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AREA DE FISIOLÓGÍA VEGETAL

CELL WALL REMODELLING OF CELLULOSE
DEFICIENT MAIZE CELLS: GOING THROUGH
THE KEY ROLE OF PHENOLICS

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INFORME DE LOS DIRECTORES DE LA TESIS
(R.D. 99/2011, de 28 de enero y Normativa de la ULE)

Los Doctores D. Antonio E. Encina García y D. José Luis Acebes Arranz, como Directores de la Tesis Doctoral titulada *"Cell wall remodelling of cellulose deficient maize cells: going through the key role of phenolics"/ "Remodelación de la pared celular en células de maíz con deficiencia en celulosa: indagando en el papel clave de los fenoles"* realizada por Dña. Romina Martínez Rubio en el programa de doctorado Biología Molecular y Biotecnología, en el Departamento de Ingeniería y Ciencias Agrarias, Área de Fisiología Vegetal, informan favorablemente el depósito de la misma, dado que reúne las condiciones necesarias para su defensa.

Lo que firmamos, en León a 21 de Junio de 2018.

Dr. Antonio E. Encina García

Dr. José Luis Acebes Arranz

A mis padres

“Todo parece imposible hasta que se hace”

(Nelson Mandela)

Scientific papers

Research carried out during the present Thesis, has led to the following publications:

-de Castro, María; **Martínez-Rubio, Romina**; Acebes, José Luis; Encina, Antonio; Fry, Stephen C. and García-Angulo, Penélope (2017). Phenolic metabolism and molecular mass distribution of polysaccharides in cellulose-deficient maize cells. *Journal of Integrative Plant Science*, 59: 475-495. doi: 10.1111/jipb.12549. **IF: 3.962/Q1.**

-**Martínez-Rubio, Romina**; Acebes, José Luis; Encina, Antonio and Kärkönen, Anna (2018). Class III peroxidases in cellulose deficient cultured maize cells during cell wall remodelling. *Physiologia Plantarum (Cell Wall Special Issue)*, doi.org/10.1111/ppl.12710. **IF: 3.330/Q1.**

Moreover, during my PhD program, additional publications (SCI indexed) has been published:

-Rebaque, Diego; **Martínez-Rubio, Romina**; Fornalé, Silvia; García-Angulo, Penélope; Alonso-Simón, Ana; Álvarez, Jesús M.; Caparrós-Ruiz, David; Acebes, José Luis and Encina, Antonio (2017). Characterization of structural cell wall polysaccharides in cattail (*Typha latifolia*): Evaluation as potential biofuel feedstock. *Carbohydrate Polymers*, 175: 679-688. doi: 10.1016/j.carbpol.2017.08.021. **IF: 4.811/Q1.**

-Hernández Altamirano, Mabel J.; Largo-Gosens, Asier; **Martínez-Rubio, Romina**; Pereda, Diego; Álvarez, Jesús M.; Acebes, José Luis; Encina, Antonio and García-Angulo, Penélope (2018). Effect of ancymidol on cell wall metabolism in growing maize cells. *Planta*, 247: 987-999. doi: 10.1007/s00425-018-2840-y. **IF: 3.361/Q1.**

International conferences

Some of the results collected from the present Thesis, and other results obtained during my PhD program, have been presented in:

-**Martínez-Rubio, Romina**; de Castro, María; Álvarez, Jesús M.; Acebes, José Luis; García-Angulo, Penélope and Encina, Antonio. (May 2016). Deciphering the cell wall phenolic metabolism of dichlobenil habituated maize cells. *XI Finnish Plant Science Days* (Turku, Finland).

-**Martínez-Rubio, Romina**; de Castro, María; Álvarez, Jesús M.; Acebes, José Luis; García-Angulo, Penélope and Encina, Antonio. (June 2016). Changes in the cell wall phenolic metabolism of cellulose deficient maize cells. *XIV Cell Wall Meeting* (Chania, Greece).

-Hernández Altamirano, Mabel J.; Largo-Gosens, Asier; **Martínez-Rubio, Romina**; Pereda, Diego; Álvarez, Jesús M.; García-Angulo, Penélope; Acebes, José Luis and Encina, Antonio (June 2016). Effect of ancymidol on cell wall metabolism in growing maize cells. *XIV Cell Wall Meeting* (Chania, Greece).

-García-Calvo, Laura; **Martínez-Rubio, Romina**; Rigau, Joan; Acebes, José Luis; Álvarez, Jesús M.; Fornalé, Silvia; Encina, Antonio; Caparrós-Ruiz, David and García-Angulo, Penélope. (June 2016). Relationship between cell wall composition and structure and resistance to lodging in maize stalks. *XIV Cell Wall Meeting* (Chania, Greece).

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-Estancia Breve del Ministerio de Educación, Cultura y Deporte dentro del Programa Estatal de Promoción del Talento y su Empleabilidad. Natural Resources Institute Finland (Luke) (Finlandia) (05.04.2017-04.05.2017) **Convocatoria 2016.**

In addition, along this Thesis I have been involved in the following research projects:

-Estudio de los polímeros estructurales de la pared celular en maíz. Uso de células deficientes en celulosa. Financiado por MINECO (2012-2014) (**Ref: AGL2011-30545-C02-02**).

-Desarrollo de herramientas biotecnológicas asociadas con la pared celular y la rotura de la caña del maíz para la mejora de la productividad y la calidad. Financiado por MICINN (2015-2018) (**Ref: AGL2014-58126-R**).

This PhD Thesis focuses on the compensation mechanisms experienced by plant cells with a reduced content of cellulose, and more specifically on the role of cell wall phenolics, considering both hydroxycinnamates and induced lignin, in such compensation mechanisms. To address this research, maize cell cultures habituated or treated with dichlobenil, a cellulose biosynthesis inhibitor, were used as model system. The general structure of the project is detailed below:

The first section is devoted to the **Introduction**, which provides a general overview of the composition, structure and biosynthesis of the maize featuring primary cell wall: the type II cell wall. Moreover, it focuses on the current knowledge of the cell wall structural plasticity and on the role of the different compounds of the cell wall, which may be modified searching for plants with an improved biotechnological potential. The Introduction is followed by its own references.

Secondly, a **general objective** chapter is presented divided into five partial objectives. To address them, **five independent chapters** are detailed, each of them written as a scientific paper and, therefore, structured with the following sections: abstract, introduction, material and methods, results, discussion and references. Two of the chapters (see Appendix; published manuscripts) have already been published in high impact journals. The remaining three chapters are currently being completed with new studies and are thought to be published in the future.

After that, a **general discussion** includes the joint comment of the aforementioned chapters followed by their own references. Finally, the **conclusions** section in which one conclusion for each objective is detailed.

Finally, a **summary in Spanish** including abstract, objectives and conclusions is added.

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Abbreviations

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

5,5'-DFA: 5-5'-dehydrodiferulate

8,5'-DFA: 8-5'-nc- dehydrodiferulate

8-O-4'-DFA: 8-O-4'- dehydrodiferulate

ABTS: 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)

AIR: alcohol insoluble residue

Ara: arabinose

AX: arabinoxylan

CA, Cou: *p*-coumaric acid

CBI: cellulose biosynthesis inhibitor

CCW: covalently bound cell wall (protein)

CesA / CESA: cellulose synthase (protein)

CIII-POX: class III peroxidase

CSC: cellulose synthase complex

Cinn: cinnamic acid

CWD: cell wall damage

DAB: 3,3'-diaminobenzidine

DCB: dichlobenil or 2,6-dichlorobenzonitrile

DFA: dehydrodiferulate

DMSO: dimethylsulfoxide

DW: dry weight

early-log: early logarithmic (growth phase)

FA, Fer: ferulic acid

FW: fresh weight

G: guaiacil (units)

GalU: galacturonic acid

GAX: glucuronoarabinoxylan

GT: glycosyltransferase (enzyme)

H: *p*-hydroxyphenyl (units)

H, Sh_{1.5}: maize suspension-cultured cells habituated to 1.5 μ M DCB

HG: homogalacturonan

[³H]SEP: soluble extracellular [³H]-polymer

ICW: ionically bound cell wall (protein)

IEF: isoelectric focusing

isoPOX: isoperoxidase

late-log: late logarithmic (growth phase)

MLG: mixed-linkage glucan

M_r: relative molecular mass

M_w: weight average relative molecular mass

NH, S_{nh}: non-habituated suspension-cultured (cells)

PCR: polymerase chain reaction

pI: isoelectric point

POX: peroxidase

RG: rhamnogalacturonan

S: syringyl (units)

SC: soluble cellular (protein)

SM: spent medium (protein)

SuSy: sucrose synthase

TFA: trichloroacetic acid

TLC: thin layer chromatography

XG: xylogalacturonan

XyG: xyloglucan

The cell wall is a dynamic structure that provides important mechanical and physiological functions for the plant. Previous studies in our group have demonstrated that a reduction in the cellulose content in maize primary cell walls leads to a modified cell wall architecture and composition, causing changes in the amount and structure of other components. Therefore, the overall aim of the present study was to have a deep insight into the knowledge of the compensation mechanisms associated to the structural plasticity of the cell wall, paying special attention to the role of the phenolic compounds. In order to achieve this goal, maize suspension-cultured cells with a reduction in the cellulose content by habituation to dichlobenil (DCB), a cellulose biosynthesis inhibitor, were used.

Cellulose impairment involved changes in the metabolism and structure of hemicelluloses. By using a radiochemical approach, it was demonstrated that cell wall bound hemicelluloses and sloughed polymers were more homogeneously sized and had a lower weight-average relative molecular mass in DCB-habituated cells when compared to non-habituated ones. Despite their lower molecular mass, hemicelluloses underwent massive cross-linking mediated by hydroxycinnamates, mainly ferulic and *p*-coumaric acids, as a part of their strategy to compensate the lack of cellulose. As previous results pointed out, it is expected for DCB-habituated cells to find a modified phenolic metabolism. The comparative study between the hydroxycinnamates esterified into protoplasmic and cell wall polysaccharides through the cell culture cycle revealed that DCB-habituated cell walls showed an enrichment in ferulic acid, *p*-coumaric acid and dehydrodiferulates indicating an extra dimerization *in muro*. Furthermore, the 8-O-4'-diferulic acid emerged as a diagnostic feature of DCB habituation process. These results support the idea that cell wall hydroxycinnamates play a key role in the remodelling of cellulose deficient cell walls.

In order to explain the molecular basis for a cell wall enriched in hydroxycinnamates that underwent a more extensive crosslinking, a study on peroxidases (POX) and genes related with cell wall feruloylation/coumaroylation was carried out. A comparative study of POX activity and isoforms associated with cell wall remodelling was addressed. POX activity was higher in DCB-habituated cells than in control ones in all cell wall compartments assayed. Moreover, the

comparison of the isoPOX profile obtained from the ionically bound cell wall proteins and spent medium proteins revealed a distinctive higher activity for certain POX isoforms in DCB-habituated cells. It is hypothesized that some of these isoPOXs could be involved in the cross-linking of arabinoxylans in DCB-habituated cells. In order to gain a further insight into the genes that could be involved in the feruloylation/coumaroylation of the cell wall, a preliminary survey into the maize BAHD genes was undertaken. Thirteen maize homologues to BAHD genes previously found in rice and *Arabidopsis* were identified by bioinformatics approaches. All the protein sequences shared the transferase domain HXXXDG characteristic of BAHD proteins involved in the acylation of arabinoxylans. From the BAHD genes analysed, *ZmBAHD1*, *ZmBAHD2* and *ZmBAHD12* were overexpressed in DCB-habituated cells. Interestingly, *ZmBAHD1* and *ZmBAHD12* showed a high coexpression level with genes related to the synthesis and remodelling of the cell wall. These results point to *ZmBAHD1* and *ZmBAHD12* as good candidates as putative feruloyl and/or coumaroyl transferases in maize cell walls.

Finally, as maize cultured cells exposed to DCB have been reported to induce the synthesis of ectopic lignin, an investigation was conducted in order to verify if a cellulose biosynthesis inhibitor with a different mode of action, such as cobtorin, also induce ectopic lignification. The negative phloroglucinol staining of cells after cobtorin treatment indicated that this compound was not inducing ectopic lignification in our experimental system. In contrast, DCB-treated cells showed positive staining for phloroglucinol, a high cell wall autofluorescence and ammonium induced fluorescence related to the accumulation of phenolics. All these three findings showed the presence of a lignin-like polymer in DCB-treated cells. Furthermore, the overexpression of *ZmBAHD5*, *ZmBAHD6*, *ZmBAHD9* and *ZmBAHD12* together with an enhancement of peroxidase activity in DCB-treated cells were found. Therefore, the induction of ectopic lignification promoted by a restriction in the synthesis of cellulose seems to be a spatiotemporal process in which changes in the expression of selected BAHD genes and in the activity of POX enzymes, together with structural modifications, contribute to reinforce the weakened cell wall entanglement.

The results obtained in this Thesis project afford new data that shed light into the key contribution of phenolics in the remodelling of cell walls with a low cellulose content. Moreover, additional information on the biochemical and genetic control of this process are provided.

INTRODUCTION

The amazing plant cell wall

The cell wall is a **dynamic and partially rigid structure** that surrounds the protoplast of the plant cells, providing structural integrity and mechanical support for the plant (Carpita et al. 2015). Cell wall provides important **structural and physiological functions**, defining the cell shape and size, controlling the metabolism exchange between protoplast and external medium (Baron-Epel et al. 1988), and taking part in recognition processes among cells and between cells and pathogens (Caffal and Mohnen 2009, Carpita, 2011, Wolf et al. 2012, Le Gall et al. 2015). In addition, cell walls are involved in the modulation of stress signalling responses, giving protection and **defence against biotic and abiotic stress factors** (Le Gall et al. 2015, Srivastava et al. 2017). The **annual production of cell walls** of land plants alone has been estimated to be 150-170 billion tons (Pauly and Keegstra 2008). Therefore, the total annual output of the chemical industry worldwide (1.2 billion tons/year) is produced by land plants in the form of cell walls in just two days, and during this process massive amounts of **atmospheric carbon dioxide** are assimilated by plants through **photosynthesis** and deposited and stored in the **cell wall**, making this structure the **dominant carbon-sequestration system on this planet**. However, the cell wall is not only a carbon storage device, but also a **sophisticated material composite** made of a number of polymer networks (Loqué et al. 2015, Wouwer et al. 2018).

The **synthesis of the cell wall** takes place during the telophase in the cell division process, with the development of the cell plate (Smertenko et al. 2018), process that leads to the synthesis of the **middle lamella**, made of pectic polysaccharides (Smith 2001, Wolf et al. 2012, Chen et al. 2018). Continuing with the development of the middle lamella, cells start to synthesise the **primary cell wall**, located between the plasma membrane and the middle lamella, constituted mainly by cellulose microfibrils and a matrix of polysaccharides and proteins, whose composition differs depending on the species and tissues (Knox 2008, Wolf et al. 2012, Carpita et al. 2015, Cosgrove 2016). In this primary cell wall the orientation of the cellulose microfibrils determines the growth direction (Wolf et

al. 2012, Chebli and Geitman 2017). In some type of cells, when the growth has ceased, a massive deposition of new materials between the plasma membrane and the primary cell wall leads to the formation of the **secondary cell wall**. This structure is characterised by the presence of higher amounts of cellulose, and the hydrophobic polyphenol **lignin** (Albersheim et al. 2010), and determines the final cellular size, shape and function (Schneider and Persson 2015, Kumar et al. 2016) (**Figure 1**).

Figure 1. Representation of plant cell wall (S1-S3, three different layers of secondary cell wall). Modified from Carpita et al. (2015) (**A**) and Encina et al. (2002) (**B**).

Humankind and plant cell walls: an interdependent story

Humans have always maintained a tricky relationship with plant cell walls by using wood for shelter, paper for writing, fibres for clothing, fuel for heating and transport, and sources for medicines and foods (Johnson et al. 2018).

In the last decades, interest has increased about considering walls as a renewable energy resource for second-generation biofuels. Particular attention has been paid to lignocellulosic biomass, defined as waste bioproducts generated from agro-based industries and composed by cellulose, hemicellulose and lignin, i.e. plant secondary cell walls (Johnson et al. 2018). Additionally, the relation with cell walls in our **diet** is also evident as diets rich in fibre are made of mainly by walls and resistant starch, containing wholegrains, fruits and vegetables. In the same way, cell walls are responsible of the organoleptic properties of fruits and vegetables, and their modifications provide their appropriate texture (Padayachee

et al. 2017). These products help to improve **health outcomes** and prevent certain **chronic diseases** such as type II diabetes and cardiovascular and inflammatory intestine diseases (Collins et al. 2010, Koropatkin et al. 2012, Padayachee et al. 2017, Johnson et al. 2018).

By better **understanding the complex structure-function** relationships among cell wall components, the optimization of foods, fibres for textiles, wood for construction and biomass for biofuels will be achieved. This understanding will contribute to a **more sustainable and efficient use of plant-based wall products**, since lignocellulosic feed-stocks do not represent a food source for humans (Carrol and Somerville 2009, Johnson et al. 2018). However, **grass cell wall constituents** play an important role in animal production by providing the major source of carbon and energy for **ruminants** which are extensively used as a human food source.

Lignocellulosic biomass represents an abundant, inexpensive and locally available **feedstock for conversion to biofuels** (Pauly and Keegstra 2008, Kootstra et al. 2009). Interestingly, the utilization of plant cell walls to partially replaced petroleum as an energy source is now a possible alternative. In fact, **by 2030**, grass lignocelluloses are expected to be a **major biomass source** for the **production of ethanol** from fermentable sugars (de O Buanafina et al. 2016).

Lignocellulosic biomass can be utilised as a feedstock for biofuel production in a number of ways. Depending on the process, different attributes in wall composition are required or desired. One approach includes degradation of the polysaccharides on the lignocellulosic feedstock to monosaccharides, and their subsequent fermentation to ethanol and other advanced biofuels (Pauly and Keegstra 2010). The major challenge in this process is that plants have cell wall structures that are recalcitrant to biological degradation (Pauly and Keegstra 2008, Carrol and Somerville 2009). Furthermore, a high amount of enzymes is required to release fermentable glucose monomers from cellulose because its tight linkage with other polymers in native walls (Pauly and Keegstra 2010). That is the reason why in order to utilise cellulose efficiently, this **complex matrix needs to be disrupted** to create more “open” cell walls in which cellulosic enzymes can easily access to cellulose (Pauly and Keegstra 2010).

In the last years, several **plant biotechnological approaches** have been successful afforded, trying to **reduce the recalcitrance** to enzymatic degradation without compromising both plant growth and biomass accumulation (Furtado et al. 2014). Three **different strategies** have been used to achieve this goal: (i) improving pre-processing (e.g. mechanical, thermochemical); (ii) improving processing, utilising more efficient enzymes and microbes capable of tolerating toxic inhibitors, withstanding high product and by-product concentrations during biomass digestion and the subsequent fermentation process, and (iii) developing less recalcitrant feedstocks by reducing/altering the lignocellulosic polymer network (Alvira et al. 2010, Balat 2011, Klein-Marcuschamer et al. 2012, Loqué et al. 2015). Hence, **genetic modification of plant cell walls** represents a powerful way to overcome biomass recalcitrance (Chen and Dixon 2007, Demura and Ye 2010, Xie and Peng 2011, Wang et al. 2016), so the different components together with the genes that are involved in the synthesis and regulation of this complex structure can be considered as **biotechnological targets** on which to act. Therefore, we will focus our attention on the composition and structure of the cell wall, in order to insight the different biotechnological targets found in the wall building.

Type II primary cell wall composition and biosynthesis

The primary cell wall is composed by cellulose embedded in a matrix of hemicelluloses, pectins and proteins. It can be distinguished **two types of primary cell walls** that differ in their chemical composition, and are associated with different plant taxa, developmental stage and cell type (Carpita and Gilbeaut 1993, Knox 2008). On the one hand, **type I primary cell wall**, characteristic of dicots and non-commelinoids monocots, in which xyloglucan is the main hemicellulosic polysaccharide and is responsible for tethering the cellulose microfibrils. Both components have similar contents and create a layer cellulose-xyloglucan which is embedded in a pectic matrix. On the other hand, **type II primary cell wall** is exclusive to commelinoid monocots (Carpita 1984, 1996) and shows lower amounts of xyloglucans than type I primary cell walls, which are mainly replaced by (glucurono)arabinoxylans bounded to cellulose microfibrils. In addition, type II

cell walls have less structural proteins and pectins, and higher amounts of phenolic compounds (mainly hydroxycinnamates) than type I primary cell walls (**Figure 2**).

Figure 2. Structural models of type I (**A**) and type II (**B**) primary cell wall (Carpita et al. 2015).

Considering that maize, the species used in this study, is featured by type II primary cell walls, we will focus on the structure and composition of its structure.

Cellulose

Cellulose is considering the **main component of plant cell walls** due to its capacity to be the scaffold for the rest of the cell wall constituents. **Cellulose content** in primary cell walls varies from 15 to 30% of its dry weight, in contrast with secondary cell walls, in which the cellulose content raise up to 50% (Carpita et al. 2015).

Cellulose is deposited in **microfibrils**, defined as a crystalline structure consisting of chains of glucose residues linked by β -(1,4) linkages arranged in parallel (Delmer 1999, Lerouxel et al. 2006, Guerriero et al. 2010). This conformation means that each glucose residue is positioned at 180° respect to the contiguous one, being the cellobiose the disaccharide which appears repeatedly in

the molecule (Saxena and Brown 2000). This configuration is the responsible for the plain spatial structure of the β -(1,4)-glucan chains, leading to the formation of inter- and intramolecular bonds among individual cellulose chains. The result is a stable crystalline microfibril structure with some amorphous (non-crystalline) regions (Somerville 2006, Taylor 2008, Guerriero et al. 2010, McFarlane et al. 2014).

For years, this **structure** was proposed to be made up of 36 chains of β -(1,4)-glucan chains disposed in parallel (Delmer 1999, Lerouxel et al. 2006). However, nowadays another structural conformation models have been proposed consisting of 18 or 24 instead 36 chains per microfibril (Vandavasi et al. 2016, Jarvis 2018, Meents et al. 2018; Turner and Kumar 2018).

Cellulose synthesis is taking part at the plasma membrane by a membrane-bound protein complex known as the **cellulose synthase complex (CSC)**, also called **rosettes** (Somerville 2006, Taylor 2008, Guerriero et al. 2010, McFarlane et al. 2014, Kumar et al. 2017, Scavuzzo-Duggan et al. 2018, Turner and Kumar 2018). The most extended model proposes that the CSC is organised in hexameric structures, which each hexamer containing six cellulose synthase proteins (CesA) (Cosgrove 2005, Somerville 2006). However, recent studies have suggested that each subunit could be arranged by only three CesA proteins leading to a 18 β -glucan chains microfibril (Gerriero et al. 2010, Hill et al. 2014, Nixon et al. 2016, Vandavasi et al. 2016). In line with this, recent studies have shown that variations in the structure of the CSC have important implications for the structure of the cellulose microfibril produced (Turner and Kumar 2018, Voiniciuc et al. 2018) (**Figure 3**).

In higher plants, **CesA proteins** are synthesised by members of **six multigene families** with a number of genes that varies among species. Different number of *CesA* genes have been identified in arabidopsis (10) and maize (12) (Kumar and Turner 2015), most of them through the study of mutants for the cellulose synthesis. These approaches showed that in arabidopsis cellulose synthesis is controlled by three *CesA* genes: *AtCesA1*, *AtCesA3* and *AtCesA6-like* (Desprez et al. 2007) in primary cell walls and other three *CesA* genes in secondary cell wall: *AtCesA4*, -7 and -8 (Taylor et al. 1999, 2003, Kumar et al. 2016).

Figure 3. Representation of the cellulose synthase complex (CSC) at the plasma membrane and some factors involved in its regulation. Cortical microtubules and contribution of UDP-Glucose (UDP-Glc) by the enzyme Sucrose Synthase (SuSy) can affect CSC movement. Membrane composition (presence/absence of sphingolipids and sterols) can also affect mobility of the CSC. Proteins interacting with CSC, such as KORRIGAN (endo β -(1,4)-glucanase) and Cesa-interactive protein (CSI1/POM2), can regulate cellulose synthesis. Extracellular proteins such as GPI anchored-protein, including COBRA protein, and Chitinases-like (CTL1 and CTL2) can influence the speed of CSC, deposition of cellulose microfibrils and synthesis of other polysaccharides (Modified from McFarlane et al. 2014).

In case of maize, three clusters each, containing three *ZmCesA* genes, are involved with the primary cell wall building, whereas the rest (*ZmCesA10*, -11 and -12) are involved in the secondary cell wall synthesis (Appenzeller et al. 2004, Kaur et al. 2016, Maleki et al. 2016).

In addition to Cesa, **other proteins** have been shown to be **involved in cellulose synthesis**, often associated with CSC, such as **sucrose synthase (SuSy)** and an **endo- β -(1,4)-glucanase (KORRIGAN)**. The first one plays an important role in cellulose synthesis providing UDP-Glc to the catalytic CSC machinery from

UDP and sucrose (McFarlane et al. 2014). The latter one has a transitory association to the CSC (Guerriero et al. 2010, McFarlane et al. 2014).

Non-cellulosic polysaccharides: hemicelluloses and pectins

Non-cellulosic polysaccharides are synthesized and assembled in the Golgi apparatus and subsequently transported to the cell wall by Golgi vesicles (Lerouxel et al. 2006, Driouich et al. 2012, Anderson et al. 2016, Hatfield et al. 2017). The proteins involved in this process are glycosyltransferases enzymes (GTs) located in the internal face of Golgi membranes (Scheller and Ulvskov 2010) which catalyse the transfer of a sugar residue from an activated nucleotide-sugar onto a specific receptor (Lairson et al. 2008, Rennie and Scheller 2014).

▪ Hemicelluloses

Hemicelluloses are a group of neutral polysaccharides with non-crystalline structure, composed of a linear chain of monosaccharides, mainly xylose, glucose or mannose, with generally short branches (Pauly et al. 2013). The main hemicelluloses in type II cell walls are **xylans** (Peña et al. 2016), whose structure consists of a backbone of 1,4 linked β -Xyl residues substituted with different residues depending on the tissue type and the species (Pauly et al. 2013) (**Figure 4**). In grasses, they are often decorated with glucuronic acid and methyl-glucuronic acid, arabinose (Ara) or a combination of acidic and neutral sugars. Thus, the type of substitution allows us to distinguish among **arabinoxylans** (AX), **(methyl)glucuronoxylans** and **glucuronoarabinoxylans** (GAX) (Peña et al. 2016, Hatfield et al. 2017). GAX are a major component of the secondary cell walls in grasses (40-50%), whereas it represents only the 20-40% of primary cell walls (Scheller and Ulvskov 2010, Peña et al. 2016). Type II cell wall xylans are featured by the presence of hydroxycinnamates, mainly ferulic acid (FA) and *p*-coumaric acid (CA) esterified on the Ara residues (Smith and Hartley 1983, Kato and Nevins 1985).

In addition, a lower amount of other hemicelluloses is found in type II primary cell walls, such as **xyloglucan (XyG)** and **mixed-linkage glucans (MLG)**.

	Chain	Frequent substitutions
Xyloglucan	(1→4)-β-D- Glc	α-D-Xil (1→6) β-D-Gal-(1→2)-α-D-Xil -(1→6) α-L-Fuc-(1→2)-β-D-Gal-(1→2)-α-D-Xil (1→6) β-D-Gal-(1→2)-α-D-Xil (1→6)
Arabinoxylan	(1→4)-β-D- Xil	α-D-Ara (1→3) / α-D-Ara (1→2)
(Glucurono)arabinoxylan	(1→4)-β-D- Xil	α-D-Ara (1→3) / α-D-Ara (1→2) (4-O-metil)-α-D-GlcU (1→2)
Mixed-linked glucan	(1→3),(1→4)-β-D- Glc	

Figure 4. Summary of molecular structures, substitutions and the most frequent types of link of the major hemicelluloses (Xyl, xylose; Glc, glucose; Gal, galactose, Fuc, fucose; GlcU, glucuronic acid). Based on Albersheim et al. (2010).

XyG consists of a lineal chain of β-(1,4)-glucan, substituted with α-(1,6)-Xyl residues, that can be branched with other substitutions changing between species and tissues (Scheller and Ulvskov 2010, Pauly and Keegstra 2016). Although xyloglucans represent only about 2-10% in type II cell walls, they have the ability to attach cellulose to other cell wall polymers forming a cellulose-xyloglucan network, playing an important role on plant growth (Pauly and Keegstra 2016).

MLG is a hemicellulose presents only in type II primary cell walls (Carpita and McCann 2010) and liverworts and some types of algae (Popper and Fry 2003, Scheller and Ulvskov 2010). The structure is an unbranched backbone of glucose residues linked by β-(1,3) and β-(1,4) linkages (Pauly et al. 2013).

These hemicelluloses bind adjacent cellulose microfibrils through the establishment of hydrogen bonds, maintaining them in the correct spatial conformation and playing a crucial role in primary cell wall expansion (Obel et al. 2002, Gibeaut et al. 2005). Additionally, adjacent AX molecules can cross-link by di-ferulate bonding, contributing to stiffen cellulose-hemicellulose network (Ishii 1997, Fry 2004).

Several studies have shed light on the hemicelluloses synthesis. Experiments carried out mainly with arabidopsis mutants have revealed the involvement of

some GTs in the process. In this sense, different protein families such as GT43, GT47, GT8 and GT61 have been identified (Scheller and Ulvskov 2010, Pauly et al. 2013).

▪ Pectins

Pectins are matrix polysaccharides enriched in galacturonic acid (GalU) with the most structural and functional complexity among the cell wall components. Their content in type II cell walls (2-10%) is lower than in type I cell walls (20-30%) (Carpita 1996, Caffall and Mohnen 2009) and are located in middle lamella and primary cell walls. In spite of their low amount in type II cell walls, they perform important physiological functions such as water absorption and retention, ion transport, cell adhesion and expansion, as well as acting in the defence against pathogens, wounds and abiotic stresses (Verhertbruggen and Knox 2006, Mohnen 2008, Boller and Felix 2009, Wolf and Greiner 2012, Anderson 2016, Bethke et al. 2016). (**Figure 5**).

	Chain	Frequent substitutions
Homogalacturonan	(1→4)-β-D- GalU	
Rhamnogalacturonan I	→4)-α-D- Gal-(1→2)-α-L- Rha-(1→	β-D- GlcU-(1→6)-β-D-Gal-(1→4)- β-D-Gal-(1→6)-[β-D-Gal-(1→4)] ₂ - α-D-Ara-(1→5) (1→2) (1→3) -β-D-Gal-(1→4)- [α-D-Ara-(1→5)] _n - (α-D-Ara) (1→4)-
Rhamnogalacturonan II	(1→4)-α-D- GalU	α-L- Rha-(1→5)-α- KDO-(2→3)- α-L- Rha-(1→5)-α- DHA-(2→3)- β-D- Gal-(1→2)-β-D- GlcU-(1→4)-α-L- Fuc- (1→2)-α-L- Rha-(1→3)-β-D- Api-(1→2)-
Xylogalacturonan	(1→4)-β-D- GalU	α-D- Xyl (1→3)-

Figure 5. Summary of molecular structures, substitutions and the most frequent types of link of pectins. Abbreviations: Gal, galactose; Fuc, fucose; GlcU, glucuronic acid, Rha, rhamnose; GalU, galacturonic acid; Api, apiose; KDO, 2-keto-3-deoxy-d-manno-2-octulosonic acid; DHA, 2-keto-3-deoxy-d-lyxo-2-heptulosaric acid. Based on Albersheim et al. (2010).

According to their structure, four structural pectin domains can be distinguished: **homogalacturonan (HG)**, **xylogalacturonan (XG)**, and **rhamnogalacturonan I and II (RG-I and RG-II)** (Scheller et al. 2007). These domains are thought to be conjoined structures (Fry 2011), supporting the idea that *in muro*, each different domain is not an independent polysaccharide (Ishii and Matsunaga 2001, Coenen et al. 2007).

HG, the most simple and abundant pectic polymer, is a linear homopolymer of α -(1,4)-linked GalU residues which can be methyl esterified or acetylated (Ridley et al. 2011). This molecule can form a stable gel structure with other pectins by calcium bridges (Liners et al. 1989). When HG is substituted with single xylose residues is known as **XG**. In addition, the backbone of HG is covalently linked to RG-I and RG-II, and is also thought to be crosslinked to xyloglucan in muro (Popper and Fry 2008).

RG-I is formed by repeating units of the disaccharide 1,2- α -Rha-(1,4)- α -GalU (Carpita et al. 2015) and is substituted with β -(1,4)-galactan, branched arabinan or arabinogalactan side chains. **RG-II** consists of a linear structure of α -(1,4)-linked GalU residues with branches points entailing 12 different types of sugars in over 20 different linkages (Mohnen 2008, Caffall and Mohnen 2009, Srivastava et al. 2017).

Due to the diversity of linkages that can be found in pectins, a large number of enzymes have been involved in their synthesis. During the last decades and through the use of different mutants, it has been estimated that methyltransferases, acetyltransferases and a high amount of GTs are involved in pectin biosynthesis (Scheller et al. 2007, Mohnen 2008, Harholt et al. 2010, Bethke et al. 2016).

Proteins

Although proteins are minor components of the cell walls, they are essential compounds, being involved in the interaction with plasma membrane proteins located at the cell wall surface, promoting the cellular cohesion and taking part in

the modification of some compounds in the cell wall (Jamet et al. 2006, Uzal et al. 2009, Albenne et al. 2013, Canut et al. 2016). Cell wall proteins can be divided into two main groups: structural proteins, usually immobilised at the cell wall, and soluble proteins, which can be located anchored or free at the apoplast, and normally have enzymatic activities (Lee et al. 2004).

