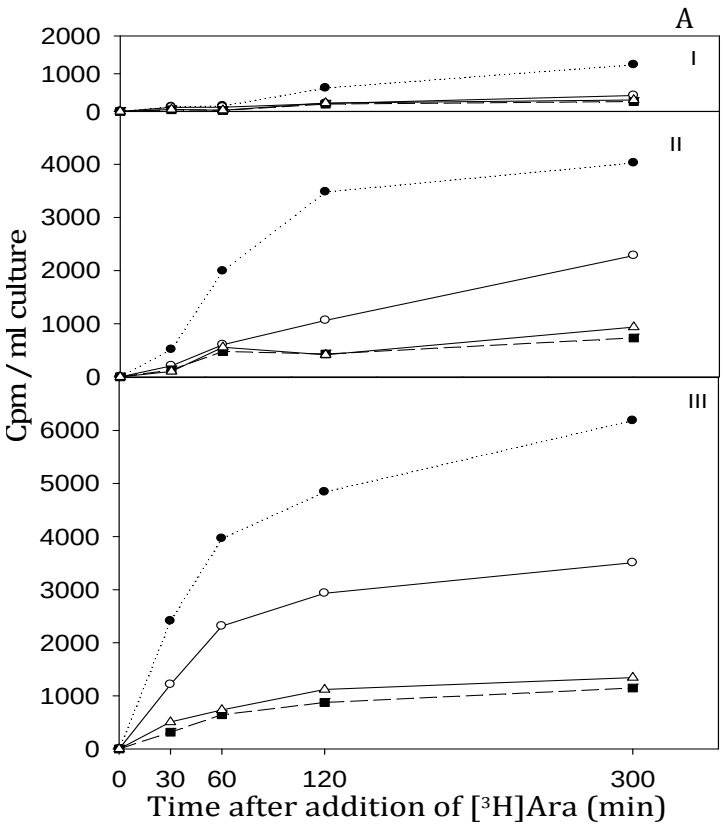


Figure 4. Pulse-chase kinetics of radiolabeling of hemicelluloses 0.1 M NaOH-extracted after Driselase enzymatic digestion of **A**: Snh and **B**: Sh1.5 maize cell suspensions. Diagnostic ^{3}H-labelled fragments were those for: polymers which have Ara (●); xylans (○); xyloglucan (■) and undigested polymers (△). Samples were taken during 5 h at different labeling time points during lag (I), early logarithmic (II) and late logarithmic (III) phases of culture cycle. Results shown are representative of two independent experiments

Both types of ^{3}H-hemicelluloses (those extracted with mild or strong alkali treatments) from Sh cells showed an increased Ara/xylan ratio when compared with those from Snh cells. However, this ratio was lower when considering to the ^{3}H-polymers in the protoplasm and [^{3}H]SEPs (Table 1).

Moreover, differences in the Ara/xylan ratios depending on the cell line were found when polysaccharides reached the cell wall as cellular fate. Likewise, ^{3}H-hemicelluloses extracted from the Snh cell wall showed lower ratios than those observed for the ^{3}H-polymers that were being synthesised in the protoplasm, regardless of the culture phase. In contrast, Sh1.5 cells showed higher ratios at the early logarithmic phase of the culture cycle, whereas a clear trend was not observed at late logarithmic phase (Table 1).



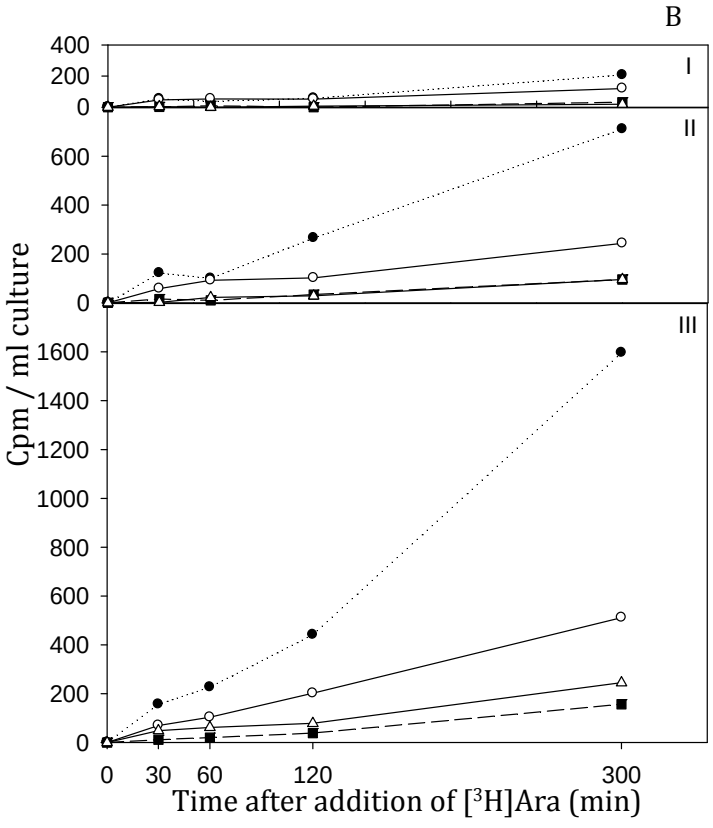


Figure 5. Pulse-chase kinetics of radiolabeling of hemicelluloses 6 M NaOH-extracted after Driselase enzymatic digestion of **A**: Snh and **B:** Sh1.5 maize cell suspensions. Diagnostic ^{3}H-labelled fragments were those for: polymers which have Ara (●); xylans (○); xyloglucan (■) and undigested polymers (△). Samples were taken during 5 h at different labeling time points during lag (I), early logarithmic (II) and late logarithmic (III) phases of culture cycle. Results shown are representative of two independent experiments

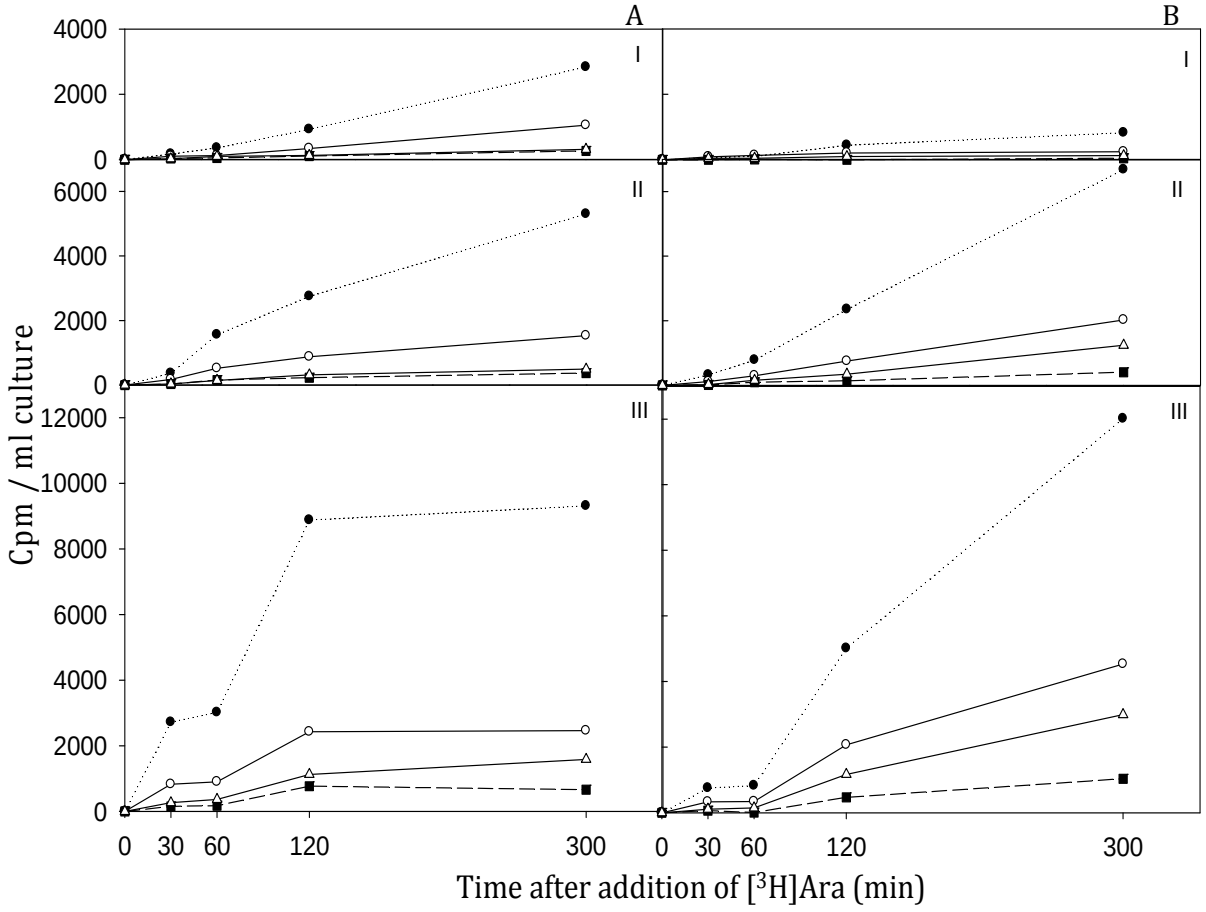


Figure 6. Pulse-chase kinetics of radiolabeling of SEPs after Driselase enzymatic digestion of **A**: Snh and **B**: Sh1.5 maize cell suspensions. Diagnostic ^{3}H-labelled fragments were those for: polymers which have Ara (●); xylans (○); xyloglucan (■) and undigested polymers (△). Samples were taken during 5 h at different labeling time points during lag (I), early logarithmic (II) and late logarithmic (III) phases of culture cycle. Results shown are representative of two independent experiments

Meanwhile, the xylan/xyloglucan ratio was particularly high in the protoplasmic ^{3}H-polymers from Sh1.5 cells, and decreased when calculated for any kind of ^{3}H-hemicellulose or for [^{3}H]SEPs. However, in general terms, this ratio was higher in Sh1.5 when compared with Snh cells (Table 1).

	Snh early logarithmic		Sh1.5 early logarithmic		Snh late logarithmic		Sh1.5 late logarithmic	
	Ara/Xylan	Xylan/XyG	Ara/Xylan	Xylan/XyG	Ara/Xylan	Xylan/XyG	Ara/Xylan	Xylan/XyG
Protoplasm	3.0	2.5	2.2	10.2	4.1	4.2	2.6	14.0
0.1 M NaOH	1.8	4.0	3.0	5.7	1.3	5.8	2.4	6.6
6 M NaOH	1.8	3.1	2.9	2.6	1.8	3.1	3.1	3.3
SEPs	3.5	4.1	3.3	5.0	3.8	3.7	2.7	4.4

Table 1. Ara/Xylan and Xylan/Xyloglucan (XyG) ratios from the different cell compartments [protoplasm, 0.1 M NaOH-extracted polymers (0.1 M NaOH), 6 M NaOH-extracted polymers (6 M NaOH) and soluble extracellular polymers (SEPs)] from Snh and Sh1.5 maize cell suspensions. Data shown are from the diagnostic ^{3}H-labelled fragments obtained after Driselase digestion of samples collected 300 min after [^{3}H]Ara was fed at early logarithmic and late logarithmic phases of culture cycle. Results shown are representative of two independent experiments

IV. DISCUSSION

Maize cell cultures habituated to different concentrations of DCB (Mélida et al., 2009; 2010a; 2010b and 2011, de Castro et al., 2013; Chapter I) are characterised by a reduction in their cellulose content, which is compensated for by a coping strategy involving an enhancement of their arabinoxylan content and/or network. However, the magnitude of this cellulose content reduction and their coping strategy depends on the DCB concentration as well as the length of time that cells are exposed to it (Alonso-Simón et al., 2004; Mélida et al., 2009). Several maize cell cultures habituated to different DCB levels have been obtained, and modifications in their walls have been characterised. Maize cells habituated to high DCB levels show a 75% of reduction in cellulose content, counteracted by a reinforced hemicellulosic network in their walls, achieved by a greater content of more cross-linked arabinoxylans with lower extractability and higher M_r and M_w. The content of other cell wall polysaccharides is not affected or even reduced (Mélida et al., 2009; 2010a; 2010b and 2011). On the other hand, maize cell suspensions habituated to low DCB levels (1.5 μM DCB) -which was the cell material studied for the present research- show a 33% reduction in their cellulose content, mainly compensated for by a slighter increase in arabinoxylan content,

molecules which are more easily extractable. In addition, other polysaccharides such as xyloglucan and rhamnogalacturonan I seem to be involved in this coping strategy (de Castro et al., 2013; Chapter I). The fact that DCB-habituated cells show plasticity in the metabolic machinery responsible for cell wall polysaccharide synthesis indicates that they constitute a good system for investigating the metabolism of heteroxylans, and their connection with the biosynthesis of other cell wall polymers.

It has been demonstrated that feeding maize cell suspensions with [^{3}H]Ara is not only a suitable but also a successful experimental methodology for tracking the *in vivo* careers of endogenous [^{3}H]xyloglucan and [^{3}H]xylan molecules in several cell pools (Kerr and Fry, 2003). Thus, in the present study, [^{3}H]Ara was added to Snh and to low level DCB-habituated cells (Sh1.5) during a set of labeling-time points at different stages of the culture cycle, and fractions from several cell compartments (protoplasmic, cell wall bound polymers extracted with 0.1 M or 6 M NaOH) as well as SEPs were obtained.

Snh and Sh1.5 cells showed differences in their capacity for [^{3}H]Ara uptake in all the culture stages analysed, with Sh1.5 cells being less efficient in all cases. For instance, at the late logarithmic phase of the culture cycle, Snh cells took up approximately 75% of exogenous [^{3}H]Ara within the first hour, which was in good agreement with data from previous studies on maize (Kerr and Fry, 2003) and rose (Edelmann and Fry, 1992; Thompson et al., 1997) cultured cells, whereas Sh1.5 cells only took up 37%. The [^{3}H]Ara uptake trends shown by Snh and Sh1.5 cells were linked to hemicellulose metabolism in both cell lines. DCB-habituated cells showed a slower and less efficient metabolism when compared with Snh or with other maize cultured cells (Kerr and Fry, 2003), and although this finding was consistent throughout all the culture phases of growth, it was especially obvious at the late logarithmic phase of the culture cycle. In order to determine whether this altered metabolism was affecting the synthesis and cellular traffic of a specific type of hemicellulose or polymer, samples were subjected to Driselase enzymatic digestion, releasing diagnostic fragments which were then separated by PC. Some of these diagnostic

fragments are exclusive of xylans ([^{3}H]xylobiose) and xyloglucan ([^{3}H]isoprimeverose), whereas others are not so highly specific and could be related to a wider range of molecules, such as Ara-containing polymers ([^{3}H]Ara) or those resistant to Driselase treatment (^{3}H-material at RF 0 on the paper chromatogram). The pulse-chase kinetics of ^{3}H-labeled diagnostic fragments mimicked the general kinetics of [^{3}H]Ara incorporation into ^{3}H-polymers and ^{3}H-hemicelluloses. This proved, firstly, the solidity of the procedure and secondly, that synthesis, grafting into the cell wall or sloughing into the culture medium of all types of polymers and hemicelluloses, [^{3}H]xylans, [^{3}H]xyloglucans, [^{3}H]Ara-containing polymers and Driselase-undigested ^{3}H-polymers, from Sh1.5 cells were delayed because of their reduced metabolic capacity. In addition, a delayed grafting of Sh1.5 arabinoxylans onto the cell wall or their sloughing into the culture medium, compared to xyloglucan molecules, could be taking place, as deduced from the reduction observed in the [^{3}H]xylan/[^{3}H]xyloglucan ratio of Sh cell wall bound fractions when compared with the protoplasm. The incorporation of [^{3}H] into [^{3}H]xylans and [^{3}H]xyloglucans occurred simultaneously. This has been observed previously in maize cultured cells, and it was theorised that both kinds of molecules probably drew from the same pool of [^{3}H]Xyl (Kerr and Fry, 2003). Likewise, the same assumption can be made about the incorporation of [^{3}H]Ara into polymers which had [^{3}H]Ara residues and the Driselase-resistant polymers. Thus, both kinds of ^{3}H-hemicelluloses ([^{3}H]xylans and [^{3}H]xyloglucan) and ^{3}H-polymers (those which contained [^{3}H]Ara and the undigested ones) left the protoplasm compartment, and were wall bound or sloughed simultaneously. However, variations in the timing of these processes were detected when Snh and Sh1.5 cells were compared, being delayed in Sh1.5 cells.

Another interesting observation concerned the hemicellulose composition of the cell wall fractions extracted with different alkali treatments, 0.1 M NaOH (able to cleave hydrogen and ester bonds) and 6 M NaOH (with sufficient strength to cleave ether-like bonds). As mentioned before, it has been demonstrated that DCB-habituated maize cells

reinforced their hemicellulose network in order to counteract cellulose deficit. The results obtained in previous studies and in the present one, reveal that arabinoxylans seem to be responsible for this effect since they were observed to have a higher radioactivity content than xyloglucan one. However, maize cultured cells have a lower xyloglucan than arabinoxylan content it cannot be ruled out that xyloglucan may play an important role, above all in cellulose-deficient walls. Thus, it could be hypothesised that Sh1.5 cells would preferentially attach [^{3}H]xyloglucan or [^{3}H]xylan in order to tightly or weakly integrate them into their walls as part of their strategy to cope with their reduced cellulose content. Our results did not show habituation-based differences in the composition of both fractions (0.1 M and 6 M NaOH-extracted), indicating instead the same kind of ^{3}H-hemicelluloses but linked by different bonds. However, results from pulse-chase kinetics and [^{3}H]xylan/[^{3}H]xyloglucan ratios showed that in both cell lines, xyloglucan molecules were preferentially extracted with 6 M NaOH, and therefore, were more strongly linked in the cell wall.

The next question to emerge was whether these ^{3}H-hemicelluloses or ^{3}H-polymers from Sh1.5 cells differed in any respect when compared to those from Snh. An increased [^{3}H]Ara/[^{3}H]xylan ratio was detected in Sh1.5 ^{3}H-hemicelluloses that might reflect the synthesis of more substituted arabinoxylans. However, taking into consideration that there are alternative sources for Ara residues besides arabinoxylans such as arabinogalactan proteins or rhamnogalacturonan I side chains (for a review see Fry, 2011), this result should be interpreted with caution. Nevertheless, the sugar analysis of 0.1 M and 6 M alkali-extracted fractions from Sh1.5 cell walls, revealed a poor content of uronic acids, Gal or Rha residues (de Castro et al., 2013; Chapter I). Therefore, it can be assumed that most of the Ara residues actually derived from arabinoxylan molecules.

When ^{3}H-polymers in synthesis left the protoplasm of DCB-habituated cells, variations in their fate were observed when compared to Snh. Results from the late logarithmic phase of the culture cycle provided a good example of this altered fate: a smaller amount of strongly-linked ^{3}H-hemicelluloses and ^{3}H-polymers firmly bound to the

α-cellulose residue were grafted into the Sh1.5 cell walls, and a relative increase in ^{3}H-hemicelluloses extracted with 0.1 M alkali treatment was detected. These findings led to the deduction that in Sh1.5 cells, strong-alkali or even alkali-resistant bonds did not play a crucial role in linking ^{3}H-hemicelluloses at any stage of the culture cycle, at least during the period of time (300 min) monitored. This is in contrast to the findings described for long term habituation of cells to high DCB levels (Mélida et al., 2009; 2011). In these studies it has been shown that these kinds of strong linkages seem to be of relevance. For instance, following a similar experimental approach to the one used in the present study, in Mélida et al. (2011) habituated cells to high DCB levels and non-habituated cells were pulse-chase fed with [^{14}C]Cinn and the appearance of radio-labelled compounds was then tracked through different cell pools. Their results showed that the proportion of radio-labelled compounds detected in both labile and stable alkali fractions was higher in the habituated cell line than in the non-habituated one. Another surprising finding related to the altered polymer and hemicellulose fates in habituated cells was the higher proportion of [^{3}H]SEPs sloughed into the culture medium when compared with Snh cells (56% *vs* 42.3% of the total radioactivity added). This result was even more interesting when percentages were compared with the proportion of cell wall-bound ^{3}H-hemicelluloses (56% *vs* 30% and 42.3% *vs* 55% for Sh1.5 and Snh cells respectively), mainly in the 6 M NaOH fraction, in which the amount of extracted molecules was much lower when compared with Snh cells. These results may indicate that this population of polysaccharides, otherwise extracted with 6 M NaOH, is sloughed into the culture medium. Polymers which are sloughed into the culture medium could represent newly synthesised polymers that pass quickly through the wall without being bound and/or undergoing any chemical change, or alternatively, wall-grafted molecules that are later loosened and therefore released into the culture medium (Kerr and Fry, 2003; Mélida et al., 2011). However, if the SEPs detected in the culture medium of Sh1.5 cells had their origin in previously wall-grafted and later loosened hemicelluloses one would be expect to find a decrease into the radioactivity incorporated into the cell wall bound

fractions concurrent with an increase of radioactivity in the culture medium, probably at the latest labeling time points monitored (120-300 min). Three possible explanations emerge: a) as Sh1.5 cells have a cellulose-impoverished wall, it is probable that newly synthesised ^{3}H-hemicelluloses pass through the wall without being bound due to the lack of linking points, b) the capacity of Sh1.5 cells to integrate [^{3}H]arabinoxylans by extraprotoplasmic phenolic cross-linking is reduced and in this case, a lower degree of arabinoxylan feruloylation, a reduced peroxidase activity or a limitation in the supply of H_2O_2 could arise as alternatives, and c) [^{3}H]arabinoxylans from the habituated cells are synthesised with a higher degree of substitution and consequently, with an increased solubility, which would reduce the possibility of establishing linkages with cellulose (as can be deduced from the [^{3}H]Ara/[^{3}H]xylan ratios when compared to protoplasm and culture medium fractions). It has previously been reported that in developing barley coleoptiles arabinoxylans were newly synthesised with 80% of their Xyl backbone substituted with Ara residues showing a substituted to unsubstituted 4-linked xylosyl unit ratio of 4:1 (Gibeaut et al., 2005), which decreased to 1:1 after 3 days, since hydrolytic enzymes remove these residues in grasses during growth (Fincher, 2009). Thus, it could be theorised that in Sh1.5 cells, these arabinosyl-hydrolytic activities were affected and therefore, [^{3}H]arabinoxylans had a greater degree of substitution and higher solubility. However, since (as previously mentioned) results for the [^{3}H]Ara/[^{3}H]xylan ratios should be interpreted with caution, it is more feasible to assume that the hypotheses described above in options a and b probably contributed to a greater or lesser extent to the altered fate observed in Sh1.5 cells. Nevertheless, further analyses are required in order to confirm this.

In sum, synthesis, grafting and sloughing of [^{3}H]xylans, [^{3}H]xyloglucans, [^{3}H]Ara-containing polymers as well as Driselase-digestion resistant ^{3}H-polymers have been tracked in three stages of culture cycle (lag, early and late logarithmic) and it was observed that DCB-habituated cells: 1) exhibited less efficiency in uptake of the radio-labelled substrate ([^{3}H]Ara), 2) presented a slower and less efficient metabolism in all the culture cycle points

analysed, and 3) showed a delayed cellular traffic of ^{3}H-hemicelluloses ([^{3}H]xylans and [^{3}H]xyloglucan) and ^{3}H-polymers ([^{3}H]Ara-contained and Driselase-digestion resistant), but this occurred simultaneously. 4) Compositional studies of hemicellulosic fractions extracted with mild or strong alkali treatments revealed that neither Snh nor Sh1.5 cells preferentially bound any kind of ^{3}H-hemicellulose (i.e [^{3}H]xyloglucan or [^{3}H]xylan) to the wall in terms of the strength of linkage. 5) Variations were found in the fate of ^{3}H-polymers from habituated cells when leaving the protoplasm, with a reduced amount of wall bound ^{3}H-hemicelluloses and an increased presence of [^{3}H]SEPs in the culture medium. The results indicate that Sh1.5 cells have a diminished capacity to graft hemicelluloses onto their walls, which could be consequence of their reduced cellulose content and/or impeded polysaccharide cross-linking.

Chapter III

Analysis of the molecular mass distribution of polysaccharides and the phenolic metabolism in maize cells with a moderate reduction in their cellulose content

ABSTRACT

As a consequence of the habituation to low levels of DCB, cultured maize cells presented an altered hemicellulose cell fate with a lower proportion of strongly wall-bound hemicelluloses and an increase in soluble extracellular polymers released into the culture medium. These findings could be related to a reduction in sites linking polysaccharides to cellulose and/or a diminished capacity to incorporate polysaccharides into the cell wall by extra-protoplasmic phenolic cross-linking. As maize cells habituated to high DCB levels have been reported to have more cross-linked arabinoxylans with lower extractability and higher M_r, the aim of this study was to investigate the M_r distribution of polysaccharides as well as phenolic metabolism in cells habituated to low levels of DCB throughout the culture cycle. Generally, cell wall bound hemicelluloses and sloughed polymers from habituated cells were more homogeneously sized and had a lower M_w. In addition, polysaccharides underwent massive cross-linking after being secreted into the cell wall, which was less pronounced in habituated cells than in non-habituated ones. These findings support the hypothesis of a reduced cross-linking capacity in habituated cells for oxidative coupling of hydroxycinnamate residues of arabinoxylan molecules. However, when relativised, Fer and *p*-Cou contents was higher in this cell line, which partially rules out this idea. Feasibly, cells habituated to low levels of DCB synthesised molecules with a lower M_w, although cross-linked, as a part of their strategy to compensate for the lack of cellulose. In addition, a possible role arises for xyloglucan in maize cells habituated to low levels of DCB. This variety of coping mechanism indicates the remarkable plasticity of DCB-habituated maize cells, which appear to adopt the appropriate strategy depending on the DCB habituation framework.

I. INTRODUCTION

Heteroxylans are the main non-cellulosic polysaccharides in the Poales primary cell wall (for general composition and structure see general introduction section) (Fincher, 2009), playing a major structural role in tethering cellulose microfibrils; hence, they are involved in cell expansion and plant growth. Heteroxylans show greater structural diversity and complexity than other types of cell wall components, since a wide range of different side-chain substitutions are found connected to the backbone (Faik, 2010). A set of genes has been described related to heteroxylan synthesis in grasses, orthologues of *IRX* from arabidopsis. Likewise, three orthologous of the arabidopsis genes *IRX9*, *IRX9L* and *IRX14* encoding putative GTs responsible for xylan backbone elongation have been reported in rice (Chiniquy et al., 2013). In wheat, the coordinated action has been described of a protein complex including a xylosyltransferase, a glucuronyltransferase and an arabinosyltransferase involved in glucuronoarabinoxylan synthesis (Zeng et al., 2010). Specifically in the case of maize, sequences have also been reported for some arabidopsis orthologues such as *IRX9*, *IRX10* and *IRX10L* (Bosch et al., 2011).

One of the unique structural features of arabinoxylan in Poales is that it contains hydroxycinnamate residues, such as Fer or *p*-Cou, esterified on the C-5 position of Ara residues (Bacic et al., 1988; Wende and Fry, 1997). The attachment of Fer residues occurs within the protoplasm, before secretion into the cell wall (Fry et al., 2000). Fer may undergo oxidative coupling when exposed to hydrogen peroxide plus peroxidase (Geissmann and Neukom, 1971). Dimerisation of feruloyl polysaccharides may occur in the protoplasm before polysaccharides are secreted into the cell wall, in the cell wall just after secretion, or later in the cell wall after binding to the wall (Fry et al., 2000; Mastrangelo et al., 2009). Larger coupling products, such as trimers or oligomers, are also formed and have been described to predominate over diferulates (Fry et al., 2000). Phenolic residues are also attached to polysaccharides as ether-like (alkali-stable) bonds (Burr and Fry, 2009). Since they are thought to be involved in the heteroxylan cross-linking, they are essential for

important biological processes such as wall assembly and restriction of cell expansion (Fry, 2004; Parker et al., 2005).

One constraint industries based on the use of grass-derived products is cell wall digestibility, which is highly related to heteroxylan content, structure and interaction with the components of the remaining cell wall forming networks. Despite notable advances in our knowledge about heteroxylan synthesis and assembly in recent years, some aspects still remain unclear. Elucidation of its metabolism is particularly challenging, and also has important economic implications. In line with this, the use of cell cultures habituated to cellulose biosynthesis inhibitors (for a review see Acebes et al., 2010) such as DCB has been demonstrated to be a valuable tool to gain insight into the mechanism responsible for the metabolic and structural plasticity exhibited by plant cell walls.

DCB habituation is a dynamic process based on the capacity of cultured cells to implement coping strategies in order to survive with a cellulose-deficient wall. In the case of maize suspension-cultured cells, a quantitatively and qualitatively a re-structured and modified arabinoxylan network is involved in many of these strategies (Mélida et al., 2009). In addition, the features and processes involved of the coping strategies exhibited in these cultured cells depend on DCB habituation level, which is highly related to the magnitude of the reduction in cellulose content (de Castro et al., 2013; see Chapter I). Hydroxycinnamates seems to play a crucial role in strengthening cellulose-deficient cell walls in maize cells habituated to high DCB levels, as they are involved in polysaccharide cross-linking (Mélida et al., 2010a; 2010b; 2011). Hemicellulose metabolism is also modified as a consequence of DCB habituation. In a previous study, maize cell suspension cultures habituated to low levels of DCB (Sh1.5; for a complete characterization of the cell line see de Castro et al., 2013; Chapter I) were fed with [^{3}H]Ara (Chapter II).

Cells suspension cultures present several advantages in *in vivo* metabolic studies performed using an isotopic approach, such as homogeneity of cell material, a lack of nutrients, O_2 or exogenous precursor gradients in the extracellular culture medium and ease

of sampling (Burr and Fry, 2009). In addition, the consistent results obtained in this study once again confirmed the suitability of radio-labelled experimental approaches based on feeding live plant cultured cell suspensions in order to track the *in vivo* fate of endogenous polysaccharides (for more details, precedents and references see Chapter II). The feeding experiments demonstrated that these cells had a less efficient and slower metabolism in all phases of the culture cycle, as well as an altered cellular fate of polysaccharides, with a lower amount of strongly wall bound ^{3}H-hemicelluloses and an increased presence of [^{3}H]SEPs. One possible explanation for this observation is that as Sh1.5 cells exhibited a reduced cellulose content, which would lead to a decrease in "binding points" for hemicelluloses. Nevertheless, another possibility also emerged, namely that Sh1.5 cells would have a diminished capacity to incorporate polysaccharides via phenolic cross-linking into the cell wall. This latter alternative is particularly interesting since an increased cross-linking of arabinoxylans leading to higher M_w of these molecules has been shown to be crucial in the habituation of cells to high levels of DCB (Mélida et al., 2009; 2011).

Thus, an extensive analysis was performed in the present study of the M_r distribution of hemicelluloses and polymers as well as the phenolic metabolism in several cell compartments of Snh and mildly cellulose-deficient maize cell suspension cultures (Sh1.5) at the early and late logarithmic phases of the culture cycle. To this end, a pulse-chase radio-labelling experimental approach was employed, which consisted of feeding both cell lines with the corresponding radio-labelled metabolic precursors ([^{3}H]Ara and [^{14}C]Cinn respectively). In addition, a preliminary study was carried out of *IRX9*, *IRX10* and *IRX10L* arabidopsis orthologue gene expression in these cell lines. The results obtained in this study contribute to enhancing our understanding of hemicellulose metabolism, providing new clues which help clarify the mechanisms involved in the structural adaptability shown by primary cell walls when exposed to environmental constraints.

II. MATERIALS AND METHODS

II.a. Cell cultures and habituation to DCB

Maize cell-suspension cultures (*Zea mays* L., Black Mexican sweet corn) from immature embryos were grown in Murashige and Skoog media (Murashige and Skoog, 1962) supplemented with 9 µM 2,4-D and 20 g L^{-1} sucrose, at 25°C under light and rotary shaken, and routinely subcultured every 15 days.

In order to obtain cell cultures habituated to DCB, Snh were stepwise subcultured in a medium supplied with increasing concentrations of DCB, dissolved in DMSO which does not affect cell growth at this range of concentrations. Likewise, in the case of cell suspensions habituated to low DCB levels, Snh cells were transferred to a medium containing 1 µM DCB, increasing the DCB concentration up to 1.5 µM DCB (Sh1.5) after seven subcultures. Cell suspensions habituated to high DCB levels were derived from maize callus cultures habituated to grow in 12 µM DCB which were transferred into liquid medium supplemented with 6 µM DCB (Sh6). Growth curves of Snh and Sh1.5 maize cell lines were obtained by measuring the increase in DW at different culture times and it was observed that Sh1.5 cells presented longer culture phases than Snh cells (de Castro et al., 2013; Chapter I, Figure 6A). Therefore, these growth curves were subsequently used to select the most appropriate days for sampling in order to ensure that Snh and Sh1.5 cell lines were at the same stage of the culture cycle.

II.b. Analysis of M_r distributions

II.b.1. Radio-labelling of liquid cultures with [^{3}H]Ara

Thirteen-ml aliquots from Snh and Sh maize cells cultures were collected at logarithmic and early stationary growth phases and each 13-ml aliquot was independently fed with 1 MBq of L-[1-^{3}H]Ara (148 GBq/mmol; Amersham International, Bucks., U.K). The days on which the 13-ml aliquot of culture was sampled and [^{3}H]Ara was added, were

adjusted depending on the cell line at early and late logarithmic phases. Thus, for the early logarithmic phase, the 4th and 8th days of culture were chosen for Snh and Sh cell lines, respectively (see de Castro et al., 2013; Chapter I). In the case of late logarithmic phase, the 8th and 12th were selected for Snh and Sh cell lines, respectively. In order to obtain an optimal quantity of radio-labelled ^{3}H-hemicelluloses and ^{3}H-polymers, samples for analysis of the M_r were collected at different labelling-time points selected according to the maximum value of incorporated [^{3}H]Ara observed in the kinetics previously described (Chapter II). Thus, in the case of protoplasmic polymers, 1-ml samples were collected from independent 13-ml aliquots of culture 60 min and 30 min after feeding [^{3}H]Ara for Snh and Sh cells, respectively. For the remaining ^{3}H-hemicelluloses and ^{3}H-polymers, sampling was carried out 300 min after feeding [^{3}H]Ara for both cell lines.

II.b.2. Obtaining ^{3}H-hemicelluloses and ^{3}H-polymers from discrete maize cell suspensions compartments

Each 1-ml sample collected at the different labelling-time points was filtered through an empty Poly-Prep column. Then, cells were rinsed with 5 ml of ice-cold fresh medium. The filtrate was pooled with the rinses and labelled as CFM.

The washed cells retained in the Poly-Prep column filter were quickly resuspended in 1 ml of homogenisation buffer [50 mM collidine acetate (pH 7.0) containing 2% (w/v) LiDS, 10% (v/v) glycerol, 5 mM sodium thiosulphate and 10 mM DTT (added freshly)] and stored at -20°C in the Poly-Prep column.

The cell suspension was then thawed at 1.5°C and transferred to the 10-ml glass mortar of a Potter–Elvehjem homogeniser by rinsing with 1 ml of chilled homogenisation buffer (x3), and then homogenised with a motor-driven Teflon pestle for 10 min. The homogenate was passed through a second empty Poly-Prep column and the filtrate collected and the cell fragments were rinsed with 1 ml H_2O (x4).

Solid KCl was added to the filtrate and rinses to a final concentration of 0.67 M in order to precipitate the dodecyl sulphate as insoluble K^+ salt. After 30 min at 4°C, the products were centrifuged at 3000 rpm for 5 min. The supernatant was removed and re-centrifuged and this procedure was repeated until the supernatant turned clear, moment in which was collected and considered the protoplasmic fraction.

Cell fragments retained in the filter of the Poly-Prep column were treated with 1 ml of 0.1 M NaOH containing 1% (w/v) $NaBH_4$ for 24 h at room temperature. The filtrate was then collected and the residue washed with 1 ml of the same extractant (x3). Filtrate and washes were pooled and labelled as 0.1 M NaOH fraction. The remaining residue was then treated with 6 M NaOH containing 1% (w/v) $NaBH_4$ for 24 h at 37°C. The filtrate was again collected and the residue washed with 1 ml of the same solution (x3). Filtrate and washes were then pooled and labelled as 6 M NaOH fraction. Both the 0.1 M NaOH and 6 M NaOH fractions were acidified to pH 4.7 by the addition of acetic acid.

II.b.3. GPC

Samples of CFM, protoplasmic and cell wall fractions were dialysed against distillated water with 0.1% (w/v) chlorobutanol at 4°C for 72 h. Then, the dialysed samples were centrifuged for 15 min at 3000 rpm in order to separate the soluble from the insoluble material in water.

To identify the void volume (V0) and totally included volume (Vi), 6 mg dextran (5–40 MDa) and 0.8 mg Glc, respectively, were added to 4 ml of this ^3H-polymer solution as markers. The polymers were then size-fractionated on Sepharose CL-4B (72 ml bed volume in a 1.5-cm-diameter column) in pyridine/acetic acid/water (1/1/23 by vol. pH 4.7 containing 0.5% chlorobutanol) at 12.5 ml/h. Fractions were assayed for total ^3H and the markers were quantified by the anthrone assay (Dische, 1962). Estimated recovery of ^3H-hemicelluloses from the Sepharose columns was routinely >80% (Kerr and Fry, 2003).

The Sepharose CL-4B column was calibrated with commercial dextrans of known M_w. Each dextran preparation was run as a mixture with a trace of very high-M_w ^{3}H-hemicellulose from maize culture medium and [^{14}C]Glc as internal markers, which defined the V0 and Vi (Kav 0 and 1) respectively. M_w was obtained using the $Kav_{(1/2)}$ method (Kerr and Fry, 2003) with the calibration curve [$\log M_w = -3.547\ Kav_{(1/2)} + 7.2048$] obtained for this column. The M_w estimates were nominal rather than absolute due to the conformational differences between dextran and hemicelluloses.

II.c. Characterisation of phenolic metabolism

II.c.1. [^{14}C]Cinn synthesis

[^{14}C]Cinn was prepared from L-[U-^{14}C]phenylalanine (Perkin-Elmer; 487 Ci/mol) following the method described by Lindsay and Fry (2008) with minor modifications.

II.c.2. [^{14}C]Cinn consumption and incorporation into different cell compartments

Fifteen-ml aliquots of Snh and Sh cell suspensions (20% of settled cell volume) at early and late logarithmic phases of the culture cycle were transferred into 100-ml glass flasks, loosely capped and shaken (150 rpm) for 1 h at 25°C, in order to adapt them to the new environmental conditions. Then, [^{14}C]Cinn (0.29 µCi) was added to each cell culture. At selected time-points, 400 µl of cell cultures was sampled and acidified by the addition of 99% formic acid, stopping the consumption and incorporation of [^{14}C]Cinn.

Each aliquot sampled at the different labelling-time points was then centrifuged for 4 min at 10000 rpm and the resultant supernatant was collected. Cells were washed with 200 µl of distilled water and subjected again to centrifugation. Supernatant and washings were then pooled, labelled and stored as CFM.

Remaining cells were then incubated for 18 h with 800 µl of 80% ethanol on a rotary wheel, the samples were subsequently centrifuged at 10000 rpm for 4 min and the

supernatant was collected. Cells were rinsed with 200 µl of 80% ethanol and centrifuged again. Supernatant and washings were then pooled and labelled as the protoplasmic-like fraction (alcohol soluble material). The residual cell fragments were considered AIR.