Structural proteins are generally glycoproteins and proteoglycans immobilized into the cell wall and they present repeated sequences enriched in one or two amino acids, being divided in four different groups: proline-rich proteins; hydroxyproline-rich glycoproteins, glycine-rich proteins and arabinogalactan proteins (Vogel 2008, Carpita et al. 2015).

Regarding the **soluble proteins**, most of them are related to processes such as cell wall extension, molecular transport, cell recognition and pathogen resistance (Rose et al. 2002, Novo-Uzal et al. 2009, Marowa et al. 2016). This group is constituted by hydrolases, transglycosylases, kinases, expansins and extracellular peroxidases. Particularly, **class III peroxidases** (CIII-POX, EC 1.11.1.7) play a crucial role in cell wall metabolism, taking part in plant growth and development, including polymerization of lignin monolignols and cross-linking the cell wall components mediated by the oxidative coupling of phenolic residues (Passardi et al. 2004, Lthje et al. 2014, Wang et al. 2015). In addition, CIII-POX have substrate specificity and it is possible to differentiate CIII-POXs involved in the oxidation of monolignols and in diferulate and lignin synthesis (**see chapter III**) (Fagerstedt et al. 2010, Shigeto et al. 2013, Fernndez-Prez et al. 2015, Li et al. 2016).

Cell wall proteins are **synthesised and assembled** in the endoplasmic reticulum and most of these proteins are encoded by multigene families. This process is highly controlled: some of them are developmentally regulated or expressed in a tissue-dependent manner. However, the synthesis of others occurs in response to wounding, infections or environmental stresses (Albersheim et al. 2010, Doblin et al. 2010).

Phenolic compounds

Hydroxycinnamates (monomeric phenolic compounds derivated from cinnamic acid) such as ferulic acid (FA) and *p*-coumaric acid (CA), are relevant compounds in grasses cell walls (Fry 1982). FA and CA can be esterified with α -Ara residues from AX, α -Xylosyl residues from XyG, β -galactosyl from RG-I and probably with glycoproteins (Ishii et al. 1990, Wende and Fry 1997, Obel et al. 2003, Harris and Trethewey 2010). Although hydroxycinnamates are minor components in the cell wall (1-5%; Vogel 2008), they play a crucial role in type II cell walls. FA is susceptible of oxidative coupling in presence of H₂O₂, process mediated by POXs (Ralph et al. 2004). The result is the synthesis of diverse dehydrodiferulates, which promote the cross-linking of the different polysaccharides which are esterified with the phenolic compounds (Ishii 1997, Fry 2004, Scheller and Ulvskov 2010, Hatfield et al. 2016). In addition, FA can be cross-linked to form ether bonds, linking chains of GAX, or GAX to lignin, modifying the degradability of the cell wall (Harris and Trethewey 2010, Scheller and Ulvskov 2010, Molinari et al. 2013, Hatfield et al. 2016), and contributing to the cell wall assembly, growth cessation and cell wall reinforcement against abiotic and biotic stresses (de O Buanafina 2009) (**see Chapter II**).

Hydroxycinnamates are synthesised in the cytosol following the first part of the **phenylpropanoids pathway** (Vogt 2010) and then transported to the Golgi apparatus and esterified on the polysaccharides due to the activity of some CoA-acyl-transferases, including the members of the BAHD acyl transferases superfamily (Pfam family PF02458) (Mitchell et al. 2007, Piston et al. 2010) (**Figure 6**).

In grasses and during cell wall deposition, **CIII-POX enzymes** mediate oxidative coupling of FA residues to obtain dimers and trimers which participate in the cross-linking of xylans (Saha 2003, Mosier et al. 2005) and also link lignin to the xylan-cellulose network via lignin-ferulate-xylan complex (de O Buanafina 2009)

structure of these primary cell walls (Hatfield et al. 2017). Furthermore, this type II primary cell wall has low amounts of pectins and structural proteins in comparison with type I cell wall (Carpita et al. 2015).

The types of **interaction among the different polymeric networks** are decisive in the remodeling of cell wall architecture during cell growth and in the reinforcement of the cell wall in response to stress factors which affect its integrity (Bashline et al. 2014, Voxeur and Höfte 2016, Hatfield et al. 2017, Bacete et al. 2018, Gigli-Bisceglia et al. 2018). In the last decades, it has been demonstrated that the modification of one of these polymeric networks usually affects the composition and the structure of the others, due to the high compositional and **structural plasticity of plant cell walls**. Much effort has been made to improve our knowledge into the interactions among the components and into the mechanisms of regulation in their biosynthesis and assembly. Accordingly, during the last years, different works by using mutants, transgenic plants and **cell cultures habituated to cellulose biosynthesis inhibitors (CBIs)** have shown that a dynamic relationship exists among the three major polymeric networks contributing to cell wall plasticity (Desprez et al. 2002, Ellis et al. 2002, Zhong et al. 2002, Caño-Delgado et al. 2003, Bischoff et al. 2009, de Castro et al. 2015, Mélida et al. 2015).

Cellulose biosynthesis inhibitors and habituation process

As mentioned before, several cultured plant cells able to grow in the presence of different compounds that affect cellulose synthesis (CBIs) have been obtained (Yamada et al. 2002, Acebes et al. 2010, Jiang and Liu 2010).

Their use has proved to be a **valuable tool** in the study of the genesis, organization, composition, structure and plasticity of the cell wall (Alonso-Simón et al. 2004, 2008, García-Angulo et al. 2006, 2009, Mélida et al. 2009, 2010a, 2010b, 2011, 2015, Encina et al. 2001, 2002, de Castro et al. 2014, 2015, 2017, Largo-Gosens et al. 2016, Martínez-Rubio et al. 2018), in the cell plate formation and cytokinesis (Vaughn et al. 2002, DeBolt et al. 2007a) and in the cell wall extension and relaxation mechanism (Hoson and Masuda 1991, Edelman and Fry 1992,

Edelmann and Köhler 1995, Montague 1995). Moreover, other aspects related to cellulose biosynthesis have been also studied using these compounds, such as the connection between cytoskeleton and cellulose (DeBolt et al. 2007a), CesA complex organization (Mizuta and Brown 1992) and the relationship between cellulose and callose synthesis (Delmer 1999).

CBIs are a structurally heterogeneous group of molecules that affects cellulose synthesis, leading to modifications in cellulose coupling or deposition in higher plants (Tateno et al. 2016). They can induce aberrant trajectories in CesA proteins, reduce their velocity or even clear them at the plasma membrane (Brabham and DeBolt 2012, Liu et al. 2017, Tran et al. 2018). Several of them, including **dichlobenil** (DCB; 2,6-dichlorobenzonitrile), the inhibitor used in the present study, isoxaben, indaziflam, flupoxam and other compounds, have been commercialised as herbicides and classified as group L by the “Herbicide Resistance Action Committee” (Menne and Köcher 2007, Acebes et al. 2010). In addition, during the last years, many other compounds that act as CBIs have been identified, such as morlin, cobtorin, quinoxifen, CESTRIN (“CESA Trafficking inhibitor”), acetoxibian and C17 compound (DeBolt et al. 2007b, Yoneda et al. 2007, Harris et al. 2012, Brabham et al. 2014, Xia et al. 2014, Worden et al. 2015, Hu 2016, Tateno et al. 2016).

The **DCB** is one of the most widely used CBIs. In agriculture, DCB is employed as a broad spectrum pre-emergent herbicide (Verloop and Nimmo 1969) and its use has been widely spread in plant research due to its capacity to induce cellulose inhibition (Corio-Costet et al. 1991, Hoson and Masuda 1991, Encina et al. 2002, Alonso-Simón et al. 2004, Mélida et al. 2009, García-Angulo et al. 2012), not affecting other cell wall polysaccharides biosynthesis (Montezinos and Delmer 1980, Francey et al. 1989) or other metabolic processes (Montezinos and Delmer 1980, Galbraith and Shields 1982, Delmer 1987).

It has been proposed that this inhibitor affects the orientation of cellulose microfibrils during their deposition into the cell wall (Sugimimoto et al. 2001), modifying the microtubule organization (Peng et al. 2013) and affecting (diminishing or interrupting) the mobility of cellulose biosynthesis complexes (Mizuta and Brown 1992, DeBolt et al. 2007b, Brabham and DeBolt 2012, Tateno

et al. 2016, Liu et al. 2017, Tran et al. 2018). As a CBI, DCB affects cell plate formation although it does not inhibit neither cell division nor cell elongation (Galbraith and Shields 1982, Sabba and Vaughn 1999, Vaughn 2002).

To date, the mode of action of DCB has not been clearly elucidated, although several putative **modes of action of DCB** have been proposed. First studies suggested that DCB blocks the cellulose biosynthesis by inhibiting the synthesis of sitosterol- β -glucoside (Peng et al. 2002). Another proposed action mechanism is that DCB alters the cellulose crystallisation when is deposited into the cell wall, probably due to the disruption of the spatial arrangement of microtubules rather than the glucan chain polymerisation (Himmelspach et al. 2003). According to this, it has been found 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). In addition, DCB does not affect the CSC number, but affects their mobility and function (DeBolt et al. 2007b, Brabham and DeBolt 2012, Peng et al. 2013, Tateno et al. 2016, Liu et al. 2017, Tran et al. 2018).

Although the **target of DCB** still remains unclear, three putative candidates have been proposed. By using a photoreactive analogue of DCB, Delmer et al. (1987) indicated that a 12-18 kDa protein loosely bound to the membrane could act as DCB target. On the other hand, Nakawaga and Sakurai (1998) suggested that DCB could specifically bind to CesA1 protein. Finally, the most plausible idea is that a microtubule-associated protein, the MAP20, is involved in cellulose synthesis being the responsible for coupling CESA proteins to microtubules, as DCB would disrupt cellulose biosynthesis by decoupling CSC microtubules through MAP20 inactivation (Rajangam et al. 2008).

▪ **Habituation to DCB**

During the last two decades it has been demonstrated that it is possible to **habituate cultured cells** to lethal levels of DCB by subculturing them in stepwise increasing concentrations of the compound in the culture medium (Encina et al. 2001, 2002, Alonso-Simón et al. 2004, García-Angulo et al. 2006, 2009, Mélida et al.

2009, 2010a, 2010b, 2011, 2015, Largo-Gosens et al. 2016). The habituation mechanism resides in the ability of the cultured cells to grow and divide with a reduction in the cellulose content. Due to this process, different changes in their composition and metabolism take place, which differs depending on the cell wall type, the plant species, and the concentration and length of exposure time to DCB (Alonso-Simón et al. 2004, Mélida et al. 2009, de Castro et al. 2014, 2015, 2017) (**Figure 7**).

Most of the studies with **cultured cells habituated to DCB** have been performed on cells with type I cell walls, 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). In general terms, and according to cell wall dependent mechanisms, habituated cells presented a reduction in cellulose and hemicellulose content, compensated by an increased amount of cross-linked pectins (Encina et al. 2002, Alonso-Simón et al. 2004, García-Angulo et al. 2006). On the other hand, and as an independent cell wall mechanism, habituated cells showed an increment in antioxidant activities to cope with the oxidative stress promoted by the DCB action (García-Angulo et al. 2009, Largo-Gosens et al. 2016).

In case of plants with type II cell wall, only barley (Shedletzky et al. 1992) and maize (Mélida et al. 2009, 2010a, 2010b, 2011, 2015, de Castro et al. 2014, 2015, 2017, Largo-Gosens et al. 2016) cultured cells have been habituated to DCB. In maize, cells habituated to high DCB concentrations (12 μ M) showed a 75% reduction in cellulose content compensated by a more extensive AXs network, more cross-linked with hydroxycinnamates, and the deposition of a lignin-like polymer (Mélida et al. 2009, 2010a, 2010b, 2011, 2015).

Figure 7. Schematic representation of DCB-habituating process. The chemical structure of DCB is included. In the green box, arrows represent a stepwise increase in the presence of DCB.

Additionally, these habituated cells showed a reduction in the antioxidant activities and a repression in the activity of the glutathione-S-transferase (Mélida et al. 2010a). On the other hand, in case of cells habituated to low levels of DCB (1.5 μM ; de Castro et al. 2014, 2015), their 33% reduction in the cellulose content was counteracted by a more extensive network of greater number of cross-linked AXs with a higher relative molecular mass (Mr) (de Castro et al. 2015, 2017).

In summary, the study of cell cultures habituated to DCB is allowing us to obtain a deep insight into the changes that take place in cell wall metabolism in response to stress situations and improve our knowledge in cell strategies to cope with a deficiency in cellulose in their cell walls. One of the most important changes experienced by DCB-habituating cells is the synthesis and deposition of a lignin-like material, process called as **ectopic lignification** (Caño-Delgado et al. 2003, Mélida et al. 2015).

The weird presence of lignin in primary cell walls

Lignin is the second most abundant biopolymer on earth (the 25% of the plant biomass is lignin; Higuchi et al. 1990) and one of the most important compounds of

secondary cell walls taking part in the lignocellulosic material. It is a complex and heterogeneous polymer and its synthesis requires a large number of enzymes involved in the phenylpropanoids pathway. This route leads to the formation of three monolignols, that differ in their methoxylation degree, *p*-coumaryl, coniferyl and sinapyl alcohols (Ros Barceló 1997, Boerjan et al. 2003, Yamada et al. 2008, Vanholme et al. 2010, Fornalé et al. 2017), and their polymerization leads to the synthesis of the units that are taking part of the lignin: *p*-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) units, respectively (Boerjan et al. 2003, Zhao 2016). Monolignols are oxidized by ClH-POX (EC. 1.11.1.7), mediated by H₂O₂ (Fagersteadt et al. 2010, Novo-Uzal et al. 2013, Zhao et al. 2013, Li et al. 2016), and/or laccases (EC. 1.10.3.2), which require the presence of O₂ (Cesarino et al. 2013, Zhao et al. 2013).

Lignin composition varies depending on the species, tissues, cell type and environmental conditions (Vanholme et al. 2010) and depends on the proportion of G, S and H units. In case of monocots, lignin is predominantly composed by S and G units and lower amounts of H units (Boerjan et al. 2003) in comparison to dicots. Furthermore, monocot lignin shows high levels of hydroxycinnamates, mainly CA and FA, monolignol acetate, hydroxycinnamyl aldehydes and *p*-hydroxybenzoates (Li et al. 2016). In addition, the flavonoid tricine has been proposed to be a new lignin monomer in monocots (Del Río et al. 2012, Fornalé et al. 2015, 2017, Lan et al. 2015).

Lignin synthesis mainly occurs in **secondary cell walls** of some cell types specialised in structural support and water and soluble transport such as xylem and fiber cells (Bonawitz and Chapple 2010). This phenolic polymer provides to these cells mechanical support, rigidity and hydrophobicity, allowing plants to grow tall and transporting water and nutrients over a long distance (Zhao 2016). In addition, lignification protects cells from mechanical and biological degradation of cellulose fibers, and also acts as a connection between cell wall polysaccharides, causing an important reinforcement of the cell wall (Pomar et al. 2002, Kärkönen and Koutaniemi 2010). But although the presence of lignin is characteristic of secondary cell walls, **lignification** can be induced in **primary cell walls**, in response to abiotic and biotic stress conditions, such as wounding, metabolic

stress, response against infection of pathogens or the presence of **CBIs** in the culture medium (Caño-Delgado et al. 2003, Miedes et al. 2014).

Ectopic lignification in cultured cells

Some cells cultured under specific conditions are able to lignify, and this **lignin-like material** can be incorporated to the cell wall or liberated to the culture medium (Kärkönen and Koutaniemi 2010, Novo-Uzal et al. 2013, Shen et al. 2013).

In some of these systems, lignification is associated with **tracheal elements differentiation** mediated (induced) by hormonal treatment (Fukuda and Komamine 1980, Simola et al. 1992, Eberhardt et al. 1993, McCann 1997, Anterola et al. 2002, Oda et al. 2005, Pauwels et al. 2008, Kärkönen and Koutaniemi 2010, Yamagishi et al. 2015). In other cases, such as cultured cells of *Picea abies*, lignification is associated with **fungal elicitors treatments** (Messner and Boll 1993, Lange et al. 1995). On the other hand, **changes in the culture medium composition** (Brunow et al. 1990, Kärkönen et al. 2002), such as modifications in sucrose levels, provoke lignin synthesis in suspension-cultured cells of *Pinus taeda* (Nose et al. 1995).

Attending to lignification in cultured cells, this process has been widely studied in **tracheal elements** of *Zinnia elegans*. To promote the tracheal elements differentiation it seems to be essential the stress caused by the explant isolation or a culture medium with high levels of cytokinins (McCann 1997). Lignin deposited in those cell walls does not differ from lignin which is found in the plant vascular tissues (Stasolla et al. 2003, Möller et al. 2006). However, when this lignin is liberated to the culture medium, known as extracellular lignin, is predominantly composed by H units and shows more condensed bonds contrary to the lignin that appears in plant (Brunow et al. 1990, 1993, Lange et al. 1995).

According to DCB, it has been proved that maize cultured cells in presence of this compound showed and **ectopic lignification** process, characterised by an enrichment of **S units** together with an **overexpression of specific genes** for the biosynthesis of monolignol units and some of the jasmonic signalling pathway genes (Mélida et al. 2015) ([see Chapter V](#)).

Cell wall components as biotechnological targets

In the last decades, the biomaterial properties of cell walls are increasingly being exploited. Therefore, a better understanding in the complex structure-function relationships between cell wall components is necessary to optimize foods, enhance fibres for textiles, wood for construction and biomass for biofuels, contributing to a more sustainable and efficient use of plant-based wall products.

In this sense, over the last years, high interest has focused on cell walls as a renewable resource, particularly lignocellulosic biomass, for second generation biofuels and/or as animal feedstock, through the development of multiple strategies to make the biomass more suitable for enzymatic saccharification. Techno-economical modelling indicates that the most important target for engineering is lignin, but properties derived from other components can also be improved with significant benefit (Klein-Marcuschamer et al. 2012), as the modification in the content, degree of polymerization and crystallinity of cellulose, the amount and the degree of xylan substitutions, the amount of pectins, and the modification in some cell wall proteins as expansins and extensins (Loqué et al. 2008). Therefore, the selection of transgenic or mutant plants that targets cell wall modifications has been considered as objectives to enhance biomass enzymatic digestibility. However, the trade off between plant growth, mechanical strength and biomass yield is still a problem, as plants need to generate a complex wall-network enabling to complete their life cycles rather than to meet biofuel or other industrial purposes (Xie and Peng 2011). To this attempt, the identification of appropriate genes is necessary for gene transformation and genetic mutagenesis, having been identified almost one thousand genes that are thought to be involved in cell wall biosynthesis, degradation and modification (Guo et al. 2014).

Each of the different cell wall components has been regarded as biotechnological targets, and below we summarize recent contributions in this field.

Regarding **cellulose**, a high cellulose level and cellulose polymerization degree are widely known to have a negative impact on biomass enzymatic saccharification in most plant species, as being correlated positively with cellulose crystallinity

(Kaida et al. 2009, Hallac and Ragauskas 2011, Huang et al. 2015, Wang et al. 2016). Increasing the biomass yield of bioenergy crops by enhancing cellulose synthesis is a major goal of bioenergy engineering. Since the first *CesA* gene was identified (Arioli et al. 1998), distinct *cesA* mutants have been identified through multiple genetic approaches. Most *cesA* mutants exhibit reduced cellulose levels and defective plant growth (Wang et al. 2016), with low amounts of crystalline cellulose and high enzymatic digestibility, providing a potential genetic engineering approach for cellulose modification (Wang et al. 2016).

According to **hemicelluloses**, their cross-linkage with cellulose and lignin is one of the main factors explaining lignocellulose recalcitrance (Himmel et al. 2007, Scheller and Ulvskov 2010, Faria-Blanc et al. 2018). Alternatively, recent studies have shown that hemicelluloses positively affect biomass enzymatic digestibility under various physical and chemical pretreatments by reducing cellulose crystallinity in species as *Miscanthus* and rice (Wang et al. 2016). Additionally, the Ara substitution degree of xylan is a main positive factor on biomass saccharification in all grass species examined (Li et al. 2014). Therefore, the removal of nearly all hemicelluloses leads to the reassembly of highly crystalline cellulose that result recalcitrant to cellulases (Wang et al. 2016). As xylan has a positive effect on biomass enzymatic digestibility by reducing crystalline cellulose (Li et al. 2014), increasing hemicellulose represents another promising strategy for enhancing both biomass yield and lignocellulose enzymatic saccharification in bioenergy crops. Recently, several genetic mutants and transgenic plants associated with hemicellulose biosynthesis modifications have exhibited a reduced biomass yield and an increased biomass digestibility in rice, wheat, maize and poplar (Yoshida et al. 2013, Biswal et al. 2015).

Pectins could play a key role in maintaining cell wall structure and biomass recalcitrance as they can form a macromolecular wall-network through molecular substitutions with side chains associated with cellulose or hemicelluloses (Marcus et al. 2008, Popper and Fry 2008, Wang et al. 2015). Although it is technically difficult to identify the specific effects of pectin in lignocellulosic enzymatic digestibility (Wang et al. 2016) due to the extremely low level and complicated structure of pectin in secondary cell walls, several transgenic plants expressing

genes involved in pectin synthesis, modification and degradation, exhibit improved biomass digestibility (Biswal et al. 2014).

Although **cell wall proteins** are considered essential components of plant cell walls, little is known about their effects on lignocellulose recalcitrance and biomass enzymatic degradability (Wang et al. 2016). Of the numerous cell wall proteins, expansins are responsible for loosening plant cell walls by targeting wall polymers and reducing hydrogen bonding (Cosgrove 2000, Nardi et al. 2015). Recent studies have shown that the mutation of extensin genes leads to reduced biomass yields, whereas its overexpression could enhance biomass production in arabidopsis (Cannon et al. 2008, Saha et al. 2013). Furthermore, the overexpression of extensins genes in tobacco cells has been demonstrated to either decrease cellulose crystallinity, increase XyG amount and cell wall hydration capacity (Tan et al. 2014).

Lignin is considered to have two effects on biomass enzymatic hydrolysis, preventing cellulose microfibril swelling and reducing cellulose accesiblity for cellulase enzymes, as well as preventing cellulase action on the cellulose surface (Keating et al. 2006). On the one hand, lignin typically has a negative impact on biomass enzymatic digestibility (Wang et al. 2016). Both effects have been observed in several species and transgenic plants, and the positive impacts could be interpreted in three different ways: (i) after lignin is extracted via physical and chemical pretreatments, the remaining non-extractable lignin-hemicellulose complexes maintain cellulose microfibrils in the state that are accessible by enzymes in amorphous regions (Wu et al. 2013, Li et al. 2014); (ii) lignins composed mainly by high proportions of G and H monomers are more extractable after alkaline pretreatment (Li et al. 2014); (iii) in case of the genetic mutants and transgenic plants, the increment in the monolignol levels may not form a complete lignin-carbohydrate complex, leading to more cell wall pores for cellulose enzyme access (Chandra et al. 2008, Li et al. 2014, Wang et al. 2014).

According to this, the **complex structure of lignin** and its **cross-linking** with other plant cell wall polymers constitute a key factor to explain the recalcitrance of plant cell walls against polysaccharide utilization, being the reason why gaining access to the carbohydrate polymers of plant cell walls for use as carbon and

energy sources requires significantly energy inputs and harsh chemical treatments (Withers et al. 2012).

Thus, **phenolic cross-linking** of cell wall components, such as that between lignin and hemicelluloses, but also between different chains of AX by FA dimers, reduces cell wall degradability by hydrolytic enzymes (Jung and Allen 1995, Grabber et al. 1998a, 1998b), and therefore reduces forage quality for ruminants and the yield of biofuel production (Grabber et al. 2004, de O Buanafina et al. 2006, 2008, 2010, 2012, 2015). In fact, a positive correlation between phenolic cross-linking and growth cessation in various monocot species has been found (Kamisaka et al. 1990, MacAdam and Grabber 2002, Azuma et al. 2005), and natural variation in FA content in different species has been inversely correlated with digestibility (Lam et al. 2003, Casler and Jung, 2006). Moreover, *in vitro* approaches have shown that FA promotes inhibition of polysaccharide saccharification to sugars (Grabber et al. 1998a, 1998b). On the contrary, screens for increased digestibility in mutant populations repeatedly correlate with low ferulate lines (Hirano et al. 2017), and the **reduction in the degree of GAX feruloylation** either by heterologous expression of feruloyl esterases (de O Buanafina et al. 2008) or by RNAi silencing of BAHD genes, has been demonstrated to decrease GAX cross-linking, improving the digestibility of grass cell walls (Grabber et al. 1998a, 1998b, Dhugga 2007, de Souza et al. 2018).

Consequently, the reduction in FA represents an interesting strategy to increase cell wall degradability. In this way, the **identification of genes and proteins** involved in grass AX feruloylation, such as BAHD genes, is a valuable tool in order to further increase cell wall digestibility of grasses as well as to enhance the efficiency of ethanol production (Piston et al. 2010, Gómez and McQueen-Mason 2018).

In summary, any progress in the knowledge of the interrelationship among cell wall components, as well as in the proteins and genes involved in the remodelling of the cell wall, could provide us useful information to obtain plants with an improved biotechnological potential in terms of biofuel production or an enhanced yield as feedstock bioresource (Kumar et al. 2016).

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Objectives

In recent years, it has been shown that plant cell walls possess high compositional and structural plasticity. Previous studies by our group have demonstrated that a reduction in cellulose content in maize cell walls leads to a modified cell wall architecture and composition, causing changes in the amount and structure of other components evidencing a functional interplay among the different polymers that form the cell wall. Therefore, the aim of this Thesis was to gain further insights into the key role of phenolics in the cell wall remodelling of cellulose deficient cells.

In order to achieve this goal, a study was conducted of maize suspension-cultured cells habituated to the cellulose biosynthesis inhibitor DCB and thus presenting reduced cellulose content.

Accordingly, we hypothesise that these cellulose-deficient cells would readjust their general phenolic metabolism affecting the cell wall composition and structure through changes in cell wall esterified hydroxycinnamates, ferulate dimerisation and the deposition of an ectopic lignin. Since cell wall hydroxycinnamates and lignin are subjected to peroxidase action, it is also expected to find changes in peroxidase activity and in the isoperoxidase profile. In addition, we hypothesise that a differential expression of the genes involved in feruloylation/coumaroylation would occur as a part of the finely-tuned regulation of cell wall remodelling.

To elucidate these mechanisms, five partial objectives will be addressed:

- To enhance our understanding of changes in the phenolic metabolism occurring in cells with reduced cellulose content, the metabolism of cell wall hydroxycinnamates in non-habituated and DCB-habituated cells will be studied. To this end, a series of radiolabeling experiments using *trans*-[¹⁴C]cinnamic acid (the precursor of cell wall hydroxycinnamates) will be carried out. The kinetics of [¹⁴C]cinnamic acid consumption and incorporation of [¹⁴C]-compounds into the protoplasm and cell wall compartments will be analysed throughout the cell culture cycle. In addition, a detailed analysis of the type of [¹⁴C]cinnamic acid derivatives incorporated into the cell wall will be attained (**Chapter I**).

- To gain further insight into the contribution of cell wall phenolics to the early remodelling of cellulose deficient cell walls, an HPLC-PAD analysis of both quantitative and qualitative changes in hydroxycinnamates esterified into nascent (protoplasmic fraction) and cell wall polysaccharides during the cell culture cycle will be carried out. Special attention to changes in the degree of dimerisation and the differential pattern of ferulate dimerisation between cell lines (non-habituated vs DCB-habituated) and cell compartments (endomembrane system vs cell wall) will be paid (**Chapter II**).

- To obtain further information on the interrelationship between peroxidases and cell wall remodelling leading to changes in the phenolic profile, a comparative study of peroxidase activity and isoforms in control and cellulose-impaired cells analysing total and specific peroxidase activity in different cellular compartments against a set of native substrates will be conducted. Additionally, a comparative analysis of the isoperoxidase profile in the different cellular compartments of non-habituated and DCB-habituated cells will be performed (**Chapter III**).

- In order to gain an initial insight into the genes putatively involved in acylation of arabinoxylans with ferulic or *p*-coumaric acids, PF02458 CoA-acyltransferase homologue genes to rice and *Arabidopsis* will be identified in maize cultured cells. A comparative analysis of the relative expression of selected genes in non-habituated and DCB-habituated cells will be carried out by RT-PCR (**Chapter IV**).

- In order to determine whether cobtorin, a CBI with a different mode of action to DCB, also triggers the ectopic lignification process in response to reduced cellulose content, maize suspension-cultured cells will be treated with cobtorin and DCB. Microscope techniques were employed to monitor the lignin induction process over the course of the cell culture cycle. Additionally, a comparative study will be conducted of the relative gene expression of selected BAHD genes, together with an analysis of peroxidase activity in control and DCB-treated cells (**Chapter V**).

Chapter I

Phenolic metabolism and molecular mass distribution of polysaccharides in cellulose-deficient maize cells*

*Chapter corresponding to the publication: **de Castro M, Martínez-Rubio R, Acebes JL, Encina A, Fry SC, García-Angulo P (2017) Phenolic metabolism and molecular mass distribution of polysaccharides in cellulose-deficient maize cells. J Integr Plant Biol 59: 475–495 (See Appendix)**

The contribution of Martínez-Rubio R. was to carry out the experiments focused on the phenolic metabolism as well as to collaborate in the preparation and revision of the manuscript.

ABSTRACT

As a consequence of the habituation to low levels of dichlobenil (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. The aim of this study was to investigate the relative molecular mass distributions of polysaccharides as well as phenolic metabolism in cells habituated to low levels of DCB (1.5 μM). Generally, cell wall bound hemicelluloses and sloughed polymers from habituated cells were more homogeneously sized and had a lower weight-average relative molecular mass. In addition, polysaccharides underwent massive cross-linking after being secreted into the cell wall, but this cross-linking was less pronounced in habituated cells than in non-habituated ones. However, when relativized, ferulic acid and *p*-coumaric acid contents were higher in this habituated cell line. Feasibly, cells habituated to low levels of DCB synthesized molecules with a lower weight-average relative molecular mass, although cross-linked, as a part of their strategy to compensate for the lack of cellulose.

INTRODUCTION

Heteroxylans are the main non-cellulosic polysaccharides in the Poales primary (type II) cell wall (Fincher 2009, Peña et al. 2016), 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). These non-cellulosic polysaccharides are predicted to be synthesized in Golgi apparatus by several glycosyltransferases whose genes have been recently identified in *A. thaliana* by using genomic approaches (Rennie and Sheller 2014 and refs. therein). Specifically in the case of maize, sequences have also been reported for some arabidopsis orthologues such as *IRX9*, *IRX10* and *IRX10-L* (Bosch et al. 2011), and recently maize genes orthologous to AtIRX10, AtIRX9 and AtIRX14 has been shown to be highly expressed in the pericarp (Chateigner-Boutin et al. 2016).

One of the unique structural features of arabinoxylan in Poales is that it contains hydroxycinnamate residues, such as ferulic acid or *p*-coumaric acid, esterified on the C-5 position of arabinose residues (Bacic et al. 1988, Wende and Fry 1997). The attachment of ferulic acid residues occurs within the protoplasm, before secretion into the cell wall (Fry et al. 2000). Ferulic acid 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 it has 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, Oliveira et al. 2015).

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

(Oliveira et al. 2015). 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 2,6-dichlorobenzonitrile (dichlobenil, 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 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. Likewise, in high DCB levels (12 μM) a 75 % of reduction is observed (Mélida et al. 2009) whereas in low DCB levels (1.5 μM) such reduction is more moderate, being of 33 % (de Castro et al. 2014) when compared to non-habituated maize cells (Shn). 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).

Cell suspension cultures present several advantages in *in vivo* metabolic studies performed using isotopes, such as homogeneity of cell wall material, uniform culture medium without nutrient gradients, access to O_2 or exogenous precursors, and ease of sampling (Burr and Fry 2009). This kind of experimental approaches is particularly useful as the substrates are endogenous and therefore the processes observed are those that naturally take place (Thompson and Fry 1997, Kerr and Fry 2003, 2004, Lindsay and Fry 2008, Mélida et al. 2011). In a previous study, maize suspension-cultured cells habituated to low levels of DCB (1.5 μM , Sh1.5) were fed with [^3H]arabinose (de Castro et al. 2015). 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 ^3H -hemicelluloses and an increased presence of soluble extracellular ^3H -polymers (^3H 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 weight-average relative molecular mass (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 relative molecular mass (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 (^3H arabinose and ^{14}C cinnamic acid respectively). The results obtained in this work 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.

MATERIALS AND METHODS

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 $9\text{ }\mu\text{M}$ 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 dimethyl sulfoxide (DMSO) which did not affect cell growth at this range of concentrations. 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 dry weight at different culture times and it was observed that Sh1.5 cells presented longer culture phases than Snh cells (de Castro et al. 2014). 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.

Analysis of M_r distributions

Radio-labelling of liquid cultures with [^3H]arabinose

Thirteen-ml aliquots from Snh and Sh1.5 maize cells cultures were collected at early logarithmic and late logarithmic growth phases and each 13-ml aliquot was independently fed with 1 MBq of L-[1- ^3H]arabinose (148 GBq/mmol; Amersham International, Bucks., U.K). The days on which the 13-ml aliquot of culture was sampled and [^3H]arabinose 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 Sh1.5 cell lines, respectively (see de Castro et al. 2014). In the case of late logarithmic phase, the 8th and 12th days were selected for Snh and Sh1.5 cell lines, respectively. In order to obtain an optimal quantity of radio-labelled ^3H -hemicelluloses and ^3H -polymers, samples for analysis of the M_r were collected at different labelling-time points selected according to the maximum value of incorporated [^3H] arabinose observed in the kinetics previously described (de Castro et al. 2015). Thus, in the case of protoplasmic polymers, 1-ml samples were collected from independent 13-ml aliquots of culture 30 min and 60 min after feeding [^3H] arabinose for Snh and Sh1.5 cells, respectively. For the remaining ^3H -hemicelluloses and ^3H -polymers, sampling was carried out 300 min after feeding [^3H]arabinose for both cell lines.

Obtaining ^3H -hemicelluloses and ^3H -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 cell-free medium fraction. 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 thiosulphate and 10 mM dithiothreitol (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 times), 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 times).

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 times). 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 times). 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.

Gel permeation chromatography

Samples of cell-free medium, protoplasmic and cell wall fractions were dialysed against distilled water with 0.1% (w/v) chlorobutanol at 4°C for 72 h, using a 12 kDa cut-off and 1.5 cm diameter dialysis membrane. 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 (V_0) and the totally included volume (V_i), 6 mg dextran (5–40 MDa) and 0.8 mg glucose, 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 ^3H -hemicellulose from maize culture medium and [^{14}C]glucose as internal markers, which defined the V_0 and V_i (K_{av} 0 and 1) respectively. M_w was obtained using the $K_{av(1/2)}$ method (Kerr and Fry 2003) with the calibration curve [$\log M_w = -3.547 K_{av(1/2)} + 7.2048$] obtained for this column. The M_w estimates were nominal rather than absolute due to the conformational differences between dextran and hemicelluloses.

Characterization of phenolic metabolism

[^{14}C]cinnamic acid synthesis

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

[^{14}C]cinnamic acid consumption and incorporation into different cell compartments

Fifteen-ml aliquots of Snh and Sh1.5 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, [¹⁴C]cinnamic acid (0.29 µCi) was added to each cell culture. At selected time-points, 400 µl of cell cultures were sampled and acidified by the addition of 15 µl 99% formic acid, stopping the consumption and incorporation of [¹⁴C]cinnamic acid.

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 cell-free medium fraction.

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 fraction (alcohol soluble material). The residual cell fragments were considered alcohol insoluble residue (AIR).

Obtaining of cinnamic acid [¹⁴C]derivatives from discrete maize cell compartments

Four hundred µl-aliquots of Snh and Sh1.5 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 [¹⁴C]cinnamic acid (0.116 µCi). At selected time-points, aliquots were acidified by the addition of 15 µl 99% formic acid, stopping [¹⁴C]cinnamic acid incorporation. Where indicated, 40 mM H₂O₂ (final concentration) was added to some of the samples 120 min after [¹⁴C]cinnamic acid and then shaken for 60 min. Aliquots of cell suspensions contained in the vials were then transferred into Poly-Prep columns and the filtrate was collected and considered the cell-free medium 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 fraction. The protoplasmic fraction was

collected, de-esterified with 0.5 M NaOH for 18 h at 25°C, acidified to pH 5.0 and further partitioned against ethyl acetate (x2 times). The ethyl acetate phases were vacuum-dried, re-dissolved in 0.5 ml of acidified water (0.01 N HCl) and further subjected to a second ethyl acetate partition (x3 times). The organic phases were collected, vacuum-dried and re-dissolved in propan-1-ol.

The AIR retained on the Poly-Prep column was de-esterified 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, acidified to pH 5.0 and collected as the 0.5 M NaOH fraction. This fraction was subjected to ethyl acetate partition as indicated above. The organic phases were collected, vacuum-dried and re-dissolved in propan-1-ol.

Assay of radioactivity

Analysis of M_r distribution

To obtain the general elution profiles of ^3H -hemicelluloses and ^3H -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 ^3H -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 trifluoroacetic acid (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 xylose 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 xylose, arabinose, glucose, isoprimeverose and xylobiose ($\approx 50 \mu\text{g}$ each) was then added to the digest, which was subjected to paper chromatography 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 scintillation liquid were added, and vials were shaken continuously for 24 h. Radioactivity was subsequently assayed by counting.

[¹⁴C]cinnamic acid consumption and incorporation into different cell compartments

To analyse the cell-free medium samples and the protoplasmic fraction, 5 and 3 ml of scintillation liquid EcoscintA (National Diagnostics) were 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.

Obtaining cinnamic acid [¹⁴C] derivatives from discrete maize cell compartments

Samples of protoplasmic and AIR fractions de-esterified with 0.5 M NaOH were subjected to thin layer chromatography (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. Standards of ferulic acid, *p*-coumaric acid 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.

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 (**Table S1**) 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.

RESULTS

M_w and M_r distribution of ³H-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 (**Figure 1**). In addition, the proportion of ³H-polymers and ³H-hemicelluloses that were eluted in the V0 was lower in Sh1.5 cells. ³H-polysaccharides from Sh1.5 cells had a lower M_w than those of Snh in all cell compartments and culture phases analysed, with the sole exception of those observed within the protoplasm at late logarithmic phase (**Figure 1**).

Protoplasmic ³H-polymers consisted of a highly poly-disperse population of molecules, which in both cell lines showed a lower M_w when compared with the cell wall bound ³H-hemicelluloses or [³H]SEPs. This result indicates that they underwent massive cross-linking once incorporated into the cell wall or sloughed into the medium. In line with this, when both cell lines were compared, Snh cells showed a higher M_w and therefore a much more pronounced degree of crosslinking than Sh1.5 cells, independently of the culture phase considered (**Figure 1**).

The general elution profiles of [³H]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 [³H]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 1**). In addition, [³H]SEPs from Sh1.5 cells had a lower M_w than Snh ones in both culture phases.

Figure 1. Mr distribution profile of ^3H -polysaccharides: protoplasmic ^3H -polymers, cell wall bound ^3H -hemicelluloses extracted with 0.1 M or 6 M NaOH and soluble extracellular ^3H -polymers (SEPs) obtained from non-habituated (Snh, purple line) and habituated to 1.5 μM DCB (Sh1.5, green line) maize cell cultures. Data shown are from the early logarithmic (A) and late logarithmic (B) phase of culture cycle. Samples used were taken 300 minutes after feeding of [^3H]arabinose, except in the case of protoplasmic ^3H -polymers, collected 30 and 60 minutes after feeding of [^3H]arabinose for Snh and Sh1.5 respectively. ^3H -polymers and ^3H -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. Mw values, derived from the median Kav(1/2) of the profiles and then converted to Mw via a calibration curve, are included in the text frame.

Both types of ^3H -hemicelluloses (0.1 M or 6 M NaOH-extracted) showed a lower M_w in Sh1.5 cells (**Figure 1**). In Snh cells, the M_w of 0.1 M NaOH-extracted wall bound ^3H -hemicelluloses did not vary according to the culture phase whereas it appeared to decrease at the late logarithmic phase in Sh1.5 cells (**Figure 2**). Likewise, the general elution profile for this type of ^3H -hemicellulose was very similar in the case of Snh cells in both culture phases, whereas Sh1.5 cells showed a slightly greater proportion of ^3H -molecules eluting in a higher K_{av} at the late logarithmic phase (**Figure 1**). In the case of 6 M NaOH-extracted ^3H -hemicelluloses, their M_w barely increased in Snh cells, whereas in Sh1.5 cells it once again decreased. These findings were repeated in the general elution profiles, where an increase in the proportion of ^3H detected at a lower K_{av} was observed at the late logarithmic phase in Snh cells, whereas it decreased in Sh1.5 cells (**Figure 1**).

M_r distribution of ^3H -labelled diagnostic fragments from ^3H -polysaccharides

M_r distribution of ^3H -labelled diagnostic fragments from ^3H -polysaccharides

After Driselase digestion, the elution profiles of polymers containing [^3H]arabinose, [^3H]xylose or [^3H]xylobiose were similar to the general ones, indicating the solidity of the experimental approach (**Figures 2-4 vs Figure 1**). In general terms, elution profiles of polymers containing [^3H]arabinose, [^3H]xylose or [^3H]xylobiose showed similar M_r distributions. This finding suggests that these diagnostic fragments were a digestion product of a unique type of polysaccharide, putatively [^3H]arabinoxylan. Reinforcing this idea was the disparity observed between these, and fragments from polymers which contained [^3H]isoprimeverose, a diagnostic fragment for [^3H]xyloglucan. Undigested ^3H -polymers were found in all digested fractions, most of them eluting in a K_{av} ranging from 0 to 0.5 (**Figures 2-4**).

Figure 2. Mr distribution profile of ^3H -labelled diagnostic fragments from ^3H -polysaccharides: cell wall bound ^3H -hemicelluloses extracted with 0.1 M NaOH obtained from non-habituated (purple line) and habituated to 1.5 μM DCB (green line) maize cell cultures. Data shown are from the early logarithmic (A) and late logarithmic (B) phase of culture cycle. Samples used were taken 300 minutes after feeding of [^3H]arabinose. Digest of each fraction were assayed for radioactive fragments characteristic of polymers which have arabinose residues {[^3H]arabinose}, xylans {[^3H]xylose} and {[^3H]xylobiose}, xyloglucans {[^3H]isoprimeverose} and polymers that Driselase does not digest {undigested ^3H -polymers}. Other details as in Figure 1.

Figure 3. Mr distribution profile of cell wall bound ^3H -hemicelluloses extracted with 6 M NaOH obtained from non-habituated (purple line) and habituated to 1.5 μM DCB (green line) maize cell cultures. Data shown are from the early logarithmic (A) and late logarithmic (B) phase of culture cycle. Samples used were taken 300 minutes after feeding of [^3H]arabinose. Digest of each fraction were assayed for radioactive fragments characteristic of polymers which have arabinose residues {[^3H]arabinose}, xylans {[^3H]xylose} and {[^3H]xylobiose}, xyloglucans {[^3H]isoprimeverose} and polymers that Driselase does not digest {undigested ^3H -polymers}. Other details as in Figure 1.

Figure 4. Mr distribution profile of soluble extracellular ^3H -polymers obtained from the culture medium of non-habituated (purple line) and habituated to $1.5\ \mu\text{M}$ DCB (green line) maize cell cultures. Data shown are from the early logarithmic (**A**) and late logarithmic (**B**) phase of culture cycle. Samples used were taken 300 minutes after feeding of $[^3\text{H}]$ arabinose. Digest of each fraction were assayed for radioactive fragments characteristic of polymers which have arabinose residues $\{[^3\text{H}]\text{arabinose}\}$, xylans $\{[^3\text{H}]\text{xylose}\}$ and $\{[^3\text{H}]\text{xylobiose}\}$, xyloglucans $\{[^3\text{H}]\text{isoprimeverose}\}$ and polymers that Driselase does not digest $\{\text{undigested } ^3\text{H-polymers}\}$. Other details as in Figure 1.

M_r distribution of 0.1 M NaOH-extracted hemicelluloses diagnostic fragments

The elution profiles of polymers containing [³H]arabinose residues and those characteristic for [³H]xylans ([³H]xylose and [³H]xylobiose) were very similar in both cell lines and culture stages. However, the elution profiles of [³H]isoprimeverose (diagnostic for [³H]xyloglucan) differed from those characteristic of [³H]xylans and [³H]arabinose-containing polymers (**Figure 2**). M_r distributions of [³H]arabinose-containing polymers and ³H-labelled diagnostic fragments for xylans in both culture phases, and those for undigested ³H-polymers at the early logarithmic phase, were very similar to the general elution profile shown in Figure 1.

M_r distributions of [³H]arabinose residues, [³H]xylose and [³H]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 [³H]isoprimeverose for [³H]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 1.

M_r distribution of 6 M NaOH-extracted hemicelluloses diagnostic fragments

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

Most of the polymers which contained [³H]arabinose, [³H]xylose and [³H]xylobiose as well as [³H]isoprimeverose and undigested ³H-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 ^3H detected at the V0 was low in both cell lines and culture phases (**Figure 3**). Nevertheless, a remarkable difference was found in the elution profile of [^3H]xyloglucan and undigested ^3H -polymers from Sh1.5 cells, in which a marked elution peak was observed near a K_{av} of 0.1 at early logarithmic phase of culture. This elution peak was not appreciable at late logarithmic phase of culture.

M_r distribution of SEPs

In both cell lines, the elution profiles of [^3H]arabinose, [^3H]xylose or [^3H]xylobiose-containing polymers were very similar (**Figure 4 vs Figure 1**), suggesting that [^3H]SEPs are mainly constituted by arabinoxylans sloughed into the culture medium. A greater proportion of ^3H -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 4**).

In Snh cells, the elution profiles of the different diagnostic fragments at early logarithmic phase were similar, mainly eluting at a K_{av} ranging from 0 to 0.5 and at the V0 (**Figure 4A**). However, M_r distribution of the undigested ^3H -polymers and the polymers from which [^3H]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 4B**). The elution profiles of [^3H]xylans, ([^3H]xylose and [^3H]xylobiose diagnostic fragments) were similar, and molecules mainly eluted in a K_{av} ranging from 0.1 to 0.4. Similarly, the elution profiles of [^3H]xyloglucan ([^3H]isoprimeverose) and undigested ^3H -polymers were alike. Surprisingly, the elution profile of ^3H -polymers which contained [^3H]arabinose residues was for the first time different to those for [^3H]xylans, and showed a wide distribution among the different K_{av} , with two well-defined “elution areas”. The first of these, in a K_{av} from 0.2 to 0.4, coincided with that observed for the [^3H]xylan diagnostic fragments, suggesting a presumable sloughing of arabinoxylans. However, the second elution area was detected in a K_{av} ranging from 0.5 to 0.8, an observation that would indicate the release into the culture medium of another type of ^3H -molecule population containing [^3H]arabinose residues. This was

not observed in the Sh1.5 cells, in which the elution profile of polymers containing [^3H]arabinose residues was very similar to those for [^3H]xylans.

In Sh1.5 cells, the digestion of [^3H]SEPs with Driselase showed that all the ^3H -polymers (those which contained [^3H]arabinose, those with diagnostic fragments for [^3H]xylans and [^3H]xyloglucan and the undigested ^3H -polymers) eluted in a higher K_{av} than those from Snh cells, and therefore had a lower M_w (**Figure 4**). This was especially clear at the early logarithmic phase (**Figure 4A**). The most remarkable difference was found in the elution profile of [^3H]xyloglucan and undigested ^3H -polymers at late logarithmic culture phase (**Figure 4B**), where a marked peak was observed close to a K_{av} of 0.3, similar to that previously described for the elution profile of 6 M NaOH-extracted [^3H]xyloglucan at early logarithmic phase.

Analysis of phenolic metabolism

Uptake of [^{14}C]cinnamic acid from the culture medium and its incorporation into cell compartments

No differences in [^{14}C]cinnamic acid consumption were observed among maize cell lines or culture phases (**Figure 5**). In general, consumption percentages of [^{14}C]cinnamic acid ranged between 40%-55% of the total [^{14}C]cinnamic acid added at time zero. Most of the [^{14}C]cinnamic acid consumption (90%) occurred during the first 15 min after [^{14}C]cinnamic acid was fed.

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

Figure 5. Kinetics of consumption and incorporation of radioactivity from exogenous [^{14}C]cinnamic acid in non-habituated (purple line) and habituated to 1.5 μM DCB (green line) maize cell suspensions. Data from the early logarithmic (A and C) and late logarithmic (B and D) phase of culture cycle are shown. The [^{14}C]cinnamic acid consumption (▼) was calculated from the radioactivity remaining in the culture medium fraction after [^{14}C]cinnamic feeding. The incorporation of ^{14}C -labelled compounds in the protoplasmic fraction (●) and Alcohol Insoluble Residue (cell wall fragments) (○) is represented. The values shown are the average $\pm$ standard deviation ($n=3$).

[^{14}C]cinnamic acid metabolism on the protoplasmic fraction

Hydroxycinnamate ^{14}C -labelled metabolites incorporated into the protoplasmic fraction of Snh and Sh1.5 cells in their early logarithmic phase (**Figures 6A and S1**), were tracked during a 180 min pulse of [^{14}C]cinnamic acid. In both cell lines, [^{14}C]cinnamic acid was quickly metabolised within the first 5 min after its addition to the culture medium. As expected, [^{14}C]cinnamic acid was rapidly converted to *p*-[^{14}C]coumaric acid and then to [^{14}C]ferulic acid. Both *p*-[^{14}C]coumaric and [^{14}C]ferulic acid, were only transiently accumulated in the protoplasm of Snh cells as their pools largely disappear after 180 min. However, protoplasmic fraction from

Sh1.5 cells was enriched in *p*-[¹⁴C]coumaric and [¹⁴C]ferulic acid when compared with Snh cells. Rf0 and low-Rf material ([¹⁴C]dimers and oligomers) was rapidly detected (5 min) after [¹⁴C]cinnamic acid feeding and gradually accumulated at the same extent in Snh and Sh1.5 cells. In both cell lines, the addition of H₂O₂ increased the extent of dimerization or oligomerisation.

Kinetics of [¹⁴C]cinnamic metabolism did not substantially change when maize suspension cultured-cells in their late logarithmic phase were assayed (**Figures 6B and S2**). As major differences, a delay in the metabolism of [¹⁴C]cinnamic acid by Sh1.5 cells, and a higher accumulation of Rf0 and low-Rf material in Snh cells can be pointed out. Finally, the addition of H₂O₂ during this culture phase did not related with an increase of dimerization or oligomerization.

[¹⁴C]cinnamic acid metabolism in the cell wall

An analysis of hydroxycinnamate ¹⁴C-labelled metabolites incorporated into the cell wall during early-logarithmic growth phase is shown in **Figures 7A and S3**. The [¹⁴C]cinnamic acid added to the culture medium was rapidly (1-3 min) metabolised to *p*-[¹⁴C]coumaric acid and then incorporated into the cell wall. In both cell lines, a decrease was observed in the relative incorporation of *p*-[¹⁴C]coumaric acid into the cell wall the first 30 min after [¹⁴C]cinnamic acid feeding. Concomitantly the [¹⁴C]ferulic acid esterified into the cell wall, abruptly increasing until reaching a plateau phase. Furthermore, at 30 to 60 min after [¹⁴C]cinnamic acid feeding a decrease in the amount of cell wall-esterified [¹⁴C]ferulic acid was detected in Snh cells.

Figure 6. Kinetics of incorporation of ^{14}C -labelled compounds (esterified and free-phenolics) into the protoplasmic polymer fraction after the addition of exogenous ^{14}C -cinnamic acid to non-habituated (purple line) and habituated to $1.5\ \mu\text{M}$ of DCB (green line) maize cell suspensions at the early logarithmic (A) and late logarithmic (B) 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. Dotted line indicates the addition of H_2O_2 (40 mM final concentration).

Figure 7. Kinetics of incorporation of ^{14}C -labelled compounds esterified of alcohol-insoluble residue (cell wall fragments) after the addition of exogenous ^{14}C cinnamic acid to non-habituated (purple line) and habituated to $1.5\ \mu\text{M}$ of DCB (green line) maize cell suspensions at the early logarithmic (A) and late logarithmic (B) 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. Dotted line indicated the addition of H_2O_2 (40 mM final concentration).

In all cases, ^{14}C ferulic acid was the main cell wall hydroxycinnamate detected. The relative quantification of ^{14}C cinnamic acid ^{14}C derivatives indicated that Sh1.5 cell walls were enriched in p - ^{14}C coumaric acid and impoverished in ^{14}C ferulic acid in comparison with Snh cell walls (**Figure 7**). ^{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 cinnamic acid 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 ferulic acid. In addition, their relative quantification indicated that ^{14}C ferulic acid, ^{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 H₂O₂ provided 120 min after [¹⁴C]cinnamic acid feeding induced an increase in the dimerisation or oligomerisation of [¹⁴C]ferulate, occurring to a similar degree in both cell lines (**Figure 7**).

No differences were found in the kinetics of Snh cell wall *p*-coumaroylation and feruloylation between growth phases (**Figures 7A and S3 vs 7B and S4**). However, some differences were observed in the case of habituated cells: Sh1.5 cell walls accumulated more *p*-[¹⁴C]coumaric acid, ¹⁴C-dimers and ¹⁴C-oligomers at the late logarithmic growth phase (**Figures 7B and S4**). Accordingly, the relative decrease in [¹⁴C]ferulic acid amount was more pronounced in the late logarithmic than in the early logarithmic growth phase. Lastly, the addition of H₂O₂ did not increase the level of [¹⁴C]ferulate dimerisation or oligomerisation.

Arabidopsis homologue *IRX* gene expression analysis

The relative expression of the maize genes *GRMZM2G100143*, *GRMZM2G059825* and *gblBT036881.1*, homologues of arabidopsis *IRX10*, *IRX10-L* and *IRX9*, respectively, was analysed by RT-PCR, shown in **Figure S5**. 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.

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. 2014). Likewise, previous studies on maize cells habituated to high DCB levels (12 µM, Sh12) 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 [^3H]arabinose revealed a lower proportion of strongly wall-bound ^3H -hemicelluloses and an increase in [^3H]SEPs, as well as a slower and less efficient metabolism of polysaccharides throughout the entire culture cycle (de Castro et al. 2015).

This finding was especially surprising, and two possible explanations arise to 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 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 ([^3H]arabinose and [^{14}C]cinnamic acid, respectively), since the use of this methodology has been successful in comparable experimental studies (Fry et al. 2000, Kerr and Fry 2003, Burr and Fry 2009, Mérida et al. 2011, de Castro et al. 2015).

The first question emerged 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 ^3H -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 [^3H]xyloglucan, [^3H]xylans, polymers which containing [^3H]arabinose and those undigested by Driselase) and were perfectly illustrated in the case of [^3H]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 [^3H]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_2O_2 and inhibitors of cross-linking (Encina and Fry 2005), also diffuse into the extracellular medium (Burr and Fry 2009). ^3H -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 ^3H -polymers and cell wall bound ^3H -hemicelluloses indicated that after secretion into the cell wall, protoplasmic ^3H -polymers underwent massive cross-linking in both cell lines, although this increase was much less pronounced in Sh1.5 cells. These results point to a reduction in the cell wall grafting ability of Sh1.5 cells.

A possible explanation for these findings is that cells habituated to low DCB levels synthesized ^3H -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 synthesized 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* 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 (**Supplementary data Figure S5**).

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-habitation levels, the M_w of 0.1 M KOH-extracted hemicelluloses increased with respect to the same fraction obtained from non-habitated 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 Mérida 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 processes 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) (Mérida et al. 2009, de Castro et al. 2014). 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. As previously mentioned, Sh1.5 cells showed a reduced amount of cell wall bound ^3H -hemicelluloses and an increased presence of [^3H]SEPs in the culture medium (de Castro et al. 2015). In addition, the timing of cross-linking may also be delayed in Sh1.5 cells due to their slower and less efficient metabolism (de Castro et al. 2015) thus preventing detection during the time window employed in this experiment (5 h). In order to explore these possibilities, an analysis was performed of cinnamic acid metabolism as well as a characterization of its derivatives on protoplasmic fraction and in the cell wall. First, no marked differences were detected in the capacity to uptake [^{14}C]cinnamic acid 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, Shn and Sh1.5 cells showed differences in their capacity to incorporate [^{14}C]cinnamic acid 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 molecules. Similar altered kinetics in Sh1.5 cells were observed when [^3H]arabinose was fed as the radioactive precursor, showing that a delayed cellular traffic and cell wall binding of ^3H -hemicelluloses and ^3H -polymers took place (de Castro et al. 2015). 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 ferulic acid. Alternatively, hemicelluloses and polymers from habituated cells were in fact shorter, albeit extensively cross-linked.

Another possibility could be that dimerisation (or oligomerisation) of ferulic acid residues contributed to the formation of intra-polymeric loops to a greater extent (Fry et al. 2000). Consequently, Sh1.5 hemicelluloses would be deposited on the cell wall as a coagulum. 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. 2014), as well as a delayed metabolism throughout the entire cycle (de Castro et al. 2015).

Generally, the elution profiles of Driselase-digested diagnostic fragments for polymers containing [^3H]arabinose and those for [^3H]xylans were very similar, although arabinose 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, galactose and rhamnose residues (de Castro et al. 2014). Therefore, it can be assumed that [^3H]arabinose, [^3H]xylose and [^3H]xylobiose are, in fact, digestion products of putative [^3H]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.

Snh cells shown faster [^{14}C]cinnamic acid metabolism than Sh1.5 and as a consequence free-phenolics and ^{14}C -labelled compounds were less persistent on the protoplasmic fraction. However the dimerization and oligomerization process were quickly in Sh1.5, especially during the late logarithmic phase. The presence of dimers and oligomers in the protoplasm of Sh1.5 cells, could be related with the M_w increase of the polymers (lower K_{av}) of this fraction at late logarithmic phase compared with early logarithmic phase. Additionally, 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 *p*-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 amount 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 Shh 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 K_{av} 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 K_{av} were compared, strongly wall bound xyloglucan from Sh1.5 cells was detected in a lower K_{av} 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. 2014), these results would partially support this hypothesis, although further experiments are required for confirmation.

In sum, the study of the M_w of [^3H]xylans, [^3H]xyloglucans, [^3H]arabinose-containing polymers and Driselase-digestion resistant ^3H -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.

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SUPPLEMENTARY DATA

Figure S1. Thin layer chromatography of ^{14}C -labelled compounds (esterified and free-phenolics) of the protoplasmic fraction after the addition of exogenous [^{14}C]cinnamic acid to non-habituated (purple line) and habituated to $1.5\ \mu\text{M}$ DCB maize cells suspensions (green line) at the early logarithmic phase of culture cycle. The position (—) of *p*-coumaric acid (Cou), ferulic acid (Fer), cinnamic acid (Cinn) and 5,5'-dehydrodiferulic acid (Di-Fer) used as external markers is shown. Compounds with low R_f are regarded as oligomers (Fry et al. 2000). + 60 min H_2O_2 shows a treatment for 60 min with $40\ \text{mM}$ H_2O_2 , 120 min after [^{14}C]cinnamic acid feeding.

Figure S2. Thin layer chromatography of ^{14}C -labelled compounds (esterified and free-phenolics) of the protoplasmic fraction after the addition of exogenous [^{14}C]cinnamic acid to non-habituated (purple line) and habituated to 1.5 μM DCB maize cell suspensions (green line) at the late logarithmic phase of culture cycle. Other details as in Figure S1.

Figure S3. Thin layer chromatography of ^{14}C -labelled compounds esterified of the alcohol-insoluble residue (cell wall fragments) after the addition of exogenous [^{14}C]cinnamic acid to non-habituated (purple line) and habituated to 1.5 μM DCB maize cells suspensions (green line) at the early logarithmic phase of culture cycle. The position (—) of *p*-coumaric acid (Cou), ferulic acid (Fer) and 5,5'-dehydrodiferulic acid (Di-Fer) used as external markers is shown. Compounds with low R_f are regarded as oligomers (Fry et al. 2000). The inset graphics show in detail the oligomers and ferulic acid dimers appearance. +60 min H₂O₂ shows a treatment for 60 min with 40 mM H₂O₂, 120 min after [^{14}C]cinnamic acid feeding.

Figure S4. Thin layer chromatography of ^{14}C -labelled compounds esterified of the alcohol-insoluble residue (cell wall fragments) after the addition of exogenous $[^{14}\text{C}]$ cinnamic acid to non-habituated (purple line) and habituated to $1.5\ \mu\text{M}$ DCB maize cell suspensions (green line) at the late logarithmic phase of culture cycle. Other details as in Figure S3.

Figure S5. Relative expression of orthologous maize genes of several *IRX* genes from arabidopsis analyzed by RT-PCR of non-habituated (Snh) and habituated to 1.5 (Sh1.5) and 6 μ M DCB (Sh6) maize cell suspensions. $\uparrow$: more mRNA accumulation than control (Snh); $\downarrow$ less mRNA accumulation than control (Snh). *ZmUbi*: Ubiquitin gene expression. Ubiquitin was used as the housekeeping gene due to its constitutive expression.

Table S1. Primers used for the analysis of maize arabinoxylan synthesis genes

Maize gene ID (arabidopsis homologous)	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')
GRMZM2G100143 (<i>IRX10</i>)	GATGCAGGCTCACCTTATCC	CGCTGCATGTCCTCGTAGTA
GRMZM2G059825 (<i>IRX10L</i>)	ACGTTGGTTCAGACCTTTGG	TCATTGCCGGTGTCATAGAA
gbIBT036881.1 (<i>IRX9</i>)	TGAACTCGAGAATGCTGTGG	GCTCAATTACCCACCCTTGA

Chapter III

Class III peroxidases in cellulose deficient cultured maize cells during cell wall remodelling

Chapter corresponding to the publication: **Martínez-Rubio R, Acebes JL, Encina A, Kärkönen A (2018) Class III peroxidases in cellulose deficient cultured maize cells during cell wall remodelling. *Physiol Plant* doi.org/10.1111/ppl.12710 (See Appendix)**

ABSTRACT

Maize (*Zea mays* L.) suspension-cultured cells habituated to a cellulose biosynthesis inhibitor 2,6-dichlorobenzonitrile (DCB) have a modified cell wall, in which the reduction in the cellulose content is compensated by a network of highly cross-linked feruloylated arabinoxylans and the deposition of lignin-like polymers. For both arabinoxylan cross-linking and lignin polymerization, class III peroxidases (POXs) have been demonstrated to have a prominent role. For the first time, a comparative study of POX activity and isoforms in control and cellulose-impaired cells has been addressed, also taking into account their cellular distribution in different compartments. Proteins from the spent medium (SM), soluble cellular (SC), ionically (ICW) and covalently bound cell wall protein fractions were assayed for total and specific peroxidase activity by using coniferyl and sinapyl alcohol and ferulic acid as substrates. The isoPOX profile was obtained by isoelectric focusing. POX activity was higher in DCB-habituated than in non-habituated cells in all protein fractions at all cell culture stages. For all substrates assayed, SC and ICW fractions showed higher activity at the early-log growth phase than at the late-log phase. However, the highest POX activity in the spent medium was found at the late-log phase. According to the isoPOX profiles, the highest diversity of isoPOXs was detected in the ICW and SM protein fractions. The latter fraction contained isoPOXs with higher activity in DCB-habituated cells. Some of the isoPOXs detected could be involved in cross-linking of arabinoxylans and in the lignin-like polymer formation in DCB-habituated cells.

INTRODUCTION

The cell wall is a dynamic structure that provides mechanical support to plant cells, playing an important role in plant growth and development, and enabling cells to cope with different biotic and abiotic stresses (Wolf et al. 2012). Primary cell walls of maize (*Zea mays* L.; type II) are complex composites made up of polysaccharides (cellulose and hemicelluloses) and proteins, with hydroxycinnamates, mainly ferulic and *p*-coumaric acids, esterified to arabinose residues of glucurono-arabinoxylans (Kato and Nevins 1985, Carpita et al. 2015). In addition, feruloyl residues are susceptible to oxidative coupling mediated by peroxidases (Geissman and Neukom 1971), leading to formation of ester-linked dehydrodiferulates and higher oligomers, which contribute to cell wall assembly by cross-linking cell wall polysaccharides (Fry 2004).

The variability in the cell wall composition reflects the **plasticity of the cell wall** structure. A valuable tool to underline the plasticity has been the habituation of plant cell cultures to grow in the presence of cellulose biosynthesis inhibitors (for a review see Acebes et al. 2010). One of these compounds, 2,6-dichlorobenzonitrile (**DCB**), specifically inhibits polymerization of glucose into β -(1,4)-linked glucans (Montezinos and Delmer 1980), causing a reduction in the cell wall cellulose content. In the last few decades, several plant cell cultures have been habituated to DCB (Shedletzky et al. 1992, Encina et al. 2001, 2002, García-Angulo et al. 2006, 2009, Mélida et al. 2009, 2015). The habituation process involves many metabolic modifications, depending on the type of the cell wall, the concentration of DCB, as well as the length of time that the cells are exposed to the inhibitor (Alonso-Simón et al. 2004, de Castro et al. 2015, Mélida et al. 2015, Largo-Gosens et al. 2016).

By compositional analysis and structural characterization of the cell walls of DCB-habituated cells, it has been demonstrated that maize cells have the capacity to cope with DCB through acquisition of a modified cell wall. In DCB-habituated cells, cellulosic scaffold was partially replaced by a more extensive network of highly cross-linked feruloylated arabinoxylans (Mélida et al. 2009, 2010a, 2010b, 2011, 2015, de Castro et al. 2014, 2015, Largo-Gosens et al. 2016). Recently, it has been shown that DCB-habituated maize cells incorporate to the cell walls lignin-like

polymers (Mélida et al. 2015), which stiffens the structure further (Pomar et al. 2002, Kärkönen and Kuchitsu 2015).

Lignin is a three-dimensional, phenolic polymer that is deposited in the secondary thickened cell walls of specialized plant cell types such as water-conducting tracheids and vessels, and support-giving sclerenchyma cells. Lignin provides structural support by giving compressive strength and hydrophobicity to the cell walls (Vanholme et al. 2010). Lignin is polymerized in muro by the oxidative coupling of monolignols: *p*-coumaryl, coniferyl, and sinapyl alcohols (Vanholme et al. 2010). The cross-coupling reactions of monolignol radicals produce a hydrophobic heteropolymer composed of *p*-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) units (Kärkönen et al. 2014, Hatfield et al. 2017). Oxidation of monolignols to phenolic radicals is carried out by H₂O₂-dependent peroxidases (Harkin and Obst 1973) and/or O₂-dependent laccases (Bao et al. 1993, Zhao et al. 2013, Koutaniemi et al. 2015). Peroxidases were earlier considered more important for lignin polymerization than laccases, because while peroxidases are able to oxidize the three monolignols to produce H, G and S units, laccases only generate G units (Berthet et al. 2011). Recent studies, however, have shown that laccases play an important role in vascular lignin formation in *Arabidopsis thaliana* being supposedly involved in the early stages of lignin polymerization making di- and oligolignols (Zhao et al. 2013, Laitinen et al. 2017). In maize, only one laccase (*ZmLac3*) of the laccase multigene family seems to be involved in polymerization of monolignols (Caparrós-Ruiz et al. 2006).