II.c.3. Obtaining of Cinn [^{14}C]derivatives from discrete maize cell compartments

Four hundred µl-aliquots of Snh and Sh cell-suspensions at early and late logarithmic phases of the culture cycle were transferred into flat-bottomed vials and shaken (75 rpm) for 1 h at 25°C to allow them to acclimatise to their new environmental conditions. Then, vials were fed with [^{14}C]Cinn (0.116 µCi). At selected time-points, aliquots were acidified by the addition of 99% formic acid, stopping [^{14}C]Cinn incorporation. Where indicated, 40 mM H_2O_2 (final concentration) was added to some of the samples 120 min after [^{14}C]Cinn and then shaken for 60 min. Aliquots of cell suspensions contained in the vials were then transferred into Poly-Preps columns and the filtrate was collected and considered the CFM fraction.

The remaining cells were resuspended in 1 ml 80% ethanol and incubated for 18 h on a rotary wheel. Cell suspensions were filtered and the alcohol-soluble fraction was considered the protoplasmic-like fraction. The AIR retained on the Poly-Prep column was then de-esterified by saponification with 0.5 M NaOH for 18 h at 25°C. The filtrate was collected and the remaining cell fragments were rinsed with 1 ml of distilled water. Filtrate and washings were then pooled and collected as the 0.5 M NaOH fraction. This fraction was acidified by addition of acetic acid and partitioned against ethyl acetate (x2). The ethyl acetate phases were vacuum-dried and re-dissolved in 0.5 ml of acidified water (0.01 N HCl). These later samples were again subjected to a second ethyl acetate partition (x3) and the organic phases were collected, vacuum-dried and re-dissolved in propan-1-ol.

II.d. Assay of radioactivity

II.d.1. Analysis of M_r distribution

To obtain the general elution profiles of ^{3}H-hemicelluloses and ^{3}H-polymers, a 200 µl aliquot of column fractions was assayed for radioactivity by liquid scintillation counting in 2 ml of OptiPhase HiSafe (Fisher).

In order to obtain the elution profiles corresponding to ^{3}H-labelled diagnostic fragments, fractions obtained from the column were pooled in pairs and vacuum-dried. Then, dried samples were subjected to a mild-hydrolysis by re-dissolving them in 500 µl of 0.1 M TFA, heated at 85°C for 1 h to cleave arabinofuranosyl linkages, and finally re-dried in vacuo to remove TFA. The dried material was re-dissolved in 20 µl 0.5% (w/v) Driselase (partially purified by the method reported by Fry, 2000) in pyridine/acetic acid/water (1/1/23 by vol. pH 4.7 containing 0.5% chlorobutanol), and incubated at 37°C for 96 h. The reaction was stopped by the addition of 15% formic acid. The mild acid pre-treatment greatly increased the yield of Xyl and xylobiose generated during subsequent digestion with Driselase and did not decrease the yield of isoprimeverose (Kerr and Fry, 2003). An internal marker mixture containing Xyl, Ara, Glc, isoprimeverose and xylobiose (≈50 µg each) was then added to the digest, which was subjected to PC on Whatman 3MM in ethyl acetate/pyridine/water (9/3/2 by vol.) for 18 h (Thompson and Fry, 1997). The internal markers were slightly stained with aniline hydrogen-phthalate and the identified spots were cut out and soaked overnight in 1 ml water [containing 0.5% (w/v) chlorobutanol]. Then, 10 ml of OptiPhase HiSafe was added, and vials were shaken continuously for 24 h. Radioactivity was subsequently assayed by counting.

II.d.2. [^{14}C]Cinn consumption and incorporation into different cell compartments

To analyse CFM samples and the protoplasmic-like fraction, 5 and 3 ml of scintillation liquid EcoscintA (National Diagnostics) was added to the sample respectively, and radioactivity was then assayed by counting.

In the case of the radioactivity incorporated into the AIR, cell fragments were firstly resuspended in 0.2 ml of distilled water; 3 ml of scintillation liquid EcoscintA was subsequently added, and the radioactivity was assayed by counting.

II.d.3. Obtaining Cinn [^{14}C]derivatives from discrete maize cell compartments

Samples of the 0.5 M NaOH cell wall fraction were subjected to TLC. TLC was carried out on plastic-backed silica-gel with a fluorescent indicator (Merck) in benzene/acetic acid (9/1) (v/v). During development, TLC plates were exposed to 312 nm, which maintains hydroxycinnamates as rapidly interconverting single spots of *cis/trans* isomers. Standars of Fer, *p*-Cou and 5-5´-Diferulate were used as external markers.

Tracks corresponding to different samples were cut off in pieces of 0.5x1.0 cm or 1.0x1.0 cm from the TLC and analysed by scintillation counting through the addition of 3 ml of scintillation liquid EcoscintA.

II.e. Arabidopsis *IRX* homologue gene expression analysis

Total RNA was extracted with Trizol Reagent (Invitrogen) and reverse-transcribed using the Superscript III first strand synthesis system for RT-PCR (Invitrogen). First-strand cDNA was generated with an oligo(dT)20 primer and used as a template in subsequent PCR reactions. For each assay, several cycles were tested to ensure that amplification was within the exponential range. Ubiquitin was used as housekeeping gene.

Primers were used for the analysis of maize genes *GRMZM2G100143*, *GRMZM2G059825* and *gbIBT036881.1*, homologues of arabidopsis *IRX10* (*AT1G27440*),

IRX10-L (*AT5G61840*) and *IRX9* (*AT2G37090*), respectively (Bosch et al., 2011). Sequences for *GRMZM2G100143* and *GRMZM2G059825* were derived from the maize genome browser and that for *gbIBT036881.1* from the Phytozome.

Maize gene ID (arabidopsis homologous)	Forward primer	Reverse primer
GRMZM2G100143 (IRX10)	GATGCAGGCTCACCTTATCC	CGCTGCATGTCCTCGTAGTA
GRMZM2G059825 (IRX10-L)	ACGTTGGTTCAGACCTTTGG	TCATTGCCGGTGTCATAGAA
gbIBT036881.1 (IRX9)	TGAACTCGAGAATGCTGTGG	GCTCAATTACCCACCCTTGA

III. RESULTS

III.a. M_w and M_r distribution of ^{3}H-polysaccharides

In general, Sh1.5 cells presented more homogeneously sized populations than those from Snh, most of them eluting at an intermediate Kav and therefore having a lower M_w. In addition, the proportion of ^{3}H-polymers and ^{3}H-hemicelluloses that were eluted in the V0 was lower in Sh1.5 cells (Figure 1). ^{3}H-polysaccharides from Sh1.5 cells had a lower M_w than those from Snh in all cell compartments and culture phases analysed, with the sole exception of those observed within the protoplasm at late logarithmic phase (Figure 2).

Protoplasmic ^{3}H-polymers were consisted of by a highly poly-disperse population of molecules, which in both cell lines showed a lower M_w when compared with the cell wall bound ^{3}H-hemicelluloses or [^{3}H]SEPs. This result indicates that they underwent massive cross-linking once incorporated into the cell wall or sloughed. In line with this, when both cell lines were compared, Snh cells showed a much more pronounced degree of crosslinking than Sh1.5 cells, independently of the culture phase considered (Figure 2).

The general elution profiles of [^{3}H]SEPs showed that those from Sh1.5 cells were almost identical in both culture phases, whereas in Snh cells a greater proportion of them eluted in the V0 at the early logarithmic phase and a lower Kav was detected (Figure 1). This observation was confirmed by the M_w data, in which the M_w of [^{3}H]SEPs did not vary

depending on the culture phase in Sh1.5 cells, whereas it decreased in the Snh cell line at the late logarithmic phase (Figure 2). In addition, [^{3}H]SEPs from Sh1.5 cells had a lower M_w than Snh ones in both culture phases.

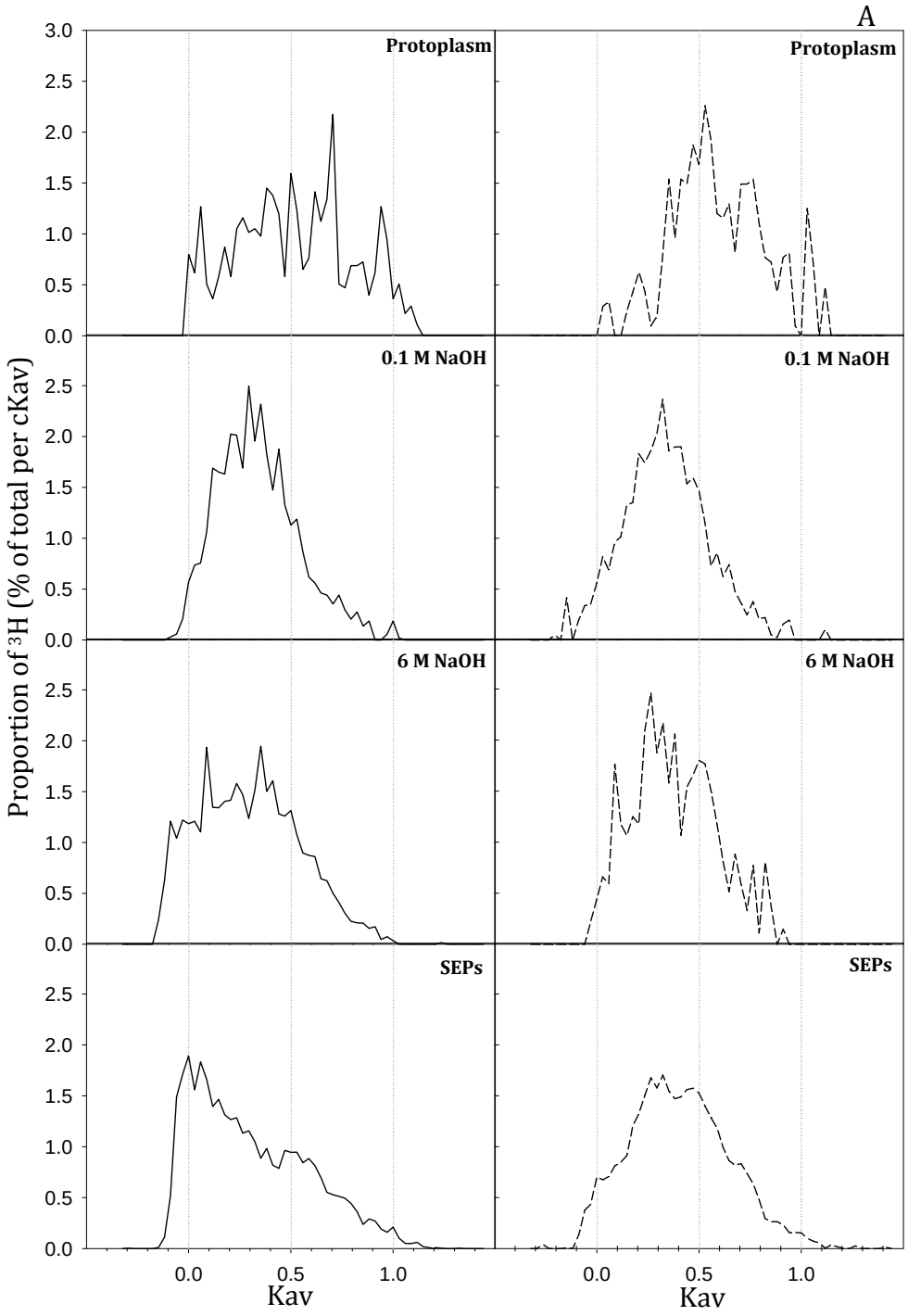
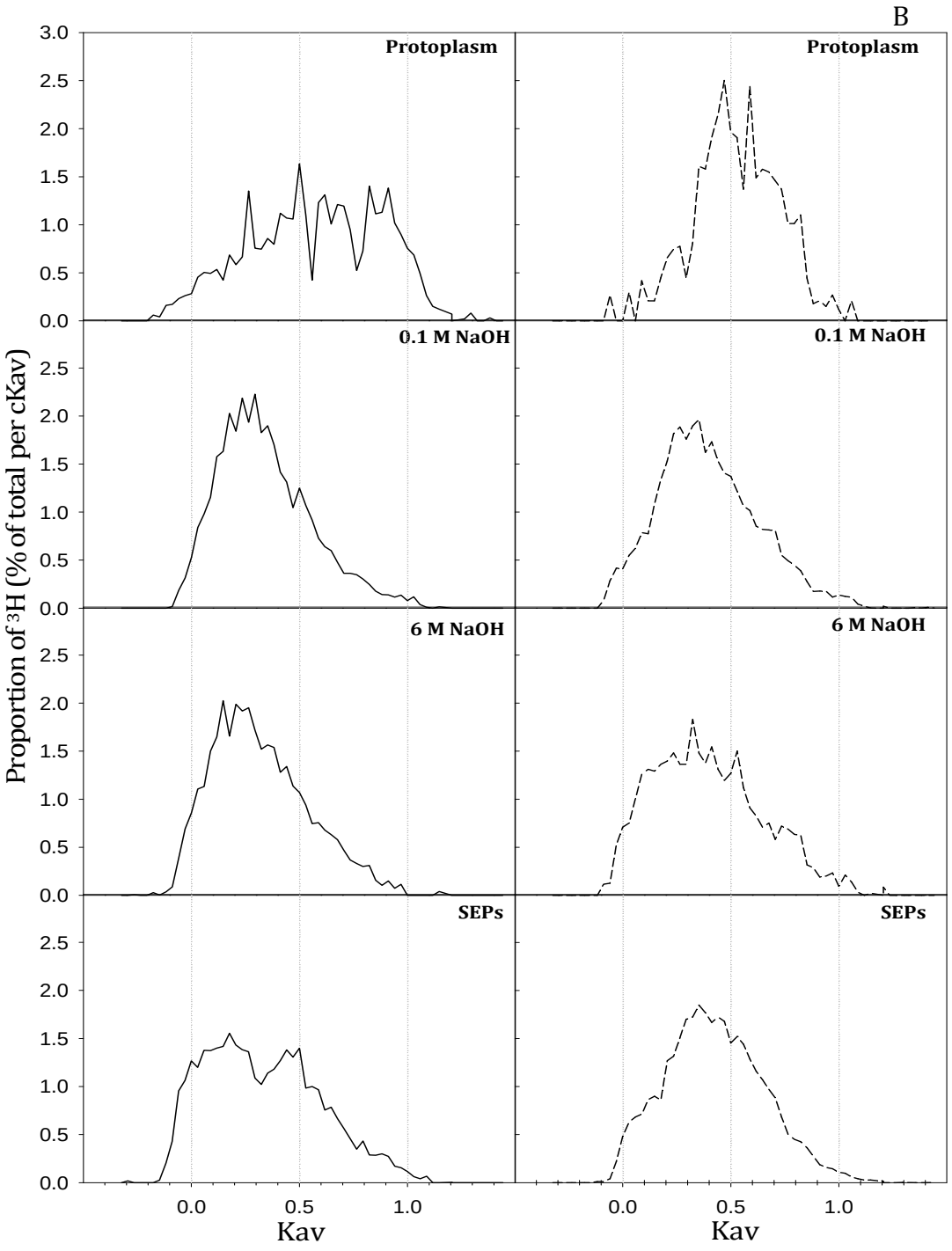


Figure 1. Molecular relative weight (M_r) distribution profile of ^{3}H-polysaccharides: protoplasmic ^{3}H-polymers, cell wall bound ^{3}H-hemicelluloses extracted with 0.1 M or 6 M NaOH and SEPs obtained from *solid line*; Snh and *dashed line*; Sh1.5 maize cell cultures. Data shown are from the **A:** early logarithmic and **B:** late logarithmic (see next page) phases of culture cycle. Samples used were taken 300 min after feeding of [^{3}H]Ara, except in the case of protoplasmic ^{3}H-polymers, collected 30 and 60 min after feeding of [^{3}H]Ara for Snh and Sh1.5 respectively. ^{3}H-polymers and ^{3}H-hemicelluloses were size-fractionated on Sepharose CL-4B. One cKav is defined as an interval of 0.01 on the *x*-axis. The profiles are scaled such that the area under the curve is equal (100%) in each frame



Both types of ^{3}H-hemicellulose (0.1 M or 6 M NaOH-extracted) showed a lower M_w in Sh1.5 cells (Figure 2). In Snh cells, the M_w of 0.1 M NaOH-extracted wall bound ^{3}H-hemicelluloses did not vary according to the culture phase whereas it appeared to decrease

at the late logarithmic in Sh1.5 cells (Figure 2). Likewise, the general elution profile for this type of ^{3}H-hemicellulose was very similar in the case of Snh cells in both culture phases, whereas Sh1.5 cells showed a slightly greater proportion of ^{3}H-molecules eluting in a higher Kav at the late logarithmic phase (Figure 1). In the case of 6 M NaOH-extracted ^{3}H-hemicelluloses, their M_w barely increased in Snh cells, whereas in Sh1.5 cells it once again decreased (Figure 2). These findings were repeated in the general elution profiles, where an increase in the proportion of ^{3}H detected at a lower Kav was observed at the late logarithmic phase in Snh cells, whereas it decreased in Sh1.5 cells (Figure 1).

III.b. M_r distribution of ^{3}H-labelled diagnostic fragments from ^{3}H-polysaccharides

After Driselase digestion, the elution profile shapes of polymers containing [^{3}H]Ara, [^{3}H]Xyl or [^{3}H]xylobiose were similar to the general ones, indicating the solidity of the experimental approach (Figures 3, 4 and 5 *vs* Figure 1). In general terms, elution profiles of polymers containing [^{3}H]Ara, [^{3}H]Xyl or [^{3}H]xylobiose showed similar M_r distributions. This finding suggests that these diagnostic fragments were a digestion product of a unique type of polysaccharide, putatively [^{3}H]arabinoxylan. Reinforcing this idea was the disparity observed between these, and fragments from polymers which contained [^{3}H]isoprimeverose, a diagnostic fragment for [^{3}H]xyloglucan. Undigested ^{3}H-polymers were found in all digested fractions, most of them eluting in a Kav ranging from 0 to 0.5 (Figures 3, 4 and 5).

III.b.1. M_r distribution of 0.1 M NaOH-extracted hemicelluloses diagnostic fragments

The elution profiles of polymers which contained [^{3}H]Ara residues and those characteristic for [^{3}H]xylans ([^{3}H]Xyl and [^{3}H]xylobiose) were very similar in both cell lines and culture stages. However, the elution profiles of [^{3}H]isoprimeverose ([^{3}H]xyloglucan) differed from those characteristic of [^{3}H]xylans and [^{3}H]Ara-containing polymers (Figure 3). M_r distributions of [^{3}H]Ara-containing polymers and ^{3}H-labeled diagnostic fragments for

xylans in both culture phases, and those for undigested ^{3}H-polymers at the early logarithmic phase, were very similar to the general elution profile shown in Figure 1.

M_r distributions of [^{3}H]Ara residues, [^{3}H]Xyl and [^{3}H]xylobiose from Snh cells did not vary substantially according to culture phase. In the case of Sh1.5 cells, the elution profiles for these diagnostic fragments as well as [^{3}H]isoprimeverose for [^{3}H]xyloglucan showed a slightly greater proportion of molecules with a low M_w (eluting Kav ranging from 0.5 to 1) at the late logarithmic phase of growth. These observations are in agreement with the results of the estimated M_w shown in Figure 2.

III.b.2. M_r distribution of 6 M NaOH-extracted hemicelluloses diagnostic fragments

In Snh cells, the elution profiles of polymers containing [^{3}H]Ara residues and those for [^{3}H]xylans ([^{3}H]Xyl and [^{3}H]xylobiose) and [^{3}H]xyloglucan ([^{3}H]isoprimeverose) were very similar in both culture stages (Figure 4). In contrast, the elution profile of [^{3}H]xyloglucan from Sh1.5 cells was different from that characteristic for [^{3}H]xylans. The undigested ^{3}H-polymers profile at early logarithmic phase was more similar to [^{3}H]xyloglucan, and at late logarithmic phase to polymers containing [^{3}H]Ara, [^{3}H]Xyl and [^{3}H]xylobiose. In both cell lines, the M_r distribution of polymers with [^{3}H]Ara residues and ^{3}H-labelled diagnostic fragments for xylans mimicked the general elution profile of 6 M NaOH-extracted hemicelluloses (Figure 4 *vs* Figure 1).

Most of the polymers which contained [^{3}H]Ara, [^{3}H]Xyl and [^{3}H]xylobiose as well as [^{3}H]isoprimeverose and undigested ^{3}H-polymers extracted from both cell lines, eluted in a Kav ranging from 0 to 0.4 in both culture phases. In addition, the proportion of ^{3}H detected at the V0 was low in both cell lines and culture phases (Figure 4). Nevertheless, a remarkable difference was found in the elution profile of [^{3}H]xyloglucan and undigested ^{3}H-polymers from Sh1.5 cells, in which a marked elution peak was observed near a Kav of 0.1 at early logarithmic phase of culture.

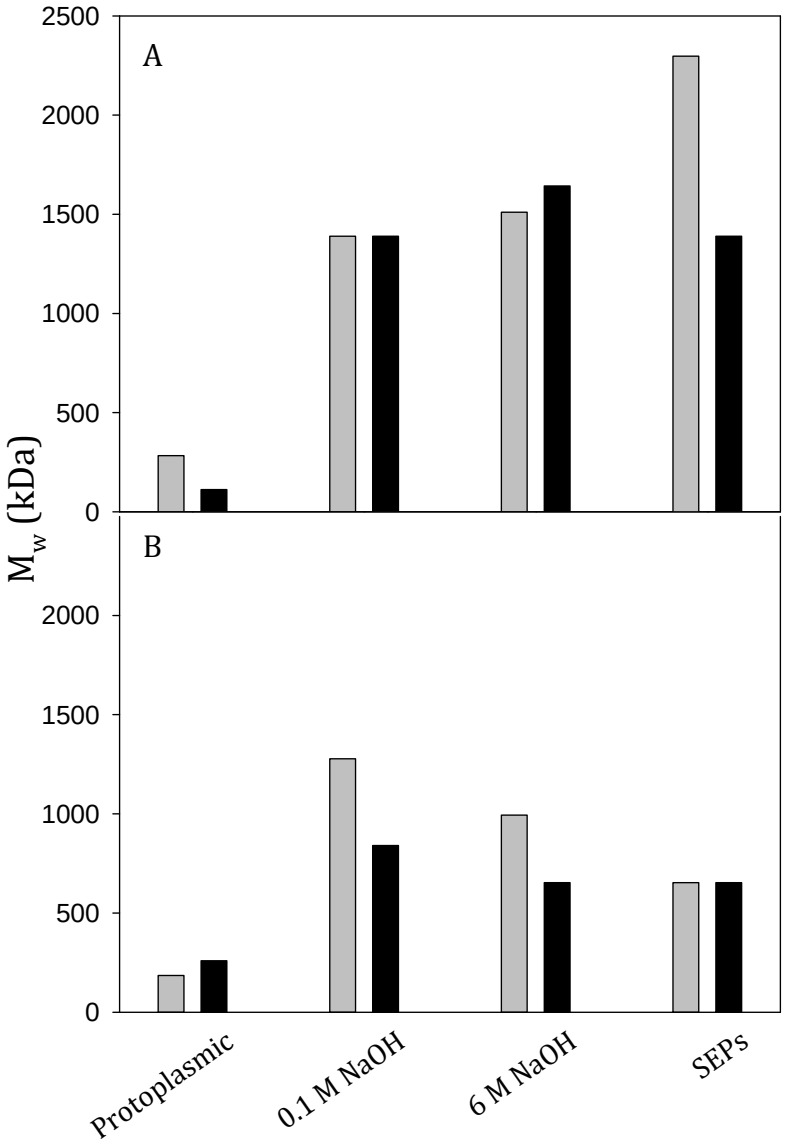


Figure 2. Relative average molecular mass (M_w) of protoplasmic ^{3}H-polymers (Protoplasmic), cell wall bound ^{3}H-hemicelluloses extracted with 0.1 M (0.1 M NaOH) or 6 M (6 M NaOH) NaOH and SEPs obtained from **A:** Snh and **B:** Sh1.5 maize cell cultures. Data shown are from the *grey bars*; early logarithmic and *black bars*; late logarithmic phases of culture cycle. Samples used were taken 300 min after feeding of [^{3}H]Ara, except in the case of protoplasmic ^{3}H-polymers, collected 30 and 60 min after feeding of [^{3}H]Ara for Snh and Sh1.5 respectively

III.b.3. M_r distribution of SEPs

In both cell lines, the elution profiles of [^{3}H]Ara, [^{3}H]Xyl or [^{3}H]xylobiose-containing polymers were very similar (Figure 5 *vs* Figure 1), suggesting that [^{3}H]SEPs are mainly constituted by arabinoxylans sloughed into the culture medium. A greater proportion of ^{3}H-molecules with a slightly higher M_w at early logarithmic phase was detected in both cell lines when compared with the late logarithmic stage of the culture cycle (Figure 5).

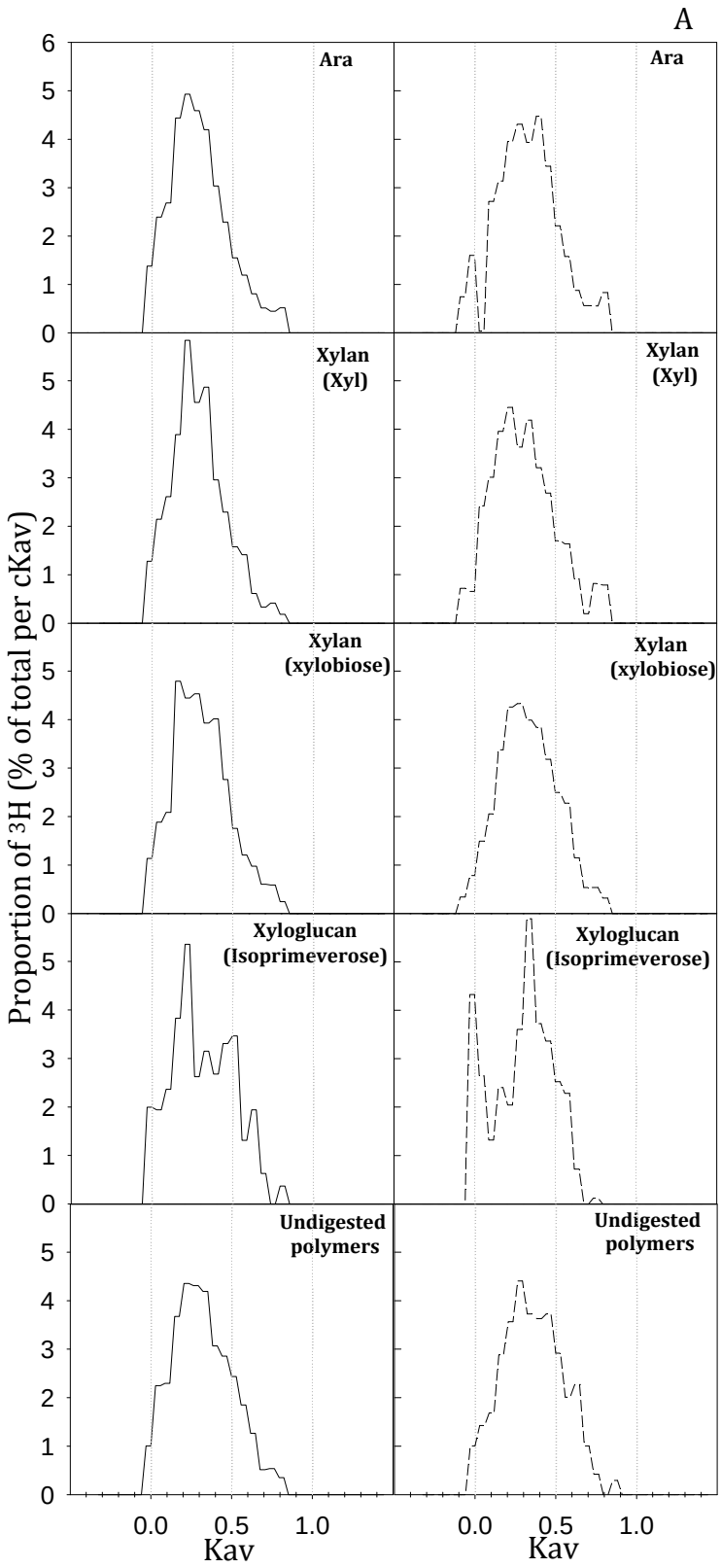
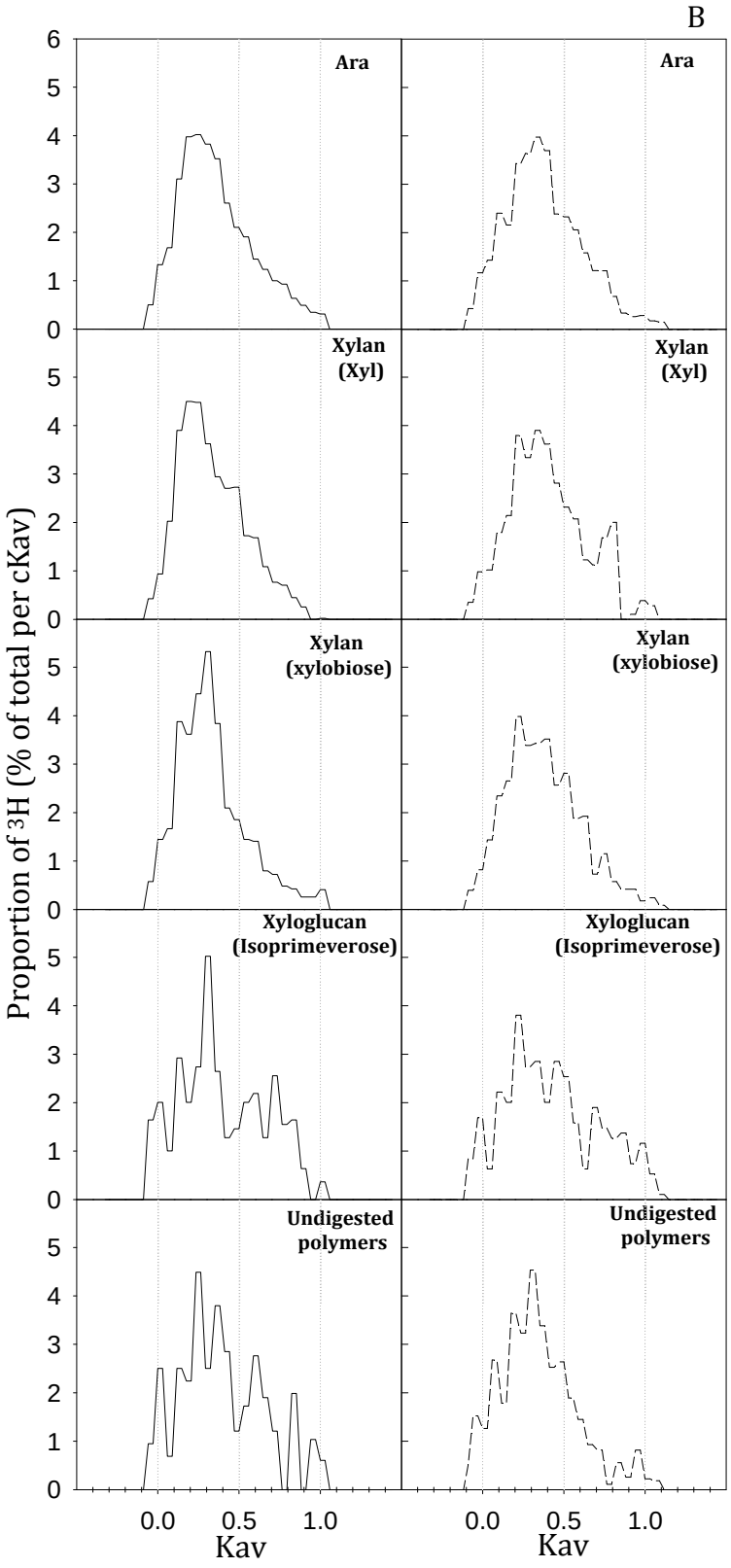


Figure 3. M_r distribution profile of ^{3}H-labeled diagnostic fragments from ^{3}H-polysaccharides: cell wall bound ^{3}H-hemicelluloses extracted with 0.1 M NaOH obtained from *solid line*; Snh and *dashed line;* Sh1.5 maize cell cultures. Data shown are from the **A:** early logarithmic and **B:** late logarithmic (see next page) phases of culture cycle. Samples used were taken 300 min after feeding of [^{3}H]Ara. Other details as in Figure 1


B
Ara
Ara
Xylan (Xyl)
Xylan (Xyl)
Xylan (xylobiose)
Xylan (xylobiose)
Xyloglucan (Isoprimeverose)
Xyloglucan (Isoprimeverose)
Undigested polymers
Undigested polymers
Proportion of ³H (% of total per cKav)
Kav
Kav
0.0
0.5
1.0
0.0
0.5
1.0
0
1
2
3
4
5
6

In Snh cells, the elution profiles of the different diagnostic fragments at early logarithmic phase were similar, mainly eluting at a Kav ranging from 0 to 0.5 and at the V0 (Figure 5A). However, M_r distribution of the undigested ^{3}H-polymers and the polymers from which [^{3}H]xylobiose was released showed a higher degree of similarity between them than with the remaining profiles.

At late logarithmic phase, Snh elution profiles differed from those observed at the early logarithmic culture stage described above (Figure 5B). The elution profiles of [^{3}H]xylans, ([^{3}H]Xyl and [^{3}H]xylobiose diagnostic fragments) were similar and molecules mainly eluted in a Kav ranging from 0.1 to 0.4. Similarly, the elution profiles of [^{3}H]xyloglucan ([^{3}H]isoprimeverose) and undigested ^{3}H-polymers were alike. Surprisingly, the elution profile of ^{3}H-polymers which contained [^{3}H]Ara residues was for the first time different to those for [^{3}H]xylans, and showed a wide distribution among the different Kav, with two well-defined "elution areas". The first of these, in a Kav from 0.2 to 0.4, coincided with that observed for the [^{3}H]xylan diagnostic fragments, suggesting a presumable sloughing of arabinoxylans. However, the second elution area was detected in a Kav ranging from 0.5 to 0.8, an observation that would indicate the release into the culture medium of another type of ^{3}H-molecule population containing [^{3}H]Ara residues. This was not observed in the Sh1.5 cells, in which the elution profile of polymers containing [^{3}H]Ara residues was very similar to those for [^{3}H]xylans.

In Sh1.5 cells, the digestion of [^{3}H]SEPs with Driselase showed that all the ^{3}H-polymers (those which contained [^{3}H]Ara, those with diagnostic fragments for [^{3}H]xylans and [^{3}H]xyloglucan and the undigested ^{3}H-polymers) eluted in a higher Kav than those from Snh cells, and therefore had a lower M_w (Figure 5). This was especially clear at the early logarithmic phase (Figure 5A). The most remarkable difference was found in the elution profile of [^{3}H]xyloglucan and undigested ^{3}H-polymers at late logarithmic culture phase (Figure 5B), where a marked peak was observed close to a Kav of 0.3, similar to that

previously described for the elution profile of 6 M NaOH-extracted [^{3}H]xyloglucan at early logarithmic phase.

III.c. Analysis of phenolic metabolism

III.c.1. Uptake of [^{14}C]Cinn from the culture medium and its incorporation into cell compartments

No differences in [^{14}C]Cinn consumption were observed among maize cell lines or culture phases (Figure 6). In general, consumption percentages of [^{14}C]Cinn ranged between 40%-55% of the total [^{14}C]Cinn added at time zero. Most of the [^{14}C]Cinn consumption (90%) occurred during the first 15 min after [^{14}C]Cinn was fed.

The kinetics of [^{14}C]Cinn incorporation into the protoplasmic-like fraction and cell wall compartment did not show major differences between either cell lines or culture phases, and as was expected, its incorporation into the protoplasmic-like fraction preceded its incorporation into the cell wall. However, relative incorporation of [^{14}C]Cinn into the Sh1.5 protoplasmic-like fraction and cell wall was, on average, half-fold reduced when compared with Snh cells (Figure 6).

III.c.2. [^{14}C]Cinn metabolism in the cell wall

An analysis of hydroxycinnamate ^{14}C-labelled metabolites incorporated into the cell wall during early logarithmic phase of growth is shown in Figures 7 and 9A. The [^{14}C]Cinn added to the culture medium was rapidly (1-3 min) metabolised to *p*-[^{14}C]Cou and then incorporated into the cell wall. In both cell lines, a decrease was observed in the relative incorporation of *p*-[^{14}C]Cou into the cell wall the first 30 min after [^{14}C]Cinn feeding. Concomitantly the [^{14}C]Fer esterified into the cell wall, abruptly increasing until reaching a plateau phase. Furthermore, at 30 to 60 min after [^{14}C]Cinn feeding a decrease was detected in the amount of cell wall-esterified [^{14}C]Fer. This was observed in both cell lines, although it was especially marked in Snh cells.