Class III secretory plant peroxidases (POX; EC 1.11.1.7; donor: hydrogen peroxide oxidoreductase) are implicated in several physiological processes including lignin and suberin formation (Christensen et al. 1998, Quiroga et al. 2000, Passardi et al. 2005), plant defence (Li et al. 2010), auxin metabolism (Cosio and Dunand 2009), cell elongation (Passardi et al. 2004), as well as generation of reactive oxygen species (Liszkay et al. 2003, Li et al. 2010, Shigeto and Tsutsumi 2016). Typically, POXs can be found in vacuoles (soluble cellular proteins) and in the cell walls. In this later case and depending on their interaction with the cell wall components, cell wall POXs can be divided into three groups: those soluble in the apoplastic fluid that permeates cell walls; ionically bound cell wall proteins (ICW)

presenting hydrophobic and ionic interactions with the matrix polysaccharides and proteins; and covalently bound cell wall proteins (CCW), which are cross-linked by covalent bonds with the cell wall components (Jamet et al. 2006, 2008). In this way, diferulate and lignin synthesis have been preferentially associated with ionically and covalently bound cell wall isoperoxidases (isoPOXs; González et al. 1999, Shigeto et al. 2013, Fernández-Pérez et al. 2015, Lin et al. 2016, Warinowski et al. 2016). In addition, it has been reported that both anionic and cationic isoPOXs play a role in lignin formation (Fagerstedt et al. 2010).

Taking into account that DCB habituation of maize cells is featured by significant changes in the cell wall components susceptible to POX action (i.e., feruloylated arabinoxylans and lignin-like compounds), the aim of this study was to obtain further information on the interrelationship between POXs and the remodelling of the cell wall. As a research material we used maize suspension-cultured cells habituated to 1.5 μ M DCB (I_{50} value for maize suspension-cultured cells is 0.5 μ M DCB; de Castro et al. 2014). **For this purpose**, proteins isolated from the maize cell cultures were fractionated into (1) culture medium proteins, (2) soluble cellular proteins (SC), (3) ionically- (ICW) and (4) covalently- (CCW) bound cell wall proteins. Total and specific POX activity in different cellular fractions against three native substrates (ferulic acid, coniferyl alcohol and sinapyl alcohol) are reported. The isoPOX profile in the different cellular compartments of non-habituated and DCB-habituated cells was obtained by isoelectric focusing followed by POX activity staining. For the first time, a comparative study of POX activity and isoforms in control and cellulose-impaired cells including their cellular distribution is shown. Both quantitative and qualitative differences in POX activity and profile were detected suggesting that POXs play a prominent role in maize cell wall remodelling as a response to reduction in cellulose content.

MATERIALS AND METHODS

Plant material and DCB-habituating process

Cell cultures

Maize (*Zea mays* L. Black Mexican sweetcorn) suspension-cultured cells were obtained as described by Lorences and Fry (1991), and subcultured every 15 days in liquid Murashige and Skoog medium (Murashige and Skoog 1962) supplemented with 9 μM 2,4-dichlorophenoxyacetic acid and 20 g l^{-1} sucrose. The cultures were cultivated at 25°C under photoperiodic conditions (16 h light: 8 h dark; 41 $\mu\text{mol m}^{-2} \text{s}^{-1}$, GRO-lux Sylvania) on a rotary shaker (120 rpm) (Mélida et al. 2011).

Habituating to DCB

Maize suspension-cultured cells were stepwise subcultured in a medium supplied with increasing concentrations of DCB (Fluka, Seelze, Germany). DCB was dissolved in dimethyl sulfoxide (DMSO), and the maximum concentration of DMSO added in the culture medium (0.015%, v/v) did not affect cell growth (de Castro et al. 2014). Non-habituating, suspension-cultured cells were transferred to a medium containing 0.3 μM DCB and increasing DCB concentrations were applied gradually up to 1.5 μM DCB (habituating cells) after seven subcultures. Cell growth kinetics for non-habituating and habituating cells were followed as described by de Castro et al. (2014), and cells were collected at early (early-log) and late logarithmic (late-log) growth phases (4 and 8 days old cells for non-habituating and 8 and 12 days old cells for DCB-habituating cell lines, respectively).

Preparation of protein fractions

Protein fractions were obtained according to the method of Kärkönen et al. (2002) with modifications.

The spent medium of suspension-cultured cells at the early-log and late-log growth phases was filtered through glass wool. Cells were collected, weighed and immediately frozen in liquid nitrogen, and stored at -80°C. All subsequent steps were carried out at 0-4°C. The medium was centrifuged (10 000 g , 30 min) and the supernatant was concentrated over 10-fold using an Amicon ultra-filtration device (Biomax membrane, cutoff 10 kDa, Darmstadt, Germany). The proteins were buffer-

exchanged prior to enzyme assays by passage through PD10 desalting columns (Sephadex G-25, GE Healthcare, Uppsala, Sweden) pre-equilibrated with 50 mM Tris-HCl buffer, pH 7.5, containing 0.5 mM CaCl_2 , 30 mM NaCl and protease inhibitors (Complete Protease Inhibitor Cocktail Tablets, EDTA-free; Roche). This fraction contained **spent culture medium proteins**.

The cells were powdered in liquid nitrogen using a mortar and pestle. The resulting fine powder was transferred into 50 mM Tris-HCl buffer, pH 7.5, supplemented with 30 mM NaCl, a protease inhibitor cocktail and 0.3% (w/v) Triton X-100 (3 ml buffer g^{-1} cells) (referred to from now on as an extraction buffer). After 30 min incubation on ice, the sample was centrifuged (17 000 g , 30 min). The supernatant containing **soluble cellular proteins (SC)** was desalted through PD10 desalting columns as described above.

The pellet precipitated by centrifugation was subsequently homogenized in a glass homogenizer with repeated centrifugation (17 000 g , 20 min) in extraction buffer without protease inhibitors (referred to from now on as a wash buffer) with 15 ml buffer in each wash for at least 8 times, and in 50 mM Tris-HCl buffer, pH 7.5, containing 0.5 mM CaCl_2 and 30 mM NaCl for at least 4 times. Washes were continued until no soluble POX activity was detected in the last wash solution as assayed with 0.55 mM 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS, Sigma-Aldrich-Aldrich, St. Louis, MO) and 0.25 mM H_2O_2 as substrates in 50 mM Na-citrate buffer pH 5.0. Cell walls were transferred into 50 mM Tris-HCl buffer, pH 7.5, supplemented with 1 M NaCl, 0.5 mM CaCl_2 and a protease inhibitor cocktail to extract **ionically bound cell wall proteins (ICW)**. After overnight extraction on ice, samples were centrifuged (10 000 g , 30 min), and the extraction repeated with gentle shaking (100 rpm for 30 min). The two fractions containing the ICW were combined, passed through PD10 columns (see above) and concentrated using a Microsep Centrifugal Device (cut off 10 kDa, Pall Corporation, Michigan, EEUU), frozen in liquid nitrogen and stored at -80°C .

The pellet from the centrifugation was homogenized in a glass homogenizer with the wash buffer (ca. 3 times) and with cold water (ca. 7 times) with repeated centrifugation (17 000 g , 20 min), until no soluble POX activity was detected in the last wash solution as assayed with ABTS.

To extract **covalently bound cell wall proteins (CCW)**, cell walls were incubated in a mixture of 1% (w/v) Cellulase Onozuka R-10 (Duchefa Biochemie) and 0.5% (w/v) Macerozyme R-10 (Duchefa Biochemie) in 100 mM Na-acetate buffer, pH 4.5, containing a protease inhibitor cocktail at 30°C for 3 h (Masuda et al. 1983). After centrifugation (10 000 *g*, 30 min, 4°C) the supernatant was buffer-exchanged in PD10 desalting columns. To release those proteins that were liberated by the enzyme treatment but were then bound ionically to the cell walls, the residue was suspended in 50 mM Tris-HCl buffer, pH 7.5, containing 0.5 mM CaCl₂ and 1 M NaCl, and incubated on ice for 30 min. After centrifugation (10 000 *g*, 30 min), the extraction was repeated. These fractions were buffer-exchanged and combined to the fraction obtained after the enzyme treatment, followed by concentration as mentioned above.

The cell wall pellet was washed with water until no soluble POX activity was detected in the last wash solution (assayed with ABTS) and lyophilized.

Three independent experiments were carried out with sample replication of at least three in each case.

Enzyme assays and quantification of proteins

All enzyme assays were carried out on protein concentrates after desalting through PD10 gel filtration columns into 50 mM Tris-HCl buffer, pH 7.5, containing 0.5 mM CaCl₂, 30 mM NaCl and protease inhibitors. To assay POX activity in the cell wall residues, the lyophilized cell walls were resuspended in the above-mentioned buffer (1 mg ml⁻¹), and 60 µl of the suspension was added to the reaction mixture. Assays were carried out in a total volume of 1.0 ml in a spectrophotometer (Shimadzu UV-1800, Kyoto, Japan) at 25°C. Coniferyl alcohol POX activity was assayed at 262 nm using 100 µM coniferyl alcohol (Fluka, Seelze, Germany; Kärkönen et al. 2002), ferulic acid POX activity was determined at 310 nm with 100 µM ferulic acid (Sigma-Aldrich, St. Louis, MO; García et al. 2002), and sinapyl alcohol POX activity was assayed at 270 nm with 100 µM sinapyl alcohol (Sigma; Koutaniemi et al. 2005) as substrates. All activities were determined by adding 250 µM H₂O₂ and up to 60 µl of sample in 45 mM Na-citrate buffer, pH 5.0. The molar absorptivity coefficients (ϵ) for coniferyl alcohol, ferulic acid and sinapyl alcohol

were taken as $14000 \text{ M}^{-1} \text{ cm}^{-1}$ (Kärkönen et al. 2002), $8400 \text{ M}^{-1} \text{ cm}^{-1}$ (García et al. 2002) and $6000 \text{ M}^{-1} \text{ cm}^{-1}$ (Koutaniemi et al. 2005), respectively.

Oxidase activity was determined at 262 nm by the method of Udagama-Randenuya and Savidge (1995) using $75 \mu\text{M}$ coniferyl alcohol in 88.5 mM Na-acetate buffer, pH 4.5, containing 125 U ml^{-1} catalase Sigma C-100 (Sigma-Aldrich, St. Louis, MO) and $100 \mu\text{l}$ of sample. Extinction coefficient for coniferyl alcohol was used for quantitative calculations.

Bio-Rad protein assay (Bio-Rad, Hercules, CA; based on Bradford 1976), scaled down to microtiter plates was used to measure protein concentration using bovine serum albumin (Sigma-Aldrich, St. Louis, MO) as a standard.

Isoelectric focusing

Isoelectric focusing (IEF) was carried out as described by Kärkönen et al. (2002) with modifications. IEF was accomplished with a Multiphor II apparatus (Pharmacia, Stockholm, Sweden) in a 4% polyacrylamide gel containing Pharmolyte with a pH range of 3-10 (GE Healthcare, Uppsala, Sweden), and 0.04 M aspartic acid and 1 M NaOH as anode and cathode solutions, respectively. Manufacturer's instructions were followed with slight modifications. Before sample application, the gel was pre-focused at 5 W for 30 min . After sample application, the gel was focused at 15 W for 1.5 h at $+10^\circ\text{C}$.

POX activity was visualized by staining the gel with 0.8% (w/v) 3,3'-diaminobenzidine (DAB, Sigma-Aldrich, St. Louis, MO) in 50 mM Na-acetate buffer, pH 5.0, supplemented with 10 mM CaCl_2 (Kärkönen et al. 2002, with some modifications). After preincubation for 10 min in a staining buffer, the reaction was started by addition of H_2O_2 . H_2O_2 was added gradually in 0.5 mM aliquots until POX activity bands became visible (final concentration of H_2O_2 ca. 2.5 mM). After at least 30 min staining at room temperature, the activity bands were recorded by photographing (Nikon F50, Tokyo, Japan). Isoelectric points (pI) were calculated based on the comparison with the pH of gel segments derived from empty lanes.

RESULTS

Peroxidase activity

To obtain a deep insight in the relationship between class III peroxidases (POXs) and the remodelling of the maize cell wall occurring due to the habituation to the cellulose biosynthesis inhibitor 2,6-dichlorobenzonitrile (DCB), POX activity against native substrates coniferyl alcohol, sinapyl alcohol and ferulic acid was measured on protein fractions obtained from non-habituated and DCB-habituated cell lines in different phases of the cell culture cycle (**Tables 1 and 2**). Regarding substrates, similar levels of POX activity (both total and specific activity) were detected with coniferyl alcohol and ferulic acid. POX activity against sinapyl alcohol was clearly smaller (7 to 57 times) than those found for the other two substrates. Because of the presence of some interfering compounds in the SC protein fraction, sinapyl alcohol POX activity could not be measured for this fraction. The nature of the interfering compound(s) is not known as the buffer-exchange in PD10 column removed the small-molecular-weight compounds.

Independently of the substrate or the cell line analyzed, the highest total POX activity was found in the spent medium, followed by soluble and cell-wall-bound fractions (**Table 1**). In all cases, a low remaining POX activity was detected in the cell wall residue. When POX activity was expressed in relation to the protein content (specific activity) (**Table 2**), the activity in the ICW fraction was highest followed by that in the spent medium and SC fractions. Because of the considerable amount of proteins provided in the commercial Cellulose-Macerozyme mixture, specific POX activities for the CCW fractions could not be calculated.

Table 1. Total peroxidase activity in soluble cellular, ionically and covalently bound cell wall and spent medium protein fractions and in cell wall residues of non-habituated (NH) and DCB-habituated (H) cell lines at the early-logarithmic (early-log) and late-logarithmic (late-log) growth phases. Data are means $\pm$ SE of at least six independent experiments. FW, cell fresh weight; n.d., not determined due to presence of interfering compounds.

Enzyme activity nkat/ g FW	Early log growth phase		Late log growth phase	
	NH	H	NH	H
Soluble cellular protein fraction				
Coniferyl alcohol	121.3 $\pm$ 3.9	145.4 $\pm$ 3.9	89.7 $\pm$ 4.0	76.8 $\pm$ 1.8
Ferulic acid	142.5 $\pm$ 3.9	197.4 $\pm$ 2.5	86.5 $\pm$ 1.7	70.6 $\pm$ 1.1
Sinapyl alcohol	n.d.	n.d.	n.d.	n.d.
Ionically bound cell wall protein fraction				
Coniferyl alcohol	55.2 $\pm$ 2.0	75.9 $\pm$ 7.5	31.9 $\pm$ 6.3	40.2 $\pm$ 0.6
Ferulic acid	56.6 $\pm$ 2.1	71.7 $\pm$ 5.1	29.0 $\pm$ 4.2	37.6 $\pm$ 0.8
Sinapyl alcohol	2.4 $\pm$ 0.1	3.9 $\pm$ 0.4	1.6 $\pm$ 0.4	2.3 $\pm$ 0.2
Covalently bound cell wall protein fraction				
Coniferyl alcohol	30.6 $\pm$ 0.8	19.9 $\pm$ 1.8	22.3 $\pm$ 2.9	28.3 $\pm$ 0.9
Ferulic acid	30.0 $\pm$ 0.9	19.7 $\pm$ 1.4	29.1 $\pm$ 3.0	32.4 $\pm$ 0.9
Sinapyl alcohol	3.3 $\pm$ 0.3	2.7 $\pm$ 0.2	2.9 $\pm$ 0.3	3.7 $\pm$ 0.1
Spent medium protein fraction				
Coniferyl alcohol	38.9 $\pm$ 2.0	235.2 $\pm$ 7.4	167.5 $\pm$ 18.5	492.7 $\pm$ 73.2
Ferulic acid	36.9 $\pm$ 1.1	233.7 $\pm$ 11.1	145.3 $\pm$ 11.2	435.8 $\pm$ 31.8
Sinapyl alcohol	1.7 $\pm$ 0.3	7.7 $\pm$ 1.9	2.8 $\pm$ 0.2	12.6 $\pm$ 0.9
Cell wall residue				
Coniferyl alcohol	0.07 $\pm$ 0.01	0.13 $\pm$ 0.01	0.13 $\pm$ 0.02	0.10 $\pm$ 0.01
Ferulic acid	0.08 $\pm$ 0.01	0.21 $\pm$ 0.03	0.19 $\pm$ 0.02	0.16 $\pm$ 0.01
Sinapyl alcohol	0.04 $\pm$ 0.01	0.05 $\pm$ 0.01	0.06 $\pm$ 0.01	0.07 $\pm$ 0.00

Table 2. Specific peroxidase activity in soluble cellular, ionically bound cell wall and spent medium protein fractions of non-habituated (NH) and DCB-habituated (H) cell lines at the early-logarithmic (early-log) and late-logarithmic (late-log) growth phases. Data represent means $\pm$ SE of at least six independent experiments. FW, cell fresh weight; n.d. not determined due to presence of interfering compounds.

Enzyme activity nkat/mg protein	Early log growth phase		Late log growth phase	
	NH	H	NH	H
Soluble cellular protein fraction				
Coniferyl alcohol	11.3 $\pm$ 0.9	15.0 $\pm$ 0.7	10.7 $\pm$ 0.5	13.0 $\pm$ 0.7
Ferulic acid	13.1 $\pm$ 0.8	20.3 $\pm$ 0.3	10.4 $\pm$ 0.4	13.2 $\pm$ 1.1
Sinapyl alcohol	n.d.	n.d.	n.d.	n.d.
Ionically bound cell wall protein fraction				
Coniferyl alcohol	364.0 $\pm$ 52.6	1338.7 $\pm$ 37.4	100.3 $\pm$ 20.5	539.9 $\pm$ 95.0
Ferulic acid	366.6 $\pm$ 46.2	1281.0 $\pm$ 7.9	91.3 $\pm$ 13.5	495.5 $\pm$ 48.2
Sinapyl alcohol	16.1 $\pm$ 2.4	69.4 $\pm$ 1.6	4.8 $\pm$ 1.1	29.2 $\pm$ 3.8
Spent medium protein fraction				
Coniferyl alcohol	441.9 $\pm$ 22.3	612.6 $\pm$ 19.3	1658.6 $\pm$ 183.0	1734.7 $\pm$ 229.2
Ferulic acid	419.7 $\pm$ 12.0	608.8 $\pm$ 29.0	1438.9 $\pm$ 110.6	1489.6 $\pm$ 108.6
Sinapyl alcohol	19.7 $\pm$ 2.9	20.0 $\pm$ 5.0	27.6 $\pm$ 1.5	43.0 $\pm$ 3.2

Both total and specific POX activities in the ICW fraction were higher at the early-log than at the late-log growth phase independently of the cell line analyzed (**Tables 1 and 2**). In the case of the spent medium, the highest POX activity was found at the late-log growth phase. No differences in the POX activity were detected in the CCW fraction when different phases of the cell culture cycle were compared.

In general, total POX activity of all protein fractions was higher in the DCB-habituated cell line than in the non-habituated cell line throughout the culture cycle. Only in the SC fraction regarding the late-log growth phase and CCW fraction regarding the early-log growth phase, higher POX activity was found in non-habituated cells compared to DCB-habituated cells. Similarly, higher specific POX activity was found for the DCB-habituated cell line (regarding both early and late-log phases) than for the non-habituated cell line regarding all fractions assayed.

Oxidase activity against coniferyl alcohol was not detected in any of the protein fractions analyzed.

IEF and in-gel activity staining

Maize suspension-cultured cells were characterized for isoperoxidases (isoPOXs) by IEF in a pH gradient of 3-10 (**Figures 1-2**). Different patterns of isoPOX were obtained for different cellular fractions, cell lines and phases of the cell culture cycle. POXs in different fractions could be classified into acidic, neutral and basic isoPOX groups. Independently of the cell lines and phases of the cell culture cycle analyzed, SC proteins' isoPOX profiles were similar, showing a diversity of isoPOXs across the whole pI range (**Figure 1A**). There were, however, some differences in the activity of individual isoPOXs (**Figure 1A**). CCW samples, on the other hand, contained several isoPOXs with neutral to slightly acidic pIs and few basic isoPOXs, but no qualitative differences could be seen in the non-habituated and DCB-habituated cell lines (**Figures 1B and C**).

In the ICW fraction, non-habituated and habituated cells showed a common pattern of basic isoPOX forms (**Figure 2A**). This was not the case for the slightly acidic isoPOXs (pIs ~5-5.7), where DCB-habituated cells lacked some of the isoforms detected in non-habituated cells. The spent medium of non-habituated and DCB-habituated cells at both culture phases contained four neutral isoPOXs (pIs ~7.5)

that became visible after staining for a few minutes (**Figures 2B (#) and C**). When stained for a longer time (up to 120 minutes), new isoPOXs appeared in the non-neutral regions. The activity level of the individual isoPOXs in these regions differed between the cell lines (**Figure 2B**). Much more active acidic isoPOXs were present in the DCB-habituated cells at both growth phases in comparison with the non-habituated cells. In addition, at the late-log phase one basic isoPOX showed higher activity in DCB-habituated cells lines than in the non-habituated cell lines (**Figure 2B**; uppermost arrowhead).

Figure 1. Peroxidase isoenzyme patterns of soluble cellular proteins(**A**) and covalently bound cell wall proteins (**B, C**) of non-habituated (NH) and DCB-habituated (H) cell lines of maize suspension-cultured cells at their early-logarithmic (early-log) and late-logarithmic (late-log) growth phases separated on an acrylamide gel by isoelectric focusing (pH 3-10). (**B**) Basic isoperoxidases after a longer staining time. (**C**) Neutral and acidic isoperoxidases. The amounts of proteins loaded onto the gel were determined as coniferyl alcohol peroxidase activity: (**A**) 600 pkat, (**B**) 3000 pkat and (**C**) 2000 pkat were used here. Peroxidase activity was visualised by DAB staining as described in Materials and methods. The pIs shown are the mean value ± sd obtained from three independent gels.

Figure 2. Peroxidase isoenzyme patterns of ionically bound cell wall proteins (**A**), and spent medium proteins (**B, C**) of non-habituated (NH) and DCB-habituated (H) cell lines of maize suspension-cultured cells at their early-logarithmic (early-log) and late-logarithmic (late-log) growth phases separated on an acrylamide gel by isoelectric focusing (pH 3-10). The amounts of proteins loaded onto the gel were determined as coniferyl alcohol peroxidase activity: (**A**) 4000 pkat and (**B, C**) 1500 pkat were used here. Peroxidase activity was visualized by DAB staining as described in Materials and methods. The pIs shown are the mean value $\pm$ sd obtained from three independent gels. Arrowheads show isoPOXs with a stronger activity in the H cell line when compared to NH one. (**C**) A close-up of the major, neutral isoPOXs (#) after a shorter staining time.

DISCUSSION

Habituation of suspension-cultured plant cells to DCB leads to a modified cell wall, which allows cells to cope with different stress situations. In previous studies, we have shown that DCB habituation of suspension-cultured maize cells causes a reduction in the cellulose content, which is compensated by a more extensive network of highly cross-linked arabinoxylans (Mélida et al. 2009). As expected, maize cells habituated to DCB presented a higher amount of cell-wall-esterified *p*-coumarates, ferulates and dehydrodiferulates, the later ones of which are arabinoxylan cross-linkers (Mélida et al. 2009). The wall-bound ferulates have been proposed to act as nucleation sites for lignin polymerization (Ralph 2010, Hatfield et al. 2017). More recently, we have demonstrated the presence of ectopic lignin or lignin-like material in the cell walls of DCB-habituated cells (Mélida et al. 2015). In summary, our previous results show that despite being minor components of maize

primary cell walls, cell wall phenolics play a major role in maintaining cell wall integrity (Mélida et al. 2009, de Castro et al. 2014).

Class III peroxidases (POXs) located in cell walls and vacuoles catalyze the oxidation of a large variety of substrates by using H_2O_2 as an oxidizing agent (Kärkönen et al. 2009). By cross-linking ferulates bound to arabinoxylans, POXs have been demonstrated to participate in cell wall remodelling (Fry 2004). Additionally, POXs promote cross-linking between ferulates and lignin contributing to further stiffening of the cell wall structure (Ralph 2010). The reported increase in the level of POX activity associated with DCB habituation of maize suspension-cultured cells (Mélida et al. 2015, Largo-Gosens, 2016) suggests that POXs play a prominent role in maize cell wall remodelling as a response to reduction in cellulose content. In agreement with this, no oxidase (laccase) activity against coniferyl alcohol was detected in the present study, whereas all fractions had strong POX activity against coniferyl alcohol, ferulic acid and sinapyl alcohol, all native substrates in the maize cell wall.

Not every isoperoxidase (isoPOX) has the ability to oxidize the three main monolignols. Coniferyl and *p*-coumaryl alcohols can be oxidized in vitro by both anionic and cationic POXs, while sinapyl alcohol is mainly oxidized by a subgroup of cationic isoPOXs (Ros Barceló et al. 2007). In the present study, the non-habituated and DCB-habituated cell lines of maize at both early-log and late-log growth stages showed similar POX activity levels against coniferyl alcohol and ferulic acid in all compartments analyzed. Thus, maize suspension-cultured cells are able to produce and secrete POXs capable of oxidizing not only the monolignol coniferyl alcohol but also ferulic acid, further supporting the broad substrate specificity of POXs (Campa 1991). The high POX activity detected in vitro against coniferyl alcohol and ferulic acid in all fractions analyzed, and the fact that the highest total POX activity was found in the spent medium fraction, suggest that POXs in this protein fraction participate in monolignol oxidation for lignin biosynthesis, and in oxidative coupling of ferulates. These data are in accordance with those previously reported for the lignin-forming Norway spruce cell culture, in which POXs activities in the spent medium fraction were higher than in other cell wall fractions (Kärkönen et al. 2002). In the present study, activity against sinapyl alcohol was detected in all apoplastic

fractions assayed. However, in comparison with activities obtained for coniferyl alcohol and ferulic acid, POX activity against sinapyl alcohol was clearly lower, indicating that maize POXs are less efficient in sinapyl alcohol oxidation (Hatfield et al. 2008). The presence of S units in maize stem lignin (S/G ratio: 1.4; Fornalé et al. 2012) and in the lignin-like material detected in the DCB-habituated suspension cultured cells (S/G ratio: 1.5; Mérida et al. 2015) requires that sinapyl alcohol is oxidized, either directly by enzymatic means or indirectly by radical transfer reaction (Takahama et al. 1996). It is possible that similarly to other plant species, only certain maize isoPOXs are capable of oxidizing sinapyl alcohol due to the presence of steric hindrances at the substrate binding site (Ros Barceló et al. 2007). This would explain the lower POX activity detected with sinapyl alcohol as a substrate.

Generally, and as expected, POX activity was higher in the DCB-habituated cells than in the non-habituated cells in all protein fractions at all cell culture stages. The high level of POX activity found in the apoplastic compartment of the DCB-habituated cells (especially in the ICW and the spent medium protein fractions) can relate to the increased level of cross-linking of arabinoxylans and the presence of ectopic lignin in this cell line (Mérida et al. 2009, 2015), both mechanisms involved in cell wall remodelling. Total POX activities in most of the protein fractions were higher at the early-log growth phase than at the late-log phase. This fact may be related to high expression of POX at this growth phase (Largo-Gosens et al. unpublished data), which predisposes habituated cells to formation of diferulates and ectopic lignin. However, the activity of the spent medium POXs was higher at the late-log growth phase than at the early-log phase.

IsoPOX profiles of SC and CCW fractions were similar among the non-habituated and habituated cell lines (**Figure 1**). However, differences in the isoPOXs were observed in the ICW and the spent medium fractions. Some slightly anionic isoPOXs (pIs ~5.0-5.7) with high activity were detected in the ICW fraction obtained from the non-habituated cell line that were clearly reduced or absent in the DCB-habituated cell line (**Figure 2A**). IsoPOXs of the corresponding pIs (~5.6) appeared in the spent medium (**Figure 2B**), showing the inability of the cell walls of DCB-habituated cells to retain those isoPOXs. One cationic isoPOX, on the other hand, was

more active in the DCB-habituated cell line (**Figure 2B**). It could be assumed that in the DCB-habituated cell line, these apoplastic isoPOXs that differ in activity between non-habituated and habituated cells are most probably involved in synthesis of lignin-like material during habituation (Mélida et al. 2015). Furthermore, the cationic isoPOX is possibly involved in S unit synthesis, since cationic isoPOXs have been hypothesized to be responsible for sinapyl alcohol oxidation (Quiroga et al. 2000, Gabaldón et al. 2005, Ros Barceló et al. 2007).

In summary, maize suspension-cultured cells habituated to DCB have a reduction in the cell wall cellulose content, and produce a more extensive network of di- and oligoferulate cross-linked arabinoxylans together with a polymeric, lignin-like material (Mélida et al. 2015). This modified cell wall architecture is a result of high structural plasticity of primary cell walls in response to the disruption of cell wall integrity. In the present study, for the first time, comparative results of POX activity and isoforms in cultured maize cells containing primary cell walls with a reduced level of cellulose have been attained. Furthermore, the cellular distribution of these POX in different compartments have been addressed.

No in-vitro coniferyl alcohol oxidase activity was detected in any of the fractions, supporting the role of POXs in cell wall cross-linking in maize. As considerable POX activity was found in all apoplastic (the spent medium, ICW and CCW) fractions, it is impossible to define a specific isoPOX(s) that is involved in the mechanisms reinforcing the cellulose-impoverished cell wall. However, both quantitative and qualitative differences in the POX activity and the isoPOX profile were detected in the spent medium and ICW fraction suggesting that these POXs play a prominent role in maize cell wall remodelling as a response to reduction in cellulose content. Therefore, this model system will be used in future studies to obtain further insight into the role of isoPOXs in cell wall cross-linking. Molecular studies will be used, focusing on identification and functional characterization of the isoPOXs that showed abundance differences between the non-habituated and DCB-habituated cell lines.

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Chapter V

Induced lignification promoted by cellulose biosynthesis inhibitors in maize suspension-cultured cells

ABSTRACT

Lignin synthesis is associated with the formation of a secondary cell wall during the specialisation of certain cell types. However, cultured cells exposed to different biotic and abiotic stresses can induce the synthesis of unexpected lignin, a process known as ectopic lignification. Our group has previously reported that treatment of cultured maize cells with dichlobenil (DCB), a group 2 cellulose biosynthesis inhibitor (CBI), induces cell wall lignification. In order to determine whether cobtorin, a group 3 CBI with a different mode of action, also induces ectopic lignification, maize suspension-cultured cells were treated with cobtorin and DCB. The I_{50} value of cobtorin for weight increase in maize cells was 2.5 μM . However, negative phloroglucinol staining of cell walls after 10 μM cobtorin treatment indicated that in contrast to DCB, cobtorin was not inducing ectopic lignification in this experimental system. Consequently, the study of ectopic lignification was only conducted in 6 μM DCB-treated cells. Microscopy analysis showed that the intensity of cellulose fluorescence in DCB-treated cells decreased slightly over the course of cell culture cycle in elongating cells. Autofluorescence associated with phenolic compounds, as well as Wiesner staining, were evident in the last phase of the cycle and were related to the accumulation of lignin-like materials in discrete areas of some cell walls. Green autofluorescence induced under ammonium associated with feruloyl esters– also increased in cell walls of these DCB-treated cells.

A previous study of the expression of the 13 *ZmBAHD* genes that were putatively involved in transferring feruloyl/coumaroyl residues to arabinoxylans showed that four of these, *ZmBAHD5*, *ZmBAHD6*, *ZmBAHD9* and *ZmBAHD12*, were overexpressed in DCB-treated cells. In addition, guaiacol-peroxidase activity in the spent medium of DCB-treated cultured cells increased as a function of time. In summary, induction of ectopic lignin seems to be CBI-dependent, as it was promoted by DCB but not by cobtorin. Furthermore, it increased steadily over the course of the cell culture cycle, and seems to be related to overexpression of a set of *ZmBAHD* genes and to enhancement of class-III peroxidase activity. Therefore, induction of ectopic lignification promoted by a reduction in the amount of

cellulose is a spatio-temporal process in which changes in the expression of selected genes and in the activity of certain enzymes, together with structural modifications, contribute to reinforce weakened cell wall entanglement.

INTRODUCTION

One of the distinctive features of plants is the presence of a complex and rigid **cell wall**. The cell wall is characterised by a set of unique traits, having a dynamic architecture and composition that determines cell shape and provides plant mechanical support during growth. As an external barrier, this structure participates in cell-cell and cell-pathogen recognition processes (Wolf et al. 2012) and is involved in modulating stress signalling responses (Driouich et al. 2012, Bashline et al. 2014, Malinovsky et al. 2014, Rao et al. 2017).

The **primary cell wall** consists of cellulose microfibrils embedded in a hemicellulosic and pectic matrix of polysaccharides together with glycoproteins (Carpita and Gibeaut 1993). Graminaceous plants, such as **maize** and other commelinoid monocots, have a **type II cell wall**, in which (glucurono)arabinoxylans and mixed-linked β -glucan (together with xyloglucan) are the main hemicelluloses (Carpita 1984, Burton and Fincher 2009). In addition, type II cell walls contain higher amounts of **hydroxycinnamates**, mainly ferulic acid and *p*-coumaric acid, which substitute arabinoxylans by ester-linking α -L-arabinosyl residues (Wende and Fry 1997, Buchanann et al. 2012). Due to their high reactivity, polysaccharide-esterified hydroxycinnamates promote **arabinoxylan cross-linking**, playing a key role in maintaining the integrity of grass cell walls (de O Buanafina 2009).