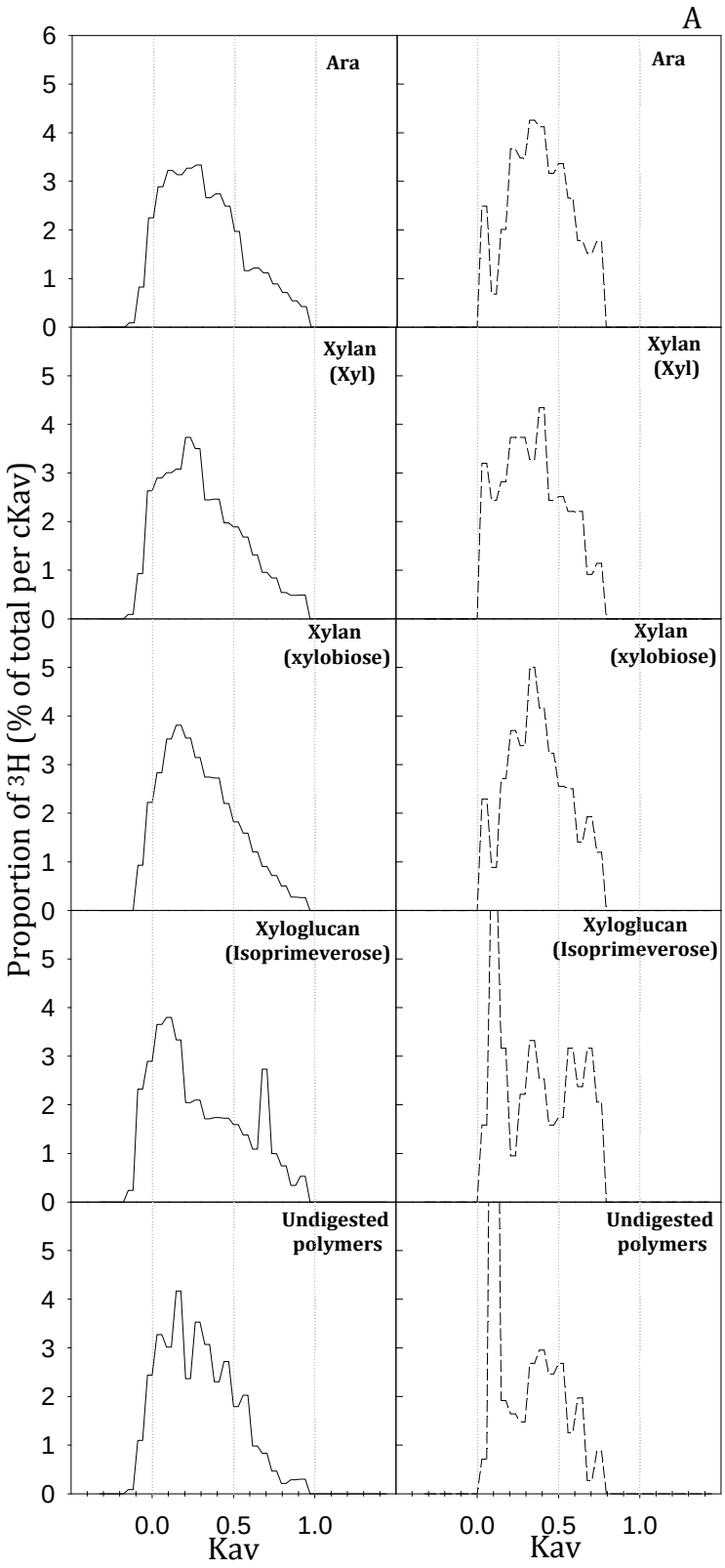
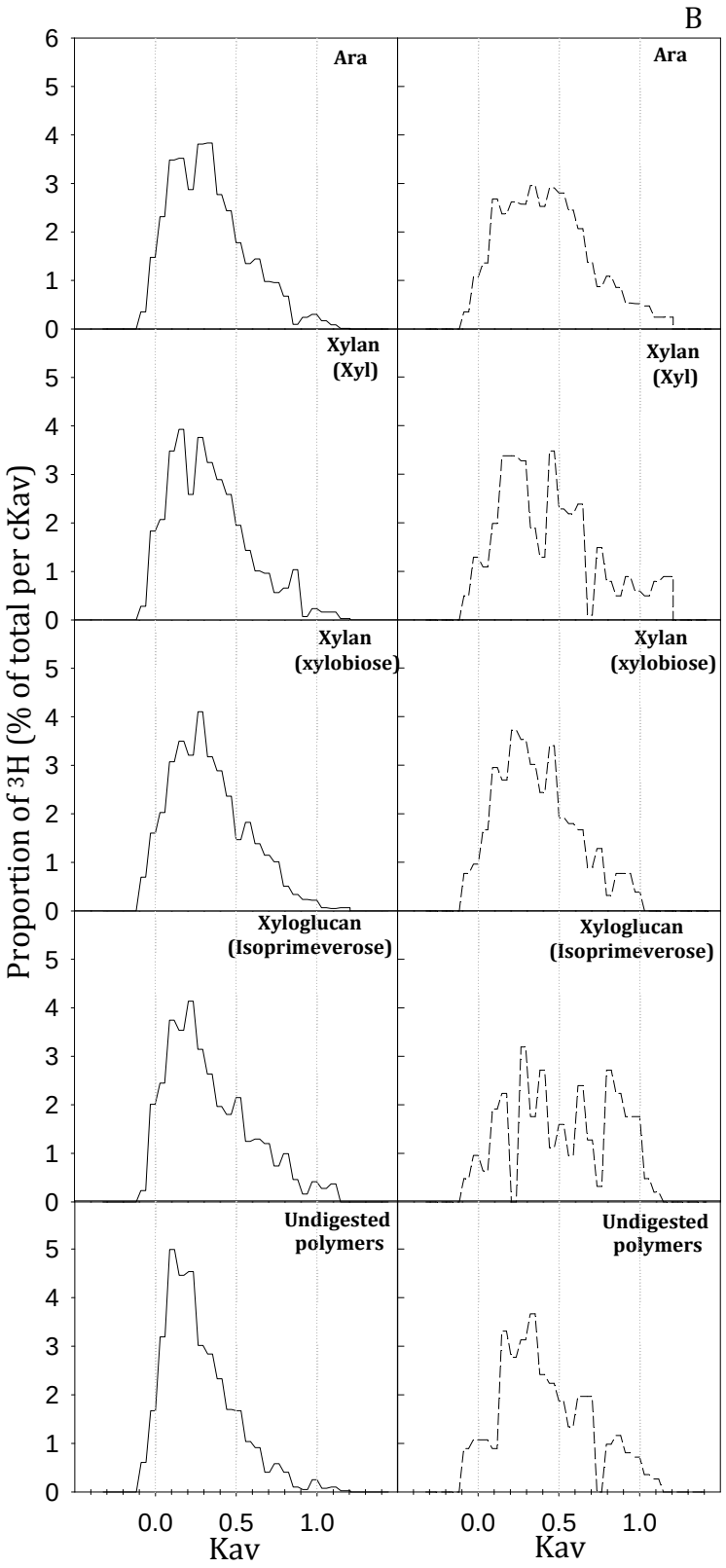


Figure 4. M_r distribution profile of cell wall bound ^{3}H-hemicelluloses extracted with 6 M NaOH obtained from *solid line*; Snh and *dashed line*; Sh1.5 maize cell cultures. Data shown are from the **A:** early logarithmic and **B:** late logarithmic (see next page) phases of culture cycle. Samples used were taken 300 min after feeding of [^{3}H]Ara. Other details as in Figure 1


B
Ara
Ara
Xylan (Xyl)
Xylan (Xyl)
Xylan (xylobiose)
Xylan (xylobiose)
Xyloglucan (Isoprimeverose)
Xyloglucan (Isoprimeverose)
Undigested polymers
Undigested polymers
Proportion of ³H (% of total per cKav)
0 1 2 3 4 5 6
0.0 0.5 1.0
Kav
0.0 0.5 1.0
Kav

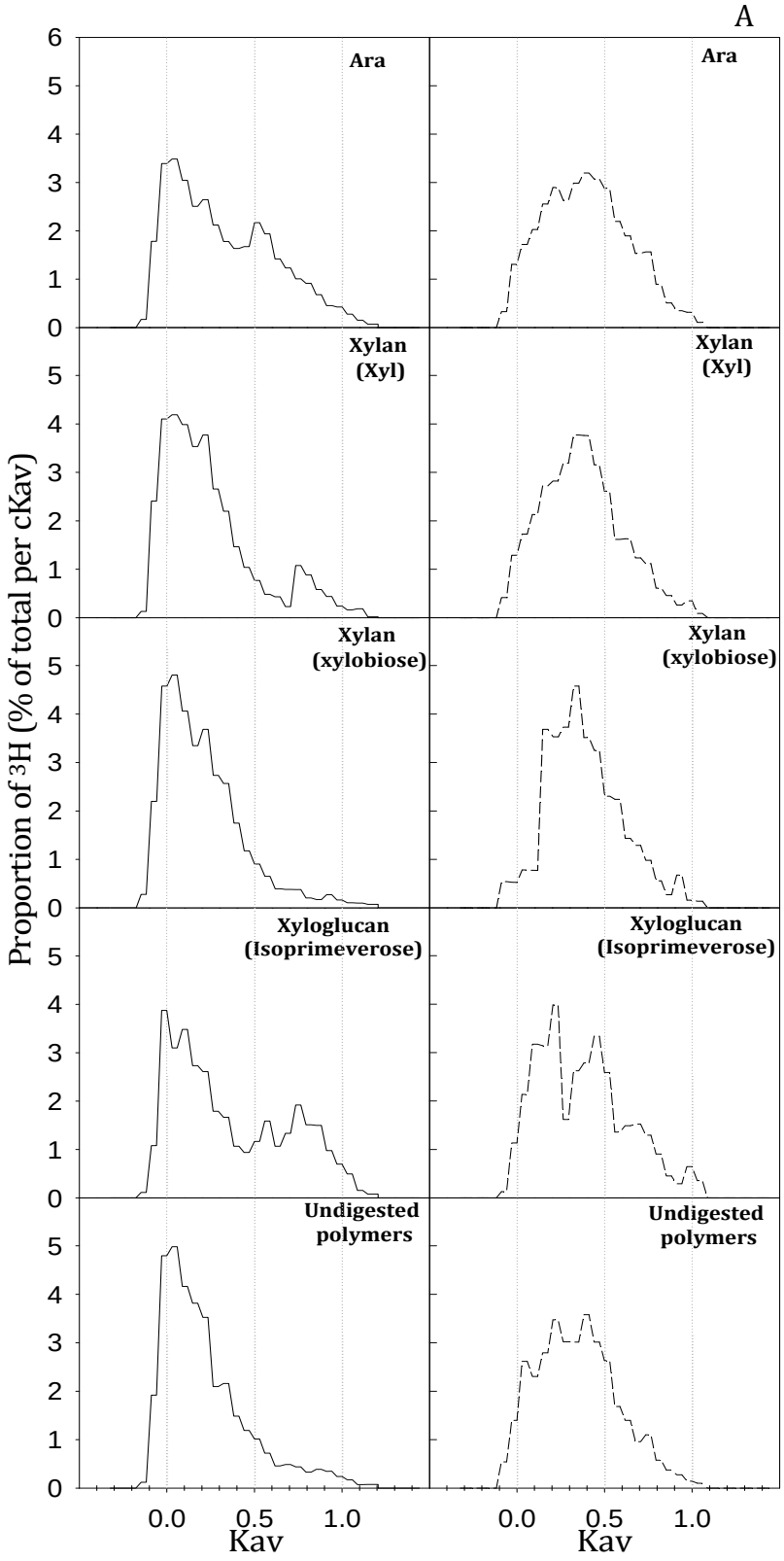
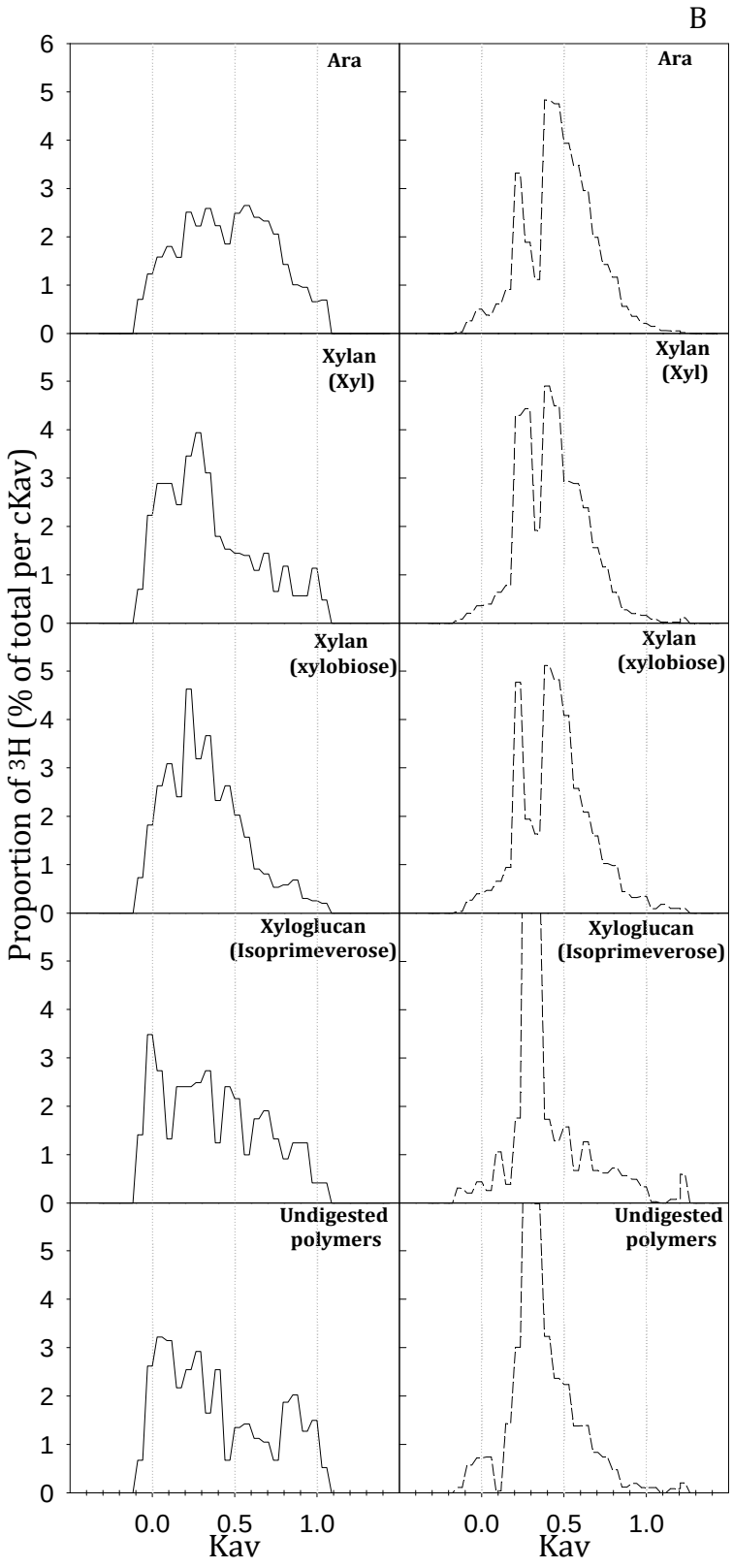


Figure 5. M_r distribution profile of [H³]SEPs obtained from the culture medium of *solid line*; Snh and *dashed line*; Sh1.5 maize cell cultures. Data shown are from the **A:** early logarithmic and **B:** late logarithmic (see next page) phases of culture cycle. Samples used were taken 300 min after feeding of [³H]Ara. Other details as in Figure 1


B
Ara
Ara
Xylan (Xyl)
Xylan (Xyl)
Xylan (xylobiose)
Xylan (xylobiose)
Xyloglucan (Isoprimeverose)
Xyloglucan (Isoprimeverose)
Undigested polymers
Undigested polymers
Proportion of ³H (% of total per cKav)
Kav
Kav
0.0
0.5
1.0
0.0
0.5
1.0
0
1
2
3
4
5
6

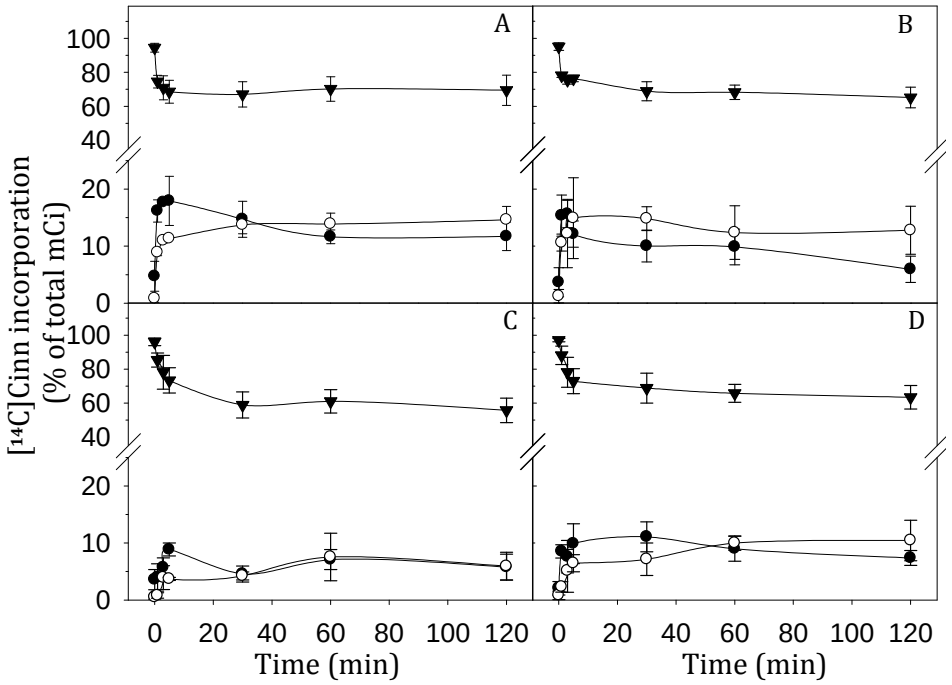


Figure 6. Kinetics of consumption and incorporation of radioactivity from exogenous [^{14}C]Cinnamic acid ([^{14}C]Cinn) in **A**, **B**: Snh and **C**, **D**: Sh1.5 maize cell suspensions. Data from the **A, C**: early logarithmic and **B, D**: late logarithmic phases of growth are shown. The [^{14}C]Cinn consumption (▼) was calculated from the radioactivity remaining in the culture medium after [^{14}C]Cinn feeding. The incorporation of [^{14}C]-labeled compounds in the protoplasmic-like fraction (●) and AIR (○) is represented. The values showed are the average ± standard deviation (n=3)

In all cases, [^{14}C]Fer was the main cell wall hydroxycinnamate detected, although when both cell lines were compared, the relative quantification of Cinn [^{14}C]derivatives indicated that Sh1.5 cell walls were enriched in *p*-[^{14}C]Cou and impoverished in [^{14}C]Fer in comparison with Snh cell walls. ^{14}C-diferuloyl groups and ^{14}C-oligomers (compounds that migrate at low RF regions) were detected in both cell lines 30-60 min after [^{14}C]Cinn feeding, depending on the cell culture phase. The increase in ^{14}C-diferulates and ^{14}C-oligomers occurred concurrently with a decrease in the amount of [^{14}C]Fer. In addition, their relative quantification indicated that [^{14}C]Fer, ^{14}C-dimers and ^{14}C-oligomers were more abundant in Sh1.5 cell walls. Lastly, and also at the early logarithmic phase, the supply of H_2O_2 provided 120 min after [^{14}C]Cinn feeding induced an increase in the dimerisation or oligomerisation of [^{14}C]ferulate, occurring to a similar degree in both cell lines.

No differences were found in the kinetics of Snh cell wall coumaroylation and feruloylation between growth phases (Figures 7 and 9A *vs* 8 and 9B). However, some differences were observed in the case of habituated cells: Sh1.5 cell walls accumulated more *p*-[^{14}C]Cou, ^{14}C-dimers and ^{14}C-oligomers at the late logarithmic phase (Figures 8 and 9B). Accordingly, the relative decrease in [^{14}C]Fer was more pronounced in the late logarithmic than in the early logarithmic phase. Lastly, the addition of H_2O_2 did not increase the level of [^{14}C]ferulate dimerisation or oligomerisation.

III.d. Arabidopsis homologue *IRX* gene expression analysis

The relative expression of the maize genes *GRMZM2G100143*, *GRMZM2G059825* and *gbIBT036881.1*, homologues of arabidopsis *IRX10*, *IRX10-L* and *IRX9*, respectively, was analysed by RT-PCR, shown in Figure 10. In Sh6 cells, the expression of arabidopsis *IRX10* and *IRX9* homologues was induced, whereas a decrease was detected in the expression of the arabidopsis *IRX10-L* homologue when compared with Snh cells. In Sh1.5 cells, a reduced expression of the arabidopsis *IRX10* homologue was observed.

IV. DISCUSSION

It has previously been reported that DCB-habituated maize cells constitute a suitable cellular model to investigate heteroxylan metabolism, since their coping strategies are based on altered arabinoxylan networks which show a certain degree of response plasticity depending on the habituation level (Mélida et al., 2009; de Castro et al., 2013; Chapter I). Likewise, previous studies on maize cells habituated to high DCB levels have described compensating strategies based on a reinforced hemicellulosic network acquired by more cross-linked arabinoxylans with a higher M_w and lower extractability (Mélida et al., 2009; 2011). These results are in contrast to those observed in maize cell suspensions habituated to low DCB levels (Sh1.5), in which the feeding of [^{3}H]Ara revealed a lower proportions of

strongly wall-bound ^{3}H-hemicelluloses and an increase in [^{3}H]SEPs, as well as a slower and less efficient metabolism of polysaccharides throughout the entire culture cycle (Chapter II).

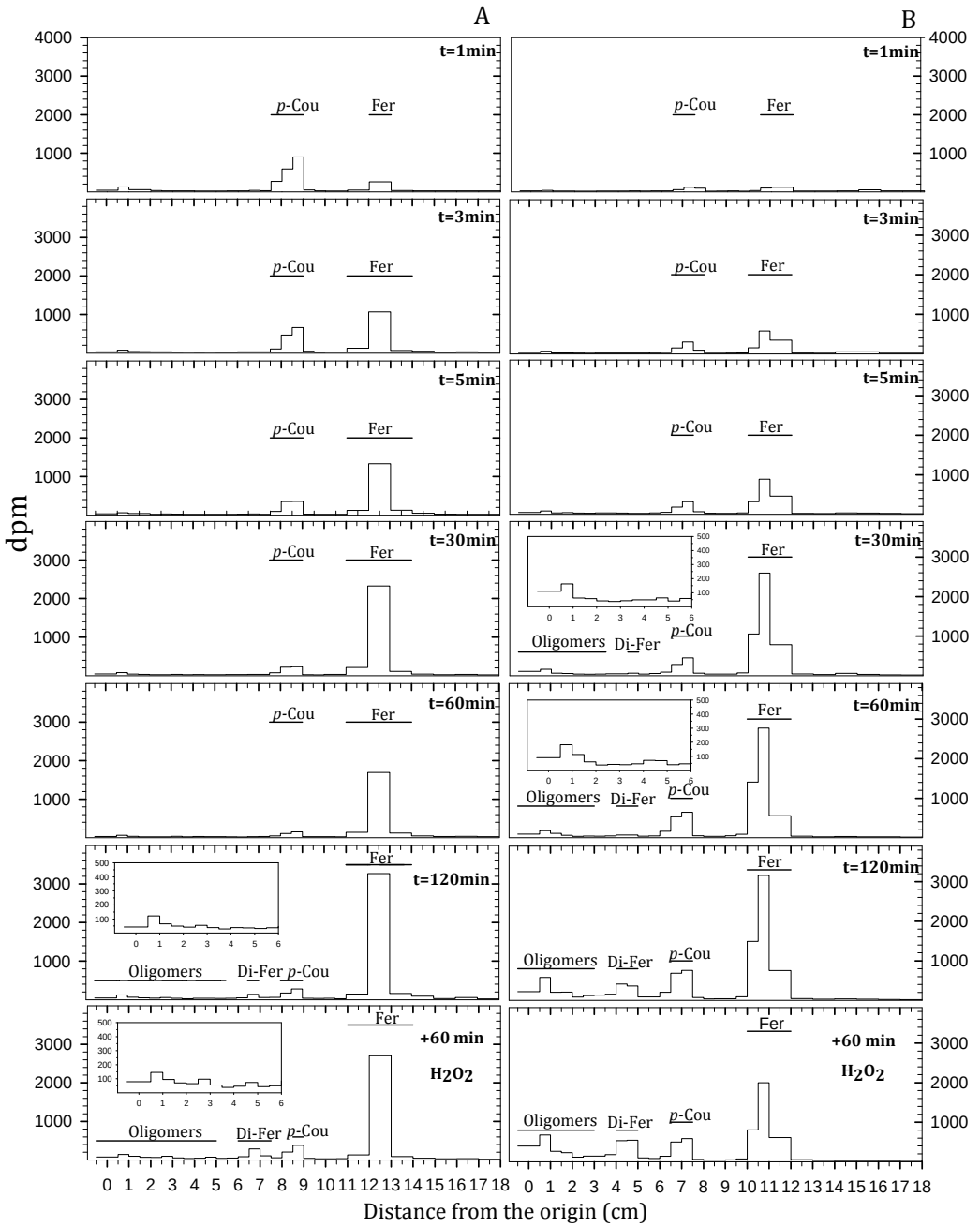


Figure 7. Thin layer chromatography (TLC) of [^{14}C]-labeled compounds esterified on the AIR after the addition of exogenous [^{14}C]Cinn to **A:** Snh and **B:** Sh1.5 maize cells suspensions at the early logarithmic phase of culture cycle. The position (—) of *p*-Coumaric acid (*p*-Cou), Fer and 5,5'-dehydrodiferulate (Di-Fer) used as external markers is shown. Compounds with low RF (Rdiferulate 0.0-0.9) are regarded as oligomers (Fry et al., 2000). The insert graphics show with detail the oligomers and Fer dimers appearance. +60 H_2O_2 min shows a treatment for 60 min with 40 mM H_2O_2, 120 min after [^{14}C]Cinn feeding

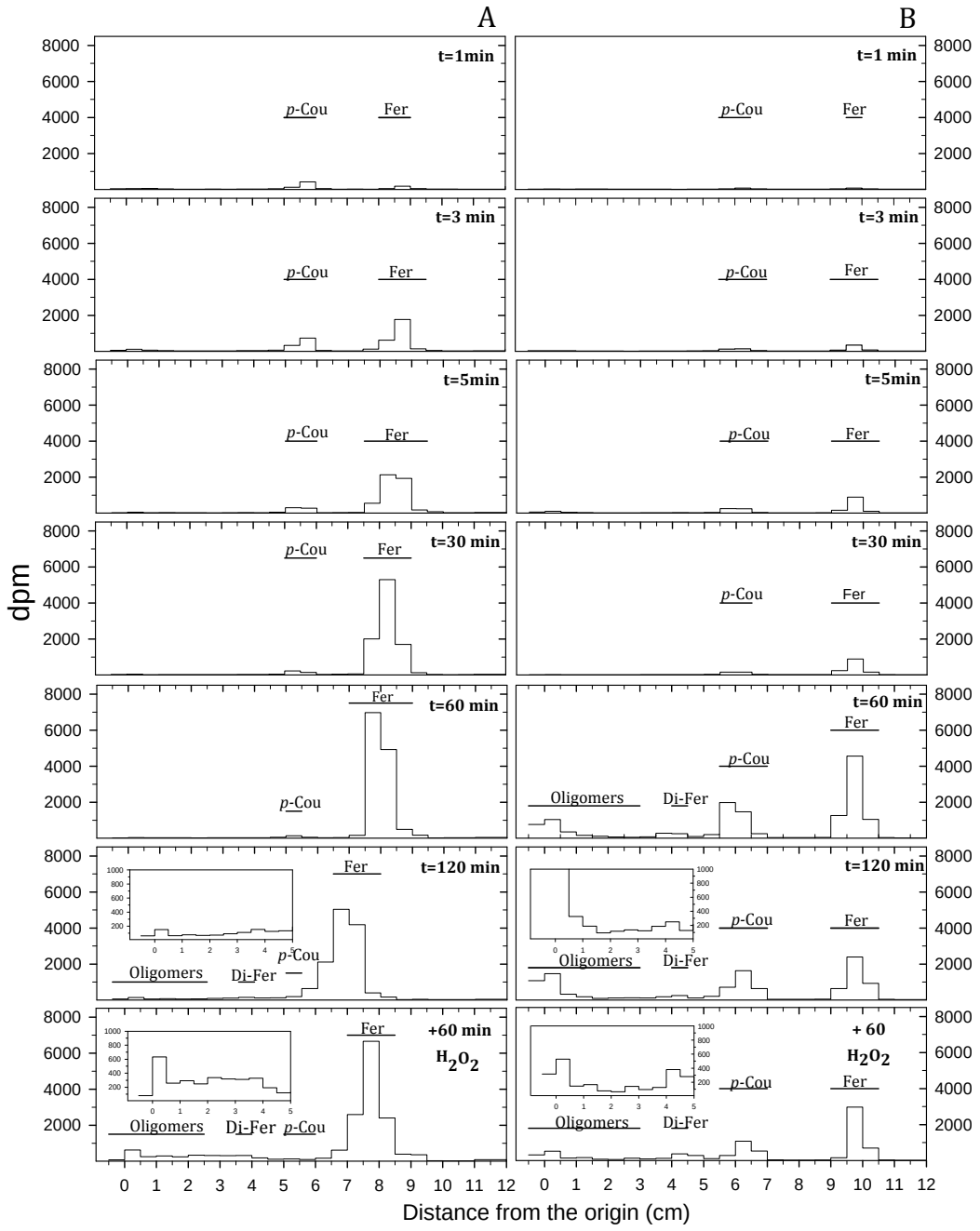


Figure 8. TLC of [^{14}C]-labeled compounds esterified on the AIR after the addition of exogenous [^{14}C]Cinn to **A:** Snh and **B:** Sh1.5 maize cell suspensions at the late logarithmic phase of culture cycle. The position (—) of *p*-Cou, Fer and Di-Fer used as external markers is shown. Compounds with low RF (Rdiferulate 0.0-0.9) are regarded as oligomers (Fry et al., 2000). The insert graphics show with detail the oligomers and Fer dimers appearance. +60 H_2O_2 min shows a treatment for 60 min with 40 mM H_2O_2, 120 min after [^{14}C]Cinn feeding

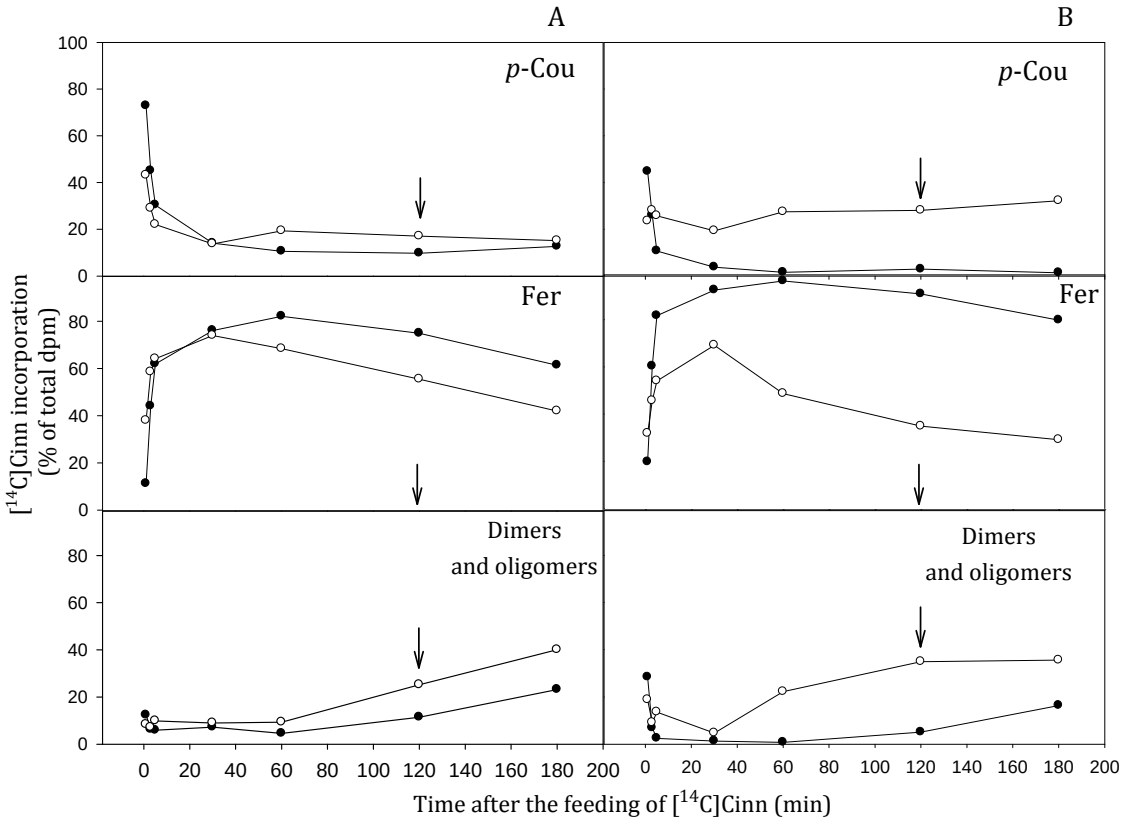


Figure 9. Kinetics of incorporation of [^{14}C]-labelled compounds esterified on AIR after the addition of exogenous [^{14}C]Cinn to *filled circle*; Snh and *open circle*; Sh1.5 maize cell suspensions at the **A:** early logarithmic and **B:** late logarithmic phase of culture cycle. [^{14}C]-labelled compounds were separated by TLC and assayed for radioactivity by scintillation counting. Data are expressed as percent of total polymer-esterified derivatives. Arrow indicated the addition of H_2O_2 (40 mM final concentration)

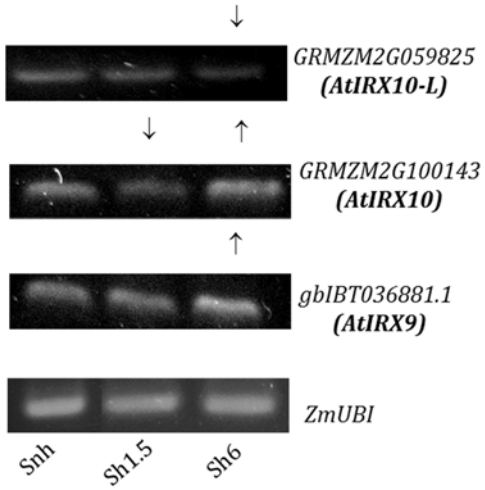


Figure 10. Relative expression of maize genes orthologues of several *IRX* genes from arabidopsis analyzed by RT–PCR of Snh, Sh1.5 and habituated to 6 µM DCB (Sh6) maize cell suspensions.↑: more mRNA accumulation than control (Snh); ↓ less mRNA accumulation than control (Snh) . *ZmUBI*: Ubiquitine gene expression. Ubiquitine was used as the housekeeping gene due to its constitutive expression

This finding was especially surprising, and there are two possible hypotheses which could explain this altered cellular fate: it may be caused by 1) a reduction in "binding points" to cellulose and/or 2) a reduced capacity to incorporate polysaccharides, thought to be mainly arabinoxylans, into the cell wall via extra-protoplasmic phenolic cross-linking.

As cross-linking is closely related to the M_w of polysaccharides and to phenolic metabolism, both were investigated in the present study, in several cell compartments of Sh1.5 cells throughout the culture cycle. This was achieved through *in vivo* feeding experiments with radio-labelled precursors ([^{3}H]Ara and [^{14}C]Cinn, respectively), since use of this methodology has been successful in comparable experimental studies (Fry et al., 2000; Kerr and Fry, 2003; Burr and Fry, 2009; Mélida et al., 2011).

The first question to arise was whether low DCB habituation levels entailed modifications in the M_w of hemicelluloses and polymers. In general, Sh1.5 cells showed more homogeneously sized and smaller ^{3}H-polysaccharides populations that were more similar among cell compartments, regardless of the culture phase, than those from Snh. These results, and the suitability of the experimental approach, were supported by the observation that these trends were consistent in the elution profiles of the different diagnostic fragments (unique for [^{3}H]xyloglucan, [^{3}H]xylans, polymers which containing [^{3}H]Ara and those undigested by Driselase) and were perfectly illustrated in the case of [^{3}H]SEPs. These latter polymers are sloughed into the extracellular medium and no extraction treatment is required in order to obtain them; their molecular integrity is maintained intact. Therefore, the data obtained from the study of [^{3}H]SEPs are especially valuable providing information about features of polysaccharides in their native structure (Kerr and Fry, 2003). In addition, some enzymes, substrates such as H_20_2 and inhibitors of cross-linking (Encina and Fry, 2005), also diffuse into the extracellular medium (Burr and Fry, 2009). ^{3}H-polysaccharides from Sh1.5 cells also had a lower M_w in all cell compartments, independently of the culture cycle stage. A comparison of the M_w from protoplasmic ^{3}H-polymers and cell wall bound ^{3}H-hemicelluloses indicated that after secretion into the cell wall, protoplasmic ^{3}H-polymers

underwent massive cross-linking in both cell lines, although this increase was much less pronounced in Sh1.5 cells. This increase in the M_w of hemicelluloses once they were integrated into cell wall has already been described in maize cell suspensions (Kerr and Fry, 2003), suggesting the occurrence of cell wall grafting processes. These processes comprise the formation of non-covalent and covalent interactions among molecules. Non-covalent cross-links include the formation of hydrogen and ionic bonds, whereas covalent interactions are mainly produced by oxidative coupling or glycosidic bonds catalysed by transglycosylation reactions (Fry, 2000). It has been reported that xyloglucan becomes glycosidic-linked to acidic pectins in rose cultured cells (Thompson and Fry, 2000) during wall binding. In addition, the existence has recently been demonstrated of heteroxylan transglycosylase in, which can use a wide range of xylans from different species as the acceptor substrate, including wheat arabinoxylans (Johnston et al., 2013). Although this heteroxylan transglycosylase was isolated from the dicot *Carica papaya,* which presents a type I cell wall, since it can use arabinoxylan from a grass like wheat (which has type II cell walls) as substrate, the occurrence of this type of transglycosylation reaction in maize cells cannot be ruled out. Nevertheless, it is to be expected that hydrogen and ester bonds or oxidative coupling cross-links would be responsible of cell wall grafting processes involving arabinoxylan molecules. However, the increase detected in the M_w of ^{3}H-hemicelluloses and ^{3}H-polymers in Sh1.5 cell walls was much less pronounced than in Snh.

A possible explanation for these findings is that cells habituated to low DCB levels synthesised ^{3}H-molecules with a lower and similar M_w as a strategy to cope with the lack of cellulose, considered as “mild” when compared with that observed in cells habituated to high DCB levels (33% *vs* 75% of reduction compared with Snh). The pattern observed in the expression of arabidopsis *IRX* orthologue genes in habituated cells (Sh1.5 and Sh6) supports this hypothesis. As mentioned above, cells habituated to high DCB levels synthesised hemicelluloses with a higher M_w in order to counteract their impoverishment in cellulose. In good agreement with this, the expression of genes involved in the xylan elongation chain

(Brown et al., 2009; Wu et al., 2009) (*GRMZM2G100143* orthologue of *AtIRX10*; Bosch et al., 2011) as well as *gbIBT036881.1*, the orthologue of *AtIRX9* (Bosch et al., 2011) involved in the initiation of the xylan synthesis (Brown et al., 2007; Pena et al., 2007) was found to be up-regulated in Sh6 cells. Interestingly, in cells habituated to low-DCB levels (Sh1.5), *GRMZM2G100143* (*AtIRX10* orthologue) was found to be repressed whereas the expression of *gbIBT036881.1* (*AtIRX9* orthologue) remained unaltered. Such preliminary results may indicate that Sh1.5 cells, do not in fact present a defective initiation of xylan synthesis but are impaired in the elongation of hemicellulose chains. This would lead to the production of shorter xylan chains in Sh1.5 cells, contrary to what happens in Sh6.