Plants detect modifications in cell wall composition caused by mutation or abiotic/biotic stresses through complex mechanisms, triggering responses to **cell wall damage** (CWD) (Denness et al. 2011, Engelsdorf and Hamann 2014). Detection of these modifications activates intracellular signalling cascades that trigger four characteristic responses: an accumulation of reactive oxygen species, induction of jasmonic and salicylic acid biosynthesis and ectopic lignification (Hamann et al. 2009, Denness et al. 2011, Gigli Bisceglia et al. 2018). This set of responses is named the cell wall damage (CWD) syndrome (Gigli Bisceglia et al. 2018).

Although lignification is commonly associated with the formation of a secondarily thickened cell wall during cell specialisation, mutants and cells exposed to different **biotic and abiotic stresses** can **induce** unexpected lignification known as **ectopic lignification** (Caño-Delgado et al. 2000, 2003, Desprez et al. 2002, Bischoff et al. 2009, Hamann et al. 2009, Moura et al. 2010, Dennes et al. 2011, Brabham et al. 2014, Miedes et al. 2014, Mélida et al. 2015). Structural lignin is mainly formed through oxidative coupling of three hydroxycinnamoyl alcohols (monolignols), *p*-coumaroyl alcohol, coniferyl alcohol and sinapyl alcohol, which constitute the *p*-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) units, respectively, (Boerjan et al. 2003, Vanholme et al. 2010, Wang et al. 2013). However, **lignin-like polymers** associated with CWD show a different composition, with a substantial enrichment of S units (Mélida et al. 2015, Rao et al. 2017).

Some **cellulose biosynthesis inhibitors (CBIs)** have been demonstrated to induce CWD. Recently, CBIs have been classified into **three groups** according to their effect on the cellulose synthase complex (CSC) or its mobility (Brabham and DeBolt 2013, Tateno et al. 2016). These are: group 1, composed of inhibitors that clear cellulose synthase (CESA) particles from the plasma membrane, such as isoxaben, quinoxifen, CGA 325'615, AE F150944, thaxtomin A, CESTRIN and acetobixan (Paredes et al. 2006, Bischoff et al. 2009, Crowel et al. 2009, Gutierrez et al. 2009, Harris et al. 2012, Xia et al. 2014, Worden et al. 2015); group 2, comprising inhibitors that increase CESA accumulation accompanied by slowed CESA movement at the plasma membrane, such as **dichlobenil (DCB)** and indaziflam (Herth, 1987, DeBolt et al. 2007a, Brabham et al. 2014); and group 3, consisting of inhibitors that modify CESA trajectory and speed at the plasma membrane focal plane, such as morlin and **cobtorin** (4-[(2-chlorophenyl)-methoxy]-1-nitrobenzene) (DeBolt et al. 2007b, Yoneda et al. 2007, 2010).

CBIs from the first and second groups have been reported to induce ectopic lignification when they are applied to arabidopsis plants. Such is the case for isoxaben (Desprez et al. 2002, Caño-Delgado et al. 2003, Bischoff et al. 2009, Hamann et al. 2009, Denness et al. 2011), quinoxifen (Harris et al. 2012), acetobixan (Xia et al. 2014) and thaxtomin A (Bischoff et al. 2009) in group 1, and

DCB (Desprez et al. 2002) in group 2. However, the possible induction of ectopic lignification by group 3 CBIs has not yet been studied.

Ectopic lignification has been induced not only in entire plants, but also in cell cultures treated with different chemical agents (Kärkönen and Koutaniemi 2010, and refs. therein). Such is the case of maize cell cultures treated with DCB (Mélida et al. 2015). In the cell walls of DCB-treated cultures, lignin-like material is composed mainly of S units, and is associated with the overexpression of several genes specific to the initial steps of monolignol unit synthesis (Mélida et al. 2015). In addition, the overexpression of some of the jasmonate and salicylic signalling pathway genes has been reported (Mélida et al. 2015). Furthermore, DCB promotes an increase in oxidative damage in these cells and concomitantly an enhancement in glutathione content, as well as several antioxidant activities, such as class III peroxidase, catalase and glutathione-S-transferase (Largo-Gosens et al. 2016, [see chapter III](#)). Therefore, the four responses associated with the CWD syndrome have been found in DCB-treated maize cells, thus rendering this material an ideal candidate for further studies.

In addition, maize cells cultured in presence of DCB show higher amounts of cell-wall-esterified *p*-coumarates, ferulates and dehydrodiferulates, the latter being responsible for arabinoxylan cross-linking (Mélida et al. 2009, [see chapter II](#)). Since *p*-coumaric acid is ester-linked mainly to lignin rather than to hemicellulose in grass cell walls, and ferulic acid is exclusively ester-linked to arabinoxylan (Grabber et al. 1995, 2000), it has been proposed that both hydroxycinnamates play a key role in the cell wall remodelling process, further strengthening this structure (Rao et al. 2017). Together with the aforementioned deposition of lignin-like material polymers, this suggests that in addition to lignin, xylans are also being esterified by these phenolic acids in this experimental system.

Recent studies have reported that acylation of monolignols may be induced by the action of different **acyltransferases** prior to polymerisation (Lu and Ralph 2008, Martínez et al. 2008, Bartley et al. 2013). These proteins are members of the **BAHD superfamily of acyltransferases**, also known as the Pfam family PF02458 (D'Auria 2006, Mitchel et al. 2007, Bartley et al. 2013, Molinari et al. 2013,

de O Buanafina et al. 2016), and some of these genes have been proposed as good candidates for **feruloyl transferases** acting on arabinoxylans (Cheteigner-Boutin et al. 2016, Barrière et al. 2017) and for *p*-coumaroyl transferases acting on lignin (Hatfield et al. 2009) ([see chapter IV](#)). Both feruloylation and coumaroylation would contribute to a more cross-linked cell wall and therefore would play a prominent role in cell wall remodelling by further strengthening this structure. It would be interesting to determine whether the expression of BAHD genes is related to the ectopic lignification process observed when maize cells are treated with CBIs.

As stated above, the CWD syndrome is associated with an accumulation of reactive oxygen species. Some of these would be involved in the lignification process, given that lignin polymerisation is preceded by the dependent activation of monolignols to free radicals mediated by peroxidase+H₂O₂ (and/or lacasse+O₂) (Fagerstedt et al. 2010). Ectopic lignification promoted by exposing maize cell cultures to DCB has been associated with an enhanced apoplastic H₂O₂ concentration (Mélida et al. 2015). It would thus also be interesting to determine whether ectopic lignification is associated with an increase in peroxidase activity in the spent medium of these DCB-treated cell cultures.

Hence, the four aims of the present study were as follows: (i) to determine whether CBIs from different groups promote the same ectopic lignification in maize cell suspensions; (ii) to monitor the development of the induced lignification process throughout the cell culture cycle; (iii) to determine if ectopic lignification is associated with higher levels of expression of some BAHD genes; and (iv) to evaluate the correlation between ectopic lignification and peroxidase activity.

To this end, maize suspension-cultured cells were treated with two different CBIs; DCB, belonging to group 2, and cobtorin, belonging to group 3. In addition, a microscopic evaluation of cell walls of CBI-treated cells, the expression levels of BAHD genes putatively involved in acylation of ferulic or *p*-coumaric acids and peroxidase activity measurement were conducted at three progressively increasing exposure times.

MATERIALS AND METHODS

Plant material

Maize cell suspension cultures (*Zea mays* L., Black Mexican sweet corn) obtained from immature embryos were grown in Murashige and Skoog medium (Murashige and Skoog, 1962) supplemented with 9 μM 2,4-D and 20 g l^{-1} sucrose, in 250 ml flasks with 100 ml of culture medium. Maize cell cultures were cultivated at 25°C under photoperiodic conditions (16 h light: 8 h dark; 41 $\mu\text{mol m}^{-2} \text{s}^{-1}$, GRO-lux Sylvania) in a rotary shaker (120 rpm) (Mélida et al. 2011), and routinely subcultured every 15 days.

Cobtorin and DCB treatments

Cobtorin and DCB were dissolved in dimethyl sulfoxide (DMSO). The maximum concentration of DMSO added to the culture medium (0.015%; v/v) did not affect cell growth (de Castro et al., 2014).

In order to evaluate the effect of cobtorin on maize cell growth, cells were exposed to different concentrations of cobtorin, up to 100 μM , and growth was expressed as relative increase in DW. The I_{50} value was calculated as the concentration of cobtorin capable of inhibiting a weight increase by 50% with respect to the control using a four parameter logistic curve.

Then, maize cells were grown for 9 days in a liquid medium containing 10 μM cobtorin (the I_{50} value for maize suspension-cultured cells is 2.9 μM) or 10 μM DCB (the I_{50} value is 0.5 μM DCB; de Castro et al., 2014), ensuring a toxic effect but not enough to kill the cells.

As cobtorin-treated cells did not show ectopic lignification, the rest of the experiments were only performed on non-treated and 6 μM DCB-treated suspension-cultured cells.

Microscopy techniques

To localise cell wall components, lignin was detected by phloroglucinol/HCl (Wiesner test): filtered cells were incubated with 1% (v/w) phloroglucinol in 70% (v/v) ethanol for 5 min. Then, the phloroglucinol solution was removed and the cells were further incubated with an 18% (w/v) HCl solution. Cell aggregates were then observed and photographed with a Nikon T1-SM microscope (Mélida et al., 2015).

Cellulose was localised using calcofluor white (fluorescent brightener 28, Sigma), adding 200 µl of 0.01% calcofluor to 500 µl of cell suspension for at least 5 min (García-Angulo et al., 2006). Cell aggregates were visualised and photographed using a Nikon SMZ1500 fluorescence microscope equipped with a Nikon DXM1200F digital camera. Phenolics esterified with cell walls were detected by adding 50 µl of a NH₄OH 0.1 M solution to 500 µl of cell suspension for at least 5 min (Harris and Hartley, 1980), and the fluorescence emitted by the cell aggregates was observed using the same microscope. Lastly, autofluorescence of cells was also observed using the same equipment.

Relative gene expression analysis

Control and DCB suspension-cultured cells at different exposure times (3, 6 and 9 days of treatment) were collected and homogenised in liquid nitrogen. Total RNA was extracted following the procedures established for Trizol reagent (Invitrogen, California, USA). The purity and integrity of the extracted RNA was quantified with a Nanodrop™ (Thermo Scientific) spectrophotometer, running the RNA in 1% agarose gels. RNA (5 µg) was reverse-transcribed with NG dART RT-PCR (EURx, Gdansk, Polonia) using oligo(dT)₂₀ as primer. Semi-quantitative expression analysis by RT-PCR was performed for the BAHD genes previously identified by Martínez-Rubio et al. (unpublished results, [see Chapter IV](#)). Primers are described in [Chapter IV \(Supplementary Table, p. 175\)](#). The ubiquitin gene was used as a reference gene for this experiment (*ZmUBI*, U29159) (Fornalé et al. 2006).

Reverse transcription-PCR agarose gels were stained with SYBER Safe DNA gel stain (Invitrogen) and gel images acquired with an Alphaimager HP system (Protein Simple 3001, San Jose, California, USA). Quantification of the bands was

performed using alpha view v3.4.0.0 software (ProteinSimple) and band intensity was expressed as relative intensity units. For each individual gene, the band intensity was normalised in relation to ubiquitin and then normalised intensity ratios were calculated for DCB-treated/control.

Peroxidase activity assay

To evaluate guaiacol peroxidase activity (CIII-POX; EC 1.11.1.7), the spent medium of suspension-cultured cells was filtered through glass wool and the cells were collected and weighed. All subsequent steps were carried out at 0-4°C. The medium was centrifuged (15 000 *g*, 2 min) and the pellet was discarded. Quantification of CIII-POX activity was performed as described by Ádám et al. (1995), based on an increase in A_{470} due to guaiacol oxidation. The reaction was performed with 3 ml of reaction buffer (100 mM sodium acetate pH 5.5 and 1mM guaiacol), 0.3 ml of 1.3 mM H_2O_2 and 0.05 ml of sample supernatant at 25°C for 2 min. Activity was calculated using the molar extinction coefficient for guaiacol at 470 nm: $\epsilon = 26.6 \text{ mM}^{-1} \text{ cm}^{-1}$.

RESULTS

Effect of cobtorin on maize cell suspension growth

Cobtorin diminished the weight increase of maize cell suspension cultures in a concentration-dependent manner (**Figure 1**). Concentrations equal to or higher than 50 μM completely prevented cell weight increase. The I_{50} value for weight increase was 2.9 μM .

Figure 1. Growth inhibition curve of maize suspension-cultured cells by increasing concentrations of cobtorin after 12 days of culture. Values are means $\pm$ SD of at least three replicates and with a correlation coefficient of 0.95.

Evaluation of ectopic lignin production in cobtorin- or dichlobenil-treated cell cultures

Cell cultures treated with 10 μ M DCB or 6 μ M cobtorin, as well as control cells, were assayed for lignin production using the phloroglucinol-HCl (Wiesner) test throughout the culture cycle. None of the three cell cultures showed phloroglucinol-positive staining after 3 or 6 days of treatment (data not shown). However, DCB-treated but not cobtorin-treated or control cells showed conspicuous pink staining indicative of the presence of lignin when treated for 9 days (**Figure 2**).

Figure 2. Control (A), cobtorin-treated (B) and DCB-treated (C) cells stained with phloroglucinol/HCl. Pink color indicates the presence of lignin.

When DCB-treated cells stained with phloroglucinol were studied under a light field microscope, dispersed accumulations of reddish materials were observed, indicating ectopic lignification (**Figure 3A**). These accumulations correlated positively with intense green fluorescence (**Figure 3C**), but negatively with the presence of cellulose (**Figure 3B**). Control cells or cobtorin-treated cells (data not shown) did not exhibit reddish accumulations indicative of the absence of ectopic lignin (or the concomitant green fluorescence). In DCB-treated cell cultures, cells were usually isodiametric in shape, and some of them were plasmolysed. Dispersed elongating cells were also observed, and these were only occasionally plasmolysed (**Figure 3A-B**).

Figure 3. Phloroglucinol/HCl (A), calcofluor white (B) and autofluorescence under NH_4OH (C) of 10 μM DCB-treated cells at 9 days of treatment. The same cell aggregates were observed with a light field microscopy (phloroglucinol/HCl), or with a fluorescence microscopy under UV light. The arrows indicate the locations of lignin-like accumulations. Bar = 100 μm .

Since ectopic lignin was accumulated after DCB but not after cobtorin treatment, the following experiments were conducted solely with DCB-treated and control cell lines. Moreover, in order to avoid the deleterious effects of high concentrations of DCB, a concentration of 6 μM instead of 10 μM was selected for this research.

Microscopic evaluation of cell walls of DCB-treated cells

Observations of 6 μM DCB-treated and control cells at 3, 6 and 9 days were performed using different microscopic techniques.

Cellulose location

When the suspension-cultured cells were stained with calcofluor and the cell aggregates observed with a fluorescence microscope, fluorescence intensity and consequently the amount of cellulose in control cells was uniform throughout the cell wall and constant throughout the cell culture cycle (**Figures 4-6B**). In contrast, the distribution of fluorescence was not uniform in DCB-treated cells and decreased over time (**Figures 4-6D**). Moreover, 9 days after DCB treatment, a substantial reduction in fluorescence intensity associated with cellulose was observed in growing –elongating– cells when compared with isodiametric cells (**Figure 6D**).

Cell autofluorescence

Phenolics, including those which contain lignin, have the property of emitting a greenish yellow autofluorescence under UV light. In order to correlate autofluorescence emission from cells with the possible presence of lignin, cell aggregates from DCB-treated and control cells were observed under a fluorescence microscope. Control cells barely emitted autofluorescence, and where this emission was observed it was unevenly distributed (**Figures 4-6F**): autofluorescence was not associated with cell walls, but with the protoplasts of plasmolysed cells, which were characterised by their brownish colouration when observed under light field microscopy, suggesting the presence of phenolics. In the case of the DCB-treated cells, autofluorescence emission was scarcely observed 3 days after treatment, and was associated with the protoplasts of plasmolysed cells (**Figure 4H**). Nevertheless, the level of autofluorescence increased considerably after 6 and 9 days of treatment and was linked both to the protoplasts and to the cell wall of certain cells (**Figures 5-6H**).

Control and DCB-treated cells were exposed to ammonium hydroxide to check the emission of bright green fluorescence due to feruloyl esters (Harris and Hartley, 1980). In contrast with the autofluorescence distribution pattern, the fluorescence associated with cell exposure to ammonium hydroxide was in both cases clearly linked to the cell wall, and not to protoplasts. Additionally, it was uniformly emitted by the entire cell wall surface (**Figures 4-6J and 4-6L**).

Scattered increments in fluorescence intensity were observed in the protoplasts of several control cells, and these were associated with brownish accumulations (**Figures 4-6**). Occasionally, this fluorescence was observed also in aggregations of the cell walls in control cells. In DCB-treated cells, the emission of fluorescence associated with the cell wall was more intense at 3 days, but additionally, fluorescence was also located in the protoplasts, exclusively in plasmolysed cells in which brownish accumulations were observed (**Figures 4L-6L**).

Figure 4. Unstained (**A, C, E, G, I and K**), calcofluor white (**B and D**), autofluorescence (**F and H**) and NH_4OH induced fluorescence (**J and L**) of control suspension-cultured cells (**A, B, E, F, I and J**) and 6 μM DCB treated cells (**C, D, G, H, K and L**) at 3 days of treatment. The arrows indicate the locations of lignin-like accumulations. Bar = 100 μm .

Figure 5. Unstained (A, C, E, G, I and K), calcofluor white (B and D), autofluorescence (F and H) and NH_4OH induced fluorescence (J and L) of control suspension-cultured cells (A, B, E, F, I and J) and 6 μM DCB treated cells (C, D, G, H, K and L) at 6 days of treatment. The arrows indicate the locations of lignin-like accumulations. Bar = 100 μm .

Figure 6. Unstained (A, C, E, G, I and K), calcofluor white (B and D), autofluorescence (F and H) and NH_4OH induced fluorescence (J and L) of control suspension-cultured cells (A, B, E, F, I and J) and 6 μM DCB treated cells (C, D, G, H, K and L) at 9 days of treatment. The arrows indicate the locations of lignin-like accumulations. Bar = 100 μm .

Analysis of BAHD gene expression

An analysis was conducted of the expression levels of the 13 BAHD genes described in maize (see Chapter IV): since these codify different acyltransferases, it was expected that at least some of them would be involved in codifying hydroxycinnamoyl transferases involved in cell wall modification, and particularly in ectopic lignification. Expression analyses were conducted on DCB-treated and control cells at 3, 6 and 9 days.

No expression was detected of *ZmBAHD1*, *ZmBAHD3*, *ZmBAHD7*, *ZmBAHD8*, *ZmBAHD10*, *ZmBAHD11* or *ZmBAHD13*. In contrast, an analysis of the bands obtained for *ZmBAHD2*, *ZmBAHD4*, *ZmBAHD5*, *ZmBAHD6*, *ZmBAHD9* and *ZmBAHD12* (Figure 7) showed differences depending on treatment and time in the cell cycle.

Figure 7. Relative expression levels of putative BAHD genes in maize analysed by reverse transcription polymerase chain reaction (RT-PCR) of control and DCB-treated cells at different exposure times (3, 6 and 9 days). The up and down arrows indicate more and less mRNA accumulation than NH cells, respectively.

Expression of *ZmBAHD1*, *ZmBAHD3*, *ZmBAHD7*, *ZmBAHD8*, *ZmBAHD10*, and *ZmBAHD13* were not detected. Primer sequences can be found in Table S1(Chapter IV, p. 175). Numerals indicate the normalised ratios of RT-PCR band

intensities calculated by dividing the band intensity corresponding to DCB-treated cells by that of control cells for each gen.

Expression of *ZmBAHD2* and *ZmBAHD4* genes was observed in 3-day old control cell cultures, whereas in the case of DCB-treated cells, expression of these genes was not observed until 6 days after subculture. The *ZmBAHD2* gene was repressed in DCB-treated cells with respect to control cells at 9 days, while the *ZmBAHD4* gene was repressed at both 6 and 9 days.

Regarding *ZmBAHD5*, *ZmBAHD6*, *ZmBAHD9* and *ZmBAHD12* genes, expression was detected at the three time points studied. *ZmBAHD5* was overexpressed in DCB-treated cells at 9 days, whereas *ZmBAHD6*, *ZmBAHD9* and *ZmBAHD12* were overexpressed at 6 days, although expression was reduced (for *ZmBAHD6*) or not detected (for *ZmBAHD9* and *ZmBAHD12*) at 9 days.

Evaluation of guaiacol-peroxidase activity

Guaiacol peroxidase activity was assayed in the spent medium of both DCB-treated and control cell suspension cultures throughout the cell culture cycle (Figure 8). At the three time points assayed, a significant increase in guaiacol peroxidase activity was detected in the spent medium of DCB-treated cells when compared with controls. The amount of peroxidase activity in the spent medium of DCB-treated cells rose steeply as a function of time, whereas in the spent medium of control cells, maximum activity reached a plateau 6 days after subculture.

Figure 8. CIII-POX activity measured in the spent medium of control (■) and DCB-treated (■) suspension-cultured cells at 3, 6 and 9 days. Data represent means $\pm$ sd of at least three replicates.

DISCUSSION

Cobtorin has been shown to act as a cellulose biosynthesis inhibitor in tobacco BY-2 cells and arabidopsis hypocotyls (Yoneda et al. 2007, 2010). However, the inhibition effect of cobtorin on cells with type II primary cell walls has not previously been reported. The present study has demonstrated that cobtorin inhibits weight increase in maize suspension-cultured cells (composed of type II cell walls) in a concentration-dependent manner, with an I_{50} value of 2.9 μM . The I_{50} value of cobtorin on arabidopsis hypocotyl growth ranged from 0.2 to 0.5 μM , depending on the arabidopsis line and the experiment (Yoneda et al. 2010). In the case of BY-2 tobacco cells, cobtorin caused strong spherical swelling at concentrations equal to or higher than 200 nM (Yoneda et al. 2007), but no I_{50} value has been reported for its effect on tobacco cell growth. Therefore, in line with previous studies, the I_{50} value of cobtorin on maize suspension-cultured cells seems to be somewhat higher than that on cells with type I primary cell walls.

Cobtorin has been included in CBI group 3 because it modifies irregular CESA deposition trajectories at the plasma membrane, in contrast to group 2 CBIs –including DCB– that halt CESA movement in the plasma membrane (Brabham and DeBolt 2013, Tateno et al. 2016). Since arabidopsis seedlings and maize cultured cells exposed to DCB show ectopic lignification (Desprez et al. 2002, Mélida et al. 2015) and this has not been reported to be promoted by cobtorin, one goal of the present study was to determine whether CBIs in different groups, such as DCB and cobtorin, induce ectopic lignification.

When maize cells were exposed to concentrations of cobtorin up to 10 μM , ectopic lignification was not detected. However, DCB induced this response at concentrations of 6 and 10 μM . Although no ectopic lignification associated with exposure to cobtorin was found in maize suspension-cultured cells, the potential induction by cobtorin of ectopic lignification in seedlings should not be ruled out since it is only possible to induce ectopic lignification in cell cultures when cells are exposed to inductive conditions (Kärkönen and Koutaniemi 2010). It is possible that some CBIs could induce lignification when cells are organised into tissues such as roots, but not in cell cultures. Related to this, previous studies have reported

that isoxaben (another group 2 CBI) induces ectopic lignification in arabidopsis seedlings (Desprez et al. 2002, Ellis et al. 2002, Caño-Delgado et al. 2003, Bischoff et al. 2009, Hamann et al. 2009, Denness et al. 2011, van de Vouwewer et al. 2016), but not in arabidopsis cell cultures (Manfield et al. 2004).

In an attempt to study the development of the induced lignification process, maize suspension-cultured cells from different stages of the cell culture cycle were collected. The results of Wiesner staining suggested that lignification was localised in discrete zones of cells that were unable to elongate, as opposed to the staining obtained with calcofluor, and therefore to the presence of cellulose. This pattern of lignification is in contrast to that shown by cells which are differentiating into tracheary elements, and seems to be more related to the pattern observed in stress conditions (Sato et al. 2011, Takeuchi et al. 2013). Although Wiesner staining did not detect the presence of lignin until 9 days (the last phase of the cell culture cycle), autofluorescence under UV light showed a putative lignin accumulation in the cell wall at 6 days, and intense fluorescence induced by ammonium hydroxide was observed as soon as 3 days. These results suggest that lignin induction occurs earlier but requires more time to be detected in the Wiesner test.

When DCB-treated maize suspension-cultured cells were exposed to ammonia hydroxide, a more intense emission of green fluorescence was detected when compared with control cells, and this fluorescence was mainly associated with the cell walls. Fluorescence emission promoted by ammonia has been reported to be a good indicator of cell wall ester-linked hydroxycinnamates (Harris and Hartley 1980). On the basis of these results, cell walls of DCB-treated cells would have higher amounts of *p*-coumaric and/or ferulic acids. These findings are in accordance with those previously reported by our group, in which maize DCB-treated cells showed a more than 130-fold increase in the amount of *p*-coumaric acid and an 8-fold increase in the amount of ferulic acid compared with non-treated cells (Mélida et al. 2015). In grasses, *p*-coumaric acid is preferentially ester-linked to lignin rather than to arabinoxylans, so increased levels of *p*-coumaric acid have been considered a lignification marker in graminaceous cell walls (Chen et al. 2013, [see Chapter II](#)). Therefore, it is probable that the increase in ammonia hydroxide-induced fluorescence observed in DCB-treated cells is

related to an enhancement of *p*-coumaric acid and the induction of lignin. In the case of ferulic acid, this seems to be exclusively ester-linked to arabinoxylans in grass cell walls (Ralph et al. 1995, Grabber et al. 2000, Rao et al. 2017), so the more intense emission of fluorescence induced by ammonia hydroxide in DCB-treated cells could also be related to the reported increment in ferulic acid (Mélida et al. 2015), together with strengthening of the cell wall structure by means of dehydrodiferulates.

Since hydroxycinnamate and lignin-like polymer content is much higher in DCB-treated than in control cells, it was expected that members of the BAHD transferase family genes would be overexpressed in the DCB-treated cells, as these genes seem to be involved in the esterification of *p*-coumaric or ferulic acids to maize cell walls (see discussion in [Chapter IV](#)). As occurred in the previous study, only 6 of the 13 *ZmBAHD* genes assayed were detected in control or DCB-treated cells ([see Chapter IV](#)). Five of these 6 genes (2, 4, 5, 6 and 12) were expressed in both studies, and *ZmBAHD4*, *ZmBAHD5* and *ZmBAHD12* showed a similar pattern of expression in DCB-treated and DCB-habituated cells. In addition, expression of the *ZmBAHD1* gene was only detected in the former study whereas *ZmBADH9* gene was only expressed in the present study.

The lack of expression of *ZmBAHD1* in the present study might seem surprising, since this gene has been considered a putative encoding hydroxycinnamoyl transferase, as proposed in [Chapter IV](#). However, it is important to bear in mind that the level of expression of this gene in control cells in the previous study was extremely low in comparison to the expression of the ubiquitin gene or the other *ZmBAHD* genes. Therefore, the main difference between the two studies was the enormously high level of expression of this gene (26-fold) in DCB-habituated cells. In this respect, it is important to note that DCB-treated and DCB-habituated cells did not show the same responses regarding lignification or hydroxycinnamoyl enhancement (Mélida et al. 2015). Therefore, *ZmBAHD1* expression may be crucial in DCB-habituated but not in DCB-treated cells.

Regarding the *ZmBAHD2* gene, the other putative hydroxycinnamoyl transferase encoding gene as proposed in [Chapter IV](#), the expression pattern for DCB-treated cells contrasted with that obtained for DCB-habituated cells. In the former,

expression of the *ZmBAHD2* gene was constant or repressed, whereas it was clearly overexpressed in DCB-habituated cells. These differences are probably linked to the abovementioned differences in lignin-like composition and/or the different hydroxycinnamate composition in the two kinds of cell (Mélida et al. 2009, 2015).

An interesting result obtained in this study was *ZmBAHD9* gene expression, which was overexpressed after 6 days of DCB treatment (whereas it was not detected in the previous study, Chapter IV). As this gene has been classified into the same cluster as *ZmBAHD6* and *ZmBAHD12* genes (cluster III) (see Chapter IV) and has been reported to present low levels of expression in different organs of maize plants (Sekhon et al. 2011), further research is necessary to determine whether its overexpression is related to changes in the phenolic component of maize cell walls.

Lignin polymerisation requires the activation of monolignols to free radicals (Fagerstedt et al. 2010), a process catalysed by peroxidases and H_2O_2 , and/or laccases and O_2 . As a considerable increment in H_2O_2 has previously been detected in the spent medium of maize cells treated with DCB (Mélida et al. 2015, Largo-Gosens et al. 2016), a study was conducted to determine whether H_2O_2 overproduction is paralleled by enhanced peroxidase activity in the spent medium of these cells. A significant increase –at least double– in peroxidase activity throughout the cell cycle was detected in DCB-treated compared to control cells. This result is in accordance with the reported enhanced peroxidase activity corresponding to cells treated with 0.5 μM or 1 μM DCB compared with non-treated cells (Largo-Gosens et al. 2016) and with the increase in peroxidase activity throughout the cell cycle reported in the spent medium of *Zinnia* cell cultures under conditions that induce stress-related lignification (Sato et al. 2011, Takeuchi et al. 2013).

Enhanced peroxidase activity in DCB-treated cells is probably a coping response to oxidative stress, as lipid peroxidation has been reported to be higher in DCB-treated than in control cells (Largo-Gosens et al. 2016). Therefore, the increment in peroxidase activity should be regarded as a common response to different stress factors, and may be rather unspecific (Passardi et al. 2005, see Chapter III).

In conclusion, the present results indicate that (i) ectopic lignification is not induced by all CBIs, as DCB but not cobtorin induced cell wall lignification; (ii) lignification increases progressively throughout the cell culture cycle; and (iii) the process seems to be related to the overexpression of a set of *ZmBAHD* genes, mainly *ZmBADH5*, *ZmBADH6*, *ZmBADH9* and *ZmBADH12*; (iv) together with enhanced class-III peroxidase activity. Therefore, the induction of ectopic lignification in response to a reduction in cellulose content seems to be a spatio-temporal process in which reinforcement of the weakened cell wall is achieved through structural modifications and changes in the expression profile of selected genes and the activity of certain enzymes such as peroxidases.

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The products obtained from plant cell walls such as fibres, wood, paper, fuel, medicines, foods and other resources have been widely used by human societies (Johnson et al. 2018). In recent decades, the increased use of plant cell wall-derived products has prompted the need to develop new strategies to convert wall polysaccharides from crop plant residues into renewable transport biofuels and to exploit the dietary benefits of wall polysaccharides for human health. To meet this need, recent technological advances have been aimed at elucidating how cell walls modulate plant growth and material properties. It is necessary to identify and characterise the genes and proteins involved in synthesising and assembling cell wall components in order to be able to build walls for specific purposes without compromising the plant's ability to grow normally and respond to biotic and abiotic stresses. Thus, the different cell wall components can be considered potential biotechnological targets on which to act in order to design walls suitable for a given biotechnological purpose.

Owing to the key role of cellulose in the cell wall structure, cellulose biosynthesis inhibitors (CBIs) such as dichlobenil (DCB) have been widely used as a valuable tool for analysing cell wall structure and biogenesis, due to their capacity to provoke changes in cell wall structure and composition through their inhibitory effect on cellulose biosynthesis (Sabba and Vaughn, 1999, Acebes et al. 2010, Álvarez et al. 2012, García-Angulo et al. 2012, Tateno et al. 2016). In the current experimental system, one of the three independent but interconnected structural networks that comprise the cell wall, the cellulose-hemicellulose network, was modified, since maize suspension-cultured cells habituated to grow in the presence of DCB show a reduction in their cellulose content. Modification of this polymeric network affects the composition and structure of the others, in which phenolic components are involved, underlining the fact that the cell wall is a highly dynamic compartment, the composition and structure of which can be modified in different ways.

Previous studies on maize cells habituated to high DCB levels (12 μ M) have described compensatory strategies based on a reinforced hemicellulosic network acquired by developing more cross-linked arabinoxylans (AXs) with a higher M_w and lower extractability (Mélida et al. 2009, 2011). These results are in contrast to those reported in maize suspension-cultured cells habituated to low DCB levels

(1.5 μ M). A pulse-chase radiolabeling experimental approach was employed to elucidate cinnamic acid metabolism and characterise its derivatives in protoplasmic and cell wall fractions. Changes were observed in phenolic metabolism in response to early DCB habituation: first, in comparison with non-habituated (NH) cells, habituated (H) cells presented a deficient capacity for the biosynthesis and incorporation into the cell wall of feruloyl(coumaroyl)-AXs. Second, the relative content in FA dehydrodimers and oligomers was higher in H cells, supporting the idea that a greater cross-linked network of hemicelluloses, mainly AXs, is crucial for the cell wall reinforcement strategy during early habituation to DCB, as previously demonstrated in long-term DCB-habituated maize cells (Mélida et al. 2010b, 2011). In light of these results, it can be concluded that the lower M_w of hemicelluloses and polymers detected in H cells was not related to a lower degree of cross-linkage through FA (**Chapter I**; de Castro et al. 2017).

Given the abovementioned results, a qualitative and quantitative study was conducted of *p*-coumaric acid (CA), ferulic acid (FA) and FA dehydrodimers (DFAs) distributed in the protoplasmic and cell wall fractions in order to shed light on the contribution of cell wall phenolics to early remodelling of cellulose deficient cell walls. Only slightly higher levels of CA and FA were detected in the protoplasmic fraction of H cells when compared to NH ones. However, H cells showed much higher levels of cell wall esterified FA and CA. In relation to a comparison of DFAs in protoplasm and cell wall fractions, 8,5'- and 5,5'-DFAs were present in the protoplasm of H cells, whereas NH protoplasm only contained 8,5'-DFA, indicating that 5,5'-DFA is the predominant dehydrodimer in the protoplasmic fraction when an extra cross-linking occurs during DCB habituation. As regards the cell wall fraction, an extra dimerisation was detected, since higher levels of FA dimers were observed in this compartment. In addition, it has been demonstrated that the 8-O-4' dehydrodimer is unique in DCB-habituated cells. In summary, cellulose impoverishment in maize suspension-cultured cells is already being compensated in the early habituation stage through the development of a more extensive network of hydroxycinnamic cross-linked AXs (**Chapter II**).

As DCB habituation of maize suspension-cultured cells is characterised by significant changes in feruloylated AXs (**Chapters I and II**) and synthesis of lignin-

like polymers mainly composed of syringyl (S) units (Mélida et al. 2009, 2010a, 2010b, 2015, de Castro et al. 2014, 2015, 2017), and given that both cell wall components are susceptible to peroxidase (POX) action, further insight was sought into the relationship between POX and cell wall remodelling. For the first time, a comparative study was conducted of POX activity and isoforms in NH and H cells, taking into account their cellular distribution in different compartments throughout the culture cycle. Proteins from the spent medium (SM), soluble cellular (SC), ionically (ICW) and covalently bound cell wall (CCW) fractions were assayed for total and specific peroxidase activity using coniferyl and sinapyl alcohol and FA as substrates, and the isoPOX profile was obtained by isoelectric focusing. First, POX activity was higher in H than in NH cells in all protein fractions at all cell culture stages. For all substrates assayed, SC and ICW fractions showed higher activity in the early than in the late log growth phase. However, the highest POX activity in the spent medium was found in the late-log phase. Second, the highest diversity of isoPOX profiles was detected in the ICW and SM protein fractions. The latter fraction contained isoPOXs with higher activity in H cells. These results suggest that some of the isoPOXs detected could be involved in AX cross-linking and lignin-like polymer formation in H cells, and therefore, in the remodelling of this complex structure in response to disruption of cell wall integrity (**Chapter III**; Martínez-Rubio et al. 2018).

In order to further elucidate the AX feruloylation/cumaroylation process in the present experimental system, a preliminary study was conducted of the genes putatively involved in acylation of these AXs. PF02458 CoA-acyltransferase (BAHD) genes in maize homologous to those previously found in rice and *Arabidopsis* (Piston et al. 2010; Bartley et al. 2013, de O Buanafina et al. 2016) were identified. In addition, their expression profile in NH and H maize suspension-cultured cells was established throughout the culture cycle by means of semi-quantitative RT-PCR. The results of gene homology studies suggest that *ZmBAHD4*, *ZmBAHD5*, *ZmBAHD6* and *ZmBAHD12* genes could function as feruloyl transferases, since they are homologues of the *OsAT7* and *BdAT1* genes, whose misregulation has an impact on the level of feruloylation in rice and induces an altered level of cell wall feruloylation in *Brachypodium*, respectively (Piston et al. 2010, de O Buanafina et al. 2016). Following the gene homology study, it has been demonstrated that *ZmBAHD1* and

ZmBAHD2 genes show a close relation to the *OsAt10* gene described by Bartley et al. (2013). Furthermore, the expression patterns of *ZmBAHD1* and *ZmBAHD2* genes in H cells, together with the fact that H cell walls were notably enriched in CA in comparison with NH ones (**Chapter III**), indicate that both genes could act as BAHD for cell wall coumaroylation (**Chapter IV**).

In summary, habituation of suspension-cultured cells to low levels of DCB has enabled a study of changes in phenolic metabolism in order to shed light on the strategies deployed to cope with cell wall cellulose deficiency. First, it has been demonstrated that H cells show differences in phenolic metabolism, presenting a relatively higher content of FA dehydrodimers, which supports the idea that greater cross-linking of cell wall AXs is essential for cell wall remodelling as a reinforcing strategy in early habituation to DCB. In this respect, higher levels of cell wall esterified CA and FA as well as an extra FA dimerisation were detected in comparison to the protoplasmic fraction in response to DCB habituation, so while FA seems to be more involved in AX cross-linking, CA may play a key role in priming lignin deposition (Santiago and Malvar 2010; Mélida et al. 2015; Hatfield et al. 2017). Additionally, it has been shown that 5,5'- and 8-O-4' dehydrodimers are unique in DCB-habituated cells, demonstrating that these cells compensate cellulose impoverishment by developing a more extensive network of dehydrodimer cross-linked AXs, a process in which POXs are involved. POX activity and diversity was higher in SC and ICW fractions of H than NH cells at all cell culture stages, indicating that some of these isoPOXs could be related to AX cross-linking and lignin-like polymer synthesis in H cells. Finally, a preliminary study of the genes putatively involved in acylation of these AXs revealed that *ZmBAHD1*, *ZmBAHD2* and *ZmBAHD12* genes are good candidates as AX feruloyl/coumaroyl transferases in primary cell walls (**Figure 1**).

Figure 1. Structural models of the type II primary cell wall of non-habituated (**A, B**) and DCB-habituated (**C, D**) cell lines of maize suspension-cultured cells at their early-logarithmic (**A, C**) and late-logarithmic (**B, D**) growth phases. The model shows the molecular interactions between cellulose, arabinoxylans and hydroxycinnamic acids (phenolic compounds). An extra dimerization and lignin synthesis, as result of increased POX and BAHD activities (**E, F**) is taking place in DCB-habituated cell lines. Based on Gómez and McQueen-Mason, 2018.

As a general conclusion, the results obtained in the present study have furthered our knowledge of how hydroxycinnamates, peroxidases and BAHD proteins participate in remodelling cellulose-impooverished cell walls, revealing a dynamic interaction between the three main components that constitute the lignocellulosic material in maize: cellulose, hemicellulose and lignin. These results provide useful information about the impact of modifying one cell wall component on the others. Future research is required in order to characterise the POX involved in cell wall remodelling and clarify the role that BAHD proteins play in the same process, affecting cell wall structure and digestibility. Thus, future research should be aimed at determining how to modify different cell wall targets in order to design cell walls suitable for a given biotechnological purpose.

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Conclusions

In light of the results obtained in this thesis, it can be concluded that:

1. Maize suspension-cultured cells habituated to grow in the presence of low concentrations of DCB (1.5 μ M) showed important changes in their phenolic metabolism profile throughout the culture cycle. Although no marked differences were detected in the capacity to uptake [14 C]cinnamic acid from the culture medium, habituated cells presented a deficient capacity for the biosynthesis and incorporation into the cell wall of [14 C]feruloylated molecules. However, despite this reduced capacity, the DCB-habituated cells showed a higher relative content of ferulate dimers and oligomers, supporting the hypothesis that a more extensive network of cross-linked arabinoxylans is crucial for the cell wall reinforcing strategy in cellulose-deficient cell walls.

2. A differential pattern of phenolic metabolism was observed in non-habituated cells and cells habituated to 1.5 μ M DCB both in protoplasm and cell wall compartment. Slightly higher levels of *p*-coumaric acid and ferulic acid were detected in the protoplasmic fraction of habituated cells in comparison to non-habituated ones. However, a significant increase was detected in *p*-coumaric acid and ferulic acid levels incorporated into habituated cell walls as the culture cycle progressed, indicating that an extra feruloylation/coumaroylation occurs *in muro* in DCB-habituated cells in response to a reduction in cellulose content. DCB-habituated cell walls showed an increase in ferulate coupling products, mainly 8,5' and 5,5' diferulates. Furthermore, 8-O-4'-diferulate emerged as a distinctive cell wall diferulate in the DCB habituation process.

3. A comparative study of peroxidase activity and peroxidase isoforms in DCB-habituated cells revealed strong peroxidase activity against ferulic acid and coniferyl and sinapyl alcohols in both protoplasmic and apoplastic fractions. A differential isoperoxidase profile was found in the ionically-bound cell wall fraction and the spent medium protein fraction of the DCB-habituated cells. Together, these results support the idea that peroxidases play a prominent role in maize cell wall remodelling in response to a reduction in cellulose content.

4. Thirteen maize genes homologues to previously reported PF02458 family genes were identified by using a bioinformatics approach. All the protein sequences shared the transferase domain HXXXDG characteristic of BAHD proteins involved in the acylation of arabinoxylans. Six of the identified genes were expressed in both non-habituated and DCB-habituated maize cell cultures. The expression pattern of three of them (*ZmBAHD1*, *ZmBAHD2* and *ZmBAHD12*) together with their coexpression profile with other cell wall related genes suggest that they are good candidates for cell wall feruloylation/coumaroylation in maize cultured cells with a reduced cellulose content.

5. Maize suspension-cultured cells treated with 10 μ M cobtorin did not show ectopic lignification, whereas those exposed to 6 or 10 μ M DCB deposited a lignin-like polymer which stained positive for phloroglucinol, related to a progressive accumulation of phenolics in cell walls. Moreover, DBC treatment was associated to the overexpression of *ZmBAHD5*, *ZmBAHD6*, *ZmBAHD9* and *ZmBAHD12* genes and the enhancement of class-III peroxidase activity. Therefore, the accumulation of ectopic lignin induced by a DCB-promoted reduction in the cellulose content seems to be a spatio-temporal process mediated by the expression of several BAHD genes and the enhancement of peroxidase activity that contributes to reinforce the weakened cell wall entanglement in response to a reduction in the cellulose content.

Phenolic metabolism and molecular mass distribution of polysaccharides in cellulose-deficient maize cells^{FA}

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Abstract As a consequence of the habituation to low levels of dichlobenil (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. The aim of this study was to investigate the relative molecular mass distributions of polysaccharides as well as phenolic metabolism in cells habituated to low levels of DCB (1.5 μ M). Generally, cell wall bound hemicelluloses and sloughed polymers from habituated cells were more homogeneously sized and had a lower weight-average relative molecular mass. In addition, polysaccharides underwent massive

cross-linking after being secreted into the cell wall, but this cross-linking was less pronounced in habituated cells than in non-habituated ones. However, when relativized, ferulic acid and *p*-coumaric acid contents were higher in this habituated cell line. Feasibly, cells habituated to low levels of DCB synthesized molecules with a lower weight-average relative molecular mass, although cross-linked, as a part of their strategy to compensate for the lack of cellulose.

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INTRODUCTION

Heteroxylans are the main non-cellulosic polysaccharides in the Poales primary (type II) cell wall (Fincher 2009; Peña et al. 2016), 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). These non-cellulosic polysaccharides are predicted to be synthesized in Golgi apparatus by several glycosyltransferases whose genes have been recently identified in *Arabidopsis thaliana* by using genomic approaches (Rennie and Scheller 2014 and refs. therein). Specifically in the case

of maize, sequences have also been reported for some *Arabidopsis* orthologues such as *IRX9*, *IRX10* and *IRX10-L* (Bosch et al. 2011), and recently maize genes orthologous to *AtIRX10*, *AtIRX9* and *AtIRX14* has been shown to be highly expressed in the pericarp (Chateigner-Boutin et al. 2016).

One of the unique structural features of arabinoxylan in Poales is that it contains hydroxycinnamate residues, such as ferulic acid or *p*-coumaric acid, esterified on the C-5 position of arabinose residues (Bacic et al. 1988; Wende and Fry 1997). The attachment of ferulic acid residues occurs within the protoplasm, before secretion into the cell wall (Fry et al. 2000). Ferulic acid may undergo oxidative coupling when exposed to hydrogen peroxide plus peroxidase (Geissmann and Neukom 1971). Dimerization 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 it has 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; Oliveira et al. 2015).

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 (Oliveira et al. 2015). 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 2,6-dichlorobenzonitrile (dichlobenil, 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.

Dichlobenil 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 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. Likewise, in high DCB levels (12 μM) a 75% of reduction is observed (Mélida et al. 2009) whereas in low DCB levels (1.5 μM) such reduction is more moderate, being of 33% (de Castro et al. 2014) when compared to non-habituated maize cells (Snh). 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).

Cell suspension cultures present several advantages in *in vivo* metabolic studies performed using isotopes, such as homogeneity of cell wall material, uniform culture medium without nutrient gradients, access to O_2 or exogenous precursors, and ease of sampling (Burr and Fry 2009). This kind of experimental approach is particularly useful as the substrates are endogenous and therefore the processes observed are those that naturally take place (Thompson et al. 1997; Kerr and Fry 2003, 2004; Lindsay and Fry 2008; Mélida et al. 2011). In a previous study, maize suspension-cultured cells habituated to low levels of DCB (1.5 μM , Sh1.5) were fed with [^3H]arabinose (de Castro et al. 2015). 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 ^3H -hemicelluloses and an increased presence of soluble extracellular [^3H]-polymers ([^3H]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 weight-average relative molecular mass (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 relative molecular mass (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-labeling experimental approach was used, which consisted of feeding both cell lines with the corresponding radio-labeled metabolic precursors ([^3H] arabinose and [^{14}C]cinnamic acid respectively). The results obtained in this work 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.

RESULTS

M_w and M_r distribution of ³H-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 (Figure 1). In addition, the proportion of ³H-polymers and ³H-hemicelluloses that were eluted in the V₀ was lower in Sh1.5 cells. ³H-polysaccharides from Sh1.5 cells had a lower M_w than those of Snh in all cell compartments and culture phases analysed, with the sole exception of those observed within the protoplasm at late logarithmic phase (Figure 1).

Protoplasmic ³H-polymers consisted of a highly poly-disperse population of molecules, which in both cell lines showed a lower M_w when compared with the cell wall bound ³H-hemicelluloses or [³H]SEPs. This result indicates that they underwent massive cross-linking once incorporated into the cell wall or sloughed into the medium. In line with this, when both cell lines were compared, Snh cells showed a higher M_w and therefore a much more pronounced degree of crosslinking than Sh1.5 cells, independently of the culture phase considered (Figure 1).

The general elution profiles of [³H]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 [³H]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 1). In addition, [³H]SEPs from Sh1.5 cells had a lower M_w than Snh ones in both culture phases.

Both types of ³H-hemicelluloses (0.1 M or 6 M NaOH-extracted) showed a lower M_w in Sh1.5 cells (Figure 1). In Snh cells, the M_w of 0.1 M NaOH-extracted wall bound ³H-hemicelluloses did not vary according to the culture phase whereas it appeared to decrease at the late logarithmic phase in Sh1.5 cells (Figure 2). Likewise, the general elution profile for this type of ³H-hemicellulose was very similar in the case of Snh cells in both culture phases, whereas Sh1.5 cells showed a

slightly greater proportion of ³H-molecules eluting in a higher Kav at the late logarithmic phase (Figure 1). In the case of 6 M NaOH-extracted ³H-hemicelluloses, their M_w barely increased in Snh cells, whereas in Sh1.5 cells it once again decreased. These findings were repeated in the general elution profiles, where an increase in the proportion of ³H detected at a lower Kav was observed at the late logarithmic phase in Snh cells, whereas it decreased in Sh1.5 cells (Figure 1).

M_r distribution of ³H-labelled diagnostic fragments from ³H-polysaccharides

After Driselase digestion, the elution profiles of polymers containing [³H]arabinose, [³H]xylose or [³H]xylobiose were similar to the general ones, indicating the solidity of the experimental approach (Figures 2–4 vs Figure 1). In general terms, elution profiles of polymers containing [³H]arabinose, [³H]xylose or [³H]xylobiose showed similar M_r distributions. This finding suggests that these diagnostic fragments were a digestion product of a unique type of polysaccharide, putatively [³H]arabinoxylan. Reinforcing this idea was the disparity observed between these, and fragments from polymers which contained [³H]isoprimeverose, a diagnostic fragment for [³H]xyloglucan. Undigested ³H-polymers were found in all digested fractions, most of them eluting in a Kav ranging from 0 to 0.5 (Figures 2–4).

M_r distribution of 0.1 M NaOH-extracted hemicelluloses diagnostic fragments

The elution profiles of polymers containing [³H]arabinose residues and those characteristic for [³H]xylans ([³H]xylose and [³H]xylobiose) were very similar in both cell lines and culture stages. However, the elution profiles of [³H]isoprimeverose (diagnostic for [³H]xyloglucan) differed from those characteristic of [³H]xylans and [³H]arabinose-containing polymers (Figure 2). M_r distributions of [³H]arabinose-containing polymers and ³H-labelled diagnostic fragments for xylans in both culture phases, and those for undigested ³H-polymers at the early logarithmic phase, were very similar to the general elution profile shown in Figure 1.

M_r distributions of [³H]arabinose residues, [³H]xylose and [³H]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 [³H]isoprimeverose for [³H]xyloglucan showed a slightly greater proportion of

Figure 1. Mr distribution profile of ^3H -polysaccharides: protoplasmic ^3H -polymers, cell wall bound ^3H -hemicelluloses extracted with 0.1 M or 6 M NaOH and soluble extracellular ^3H -polymers (SEPs) obtained from non-habituated (Snh, purple line) and habituated to 1.5 μM DCB (Sh1.5, green line) maize cell cultures Data shown are from the early logarithmic (A) and late logarithmic (B) phase of culture cycle. Samples used were taken 300 min after feeding of [^3H]arabinose, except in the case of protoplasmic ^3H -polymers, collected 30 and 60 min after feeding of [^3H]arabinose for Snh and Sh1.5 respectively. ^3H -polymers and ^3H -hemicelluloses were

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 1](#).

M_r distribution of 6 M NaOH-extracted hemicelluloses diagnostic fragments

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

Most of the polymers which contained [^3H]arabinose, [^3H]xylose and [^3H]xylobiose as well as [^3H]isoprimeverose and undigested ^3H -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 ^3H detected at the Vo was low in both cell lines and culture phases ([Figure 3](#)). Nevertheless, a remarkable difference was found in the elution profile of [^3H]xyloglucan and undigested ^3H -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. This elution peak was not appreciable at late logarithmic phase of culture.

M_r distribution of SEPs

In both cell lines, the elution profiles of [^3H]arabinose, [^3H]xylose or [^3H]xylobiose-containing polymers were very similar ([Figure 4](#) vs [Figure 1](#)), suggesting that [^3H]SEPs are mainly constituted by arabinoxylans sloughed into the culture medium. A greater proportion of ^3H -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 4](#)).

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 Vo ([Figure 4A](#)). However, M_r distribution of the undigested ^3H -polymers and the polymers from which [^3H]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 4B](#)). The elution profiles of [^3H]xylans, ([^3H]xylose and [^3H]xylobiose diagnostic fragments) were similar, and molecules mainly eluted in a Kav ranging from 0.1 to 0.4. Similarly, the elution profiles of [^3H]xyloglucan ([^3H]isoprimeverose) and undigested ^3H -polymers were alike. Surprisingly, the elution profile of ^3H -polymers which contained [^3H]arabinose residues was for the first time different to those for [^3H]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 [^3H]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 ^3H -molecule population containing [^3H]arabinose residues. This was not observed in the Sh1.5 cells, in which the elution profile of polymers containing [^3H]arabinose residues was very similar to those for [^3H]xylans.

In Sh1.5 cells, the digestion of [^3H]SEPs with Driselase showed that all the ^3H -polymers (those which contained [^3H]arabinose, those with diagnostic fragments for [^3H]xylans and [^3H]xyloglucan and the undigested ^3H -polymers) eluted in a higher Kav than those from Snh cells, and therefore had a lower M_w ([Figure 4](#)). This was especially clear at the early logarithmic phase ([Figure 4A](#)). The most remarkable difference was found in the elution profile of [^3H]xyloglucan and

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. Mw values, derived from the median Kav(1/2) of the profiles and then converted to Mw via a calibration curve, are included in the text frame.

Figure 2. Mr distribution profile of ^3H -labelled diagnostic fragments from ^3H -polysaccharides: Cell wall bound ^3H -hemicelluloses extracted with 0.1M NaOH obtained from non-habituated (purple line) and habituated to 1.5 μM DCB (green line) maize cell cultures

Data shown are from the early logarithmic (**A**) and late logarithmic (**B**) phase of culture cycle. Samples used were taken 300 min after feeding of [^3H]arabinose. Digest of each fraction were assayed for radioactive fragments characteristic of polymers which have arabinose residues ([^3H]arabinose), xylans ([^3H]xylose) and ([^3H]xylobiose), xyloglucans ([^3H]isoprimeverose) and polymers that Driselase does not digest (undigested ^3H -polymers). Other details as in Figure 1.

Figure 3. Mr distribution profile of cell wall bound ^3H -hemicelluloses extracted with 6 M NaOH obtained from non-habituated (purple line) and habituated to 1.5 μM DCB (green line) maize cell cultures

Data shown are from the early logarithmic (A) and late logarithmic (B) phase of culture cycle. Samples used were taken 300 min after feeding of [^3H]arabinose. Digest of each fraction were assayed for radioactive fragments characteristic of polymers which have arabinose residues ([^3H]arabinose), xylans ([^3H]xylose) and ([^3H]xylobiose), xyloglucans ([^3H]isoprimeverose) and polymers that Driselase does not digest (undigested ^3H -polymers). Other details as in Figure 1.

Figure 4. Mr distribution profile of soluble extracellular ^3H -polymers obtained from the culture medium of non-habituated (purple line) and habituated to $1.5\ \mu\text{M}$ DCB (green line) maize cell cultures

Data shown are from the early logarithmic (A) and late logarithmic (B) phase of culture cycle. Samples used were taken 300 min after feeding of $[^3\text{H}]$ arabinose. Digest of each fraction were assayed for radioactive fragments characteristic of polymers which have arabinose residues ($[^3\text{H}]$ arabinose), xylans ($[^3\text{H}]$ xylose) and ($[^3\text{H}]$ xylobiose), xyloglucans ($[^3\text{H}]$ isoprimeverose) and polymers that Driselase does not digest (undigested ^3H -polymers). Other details as in Figure 1.

Figure 5. Kinetics of consumption and incorporation of radioactivity from exogenous [^{14}C]cinnamic acid in non-habituated (purple line) and habituated to $1.5\ \mu\text{M}$ DCB (green line) maize cell suspensions

Data from the early logarithmic (A, C) and late logarithmic (B, D) phase of culture cycle are shown. The [^{14}C]cinnamic acid consumption (▼) was calculated from the radioactivity remaining in the culture medium fraction after [^{14}C]cinnamic feeding. The incorporation of ^{14}C -labelled compounds in the protoplasmic fraction (●) and Alcohol Insoluble Residue (cell wall fragments) (○) is represented. The values shown are the average $\pm$ standard deviation ($n = 3$).

undigested ^3H -polymers at late logarithmic culture phase (Figure 4B), where a marked peak was observed close to a K_{av} of 0.3, similar to that previously described for the elution profile of 6 M NaOH-extracted [^3H]xyloglucan at early logarithmic phase.

Analysis of phenolic metabolism

Uptake of [^{14}C]cinnamic acid from the culture medium and its incorporation into cell compartments

No differences in [^{14}C]cinnamic acid consumption were observed among maize cell lines or culture phases (Figure 5). In general, consumption percentages of [^{14}C]cinnamic acid ranged between 40%–55% of the total [^{14}C]cinnamic acid added at time zero. Most of the [^{14}C]cinnamic acid consumption (90%)

occurred during the first 15 min after [^{14}C]cinnamic acid was fed.

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

[^{14}C]cinnamic acid metabolism on the protoplasmic fraction

Hydroxycinnamate ^{14}C -labelled metabolites incorporated into the protoplasmic fraction of Snh and Sh1.5

Figure 6. Kinetics of incorporation of ^{14}C -labelled compounds (esterified and free-phenolics) into the protoplasmic polymer fraction after the addition of exogenous ^{14}C -cinnamic acid to non-habituated (purple line) and habituated to $1.5\ \mu\text{M}$ of DCB (green line) maize cell suspensions at the early logarithmic (A) and late logarithmic (B) 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. Dotted line indicates the addition of H_2O_2 (40 mmol/L final concentration).

cells in their early logarithmic phase (Figures 6A, S1), were tracked during a 180 min pulse of ^{14}C -cinnamic acid. In both cell lines, ^{14}C -cinnamic acid was quickly metabolized within the first 5 min after its addition to the culture medium. As expected, ^{14}C -cinnamic acid was rapidly converted to p - ^{14}C -coumaric acid and then

to ^{14}C -ferulic acid. Both p - ^{14}C -coumaric and ^{14}C -ferulic acid, were only transiently accumulated in the protoplasm of Snh cells as their pools largely disappear after 180 min. However, protoplasmic fraction from Sh1.5 cells was enriched in p - ^{14}C -coumaric and ^{14}C -ferulic acid when compared with Snh cells. Rfo and low-Rf

material ($[^{14}\text{C}]$ dimers and oligomers) was rapidly detected (5 min) after $[^{14}\text{C}]$ cinnamic acid feeding and gradually accumulated at the same extent in Snh and Sh1.5 cells. In both cell lines, the addition of H_2O_2 increased the extent of dimerization or oligomerization.

Kinetics of $[^{14}\text{C}]$ cinnamic metabolism did not substantially change when maize suspension cultured-cells in their late logarithmic phase were assayed (Figures 6B, S2). As major differences, a delay in the metabolism of $[^{14}\text{C}]$ cinnamic acid by Sh1.5 cells, and a higher accumulation of Rfo and low-Rf material in Snh cells can be pointed out. Finally, the addition of H_2O_2 during this culture phase did not related with an increase of dimerization or oligomerization.

$[^{14}\text{C}]$ cinnamic acid metabolism in the cell wall

An analysis of hydroxycinnamate ^{14}C -labelled metabolites incorporated into the cell wall during early-logarithmic growth phase is shown in Figures 7A and S3. The $[^{14}\text{C}]$ cinnamic acid added to the culture medium was rapidly (1–3 min) metabolized to p - $[^{14}\text{C}]$ coumaric acid and then incorporated into the cell wall. In both cell lines, a decrease was observed in the relative incorporation of p - $[^{14}\text{C}]$ coumaric acid into the cell wall the first 30 min after $[^{14}\text{C}]$ cinnamic acid feeding. Concomitantly the $[^{14}\text{C}]$ ferulic acid esterified into the cell wall, abruptly increasing until reaching a plateau phase. Furthermore, at 30 to 60 min after $[^{14}\text{C}]$ cinnamic acid feeding a decrease in the amount of cell wall-esterified $[^{14}\text{C}]$ ferulic acid was detected in Snh cells.

In all cases, $[^{14}\text{C}]$ ferulic acid was the main cell wall hydroxycinnamate detected. The relative quantification of $[^{14}\text{C}]$ cinnamic acid $[^{14}\text{C}]$ derivatives indicated that Sh1.5 cell walls were enriched in p - $[^{14}\text{C}]$ coumaric acid and impoverished in $[^{14}\text{C}]$ ferulic acid in comparison with Snh cell walls (Figure 7). ^{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 $[^{14}\text{C}]$ cinnamic acid 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 $[^{14}\text{C}]$ ferulic acid. In addition, their relative quantification indicated that $[^{14}\text{C}]$ ferulic acid, ^{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 $[^{14}\text{C}]$ cinnamic acid feeding induced an increase in the

dimerization or oligomerization of $[^{14}\text{C}]$ ferulate, occurring to a similar degree in both cell lines (Figure 7).

No differences were found in the kinetics of Snh cell wall p -coumaroylation and feruloylation between growth phases (Figures 7A, S3 vs 7B, S4). However, some differences were observed in the case of habituated cells: Sh1.5 cell walls accumulated more p - $[^{14}\text{C}]$ coumaric acid, ^{14}C -dimers and ^{14}C -oligomers at the late logarithmic growth phase (Figures 7B, S4). Accordingly, the relative decrease in $[^{14}\text{C}]$ ferulic acid amount was more pronounced in the late logarithmic than in the early logarithmic growth phase. Lastly, the addition of H_2O_2 did not increase the level of $[^{14}\text{C}]$ ferulate dimerization or oligomerization.

***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 analyzed by RT-PCR, shown in Figure S5. 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.

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. 2014). Likewise, previous studies on maize cells habituated to high DCB levels (12 μM , Sh12) 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 $[^3\text{H}]$ arabinose revealed a lower proportion of strongly wall-bound ^3H -hemicelluloses and an increase in $[^3\text{H}]$ SEPs, as well as a slower and less efficient metabolism of

Figure 7. Kinetics of incorporation of ^{14}C -labelled compounds esterified of alcohol-insoluble residue (cell wall fragments) after the addition of exogenous ^{14}C -cinnamic acid to non-habituated (purple line) and habituated to $1.5\ \mu\text{M}$ of DCB (green line) maize cell suspensions at the early logarithmic (A) and late logarithmic (B) 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. Dotted line indicated the addition of H_2O_2 (40 mmol/L final concentration).

polysaccharides throughout the entire culture cycle (de Castro et al. 2015).

This finding was especially surprising, and two possible explanations arise to explain this altered cellular fate: it may be caused by (i) a reduction in “binding points” to cellulose; and/or (ii) a reduced capacity to incorporate 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-labeled precursors (^3H arabinose and ^{14}C cinnamic acid, respectively), since the use of this methodology has been successful in comparable experimental studies (Fry et al. 2000; Kerr and Fry 2003; Burr and Fry 2009; Mérida et al. 2011; de Castro et al. 2015).

The first question that emerged 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 ^3H -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 [^3H]xyloglucan, [^3H]xylans, polymers which containing [^3H]arabinose and those undigested by Driselase) and were perfectly illustrated in the case of [^3H]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 [^3H]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_2O_2 and inhibitors of cross-linking (Encina and Fry 2005), also diffuse into the extracellular medium (Burr and Fry 2009). ^3H -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 ^3H -polymers and cell wall bound ^3H -hemicelluloses indicated that after secretion into the cell wall, protoplasmic ^3H -polymers underwent massive cross-linking in both cell lines, although this increase was much less pronounced in Sh1.5 cells. These results point to a reduction in the cell wall grafting ability of Sh1.5 cells.

A possible explanation for these findings is that cells habituated to low DCB levels synthesized ^3H -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 Shh). 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 synthesized 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* as well as *gblBT036881.1*, the orthologue of *AtIRX9* (Bosch et al. 2011) involved in the initiation of the xylan synthesis (Brown et al. 2007; Peña et al. 2007) was found to be upregulated 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 *gblBT036881.1* (*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 (Figure S5).

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 solubilize weakly- and firmly-bound polysaccharide populations, respectively. The results showed that in medium DCB-habitation levels, the M_w of 0.1 M KOH-extracted hemicelluloses increased with respect to the same fraction obtained from non-habitated 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-habitated cells, cells habituated to medium DCB levels and to those solubilized 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 processes 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 d 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. 2014). 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 hypothesized 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. As previously mentioned, Sh1.5 cells showed a reduced amount of cell wall bound ^3H -hemicelluloses and an increased presence of ^3H SEPs in the culture medium (de Castro et al. 2015). In addition, the timing of cross-linking may also be delayed in Sh1.5 cells due to their slower and less efficient metabolism (de Castro et al. 2015) thus preventing detection during the time window used in this experiment (5 h). In order to explore these possibilities, an analysis was performed of cinnamic acid metabolism as well as a characterization of its derivatives on protoplasmic fraction and in the cell wall. First, no marked differences were detected in the capacity to uptake ^{14}C -cinnamic acid 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 -cinnamic acid 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 molecules. Similar altered kinetics in Sh1.5 cells were observed when ^3H -arabinose was fed as the radioactive precursor, showing that a delayed cellular traffic and cell wall binding of ^3H -hemicelluloses and ^3H -polymers took place (de Castro et al. 2015). 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 ferulic acid. Alternatively, hemicelluloses and polymers from habituated cells were in fact shorter, albeit extensively cross-linked.

Another possibility could be that dimerization (or oligomerization) of ferulic acid residues contributed to the formation of intra-polymeric loops to a greater extent (Fry et al. 2000). Consequently, Sh1.5 hemicelluloses would be deposited on the cell wall as a coagulum. 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. 2014), as well as a delayed metabolism throughout the entire cycle (de Castro et al. 2015).

Generally, the elution profiles of Driselase-digested diagnostic fragments for polymers containing [^3H] arabinose and those for [^3H]xylans were very similar, although arabinose 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, galactose and rhamnose residues (de Castro et al. 2014). Therefore, it can be assumed that [^3H]arabinose, [^3H]xylose and [^3H]xylobiose are, in fact, digestion products of putative [^3H]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 polymerization (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.

Snh cells shown faster [^{14}C]cinnamic acid metabolism than Sh1.5 and as a consequence free-phenolics and

^{14}C -labelled compounds were less persistent on the protoplasmic fraction. However the dimerization and oligomerization process were quickly in Sh1.5, especially during the late logarithmic phase. The presence of dimers and oligomers in the protoplasm of Sh1.5 cells, could be related with the M_w increase of the polymers (lower K_{av}) of this fraction at late logarithmic phase compared with early logarithmic phase. Additionally, 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 *p*-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 amount 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 dimerization and oligomerization 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 dimerization. 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 K_{av} 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 V_0 , where molecules with the highest M_w would elute. However, when both K_{av} were compared, strongly wall bound xyloglucan from Sh1.5 cells was detected in a lower K_{av} 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. 2014), these results would partially support this hypothesis, although further experiments are required for confirmation.