In addition, a previous study on maize cells habituated to medium and high DCB levels revealed differences in the M_w of wall-bound hemicelluloses (Mélida et al., 2009). In this study, polysaccharides were subsequently extracted from the cell wall using two alkali-treatments, 0.1 M KOH and 4 M KOH, known to solubilise weakly- and firmly-bound polysaccharide populations respectively. The results showed that in medium DCB-habituation levels, the M_w of 0.1 M KOH-extracted hemicelluloses increased with respect to the same fraction obtained from non-habituated cells, cells habituated to high DCB levels, and even with respect to 4 M KOH-extracted hemicelluloses. Interestingly, in maize cells habituated to high DCB levels, the M_w of 4 M KOH-extracted hemicelluloses increased with respect to non-habituated cells, cells habituated to medium DCB levels and to those solubilised by 0.1 M KOH treatment. It is probable that, as the level of DCB habituation increases, a progressive input of higher M_w hemicelluloses that are more tightly bound takes place in order to reinforce the cell walls. However, it should be borne in mind that these results were obtained using a different experimental approach to ours. The maize cells used in Mélida et al. (2009) were collected at an advanced stage of the culture cycle. Thus, it is feasible to assume that hemicelluloses in such walls have been there for a longer period of time, and have presumable been subjected to grafting process with other molecules, which would lead to an increase in their M_w. Furthermore, it is important to note that massive

cross-linking of hemicelluloses in maize cell suspension cultures occurred 11-days after sub-culturing (Burr and Fry, 2009). In contrast, our experimental approach involved a pulse-chase experiment, in which the screened molecules were formed, integrated into the wall or sloughed over a short period of time (5 h). Despite this, the idea that maize cells gradually require less extractable and higher M_w hemicelluloses as the DCB habituation level increases is in good agreement with observations in previous studies, where cells habituated to low and medium DCB levels seemed to compensate for their lack in cellulose through an increase in hemicelluloses extracted with mild alkali treatment (0.1 M KOH) (Mélida et al., 2009; de Castro et al., 2013; Chapter I). The finding that Sh1.5 cells had molecules, and more specifically hemicelluloses, with a lower M_w than those from Snh may indicate that cells habituated to low DCB levels increase the amount of low M_w hemicelluloses as a part of their coping strategy. In this respect, it has previously been suggested that a decrease in the M_w of xyloglucan improves its capacity to bind to cellulose (Lima et al., 2004), and in accordance with this, it has been found that xyloglucan from cellulose-deficient bean cells has a lower M_w than that of control cells (Alonso-Simón et al., 2007). In this latter case, it was hypothesised that smaller xyloglucan molecules together with an increased xyloglucan-endo-transglucosylase activity would produce a more rigidified cell wall (Alonso-Simón et al., 2007). However, it should be borne in mind that this finding was described in bean cells, which have a type I cell wall. Therefore, it still remains unclear whether this mechanism could play any kind of role in cells with a type II cell wall.

Another possible explanation is closely related to the capacity of Sh1.5 cells to cross-link cell wall hemicelluloses and SEPs. It should be borne in mind that, as previously mentioned, Sh1.5 cells showed a reduced amount of cell wall bound ^{3}H-hemicelluloses and an increased presence of [^{3}H]SEPs in the culture medium (Chapter II). In addition, the timing of cross-linking may also be delayed in Sh1.5 cells due to their slower and less efficient metabolism (Chapter II) thus preventing detection during the time window employed in this experiment (5 h). In order to explore these possibilities, an analysis was performed of Cinn

metabolism as well as a characterisation of its derivatives in the cell wall. First, no marked differences were detected in the capacity to uptake [^{14}C]Cinn from the culture medium depending on the cell line, a finding that it is in agreement with observations in a previous study on maize cells habituated to high DCB levels and employing a similar experimental approach (Mélida et al., 2011). Nevertheless, Snh and Sh1.5 cells showed differences in their capacity to incorporate [^{14}C]Cinn into the protoplasm and cell wall; habituated cells presented a deficient capacity for the biosynthesis and incorporation into the cell wall of [^{14}C]feruloylated/coumaroylated molecules. Similar altered kinetics in Sh1.5 cells were observed when [^{3}H]Ara was fed as the radioactive precursor, showing that a delayed cellular traffic and cell wall binding of ^{3}H-hemicelluloses and ^{3}H-polymers took place (Chapter II). However, and despite this reduced capacity, the relative content in ferulate dimers and oligomers was higher in Sh1.5 cells. These results are in accordance with those previously observed in maize cells habituated to high DCB levels (Mélida et al., 2010b; 2011) supporting the hypothesis that a greater cross-linked network of hemicelluloses, mainly arabinoxylans, would be crucial for the cell wall reinforcing strategy in DCB-habituated maize cells. Thus, the results suggest that the lower M_w of hemicelluloses and polymers detected in Sh1.5 cells was not related to a lower degree of cross-linkage through Fer. Alternatively, hemicelluloses and polymers from habituated cells were in fact shorter, albeit extensively cross-linked.

Another possibility could be that dimerisation (or oligomerisation) of Fer residues contributed to the formation of intra-polymeric loops to a greater extent. Consequently, Sh1.5 hemicelluloses would be deposited on the cell wall as a coagulum (Fry et al., 2000). However, a negative association between intra-polymeric arabinoxylan cross-linking and cell wall reinforcement has been suggested (Fry et al., 2000). Consequently, this latter finding would render this possibility unlikely in the scenario of cell wall rigidification.

Changes in the M_w of maize cell wall hemicelluloses and polymers during the culture cycle have been demonstrated, which is not surprising since cells would need to modify them in order to allow or restrict cell expansion (Kerr and Fry, 2003). In addition, ferulate cross-

linking in maize cells has also been shown to vary throughout the culture cycle, according to the physiological condition of the cells (Burr and Fry, 2009). In this respect, and as a precedent, differences in the phenolic profile and metabolism of cells habituated to high DCB levels have previously been reported as the culture cycle progresses (Mélida et al., 2011). Thus, the study of how the M_w of hemicelluloses and polymers as well as the phenolic metabolism vary during the culture cycle would be of interest in the case of maize cells habituated to low DCB levels, which have been demonstrated to present altered patterns and kinetics of growth (de Castro et al., 2013; Chapter I), as well as a delayed metabolism throughout the entire cycle (Chapter II).

Generally, the elution profiles of Driselase-digested diagnostic fragments for polymers containing [^{3}H]Ara and those for [^{3}H]xylans were very similar, although Ara residues can derive from other polymers besides arabinoxylans, such as rhamnogalacturonan type I, or arabinogalactan proteins. However, a sugar analysis of 0.1 M- and 6 M alkali-extracted fractions from Sh1.5 cell walls revealed a low content of uronic acid, Gal and Rha residues (de Castro et al., 2013; Chapter I). Therefore, it can be assumed that [^{3}H]Ara, [^{3}H]Xyl and [^{3}H]xylobiose are, in fact, digestion products of putative [^{3}H]arabinoxylan molecules.

In both, Snh and Sh1.5 cell lines, a greater proportion of smaller molecule populations were detected at the late logarithmic phase compared with the early logarithmic phase. In general, this trend was observed for arabinoxylans, xyloglucan and Driselase-undigested elution profiles regardless of the cell compartment, and was confirmed by the data from the M_w calculation. Some of the main factors controlling ferulate cross-linking (and therefore involved in changes in the M_w of hemicelluloses), such as peroxidase action and H_2O_2 availability, varied during the culture cycle (Burr and Fry, 2009). Thus, it is feasible that changes in these factors were responsible for the lower M_w detected at the late logarithmic phase of culture in both cell lines.

The increased M_w of SEPs compared with cell wall bound hemicelluloses from Snh at the early logarithmic phase may indicate that polysaccharides sloughed into the culture medium underwent additional cross-linking. Indeed, SEPs differ from wall bound hemicelluloses in having greater conformational freedom and mobility, rendering them more accessible to enzymes, which would increase the degree of ferulate polymerisation (Burr and Fry, 2009). Nevertheless, the trend described was not observed either in Snh cells at late-logarithmic phase or in Sh1.5 cells independently of the cell culture phase.

In the cell wall compartment, the M_w of polysaccharides in Snh cells was very similar in both culture stages and the same trend of similarity between culture phases was observed in Snh kinetics of cell wall coumaroylation and feruloylation. In contrast, the M_w of Sh1.5 cell wall hemicelluloses and polymers decreased in the late logarithmic phase, but surprisingly, greater amounts of ferulate ^{14}C-dimers and ^{14}C-oligomers were detected. In line with this, exogenous H_2O_2 was observed to stimulate feruloyl dimerisation in Snh cells independently of the growth phase, suggesting H_2O_2 availability as the limiting factor for ferulate dimerisation and oligomerisation in the case of Snh cells. In contrast, since exogenous H_2O_2 in Sh1.5 cells at the late logarithmic growth phase did not produce any change, the limiting factor seems to be the availability of free ferulate for an extra dimerisation. This latter finding once again demonstrated that hemicelluloses and polymers from habituated cells would in fact be shorter, albeit widely cross-linked, and/or underwent an additional trimming process that did not occur in Snh cells.

Another interesting finding was the marked elution peak in the Sh1.5 xyloglucan molecules extracted with 6 M NaOH as well as in those sloughed into the culture medium observed at Kav 0.1 and 0.3 respectively. A very high M_w of wall bound xyloglucan molecules in maize suspension cells has previously been reported, suggesting that a high M_w complex of xyloglucan, possibly linked to other polysaccharides, may play a more effective tethering role. This finding would partially explain why graminaceous monocots can survive with much smaller quantities of xyloglucan than dicots (Kerr and Fry, 2003). Indeed, neither of

the marked elution peaks of Sh1.5 xyloglucan was detected in the V0, where molecules with the highest M_w would elute. However, when both Kav were compared, strongly wall bound xyloglucan from Sh1.5 cells was detected in a lower Kav than that for the sloughed xyloglucan (0.1 *vs* 0.3). This would in fact indicate that M_w increased in wall bound xyloglucan in Sh1.5, probably through an association with other polysaccharides or polymers. Since it has previously been suggested that xyloglucan could be involved in the coping response induced at low DCB levels (de Castro et al., 2013; Chapter I), these results would partially support this hypothesis, although further experiments are required for confirmation.

In sum, the study of the M_w of [^{3}H]xylans, [^{3}H]xyloglucans, [^{3}H]Ara-containing polymers as well as Driselase-digestion resistant ^{3}H-polymers as well as the phenolic metabolism in maize cells habituated to low DCB levels revealed that: a) Sh1.5 cells synthesised molecules with a lower M_w and a more homogeneous size throughout the entire culture cycle, b) although hemicelluloses were shorter than those observed in the non-habituated cells, this was not related to a diminished capacity for polysaccharide cross-linking, c) both cell lines synthesised molecules with a lower M_w at the late logarithmic phase of the culture cycle, probably associated with the cessation of growth, and d) the hypothesis of a significant role for xyloglucan in habituation to low levels of DCB was supported by the results.

Our results indicate that Sh1.5 cells synthesise shorter, albeit extensively cross-linked, molecules as part of their coping strategy. These results demonstrate that DCB habituation is a gradual and dynamic process in which maize cells show highly plastic coping responses depending on the DCB habituation framework or, the equivalent, on the level of cellulose deficiency.

Chapter IV

Study of the Golgi proteome

in DCB-habituated cells

ABSTRACT

Cultured maize cells habituated to DCB have the capacity to adopt different mechanism to counteract the reduction in wall cellulose content, including metabolic adaptations. Since it is well known that the Golgi is an important organelle in polysaccharide and protein metabolism, the aim in the present study was to obtain a proteomic picture of Golgi-enriched fractions of habituated maize cell suspensions in order to gain a better understanding of their metabolic activity. To this end, a protocol was developed for obtaining Golgi-enriched fractions and conducting a 2-D proteomic study. Using MALDI-TOF/MS and ESI-MS/MS, a total of 13 proteins detected as unique or mis-regulated in habituated cells were sequenced. Some of them were probably mis-regulated as a consequence of the DCB action, such as chaperonin 60 and the reversible glycosylation polypeptide α-1,4-glucan protein synthase (UDP-forming), whereas others may be involved in various coping strategies. This latter group included enzymes related to stress signalling when cell wall integrity is impaired, such as enolase 1 or 1-aminocyclopropane-1-carboxylate oxidase 1; and others such as caffeoyl-CoA *O*-methyltransferase 1, which is related to qualitative and quantitative changes in lignin composition, and ascorbate peroxidase, in turn related to an oxidative coping mechanism. The results of this study shed light not only on the DCB-habituation process but also on the counteracting mechanisms that maize cells adopt to survive with an impaired cell wall.

I. INTRODUCTION

Cell walls are responsible for crucial functions in plants, such as growth, morphogenesis and modulation of stress signalling responses to abiotic and biotic stresses (Hamann et al., 2009; Driouich et al., 2012). Basically, the cell wall structure consists of cellulose acting as a scaffold to which hemicelluloses are attached, and the entire arrangement is surrounded by a pectin matrix (Carpita and Gibeaut, 1993). In addition to their physiological importance, cell walls also have significant economic implications. Their components are important constituents of dietary fibre, with notable implications for industry as well as having a positive impact on health. Another promising role arose when they began to be considered as a source of energy from plant materials in biofuel production (Burton and Fincher, 2012).

Primary cell wall composition in Poales differs from that in dicots or other monocots, essentially in the non-cellulosic polysaccharide portion, with lower amounts of xyloglucans and pectins which are predominantly substituted by heteroxylans (Carpita and McCann, 2000). Those in grasses have the basic structure of all xylans, a backbone of Xyl residues with Ara, GlcA, and MeGlcA residues attached as well as *p*-Cou and Fer rests esterified on Ara residues (Wende and Fry, 1997; Faik, 2010), but they also present unique features, such as the presence of Xyl-Ara-furanosyl side chains (Ebringerova et al., 2005).

Cell wall polysaccharides are synthesised in the Golgi apparatus, a fundamental organelle in the eukaryotic secretory pathway. As a component of the endomembrane system, it is highly involved in membrane signalling processes and vesicular trafficking, and this organelle is where post-translational modification, processing of proteins and synthesis of complex carbohydrates takes place, endowing the Golgi apparatus with multiple regulatory functions in complex metabolic processes (Parsons et al., 2012). In higher plants, vesicles secreted by the Golgi apparatus are responsible for cell plate formation during cell division (Driouich et al., 2012). Furthermore, the Golgi apparatus plays an important role during plant cell growth: newly synthesised polysaccharides in the Golgi are transported into

vesicles until they coalesce with the plasma membrane to generate the growing cell wall (Cosgrove, 2005).

When maize cells are habituated to grow with cellulose-impoverished cell walls, a modified and remodelled heteroxylan (predominantly arabinoxylan) network compensates for this deficiency (Mélida et al., 2009). In addition, previous studies performed using a biochemical approach have demonstrated that metabolism in habituated cells showed an overall alteration (Chapter II). Thus, habituated maize cell cultures would be a suitable starting cellular material for the study of metabolic modifications, and more specifically those concerning heteroxylan synthesis.

Therefore, the aim of the present study was to determine whether DCB habituation entails metabolic modifications from a proteomic point of view. Important metabolic enzymes are known to be Golgi-resident proteins, so taking advantage of our habituated maize cultured cells as a cellular model, Golgi-enriched fractions were obtained from Snh and habituated to low (Sh1.5) and high (Sh6) DCB concentrations maize cultured cells. Then, a comparative proteomic expression analysis was performed of their Golgi-enriched snapshots. The results shed light on the metabolism of maize cultured cells, as well as clarifying unknown aspects related to DCB coping strategies from a proteomic perspective.

II. MATERIALS AND METHODS

II.a. Cell cultures and habituation to DCB

Maize cell-suspension cultures (*Zea mays* L., Black Mexican sweet corn) from immature embryos were grown in Murashige and Skoog media (Murashige and Skoog, 1962) supplemented with 9 µM 2,4-D and 20 g L^{-1} sucrose, at 25°C under light and rotary shaken, and routinely subcultured every 15 days.

In order to obtain habituated cell cultures to DCB, Snh cells were cultured in a medium supplied with DCB increased concentrations, dissolved in DMSO which does not

affect cell growth at this range of concentrations. Likewise, in the case of cell suspensions habituated to low DCB levels, Snh cells were transferred in a medium containing 1 µM DCB, and after seven subcultures the DCB concentration was increased up to 1.5 µM DCB (Sh1.5). Habituated cells suspensions to high DCB levels were derived from maize callus cultures habituated to grow in 12 µM DCB which were transferred into liquid medium supplemented with 6 µM DCB (Sh6).

II.b. Obtaining Golgi-enriched fractions

Cells from Snh, Sh1.5 and Sh6 suspension cultures were collected at the logarithmic phase of growth and frozen at -80°C until used. Since habituated cells (Sh1.5 and Sh6) presented longer culture phases than Snh cells, the days of sampling were adjusted depending on the growth kinetics showed by the different cell lines (de Castro et al., 2013; Chapter I), in order to have synchronized at the stage of the culture cycle all the cell lines. Thus, the 4^{th}, the 8^{th} and the 12^{th} days of the culture cycle were selected for the harvest of Snh, Sh1.5 and Sh6 cells, respectively.

The entire procedure was carried out at 4°C, and all the sucrose buffers were dissolved in 0.1 M HEPES- (2-[4-(2-hydroxyethyl)-l-piperazinyl]-ethanesulfonic acid) KOH pH 7 supplemented with 1 mM DTT. Cell material was first pulverised in liquid nitrogen with a mortar and pestle and then ground for 20 min with an omni-mixer in extraction buffer (0.1 M HEPES-KOH pH 7 containing 0.4 M sucrose, 0.1% (w/v) bovine serum albumin, 1 mM DTT [added freshly], 5mM $MgCl_2$, 5 mM $MnCl_2$, 1 mM phenylmethylsulphonyl fluoride and one Roche complete protease inhibitor cocktail tablet ; Zeng et al., 2008) in a proportion of 1.5 ml extraction buffer/1 g fresh weight. The homogenate was then centrifuged at 4000 rpm for 20 min (x2) in order to discard cell debris, and the resultant supernatant was collected to obtain Golgi-enriched fractions as described in Porchia et al. (2002) with minor modifications. Briefly, the supernatant was loaded on top of 6 ml of 2 M sucrose buffer (also referred to throughout the text as cushion buffer) and centrifuged at 100000 g for 90 min in order to

separate the microsomal membranes, which after centrifugation were located on top of the cushion buffer. The upper phase was then discarded without disturbing the crude microsomal interface with the cushion buffer, and afterwards, a discontinuous sucrose gradient was developed by overlaying 5 ml of 1.3 M sucrose buffer on top of the microsomes, followed by another 5 ml of 1.1 M sucrose and 5 ml of 0.25 M sucrose. The discontinuous sucrose gradient was centrifuged at 100000 g for 2 h, and finally the 0.25 M/1.1 M sucrose interface was collected as a Golgi-enriched fraction, and stored at -80°C until use.

The Golgi-enriched fractions were then subjected to routine analysis. Golgi-membrane marker activity assays were performed using Triton-dependent IDPase (Morré et al., 1977). Total protein content was determined by using Bradford reagent (Sigma) and bovine albumin serum as standard. The Triton-dependent IDPase activity assay was carried out by diluting 200 µl of the sample in 200 µl of 50 mM Tris buffer pH 7.2 [adjusted by the addition of 2-morpholino-ethanesulfonic acid (MES)], containing: 3 mM inosine-5-diphosphate (IDP), 1 mM $MgCl_2$ and 0.01% Triton X-100. The reaction mixture was incubated at 37°C for 1 h (Weinecke et al., 1982) and released phosphate was then determined by the Ames method (Ames, 1966).

II.c. Test for protein solubilisation from transmembrane complex

Golgi-enriched fractions were incubated for 10 min on ice with two different detergents (0.5% Triton X-100 and 1% digitonine) in order to determine whether any of the treatments partially solubilised them from trans-membrane complex (Zeng et al., 2010).

II.d. SDS-PAGE analysis

Prior to SDS-PAGE, proteins were incubated for 10 min on ice with 0.5% Triton X-100. For SDS-PAGE separation, proteins were treated under reducing conditions (boiled samples) or partial non-reducing conditions (not boiled samples) and separated by standard SDS-PAGE on a 7.5%, 10% or 12.5% gel. Under non-reducing conditions, SDS was substituted

by 0.1% Triton X-100 on the stacking and separating gels to facilitate the mobility of protein complexes. The resultant gels were stained with CBB R-250 (Bio-Rad) following manufacturer's instructions.

II.e. Preparation of Golgi-enriched fractions and protein extraction for 2-D PAGE

In order to solubilise proteins from the Golgi-enriched fraction membranes as well as to shift the buffer (samples were dissolved in extraction buffer) to the appropriate for carrying out 2-D electrophoresis [lysis buffer: 20 mM Tris-HCl pH 8.8, 7 M urea, 2 M thiourea, 4% 3-3-(3-cholamidopropyl) diethyl-ammonio-1-propanesulfonate (CHAPS)], an aliquot of each sample containing approximately 4.2 μg/μl of protein was freeze-dried. The resultant freeze-dried samples were then dissolved in 2 ml of lysis buffer, incubated for 5 min in an ultrasound bath and centrifuged at 8000 rpm for 20 min. The soluble fraction (supernatant) was collected and dialysed against 20 mM Tris-HCl pH 8.8, 7 M urea and 2 M thiourea. Dialysed samples were finally concentrated by Vivaspin columns (supplied by Sartorius) until the required amount of protein for 2-D electrophoresis was obtained.

II.f. 2-D PAGE analysis

Two independent but complementary analyses of the samples were carried out by resolving the resultant gels with different stains, CBB G-250 (Bio-Rad) (Campos et al., 2010) and silver stain (Shevchenko et al., 1996; Irar et al., 2006), and performing 4 and 3 technical replicates of each analysis, respectively. For the CBB G-250 stain, gels were initially fixed in 40% methanol and 10% acetic acid, for 3 h (or alternatively overnight). Then they were incubated in a mixture of two different solutions: 98% of solution A, containing 2% orthophosphoric acid and 10% ammonium sulphate, and 2% of solution B composed of 5% CBB G-250, for 3 h (or alternatively overnight). Finally, gels were washed in Milli-Q water for 2 h (x3). In the case of silver nitrate, the procedure was carried out as follows: firstly, gels were fixed in 40% ethanol and 10% acetic acid for 3h (or alternatively overnight) and then

rinsed with Milli-Q water for 5 min. They were then soaked in 6.8% sodium acetate, 0.2% sodium thiosulfate and 30% ethanol for 30 min and washed with Milli-Q water for 5 min (x3). Subsequently, they were incubated for 20 min in a solution containing 0.1% silver nitrate and washed in Milli-Q water for 1 min (x2). Colour was developed for 3-5 min by adding 25 µl of 37% formaldehyde in a 3% sodium carbonate solution. Afterwards, gels were rinsed in Milli-Q water for 20 s, soaked in 1.46% di-sodium ethylene-di-amine-tetra-acetic acid (EDTA) in order to stop the stain and finally, washed again in Milli-Q water for 5 min (x3).

Depending on the sensitivity of the stain used, the protein concentration required varied, using 400 µg and 100 µg in for CBB G-250 and silver stain, respectively. Thus, the required amount of protein was diluted in a final volume of 350 µl of 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), and loaded onto non-linear pH 4–7, 18-cm IPG strips to carry out the first dimension. Isoelectric focusing was performed using an Ettan™ IPGphor Isoelectric Focusing System (Amersham Biosciences) with the following settings: 50 V for 10 h, 500 V in gradient for 1.5 h, 1000 V in gradient for 1.5 h, 2000 V in gradient for 1.5 h, 4000 V in gradient for 1.5 h, 8000 V in gradient for 2 h, and 8000 V holding for 10 h. Afterwards, IPG strips were collected and equilibrated. First, a 15 min treatment with 10 mg/ml of DTT dissolved in equilibration buffer (50 mM Tris-HCl (pH 8.8), 6 M urea, 30% (v/v) glycerol, 2% SDS and a trace of Bromophenol Blue), was followed by a second 15 min equilibration step of 25 mg/ml of iodoacetamide dissolved in the equilibration buffer, both of them in shacking conditions (at 180 rpm). The focused strips were then loaded on top of a SDS–PAGE 10% polyacrylamide gel (26 x 20 x 0.1 cm) and subjected to the second dimension using an Ettan DALTsix System (Amersham Biosciences) and the following running conditions: 2.5 W/gel for 30 min, followed by 20 W/gel for 4 h. Gels were finally resolved with the corresponding stain (CBB G-250 or silver stain). The experiment was carried out with three technical

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, and saved as TIFF (Tagged Image File Format) files. Image analysis was performed using ImageMaster™ 2D Platinum 5.0 Software (Amersham Biosciences) (Farinha et al., 2011). 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 normalised value and represented 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 accumulated. Statistically significant protein abundance variation was validated by Student's t-test (p, 0.05) (Farinha et al., 2011). The selected differential spots were excised from the gels and identified either by PMF using MALDI–TOF/MS or ESI-MS/MS on the Proteomics Platform (Barcelona Science Park, Barcelona, Spain). The MALDI–TOF/MS analysis was performed using a Voyager DEPRO (Applied Biosystems) instrument in 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} cyano-4-hydroxycinnamic acid (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 sufficient intensities, an automatic internal calibration of the spectra was performed. Data were generated in PKL file format and were submitted for database searching in the MASCOT server. The software packages Protein Prospector v 3.4.1 from the University of California San Francisco and MASCOT were used to identify the proteins from the PMF data (Carrascal et al., 2002). 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 mass (MM) and pI. No species restriction was applied. When an identity search produced no matches, the homology mode was used.

III. RESULTS

III.a. Obtaining Golgi-enriched fractions

Golgi-enriched fractions from Snh, Sh1.5 and Sh6 maize cultured cells were obtained by ultracentrifugation on a discontinuous sucrose gradient (Figure 1). Different combinations of sucrose concentrations for the gradient were tested in order to select the most suitable for an optimum separation of Golgi membranes from Snh, Sh1.5 and Sh6 maize cells. To determine this, gradient faces and interfaces were subjected to the IDPase activity assay, finding that the best results for separation of the Golgi bodies were achieved by using 2 M, 1.3 M, 1.1 M and 0.25 M discontinuous sucrose gradient (data not shown). In addition, the highest value of IDPase activity was detected at the 0.25 M/1.1 M sucrose interface in all the cell lines (Figure 1F), and therefore it was considered the Golgi-enriched fraction. Moreover, whereas the total protein concentration was lower in the Sh Golgi-enriched fractions than in those from Snh, the IDPase activity showed higher values in the habituated cell lines (Table 1).

III.b. Analysis of Golgi proteome

III.b.1. Procedure

First since some of the proteins contained within Golgi stacks are known to have trans-membrane domains, Golgi-enriched fractions were incubated with Triton X-100 or digitonin in order to determine whether any of these treatments would improve their solubility. No differences, at least in terms of protein populations, were found between the

detergent treatments and the control (Figure 2). However, a previous treatment with Triton X-100 was performed for the following assays as a preventive measure.

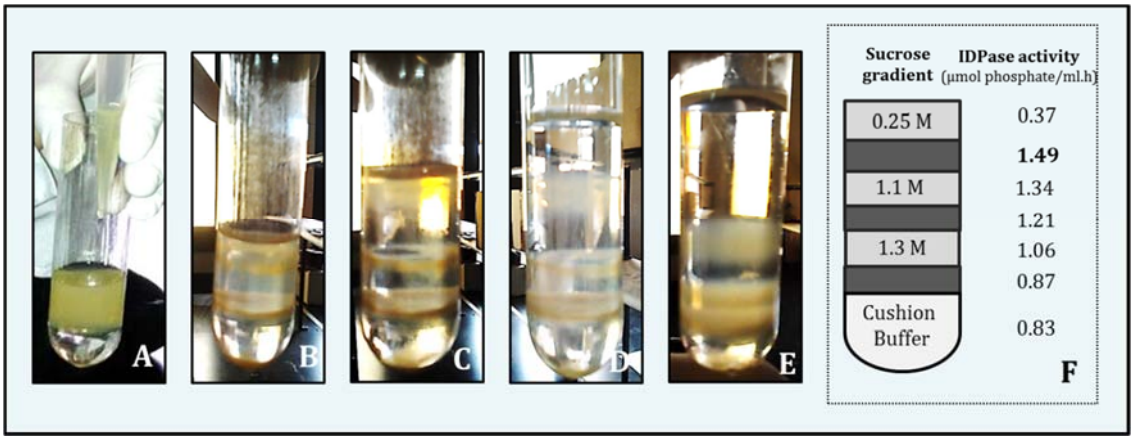


Figure 1. Performing a sucrose discontinuous gradient for the obtaining of Golgi-enriched fractions. An extract from Snh maize cell suspensions was loaded on top of the cushion buffer (**A**) and ultracentrifuged in order to get the microsomal fraction. After discarding the upper phase, 1.3 M (**B**), 1.1 M (**C**) and 0.25 M (**D**) sucrose buffers were loaded on top of the crude microsomes forming a discontinuous sucrose gradient, then subjected again to ultracentrifugation. Afterwards, triton-dependent ionidine di-phosphatase activity (IDPase) activity was tested in every interface and face (**E**), selecting the interface located between 1.1 M and 0.25 M sucrose as a Golgi enriched fraction since it showed the highest Golgi enzymatic activity marker values (**F**)

Cell line	Protein (µg/µl)	IDPase activity (µmol phosphate/mg protein)
Snh	1.90	2.1
Sh1.5	1.26	2.6
Sh6	1.10	2.7

Table 1. Protein content and IDPase specific activity on Golgi-enriched fractions from Snh, Sh1.5 and Sh6 maize cells

2-D electrophoresis was used for differential proteomic expression analyses. Since these are comparative analyses, a minimum degree of homology (≈60%) among the protein populations of the Golgi proteome from the different cell lines was required. In order to verify this, samples were previously subjected to mono-dimensional PAGE, showing that the

degree of protein population homology for both high and low MM, was satisfactory among Snh, Sh6 (Figure 3) and Sh1.5 (an exact test as in Figure 3 was performed with this cell line: data not shown) cell lines.

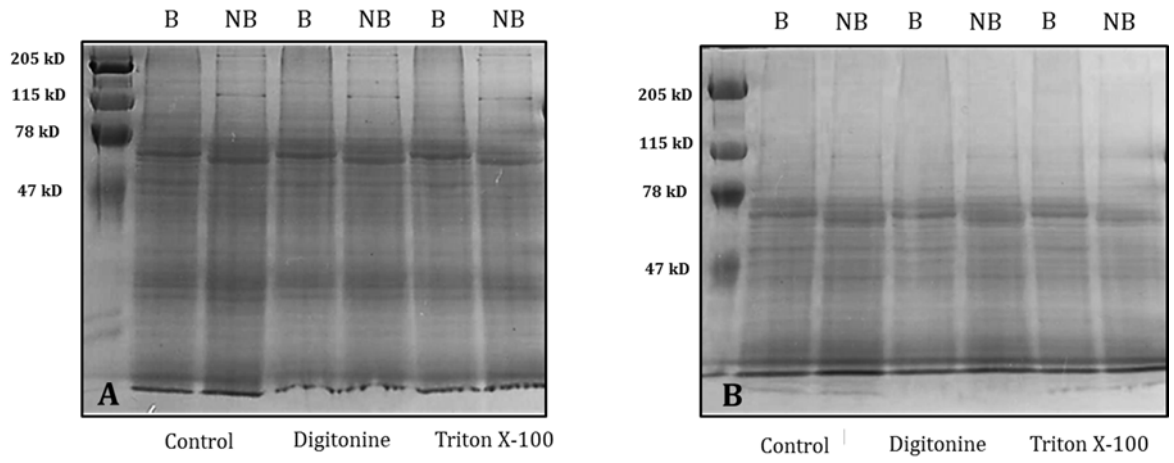


Figure 2. Incubation of Golgi-enriched fractions obtained from Snh maize cells with different detergents. Assays were carried out by incubating them on ice for 10 min with 1% digitonine or 0.5% Triton X-100. To control samples no detergent was added. Samples were boiled (B) or not boiled (NB) and subsequently subjected to (**A**) 10% or (**B**) 7.5% polyacrylamide gel electrophoresis (PAGE). 0.1% Triton X-100 was added on gels in substitution of sodium dodecyl sulphate (SDS). Gels were coomassie brilliant blue (CBB) R-250 stained. Molecular mass (MM) markers in kD are indicated at the right of the gels

Other adjustments were necessary before conducting the 2-D analysis due to the concurrence of three circumstances: a) to obtain Golgi-enriched fractions, Snh, Sh1.5 and Sh6 maize cultured cells had been homogenised in extraction buffer, which differed in composition (see materials and methods) from that used to carry out the 2-D electrophoresis, b) as previously mentioned, some of the proteins within the Golgi bodies are known to have trans-membrane domains or to form trans-membrane complexes, and such structural features rendered it indispensable to solubilise them in order to perform the 2-D analysis, requiring treatment with a powerful detergent such as CHAPS, which is typically used for this technique, and c) the required total amount of protein for the 2-D analysis had to be concentrated in a specific volume to be loaded on the IPG strip; thus, concentration of

samples was required. Therefore, a protocol for meeting these requisites was developed, and checking analyses were routinely performed after each key step of the protocol in order to detect variations in the total amount of protein as well as to determine whether any protein population was being lost as a consequence of the procedure. Neither the Golgi-enriched fraction from Snh or from Sh1.5 and Sh6 cells suffered any loss of a specific protein population (Figure 4). However, as expected, a lower total protein value was obtained when a Vivaspin concentration step was included (Table 2). Throughout the entire procedure, this total protein loss was greater in the case of the habituated cell lines (Table 2).

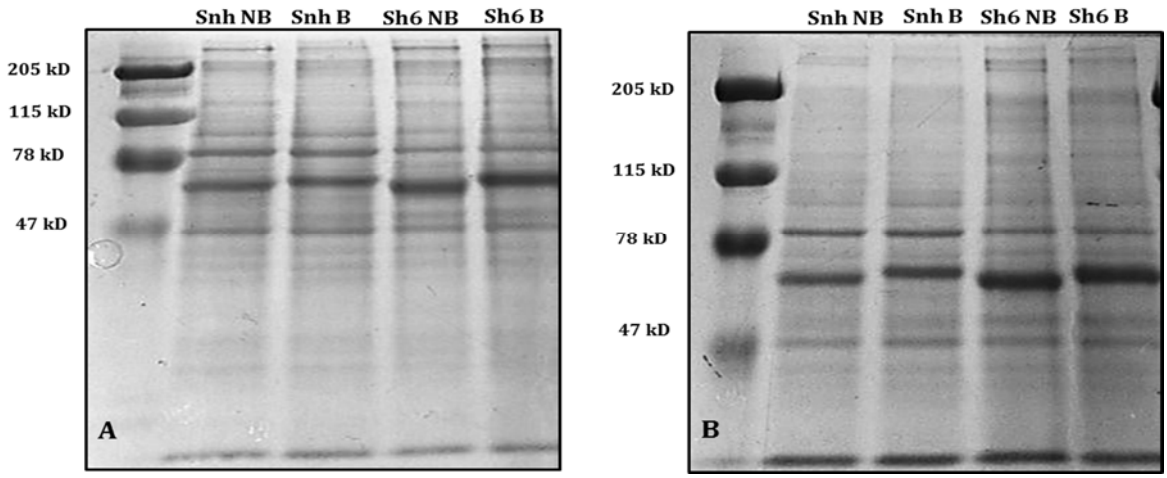


Figure 3. Golgi-enriched fractions obtained from Snh or Sh6 maize cells suspensions, incubated on ice for 10 min with 0.5% (v/v) Triton X-100. Samples were boiled (B) or not boiled (NB) and subsequently subjected to **A:** 10% or **B**: 7.5% PAGE. 0.1% Triton X-100 was added on gels in substitution of SDS. Gels were CBB R-250 stained. MM markers in kD are indicated at the right of the gel

III.b.2. 2-D electrophoresis

Once those critical points were successfully resolved, 2-D electrophoresis was performed and the resultant gels were subsequently CBB G-250 or silver nitrate stained, obtaining two complementary but independent studies of the Golgi-proteome (Figure 5). Stained gels were then analysed in order to detect differentially accumulated proteins among cell lines. In the case of the CBB G-250 study, Sh6 showed the lowest values in the number of total protein spots detected on the gels, exhibiting an 80% reduction when compared with

Snh. Although the number of total proteins observed was also lower in the case of Sh1.5 cells, this value was more similar to Snh showing only a 22% reduction when both cell lines were compared (Table 3A). As was expected, the greatest number of mis-regulated proteins was observed in the Snh *vs* Sh6 comparative study, regardless of the stain used. The number of proteins that were only present in one cell line was considerable in comparative studies in which Sh6 cells were involved. The silver nitrate study confirmed these trends in the CBB G-250 analysis (Table 3B).

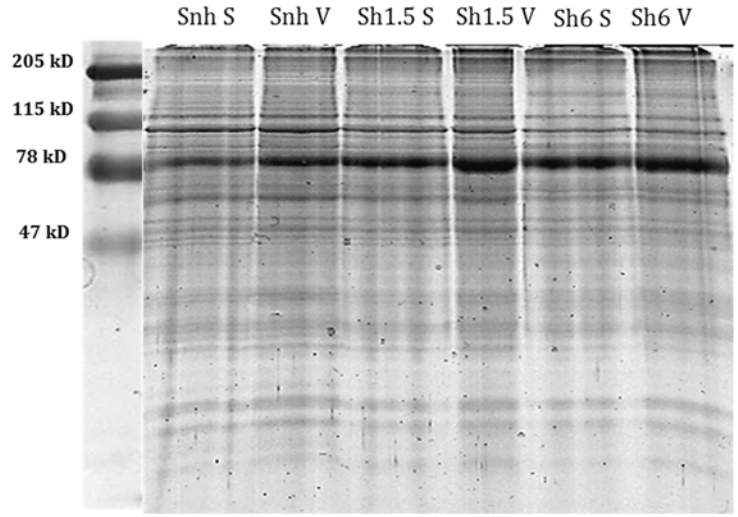


Figure 4. Verification of presence and integrity of Golgi-enriched proteins populations from Snh, Sh1.5 and Sh6 maize cell suspensions by comparing the first (solubilization: S) with the last (vivaspin concentration: V) step of the preparation process for the two dimensional (2-D) electrophoresis. Samples were boiled and subjected to 12.5% SDS-PAGE. Gel was CBB R-250 stained. MM markers in kD are indicated at the right of the gels

	Protein content in Golgi-enriched fractions (mg)		
	Snh	**Sh1.5**	**Sh6**
Re-suspension	13.3	12.8	8.9
Solubilization	13.1	12.2	8.7
Dialysis	12	9.6	7.6
Vivaspin	3.5	3.2	1.5

Table 2. Total protein content of Golgi-enriched fractions from Snh, Sh1.5 or Sh6 maize cells suspensions throughout the different steps (resuspension, solubilization, dialysis and vivaspin samples concentration) of the preparation process for the 2-D electrophoresis. Values shown were stimated in a pool of four-technical replicates

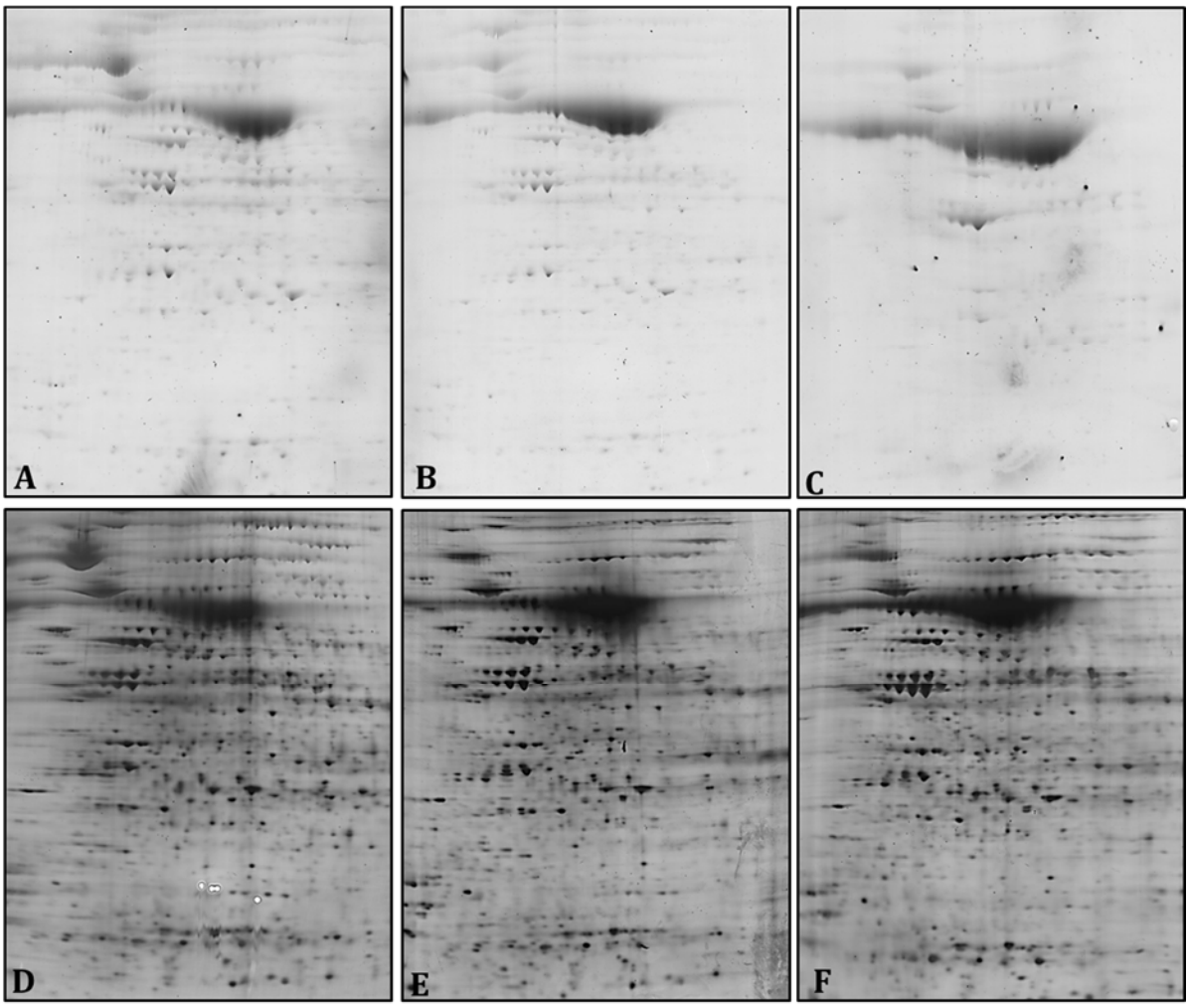


Figure 5. 2-D electrophoresis CBB G-250 (**A**, **B**, **C**) and silver-nitrate (**D**, **E**, **F**) stained gels of Golgi-enriched fractions from **A**, **D**: Snh; **B**, **E**: Sh1.5 and **C**, **F**: Sh6 maize cell suspensions

III.b.3. Sequencing

Subsequently unique and mis-regulated proteins were selected and excised from the gel, and a total of 13 proteins were identified using MALDI–TOF/MS or ESI-MS/MS (Table 4). Enzymes related to carbohydrate metabolism (enolase 1) and ethylene biosynthesis (1-aminocyclopropane-1-carboxylate oxidase 1: ACO 1 and adenosylhomocysteinase) were affected in the habituated cells. Habituation to DCB also entailed changes in the level of expression of caffeoyl-CoA *O*-methyltransferase 1 (CCoAOMT 1), an enzyme involved in lignin biosynthesis, which was found to be down-regulated. Surprisingly, in Sh1.5 and Sh6

cells, proteins related to stress responses, such as ascorbate peroxidase and chaperonin 60, were also found to be down-regulated. Another interesting finding was that an alpha-1,4-glucan-protein synthase responsible for UDP-forming was repressed in the habituated cell lines.