In sum, the study of the M_w of [^3H]xylans, [^3H]xyloglucans, [^3H]arabinose-containing polymers and Driselase-digestion resistant ^3H -polymers as well as the phenolic metabolism in maize cells habituated to low DCB levels revealed that: (i) Sh1.5 cells synthesized molecules with a lower M_w and a more homogeneous size throughout the entire culture cycle, (ii) although hemicelluloses were shorter than those observed in the non-habituated cells, this was not related to a diminished capacity for polysaccharide cross-linking; (iii) both cell lines synthesized molecules with a lower M_w at the late logarithmic phase of the culture cycle, probably associated with the cessation of growth; and (iv) 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.

MATERIALS AND METHODS

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 sucrose, at 25°C under light and rotary shaken, and routinely subcultured every 15 d.

In order to obtain cell cultures habituated to DCB, Snh were stepwise subcultured in a medium supplied with increasing concentrations of DCB, dissolved in dimethyl sulfoxide (DMSO) which did not affect cell growth at this range of concentrations. 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 dry weight at different culture times and it was observed that Sh1.5 cells presented longer culture phases than Snh cells (de Castro et al. 2014). 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.

Analysis of M_r distributions

Radio-labeling of liquid cultures with [^3H]arabinose
Thirteen milliliter aliquots from Snh and Sh1.5 maize cells cultures were collected at early logarithmic and late logarithmic growth phases and each 13 mL aliquot was independently fed with 1 MBq of L-[1- ^3H]arabinose (148 GBq/mmol; Amersham International, Bucks., UK). The days on which the 13 mL aliquot of culture was sampled and [^3H]arabinose 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 d of culture were chosen for Snh and Sh1.5 cell lines, respectively (see de Castro et al. 2014). In the case of late logarithmic phase, the 8th and 12th d were selected for Snh and Sh1.5 cell lines, respectively. In order to obtain an optimal quantity of radio-labelled ^3H -hemicelluloses and ^3H -polymers, samples for analysis of the M_r were collected at different labeling-time points selected according to the maximum value of incorporated [^3H]arabinose observed in the kinetics previously described (de Castro et al. 2015). Thus, in the case of protoplasmic polymers, 1 mL samples were collected from independent 13 mL aliquots of culture 30 min and 60 min after feeding [^3H]arabinose for Snh and Sh1.5 cells, respectively. For the remaining ^3H -hemicelluloses and ^3H -polymers, sampling was carried out 300 min after feeding [^3H]arabinose for both cell lines.

Obtaining ^3H -hemicelluloses and ^3H -polymers from discrete maize cell suspensions compartments
Each 1 mL sample collected at the different labeling-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 labeled as cell-free medium fraction.

The washed cells retained in the Poly-Prep column filter were quickly resuspended in 1 mL of homogenization buffer (50 mmol/L collidine acetate (pH 7.0) containing 2% (w/v) lithium dodecyl sulfate (LiDS), 10% (v/v) glycerol, 5 mmol/L sodium thiosulphate and 10 mmol/L dithiothreitol (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 homogenizer by rinsing with 1 mL of chilled homogenization buffer ($\times 3$ times), and then homogenized 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 ($\times 4$ times).

Solid KCl was added to the filtrate and rinses to a final concentration of 0.67 M in order to precipitate the dodecyl sulfate as insoluble K^+ salt. After 30 min at 4°C , the products were centrifuged at 3,000 rpm (1,814 rcf) for 5 min. The supernatant was removed and re-centrifuged and this procedure was repeated until the supernatant turned clear, the moment at which it 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 ($\times 3$ times). Filtrate and washes were pooled and labeled 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 ($\times 3$ times). 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.

Gel permeation chromatography

Samples of cell-free medium, protoplasmic and cell wall fractions were dialyzed against distilled water with 0.1% (w/v) chlorobutanol at 4°C for 72 h, using a 12 kDa cut-off and 1.5 cm diameter dialysis membrane. Then, the dialyzed samples were centrifuged for 15 min at 3,000 rpm (1,814 rcf) in order to separate the soluble from the insoluble material in water.

To identify the void volume (V_0) and the totally included volume (V_i), 6 mg dextran (5–40 MDa) and

0.8 mg glucose, 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 ^3H -hemicellulose from maize culture medium and $[^{14}\text{C}]$ glucose as internal markers, which defined the V_0 and V_i (K_{av} 0 and 1) respectively. M_w was obtained using the $K_{av(1/2)}$ method (Kerr and Fry 2003) with the calibration curve ($\log M_w = -3.547 K_{av(1/2)} + 7.2048$) obtained for this column. The M_w estimates were nominal rather than absolute due to the conformational differences between dextran and hemicelluloses.

Characterization of phenolic metabolism

$[^{14}\text{C}]$ cinnamic acid synthesis

$[^{14}\text{C}]$ cinnamic acid was prepared from L- $[U-^{14}\text{C}]$ phenylalanine (Perkin-Elmer; 487 Ci/mol) following the method described by Lindsay and Fry (2008).

$[^{14}\text{C}]$ cinnamic acid consumption and incorporation into different cell compartments

Fifteen mL aliquots of Snh and Sh1.5 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, $[^{14}\text{C}]$ cinnamic acid (0.29 μCi) was added to each cell culture. At selected time-points, 400 μL of cell cultures were sampled and acidified by the addition of 15 μL 99% formic acid, stopping the consumption and incorporation of $[^{14}\text{C}]$ cinnamic acid.

Each aliquot sampled at the different labelling-time points was then centrifuged for 4 min at 10,000 rpm (10,640 rcf) 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, labeled and stored as cell-free medium fraction.

Remaining cells were then incubated for 18 h with 800 μ L of 80% ethanol on a rotary wheel, the samples were subsequently centrifuged at 10,000 rpm (10,640 rcf) for 4 min and the supernatant was collected. Cells were rinsed with 200 μ L of 80% ethanol and centrifuged again. Supernatant and washings were then pooled and labeled as the protoplasmic fraction (alcohol soluble material). The residual cell fragments were considered alcohol insoluble residue (AIR).

Obtaining of cinnamic acid [14 C]derivatives from discrete maize cell compartments

Four hundred microliter aliquots of Snh and Sh1.5 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 acclimatize to their new environmental conditions. Then, vials were fed with [14 C]cinnamic acid (0.116 μ Ci). At selected time-points, aliquots were acidified by the addition of 15 μ L 99% formic acid, stopping [14 C] cinnamic acid incorporation. Where indicated, 40 mmol/L H₂O₂ (final concentration) was added to some of the samples 120 min after [14 C]cinnamic acid and then shaken for 60 min. Aliquots of cell suspensions contained in the vials were then transferred into Poly-Prep columns and the filtrate was collected and considered the cell-free medium 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 fraction. The protoplasmic fraction was collected, de-esterified with 0.5 M NaOH for 18 h at 25°C, acidified to pH 5.0 and further partitioned against ethyl acetate ($\times 2$ times). The ethyl acetate phases were vacuum-dried, re-dissolved in 0.5 mL of acidified water (0.01 N HCl) and further subjected to a second ethyl acetate partition ($\times 3$ times). The organic phases were collected, vacuum-dried and re-dissolved in propan-1-ol.

The AIR retained on the Poly-Prep column was de-esterified 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, acidified to pH 5.0 and collected as the 0.5 M NaOH fraction. This fraction was subjected to ethyl acetate partition as indicated above. The organic phases were collected, vacuum-dried and re-dissolved in propan-1-ol.

Assay of radioactivity

Analysis of M_r distribution

To obtain the general elution profiles of 3 H-hemicelluloses and 3 H-polymers, a 200 μ L 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 μ L of 0.1 M trifluoroacetic acid (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 μ L 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 xylose 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 xylose, arabinose, glucose, isoprimeverose and xylobiose (≈ 50 μ g each) was then added to the digest, which was subjected to paper chromatography on Whatman 3MM in ethyl acetate/pyridine/water (9/3/2 by vol.) for 18 h (Thompson et al. 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 scintillation liquid were added, and vials were shaken continuously for 24 h. Radioactivity was subsequently assayed by counting.

[14 C]cinnamic acid consumption and incorporation into different cell compartments

To analyse the cell-free medium samples and the protoplasmic fraction, 5 and 3 mL of scintillation liquid EcoscintA (National Diagnostics) were 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.

Obtaining cinnamic acid [^{14}C] derivatives from discrete maize cell compartments

Samples of protoplasmic and AIR fractions de-esterified with 0.5 M NaOH were subjected to thin layer chromatography (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 inter-converting single spots of *cis/trans* isomers. Standards of ferulic acid, *p*-coumaric acid and 5-5'-diferulate were used as external markers.

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

***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 (Table S1) 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.

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AUTHOR CONTRIBUTIONS

M.d.C. and R.M.-R. performed most of the research. S.C.F. supervised and designed the polysaccharides metabolism experiment which was carried out by M.d.C. A.E. supervised and designed the phenolic metabolism experiments which was carried out by R.M.-R. P.G.-A. and M.d.C. drafted the manuscript and made the gene expression analysis. J.L.A. provided the research environment and supervised the project. All the authors revised the manuscript. All the work was coordinated and supervised by P.G.-A.

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SUPPORTING INFORMATION

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Figure S1. Thin layer chromatography of ^{14}C -labelled compounds (esterified and free-phenolics) of the protoplasmic fraction after the addition of exogenous ^{14}C -cinnamic acid to non-habituated (purple line) and habituated to $1.5\ \mu\text{M}$ DCB maize cells suspensions (green line) at the early logarithmic phase of culture cycle

The position (—) of *p*-coumaric acid (Cou), ferulic acid (Fer), cinnamic acid (Cinn) and 5,5'-dehydrodiferulic acid (Di-Fer) used as external markers is shown. Compounds with low R_f are regarded as oligomers (Fry et al. 2000). +60 min H_2O_2 shows a treatment for 60 min with $40\ \text{mmol/L}$ H_2O_2 , 120 min after ^{14}C -cinnamic acid feeding.

Figure S2. Thin layer chromatography of ^{14}C -labelled compounds (esterified and free-phenolics) of the protoplasmic fraction after the addition of exogenous ^{14}C -cinnamic acid to non-habituated (purple line) and habituated to $1.5\ \mu\text{M}$ DCB maize cell suspensions (green line) at the late logarithmic phase of culture cycle

Other details as in Figure S1.

Figure S3. Thin layer chromatography of ^{14}C -labelled compounds esterified of the alcohol-insoluble residue (cell wall fragments) after the addition of exogenous

^{14}C -cinnamic acid to non-habituated (purple line) and habituated to $1.5\ \mu\text{M}$ DCB maize cells suspensions (green line) at the early logarithmic phase of culture cycle

The position (—) of *p*-coumaric acid (Cou), ferulic acid (Fer) and 5,5'-dehydrodiferulic acid (Di-Fer) used as external markers is shown. Compounds with low R_f are regarded as oligomers (Fry et al. 2000). The inset graphics show in detail the oligomers and ferulic acid dimers appearance. +60 min H_2O_2 shows a treatment for 60 min with $40\ \text{mmol/L}$ H_2O_2 , 120 min after ^{14}C -cinnamic acid feeding.

Figure S4. Thin layer chromatography of ^{14}C -labelled compounds esterified of the alcohol-insoluble residue (cell wall fragments) after the addition of exogenous ^{14}C -cinnamic acid to non-habituated (purple line) and habituated to $1.5\ \mu\text{M}$ DCB maize cell suspensions (green line) at the late logarithmic phase of culture cycle

Other details as in Figure S3.

Figure S5. Relative expression of orthologous maize genes of several IRX genes from arabidopsis analyzed by RT-PCR of non-habituated (Snh) and habituated to 1.5 (Sh1.5) and $6\ \mu\text{M}$ DCB (Sh6) maize cell suspensions $\uparrow$, more mRNA accumulation than control (Snh); $\downarrow$, less mRNA accumulation than control (Snh). *ZmUbi*: Ubiquitin gene expression. Ubiquitin was used as the housekeeping gene due to its constitutive expression.

Table S1. Primers used for the analysis of maize arabinoxylan synthesis genes

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Class III peroxidases in cellulose deficient cultured maize cells during cell wall remodeling

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Maize (*Zea mays* L.) suspension-cultured cells habituated to a cellulose biosynthesis inhibitor 2,6-dichlorobenzonitrile (DCB) have a modified cell wall, in which the reduction in the cellulose content is compensated by a network of highly cross-linked feruloylated arabinoxylans and the deposition of lignin-like polymers. For both arabinoxylan cross-linking and lignin polymerization, class III peroxidases (POXs) have been demonstrated to have a prominent role. For the first time, a comparative study of POX activity and isoforms in control and cellulose-impaired cells has been addressed, also taking into account their cellular distribution in different compartments. Proteins from the spent medium (SM), soluble cellular (SC), ionically (ICW) and covalently bound cell wall protein fractions were assayed for total and specific peroxidase activity by using coniferyl and sinapyl alcohol and ferulic acid as substrates. The isoPOX profile was obtained by isoelectric focusing. POX activity was higher in DCB-habituated than in non-habituated cells in all protein fractions at all cell culture stages. For all substrates assayed, SC and ICW fractions showed higher activity at the early log growth phase than at the late log phase. However, the highest POX activity in the spent medium was found at the late log phase. According to the isoPOX profiles, the highest diversity of isoPOXs was detected in the ICW and SM protein fractions. The latter fraction contained isoPOXs with higher activity in DCB-habituated cells. Some of the isoPOXs detected could be involved in cross-linking of arabinoxylans and in the lignin-like polymer formation in DCB-habituated cells.

Introduction

The cell wall is a dynamic structure that provides mechanical support to plant cells, playing an important role in plant growth and development, and enabling cells to cope with different biotic and abiotic stresses (Wolf et al. 2012). Primary cell walls of maize (*Zea mays* L.; type II) are complex composites made up of

polysaccharides (cellulose and hemicelluloses) and proteins, with hydroxycinnamates, mainly ferulic and *p*-coumaric acids, esterified to arabinose residues of glucurono-arabinoxylans (Kato and Nevins 1985, Carpita et al. 2015). In addition, feruloyl residues are susceptible to oxidative coupling mediated by peroxidases (Geissman and Neukom 1971), leading to formation of ester-linked dehydroferulates and higher

Abbreviations – ABTS, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); CCW, covalently bound cell wall proteins; DAB, 3,3'-diaminobenzidine; DCB, 2,6-dichlorobenzonitrile; DMSO, dissolved in dimethyl sulfoxide; early log, early logarithmic growth phase; ICW, ionically bound cell wall proteins; IEF, isoelectric focusing; isoPOX, isoperoxidase; late log, late logarithmic growth phase; pl, isoelectric point; POX, class III peroxidase; SC, soluble cellular proteins.

oligomers, which contribute to cell wall assembly by cross-linking cell wall polysaccharides (Fry 2004).

The variability in the cell wall composition reflects the plasticity of the cell wall structure. A valuable tool to underline the plasticity has been the habituation of plant cell cultures to grow in the presence of cellulose biosynthesis inhibitors (for a review see Acebes et al. 2010). One of these compounds, 2,6-dichlorobenzonitrile (DCB), specifically inhibits polymerization of glucose into β -1,4-linked glucans (Montezinos and Delmer 1980), causing a reduction in the cell wall cellulose content. In the last few decades, several plant cell cultures have been habituated to DCB (Shedletzky et al. 1992, Encina et al. 2001, 2002, García-Angulo et al. 2006, 2009, Mérida et al. 2009, 2015). The habituation process involves many metabolic modifications, depending on the type of the cell wall, the concentration of DCB, as well as the length of time that the cells are exposed to the inhibitor (Alonso-Simón et al. 2004, de Castro et al. 2015, Mérida et al. 2015, Largo-Gosens et al. 2016).

By compositional analysis and structural characterization of the cell walls of DCB-habituated cells, it has been demonstrated that maize cells have the capacity to cope with DCB through acquisition of a modified cell wall. In DCB-habituated cells, cellulosic scaffold was partially replaced by a more extensive network of highly cross-linked feruloylated arabinoxylans (Mérida et al. 2009, 2010a, 2010b, 2011, 2015, de Castro et al. 2014, 2015, Largo-Gosens et al. 2016). Recently, it has been shown that DCB-habituated maize cells incorporate to the cell walls lignin-like polymers (Mérida et al. 2015), which stiffens the structure further (Pomar et al. 2002, Kärkönen and Kuchitsu 2015).

Lignin is a three-dimensional, phenolic polymer that is deposited in the secondary thickened cell walls of specialized plant cell types such as water-conducting tracheids and vessels, and support-giving sclerenchyma cells. Lignin provides structural support by giving compressive strength and hydrophobicity to the cell walls (Vanholme et al. 2010). Lignin is polymerized in muro by the oxidative coupling of monolignols: *p*-coumaryl, coniferyl, and sinapyl alcohols (Vanholme et al. 2010). The cross-coupling reactions of monolignol radicals produce a hydrophobic heteropolymer composed of *p*-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) units (Kärkönen et al. 2014, Hatfield et al. 2017). Oxidation of monolignols to phenolic radicals is carried out by H₂O₂-dependent peroxidases (Harkin and Obst 1973) and/or O₂-dependent laccases (Bao et al. 1993, Zhao et al. 2013, Koutaniemi et al. 2015). Peroxidases were earlier considered more important for lignin polymerization than laccases, because while peroxidases are able

to oxidize the three monolignols to produce H, G and S units, laccases only generate G units (Berthet et al. 2011). Recent studies, however, have shown that laccases play an important role in vascular lignin formation in *Arabidopsis thaliana* being supposedly involved in the early stages of lignin polymerization making di- and oligolignols (Zhao et al. 2013, Laitinen et al. 2017). In maize, only one laccase (*ZmLac3*) of the laccase multigene family seems to be involved in polymerization of monolignols (Caparrós-Ruiz et al. 2006).

Class III secretory plant peroxidases (POX; EC 1.11.1.7; donor: hydrogen peroxide oxidoreductase) are implicated in several physiological processes including lignin and suberin formation (Christensen et al. 1998, Quiroga et al. 2000, Passardi et al. 2005), plant defense (Li et al. 2010), auxin metabolism (Cosio and Dunand 2009), cell elongation (Passardi et al. 2004), as well as generation of reactive oxygen species (Liszkay et al. 2003, Li et al. 2010, Shigeto and Tsutsumi 2016). Typically, POXs can be found in vacuoles (soluble cellular proteins) and in the cell walls. In this later case and depending on their interaction with the cell wall components, cell wall POXs can be divided into three groups: those soluble in the apoplastic fluid that permeates cell walls; ionically bound cell wall proteins (ICW) presenting hydrophobic and ionic interactions with the matrix polysaccharides and proteins; and covalently bound cell wall proteins (CCW), which are cross-linked by covalent bonds with the cell wall components (Jamet et al. 2006, 2008). In this way, diferulate and lignin synthesis have been preferentially associated with ionically and covalently bound cell wall isoperoxidases (isoPOXs; González et al. 1999, Shigeto et al. 2013, Fernández-Pérez et al. 2015, Lin et al. 2016, Wariowski et al. 2016). In addition, it has been reported that both anionic and cationic isoPOXs play a role in lignin formation (Fagerstedt et al. 2010).

Taking into account that DCB-habituation of maize cells is featured by significant changes in the cell wall components susceptible to POX action (i.e. feruloylated arabinoxylans and lignin-like compounds), the aim of this study was to obtain further information on the interrelationship between POXs and the remodeling of the cell wall. As a research material, we used maize suspension-cultured cells habituated to 1.5 μ M DCB (*I*₅₀ value for maize suspension-cultured cells is 0.5 μ M DCB; de Castro et al. 2014). For this purpose, proteins isolated from the maize cell cultures were fractionated into (1) culture medium proteins, (2) soluble cellular proteins (SC), (3) ionically (ICW) and (4) covalently (CCW) bound cell wall proteins. Total and specific POX activities in different cellular fractions against three native substrates (ferulic acid, coniferyl alcohol and

sinapyl alcohol) are reported. The isoPOX profile in the different cellular compartments of non-habituated and DCB-habituated cells was obtained by isoelectric focusing followed by POX activity staining. For the first time, a comparative study of POX activity and isoforms in control and cellulose-impaired cells including their cellular distribution is shown. Both quantitative and qualitative differences in POX activity and profile were detected suggesting that POXs play a prominent role in maize cell wall remodeling as a response to reduction in cellulose content.

Materials and methods

Plant material and DCB-habituation process

Cell cultures

Maize (*Zea mays* L. Black Mexican sweet corn) suspension-cultured cells were obtained as described by Lorences and Fry (1991), and subcultured every 15 days in liquid Murashige and Skoog (1962) medium supplemented with 9 μM 2,4-dichlorophenoxyacetic acid and 20 g l⁻¹ sucrose. The cultures were cultivated at 25°C under photoperiodic conditions (16 h light: 8 h dark; 41 $\mu\text{mol m}^{-2} \text{s}^{-1}$, GRO-lux Sylvania) on a rotary shaker (120 rpm) (Mélida et al. 2011).

Habituation to DCB

Maize suspension-cultured cells were stepwise subcultured in a medium supplied with increasing concentrations of DCB (Fluka, Seelze, Germany). DCB was dissolved in dimethyl sulfoxide (DMSO), and the maximum concentration of DMSO added in the culture medium (0.015% v/v) did not affect cell growth (de Castro et al. 2014). Non-habituated, suspension-cultured cells were transferred to a medium containing 0.3 μM DCB and increasing DCB concentrations were applied gradually up to 1.5 μM DCB (habituated cells) after seven subcultures. Cell growth kinetics for non-habituated and habituated cells were followed as described by de Castro et al. (2014), and cells were collected at early (early log) and late logarithmic (late log) growth phases (4 and 8 days old cells for non-habituated and 8 and 12 days old cells for DCB-habituated cell lines, respectively).

Preparation of protein fractions

Protein fractions were obtained according to the method of Kärkönen et al. (2002) with modifications.

The spent medium of suspension-cultured cells at the early log and late log growth phases was filtered through glass wool. Cells were collected, weighed and

immediately frozen in liquid nitrogen, and stored at -80°C. All subsequent steps were carried out at 0–4°C. The medium was centrifuged (10 000g, 30 min) and the supernatant was concentrated over 10-fold using an Amicon ultra-filtration device (Biomax membrane, cutoff 10 kDa, Darmstadt, Germany). The proteins were buffer-exchanged prior to enzyme assays by passage through PD10 desalting columns (Sephadex G-25, GE Healthcare, Uppsala, Sweden) pre-equilibrated with 50 mM Tris-HCl buffer, pH 7.5, containing 0.5 mM CaCl₂, 30 mM NaCl and protease inhibitors (Complete Protease Inhibitor Cocktail Tablets, EDTA-free; Roche, Basel, Switzerland). This fraction contained spent culture medium proteins.

The cells were powdered in liquid nitrogen using a mortar and pestle. The resulting fine powder was transferred into 50 mM Tris-HCl buffer, pH 7.5, supplemented with 30 mM NaCl, a protease inhibitor cocktail and 0.3% (w/v) Triton X-100 (3 ml buffer g⁻¹ cells) (referred to from now on as an extraction buffer). After 30 min incubation on ice, the sample was centrifuged (17 000g, 30 min). The supernatant containing SC was desalted through PD10 desalting columns as described above.

The pellet precipitated by centrifugation was subsequently homogenized in a glass homogenizer with repeated centrifugation (17 000g, 20 min) in extraction buffer without protease inhibitors (referred to from now on as a wash buffer) with 15 ml buffer in each wash for at least eight times, and in 50 mM Tris-HCl buffer, pH 7.5, containing 0.5 mM CaCl₂ and 30 mM NaCl for at least four times. Washes were continued until no soluble POX activity was detected in the last wash solution as assayed with 0.55 mM 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS, Sigma-Aldrich-Aldrich, St. Louis, MO) and 0.25 mM H₂O₂ as substrates in 50 mM Na-citrate buffer pH 5.0. Cell walls were transferred into 50 mM Tris-HCl buffer, pH 7.5, supplemented with 1 M NaCl, 0.5 mM CaCl₂ and a protease inhibitor cocktail to extract ionically bound cell wall proteins (ICW). After overnight extraction on ice, samples were centrifuged (10 000g, 30 min), and the extraction repeated with gentle shaking (100 rpm for 30 min). The two fractions containing the ICW were combined, passed through PD10 columns (see above) and concentrated using a Microsep Centrifugal Device (cut off 10 kDa, Pall Corporation, New York, NY), frozen in liquid nitrogen and stored at -80°C.

The pellet from the centrifugation was homogenized in a glass homogenizer with the wash buffer (ca. three times) and with cold water (ca. seven times) with repeated centrifugation (17 000g, 20 min), until

no soluble POX activity was detected in the last wash solution as assayed with ABTS.

To extract covalently bound cell wall proteins (CCW), cell walls were incubated in a mixture of 1% (w/v) Cellulase Onozuka R-10 (Duchefa Biochemie B.V, Haarlem The Netherlands) and 0.5% (w/v) Macerozyme R-10 (Duchefa Biochemie B.V, Haarlem The Netherlands) in 100 mM Na-acetate buffer, pH 4.5, containing a protease inhibitor cocktail at 30°C for 3 h (Masuda et al. 1983). After centrifugation (10 000 g, 30 min, 4°C) the supernatant was buffer-exchanged in PD10 desalting columns. To release those proteins that were liberated by the enzyme treatment but were then bound ionically to the cell walls, the residue was suspended in 50 mM Tris–HCl buffer, pH 7.5, containing 0.5 mM CaCl₂ and 1 M NaCl, and incubated on ice for 30 min. After centrifugation (10 000 g, 30 min), the extraction was repeated. These fractions were buffer-exchanged and combined to the fraction obtained after the enzyme treatment, followed by concentration as mentioned above.

The cell wall pellet was washed with water until no soluble POX activity was detected in the last wash solution (assayed with ABTS) and lyophilized.

Three independent experiments were carried out with sample replication of at least three in each case.

Enzyme assays and quantification of proteins

All enzyme assays were carried out on protein concentrates after desalting through PD10 gel filtration columns into 50 mM Tris–HCl buffer, pH 7.5, containing 0.5 mM CaCl₂, 30 mM NaCl and protease inhibitors. To assay POX activity in the cell wall residues, the lyophilized cell walls were resuspended in the above-mentioned buffer (1 mg ml⁻¹), and 60 µl of the suspension was added to the reaction mixture. Assays were carried out in a total volume of 1.0 ml in a spectrophotometer (Shimadzu UV-1800, Kyoto, Japan) at 25°C. Coniferyl alcohol POX activity was assayed at 262 nm using 100 µM coniferyl alcohol (Fluka, Seelze, Germany; Kärkönen et al. 2002), ferulic acid POX activity was determined at 310 nm with 100 µM ferulic acid (Sigma-Aldrich, St. Louis, MO; García et al. 2002), and sinapyl alcohol POX activity was assayed at 270 nm with 100 µM sinapyl alcohol (Sigma; Koutaniemi et al. 2005) as substrates. All activities were determined by adding 250 µM H₂O₂ and up to 60 µl of sample in 45 mM Na-citrate buffer, pH 5.0. The molar absorptivity coefficients (ϵ) for coniferyl alcohol, ferulic acid and sinapyl alcohol were taken as 14 000 M⁻¹ cm⁻¹ (Kärkönen et al. 2002), 8400 M⁻¹ cm⁻¹ (García et al. 2002) and 6000 M⁻¹ cm⁻¹ (Koutaniemi et al. 2005), respectively.

Oxidase activity was determined at 262 nm by the method of Udagama-Randeniya and Savidge (1995) using 75 µM coniferyl alcohol in 88.5 mM Na-acetate buffer, pH 4.5, containing 125 U ml⁻¹ catalase Sigma C-100 (Sigma-Aldrich, St. Louis, MO) and 100 µl of sample. Extinction coefficient for coniferyl alcohol was used for quantitative calculations.

Bio-Rad protein assay (Bio-Rad, Hercules, CA; based on Bradford 1976), scaled down to microtiter plates was used to measure protein concentration using bovine serum albumin (Sigma-Aldrich, St. Louis, MO) as a standard.

Isoelectric focusing

Isoelectric focusing (IEF) was carried out as described by Kärkönen et al. (2002) with modifications. IEF was accomplished with a Multiphor II apparatus (Pharmacia, Stockholm, Sweden) in a 4% polyacrylamide gel containing Pharmolyte with a pH range of 3–10 (GE Healthcare, Uppsala, Sweden), and 0.04 M aspartic acid and 1 M NaOH as anode and cathode solutions, respectively. Manufacturer's instructions were followed with slight modifications. Before sample application, the gel was pre-focused at 5 W for 30 min. After sample application, the gel was focused at 15 W for 1.5 h at +10°C.

POX activity was visualized by staining the gel with 0.8% (w/v) 3,3'-diaminobenzidine (DAB, Sigma-Aldrich, St. Louis, MO) in 50 mM Na-acetate buffer, pH 5.0, supplemented with 10 mM CaCl₂ (Kärkönen et al. 2002, with some modifications). After preincubation for 10 min in a staining buffer, the reaction was started by addition of H₂O₂. H₂O₂ was added gradually in 0.5 mM aliquots until POX activity bands became visible (final concentration of H₂O₂ ca. 2.5 mM). After at least 30 min staining at room temperature, the activity bands were recorded by photographing (Nikon F50, Tokyo, Japan). Isoelectric points (pI) were calculated based on the comparison with the pH of gel segments derived from empty lanes.

Results

Peroxidase activity

To obtain a deep insight in the relationship between class III peroxidases (POXs) and the remodeling of the maize cell wall occurring due to the habituation to the cellulose biosynthesis inhibitor DCB, POX activity against native substrates coniferyl alcohol, sinapyl alcohol and ferulic acid was measured on protein fractions obtained from non-habituated and DCB-habituated cell lines in different phases of the cell culture cycle (Tables 1 and 2). Regarding substrates, similar levels of POX activity (both

Table 1. Total peroxidase activity in soluble cellular, ionically and covalently bound cell wall and spent medium protein fractions and in cell wall residues of non-habituated (NH) and DCB-habituated (H) cell lines at the early logarithmic (early log) and late logarithmic (late log) growth phases. Data are means $\pm$ SE of at least six independent experiments. FW, cell fresh weight; n.d., not determined due to presence of interfering compounds.

Enzyme activity nkat/ g FW	Early log growth phase		Late log growth phase	
	NH	H	NH	H
Soluble cellular protein fraction				
Coniferyl alcohol	121.3 $\pm$ 3.9	145.4 $\pm$ 3.9	89.7 $\pm$ 4.0	76.8 $\pm$ 1.8
Ferulic acid	142.5 $\pm$ 3.9	197.4 $\pm$ 2.5	86.5 $\pm$ 1.7	70.6 $\pm$ 1.1
Sinapyl alcohol	n.d.	n.d.	n.d.	n.d.
Ionically bound cell wall protein fraction				
Coniferyl alcohol	55.2 $\pm$ 2.0	75.9 $\pm$ 7.5	31.9 $\pm$ 6.3	40.2 $\pm$ 0.6
Ferulic acid	56.6 $\pm$ 2.1	71.7 $\pm$ 5.1	29.0 $\pm$ 4.2	37.6 $\pm$ 0.8
Sinapyl alcohol	2.4 $\pm$ 0.1	3.9 $\pm$ 0.4	1.6 $\pm$ 0.4	2.3 $\pm$ 0.2
Covalently bound cell wall protein fraction				
Coniferyl alcohol	30.6 $\pm$ 0.8	19.9 $\pm$ 1.8	22.3 $\pm$ 2.9	28.3 $\pm$ 0.9
Ferulic acid	30.0 $\pm$ 0.9	19.7 $\pm$ 1.4	29.1 $\pm$ 3.0	32.4 $\pm$ 0.9
Sinapyl alcohol	3.3 $\pm$ 0.3	2.7 $\pm$ 0.2	2.9 $\pm$ 0.3	3.7 $\pm$ 0.1
Spent medium protein fraction				
Coniferyl alcohol	38.9 $\pm$ 2.0	235.2 $\pm$ 7.4	167.5 $\pm$ 18.5	492.7 $\pm$ 73.2
Ferulic acid	36.9 $\pm$ 1.1	233.7 $\pm$ 11.1	145.3 $\pm$ 11.2	435.8 $\pm$ 31.8
Sinapyl alcohol	1.7 $\pm$ 0.3	7.7 $\pm$ 1.9	2.8 $\pm$ 0.2	12.6 $\pm$ 0.9
Cell wall residue				
Coniferyl alcohol	0.07 $\pm$ 0.01	0.13 $\pm$ 0.01	0.13 $\pm$ 0.02	0.10 $\pm$ 0.01
Ferulic acid	0.08 $\pm$ 0.01	0.21 $\pm$ 0.03	0.19 $\pm$ 0.02	0.16 $\pm$ 0.01
Sinapyl alcohol	0.04 $\pm$ 0.01	0.05 $\pm$ 0.01	0.06 $\pm$ 0.01	0.07 $\pm$ 0.00

total and specific activity) were detected with coniferyl alcohol and ferulic acid. POX activity against sinapyl alcohol was clearly smaller (7 to 57 times) than those found for the other two substrates. Because of the presence of some interfering compounds in the SC protein fraction, sinapyl alcohol POX activity could not be measured for this fraction. The nature of the interfering compound(s) is not known as the buffer-exchange in PD10 column removed the small-molecular-weight compounds.