A

	Proteins (n)	
	CBB G-250	Silver Nitrate
Snh	652	625
Sh1.5	511	576
Sh6	128	992

B

	Comparative study	Unique proteins Snh	Unique proteins Sh1.5	Unique proteins Sh6	Mis-regulated proteins
CBB G-250	Snh *vs* Sh1.5	1	0	–	14
	Snh *vs* Sh6	7	–	0	18
	Sh1.5 *vs* Sh6	–	1	6	12
Silver nitrate	Snh *vs* Sh1.5	1	0	–	9
	Snh *vs* Sh6	4	–	1	26
	Sh1.5 *vs* Sh6	–	0	6	11

Table 3. A general overview of Golgi-enriched fractions proteome from Snh, Sh1.5 and Sh6 maize cultured cells. **A:** number of total proteins in CBB G-250 and silver nitrate proteomic studies in each cell line. **B:** number of mis-regulated and exclusive proteins detected in the comparative proteomic studies among cell lines (Snh *vs* Sh1.5 or Sh6, and Sh1.5 *vs* Sh6) in CBB G-250 and silver nitrate stained 2-D gels. Results from the CBB G-250 study were obtained from four technical replicates. Those from the silver nitrate for Snh and Sh1.5 cells were obtained from three technical replicates, whereas the value for Sh6 cells was from just two replicates

IV. DISCUSSION

Previous studies performed using a biochemical approach have demonstrated that metabolism in cellulose-impoverished cells is altered (Chapter II). Therefore, it is feasible to

hypothesise that metabolic alterations from a proteomic perspective could also be taking place.

As the relevant metabolic enzymes are Golgi-resident proteins, it was necessary to obtain Golgi-enriched fractions from the different cell lines. Typically, the isolation of such Golgi-enriched fractions has been carried out by ultracentrifugation on a discontinuous sucrose gradient. The different cellular organelles spread over the gradient, stabilising at the interfaces (among the different sucrose concentrations) depending on its density. In the present study, a protocol for obtaining Golgi-enriched fractions from Snh, Sh1.5 and Sh6 maize cultured cells was optimised based on those previously reported in other species, such as wheat (Porchia et al., 2002; Faik et al., 2002; Zeng et al., 2008), arabidopsis (Brown et al., 2007) and others (Leelavathi et al., 1970). This procedure led to two main conclusions: a) an improved separation of Golgi stacks was achieved by using 2 M, 1.3 M, 1.1 M and 0.25 M as sucrose concentrations in the gradient, and b) the Golgi-enriched fraction was the one located at the 0.25 M/1.1 M sucrose interface in all the cell lines.

Differences were found in Golgi-enriched fractions from Sh and Snh cells. An interesting finding was that the highest values of IDPase activity were detected in Sh, mainly Sh6, Golgi-enriched fractions, compared to those obtained from Snh cells. Furthermore, the amount of total protein estimated in the Sh cell lines was lower than in Snh cells. IDPase activity is a well-known and widely used Golgi-enzymatic marker (Gibeaut and Carpita, 1990; Turner et al., 1998; Zeng et al., 2010). It is considered a nucleoside diphosphatase reaction showing specificity mainly for IDP, UDP and guanosine-5-diphosphate substrates (Goldfischer et al., 1964). Thus, reactions in which UDP substrates are involved, such as polysaccharide synthesis, could be co-related with this marker activity. A previous study has demonstrated a concomitant increase in IDPase activity in maize tissues, where the carbohydrate synthesis within the Golgi apparatus and its secretion were very active (such as root cap and the epidermis) (Dauwalder et al., 1969). In contrast, in tissues where such secretory and synthesis activities had ceased (such as the vacuolated outermost cap cells of

the roots) and, therefore Golgi apparatus had reverted to a compact form, the values observed for IDPase were residual. These findings evidenced a correlation between polysaccharide synthesis and morphological differentiation for secretion of the Golgi apparatus and IDPase activity, endowing this enzymatic marker activity with a considerable functional significance. We hypothesised that a more active synthesis of polysaccharides would occur in cells habituated to DCB, in order to counteract their cellulose deficit. Thus, it would be feasible to suggest that the highest IDPase values exhibited by Sh cells could be related to an enhanced polysaccharide synthesis activity within the Golgi apparatus and/or its morphological specialisation for a secretory function.

Protein name	Behaviour	pI / MM
Chaperonine 60	Down-regulated in Sh1.5 and Sh6 when compared to Snh (C)	4.8 / 63
Chaperonine 60	Down-regulated in Sh1.5 and Sh6 when compared to Snh (S)	4.9 / 40
alpha-1,4-glucan-protein synthase [UDP-forming]	Down-regulated in Sh6 when compared to Sh1.5 (C) and Snh (S)	6.2 / 27
1-aminocyclopropane-1-carboxylate oxidase 1	Down-regulated in Sh6 when compared to Snh and Sh1.5 (C and S)	5.1 / 29
Caffeoyl-CoA O-methyltransferase 1	Down-regulated in Sh1.5 and Sh6 when compared to Snh (S)	5.1 / 25
Ascorbate peroxidase	Down-regulated in Sh6 when compared to Snh (C and S)	5.9 / 20
Enolase 1	Up-regulated in Sh6 when compared to Snh y Sh1.5 (C)	5.3 / 36
Enolase 1	Up-regulated in Sh6 when compared to Snh (S)	5.3 / 39
ATP synthase subunit alpha	Up-regulated in Sh6 when compared to Snh and Sh1.5 (S)	6.1 / 38
T-cytoplasm male sterility restorer factor 2 [*Zea mays*]	Up-regulated in Sh6 when compared to Sh1.5 (S)	6.2 / 37
Enolase 1	Only present in Sh6 when compared to Sh1.5 (S)	5.2 / 33
Enolase 1	Only present in Sh6 when compared to Sh1.5 (S)	5.5 / 30
Adenosylhomocysteinase [*Zea mays*]	Only present in Sh1.5 when compared to Sh6 (C and S)	5.7 / 35

Table 4. Identification of proteins by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF/MS) or by electrospray ionization with tandem mass spectrometry (ESI-MS/MS) after spot excision from 2-D gels. It is indicated: mis-regulation behaviour; study in which protein appears CBB G-250 (C) or silver nitrate (S); isoelectric point (pI) and MM

Despite the differences observed in the IDPase activity values, no disparity was detected in the different cell lines in terms of protein populations. This was especially relevant to ensure success in carrying out the subsequent comparative proteomic analysis of the fractions, which was performed by 2-D electrophoresis. A crucial requisite for this technique is that different samples must show the degree of homology required for the comparison, essential for the accuracy of the subsequent statistical tests for validating the study. Therefore, the fact that no qualitative differences were found in our different samples regarding protein populations guaranteed the minimum degree of homology required to conduct the 2-D analysis.

In spite of the structural complexity of the Golgi apparatus, which renders it a complicated subcellular compartment to study using modern proteomic techniques (Parsons et al., 2012), several studies have used 2-D electrophoresis to carry out an analysis of the Golgi proteome (Taylor et al., 2000; Wu et al., 2000). The proteome of a cell reflects the proteins expressed in a given moment. Undoubtedly, expression could vary depending on a wide range of factors, such as development stage or response to stress conditions, for instance, DCB-habituation itself. Therefore, the information contained in a proteome provides crucial clues to the metabolic and physiological changes occurring in a cell at a particular time (Faik, 2012). In this study, two independent but complementary analyses were conducted to examine the proteomes from the different cell lines, using two different stains for the gels: CBB G-250 and silver nitrate. The protein detection threshold of these compounds differs: whereas CBB G-250 stains the most abundant proteins, silver nitrate is more sensitive, allowing the detection of proteins in lower quantities. In addition, the stains may even detect different proteins. The number of total proteins observed in the Snh Golgi-enriched fraction was much lower than observed in the total proteome of non-habituated maize cultured cells (Mélida et al., 2010a). This finding was predictable, as we were working with a partially purified fraction of the proteome. Sh cells showed lower quantities of total proteins on the gels, and this circumstance was especially pronounced in the Sh6 cells, which

exhibited an 80% reduction when compared to Snh. Again, this result was not surprising since this trend had previously been observed in the determination of the total protein concentration assayed by the Bradford method. It should be noted that the Sh6 cells had been habituated to a high level of DCB, which could be expected to provoke drastic metabolic and physiological alterations. This would also explain the greater number of unique or mis-regulated proteins detected in comparative studies involving Sh6 cells.

Differentially accumulated and unique spots and were identified by MALDI-TOF/MS and ESI-MS/MS. The magnitude of mis-regulation seemed to have a positive correlation with DCB habituation level, showing higher values in the Sh6 cell Golgi-enriched fractions. Some of the sequenced proteins are known not to be Golgi-resident proteins; however, it should be borne in mind that 1) the study was conducted with Golgi-enriched (not purified) fractions therefore, contamination with other endomembrane system organelles was impossible to avoid since they are highly interconnected and have similar densities or sizes (Hanton et al., 2005; Parsons et al., 2012), 2) it is well known that many protein modifications take place within Golgi apparatus, making it very complicated to distinguish between Golgi-resident proteins and transient proteins, 3) one of the methodological restrictions of 2-D electrophoresis is that abundant proteins can mask minority ones (Timperio et al., 2008). This means that if a non-specific Golgi protein undergoes any kind of mis-regulation and is present in a greater amount than Golgi-resident ones, the magnitude of its mis-regulation would probably be higher than that exhibited by minority Golgi-resident proteins, which would make it a firm candidate for sequencing. This could have been the case of the chaperonins, known to be involved in a wide range of protein-assisted functions and therefore their presence is to be expected in an organelle in which such an extensive modification of proteins is taking place.

Interestingly, several of the mis-regulated proteins reported here, such as enolase 1, chaperonin 60 and ACO 1, were also detected as being mis-regulated in a previous analysis of the total proteome of maize DCB-habituated cells (Mélida et al., 2010a). This indicates that

they play an important metabolic role in cellulose-deficient maize cells and confirms the reliability of the methodological procedure designed for the present study as well as the results obtained. Another concurrence when comparing both studies was related to ascorbate peroxidase. Assays of its detoxifying activity in habituated cells revealed that this was slightly lower when compared with non-habituated cells (Mélida et al., 2010a). Thus, this finding is in good agreement with its down-regulated expression detected in this study.

Enolase 1 was found to be up-regulated in the habituated cells and its level of mis-regulation was correlated with DCB habituation level, being higher in Sh6 cells. Although enolase is involved in carbohydrate metabolism, through catalysing the reversal conversion of the hexose glucose to pyruvate, it has also been related in maize to different stressors, such as anaerobic conditions (Sachs et al., 1996). More interestingly, a series of studies have indicated that hexoses may act as stress indicators when cell walls are damaged (Hamann et al., 2003; Wolf et al., 2012). This concurs with our observation of a higher level of up-regulation in maize cells habituated to high DCB concentrations (Sh6) when compared with low-level habituation (Sh1.5) and Snh. Sh6 cell walls were more seriously impaired, with a 70% impoverishment in cellulose content (Mélida et al., 2009), whereas this figure was the 33% for Sh1.5 (de Castro et al., 2013; Chapter I), when compared with Snh. Since enolase 1 was identified several times (and was probably the same protein but with different post-transcriptional modifications), it would be reasonable to assume that it plays an important role as a stress indicator.

Another coincident protein was chaperonin 60, which was down-regulated in Sh1.5 and Sh6 cell lines. Chaperonins are known to play an active part in protein folding and re-folding, assembly, translocation and degradation in many cellular processes, and because of these functions they can re-establish cellular homeostasis when plants are under stress conditions (Wang et al., 2004; Hu et al., 2009). It has been described elsewhere that some members of chaperonin 60 class are involved in cell death and activating systemic acquired resistance (Ishikawa et al., 2003). Focusing on the context of stress conditions, such as DCB

habituation, one could expect to find them up-regulated. Nevertheless, members of the chaperonin 60 class have also been reported to be involved in the folding of tubulin and actin (Gutsche et al., 1999), and it should be noted that the DCB action mechanism affects microtubul integrity (Bisgrove and Kropf, 2001; Himmelspach et al., 2003; Rajangam et al., 2008). Thus, this finding would be a possible hypothesis for the repression observed in the habituated cells, although further evidence is needed.

Another down-regulated protein found in the habituated cells was CCoAOMT 1, involved in lignin biosynthesis, more specifically in the G and S units. Two enzymes may catalyse the production of both units, but differences in their catalytic preferences have been suggested although not proven: whereas CCoAOMT preferentially synthesises G units, caffeic acid 3-*O*-methyltransferase (COMT) tends towards S ones (Guo et al., 2001). In addition, it has been suggested that CCoAOMT is partially replaced in DCB-habituated cells by COMT (Mélida et al., 2010a), and its down-regulation has been related to qualitative and quantitative changes in lignin composition in alfalfa, showing a decrease in G units with no apparent effect on S ones (Guo et al., 2001). These qualitative differences are consistent with unpublished results obtained in our laboratory, which showed that the DCB-habituation entailed a reduction in the amount of G lignin units whereas S levels remained unaltered. Thus, the CCoAOMT 1 down-regulation exhibited by habituated cells in this study is in agreement with these findings, suggesting a role for this protein in the DCB coping strategy.

An α-1,4-glucan protein synthase (UDP-forming) was another of the mis-regulated proteins, showing a higher level of repression in the Sh6 cell line. This enzyme is a reversible glycosylation polypeptide (RGP), a group of Golgi-resident proteins (Dhugga et al., 1991) involved in cell wall polysaccharide synthesis (Dhugga et al., 1997; Langeveld et al., 2002) and it has been suggested that it forms part of a large Golgi-complex which includes not only GTs but also RGPs and multimembrane-spanning transporter proteins (Oikawa et al., 2012). Consistent with this statement, its involvement in xyloglucan synthesis had been described in pea and potato (Dhugga et al., 1997; Wald et al., 2003). A previous study carried out on

arabidopsis with a double mutant for two RGP genes showed that its pollen development was extremely abnormal, exhibiting a poorly defined inner cell wall layer (Drakakaki et al., 2006). This would suggest that the absence of RGPs is related to a weakened cell wall, which is in good agreement with the down-regulation of the α-1,4-glucan protein synthase observed in the cellulose-deficient walls in habituated cells.

The enzymes related to ethylene metabolism, ACO 1 and adenosylhomocysteinase, were also found to be differentially accumulated in the habituated cells. Focusing on the former, ACO 1, which is involved in ethylene biosynthesis, it was found to be down-regulated in the habituated cell lines. Ethylene synthesis requires the conversion of methionine to adenosylmethionine by the adenosylmethionine synthetase (ACS) to 1-aminocyclopropane-1-carboxylic acid (ACC), which is then used as a substrate of ACO to produce ethylene. Pathway regulation is normally conducted by ACS, but in conditions of excessive ethylene concentrations, ACO could also act as a regulating enzyme. A role for ACC in signalling, independently of its involvement in ethylene biosynthesis, has also been reported (Vandenbussche et al., 2012; Yoon and Kieber, 2013). Interestingly, this suggestion arises from two studies in which reduced cellulose content conditions were involved (Xu et al., 2008; Tsang et al., 2011). Furthermore, the later one hypothesised a role for ACC in the regulation of the cell wall sensing system when its integrity is impaired, based on the results obtained from the inhibition of cellulose biosynthesis by isoxaben in root epidermal cells (Tsang et al., 2011). This connection between reduced cellulose content and ACC as a signalling molecule renders it feasible to hypothesise that in our cellulose-deficient cells, a down-regulation of ACO 1 would lead to an increase in ACC content, activating signalling pathways for the subsequent triggering of coping strategies.

Overall, our results strongly suggest that some of the identified proteins were differentially accumulated because they play a role in the DCB-coping strategy adopted by habituated cells. However, in other cases, the mis-regulation of protein expression observed seemed to be related to DCB effects, probably not having any specific “tactical” function as

part of a response against the inhibitor. Nevertheless, recent studies have demonstrated the existence of Golgi protein interaction. Furthermore, when protein members of this complex vary, their biochemical and catalytic functions also differ (Oikawa et al., 2012). Thus, the possibility cannot be ruled out that some of the mis-regulated proteins observed in this study, although at first glance not clearly involved in any expected physiological response to DCB, may play a role through their interaction with others.

Discussion

Habituation to DCB involves various strategies for modifying cell wall architecture and thus overcoming the constraint implied by having cellulose-deficient walls. In the specific case of maize cells, it has previously been reported that in cultures habituated to otherwise lethal DCB concentrations, a quantitatively and qualitatively re-modelled and modified arabinoxylan network plays a key role in many of these strategies (Mélida et al., 2009; 2010a; 2010b; 2011). However, it has also been demonstrated that the variety of coping responses depends on the cell wall type as well as on the DCB concentration and length of exposure time to it (Alonso-Simón et al., 2004; Mélida et al., 2009). Since previous studies on DCB-habituated maize cultured cells have been achieved by the use of high DCB concentrations and long-term habituation periods, data about the early modifications taking place during the habituation process are lost. Such information would be highly valuable because in the earliest stages of DCB habituation, cells would present a wide variety of responses. In addition to cell wall alterations, DCB-habituated cells are likely to have a modified metabolism. Therefore, the general aim pursued in the present thesis was to clarify the mechanisms responsible for the structural plasticity of maize cell walls during early DCB-habituation, whilst also focusing on metabolic strategies.

However, it was first necessary to obtain a cell line with these features. Extensive monitoring was performed of maize cell lines habituated to low DCB levels, using a set of techniques such as FTIR spectroscopy and cellulose content determination (de Castro et al., 2013; Chapter I, Figures 1 to 4). The results obtained from this preliminary stage revealed the Sh1.5 cell line was the most suitable for use in order to accomplish the objective.

As expected, the main modification detected in the walls of Sh1.5 cells pertained to cellulose content, with a 33% reduction. When compared to the value reported for maize cells habituated to high DCB levels (75%; Mélida et al., 2009), the cellulose reduction in our cells can be considered mild. Likewise, our results showed an induction of *ZmCESA7* and *ZmCESA8* genes in several short-term habituated maize cells (de Castro et al., 2013; Chapter I, Figure 5), as was also observed in the maize cells subjected to long-term habituation to

high DCB concentrations (Mélida et al., 2010a). This coincidence could indicate that CESA7 and CESA8 may be more resistant to the effects of DCB, playing a role in habituation at all levels. Characterisation of the Sh1.5 cell line and walls revealed features common to all levels of DCB habituation in maize cells, such as delayed growth kinetics (de Castro et al., 2013; Chapter I, Figure 6), cellular development in larger clusters (de Castro et al., 2013; Chapter I, Figure 7) as well as a net increase in arabinoxylans (de Castro et al., 2013; Chapter I, Figure 8). However, interesting differences were also found.

In our mildly cellulose-deficient cells an increase was observed in the amount of easily extractable arabinoxylan populations when compared to those extracted from Snh cells walls (de Castro et al., 2013; Chapter I, Figure 8). This was in clear contrast to the lower extractability degree reported for arabinoxylans from maize cells habituated to high DCB levels (Mélida et al., 2009; 2011), indicating that the degree of arabinoxylan extractability from the cell wall differed depending on habituation level. Furthermore and surprisingly, the M_r and M_w analyses of recently synthesised arabinoxylans in Sh1.5 cells revealed smaller molecules and a more homogeneously sized population than arabinoxylans observed in the Snh cell line (Chapter III, Figures 1 and 2). This trend was consistent throughout the culture cycle, and was applicable to all hemicelluloses and polymers obtained from the protoplasm, cell wall and extracellular medium. When the M_w of protoplasmic polymers and cell wall bound hemicelluloses was compared, the results showed that newly synthesised protoplasmic polymers underwent extensive cross-linking when bound to the cell wall (Chapter III, Figure 2). This finding has been reported previously in cultured maize cell suspensions (Kerr and Fry, 2003), and the most plausible explanation is that polysaccharides undergo a grafting process after being secreted into the cell wall. In maize cells the dimerisation or oligomerisation by peroxidase action of Fer residues attached to the arabinoxylan chains seems to be mainly responsible for this arabinoxylan grafting process (Fry et al., 2000). However, Sh1.5 cells showed a less pronounced degree of cross-linking when compared with Snh cells, and this finding is in contrast to the crucial role attributed to

this type of molecular interaction in maize cells habituated to high DCB concentrations (Mélida et al., 2011). An analysis of Cinn metabolism and characterisation of its derivatives in the cell wall (Chapter III, Figures 6 to 9) showed that Sh1.5 cells exhibited a poor capacity to synthesise polysaccharides bearing Fer or *p*-Cou residues or graft them to the wall. Furthermore, arabinoxylans and polysaccharides in general had an altered cellular fate in Sh1.5 cells, showing a reduced amount of strongly wall bound hemicelluloses and an increased presence of SEPs in the culture medium (Chapter II, Figures 2, 5 and 6). Taken together these results support the hypothesis of a diminished capacity of Sh1.5 to carry out cross-linking of polysaccharides. However, when Fer dimer and oligomer content was relativised, it was observed to be higher in Sh1.5 cells. This finding indicated that the lower M_w detected in arabinoxylans from Sh1.5 cells was not related to a deficient capacity to carry out phenolic oxidative coupling of arabinoxylans. Thus, it is feasible to assume that although they have a lower M_w, arabinoxylans from Sh1.5 cells are actually oxidative cross-linked. Therefore, the coupling of arabinoxylans by peroxidase action seems to be another shared feature of DCB-habituation in maize cells to all concentrations, despite the M_w of the molecule.

Regarding the increased presence of SEPs detected in the extracellular medium of Sh1.5 cells, it should be noted that this polymer population may have its origin in a) polymers that are sloughed into the culture medium immediately after synthesis without being grafted onto the cell wall, or b) in polymers that were actually integrated into the wall but later loosened and were released into the culture medium (Kerr and Fry, 2003; Mélida et al., 2011). However, this latter possibility can be ruled out since wall bound hemicelluloses were not observed to decrease simultaneously with an increase of SEPs in the culture medium. Hence, the most probable explanation is that due to the reduction in cellulose content, and therefore in polysaccharide-cellulose linking points, Sh1.5 cells sloughed an increased amount of polysaccharides into the culture medium because of the impossibility of grafting them onto the cell wall.

Since the reduction in the cellulose content detected in cells habituated to low concentrations of DCB was considerably lower than in those habituated to high concentrations (33% *vs* 75%), the possibility arises that Sh1.5 cells synthesised an increased amount of hemicelluloses with a lower M_w as part of their coping strategy. In this respect, it has previously been reported that a lower M_w would facilitate binding to cellulose in hemicelluloses, and more specifically in xyloglucan (Lima et al., 2004). Furthermore, it has been suggested that lower M_w xyloglucan molecules in conjunction with an increased xyloglucan-endo-transglucosylase activity would contribute to rigidifying the cellulose-deficient walls of DCB-habituated bean cells (Alonso-Simón et al., 2007). Previous data from the analysis of the M_w of hemicelluloses in maize cells habituated to medium and high DCB levels (Mélida et al., 2009), suggest that as the level of DCB habituation rises (and therefore cellulose content decreases) the M_w of wall bound hemicelluloses also increases progressively. The results obtained in this study from an analysis of the expression of arabidopsis *IRX* orthologue genes are consistent with this hypothesis. Likewise, the genes involved in initiation and elongation of xylan chains were found to be induced in maize cells habituated to high DCB levels. In contrast, initiation of xylan synthesis was unaffected in Sh1.5 cells, whereas elongation was impaired, which would partially explain why Sh1.5 cells had shorter hemicellulose chains (Chapter III, Figure 10).

The next question to arise was whether any feature of arabinoxylans from Sh1.5 cells differed when compared with those from Snh. The increased Ara/xylan ratio detected (Chapter II, Table 1) would suggest the synthesis of more substituted arabinoxylans. Certainly, other sources for Ara residues besides arabinoxylans, such as arabinogalactan proteins or rhamnogalacturonan side chains (for a review see Fry, 2011), can exist. Nevertheless, a sugar analysis of 0.1 M and 6 M alkali-extracted fractions from Sh1.5 cell walls, revealed a poor content of uronic acids, Gal or Rha residues (de Castro et al., 2013; Chapter I, Figure 8). Therefore, it can be assumed that most of the Ara residues actually derived from arabinoxylan molecules.

Another interesting finding was that other polysaccharides, such as xyloglucan, rhamnogalacturonan I and homogalacturonan with a low degree of esterification, seem to be involved in the early events of DCB habituation (de Castro et al., 2013; Chapter I, Figure 9B). The reduction in cellulose content in DCB-habituated bean cells was compensated for by an increase in pectic polysaccharides (Encina et al., 2001; García-Angulo et al., 2006). Interestingly, although habituated maize cells have type II walls in which xyloglucan and pectin polysaccharides are not major components, they nevertheless shared features with habituated bean cells which have type I walls where these polysaccharides are more abundant. In addition, pectin or xyloglucan content in maize cells habituated to high DCB levels generally remained unmodified, but in some cases was even reduced (Mélida et al., 2009).

The continued albeit reduced presence of xyloglucan in the cell walls of graminaceous monocots plants, suggests it plays an important role. Thus, a high-M_w complex of xyloglucan thought to be linked to other polysaccharides would explain, at least in part, why graminaceous monocots can survive with considerably smaller amounts of this type of hemicellulose than dicots (Kerr and Fry, 2003). It was therefore remarkable to find a higher M_w population of strongly wall bound xyloglucan in Sh1.5 cells (de Castro et al., 2013; Chapter I, Figures 9B and 10; Chapter III, Figure 5B). Hence, a role for xyloglucan in the coping strategies of mildly cellulose-deficient cells cannot be ruled out. Since the compositional analysis of cell wall fractions revealed that neither Snh or Sh1.5 cells were preferentially tightly or weakly-grafting xyloglucan or arabinoxylans onto the cell wall (Chapter II, Figures 4 and 5), it could be hypothesised that the reinforcing strategy is based on changes in their M_w rather than in the strength of their bond to the cell wall.

The observation that epitopes of other cell wall polymers, such as homogalacturonan with a higher degree of esterification or the arabinan side chains of rhamnogalacturonan I, did not appear to be modified in Sh1.5 cells indicates that rather than an overall reshuffle of

the cell wall taking place, only some specific subsets of polysaccharides were affected during early DCB habituation.

Therefore, these results demonstrate the capacity of maize cell cultures to modify their cell wall architecture in order to counteract different DCB habituation frameworks. However, reduced cellulose content coping strategies were not limited to cell wall architecture alone. DCB-habituated maize cells also showed general metabolic changes, not only pertaining to the synthesis and cellular fate of hemicelluloses and polymers, which were delayed, less efficient and altered (Chapter II and III), but also to other important cellular processes (Mélida et al., 2010a). With regard to metabolism, the Golgi apparatus is known to be a one of the most important organelles in the eukaryotes; it is where polysaccharides in plants are synthesised and proteins are processed and undergo post-translational modifications (Parsons et al., 2012). Thus, information about the Golgi proteomic picture from cellulose-deficient cells would provide valuable information about how proteins may contribute to the plasticity of responses shown by such cell lines. To this end, a protocol for obtaining of Golgi-enriched fractions from maize cells habituated to high and low DCB levels and non-habituated, was optimised using discontinuous sucrose gradient ultracentrifugation (Chapter IV, Figure 1). A general characterisation of these fractions revealed that although they contained a lower amount of total proteins, habituated cells exhibited higher IDPase activity (a Golgi-enzymatic marker) values (Chapter IV, Table 1). The Golgi apparatus was observed to undergo morphological differentiation when synthesis and secretion of polysaccharides took place, but reverted to its compact morphology when both processes ceased (Dauwalder et al., 1969). Interestingly, in this latter study, higher IDPase activity values were detected in tissues where polysaccharide synthesis and secretion was occurring. In contrast residual IDPase activity values were observed once the processes had halted. These findings suggest that IDPase activity is co-related to polysaccharide synthesis and Golgi-morphological differentiation for secretion. Therefore, our results may indicate an enhanced synthesis of polysaccharides in cellulose-deficient cells in order to counteract this

lack. Golgi-enriched fractions were then subjected to 2-D electrophoresis and the mis-regulated proteins detected were studied (Chapter IV, Table 4).

Coincidences were found between some mis-regulated proteins (enolase 1, chaperonine 60 and ACO 1) from Golgi-enriched fractions and a previous study of the total proteome of habituated maize cells (Mélida et al., 2010a). These concurrences suggest they play an important role in cellulose-deficient cells. In addition, ascorbate peroxidase was found to be down-regulated in accordance with its lower activity detected in DCB-habituated cells (Mélida et al., 2010a). Mis-regulation of some of the sequenced proteins would be related to some of the effects provoked by DCB. For instance, chaperonin 60 has been described to be involved in actin and tubuline folding (Gutsche et al., 1999), and since DCB disrupts microtubule structure (Bisgrove and Kropf, 2001; Himmelspach et al., 2003; Rajangam et al., 2008), this would be a possible explanation for its down-regulation in habituated cells.

Another example would be the repression observed in habituated cells of the RGP α-1,4-glucan protein synthase (UDP-forming). Although some RGPs are involved in cell wall polysaccharide synthesis (Dhugga et al., 1997; Langeveld et al., 2002), their absence could be related to a weakened cell wall, as in the case of an arabidopsis double mutant for two RGP genes reported in Drakakaki et al. (2006). In contrast, other differentially accumulated proteins in habituated cells seem to participate in DCB coping strategies, whilst others act as indicators when cell wall integrity is damaged. For example, enolase 1, which was up-regulated in habituated cells, has been reported to act as a stress indicator in maize (Sachs et al., 1996), and also as its substrate (hexoses) when cell wall integrity is impaired (Hamann et al., 2003; Wolf et al., 2012). In addition, another enzyme found to be down-regulated in habituated cells was ACO 1, which participates in ethylene biosynthesis. But more than the protein, it is its substrate, ACC, which is of considerable physiological importance when cellulose biosynthesis is inhibited, as it is involved in cell wall sensing when its integrity is damaged (Tsang et al., 2011). Therefore, a possible explanation for the down-regulation of

ACO 1 in our habituated cells would be that it provokes an increase in ACC content which would induce the activation of the corresponding signalling pathways to initiate the coping strategy.

CCoAOMT 1 involved in the G and S units of lignin, was found to be down-regulated in the DCB-habituated cells. This enzyme was previously thought to be substituted to some extent in DCB habituated cells by COMT (involved in S unit synthesis) (Mélida et al., 2010a). Furthermore, qualitative and quantitative changes in lignin composition in alfalfa have been related to CCoAOMT repression (Guo et al., 2001). Interestingly, similar qualitative differences such as those reported in the previously mentioned study have also been demonstrated in unpublished results from our laboratory. This study showed that the proportion of G lignin units was reduced as a consequence of the DCB-habituation, with no apparent effect on S units.

It is possible that not all the sequenced proteins were Golgi-resident. Nevertheless, the study was carried out on Golgi-enriched fractions, which could be expected to be contaminated with other, extremely difficult to avoid organelles (Hanton et al., 2005; Parsons et al., 2012). In addition, since one of the most important functions of the Golgi apparatus is the processing of protein modifications, it is difficult to discriminate between Golgi-resident and others merely in transit. Although the methodology used, 2-D electrophoresis, has been demonstrated to be successful in the analysis of similar samples in other species (Taylor et al., 2000; Wu et al., 2000), it also presents an important limitation, namely that abundant proteins can mask minority ones (Timperio et al., 2008). Nevertheless, an interaction has recently been reported in the Golgi protein forming complex and, depending on the protein complex composition, its catalytic function also differs (Oikawa et al., 2012). Therefore, although not expected to have a clear physiologic role in the response to DCB, the possibility that some of the mis-regulated proteins may be involved through interaction with others should be taken into consideration.

Since habituated cells showed modified arabinoxylan networks, it is feasible to assume some degree of mis-regulation in their synthesising-enzymes. Cell wall polysaccharides synthesis and maturation has recently been discovered to initiate in different Golgi cisternae, and localisation varies depending on the cell type (Driouich et al., 2012; Worden et al., 2012). Thus, it is to be expected that the GTs responsible for a particular step of the synthesis and/or maturation would also be located in their corresponding Golgi-cisternae. Therefore, it would be of interest in future experiments to perform a further fractionation of the membranes contained in the Golgi-enriched fractions in order to obtain glucuronoarabinoxylan synthase-enriched fractions and assay their enzymatic activities.

The results obtained in the present study confirm that maize cell cultures have the capacity to adopt different coping strategies depending on the DCB habituation framework, using mechanisms based not only on modification of cell wall composition but also on important variations in metabolic and gene expression. These strategies showed common features throughout the different DCB habituation levels, such as a modified arabinoxylan network, changes in the level of expression of genes involved in cellulose or xylan biosynthesis, and the mis-regulation of metabolic proteins; however, alterations which were exclusively associated with particular DCB-habituation levels were also found, thus demonstrating the remarkable dynamic plasticity of maize cells in coping with different degrees of cellulose content reduction.

Conclusions

V. CONCLUSIONS

1. A maize cell suspension line habituated to 1.5 µM dichlobenil -DCB- (Sh1.5) showed features of early DCB-habituation. Sh1.5 cells presented alterations in their growth pattern, with delayed growth kinetics, longer doubling times and lower growth rates than non-habituated cells (Snh). In addition, Sh1.5 cells developed larger cell clusters. Their cell wall composition was also modified, with a mild reduction (33%) in cellulose content that was partially compensated for by an increase in arabinoxylans. However, other polysaccharides such as xyloglucan or rhamnogalacturonan I also seemed to be involved, although to a lesser extent.

2. Arabinoxylans from Sh1.5 cells were more homogeneously sized with higher extractability and lower relative molecular mass and weight-average than those from Snh cells. This finding was consistent throughout the culture cycle in both the protoplasm and cell wall, as well as in the extracellular medium. The cellular fate of polysaccharides also modified, with reduced proportions of strongly wall bound ones and an increased presence of the polysaccharides sloughed into the culture medium. However, an analysis of phenolic metabolism revealed that this latter finding was not related to a diminished capacity for cross-linking.

3. Expression of the genes involved in cellulose biosynthesis was also affected during short-term habituation to DCB. *ZmCESA7* and *ZmCESA8* isoforms were induced when the number of culture cycles grown in the presence of DCB was increased or when the inhibitor concentration exceeded a given threshold, leading to the hypothesis that although less efficient in the cellulose synthesis, CESA7 and CESA8 proteins would be more resistant to the effects of DCB. Furthermore, the lower expression of arabidopsis *IRX* orthologues, involved in initiating the synthesis and elongation of

xylan chains, suggested that only elongation was impaired in Sh1.5 cells, which would partially explain the lower molecular relative weights detected.

4. Low-level DCB habituation also entailed modifications in cultured cell metabolism, which was slower and less efficient throughout the entirety of the culture cycle. Nevertheless, although delayed with respect to Snh cells, synthesis, wall-grafting and sloughing of the different types of polysaccharides and polymers occurred synchrony.

5. Important metabolic enzymes related to stress conditions, ethylene biosynthesis and carbohydrate and lignin metabolism were also differentially accumulated in Golgi-enriched fractions from DCB-habituated cells. The mis-regulation observed could be related to the direct effects of DCB on the cells, as in the case of chaperonin 60 and the reversible glycosylation polypeptide α-1,4-glucan protein synthase (UDP-forming), or may have reflected a role in the DCB-coping strategies adopted by the habituated cells such as enolase 1, 1-aminocyclopropane-1-carboxylate oxidase 1, ascorbate peroxidase and caffeoyl-CoA *O*-methyltransferase 1.

Most of these results show that some of the changes that occurred in the cell walls in order to compensate for the lack of cellulose 1) differed according to the DCB-habituation level, 2) implied modifications of cell wall architecture and several metabolic features, and 3) illustrate the capacity of plant cells to adopt appropriate coping strategies depending on the herbicide concentration and length of exposure time.

Resumen

INTRODUCCIÓN

La pared celular de las plantas

Los protoplastos de las células de las plantas terrestres están rodeados por una capa semirrígida: la pared celular. A pesar de que el término para designarla induzca a pensar en una estructura impenetrable y estática, se trata de un compartimento celular muy dinámico con importantes funciones tanto estructurales como fisiológicas. Las paredes celulares guían, restringen y determinan el crecimiento celular y tienen un papel crucial en la especialización funcional de los diferentes tipos celulares. Al constituir la barrera externa entre la célula y el medio se encuentran implicadas en la señalización de respuestas a situaciones de estrés (Roberts, 2001; Hückelhoven, 2007; Driouich y col., 2012) y procesos de reconocimiento célula-célula así como célula-patógeno (Vorwerk y col., 2004; Seifert y Blaukopf, 2010; Wolf y col., 2012). Además, presentan una elevada plasticidad estructural y de composición, que permite a las células adaptarse a condiciones de estrés, tanto abióticos como bióticos (Hamann y col., 2009). A parte de sus funciones fisiológicas, las paredes celulares también tienen un importante valor económico, ya que influyen en la textura y el valor nutricional de la mayor parte de los productos que se obtienen de plantas, son un factor clave en su procesamiento, y se consideran fuente y reservorio de energía en plantas para la producción de biocombustibles (Burton y Fincher, 2012).

La pared celular primaria es exclusiva de células que aún mantienen la capacidad de dividirse y/o elongarse. Finalmente, en algunas células especializadas aparece una estructura multicapa, que es la pared secundaria, de mayor espesor y ordenación más regular de las microfibrillas de celulosa y que suele presentar depósitos de lignina y suberina.

La síntesis de los diferentes componentes de la pared celular ocurre en distintos compartimentos celulares. Las microfibrillas de celulosa se sintetizan en la membrana plasmática por acción del complejo enzimático celulosa sintasa (CSC), y se depositan

directamente en la pared. Los polisacáridos no celulósicos (hemicelulosas y pectinas) se sintetizan en el aparato de Golgi, y las diferentes proteínas, tanto estructurales como enzimáticas, se sintetizan en el retículo endoplásmico rugoso, aunque con frecuencia las proteínas estructurales sufren glicosilaciones y otras modificaciones en el Golgi. Los distintos componentes no celulósicos de la pared celular se empaquetan en vesículas secretoras y se transportan a la superficie celular, donde se integran con las microfibrillas de celulosa sintetizadas *de novo* (Carpita y McCann, 2000).

Arquitectura de la pared celular primaria

La pared celular primaria se compone de tres redes estructuralmente independientes pero interconectadas (Carpita y McCann, 2000). La primera de estas redes está conformada por un armazón de celulosa-hemicelulosas, la segunda red está constituida por polisacáridos pécticos, y la tercera red consiste en proteínas estructurales y compuestos fenólicos.

En las plantas superiores se distinguen dos tipos de paredes celulares primarias (Carpita y Gibeaut, 1993) que difieren en la composición química y en la asociación a diferentes taxones: la pared celular primaria tipo I, que es característica de dicotiledóneas y algunas monocotiledóneas (no commelinoides), y la pared celular tipo II presente en algunas monocotiledóneas (commelinoides) (Popper, 2008).

Composición de la pared celular tipo II

Celulosa

El contenido de celulosa en las paredes celulares primarias, tanto tipo I como tipo II, varía entre el 15 % y el 30 %, siendo este porcentaje mayor en la pared celular secundaria (Carpita y McCann, 2000). En el caso de células cultivadas *in vitro*, la proporción de celulosa se sitúa en torno a un 20% (Blaschek y col., 1981).

La celulosa en las paredes celulares primarias se dispone formando microfibrillas,

que son estructuras insolubles compuestas por 36 cadenas de β-1,4-glucano dispuestas en paralelo (Delmer, 1999; Saxena y Brown, 2000; Lerouxel y col., 2006). Las cadenas de β-1,4-glucano adquieren una estructura espacial plana que permite la interacción de unas con otras mediante la formación de puentes de hidrogeno intra e intercatenarios, que dan lugar a una estructura cristalina muy estable (Somerville, 2006).

La síntesis de la celulosa ocurre en la membrana plasmática, siendo depositada en la pared celular (Guerriero y col., 2010). La maquinaria encargada de su síntesis es un complejo proteico CSC o rosetas (Brown, 1996; Kimura y col., 1999). En plantas, los CSCs se encuentran organizados en hexámeros constituido por proteínas denominadas celulosa sintasa (CESA).

Las proteínas CESA son codificadas por familias multigénicas, con un número variable de genes dependiendo de la especie. En arabidopsis se han identificado 10 genes *CESA*, mientras que en maíz han sido 12 (Holland y col., 2000; Appenzeller y col., 2004). En maíz los genes *ZmCESA1* al *9* se encuentran implicados en la síntesis de celulosa en paredes primarias y *ZmCESA10, 11* y *12* en la correspondiente a las secundarias. Sin embargo debido a su homología con el gen *ZmCESA12* más que con *ZmCESA10* y *11*, se ha propuesto que están implicados en la síntesis de celulosa en paredes celulares primarias durante los estadios tardíos del desarrollo (Appenzeller y col., 2004).

Además de las CESA, existen otras proteínas implicadas en la síntesis de celulosa, como la sacarosa sintasa (Amor y col., 1995), la proteína KORRIGAN (Nicol y col., 1998) o las proteínas asociadas a microtúbulos (MAP) (Sedbrook, 2004).

Polisacáridos no celulósicos: pectinas y hemicelulosas

Las pectinas son polisacáridos matriciales ricos en ácido galacturónico. Las paredes tipo II presentan menor contenido en pectinas que las paredes tipo I. Aun siendo un componente minoritario, las pectinas juegan un importante papel en la retención de agua, transporte de iones, adhesión celular y determinación de tamaño de poro de la pared.

Además, están implicadas en mecanismos de defensa frente a patógenos, heridas y estreses abióticos (Ridley y col., 2001; Jarvis y col., 2003; Verhertbrugger y Knox, 2007). Entre los polisacáridos pécticos se encuentran el homogalacturonano, el ramnogalacturonano I y el ramnogalacturonano II.

El homogalacturonano es un homopolímero de restos de ácido galacturónico que puede presentar diferentes grados de metilesterificación. En principio, se deposita en la pared celular con un alto grado de metilesterificación, y una vez allí sufre la pérdida de restos metilo por la acción de pectin metilesterasas. Esta pérdida de metilaciones es necesaria para que se establezcan puentes de calcio entre cadenas antiparalelas de homogalacturonano (Liners y col., 1989). La cadena principal del homogalacturonano se encuentra covalentemente unida a ambos tipos de ramnogalacturonano, y además se cree que también puede interaccionar *in muro* con el xiloglucano (Popper y Fry, 2008).

El ramnogalacturonano tipo I se compone de una cadena formada por la repetición del disacárido α-1,4-ácido-galacturónico-α-1,2-ramnosa (Carpita y McCann, 2000). Los restos de ramnosa pueden tener unidas cadenas laterales de galactano, arabinano o arabinogalactano.

El ramnogalacturonano tipo II es un galacturonano con una gran diversidad de sustituciones. Las moléculas de ramnogalacturonano tipo II se pueden asociar entre sí formando dímeros mediante el enlace a través de restos borato (Albersheim y col., 2011).

Las hemicelulosas son un grupo de polisacáridos neutros constituidos por una cadena lineal de monosacáridos, principalmente xilosa, glucosa o manosa, en general con ramificaciones cortas y estructura no cristalina. La pared celular tipo II contiene como principales polisacáridos hemicelulósicos xilanos, y glucano mixto. Además, aparece una pequeña proporción de xiloglucano (Carpita y McCann, 2000).

Los xilanos y el glucano mixto unen microfibrillas de celulosa adyacentes mediante el establecimiento de puentes de hidrogeno, y las mantienen en su disposición espacial

correcta, asumiendo las funciones que el xiloglucano desempeña en las paredes celulares tipo I.

Los xilanos son los principales polisacáridos hemicelulósicos en paredes celulares tipo II (Fincher, 2009) constituyendo alrededor del 20-40% del peso de la pared (Vogel, 2008). Su estructura se basa en una cadena principal de β-1,4-xilosas, y pueden presentar sustituciones de arabinosa (arabinoxilanos) y en menor medida de ácido glucurónico (glucuronoarabinoxilanos). En paredes celulares tipo II muy frecuentemente las unidades de arabinosa del arabinoxilano/glucurono-arabinoxilano pueden encontrarse sustituidas por ácidos hidroxicinámicos (principalmente ácido ferúlico y en menor medida ácido cumárico) unidos mediante enlace éster (Wende y Fry, 1997). Estos restos de ácidos hidroxicinámicos, mediante acoplamiento oxidativo mediado por peroxidasas (Geissmann y Neukom, 1971), son susceptibles de unirse de manera que entrecruzan las cadenas de arabinoxilanos sobre las que se encuentran esterificados (Fry, 2004).

El xiloglucano es un β-1,4-glucano con numerosas ramificaciones de α-1,6-xilosa que presentan a su vez sustituciones de arabinosa, galactosa y/o fucosa (Hayashi, 1989). En paredes celulares tipo II es un polisacárido minoritario, en el que la fucosa no está presente (Carpita, 1996).

La biosíntesis así como el ensamblaje de los polisacáridos no celulósicos tiene lugar en el aparato de Golgi. Posteriormente, son transportados en vesículas derivadas de este orgánulo hasta la membrana plasmática, donde se produce la fusión de las vesículas y el consecuente vertido de su contenido hacia la pared celular (Driouich y col., 1993; 2012; Lerouxel y col., 2006; Day y col., 2013). Su síntesis corre a cargo de una serie de enzimas denominadas glicosiltransferasas (GTs), clasificadas en 92 familias en la base de datos CAZy.

Proteínas

En la pared primaria se pueden encontrar tanto proteínas estructurales como solubles. En cuanto a las proteínas estructurales, las paredes tipo II presentan también

contenidos reducidos (1%) en comparación con las tipo I (10%) (Vogel, 2008). Este tipo de proteínas se encuentran inmovilizadas en la pared, y frecuentemente son glicoproteínas con secuencias repetidas de uno o dos aminoácidos.

Respecto a las *proteínas solubles*, la mayor parte de ellas son enzimas relacionadas con la extensión de la pared celular, el transporte molecular, el reconocimiento celular o la resistencia a patógenos (Rose y col., 2002). Dentro de ellas encontramos hidrolasas, transglicosilasas, peroxidasas, expansinas y kinasas.

Fenoles

Los ácidos ferúlico y *p*-cumárico son los principales fenilpropanoides o hidroxicinamatos de la pared celular (Wallace y Fry, 1994) y, aunque son cuantitativamente minoritarios, son importantes. Las cadenas de arabinoxilano pueden entrecruzarse gracias a la polimerización oxidativa de estos restos de ácido ferúlico, generando dímeros (dehidroferulatos) o incluso trímeros u oligómeros, unidos por enlace fenil-fenil o fenil-éter. El proceso de esterificación y entrecruzamiento se produce de manera mayoritaria en el Golgi pero también puede realizarse *in muro*. Estas uniones conducen a un reforzamiento de la estructura de la pared celular, promueven la cohesión celular, restringen la expansión celular, contribuyen al ensamblaje de la pared y participan en el reforzamiento de esta estructura en respuesta a factores abióticos y bióticos (Buanafina, 2009).

Plasticidad estructural de la pared celular

Las células pueden modificar la composición y estructura de sus paredes celulares en condiciones de estrés, tanto abióticos como bióticos. El grado de flexibilidad de las paredes depende de la especie de que se trate, del tipo de tejido e incluso del grado de diferenciación del tipo celular dado. El estudio de la plasticidad estructural de la pared celular adquiere una gran importancia, no sólo desde un punto de vista de investigación básica con el fin de conocer cómo se regula la síntesis y el ensamblaje de sus componentes, sino también en

investigación aplicada, con el fin de obtener productos de interés en las industrias textil, papelera, maderera, o de biocombustibles.

En los últimos años el empleo de técnicas de cultivo *in vitro* de células, tejidos y órganos vegetales ha permitido avanzar considerablemente en la caracterización de paredes celulares alteradas por modificaciones genéticas (mutaciones) y por adaptaciones a estreses abióticos, como salinos (Binzel y col., 1988), metales pesados (Carpena y col., 2000), bajas temperaturas (Yamada y col., 2002) o desecación (Moore y col., 2013), así como por la habituación a inhibidores de la biosíntesis de la pared celular (Satiat-Jeunemaitre y Darzens, 1986; Suzuki y col., 1992; Vaughn y Turley, 1999; Encina y col., 2001; García-Angulo y col., 2006; 2009; Mélida y col., 2009).

Habituación a herbicidas inhibidores de la biosíntesis de celulosa

La habituación de cultivos celulares a inhibidores de la biosíntesis de la pared celular refleja la capacidad de las células de sobrevivir con una pared celular modificada, siendo por tanto un sistema muy útil para avanzar en el conocimiento de la plasticidad estructural de la pared celular. Varios compuestos han sido descritos como inhibidores de algunos de los componentes de la pared celular primaria (Acebes y col., 2010; García-Angulo y col., 2012), entre los que se encuentra el diclobenil (2,6-diclorobenzonitrilo o DCB), que inhibe específicamente la síntesis de celulosa en plantas superiores (Vaughn, 2002), sin afectar a otros procesos fisiológicos como la síntesis de ADN o proteínas (Galbraith y Shields, 1982), la respiración (Montezinos y Delmer, 1980) o el mantenimiento de los niveles de UDP-Glucosa, fosfolípidos o nucleósidos mono o trifosfato (Delmer, 1987). Aunque la diana del DCB aún permanece desconocida, parece tratarse de una proteína MAP, en concreto la MAP20 (Rajangam y col., 2008). Estos autores postularon que el DCB interaccionaría con MAP20, bloqueando el ensamblaje de las proteínas CESA a los microtúbulos y por tanto la síntesis de celulosa.

En las dos últimas décadas se ha demostrado que es posible habituar cultivos de células indiferenciadas (callos y suspensiones celulares) a concentraciones letales de DCB aumentando paulatinamente su concentración en el medio de cultivo (Shedletzky y col., 1992; Wells y col., 1994; Nakagawa y Sakurai, 1998; Sabba y col., 1999; Encina y col., 2001; 2002; Alonso-Simón y col., 2004; García-Angulo y col., 2006; 2009; Mélida y col., 2009; 2010a; 2010b; 2011).

La habituación a DCB reside en la capacidad que las células tienen de dividirse y crecer con un contenido reducido en celulosa en la pared celular. En este proceso se produce una serie de cambios estables, que difieren en cuanto al tipo de pared celular que presentan las células. En el caso de cultivos de maíz habituados a concentraciones letales de DCB, los cambios que acompañan a la habituación residen, sobre todo, en la compensación de la pérdida de celulosa con incrementos en el contenido de ácidos fenólicos y arabinoxilanos. Además estos arabinoxilanos difieren de los que presentan las células no habituadas, ya que tienen mayor masa molecular, se unen más fuertemente a la pared y se encuentran más entrelazados por dehidroferulatos (Mélida y col., 2009; 2010a; 2010b y 2011). Los cambios asociados a la habituación a altas concentraciones de DCB son muy drásticos y se han observado tras largos periodos en presencia del inhibidor. Sin embargo, es posible que estas modificaciones tengan lugar de manera paulatina y que por lo tanto estemos perdiendo valiosa información acerca del mecanismo de habituación durante los primeros estadios. Además, ha sido previamente demostrado en cultivos celulares de alubia que los cambios producidos por la habituación a DCB difieren (tanto cualitativamente como cuantitativamente) dependiendo de su concentración en el medio como del periodo de tiempo que las células permanecen expuestas a él (Alonso-Simón y col., 2004; Mélida y col., 2009). Por otro lado, el hecho de que altas concentraciones del inhibidor provoquen importantes modificaciones celulares podría limitar la realización de determinados tipos de análisis, como los estudios metabólicos *in vivo*, entre otros. Por tanto, el empleo de suspensiones celulares de maíz habituadas a bajas concentraciones de DCB es una valiosa

herramienta para estudiar en profundidad la plasticidad estructural de las paredes celulares tipo II, particularmente desde un punto de vista metabólico.

OBJETIVOS

El principal objetivo de esta tesis es esclarecer los mecanismos subyacentes a la plasticidad estructural de las paredes celulares primarias tipo II, haciendo énfasis en las estrategias metabólicas. Para llevarlo a cabo, se utilizarán cultivos celulares de maíz habituados a bajos niveles de DCB. Este objetivo principal se divide a su vez en cuatro sub-objetivos parciales, enumerados a continuación:

Objetivo I

Monitorización y caracterización de las modificaciones que suceden durante los primeros pasos del proceso de habituación a DCB

Las células habituadas a DCB son capaces de desarrollar estrategias para sobrevivir en presencia del inhibidor, que varían dependiendo del tipo de pared celular, la concentración de DCB o el periodo de tiempo en el cual las células se encuentran creciendo en contacto con el (Alonso-Simón y col., 2004). Las modificaciones producidas en las paredes de células de maíz habituadas a DCB han sido previamente estudiadas mediante el empleo de concentraciones letales del inhibidor y tiempos prolongados de habituación (Mélida y col., 2009). Sin embargo, se desconocen los cambios que acontecen durante los primeros momentos del proceso de habituación. Por este motivo, el primer objetivo del presente estudio es a) la monitorización de las modificaciones que tienen lugar durante la habituación temprana a DCB, b) la selección de una línea celular que muestre tanto características de esta habituación incipiente, como una reducción moderada en el contenido en celulosa y, c) la consiguiente caracterización de sus patrones de crecimiento y composición de sus paredes

celulares. Para acometerlo, se emplearan técnicas tanto espectrofotométricas como cromatográficas y de inmunoanálisis.

Objetivo II

Análisis del metabolismo hemicelulósico de células con niveles incipientes de habituación a DCB

Las suspensiones celulares de maíz habituadas a DCB son capaces de contrarrestar el empobrecimiento en celulosa presente en sus paredes mediante la adquisición de una red modificada de arabinoxilanos tanto a nivel cuantitativo como cualitativo (Mélida y col., 2009; 2010a; 2010b; 2011). A raíz de estos antecedentes, surge el segundo objetivo de este trabajo, que será el estudio del metabolismo de polisacáridos en células de maíz con una reducción moderada en el contenido en celulosa. Este estudio se realizará a lo largo del ciclo de cultivo a través experimentos de radio-marcaje *in vivo,* suministrando a las células [^{3}H]arabinosa como precursor metabólico en fase de acomodación, exponencial y estacionaria. Posteriormente, se rastreará tanto la síntesis como el destino celular de hemicelulosas y polímeros marcados radioactivamente, en varios compartimentos celulares durante 5 h.

Objetivo III

Estudio de la distribución de masas moleculares de polisacáridos y metabolismo fenólico en células de maíz con una reducción moderada en el contenido en celulosa

En estudios previos realizados en células de maíz habituadas a DCB, se han descrito paredes celulares reforzadas a través del desarrollo de una red de arabinoxylanos con mayores masas moleculares y más entrecruzados, que además presentaban un menor grado de extractabilidad de la pared (Mélida y col., 2009; 2011). Sin embargo, estos estudios fueron realizados en arabinoxilanos aislados únicamente de la pared celular y en una fase final del

ciclo de cultivo. Por esta razón, el tercer objetivo del presente trabajo será el análisis, en células de maíz con una reducción leve en el contenido en celulosa, de la distribución de masas moleculares de polisacáridos en varios compartimentos celulares a lo largo del ciclo de cultivo. Para llevarlo a cabo, empleando como metodología experimentos de radio-marcaje *in vivo,* se administrará a las suspensiones celulares [^{3}H]arabinosa y ácido [^{14}C]cinámico como precursores metabólicos para hemicelulosas e hidroxicinamatos, respectivamente.

Objetivo IV

Análisis del proteoma de Golgi en células de maíz habituadas a DCB

La plasticidad estructural mostrada por las paredes de células de maíz cuando son habituadas a DCB innegablemente incurre en su metabolismo y por ende, en las proteínas responsables de las respuestas exhibidas. Teniendo en cuenta que importantes enzimas metabólicas son proteínas residentes en el aparato de Golgi, se obtendrán fracciones enriquecidas en dicho orgánulo tanto de células de maíz no habituadas como habituadas a diferentes niveles de DCB. En primer lugar, se realizará un análisis comparativo del proteoma de Golgi de las diferentes líneas celulares mediante electroforesis bidimensional. Posteriormente, aquellas proteínas de interés que se acumulen diferencialmente entre líneas se secuenciarán mediante MALDI-TOF/MS (matrix-assisted laser desorption ionization-time of flight mass spectrometry) o ESI-MS/MS (electrospray ionization with tandem mass spectrometry).

MATERIALES Y MÉTODOS

Cultivos celulares

Las suspensiones celulares se obtuvieron a partir de callos de maíz (*Zea mays* L., Black Mexican) procedentes de embriones inmaduros, en medio líquido Murashige y Skoog que contenía 2,4-D 9 µM, sacarosa 20 g L^{-1} (pH 5,6). Las suspensiones se mantuvieron en agitación orbital, a 25°C, fotoperiodo de 16 h y fueron subcultivadas cada 15 días.

Habituación a DCB

El proceso de habituación a DCB se realizó cultivando suspensiones celulares de maíz no habituadas (Snh) en presencia del inhibidor. En el caso de suspensiones habituadas a bajos niveles de DCB, células Snh se subcultivaron y fueron mantenidas en tres concentraciones iniciales de DCB: 0,3 µM (Sh0,3), 0,5 µM (Sh0,5) y 1 µM (Sh1). Después de varios subcultivos, parte de las células procedentes de las suspensiones mantenidas en DCB 1 µM se transfirieron a un medio que contenía DCB 1,5 µM (Sh1,5).

Las células de maíz habituadas a altas concentraciones de DCB se obtuvieron a partir de callos de maíz habituados a DCB 12 µM, que se transfirieron a medio líquido conteniendo DCB 6 µM (Sh6).

Caracterización de los cultivos celulares

Se realizó una cinética de crecimiento de las suspensiones celulares de maíz midiendo el incremento en peso seco para cada intervalo de tiempo a lo largo de todo el ciclo de cultivo, y se estimaron varios parámetros de crecimiento como el tiempo de duplicación, la tasa relativa y la tasa máxima de crecimiento.

Para la determinación del tamaño de agregado, las suspensiones celulares de maíz se filtraron a través de filtros de nylon de diferente tamaño de poro, expresando el resultado final como porcentaje de peso seco retenido en cada filtro respecto al total.

Aislamiento de fracciones de diversos compartimentos celulares de suspensiones celulares de maíz

Obtención del medio extracelular y extracción del contenido protoplasmático

Alícuotas de suspensiones celulares se filtraron a través de columnas Poly-Prep. El filtrado resultante libre de células, se recogió y consideró la fracción correspondiente al medio extracelular (CFM). En los estudios llevados a cabo con [^{3}H]arabinosa como precursor metabólico, las células que quedaron retenidas en el filtro de la columna Poly-Prep se resuspendieron en un tampón de extracción del contenido protoplasmático y fueron homogeneizadas. A continuación, el homogeneizado resultante se hizo pasar a través de una segunda columna Poly-Prep para recoger el filtrado correspondiente, que fue centrifugado varias veces hasta que el sobrenadante se tornó transparente, momento en el cual se consideró como la fracción protoplásmica.

En el caso de los estudios realizados con ácido [^{14}C]cinámico como precursor metabólico, se tomaron alícuotas de suspensiones celulares que se filtraron a través de columnas Poly-Prep o, alternativamente, se sometieron a centrifugación. El filtrado o sobrenadante se descartó, y las células se incubaron con etanol al 80%. Posteriormente, las células se filtraron o centrifugaron y el filtrado o sobrenadante se recogió y se consideró como contenido similar al protoplásmico.

Obtención de fracciones enriquecidas en Golgi

Células procedentes de suspensiones celulares de maíz se pulverizaron en nitrógeno líquido y se homogeneizaron en tampón de extracción (Zeng y col., 2008). El homogeneizado se centrifugó y se recogió el sobrenadante. Este sobrenadante se cargó encima de tampón de sacarosa 2 M y fue sometido a ultracentrifugación para la obtención de la fracción microsomal. Después de esta primera ultracentrifugación, los microsomas quedaron localizados en una banda definida inmediatamente por encima de la fase correspondiente al tampón de sacarosa 2 M. La fracción superior a esta banda fue retirada y sobre la fracción

microsomal se añadieron superpuestos una serie de tampones de sacarosa de concentraciones 1,3 M, 1,1 M y 0,25 M, con objeto de crear un gradiente discontinuo. Este gradiente discontinuo se sometió de nuevo a ultracentrifugación, y la interfase generada entre los tampones de concentraciones 0,25 M y 1,1 M se recogió como fracción enriquecida en Golgi.

Extracción y fraccionamiento de paredes celulares

Dependiendo del enfoque experimental, se siguieron dos metodologías diferentes para la obtención de las paredes celulares y su posterior fraccionamiento. De esta manera, cuando los experimentos realizados no incluían el uso de compuestos radio-marcados, se llevó a cabo un protocolo más exhaustivo (protocolo A). En cambio, cuando la metodología incluía el uso y manejo de compuestos radioactivos, se empleó una versión simplificada por razones de seguridad (protocolos B1 y B2).

Protocolo A

Para la obtención de paredes celulares, las suspensiones celulares de maíz se sometieron a una homogeneización con nitrógeno líquido hasta obtener un polvo fino, y el homogeneizado se trató con etanol al 70% durante 5 días. El residuo resultante se trató con etanol al 70% y acetona y se dejó secar a temperatura ambiente para obtener el residuo insoluble en alcohol (AIR). Posteriormente el AIR fue tratado con dimetilsulfóxido y el residuo obtenido se incubó con α-amilasa de páncreas porcino disuelta en tampón fosfato. Tras retirar la solución enzimática, el residuo se lavó con etanol al 70% y acetona y se dejó secar a temperatura ambiente.

Posteriormente, se trató con fenol/acético/agua y finalmente se lavó con etanol al 70% y acetona y se dejó secar a temperatura ambiente. Se consideró que este residuo eran las paredes celulares.

Las paredes celulares fueron sometidas a una serie de tratamientos para extraer de manera secuencial distintos tipos de polímeros. Primeramente, fueron tratadas con ácido t-1,2-diaminociclohexano-N,N,N´,N´-tetra-acético (CDTA) y el residuo insoluble obtenido se incubó con KOH 0,1 M. Al residuo resistente a esta extracción se le añadió KOH 4 M y de nuevo, el residuo obtenido de la fracción anterior fue sometido a un último tratamiento con KOH 6 M. Los sobrenadantes de cada uno de los tratamientos constituyeron las fracciones CDTA; KOH 0,1 M; KOH 4 M y KOH 6 M.

Protocolo B1: estudios que implicaron el uso de [^{3}H]arabinosa como precursor metabólico

En este caso, células procedentes de las suspensiones celulares de maíz se homogeneizaron y el homogeneizado resultante se filtró a través de una columna Poly-Prep. A los fragmentos celulares retenidos en el filtro se les trató con una solución de NaOH 0,1 M que contenía $NaBH_4$. El residuo insoluble fue sometido a un segundo tratamiento con NaOH 6 M conteniendo $NaBH_4$, y al material resistente se le consideró como polímeros fuertemente unidos a la celulosa.

Protocolo B2: estudios que implicaron el uso de ácido [^{14}C]cinámico como precursor metabólico

Las células de suspensiones de maíz se re-suspendieron e incubaron en etanol al 80%. Posteriormente, se filtraron o, alternativamente, centrifugaron y el correspondiente filtrado o sobrenadante se descartó, considerando a los fragmentos celulares residuales el AIR. El AIR se saponificó mediante tratamiento con NaOH 0,5 M y la fracción soluble resultante del mismo se consideró la fracción NaOH 0,5 M. Dicha fracción fue acidificada con ácido acético y se procedió a realizar una primera partición en acetato de etilo. Consecutivamente, las muestras se secaron mediante

vacío, y se re-disolvieron en agua acidificada, para volver a ser sometidas a una segunda partición en acetato de etilo. Las fases orgánicas se recogieron, secaron en vacío y re-disolvieron en propanol.

Las fracciones solubles obtenidas de los diferentes tratamientos fueron neutralizadas a pH 5 con ácido acético, dializadas y etiquetadas con el nombre del tratamiento correspondiente utilizado para su extracción.

Análisis de las paredes celulares

Valoración del contenido en celulosa

La cantidad de celulosa se cuantificó espectrofotométricamente mediante el método de Updegraff (Updegraff, 1969) con las condiciones hidrolíticas descritas por Saeman (Saeman y col., 1963). La glucosa liberada se valoró por el método de la antrona (Dische, 1962).

Valoración del contenido en azúcares

La determinación del contenido de azúcares totales de cada fracción se realizó espectrofotométricamente mediante el método del fenol-sulfúrico (Dubois y col., 1956) y la de ácidos urónicos mediante el método del *m*-hidroxibifenil (Blumenkrantz y Asboe-Hansen, 1963) utilizando como estándares glucosa y ácido galacturónico respectivamente. El contenido en azúcares neutros se realizó mediante cromatografía de gases según el método descrito por Albersheim y col. (1967).

Espectroscopía de infrarrojo por transformada de Fourier (FTIR)

Con objeto de monitorizar los cambios ocurridos en las paredes celulares de las diferentes líneas de maíz, se utilizó FTIR. Para ello, las paredes celulares procedentes de suspensiones, se mezclaron con KBr y se homogeneizaron en un mortero de ágata, hasta

conseguir un polvo fino. Posteriormente se comprimieron en una prensa Graseby-Specac, obteniéndose las pastillas necesarias para el análisis y que se realizó mediante el empleo de un espectroscopio Perkin-Elmer. Una vez obtenidos los espectros FTIR se corrigió su línea base y se normalizaron sus áreas.

Inmuno-ensayos

La composición de las diferentes fracciones obtenidas en el fraccionamiento de las paredes celulares se analizó mediante ensayos de inmunodot. Para ello, se tomaron alícuotas de 1 µl de distintas diluciones de las fracciones, y se colocaron en orden decreciente en membranas de nitrocelulosa. Posteriormente, las membranas se incubaron con una endo-poligalacturonanasa, una β-xilanasa y una endo-arabinanasa (para más detalle ver el apartado de digestiones enzimáticas) aplicando la metodología descrita en Øbro y col. (2007) con ligeras modificaciones. Después de los tratamientos enzimáticos, las membranas de nitrocelulosa fueron bloqueadas con tampón fosfato salino (PBS) con leche en polvo, y se incubaron con anticuerpos primarios específicos para diferentes tipos de polisacáridos pécticos y hemicelulósicos. Se lavaron las membranas con agua y PBS para eliminar el exceso de anticuerpo primario y se incubaron con un anticuerpo secundario anti-rata conjugado con peroxidasa de rábano. Después de lavar las membranas con PBS y agua milli-Q para eliminar el exceso de anticuerpo secundario, se añadió la solución sustrato para proceder al revelado de las membranas.

La cuantificación del inmuno-marcaje mostrado en cada fracción se realizó otorgando valores correspondientes al número de diluciones que exhibían marcaje. Estos valores fueron después utilizados para generar los heatmaps (para más detalle ver el apartado correspondiente al análisis estadístico).

Análisis de expresión génica: aislamiento de ARN total, RT-PCR y PCR

El ARN total se extrajo empleando el reactivo comercial Trizol, y fue posteriormente retro-transcrito mediante el sistema "Superscript III first strand synthesis system". El ADN complementario se generó usando un cebador oligo(dT)20, que fue utilizado como cadena molde en las consiguientes reacciones de PCR. Para cada análisis de expresión se hicieron pruebas variando el número de ciclos de las reacciones de PCR con objeto de determinar aquellas condiciones óptimas en las que la amplificación se encontrase dentro del rango exponencial. Como gen control se empleó el de la ubiquitina.

Análisis de expresión de genes *ZmCESA*

Para llevar a cabo el análisis de los genes *ZmCESA1* (*AF200525*), *ZmCESA2* (*AF200526*), *ZmCESA3* (*AF200527*), *ZmCESA5* (*AF200529*), *ZmCESA6* (*AF200530*) y *ZmCESA7* (*AF200531*) se usaron los cebadores específicos para ellos descritos en Holland y col. (2000). Debido a que los genes *ZmCESA1* y *ZmCESA2* poseen una alta homología de secuencia, se utilizó el mismo cebador para estudiar la expresión de ambos genes. En el caso de *ZmCESA4* (*AF200528*) y *ZmCESA8* (*AF200532*), se emplearon los cebadores descritos en Mélida y col. (2010a).

Análisis de expresión de genes homólogos *IRX* de arabidopsis

Se diseñaron y emplearon cebadores específicos para el análisis en maíz de los genes *GRMZM2G100143*, *GRMZM2G059825* y *gbIBT036881.1* homólogos de *IRX10* (*AT1G27440*), *IRX10-L* (*AT5G61840*) y *IRX9* (*AT2G37090*) respectivamente en arabidopsis (Bosch y col., 2011). Las secuencias de dichos cebadores derivaron de la base de datos "maize genome browser" para el caso de *GRMZM2G100143* y *GRMZM2G059825* y "Phytozome" para *gbIBT036881.1*.

Experimentos de radiomarcaje *in vivo*

Estudios que implicaron con [^{3}H]arabinosa como precursor metabólico

Se recogieron alícuotas de suspensiones celulares de maíz en diferentes etapas del ciclo de cultivo y se les suministró L-[1-^{3}H]arabinosa. La incorporación de [^{3}H]arabinosa en los diferentes compartimentos celulares se midió a distintos tiempos en cada etapa de cultivo.

Estudios con ácido [^{14}C]cinámico como precursor metabólico

El ácido [^{14}C]cinámico se sintetizó partiendo de L-[U-^{14}C]fenilalanina, siguiendo básicamente el método descrito en Lindsay y Fry. (2008). A las alícuotas de suspensiones de maíz se les suministró ácido [^{14}C]cinámico en diferentes puntos del ciclo de cultivo y, tanto la incorporación del precursor como su consumo, se midieron a diferentes tiempos.

Cuando fue requerido, a algunas de las muestras se les añadió H_2O_2 120 min después de la administración de ácido [^{14}C]cinámico, y se mantuvieron durante 60 min más en condiciones de agitación.

La radioactividad presente en las muestras se analizó en un contador de centelleo.

Digestiones enzimáticas

Previamente al tratamiento enzimático con Driselasa las muestras se secaron en vacío y se sometieron a una hidrólisis suave en ácido trifluoroacético (TFA). Finalmente se volvieron a secar y el material seco se redisolvió en una solución de Driselasa al 0,5% (p/v) en piridina/ácido acético/agua y se incubó a 37°C durante 96 h. La reacción se paró mediante la adición de ácido fórmico al 15%.

Los tratamientos con endo-poligalacturonanasa (M2) extraída de *Aspergillus aculeatus* (E-PGALUSP) (EC 3.2.1.15), β-xilanasa recombinante de *Neocallimastix patriciarum* (E-XYLNP) (EC 3.2.1.8) y endo-arabinanasa de *Aspergillus niger* (E-EARAB) (EC 3.2.1.99) se

llevaron a cabo a 37°C durante 24 h. Para ello, las enzimas se disolvieron en tampón acetato 350 mM pH 4.7 a una concentración de 1 U/ml.

Cromatografía

Cromatografía en papel

Para llevar a cabo la cromatografía en papel se utilizó papel Whatman 3 MM, y el tipo de solvente y la duración variaron dependiendo de los componentes a separar. De esta manera, para la separación de monosacáridos, se realizó en butanol/ácido acético/agua durante 16 h. En aquellos casos en que fue necesaria la separación de monosacáridos, disacáridos y material polimérico a RF 0, la cromatografía en papel se llevó a cabo empleando acetato de etilo/piridina/agua durante 18 h (Thompson y Fry, 1997). Posteriormente, se procedió a la tinción de los cromatogramas con ftalato de anilina (Fry, 2000) y se revelaron a 105°C.

Cromatografía de Filtración en Gel

Los polímeros se fraccionaron por tamaños usando una columna de Sefarosa CL-4B por la que se hizo pasar un flujo de piridina/ácido acético/agua de 12,5 ml/h. La columna fue previamente calibrada con dextranos de masas moleculares relativas medias (M_w) conocidas. Mediante el método del $Kav_{(1/2)}$ (Kerr y Fry, 2003) se obtuvo la ecuación de calibración [$\log M_w = -3{,}547\ Kav_{(1/2)} + 7{,}2048$] que se empleó posteriormente para los cálculos necesarios.

Cromatografía de Gases

Las muestras fueron previamente liofilizadas e hidrolizadas en TFA 2 M a 121°C durante 1 h. Los azúcares resultantes se derivatizaron a alditol acetatos y se analizaron mediante una columna Supelco SP-2330 (Albersheim y col., 1967).

Cromatografía en Capa Fina

Se realizó en placas de plástico con silica gel como fase inerte, conteniendo indicadores de fluorescencia. Como solvente se empleó benceno/ácido acético y para su revelado las placas se expusieron a 321 nm.

Electroforesis

Electroforesis en poliacrilamida (SDS-PAGE)

Los análisis se llevaron a cabo tanto en condiciones semi-nativas como en desnaturalizantes, no hirviendo o hirviendo, respectivamente, las muestras. Posteriormente se separaron en geles de acrilamida del 7,5%, 10% o 12,5%. Como tinciones se emplearon coomassie brilliant blue (CBB) R-250 o nitrato de plata.

Electroforesis bidimensional

Antes de la realización de la electroforesis bidimensional, fueron necesarios ciertos pasos previos para la preparación de las muestras. Para ello, se liofilizaron alícuotas de las mismas que posteriormente se disolvieron en el tampón específico para la electroforesis bidimensional, el tampón de lisis. Una vez disueltas, se incubaron en un baño de ultrasonidos, se centrifugaron y se recogió el sobrenadante, que fue dializado. A continuación, las muestras se concentraron mediante el sistema de columnas Vivaspin, hasta que se obtuvo la concentración de proteína necesaria.

Subsiguientemente, con objeto de realizar la separación en la primera dimensión se utilizaron gradientes inmovilizados con un rango de pH 4-7. La separación en la segunda dimensión se realizó en geles SDS-PAGE al 10%. Los geles resultantes se tiñeron empleando CBB G-250 (Campos y col., 2010) o mediante tinción de plata (Shevchenko y col., 1996; Irar y col., 2006) en función de la cantidad de proteínas a resolver, siendo este último de mayor sensibilidad. Una vez teñidos, los geles fueron escaneados y la adquisición de las imágenes se realizó a 16-bits/canal, 300 dpi (en escala de grises) y en formato TIFF. Las imágenes

generadas se utilizaron para realizar el análisis proteómico (Farinha y col., 2011), fueron integradas y los spots identificados. A estos spots de manera automática les fueron asignados una intensidad, volumen, área y el parámetro saliency, que integra a todos ellos. En este caso el parámetro normalizado utilizado fue el %Volumen o volumen normalizado. Cuando los datos mostraron variaciones superiores a un valor del 0,5 del volumen normalizado y 1 del ratio se realizó el análisis estadístico, con la consecuente validación de los resultados mediante el Test-t con un valor p del 0,05 (Farinha y col., 2011). Los spots de interés fueron cortados y se procedió a su identificación mediante MALDI-TOF y por ESI-MS/MS. Para la identificación de proteínas se utilizaron las bases de datos de NCBI y Swissprot. Los programas de búsquedas utilizados fueron SEQUEST y MASCOT.

Análisis estadísticos

El análisis de componentes principales (PCA) se realizó con un máximo de cinco componentes principales en el software Statistica 6.0.

Para el análisis de diferencias significativas se realizó un test ANOVA de una vía con una prueba de Tukey's como análisis post-hoc en el software PASW Statistics 18 o bien el Test-t con un valor p del 0,05.

Los heatmaps se realizaron empleando el software RKWard.

RESULTADOS Y DISCUSIÓN

La habituación a DCB se basa en el desarrollo de estrategias que implican cambios tanto en el metabolismo como en la arquitectura de la pared celular, para superar la limitación que supone el hecho de tener paredes deficitarias en celulosa. En el caso específico de las células de maíz habituadas a crecer en concentraciones letales de DCB, se ha observado que una remodelación cuantitativa y cualitativa de la red de arabinoxilanos juega un papel clave en una gran parte de estas estrategias (Mélida y col., 2009; 2010a; 2010b; 2011). Sin embargo, también se ha demostrado que la variedad de respuestas de adaptación

depende del tipo de pared celular, así como de la concentración de DCB y el tiempo de exposición al inhibidor (Alonso-Simón y col., 2004; Mélida y col., 2009). Dado que los estudios previos en células de maíz habituadas a DCB se han llevado a cabo con altas concentraciones del inhibidor y períodos de habituación a largo plazo, la información acerca de las primeras modificaciones que tienen lugar durante el proceso de habituación se pierde. Tal información puede resultar muy valiosa ya que durante los primeros estadios de la habituación las células podrían presentar una gran variedad de respuestas. Por lo tanto, en la presente tesis se ha realizado un extenso análisis de las modificaciones que tienen lugar en los primeros pasos del proceso de habituación a DCB.

Monitorización de los cambios acontecidos en los primeros pasos del proceso de habituación a DCB

En primer lugar, se procedió a identificar una línea celular de maíz que mostrara características de una temprana o incipiente habituación a DCB (de Castro et al., 2013; Capítulo I). Por esta razón, suspensiones celulares de maíz Snh fueron sub-cultivadas durante varios ciclos de cultivo en diferentes concentraciones de DCB (partiendo de 0,3 µM hasta 1,5 µM), y las modificaciones en la pared celular se monitorizaron mediante espectroscopía FTIR (Figuras I.1 a 3) y valoraciones del contenido en celulosa (Figura I.4) Los resultados revelaron que la principal modificación de la pared celular durante la habituación a DCB está relacionada con el contenido en celulosa. Las células que crecían en las concentraciones más bajas de DCB presentaron inicialmente una reducción en el contenido en celulosa que posteriormente revirtió hasta valores próximos a los de Snh, a medida que se incrementaban los ciclos de cultivo en presencia del inhibidor (Figura I.4). Dado que se había observado previamente que las células habituadas a altas concentraciones de DCB presentaban fluctuaciones en el nivel de expresión de algunos genes *ZmCESA* (Mélida y col., 2010a) se procedió a evaluar su expresión también en células habituadas a bajas concentraciones de DCB (Figura I.5). Al igual que en los procesos de habituación a altos

niveles (Mélida y col., 2010a), los resultados mostraron una inducción de *ZmCESA7 y ZmCESA8* en células de maíz habituadas a bajos niveles. Dicha coincidencia podría indicar que CESA7 y CESA8 actúan de manera más eficiente en presencia de DCB, por lo que podrían tener un papel en la habituación a todos los niveles.

Los resultados de la monitorización dieron lugar a la selección de una línea Sh1,5 como la más adecuada para los posteriores análisis, basándonos en los siguientes criterios: a) presentó una reducción no reversible de un 33% del contenido en celulosa respecto al control (Figura I.4), dicha reducción puede considerarse leve si se compara con el 75% de reducción que presentan las líneas habituadas a largos tiempos y elevadas concentraciones de DCB (Mélida y col., 2009); b) los espectros FTIR mostraron que, además de la celulosa, también otros polisacáridos estaban afectados en esta línea (Figuras I.1 y 2); c) los resultados de PCA apuntan a diferencias no solo con las células control sino también con respecto al resto de las líneas habituadas monitorizadas (Figura I.3), lo que indicaría características de una habituación incipiente.

Caracterización de la línea celular de maíz habituada a bajos niveles de DCB

La amplia caracterización de la línea Sh1,5 y de sus paredes celulares reveló factores comunes en la habituación de maíz a DCB a todos los niveles. Algunas de las características comunes de las células habituadas a DCB es que muestran cinéticas de crecimiento retrasadas y parámetros de crecimiento alterados respecto a las de las células control (Figura I.6), desarrollo celular en forma de grandes agregados (Figura I.7) y un incremento neto en arabinoxilanos (Figura I.8). Sin embargo, también se han encontrado diferencias en los tipos de modificaciones de la pared celular. En primer lugar, durante los primeros estadios de la habituación, el incremento en arabinoxilanos se produjo en fracciones de polisacáridos fácilmente extraíbles (Figura I.8), contrastando con los arabinoxilanos más fuertemente unidos que presentaban las líneas habituadas a altos niveles de DCB (Mélida y col., 2009). Además, otros polisacáridos como el xiloglucano, el ramnogalacturonano I y el

homogalacturonano con bajo grado de metil esterificación parecen estar involucrados en los primeros pasos de la habituación (Figura I.9B). Los tratamientos enzimáticos específicos para pectinas, hemicelulosas y cadenas laterales de algunos polisacáridos, afectaron de manera diferencial a las líneas celulares control y estas líneas habituadas (Figura I.10). Esto podría indicar diferencias estructurales en los polisacáridos de ambas líneas, lo cual permitiría una acción enzimática más o menos eficaz. No obstante, son necesarios análisis más profundos para verificar esta hipótesis.

Estudios similares realizados en cultivos celulares de alubia habituados a DCB mostraron que estas células con pared celular tipo I compensaban su pérdida en celulosa modificando la red péctica y hemicelulósica (Encina y col., 2001; García-Angulo y col., 2006). Las células de maíz presentan paredes de tipo II en las que el xiloglucano y las pectinas no son componentes mayoritarios. Sin embargo, es de destacar que cuando son habituadas a DCB, comparten características con células de alubia. En sus paredes celulares tipo I, xiloglucano y pectinas sí son componentes principales y tienen un papel relevante en la habituación a DCB (Encina y col., 2001; 2002; Alonso-Simón y col., 2004; 2011; García-Angulo y col., 2006; 2009). Por otro lado, en las células de maíz habituadas a altos niveles de DCB el contenido en pectinas o xiloglucano no se vio modificado o incluso fue reducido (Mélida y col., 2009).

Estos resultados ponen en evidencia la elevada capacidad de los cultivos celulares de maíz para modificar la arquitectura de la pared celular, con el fin de adaptarse a los distintos niveles de habituación a DCB.

Análisis del metabolismo de polisacáridos de células de maíz habituadas a bajos niveles de DCB

Dado que es esperable que tales respuestas de plasticidad estructural estén relacionadas con el metabolismo, se eligieron células habituadas a DCB como sistema experimental para indagar en el metabolismo de los polisacáridos de la pared celular y en las

interacciones que entre ellos tengan lugar. De este modo, células de maíz Snh y Sh1,5 fueron cultivadas en presencia de [^{3}H]arabinosa como precursor metabólico en diferentes etapas del ciclo de cultivo, con el objetivo de aislar distintos compartimentos celulares y fracciones de pared para su posterior análisis (Capítulo II). Además, con el fin de comprobar si estaba siendo particularmente afectado un tipo específico de polisacárido, las muestras se sometieron a digestión enzimática con Driselasa.

Tráfico celular y caracterización de hemicelulosas y otros polímeros

Se realizó un seguimiento del metabolismo, así como del tráfico celular de hemicelulosas ([^{3}H]arabinoxilanos y [^{3}H]xiloglucano) y otros polímeros ([^{3}H]polímeros que contienen arabinosa y polímeros sin digerir por Driselasa). En primer lugar, se observó que ambas líneas celulares diferían en la toma del precursor radio-marcado. Mientras que las células Snh mostraron una tendencia similar a la observada previamente en cultivos celulares de maíz (Kerr y Fry, 2003) y rosa (Edelmann y Fry, 1992; Thompson y col., 1997), las células Sh1,5 mostraron una capacidad menor en la toma de [^{3}H]arabinosa (Figura II.1). Además, en todas las fases de cultivo analizadas (Figura II.2) las células Sh1,5 fueron metabólicamente más lentas y menos eficientes que las células Snh y que otros cultivos celulares de maíz (Kerr y Fry, 2003). Dicha reducción en la capacidad metabólica afectó por igual a la síntesis, la incorporación a pared celular o la liberación al medio de cultivo de arabinoxilanos, xiloglucano, polímeros que contienen arabinosa y polímeros no digeridos por Driselasa (Figuras II.3 a 6).

La extractabilidad de los arabinoxilanos de la pared celular también se vio modificada en función del nivel de habituación. Los cultivos celulares habituados a altos niveles de DCB presentaron arabinoxilanos más difícilmente extraíbles que las líneas Snh (Mélida y col., 2009; 2011) y Sh1,5.

La siguiente cuestión que se planteó fue si las ^{3}H-hemicelulosas o los ^{3}H-polímeros de las células Sh difieren de alguna manera de los correspondientes a las células Snh. Las ^{3}H-

hemicelulosas de las células Sh presentaron un incremento en la relación [^{3}H]arabinosa/[^{3}H]xilano lo cual podría estar indicando la presencia de arabinoxilanos más sustituidos en estos cultivos (Tabla II.1). No obstante, este resultado se debe tomar con cautela ya que otros polisacáridos también pueden aportar restos de arabinosa, como los arabinogalactanos de las arabinogalactano proteínas y determinadas cadenas laterales del ramnogalacturonano (para más detalle ver Fry, 2011). Sin embargo, el análisis de las fracciones obtenidas de la pared celular de células Sh1,5 reveló bajos contenidos de ácidos urónicos así como de restos de galactosa y ramnosa (Figura I.8). Por lo tanto, se puede asumir que la mayor parte de los restos de arabinosa derivan de moléculas de arabinoxilanos.

Destino celular de hemicelulosas y otros polímeros

El destino celular de los ^{3}H-polímeros cuando abandonan el protoplasma también fue distinto en Sh1,5 (Figura II.2). Al contrario de lo observado en altos niveles de habituación (Mélida y col., 2009; 2011), las células Sh1,5 presentaron una reducción en la cantidad de hemicelulosas fuertemente unidas a la pared (las extraídas con NaOH 6 M). Además, las células Sh1,5 mostraron un incremento relativo en hemicelulosas extraídas con tratamientos suaves (NaOH 0,1 M) (Figura II.4), lo cual coincide con resultados anteriores (Figura I.8). Por último, se observó un incremento de polisacáridos extracelulares (SEPs) liberados al medio de cultivo (Figura II.6). Estos SEPs pueden incluir polímeros que han sido sintetizados recientemente y excretados al medio sin unirse a la pared, y polímeros que fueron integrados a la pared y más tarde han sido liberados al medio de cultivo (Kerr y Fry, 2003; Mélida y col., 2011). Sin embargo esta última posibilidad puede descartarse, ya que no se observó en las células Sh1,5 una disminución en la incorporación de radiactividad en las fracciones obtenidas de la pared, sincrónicamente con un aumento de la misma en el medio de cultivo.

Este hecho puede tener dos posibles explicaciones: a) dado que las células Sh1,5 contienen menos celulosa en sus paredes, se produciría un descenso en los posibles puntos

de unión para otros polisacáridos, y/o b) las líneas Sh1,5 podrían tener una menor capacidad para incorporar polisacáridos a través de enlaces fenólicos. Esta alternativa es particularmente interesante ya que en la habituación de células de maíz a niveles altos de DCB, se ha demostrado que la presencia de una red más desarrollada de arabinoxilanos unidos mediante acoplamiento fenólico oxidativo es decisiva en las estrategias de reforzamiento de la pared (Mélida y col., 2009; 2011). La importancia del incremento del acoplamiento oxidativo reside en que produce moléculas de arabinoxilano con mayores M_w, cruciales para reforzar unas paredes celulares que muestran un 75% de reducción en celulosa.

Análisis de la distribución de masas moleculares y M_w de hemicelulosas y otros polímeros

Las M_w de hemicelulosas y polímeros recién sintetizados en células Sh1,5 fueron menores que los de Snh (Capítulo III), lo cual apoya la hipótesis de que estas líneas pudieran presentan menor capacidad para entrecruzar polisacáridos (Figuras III.1 y 2). Esta tendencia fue consistente en todo el ciclo de cultivo y en todos los compartimentos celulares (protoplasma y pared celular), así como en el medio extracelular para los diferentes tipos de polisacáridos y polímeros (Figuras III.1 a 5). Además, cuando se compararon las M_w de polímeros protoplásmicos con los de hemicelulosas unidas a la pared, se comprobó que los polímeros protoplásmicos recién sintetizados sufrían un intenso entrecruzamiento una vez incorporados a la pared (Figura III.2). Este comportamiento ya se había observado con anterioridad en suspensiones de maíz (Kerr y Fry, 2003), aunque fue menos marcado en Sh1,5 que en Snh. La explicación más plausible es que los polisacáridos son sometidos a un proceso de unión después de ser secretados en la pared celular. Tales mecanismos incluyen interacciones entre moléculas tanto de tipo no covalente como de tipo covalente, tales como formación de puentes iónicos, de hidrógeno, glicosídicos o acoplamiento oxidativo (Fry, 2000). Aunque conviene considerar todo tipo de interacciones, es esperable que los puentes

de hidrógeno o el acoplamiento oxidativo sean especialmente importantes en el proceso de entrecruzamiento entre arabinoxilanos.

Estudio del metabolismo fenólico

En maíz, se sabe que la formación de dímeros u oligómeros de ácido ferúlico que entrelazan arabinoxilanos por acción de peroxidasas es el principal mecanismo por el cual aumenta la M_w de estas hemicelulosas (Fry y col., 2000). Por otra parte, y como se mencionó anteriormente, en las células de maíz habituadas a altas concentraciones de DCB este tipo de entrecruzamiento fenólico tiene un papel crucial en las estrategias de reforzamiento de la pared (Mélida y col., 2011). Por este motivo, se realizó un análisis del metabolismo del ácido cinámico, así como una caracterización de sus derivados en la pared celular, en células habituadas a bajos niveles de DCB (Figuras III.6 a 9). Las células Sh1,5 mostraron una escasa capacidad para incorporar en la pared celular polisacáridos con restos de ácido ferúlico o *p*-cumárico (Figuras III.6 a 9), lo cual apoyaría los resultados obtenidos previamente (Figura II.2). Los resultados que se han expuesto hasta ahora reforzarían la hipótesis de una disminución en la capacidad de las células Sh1,5 para llevar a cabo el entrecruzamiento de los polisacáridos. Sin embargo, en términos relativos, el contenido en dímeros y oligómeros de ácido ferúlico fue mayor en células Sh1,5. Este hecho podría indicar que las M_w menores observadas en estas líneas no están relacionadas con un menor acoplamiento fenólico de los arabinoxilanos.

Dado que la reducción en el contenido de celulosa en las células habituadas a bajos niveles es considerablemente menor que en las habituadas a concentraciones altas de DCB (33% *vs* 75%), surge la posibilidad de que las células Sh1,5 estén sintetizando una mayor cantidad de moléculas con menor M_w como parte de su estrategia de habituación. En este sentido, se ha descrito con anterioridad que las hemicelulosas, específicamente el xiloglucano, con menores M_w pueden unirse más fácilmente a la celulosa (Lima y col., 2004). Además, Alonso-Simón y col. (2007) propusieron que un xiloglucano con menor M_w, junto

con un incremento de la actividad xiloglucano-endo-transglucosilasa, podría contribuir a aumentar la rigidez de la pared en células de alubia habituadas a DCB. Estudios previos, realizados en niveles de habituación a DCB medios y altos, parecen indicar que a medida que disminuye el contenido en celulosa en las paredes aumenta progresivamente el tamaño (M_w) de las hemicelulosas unidas a pared (Mélida y col., 2009). En concordancia con esto se comprobó que las células de maíz habituadas a altos niveles de DCB presentaban un mayor nivel de expresión de los genes *IRX* ortólogos de arabidopsis implicados tanto en la iniciación como en la elongación de las cadenas de xilano (Figura III.10). Sin embargo las células Sh1,5 no mostraron cambios en la expresión de los genes implicados en la iniciación de la síntesis de xilanos, pero sí presentaron una menor expresión para los genes involucrados en la elongación. Este hecho podría explicar por qué las células Sh1,5 contienen hemicelulosas de cadenas más cortas.

Como consecuencia, es factible suponer que a pesar de tener menores M_w, las hemicelulosas y polímeros de células Sh1,5 están más entrelazados. Por lo tanto, la contribución de este tipo de enlaces parece ser otra característica común en el proceso de habituación a DCB en células de maíz.

Estudio del proteoma de Golgi en células de maíz habituadas a DCB

Como hemos visto, las células habituadas a crecer en presencia de DCB presentan alteraciones metabólicas que afectan entre otras cosas a la síntesis de polisacáridos de la pared. En lo referente al metabolismo de polisacáridos, el aparato de Golgi es uno de los orgánulos más importantes en eucariotas. En plantas es el orgánulo encargado no solo de la síntesis de polisacáridos sino también del procesamiento de algunas proteínas (Parsons y col., 2012). Por lo tanto, un estudio del proteoma de Golgi podría proporcionar una valiosa información sobre cómo las proteínas contribuyen a la plasticidad de las respuestas que muestran las células habituadas a DCB (Capítulo IV).

Obtención de fracciones enriquecidas en Golgi

Para abordar este objetivo se desarrolló un protocolo de obtención de fracciones enriquecidas en Golgi de células de maíz, no habituadas y habituadas a niveles bajos y altos de DCB, empleando un gradiente discontinuo de sacarosa (Figura IV.1). La caracterización general mostró que las fracciones obtenidas de células habituadas presentaban menor cantidad de proteína total pero mayor actividad IDPasa (actividad marcadora de Golgi; Tabla IV.1). Cuando la síntesis y secreción de polisacáridos tiene lugar, el aparato de Golgi experimenta ciertas diferenciaciones morfológicas, revirtiendo a su estado compacto cuando estas actividades cesan (Dauwalder y col., 1969). En este estudio se observó un aumento en los valores de actividad IDPasa en tejidos donde la síntesis y secreción de polisacáridos eran muy activas, valores que revirtieron a basales cuando ambos procesos dejaron de producirse. Por lo tanto, estos resultados sugieren que la actividad IDPasa está correlacionada con la síntesis de polisacáridos, más concretamente con los cambios morfológicos que experimenta el aparato de Golgi durante la secreción de este tipo de moléculas. Consecuentemente, nuestros resultados podrían apuntar a una síntesis potenciada de polisacáridos no celulósicos en las líneas habituadas, precisamente para contrarrestar la pérdida en celulosa.

Tras varios pasos de preparación de las muestras (Figuras IV.2 a 4 y Tabla 2), la fracción enriquecida en Golgi de cada una de las líneas fue sometida a electroforesis 2-D (Figura IV.5), ya que este tipo de metodología ha demostrado ser adecuada en estudios similares realizados con otras especies (Taylor y col., 2000; Wu y col., 2000). De todas las proteínas desreguladas (Tabla IV.3) aquellas de mayor interés fueron extraídas del gel para su posterior identificación.

Identificación de proteínas desreguladas

Los resultados de la secuenciación de dichas proteínas se muestran en la Tabla IV.4. Algunas de las proteínas secuenciadas (enolasa 1, chaperonina 60 y 1-aminociclopropano-1-

carboxílico oxidasa 1 -ACO 1-) fueron coincidentes con otras encontradas en un estudio de proteoma total llevado a cabo anteriormente por nuestro grupo en células habituadas (Mélida y col., 2010a). Estos resultados parecen sugerir que estas proteínas juegan un importante papel en la habituación a DCB. Además, se detectó una ascorbato peroxidasa menos diferencialmente acumulada en las líneas habituadas, lo cual coincide con la menor actividad que presenta esta enzima en dichas líneas (Mélida y col., 2010a).

Los resultados apuntan a que algunas de las proteínas secuenciadas se encuentran desreguladas por efecto directo del DCB, y no tanto por la habituación al mismo. Por ejemplo, se ha descrito que la chaperonina 60 está implicada en el ensamblaje de la actina y la tubulina (Gutsche y col., 1999). Dado que el DCB actúa impidiendo la polimerización de los microtúbulos (Bisgrove y Kropf, 2001; Himmelspach y col., 2003; Rajangam y col., 2008), este hecho podría explicar por qué en células habituadas la chaperonina 60 aparece menos diferencialmente acumulada. Otro caso puede ser la represión observada en células habituadas para la α-1,4-glucano sintasa, clasificada dentro del grupo de polipéptidos de glicosilación reversible (RGP). Aunque algunos RGPs están involucrados en la síntesis de polisacáridos de pared (Dhugga y col., 1997; Langeveld y col., 2002), su ausencia puede estar relacionada con una pared celular más debilitada, tal y como han demostrado para dobles mutantes de genes RGPs Drakakaki y col. (2006).

Por otro lado, otras proteínas diferencialmente acumuladas en las células habituadas parecen formar parte de la estrategia de adaptación a DCB. Algunas de ellas podrían participar como indicadores relacionados con la integridad de la pared celular cuando ésta se ve dañada. La enolasa 1, que aparece más acumulada en las células habituadas, se ha descrito como una señal de estrés en maíz (Sachs y col., 1996), así como su sustrato (hexosas) cuando la integridad de la pared celular se ve dañada (Hamann y col., 2003; Wolf y col., 2012). La ACO 1, enzima implicada en la síntesis de etileno, también se acumuló menos en las líneas habituadas. En este caso, es su sustrato, amino-ciclopropano-carboxilico (ACC), más que la enzima, el que presenta una relevancia fisiológica cuando la síntesis de celulosa

se ve afectada (Tsang y col., 2011). Si los niveles de ACO 1 son menores en las células habituadas, su sustrato ACC sufrirá una acumulación en las células, lo cual podría activar la correspondiente ruta de señalización para comenzar con las estrategias de habituación.

La enzima cafeoil-CoA *O*-metiltransferasa 1 (CCoAOMT 1), que participa en la síntesis de unidades G y S de la lignina, apareció menos acumulada en las células habituadas a DCB. Ya se había descrito anteriormente que esta enzima era sustituida durante el proceso de habituación por la ácido cafeico 3-*O*-metiltransferasa (COMT), que participa en la síntesis de unidades S (Mélida y col., 2010a). Además, se ha visto en alfalfa que los cambios cualitativos y cuantitativos en la composición de la lignina están relacionados con la represión de la enzima CCoAOMT (Guo y col., 2001). Curiosamente, resultados preliminares de nuestro laboratorio indican que nuestras líneas celulares habituadas a DCB presentan diferencias cualitativas en la lignina, ya que ésta presenta un menor contenido en unidades G sin tener afectado el contenido de las unidades S.

No todas las proteínas que fueron secuenciadas son residentes de Golgi. Sin embargo, hay que considerar que el estudio se ha llevado a cabo con fracciones enriquecidas en Golgi, por lo que es esperable que puedan tener contaminaciones con otros orgánulos celulares (Hanton y col., 2005; Parsons y col., 2012). Además, dado que una de las funciones más importantes del aparato de Golgi es realizar modificaciones post-traduccionales en muchas proteínas, resulta difícil discriminar entre proteínas en tránsito o residentes.

Aunque la metodología empleada ha demostrado ser efectiva para el análisis de muestras similares en otras especies (Taylor y col., 2000; Wu y col., 2000), tiene la restricción de que las proteínas más abundantes enmascaran a las minoritarias (Timperio y col., 2008). Por otro lado, recientemente se ha visto que la interacción entre proteínas que forman parte de los complejos proteicos del Golgi puede diferir, y que este hecho afecta también a la función catalítica de los mismos (Oikawa y col., 2012). Por lo tanto, hay que considerar la posibilidad de que algunas de las proteínas desreguladas, aunque no tengan un

papel fisiológico claro en la respuesta de habituación a DCB, podrían participar a través de su interacción con otras.

Puesto que las células habituadas tienen modificada la red de arabinoxilanos, es plausible asumir que también presenten algún tipo de desregulación en las enzimas que los sintetizan. Se ha descubierto recientemente que la iniciación de la síntesis de polisacáridos de la pared celular y su maduración se produce en diferentes cisternas de Golgi, y que dicha localización varía dependiendo del tipo de célula (Driouich y col., 2012; Worden y col., 2012). Por lo tanto, sería esperable que las enzimas GTs encargadas de dicha síntesis y/o maduración estuvieran también ubicadas en distintas cisternas del aparato de Golgi. En futuros estudios sería interesante realizar un análisis más fino de las membranas contenidas en la fracción enriquecida en Golgi y comprobar en ellas distintas actividades GTs.

CONCLUSIONES

1. La línea celular habituada a DCB 1,5 µM (Sh1,5) mostró las características de una habituación incipiente al inhibidor. Estas células presentaron patrones de crecimiento alterados, entre los que se observaron cinéticas de crecimiento más lentas, tiempos de duplicación más largos y tasas de crecimiento más bajas que las células no habituadas (Snh). Además, crecieron desarrollando agregados celulares de mayores tamaños. La composición de sus paredes celulares también resultó modificada como consecuencia de la habituación a DCB: se observó una reducción del 33% en el contenido en celulosa que fue parcialmente compensado por un incremento en arabinoxilanos. Además, aunque en menor medida, los contenidos de otros polisacáridos como el xiloglucano y el ramnogalacturonano tipo I parecen encontrarse modificados.

2. Los arabinoxilanos de las células Sh1,5 constituyeron poblaciones más homogéneas en términos de masas moleculares, con tamaños medios más bajos y mayor

grado de extractabilidad que los de las células Snh. Estas características fueron consistentes en todas las fases del ciclo de cultivo así como en los diferentes compartimentos celulares analizados: protoplasma, pared celular y en el medio extracelular. El destino celular de los polisacáridos se encontró también modificado en las células habituadas a DCB, que mostraron proporciones reducidas de hemicelulosas fuertemente unidas a la pared y un incremento de los polímeros liberados al medio de cultivo. El análisis del metabolismo fenólico descartó que este último hecho fuese debido a una menor capacidad de las células Sh1,5 para entrelazar dichos polisacáridos.

3. La expresión de algunos de los genes implicados en la biosíntesis de celulosa y de xilanos resultó alterada durante la habituación a bajos niveles de DCB. De esta manera, según aumentaba el número de ciclos de cultivo en el que las células crecían en presencia de DCB, o cuando la concentración del mismo superó cierto umbral, las isoformas *ZmCESA7* y *ZmCESA8* resultaron inducidas. Este hallazgo da lugar a la hipótesis de que aunque menos eficientes en la síntesis de celulosa, las proteínas CESA7 y CESA8 serían más resistentes a los efectos de DCB. Los resultados del análisis de expresión de los genes ortólogos *IRX* de arabidopsis, implicados tanto en la iniciación de la síntesis como en la elongación de las cadenas de xilanos, sugieren que solo ésta última se encontraría afectada en las células Sh1,5, lo cual contribuiría parcialmente a explicar las menores masas moleculares medias de estas moléculas observadas en esta línea celular.

4. Como consecuencia de la habituación a bajos niveles de DCB, el metabolismo de las células de maíz también resultó alterado, siendo menos eficiente y más lento a lo largo de todo el ciclo de cultivo. No obstante, aunque la síntesis, integración en la pared así como la liberación de los distintos tipos de polisacáridos al medio de cultivo se encontró retrasada con respecto a los de las células Snh, ocurrió de manera sincronizada.

5. Importantes enzimas metabólicas relacionadas con condiciones de estrés, síntesis de etileno o metabolismo de carbohidratos y lignina se encontraron diferencialmente acumuladas en las fracciones enriquecidas en Golgi de las células habituadas a DCB. Algunas de las proteínas desreguladas parecen estarlo debido a efectos directos del DCB, como la chaperonina 60 y la α-1,4-glucano sintasa. Sin embargo otras parecen estar implicadas en las estrategias de habituación a DCB, como la enolasa 1, la 1-aminociclopropano-1-carboxilato oxidasa 1, la ascorbato peroxidasa y la cafeoil-CoA *O*-metiltransferasa 1.

Los resultados del presente trabajo confirman que los cultivos celulares de maíz son capaces de adoptar distintas estrategias en función del nivel de habituación a DCB, lo que implica no sólo cambios en la composición de la pared celular, sino también importantes alteraciones metabólicas. Estas estrategias mostraron rasgos comunes a todos los niveles de habituación, como la presencia de una red de arabinoxilano modificada, cambios en la expresión de genes relacionados con la biosíntesis de celulosa o xilanos, o la desregulación en el metabolismo de algunas proteínas. Sin embargo, también se han observado alteraciones exclusivas asociadas a los distintos procesos de adaptación a DCB. Todos estos resultados ponen de manifiesto la notable plasticidad estructural de las células de maíz para hacer frente a diferentes grados de reducción en su contenido de celulosa.

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Physiology

Early cell-wall modifications of maize cell cultures during habituation to dichlobenil

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ABSTRACT

Studies involving the habituation of plant cell cultures to cellulose biosynthesis inhibitors have achieved significant progress as regards understanding the structural plasticity of cell walls. However, since habituation studies have typically used high concentrations of inhibitors and long-term habituation periods, information on initial changes associated with habituation has usually been lost. This study focuses on monitoring and characterizing the short-term habituation process of maize (*Zea mays*) cell suspensions to dichlobenil (DCB). Cellulose quantification and FTIR spectroscopy of cell walls from 20 cell lines obtained during an incipient DCB-habituation process showed a reduction in cellulose levels which tended to revert depending on the inhibitor concentration and the length of time that cells were in contact with it. Variations in the cellulose content were concomitant with changes in the expression of several *ZmCesA* genes, mainly involving overexpression of *ZmCesA7* and *ZmCesA8*. In order to explore these changes in more depth, a cell line habituated to 1.5 μM DCB was identified as representative of incipient DCB habituation and selected for further analysis. The cells of this habituated cell line grew more slowly and formed larger clusters. Their cell walls were modified, showing a 33% reduction in cellulose content, that was mainly counteracted by an increase in arabinoxylans, which presented increased extractability. This result was confirmed by immunodot assays graphically plotted by heatmaps, since habituated cell walls had a more extensive presence of epitopes for arabinoxylans and xylans, but also for homogalacturonan with a low degree of esterification and for galactan side chains of rhamnogalacturonan I. Furthermore, a partial shift of xyloglucan epitopes toward more easily extractable fractions was found. However, other epitopes, such as these specific for arabinan side chains of rhamnogalacturonan I or homogalacturonan with a high degree of esterification, seemed to be not affected.

In conclusion, the early modifications occurring in maize cell walls as a consequence of DCB-habituation involved quantitative and qualitative changes of arabinoxylans, but also other polysaccharides. Thereby some of the changes that took place in the cell walls in order to compensate for the lack of cellulose differed according to the DCB-habituation level, and illustrate the ability of plant cells to adopt appropriate coping strategies depending on the herbicide concentration and length of exposure time.

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Introduction

Plant cell walls are dynamic structures whose importance resides principally in the key role they play in plant growth and development, enabling cells to adapt to abiotic or biotic stresses (Wolf et al., 2012). Cell walls influence the properties of most plant-based products, including their texture and nutritional value, and condition the processing properties of plant-based foods for human and animal consumption (Doblin et al., 2010). Cell walls are mainly composed of polysaccharides, which are classified as cellulose, hemicelluloses and pectins, and have lower amounts of proteins, phenolics and other minor components. Cell wall composition varies according to the plant type and species (Sarkar et al., 2009), cell type and position within the plant, developmental stage and history of responses to stresses (Doblin et al., 2010). This variability in the composition of cell walls reflects a certain degree of plasticity as regards cell wall structure and composition. One suitable method to study the mechanisms underlying the plasticity of plant cell wall structure and composition consists in habituating cell cultures to grow in the presence of high concentrations of different cellulose biosynthesis inhibitors (CBIs) (for a review see Acebes et al., 2010). CBIs are a structurally heterogeneous group of compounds that affects cellulose synthesis, acting specifically on cellulose coupling or deposition in higher plants. They can induce

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aberrant trajectories in CESA proteins, reduce their velocity or even clear them at the plasma membrane (Acebes et al., 2010; Brabham and DeBolt, 2013). In the last two decades it has been shown that it is possible to habituate undifferentiated cell cultures (calluses and cell suspensions) of various dicotyledonous plants, such as arabidopsis, tobacco, tomato, bean or poplar, to lethal concentrations of diverse cellulose biosynthesis inhibitors and related compounds, such as isoxaben, thaxtomin A, dichlobenil (DCB) or quinclorac, by gradually increasing the concentration in the culture medium. In general terms, in order to cope with the stressful conditions, the cell cultures habituated to these inhibitors modify the characteristic architecture of the type I cell wall (typical of gymnosperms, dicots and most monocots), having reduced levels of cellulose accompanied by a decrease in the hemicellulose content and a significant increase in pectins (Shedletzky et al., 1992; Díaz-Cacho et al., 1999; Encina et al., 2001, 2002; García-Angulo et al., 2006, 2009). The modifications produced in cells habituated to these inhibitors not only depend on the inhibitor concentration but also on the period of time that cells grow in contact with the inhibitor (Alonso-Simón et al., 2004).

There are only two reported studies in which cell cultures from plants with type II cell walls (characteristic of graminaceous plants, together with the other commelinoid monocots) have been habituated to a CBI. These were barley cell suspensions (Shedletzky et al., 1992) and maize callus cultures (Mélida et al., 2009, 2010a,b), habituated to DCB in both cases. The modifications found in these type II cell walls differed considerably from those described for type I cell walls, since the drastic reduction in cellulose content was compensated for an increase in the content of heteroxylans, which had lower extractability and higher relative molecular mass. Furthermore, in the case of maize cell cultures obtained in our laboratory, DCB habituation has implied an enrichment of hydroxycinnamates and dehydroferulates esterified on arabinoxylans. The content of other polymers, such as mixed glucan, xyloglucan, mannan, pectins and proteins, was unchanged or reduced (Mélida et al., 2009). As these characteristics differed from those described for DCB-habituated barley cell cultures, and did not look like any other structure previously described for type II cell walls, we proposed that our DCB-habituated maize cells had a unique structure, which was particularly interesting in order to gain a deeper understanding of the structural plasticity of the type II cell wall (Mélida et al., 2009).

Changes associated with the habituation of cell cultures to CBIs have commonly been analyzed using high concentrations of herbicide and long-term habituation periods. These kinds of study take advantage of the fact that the cell culture characteristics have been "fixed". However, information about the initial changes that take place during the process of habituation is lost, although cells at these stages probably present a huge variability in relation to their cell wall composition, properties and ability to habituate. A previous study conducted in order to monitor cell wall changes during DCB-habituation in bean calluses showed that when them were cultured in 0.5 μM DCB for less than 7 culture cycles no appreciable changes in cell wall FTIR spectra were detected, but when the number of culture cycles growing in DCB presence increased up to 13, the spectra of the cell walls underwent several changes, including an attenuation of the peaks related to cellulose (Alonso-Simón et al., 2004). So, this study established that (a) an initial period would be necessary until cell wall modifications arise and (b) that a set of cell wall modifications, not only a reduction in cellulose content, would be taking place.

Accordingly to these results, the aim of this study was to monitor and characterize the early changes happening during the DCB-habituation of cells with type II walls using maize cell suspensions as a cellular model. First of all, we monitored the changes produced throughout the DCB-habituation process, in order to select a cell line that showed an initial contrastable modification in some cell wall features. Next, we compared the cell growth pattern and cell wall composition of 1.5 μM DCB-habituated and non-habituated cells, paying special attention to the variation in composition of polysaccharides, using a set of techniques such as cell wall fractionation, gas-chromatography, immunodot assays and heatmaps.

Materials and methods

Cell cultures

Maize cell suspension cultures (*Zea mays* L., Black Mexican sweet corn, donated by Dr. S. C. Fry, Institute of Molecular Plant Sciences, University of Edinburgh, UK) from calluses obtained from immature embryo explants were grown in Murashige and Skoog media (Murashige and Skoog, 1962) supplemented with 9 μM 2,4-D and 20 g L^{-1} sucrose, at 25 °C under light and rotary shaken, and routinely subcultured every 15 days.

Habituation to DCB

Maize cell suspensions were cultured in DCB (supplied by Fluka) concentrations ranging from 0.3 to 1.5 μM. DCB was dissolved in dimethyl sulfoxide (DMSO), which did not affect cell growth at this range of concentrations.

Initially, non-habituated maize cells were cultured in media containing three DCB concentrations: 0.3 μM (lower than the I_{50}-concentration of DCB capable of inhibiting dry weight (DW) increase by 50% with respect to the control-value), 0.5 μM (the I_{50} value) and 1 μM (higher than the I_{50} value). After seven subcultures, some of the cells habituated to growth in 1 μM DCB were transferred into media containing 1.5 μM DCB. Habituated suspension cultures are referred to as Shx (n), where '*x*' indicates DCB concentration (μM) and (*n*) number of subcultures in that DCB concentration.

Growth measurements

Growth curves of non-habituated and 1.5 μM DCB-habituated cell lines were obtained by measuring the increase in DW at different culture times. Doubling time (dt) was defined as the time required for a cell to divide or a cell population to double in size in the logarithmic phase of growth, and was estimated by using the equation $\ln Wt = \ln Wo + 0.693t/dt$ (in which Wt: suspension cell culture DW at time '*t*' of the culture cycle; and Wo: suspension cell culture DW at the beginning of the culture cycle). Thus, Wt or Wo logarithmic plotting versus culture time is a straight line where the ordinate at the origin corresponds to the Wt or Wo natural logarithm. Relative growth rate (μ) was determined by the slope of the straight line ($0.693t/dt$). Maximum growth rate was the maximum difference in DW per time unit between two consecutive measurements in growth kinetics.

Cell cluster size was determined after vacuum filtration of cell suspensions that were sequentially passed through different pore-size meshes, and the relative abundance of the clusters was expressed as the percentage of DW retained in each filter.

ZmCesA gene expression analysis: 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 the Superscript III first strand synthesis system for RT-PCR (Invitrogen). First-strand cDNA was generated using an oligo (dT) 20 primer, and 1 μl of the first-strand cDNA was used as a template in subsequent PCR

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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 within 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) genes 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) and *ZmCesA8* (AF200532) were those described in Mélida et al. (2010a).

Preparation and fractionation of cell walls

Cells collected from suspension cultures in the stationary phase of growth were frozen and homogenized with liquid nitrogen using a pestle and mortar, and treated with 70% ethanol for 5 days at room temperature. The suspension obtained was centrifuged and the pellet was washed with 70% ethanol (x6) and acetone (x6), and subsequently air dried 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 incubated with 2.5 U ml^{-1} α-amylase 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 fiber filter, and the residue washed with 70% ethanol (x6) and acetone (x6), air dried and then treated with phenol–acetic acid–water (2:1:1 by vol.) for 8 h at room temperature (x2). This residue was finally washed with 70% ethanol (x6) and acetone (x6) and then air dried to obtain the cell walls.

In order to carry out a cell wall fractionation, dried cell walls were extracted at room temperature with 50 mM cyclohexane-*trans*-1,2-diamine-*N*,*N*,*N*′,*N*′-tetraacetic sodium salt (CDTA; 10 mg ml^{-1}) and then washed with distilled water. The resulting pellet was treated with 0.1 M KOH (10 mg ml^{-1}) for 2 h (x2) and washed again with distilled water. The remaining residue was then treated with 4 M KOH (10 mg ml^{-1}) for 4 h (x2) and subsequently washed with distilled water. Then 6 M KOH (10 mg ml^{-1}) was finally added to the pellet and treated at 37 °C for 24 h. KOH extracts were neutralized with acetic acid to pH 5.0.

Extracts of each treatment were dialyzed (48 h) and lyophilized, and referred to CDTA, 0.1 M KOH, 4 M KOH and 6 M KOH fractions, respectively.

Cell wall analyses

Q2 Cellulose content was quantified in crude cell walls by the Updegraff method (Updegraff, 1962) using 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 using cell wall samples (2 mg) mixed with KBr (1:100, w/w). Spectra were obtained on a Perkin-Elmer instrument at a resolution of 1 cm^{-1} (Online Resource 1). A window between 800 and 1800 cm^{-1}, containing information about characteristic polysaccharides, was selected in order to monitor cell wall structure modifications. All the spectra were normalized and baseline-corrected with Spectrum v 5.3.1 software, by Perkin-Elmer. Data were then exported to Microsoft Excel 2003 and all spectra were area-normalized. Difference spectra were obtained by digital subtraction of non-habituated and habituated cell wall FTIR spectra.

Total sugar content of each fraction was determined by the phenol–sulfuric acid method (Dubois et al., 1956) and was expressed as the glucose equivalent. Uronic acid content was determined by the *m*-hydroxydiphenyl method (Blumenkrantz and Asboe-Hansen, 1973) using galacturonic acid as standard. Neutral sugar content analysis was performed as described by Albersheim et al. (1967). Briefly, a lyophilized sample of each fraction was hydrolyzed with 2 M trifluoroacetic acid at 121 °C for 1 h and the resulting sugars were derivatized to alditol acetates and analyzed by gas chromatography (GC) using a Supelco SP-2330 column.

Analysis of polysaccharides by immunodot assays

An extensive analysis of polysaccharide distribution and composition of the cell wall fractions was carried out. Briefly, aliquots of 1 μl from each fraction were spotted onto nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany) as a replicated 1/5 dilution series of the preceding one, with 5 different dilutions for each fraction. Then, nitrocellulose membranes were blocked with phosphate-buffered saline (PBS, 0.14 M NaCl, 2.7 mM KCl, 7.8 mM $Na_2HPO_4{\cdot}12H_2O$, 1.5 mM KH_2PO_4, pH 7.2) containing 4% fat-free milk powder (MPBS) prior to incubation in primary antibody (hybridoma supernatants diluted 1/10 in MPBS) for 1.5 h at room temperature. After washing extensively under running tap water and PBS, membranes were incubated in secondary antibody (anti-rat horseradish peroxidase conjugate, Sigma) diluted to 1/1000 in MPBS for 1.5 h at room temperature. Membranes were washed as described above prior to color development in substrate solution [25 ml de-ionized water, 5 ml methanol containing 10 mg ml^{-1} 4-chloro-1-naphtol, 30 ml 6% (v/v) H_2O_2]. Color development was stopped by washing the membranes. Samples of commercial pectin (10 mg/ml; P41, Danisco), xyloglucan (10 mg/ml; kindly donated by Dr. T. Hayashi) and an arabinoxylan-enriched fraction (10 mg/ml; obtained from maize calluses habituated to 12 μM DCB, kindly donated by Dr. H. Mélida) were used as reference compounds.

Monoclonal antibodies were assayed and their corresponding recognized epitopes were: JIM5 (homogalacturonan with a low degree of methyl esterification; Clausen et al., 2003), JIM7 (homogalacturonan with a high degree of methyl esterification; Knox et al., 1990), LM5 (β-1,4-galactan side chain of rhamnogalacturonan type I; Jones et al., 1997), LM6 (α-1,5-arabinan side chain of rhamnogalacturonan type I and arabinogalactan proteins; Willats et al., 1998), LM10 (unsubstituted and relatively low substituted (1,4)-β-D-xylan; McCartney et al., 2005), LM11 (xylans and arabinoxylans; McCartney et al., 2005), and LM15 (non-fucosylated xyloglucan; Marcus et al., 2008).

Immunodot assay related heatmaps

Immunolabeling semi-quantification for each fraction was performed by scoring the number of dilutions labeled. Thus, a value ranging from 1 to 5 was assigned depending on the number of colored spots which appeared after incubation with the secondary antibody. These values were then entered into the statistical software (see Statistical analysis section for details) in order to obtain heatmaps.

Statistical analysis

Principal component analysis (PCA) was performed using a maximum of five principal components. All these analyses were carried out using the Statistica 6.0 software package.

Significant differences in the cellulose content were tested applying a one-factor ANOVA (performing Tukey's test as post hoc analysis), using the PASW Statistics 18 software package.

Heatmaps were performed by using RKWard statistical software.

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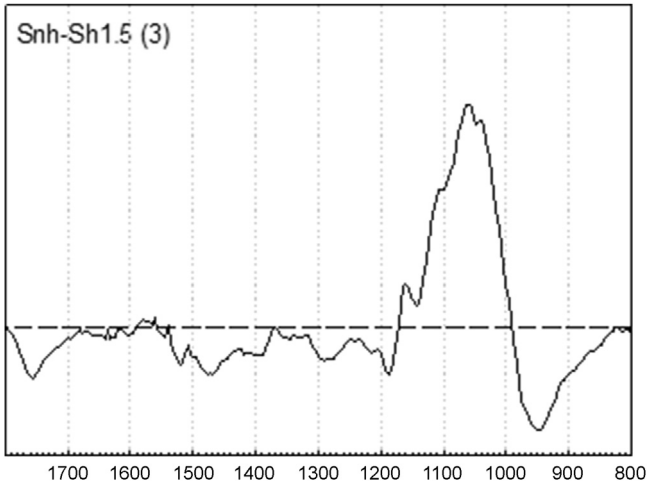

Fig. 1. Difference spectrum obtained from the digital subtraction of the FTIR spectra of cell walls from non-habituated (Snh) and habituated to 1.5 μM DCB (Sh1.5) maize cell suspensions, growing in that DCB concentration for three subcultures.

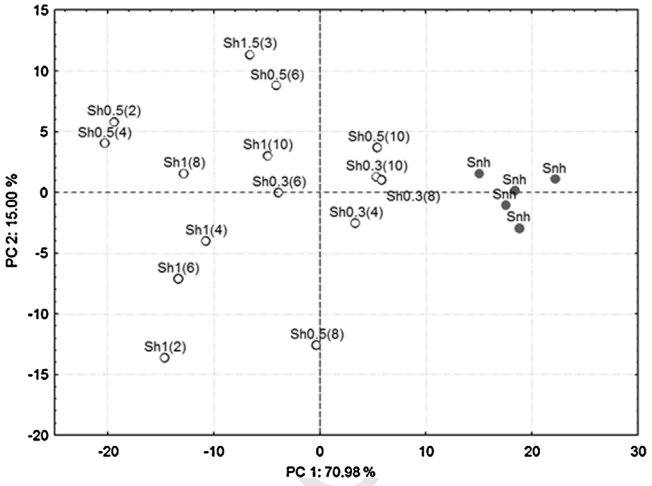

Fig. 2. Principal components analysis (PCA) of maize cell suspensions FTIR spectra. A plot of the first and second principal components (PCs) is represented based on the FTIR spectra of cell walls from non-habituated (Snh) and DCB-habituated (Sh) maize cell suspensions along several culture cycles. (Shx (n); x indicates DCB concentration (μM) and (n) number of subcultures in that DCB concentration).

Results

Monitoring of early cell wall modifications during DCB-habituation

FTIR spectroscopy and multivariate analysis

FTIR spectra from cell walls corresponding to non-habituated (Snh) and habituated (Sh) to 0.3 (Sh0.3), 0.5 (Sh0.5), 1 (Sh1) and 1.5 (Sh1.5) μM DCB maize cell suspensions which had been for different number of culture cycles growing in the presence of DCB were obtained and analyzed (Supplementary Data 1). The main differences among cell lines were detected in the fingerprint area (900–1200 cm^{-1}). In this area of the spectra, many polysaccharides absorb IR, including cellulose. In order to better appreciate the variations between Sh and Snh cell wall spectra, difference spectra for each cell line were obtained (Supplementary Data 2; Fig. 1). These spectra showed noticeable variation among Snh and most of the Sh cell lines subjected to short-term DCB habituation [i.e. see Snh-Sh0.3(2); Snh-Sh0.5(2) and Snh-Sh1(1)]. Spectra obtained from Sh cells showed decreased peaks in wavenumbers associated with cellulose (1039, 1056, 1060, 1105 and 1160 cm^{-1}) (Alonso-Simón et al., 2004, 2011). However, these variations were gradually attenuated in the difference spectra corresponding to cell walls of Sh0.3 and Sh0.5 cell lines as the number of culture cycles in presence of DCB was increased [see Snh-Sh0.3(4–10); Snh-Sh0.5(4–10)]. In contrast, the differences observed in the Sh1 cell line were more marked and they did not revert as the number of subcultures was increased [see Snh-Sh1(4–10)]. In addition, other wavenumbers not associated to cellulose such as 950 cm^{-1} – ascribed to pectins – or 1520 cm^{-1} -attributed to phenolic rings (Alonso-Simón et al., 2011), and others unattributed such as 1190 and 1480 cm^{-1}, were also affected indicating that other cell wall components have been modified.

Principal component analysis (PCA) of FTIR spectra showed two groups (Fig. 2) and the spectra were mainly discriminated by PC1 (approx. 71% of total variance explained). Spectra obtained from cell walls corresponding to Snh and suspensions habituated to lower DCB concentrations (Sh0.3 and Sh0.5) which had been growing in presence of the inhibitor for a greater number of culture cycles were located on the positive side of PC1. In contrast, spectra corresponding to cells habituated to higher DCB concentrations (Sh1 and Sh1.5) and those corresponding to cells which had been growing for a lower number of culture cycles in contact with lower concentrations of the inhibitor were grouped on the negative side of PC1.

Cellulose content

Cellulose content was influenced by DCB concentration as well as by the time that maize cells were growing in its presence (Fig. 3). In Snh cell walls, cellulose represented roughly 27% of the cell wall weight. However, a reduction in the cellulose level occurred when maize cells were exposed to DCB for one or two culture cycles. As the number of subcultures in presence of DCB was increased, the cellulose content of cell walls from suspensions habituated to lower DCB concentrations (Sh0.3 and Sh0.5) gradually reverted to that of the control level. In fact, after growing in contact with the inhibitor for ten culture cycles, their cellulose content reached values that did not differ significantly from those of the Snh. In contrast, this complete reversion did not occur in the Sh1 cell line, at least in the same number of culture cycles as Sh0.3 and Sh0.5 cell lines did.

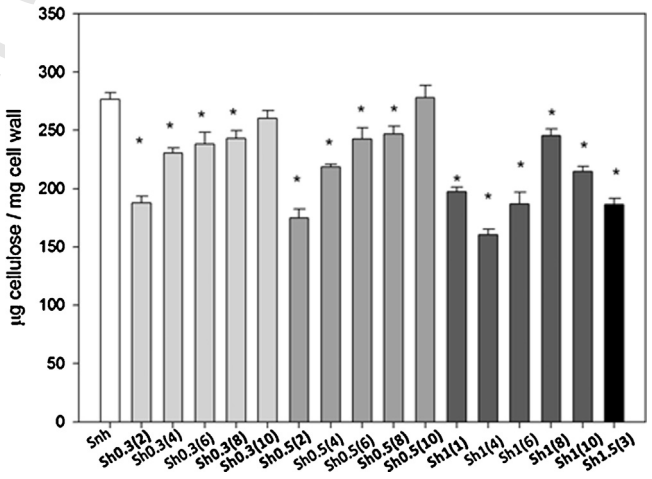

Fig. 3. Cellulose content of cell walls from non-habituated maize cell suspensions (Snh) and different DCB-habituated (Sh) maize cell suspensions (legend as Fig. 1). The asterisk reflects significant differences among non-habituated and DCB-habituated cell lines according to Tukey test ($p < 0.05$). Values represented are mean ± SE of nine measurements ($n = 9$). References in bold refer to the cell lines which were selected for further *ZmCesA* genes expression analysis (Fig. 4).

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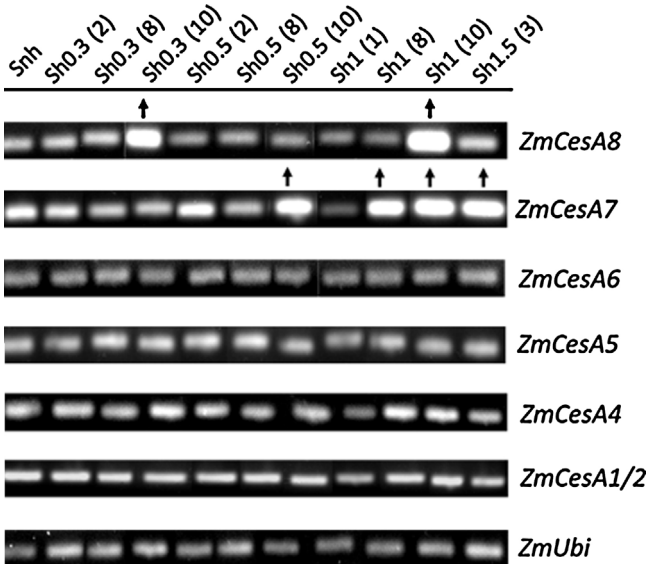


Fig. 4. Relative *ZmCesA* gene expression analyzed by RT-PCR of different maize cell suspensions (legend as Fig. 1). ↑; more mRNA accumulation than control (Snh); *ZmCesA3* was not included because no expression was detected. *ZmUbi*: ubiquitine gene expression. Ubiquitine was used as the housekeeping gene due to its constitutive expression.

ZmCesA genes expression

ZmCesA8 and *ZmCesA7* gene expression was induced in those cell cultures which were grown for a high number of culture cycles in the presence of DCB -Sh0.3(10) and Sh0.5(10), respectively (Fig. 4). An interesting trend was observed in cells habituated to 1 μM DCB: an induction of *ZmCesA7* expression was detected after 8 and 10 culture cycles – Sh1(8) and Sh1(10), and in the former line, *ZmCesA8* expression was also enhanced. However, none of these genes were induced in DCB habituated cells in their first culture cycle – Sh1(1). Lastly, the expression of *ZmCesA7* was also induced in Sh1.5(3). No expression of *ZmCesA3* was observed in any of the cell lines analyzed, and no differences in mRNA accumulation in *ZmCesA1/2*, *ZmCesA4*, *ZmCesA5* and *ZmCesA6* with respect to the Snh were detected.

The Sh1.5 cell line was selected for further analysis since it was representative of an early DCB-habituation based on the following criteria: (a) a 33% reduction of cellulose content, later demonstrated to be irreversible (data not shown) was observed (b) FTIR spectrum indicated that cellulose, as well as other polysaccharides, were affected (c) PCA results pointed to differences with Snh cells but also with the remaining Sh cells, indicating features of an incipient DCB habituation.

Culture features

Growth of Snh and Sh1.5 maize cell suspensions throughout the culture cycle was analyzed (Fig. 5a). Sh1.5 cells showed a longer lag phase and a less pronounced logarithmic phase of growth when compared with the Snh cells, which determined longer doubling times and lower relative and maximum growth rates (Fig. 5b).

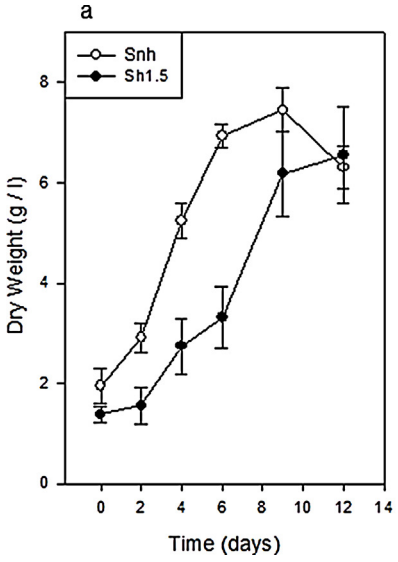
Snh cells showed homogeneous cluster-size since 88% of their DW was found to have diameters ranging between 710 and 400 μm. In contrast, a larger average size and greater variety of sizes was observed in the Sh1.5 cells, and 73% of their DW was retained in filters with pore sizes ranging from 3000 to 710 μm (Fig. 6).

Cell wall characterization

Cell wall fractionation and sugar analysis

Most polysaccharides were extracted by alkali treatments, especially in 4 M KOH and 6 M KOH fractions. Sh1.5 cell walls were enriched in total sugars, mainly extracted with 4 M KOH followed by 0.1 M KOH (Fig. 7a).

Gas chromatography and uronic acid analysis of cell wall fractions (Fig. 7b) revealed that alkali fractions were composed mainly of arabinose and xylose, followed by glucose, uronic acids and galactose, whereas the CDTA fraction was enriched in uronic acids and arabinose. Arabinose and xylose were responsible for the increase in neutral sugars detected in the 0.1 M KOH and 4 M KOH fractions in Sh1.5 cell walls. A decrease in glucose, especially in fractions extracted with 4 M KOH and 6 M KOH, was observed in Sh1.5 cells. Finally, an slight increase in uronic acids, mainly in alkali-extracted fractions, was shown by Sh1.5 cells.



b

	Doubling time	Relative growth rate	Maximum growth rate (g / l day)
Snh	2.3	0.3	1.17
Sh1.5	3.6	0.19	0.96

Fig. 5. (a) Growth of maize cell suspensions during the culture cycle. *Open circle*; non-habituated maize cell suspensions (Snh). *Filled circle*; habituated to 1.5 μM DCB maize cell suspensions (Sh1.5). Values represented are mean ± SD of nine measurements ($n = 9$); (b) growth parameters obtained from the growth curves of non-habituated (Snh) and habituated to 1.5 μM DCB (Sh1.5) maize cell suspensions.

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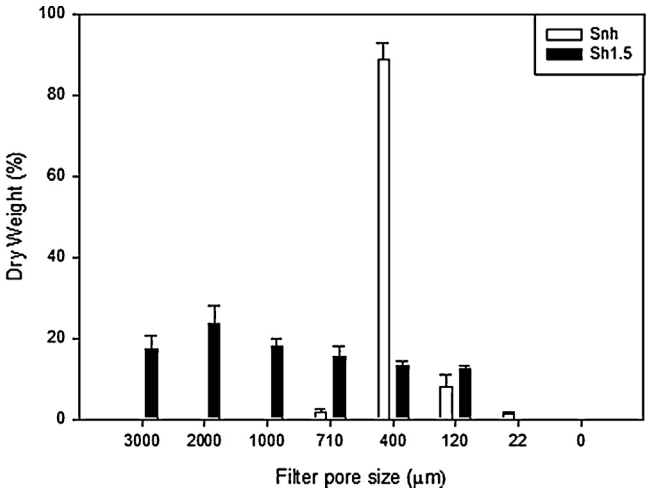

Fig. 6. Size of cell clusters of maize cell suspensions at the stationary phase. *White bars*; non-habituated maize cell suspensions (Snh). *Black bars*; habituated to 1.5 μM DCB maize cell suspensions (Sh1.5). Values represented are mean ± SE of three measurements ($n = 3$).

Polysaccharide epitope screening

In order to detect cell wall changes in the distribution of epitopes in short-term DCB-habituated cells, aliquots of cell wall fractions of Snh and Sh1.5 cell suspensions were loaded onto nitrocellulose membranes as 1/5 dilutions and subjected to immunodot assays (Fig. 8a). Monoclonal antibodies for different pectic and hemicellulosic epitopes were probed. Regarding to the specific antibodies probed for hemicelluloses epitopes for β-1,4-xylans, recognized by LM10, were exclusively found in all the alkali-extracted cell wall fractions from Sh1.5. The pattern of LM11 labeling -which detected specifically epitopes for xylans and arabinoxylans- was similar in both cell lines, with the exception that the Sh1.5 6 M KOH cell wall fraction showed an increased intensity of labeling. Lastly, the degree of labeling for non-fucosylated xyloglucan epitopes (LM15) was revealed to be stronger in the Sh1.5 4 M KOH cell wall fraction, whereas the opposite trend was observed in the 6 M KOH fraction.

Respect to pectic polysaccharides, cell wall fractions from both cell lines were probed with specific antibodies for homogalacturonan and rhamnogalacturonan I. Results obtained for JIM5 and JIM7, antibodies for homogalacturonan with low and high degree of methylesterification respectively, showed that no differences in the pattern of labeling were found among cell wall-fractions from the Snh and Sh1.5 cell lines for JIM7, whereas a greater intensity of labeling for JIM5 was found in CDTA and 0.1 M KOH Sh1.5 cell line wall-fractions when compared to Snh.

LM5 and LM6 were probed in order to detect β-1,4-galactan and α-1,5-arabinan side chains of rhamnogalacturonan I. A more intense label for LM5 was found in all the alkali extracted-fractions from Sh1.5 cells. In contrast, no differences between cell lines were

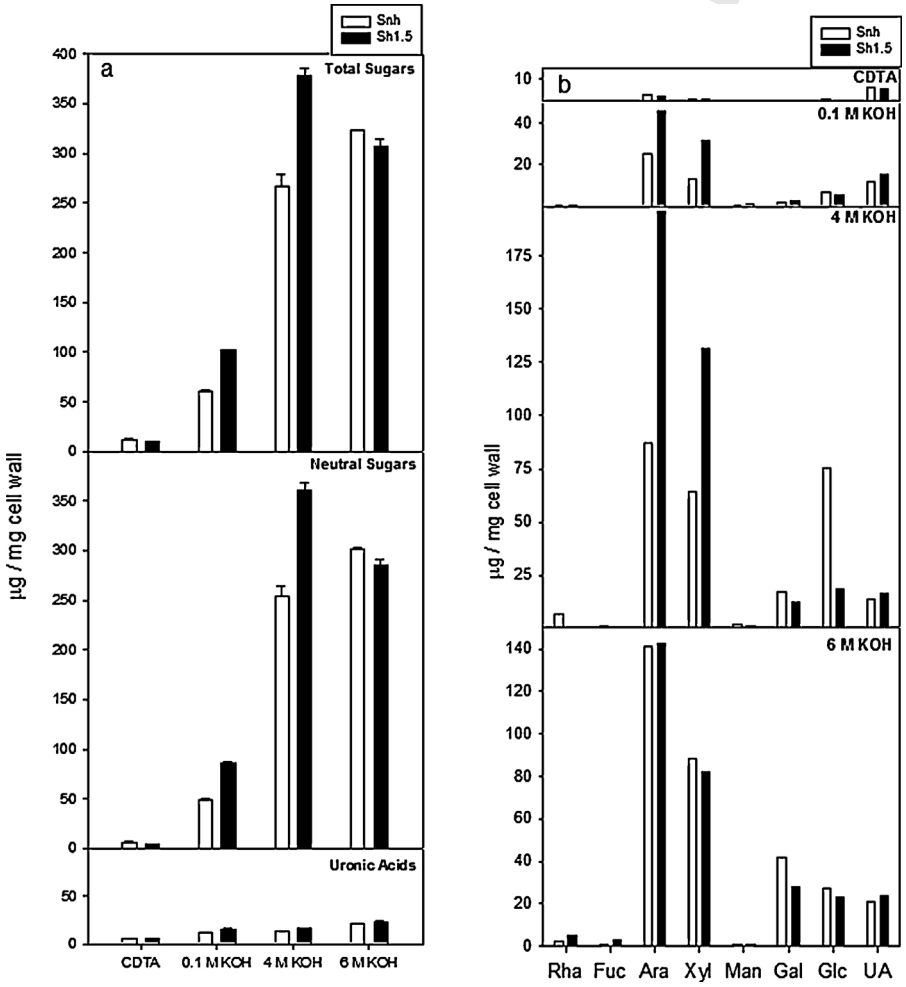

Fig. 7. (a) Total sugars, neutral sugars and uronic acids contents of fractions from cell walls of non-habituated (*White bars*) and habituated to 1.5 μM DCB (*Black bars*) maize cell suspensions. Values represented are mean ± SE of three measurements ($n = 3$); (b) monosaccharide composition of fractions from cell walls of non-habituated (*White bars*) and habituated to 1.5 μM DCB (*Black bars*) maize cell suspensions. Rha: rhamnose, Fuc: fucose, Ara: arabinose, Xyl: xylose, Man: mannose, Gal: galactose, Glc: glucose, UA: uronic acids.

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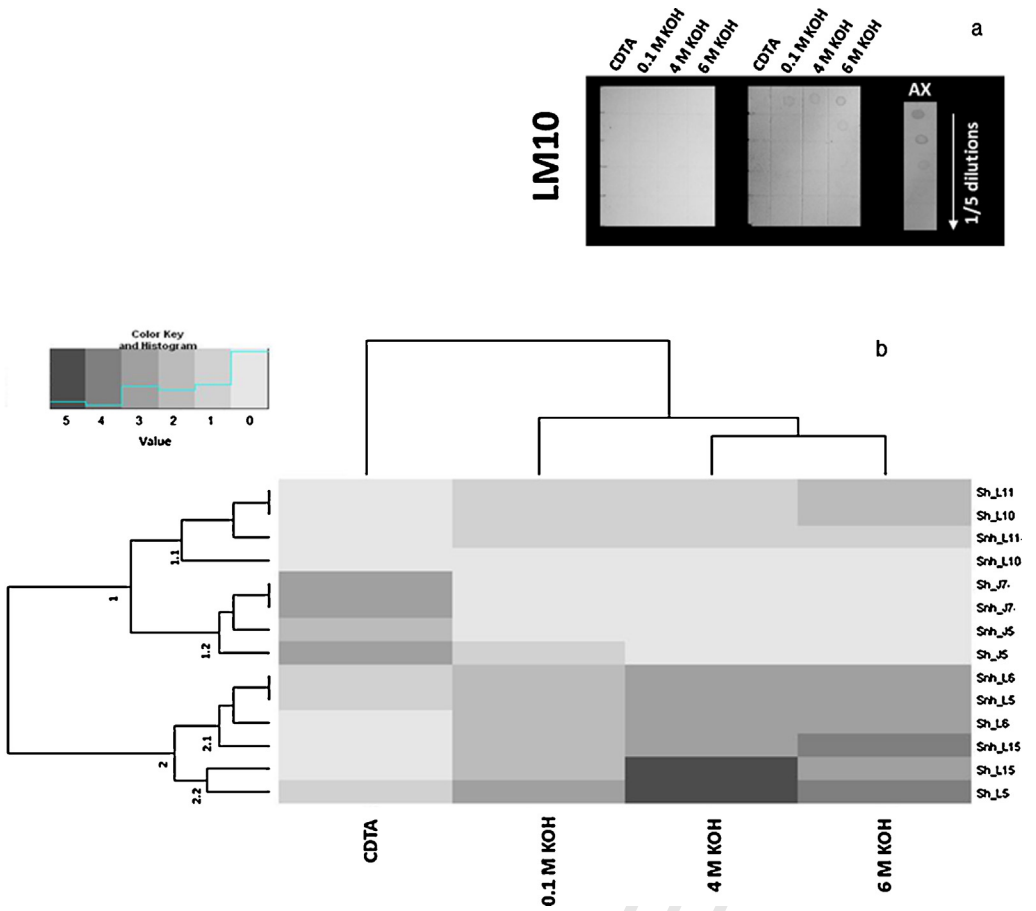

Fig. 8. (a) Representative immunodot assay (IDA). Cell wall fractions obtained from non-habituated cells (Snh) and habituated to 1.5 μM DCB (Sh1.5) were probed with LM10, monoclonal antibody specific for xylans. Any labeling was observed for Snh fractions, whereas 0, 1, 1 and 2 colored spots were detected respectively in CDTA, 0.1 M KOH, 4 M KOH and 6 M KOH fractions from Sh1.5 cells. AX: arabinoxylan-enriched fraction used as standard. (b) Heatmap of data obtained from IDAs of non-habituated (Snh) and habituated to 1.5 μM DCB (Sh) cell wall fractions. A value ranging from 0 (no colored spot detected) to 5 (5 colored spots detected) was assigned to each antibody (J5: JIM5, J7: JIM7, L5: LM5, L6; LM5, L10:LM10, L11:LM11, L15:LM15) and in each cell wall fraction (CDTA, 0.1 M KOH, 4 M KOH, 6 M KOH), depending on the amount of colored spots (corresponding to dilutions) that were shown after revealing.

found in these fractions when LM6 was probed. Nevertheless, α-1,5-arabinan side chains of rhamnogalacturonan I epitopes were solely detected in the CDTA fraction of Snh cells.

A heatmap was generated with the resulting pool of data (Fig. 8b). As the total number of dilutions assayed was 5, a value ranging from 0 to 5 was assigned. This value depended on the sum of colored spots (corresponding to labeled epitopes in each dilution) found in the heatmap (see methods). In order to illustrate this process, a representative immunodot assay for LM10 is shown in Fig. 8a. Likewise, when LM10 was assayed, the scoring values assigned were 0 for all the Snh cell wall fractions and 0, 1, 1 and 2 for Sh1.5 CDTA, 0.1 M KOH, 4 M KOH and 6 M KOH fractions, respectively.

Two different types of groupings appeared in the heatmap: a dendrogram with cell wall fractions and a dendrogram with the different antibodies probed. These groupings depended on the distribution pattern of labeled epitopes in the fractions.

The closest cell wall fractions in the Snh and Sh1.5 cell lines, and consequently the most similar in terms of the distribution of labeled epitopes, were 6 M KOH and 4 M KOH, followed by 0.1 M KOH and CDTA. In the dendrogram generated from the antibodies probed, two main branches were formed, each of which then divided again into another two principal sub-branches. Branch 1.1 grouped antibodies which recognized xylans (LM10) and arabinoxylans (LM11). For these, a darker intensity of color was observed in the heatmap for Sh1.5 cells when compared with that for Snh cells, indicating a more widespread presence of these polysaccharides in the alkali-extracted cell wall fractions of the habituated cell line. Although not so marked, a similar trend appeared in the labeling pattern of antibodies which recognized β-1,4-galactan (LM5) side chains of rhamnogalacturonan I, non-fucosylated xyloglucan (LM15) (branch 2.1 vs. 2.2) and homogalacturonan with a low degree of esterification (JIM5) (branch 1.2), pointing again to a more extensive occurrence of epitopes for these polysaccharides throughout the cell wall fractions of the Sh1.5 cells.

Discussion

One of the most conspicuous changes detected by FTIR monitoring of the cell walls from the cell lines studied was a reduction in the cellulose content. In fact, the main differences among spectra of non-habituated (Snh) and DCB-habituated (Sh) (Fig. 1 and Supplementary Data 1, 2) maize cell walls appeared in the fingerprint region (900–1200 cm^{-1}), in which a wide range of polysaccharides absorb IR radiation, including cellulose. These differences became more marked as the DCB concentration in the culture medium was increased. However, differences among the spectra of Snh and Sh exposed to lower DCB concentrations (Sh0.3 and Sh0.5) were attenuated when the number of culture cycles that cells were grown in contact the inhibitor was increased. These data were confirmed by the results obtained from the analysis of cellulose content (Fig. 3). A 23% and 27% reduction in the cellulose content was observed

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when maize cells were exposed to 0.3 and 0.5 μM DCB, respectively, but after 10 culture cycles growing in presence of the inhibitor, these levels reverted to values close to the Snh value. At higher DCB concentrations (Sh1 and Sh1.5), differences in the fingerprint region among spectra (Fig. 1 and see Supplementary Data 1, 2) were greater with respect to the Snh spectra and eventual recovery of the cellulose content was not observed (Fig. 3). The gradual reversion in cellulose content produced when cells were growing in low DCB concentrations and the number of culture cycles in its presence was increased, had previously been described in bean cell suspensions (García-Angulo et al., 2006) and callus-cultured cells (Alonso-Simón et al., 2004), but interestingly, in these cells with type I walls, an initial period of about seven subcultures was required previously to the detection of any changes in their cell walls FTIR profiles, whereas in our maize cells, which have type II cell walls, this lag period has not been observed.

One possible explanation for this observed transient reversion in cellulose content at the initial steps of the DCB-habituation process could be that increasing concentrations of herbicide may promote some change in the expression level of *ZmCesA* genes. In fact, it has been found that *ZmCesA* gene expression was mis-regulated in maize cultured cells habituated to high (12 μM) DCB concentrations (Mélida et al., 2010a). An induction of *ZmCesA7* and *ZmCesA8*, thought to be involved in producing the primary cell wall before the onset of secondary wall formation (Appenzeller et al., 2004; Bosch et al., 2011), was detected in some of our short-term habituated cell lines (Fig. 4). It seems that maize cells start to overexpress these isoforms when the number of culture cycles growing in presence of DCB is increased or when the concentration of the inhibitor exceeds a threshold. Similarly maize cells subjected to long-term habituation to high DCB concentrations also showed an induction of *ZmCesA7* and *ZmCesA8* genes (Mélida et al., 2010a). This could be indicative of an important role for these two *CesA* isoforms in habituation to all DCB levels. It could be hypothesized that, although less efficient in cellulose synthesis, *ZmCesA7* and *ZmCesA8* may be more resistant to the effects of DCB.

In addition to cellulose, FTIR monitoring revealed that other polysaccharides seem to be also affected by DCB-habituation. So, in order to unmask other actors playing a role in early DCB-habituation events, Sh1.5 cell line was selected for further studies.

In the Sh1.5 cell line the observed 33% reduction in cellulose content was compensated by a net increase in 0.1 M KOH and 4 M KOH extracted arabinoxylans (Figs. 7a and b). Immunodot assays also showed in this cell line an increase in the proportion of epitopes for arabinoxylan/xylan (LM10 and LM11). Interestingly, no LM10 labeling was observed neither in Snh cell walls in our work, nor in maize calluses habituated to high DCB concentrations – 4, 6 or 12 μM (Mélida et al., 2009). The divergence between both studies could be related to the difference of plant material (calluses or suspension cell cultures), but would be also linked to a transient modification in chemical composition in some subsets of arabinoxylans populations at the first steps of the habituation.

Other hemicelluloses, such as xyloglucan, seems also to be involved in the early events of DCB-habituation, since an increase in the detection of LM15 epitopes was observed in the Sh1.5 4 M KOH fraction, pointing to more easily extractable xyloglucan molecules. Reinforcing this finding, a lower presence of LM15 epitopes was found in the in 6 M KOH fraction from Sh1.5 cells, treatment which would extract strongly linked molecules.

Furthermore, sugar composition analysis points to an increase of uronic acid-rich polysaccharides, especially in alkali-extracted fractions, in the early steps of habituation. Likewise, in such alkali-extracted fractions, immunodot assays revealed an enhancement of LM5 labeling and, therefore, an increase in galactan side chains of rhamnogalacturonan I in the habituated cell line. Also, an enrichment of epitopes for JIM5 (homogalacturonan with a low degree of esterification) was found in the mild-alkali (0.1 M KOH) extracted fraction, as well as in the CDTA-extracted fraction.

The fact that other related epitopes such as those for homogalacturonan with a higher degree of esterification (JIM7) or for arabinan (LM6) did not change between cell lines, indicates that only some specific subsets of polysaccharides were affected during the early DCB-habituation.

It is interesting to note how in the type II cell walls of our cell cultures, characterized by a rather low amount of pectic polysaccharides, an increase in such type of polysaccharides is enhanced in the first steps of DCB-habituation. This fact is comparable with the observed trend in DCB-habituated plant cultured cells having type I walls, as bean, in which the reduction in the cellulose content is mainly counteracted by an increase in pectic polysaccharides (Encina et al., 2001; García-Angulo et al., 2006). Previous work carried out in DCB or isoxaben habituated cultured cells having type I cell walls, are characterized by a substantial increase in JIM5-reactive low-esterified pectins, which would point to more Ca^{+2}-cross-linked pectins (Wells et al., 1994, Sabba et al., 1999, Manfield et al., 2004; García-Angulo et al., 2006). As pectic polysaccharides are involved in cell-cell adhesion (Willats et al., 2001), the higher amount of these polysaccharides observed in our habituated cells could explain the fact that these cultured cells developed larger cell clusters than the Snh cultures (Fig. 6), resembling to that described for DCB-habituated bean cell suspensions (Encina et al., 2001; García-Angulo et al., 2009). Nevertheless, and as a supplementary explanation, such slower and altered growth patterns detected in Sh1.5 cells could be also related with their cellulose-deficient wall which would therefore, cause an impaired cell division. On the other hand, and although xyloglucan is known not to be an abundant hemicellulose in type II cell walls (Fry, 2000), our results shown that these hemicelluloses may play some role during this early stress situation, as the detection of LM15 epitopes increased (at least in 4 M KOH fraction) during the first steps of DCB habituation.

Interesting differences in cell wall modifications appear when early DCB-habituation events are compared with those described for maize calluses habituated to intermediate and high DCB levels (Mélida et al., 2009, 2010a,b). In long-term DCB habituated cells, a 75% of reduction in cellulose content was detected, and this value remained stable even when DCB concentration in the culture medium increased from 6 to 12 μM. In contrast, in short term DCB-habituation, the reduction in cellulose content was lower (23–33%) and tended to revert when DCB concentration in the culture medium ranged from 0.3 and 0.5 μM. Moreover, in long-term DCB-habituation the reduction in the amount of cellulose was compensated by an increase in arabinoxylans, but the content of other polysaccharides, such as pectins or xyloglucan, was not affected or even was reduced. However, as mentioned above, in Sh1.5 cultures besides the not so drastic increase in the arabinoxylan amount, other polysaccharides such as rhamnogalacturonan I, homogalacturonan with a low degree of esterification, and even xyloglucan seemed to be involved. Finally, although further studies are required, our preliminary results suggest that antioxidant activities play an important role during the first steps of the DCB habituation process (Largo, personal communication), in contrast to those described in cultures habituated long-term to high DCB concentrations (Mélida et al., 2010a).

Concluding remarks

In sum, monitoring of short-term DCB habituation of maize cell suspensions has revealed that the main modification produced during the first steps of this process consisted in the reduction of cellulose content, and it has been confirmed that changes in these

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cellulose levels were promoted by two main factors: the inhibitor concentration and the number of culture cycles that cells were in its presence. Wall composition of Sh1.5 cells was modified as a consequence of habituation to low DCB levels, showing a reduction of 33% in the cellulose content. Furthermore, an induction of *ZmCesA7* and *ZmCesA8* took place and this effect was revealed as a constant feature of DCB habituation. Sh1.5 cells counteracted the lack of cellulose with an increase in arabinoxylans. In addition to arabinoxylans, other polysaccharides, such as galactan side chains of rhamnogalacturonan I, homogalacturonan with a low degree of esterification and xyloglucan, seemed to have a role in the first steps of DCB-habituation. Thereby some of the modifications occurred in the cell walls in order to compensate for the lack of cellulose differed according to the DCB-habituation level, and demonstrates the remarkable dynamic plasticity of maize cell cultures, which enables them to cope with different DCB habituation conditions by altering their cell wall composition.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jplph.2013.10.010.

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