Independently of the substrate or the cell line analyzed, the highest total POX activity was found in the spent medium, followed by soluble and cell wall-bound fractions (Table 1). In all cases, a low remaining POX activity was detected in the cell wall residue. When POX activity was expressed in relation to the protein content (specific activity) (Table 2), the activity was highest in the ICW fraction followed by that in the spent medium and SC fractions. Because of the considerable amount of proteins provided in the commercial Cellulose-Macerozyme mixture, specific POX activities for the CCW fractions could not be calculated.

Both total and specific POX activities in the ICW fraction were higher at the early log than at the late log growth phase independently of the cell line analyzed (Tables 1 and 2). In the case of the spent medium, the highest POX activity was found at the late log growth phase. No differences in the POX activity were detected

in the CCW fraction when different phases of the cell culture cycle were compared.

In general, total POX activity of all protein fractions was higher in the DCB-habituated cell line than in the non-habituated cell line throughout the culture cycle. Only in the SC fraction regarding the late log growth phase and CCW fraction regarding the early log growth phase, higher POX activity was found in non-habituated cells compared to DCB-habituated cells. Similarly, higher specific POX activity was found for the DCB-habituated cell line (regarding both early and late log phases) than for the non-habituated cell line regarding all fractions assayed.

Oxidase activity against coniferyl alcohol was not detected in any of the protein fractions analyzed.

IEF and in-gel activity staining

Maize suspension-cultured cells were characterized for isoPOXs by IEF in a pH gradient of 3–10 (Figs 1 and 2). Different patterns of isoPOX were obtained for different cellular fractions, cell lines and phases of the cell culture cycle. POXs in different fractions could be classified into acidic, neutral and basic isoPOX groups. Independently of the cell lines and phases of the cell culture cycle analyzed, SC proteins' isoPOX profiles were similar, showing a diversity of isoPOXs across the whole pI range (Fig. 1A). There were, however, some

Table 2. Specific peroxidase activity in soluble cellular, ionically bound cell wall and spent medium protein fractions of non-habituated (NH) and DCB-habituated (H) cell lines at the early logarithmic (early log) and late logarithmic (late log) growth phases. Data represent means $\pm$ SE of at least six independent experiments. FW, cell fresh weight; n.d., not determined due to presence of interfering compounds.

Enzyme activity nkat/mg protein	Early log growth phase		Late log growth phase	
	NH	H	NH	H
Soluble cellular protein fraction				
Coniferyl alcohol	11.3 $\pm$ 0.9	15.0 $\pm$ 0.7	10.7 $\pm$ 0.5	13.0 $\pm$ 0.7
Ferulic acid	13.1 $\pm$ 0.8	20.3 $\pm$ 0.3	10.4 $\pm$ 0.4	13.2 $\pm$ 1.1
Sinapyl alcohol	n.d.	n.d.	n.d.	n.d.
Ionically bound cell wall protein fraction				
Coniferyl alcohol	364.0 $\pm$ 52.6	1338.7 $\pm$ 37.4	100.3 $\pm$ 20.5	539.9 $\pm$ 95.0
Ferulic acid	366.6 $\pm$ 46.2	1281.0 $\pm$ 7.9	91.3 $\pm$ 13.5	495.5 $\pm$ 48.2
Sinapyl alcohol	16.1 $\pm$ 2.4	69.4 $\pm$ 1.6	4.8 $\pm$ 1.1	29.2 $\pm$ 3.8
Spent medium protein fraction				
Coniferyl alcohol	441.9 $\pm$ 22.3	612.6 $\pm$ 19.3	1658.6 $\pm$ 183.0	1734.7 $\pm$ 229.2
Ferulic acid	419.7 $\pm$ 12.0	608.8 $\pm$ 29.0	1438.9 $\pm$ 110.6	1489.6 $\pm$ 108.6
Sinapyl alcohol	19.7 $\pm$ 2.9	20.0 $\pm$ 5.0	27.6 $\pm$ 1.5	43.0 $\pm$ 3.2

differences in the activity of individual isoPOXs (Fig. 1A). CCW samples, on the other hand, contained several isoPOXs with neutral to slightly acidic pIs and few basic isoPOXs, but no qualitative differences could be seen in the non-habituated and DCB-habituated cell lines (Fig. 1B and C). In the ICW fraction, non-habituated and habituated cells showed a common pattern of basic isoPOX forms (Fig. 2A). This was not the case for the slightly acidic isoPOXs (pIs approximately 5–5.7), where DCB-habituated cells lacked some of the isoforms detected in non-habituated cells. The spent medium of non-habituated and DCB-habituated cells at both culture phases contained four neutral isoPOXs (pIs approximately 7.5) that became visible after staining for a few minutes (Fig. 2B, #, C). When stained for a longer time (up to 120 min), new isoPOXs appeared in the non-neutral regions. The activity level of the individual isoPOXs in these regions differed between the cell lines (Fig. 2B). Much more active acidic isoPOXs were present in the DCB-habituated cells at both growth phases in comparison with the non-habituated cells. In addition, at the late log phase one basic isoPOX showed higher activity in DCB-habituated cells lines than in the non-habituated cell lines (Fig. 2B; uppermost arrowhead).

Discussion

Habituation of suspension-cultured plant cells to DCB leads to a modified cell wall, which allows cells to cope with different stress situations. In previous studies, we have shown that DCB-habituation of suspension-cultured maize cells causes a reduction in the cellulose content, which is compensated by a more extensive network of highly cross-linked arabinoxylans (Mélida et al. 2009). As expected, maize cells habituated

to DCB presented a higher amount of cell wall-esterified *p*-coumarates, ferulates and dehydroferulates, the later ones of which are arabinoxylan cross-linkers (Mélida et al. 2009). The wall-bound ferulates have been proposed to act as nucleation sites for lignin polymerization (Ralph 2010, Hatfield et al. 2017). More recently, we have demonstrated the presence of ectopic lignin or lignin-like material in the cell walls of DCB-habituated cells (Mélida et al. 2015). In summary, our previous results show that despite being minor components of maize primary cell walls, cell wall phenolics play a major role in maintaining cell wall integrity (Mélida et al. 2009, de Castro et al. 2014).

POXs located in cell walls and vacuoles catalyze the oxidation of a large variety of substrates by using H₂O₂ as an oxidizing agent (Kärkönen et al. 2009). By cross-linking ferulates bound to arabinoxylans, POXs have been demonstrated to participate in cell wall remodeling (Fry 2004). Additionally, POXs promote cross-linking between ferulates and lignin contributing to further stiffening of the cell wall structure (Ralph 2010). The reported increase in the level of POX activity associated with DCB-habituation of maize suspension-cultured cells (Mélida et al. 2015, Largo-Gosens et al. 2016) suggests that POXs play a prominent role in maize cell wall remodeling as a response to reduction in cellulose content. In agreement with this, no oxidase (laccase) activity against coniferyl alcohol was detected in the present study, whereas all fractions had strong POX activity against coniferyl alcohol, ferulic acid and sinapyl alcohol, all native substrates in the maize cell wall.

Not every isoPOX has the ability to oxidize the three main monolignols. Coniferyl and *p*-coumaryl alcohols can be oxidized in vitro by both anionic and cationic POXs, while sinapyl alcohol is mainly oxidized

Fig. 1. Peroxidase isoenzyme patterns of soluble cellular proteins (A) and covalently bound cell wall proteins (B, C) of non-habituated (NH) and DCB-habituated (H) cell lines of maize suspension-cultured cells at their early logarithmic (early log) and late logarithmic (late log) growth phases separated on an acrylamide gel by isoelectric focusing (pH 3–10). (B) Basic isoPOXs after a longer staining time. (C) Neutral and acidic isoPOXs. The amounts of proteins loaded onto the gel were determined as coniferyl alcohol peroxidase activity: (A) 600 pkat, (B) 3000 pkat and (C) 2000 pkat were used here. Peroxidase activity was visualized by the DAB staining as described in materials and methods. The pIs shown are the mean value $\pm$ *sd* obtained from three independent gels.

by a subgroup of cationic isoPOXs (Ros Barceló et al. 2007). In the present study, the non-habituated and DCB-habituated cell lines of maize at both early log and late log growth stages showed similar POX activity levels against coniferyl alcohol and ferulic acid in all compartments analyzed. Thus, maize suspension-cultured cells are able to produce and secrete POXs capable of oxidizing not only the monolignol coniferyl alcohol but also ferulic acid, further supporting the broad substrate specificity of POXs (Campa 1991). The high POX activity detected in vitro against coniferyl alcohol and ferulic acid in all fractions analyzed, and the fact that the highest total POX activity was found in the spent medium fraction, suggest that POXs in this protein fraction participate in monolignol oxidation for lignin biosynthesis, and in oxidative coupling of ferulates. These data are in accordance with those previously reported for the lignin-forming Norway spruce cell culture, in which POXs activities in the spent medium fraction were higher than in other cell wall fractions (Kärkönen et al. 2002). In the present study, activity against sinapyl alcohol was detected in all apoplastic fractions assayed. However, in comparison with activities obtained for coniferyl alcohol and ferulic acid, POX activity against sinapyl alcohol was clearly lower, indicating that maize POXs are

less efficient in sinapyl alcohol oxidation (Hatfield et al. 2008). The presence of S units in maize stem lignin (S/G ratio: 1.4; Fornalé et al. 2012) and in the lignin-like material detected in the DCB-habituated suspension cultured cells (S/G ratio: 1.5; Mérida et al. 2015) requires that sinapyl alcohol is oxidized, either directly by enzymatic means or indirectly by radical transfer reaction (Takahama et al. 1996). It is possible that similarly to other plant species, only certain maize isoPOXs are capable of oxidizing sinapyl alcohol due to the presence of steric hindrances at the substrate binding site (Ros Barceló et al. 2007). This would explain the lower POX activity detected with sinapyl alcohol as a substrate.

Generally, and as expected, POX activity was higher in the DCB-habituated cells than in the non-habituated cells in all protein fractions at all cell culture stages. The high level of POX activity found in the apoplastic compartment of the DCB-habituated cells (especially in the ICW and the spent medium protein fractions) can relate to the increased level of cross-linking of arabinoxylans and the presence of ectopic lignin in this cell line (Mérida et al. 2009, 2015), both mechanisms involved in cell wall remodeling. Total POX activities in most of the protein fractions were higher at the early log growth phase than at the late log phase. This fact

Fig. 2. Peroxidase isoenzyme patterns of ionically bound cell wall proteins (A), and spent medium proteins (B, C) of non-habituated (NH) and DCB-habituated (H) cell lines of maize suspension-cultured cells at their early logarithmic (early log) and late logarithmic (late log) growth phases separated on an acrylamide gel by isoelectric focusing (pH 3–10). The amounts of proteins loaded onto the gel were determined as coniferyl alcohol peroxidase activity: (A) 4000 pkat and (B, C) 1500 pkat were used here. Peroxidase activity was visualized by the DAB staining as described in materials and methods. The pIs shown are the mean value $\pm$ SD obtained from three independent gels. Arrowheads show isoPOXs with a stronger activity in the H cell line when compared to NH one. (C) A close-up of the major, neutral isoPOXs (#) after a shorter staining time.

may be related to high expression of POX at this growth phase (Largo-Gosens et al., unpublished data), which predisposes habituated cells to formation of diferulates and ectopic lignin. However, the activity of the spent medium POXs was higher at the late log growth phase than at the early log phase.

IsoPOX profiles of SC and CCW fractions were similar among the non-habituated and habituated cell lines (Fig. 1). However, differences in the isoPOXs were observed in the ICW and the spent medium fractions. Some slightly anionic isoPOXs (pIs approximately 5.0–5.7) with high activity were detected in the ICW fraction obtained from the non-habituated cell line that were clearly reduced or absent in the DCB-habituated cell line (Fig. 2A). IsoPOXs of the corresponding pIs (approximately 5.6) appeared in the spent medium (Fig. 2B), showing the inability of the cell walls of DCB-habituated cells to retain those isoPOXs. One cationic isoPOX, on the other hand, was more active in the DCB-habituated cell line (Fig. 2B). It could be assumed that in the DCB-habituated cell line, these apoplastic isoPOXs that differ in activity between non-habituated and habituated cells are most probably involved in synthesis of lignin-like material during habituation (Mélida et al. 2015). Furthermore, the cationic isoPOX is possibly involved in S unit synthesis,

since cationic isoPOXs have been hypothesized to be responsible for sinapyl alcohol oxidation (Quiroga et al. 2000, Gabaldón et al. 2005, Ros Barceló et al. 2007).

In summary, maize suspension-cultured cells habituated to DCB have a reduction in the cell wall cellulose content, and produce a more extensive network of di- and oligoferulate cross-linked arabinoxylans together with a polymeric, lignin-like material (Mélida et al. 2015). This modified cell wall architecture is a result of high structural plasticity of primary cell walls in response to the disruption of cell wall integrity. In the present study, for the first time, comparative results of POX activity and isoforms in cultured maize cells containing primary cell walls with a reduced level of cellulose have been attained. Furthermore, the cellular distribution of these POXs in different compartments has been addressed.

No *in vitro* coniferyl alcohol oxidase activity was detected in any of the fractions, supporting the role of POXs in cell wall cross-linking in maize. As considerable POX activity was found in all apoplastic (the spent medium, ICW and CCW) fractions, it is impossible to define a specific isoPOX(s) that is involved in the mechanisms reinforcing the cellulose-impooverished cell wall. However, both quantitative and qualitative differences in the POX activity and the isoPOX profile were detected in the spent medium and ICW fraction suggesting that these

POXs play a prominent role in maize cell wall remodeling as a response to reduction in cellulose content. Therefore, this model system will be used in future studies to obtain further insight into the role of isoPOXs in cell wall cross-linking. Molecular studies will be used, focusing on identification and functional characterization of the isoPOXs that showed abundance differences between the non-habituated and DCB-habituated cell lines.

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Author contributions

M.-R.R. designed research, performed research, analyzed the data and wrote the manuscript. E.A., A.J.L. and K.A. provided research opportunity, designed research and wrote the manuscript.

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The doctoral thesis entitled "Cell wall remodelling of cellulose deficient maize cells: Going through the key role of phenolics" presented by Romina Martinez Rubio to aspire to the degree of Doctor in the Department of Engineering and AgroSciences-Area of Plant Physiology, Faculty of Biology (University of Leon) provides a comprehensive study on the molecular basis of how phenolics, especially hydroxycinnamates, and enzymes as peroxidases influence the cell wall remodelling of maize cells with deficiency in cellulose, putting in value the dynamic interaction between the three parts in the cell wall: cellulose, hemicellulose and lignin.

Cell Wall is a structure that confers rigidity, and other functions, to the plant protoplast. Besides being an important factor in the structure and rigidity of the cell, it also confers defense functions against possible pathogens that want to enter the plant cell. The cell wall can be divided into primary and secondary cell walls with the middle lamina between them. The primary cell wall is formed by high concentrations of cellulose, hemicellulose and pectin contents. The composition of each of these parts depends on the type of plant and we can find mainly different types of polysaccharides such as arabinoxylans with hydroxycinnamates in their molecular structure. The conversion of cell walls into biomass by humans has important challenges since cellulose is a very recalcitrant polysaccharide, with which researchers have put their effort in finding new techniques to improve the yield in the obtaining of this biomass. One of these techniques is to find plants with deficiency in cellulose in their structure. However, it has been shown that this deficiency involves compensation mechanisms on the part of the plant such as the general metabolism of phenolic acids.

The PhD student combined with great skill and ability numerous multidisciplinary techniques of Cellular Biology, Molecular Biology and Biochemistry plus different histochemical and Bioinformatic techniques. In this Thesis, numerous figures and illustrations of high quality are included with an easy scientific language to follow even for people not introduced in the research topic.

In this thesis, the author focuses first on the study of the general metabolism of hydroxycinnamates, highlighting cumaric acid and ferulic acid, in maize plants with cellulose deficiency. It also focuses on the content of peroxidase and how it intervenes in the general process described above and, finally, try to determine the genes involved in the acetylation of the cell wall. The doctoral student demonstrates, by radiokinetics, the existence of a more accentuated connection in the arabinoxylans of walls with lower cellulose content, strengthening said connections in this type of plants. In this sense, she demonstrates a high proportion of ferulic and coumaric acids in the wall of these plants deficient in cellulose. In addition, she demonstrates a potent activity of peroxidase when acting on these hydroxycinnamates, highlighting the important role of this enzyme in the remodeling of the cell wall. Finally, the author identifies ZmBAHD1, ZmBAHD2 and ZmBAHD12 as the genes involved in the transfer of cumaric and ferulic acids to the arabinoxylans of cell walls of cellulose-deficient plants.

The quality of the results is demonstrated by the different publications in prestigious scientific journals and they have also been discussed at various national and international conferences.

These findings reveal the mechanisms of intervention of hydroxycinnamates, peroxidases and other proteins in the dynamic remodeling of the cell walls of maize plants with deficiency in the cellulose content, opening new strategies to increase the yield in the conversion of the biomass.

Therefore, the Doctoral Thesis " Cell wall remodelling of cellulose deficient maize cells: Going through the key role of phenolics " presents all the criteria without any doubt for the recognition of the degree of Romina Martinez Rubio.

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A handwritten signature in blue ink, appearing to be 'J. Muñoz', with a stylized flourish at the end.

Newcastle. 19 de Abril de 2018

Object: External referee report

Title of the thesis: *Cell wall remodeling of cellulose maize cells: going through the key role of phenolics*

Name of the student presenting the thesis: *Romina MARTINEZ-RUBIO*

The manuscript includes first a general introduction, followed by objectives of the thesis. The different results are then presented in 4 main chapters: 1) **Chapter I** – *Phenolic metabolism and molecular mass distribution of polysaccharides in cellulose-deficient maize cells*, 2) **Chapter II** – *Changes in the cell wall phenolic profile associated to short-term DCB habituation of maize cells*, 3) **Chapter III** – *Class III peroxidases in cellulose deficient cultured maize cells during cell wall remodeling*, 4) **Chapter IV** – *A preliminary survey into the putative BAHD genes involved in the acylation of arabinoxylans in cellulose deficient maize cells*; where each chapter includes an introduction - Material and methods - Results - Discussion and References. The manuscript is enclosed by a general discussion and conclusions and appendix.

The manuscript submitted is well written and well presented, in a progressive and continuous manner. The introduction described well the importance of the plant cell walls, its structure, composition, plasticity and the consideration of the cell wall components as biotech potentials. This work is based on the use of cellulose biosynthesis inhibitor, such as dichlobenil (DCB; 2,6-dichlorobenzonitrile), as a convenient tool to study cell wall plasticity in maize cell cultures. Indeed, habituated DCB cell cultures presented altered cellulose content.

Chapter I: The authors showed that DCB habituated cells compensate the cellulose deficiency by 1) increasing their phenolic metabolism, in particularly ferulic acid and *p*-coumaric acid contents and 2) the synthesis of lower weight-average relative molecular mass molecules.

Chapter II: A quantitative and qualitative analysis of the hydroxycinnamates esterified into nascent and cell wall polysaccharides has been performed by HPLC-PAD along the cell culture cycle, between cell lines (DCB habituated cell and control lines) and cell compartments (endomembrane system and cell wall). Authors showed in particularly that DCB-treated cells remodeled their cell wall by modifying extensively their di- and oligoferulate crossed-linked arabinoxylans in order to compensate the reduction in the cell wall cellulose content.

Chapter III: Class III peroxidases (POX) have been demonstrated to play important roles in arabinoxylan cross-linking and lignin formation. This results are the first comparative analysis of POX activity and isoforms in control and cellulose-impaired cells, taking also into account their cellular distribution. The results suggest that some of the isoPOXs detected could be involved in the arabinoxylan cross-linking and lignin-like polymer formation in DCB habituated cells. This work has been done in collaboration of Dr. Anna Kärkönen (University of Helsinki, Finland).

Chapter IV: The last part addressed a preliminary view into the putative genes involved in the acylation of arabinoxylans with ferulic and *p*-coumaric acids, in DCB habituated maize cells.

Overall, by the quality of this manuscript (concise, precise, well written, fluent in English) and the quality and the number of the obtained results, Romina has done an excellent work and being really efficient. Indeed Romina has already published **two articles as first and 2nd author in high quality scientific journals** (Chapter I and III), but also has been involved during her PhD project to side projects where she is also **author of two other articles** including one as **2nd author** (Rebarque *et al.*, 2017 and Hernández-Altamirano *et al.*, 2017). Romina presented also her work in two international conferences (Finland and Greece). Romina acquired an excellent expertise on the plant cell wall

By a multidisciplinary approach (cell biology, biochemistry, molecular biology, bioinformatics), Romina thesis succeeded perfectly to bring new and interesting clues to understand the mechanisms underlying the compensation of cellulose content and opens new potential paths to understand the plant cell plasticity.

In consideration of all the above, Romina MARTINEZ-RUBIO doctoral thesis entitled **“Cell wall remodeling of cellulose maize cells: going through the key role of phenolics”** is totally suitable, without any doubt, for public reading and defense.

Date, place, signature

10th of June 2018 , Rouen

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UNIVERSIDAD DE LEÓN
FACULTAD DE CIENCIAS BIOLÓGICAS Y
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DEPARTAMENTO DE INGENIERÍA Y CIENCIAS AGRARIAS

AREA DE FISIOLÓGÍA VEGETAL

REMODELACIÓN DE LA PARED CELULAR EN
CÉLULAS DE MAÍZ CON DEFICIENCIA EN
CELULOSA: INDAGANDO EN EL PAPEL CLAVE
DE LOS FENOLES

D^a. ROMINA MARTÍNEZ RUBIO

León, Junio 2018

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La pared celular es una estructura dinámica que confiere importantes funciones mecánicas y fisiológicas a la planta. Estudios previos realizados por nuestro grupo de investigación han demostrado que la reducción en el contenido en celulosa en las paredes celulares primarias de maíz da lugar a modificaciones estructurales y de composición en la pared celular. En este contexto, el objetivo general de la Tesis fue profundizar en el conocimiento de los mecanismos de compensación asociados con la plasticidad estructural de la pared celular, prestando especial atención al papel de los compuestos fenólicos. Para ello, se emplearon suspensiones celulares de maíz que presentan una reducción en el contenido en celulosa al estar habituadas a crecer en presencia de diclobenil (DCB), un inhibidor de la biosíntesis de celulosa.

Mediante el uso de precursores marcados radiactivamente, se comprobó que la deficiencia en el contenido en celulosa promovió cambios en el metabolismo y estructura de la red de hemicelulosas, ya que las hemicelulosas unidas a la pared celular así como los polímeros liberados al medio de cultivo presentaban un tamaño más homogéneo y una menor masa molecular en células habituadas a DCB en comparación con las no habituadas. A pesar de su menor masa molecular, estas hemicelulosas estaban más entrecruzadas entre sí a través de ácidos hidroxicinámicos, principalmente ácido ferúlico y *p*-cumárico, posiblemente como estrategia de compensación para contrarrestar la deficiencia en el contenido en celulosa.

El estudio comparativo de los ácidos hidroxicinámicos esterificados a polisacáridos en las fracciones protoplásmica y de pared celular a lo largo del ciclo de cultivo revelaron que las células habituadas mostraban un enriquecimiento en los ácidos ferúlico y *p*-cumárico así como en dímeros de ácido ferúlico. A su vez, el dímero 8-O-4' apareció como un dímero exclusivo del proceso de habituación. Estos resultados apoyan la idea de que los ácidos hidroxicinámicos juegan un papel relevante en la remodelación de paredes celulares con deficiencia en celulosa.

Para explicar las bases moleculares que explican una pared celular enriquecida en ácidos hidroxicinámicos extensamente entrecruzados, se fijó el objetivo sobre las peroxidasas, responsables de la dimerización, y sobre los genes BAHD, responsables de la feruloilación/cumaroilación de arabinoxilanos. Para ello, se

realizó un estudio comparativo de la actividad peroxidasa y sus isoformas relacionadas con la remodelación de la pared celular. La actividad peroxidasa fue mayor en las líneas celulares habituadas con respecto a las control en todos los compartimentos celulares analizados. Además, la comparación del perfil de isoperoxidasas obtenido en las fracciones de proteínas unidas iónicamente a la pared celular y las liberadas al medio de cultivo indicó una mayor actividad en ciertas isoformas en líneas celulares habituadas.

Con el objetivo de profundizar en el conocimiento de los genes relacionados con la feruloilación y cumaroilación de la pared celular, se llevó a cabo un estudio preliminar de los genes BAHD en maíz. Mediante análisis bioinformáticos se identificaron trece genes BAHD homólogos a los encontrados previamente en arroz y arabidopsis, de tal forma que todas las secuencias proteicas compartían el dominio transferasa HXXXDG característico de las proteínas BAHD relacionadas con la acilación de arabinosilanos. De todos los genes BAHD analizados, los *ZmBAHD1*, *ZmBAHD2* y *ZmBAHD12* estaban sobreexpresados en líneas celulares habituadas. Es de destacar que los genes *ZmBAHD1* y *ZmBAHD12* mostraron niveles de co-expresión elevados con genes relacionados con la síntesis y remodelación de la pared celular. Por lo tanto, estos resultados apuntan a que los genes *ZmBAHD1* y *ZmBAHD12* son buenos candidatos como feruloil/cumaroil transferasas en paredes celulares de maíz en un contexto de remodelación como respuesta a una reducción en el contenido en celulosa.

Por último, se trató de verificar si la cobtorina, un inhibidor de la biosíntesis de celulosa con un mecanismo de acción diferente al del DCB, induce lignificación ectópica, ya que en suspensiones celulares expuestas a DCB se había comprobado dicha respuesta. La ausencia de tinción con floroglucinol en las células tratadas con cobtorina indicó que este compuesto no induce lignificación ectópica en nuestro sistema experimental. Por el contrario, las células tratadas con DCB mostraron tinción positiva para floroglucinol, una intensa autofluorescencia y fluorescencia inducida por amonio relacionada con la acumulación de compuestos fenólicos. Todos estos hallazgos indicaron la presencia de un polímero tipo lignina en células tratadas con DCB. A su vez, se detectó en estas células la sobreexpresión de los genes *ZmBAHD5*, *ZmBAHD6*, *ZmBAHD9* y *ZmBAHD12*, junto con un incremento de

la actividad peroxidasa. Por tanto, la inducción de lignificación ectópica promovida por una reducción en el contenido en celulosa parece ser un proceso espacio-temporal en el que los cambios en la expresión de ciertos genes BAHD y en la actividad peroxidasa, junto con modificaciones estructurales, contribuyen al reforzamiento de la pared celular.

Los resultados obtenidos en la presente Tesis proporcionan nueva información que ahonda en la contribución clave de los compuestos fenólicos en la remodelación de paredes celulares con un bajo contenido en celulosa. Además, se proporciona información adicional sobre el control bioquímico y génico de este proceso.

Objetivos

En los últimos años se ha puesto de manifiesto que las paredes celulares de las plantas poseen una elevada plasticidad estructural y de composición. Estudios previos llevados a cabo en nuestro grupo de investigación han demostrado que una reducción en el contenido en celulosa en paredes celulares de maíz da lugar a una pared celular con una arquitectura y composición modificadas, en la que se provocan cambios en la cantidad y estructura del resto de componentes de la pared celular, poniendo de manifiesto la relación funcional existente entre los diferentes polímeros que componen tal estructura. En este contexto, el objetivo de esta Tesis fue ahondar en el papel que los compuestos fenólicos desempeñan en la remodelación de paredes celulares con deficiencia en celulosa.

Para lograr este objetivo, se realizó un estudio con suspensiones celulares de maíz habituadas a crecer en presencia del inhibidor de la biosíntesis de celulosa DCB, y que como consecuencia sufren una reducción en el contenido de este polisacárido.

Por consiguiente, la hipótesis de este trabajo es que las células con deficiencia en celulosa readaptan su metabolismo fenólico, lo cual afecta a la composición y estructura de la pared celular al modificar el contenido de ácidos hidroxicinámicos esterificados a la pared, el patrón de dimerización del ácido ferúlico y al promover la deposición de lignina ectópica. Como los ácidos hidroxicinámicos y la lignina son susceptibles de la acción de las peroxidasas, caben esperar cambios en la actividad peroxidasa y en su perfil de isoformas. Así mismo, esperamos que exista una expresión diferencial en los genes relacionados con la feruolailación/cumaroilación como parte del proceso de remodelación celular.

Para dilucidar estos mecanismos, se han propuesto cinco objetivos parciales:

- Para comprender mejor los cambios que se producen en el metabolismo fenólico de células con una reducción en el contenido en celulosa, se estudiará el metabolismo de los ácidos hidroxicinámicos de la pared celular en líneas celulares no habituadas y habituadas a DCB. Para ello, se llevará a cabo un conjunto de experimentos de radiomarcaje empleando ácido *trans*-[¹⁴C]cinámico (precursor de los ácidos hidroxicinámicos). Se analizarán las cinéticas de consumo e incorporación de ácido [¹⁴C]cinámico y de los compuestos marcados con ¹⁴C en las

fracciones protoplásmica y de pared celular a lo largo del ciclo de cultivo. Así mismo, se realizará un análisis detallado del tipo de compuestos derivados del ácido [^{14}C]cinámico que se incorporen a la pared celular (**Capítulo I**).

- Para obtener más información acerca de la contribución que los fenoles de la pared celular tienen en la remodelación de esta estructura en células con deficiencia en celulosa, se llevará a cabo un estudio de los cambios tanto cualitativos como cuantitativos que se producen en los ácidos hidroxycinámicos esterificados en polisacáridos en formación (fracción protoplásmica) y en la pared celular a lo largo del ciclo de cultivo. Se prestará especial atención a los cambios que se produzcan en el grado de dimerización y en el patrón diferencial de dímeros de ácido ferúlico entre líneas celulares (no habituadas y habituadas a DCB) y compartimentos analizados (sistema endomembrana *versus* pared celular) (**Capítulo II**).

- Para ahondar en la relación existente entre las peroxidasas y la remodelación de la pared celular que conduce a cambios en el perfil fenólico, se realizará un estudio comparativo de la actividad peroxidasa y de sus isoformas en células control y con deficiencia en celulosa, analizando la actividad peroxidasa total y específica frente a distintos sustratos naturales en diferentes compartimentos celulares. A su vez, se llevará a cabo un análisis comparativo del perfil de isoperoxidasas entre los diferentes compartimentos celulares en células no habituadas y habituadas a DCB (**Capítulo III**).

- Como punto de partida para el estudio de los genes candidatos a ser responsables de la acilación de arabinoxilanos con los ácidos ferúlico y *p*-cumárico, se identificarán los genes de maíz CoA-aciltransferasa pertenecientes a la familia Pfam PF02458 homólogos a genes de arroz y *Arabidopsis*. Se realizará un análisis comparativo de la expresión relativa mediante RT-PCR de genes seleccionados en líneas celulares no habituadas y habituadas a DCB (**Capítulo IV**).

- Con el objetivo de determinar si la cobtorina, un inhibidor de la biosíntesis de celulosa con un mecanismo de acción diferente al del DCB, también desencadena lignificación ectópica en respuesta a una reducción en el contenido en celulosa, se tratarán suspensiones celulares de maíz con cobtorina y DCB. Se monitorizará el

proceso de lignificación ectópica a lo largo del ciclo de cultivo mediante el empleo de técnicas microscópicas. Adicionalmente, se llevará a cabo un estudio comparativo de la expresión relativa de genes BAHD seleccionados junto con un análisis de la actividad peroxidasa en células control y tratadas con DCB (**Capítulo V**).

Conclusiones

En base a los resultados obtenidos en esta Tesis podemos concluir que:

1. Las suspensiones celulares de maíz habituadas a crecer en presencia de bajas concentraciones de DCB (1.5 μ M) mostraron importantes cambios en su metabolismo fenólico a lo largo del ciclo de cultivo. Aunque no se detectaron diferencias en el consumo del ácido [14 C]cinámico del medio de cultivo, las células habituadas presentaron una deficiencia en la capacidad de síntesis e incorporación a la pared celular de moléculas feruloiladas marcadas con 14 C. Sin embargo, a pesar de esta capacidad reducida, las células habituadas a DCB mostraron un incremento relativo en el contenido de dímeros de ácido ferúlico, apoyando la hipótesis de que una red más entrecruzada de arabinoxilanos es crucial como estrategia de reforzamiento de paredes celulares con deficiencia en celulosa.

2. Se observó un patrón diferencial en el metabolismo fenólico en células no habituadas y habituadas a DCB 1.5 μ M en el protoplasma y en la pared celular. En la fracción protoplásmica de células habituadas se detectaron niveles ligeramente más altos de los ácidos *p*-cumárico y ferúlico en comparación con las no habituadas. Sin embargo, en las células habituadas el incremento en los niveles de los ácidos *p*-cumárico y ferúlico incorporados a las paredes celulares fue muy elevado a lo largo de todo el ciclo de cultivo, indicando que se produce una extra feruloilación/cumaroilación *in muro* en respuesta a una reducción en el contenido en celulosa. Las suspensiones celulares habituadas a DCB mostraron un incremento en los dímeros de ácido ferúlico, especialmente en los diferulatos 8,5' y 5,5'. Así mismo, el dímero 8-O-4' apareció como específico del proceso de habituación a DCB.

3. El estudio comparativo de la actividad peroxidasa y sus isoformas en células habituadas a DCB reveló una fuerte actividad peroxidasa frente a ácido ferúlico y a los alcoholes coniferil y sinapil tanto en la fracción protoplásmica como en las apoplásticas. Se encontró un perfil diferencial de isoperoxidasas en las fracciones de proteínas unidas iónicamente a la pared celular y en las liberadas al medio de cultivo en líneas celulares habituadas a DCB. Todos estos resultados apoyan la idea de que las peroxidasas juegan un papel relevante en la remodelación de paredes celulares de maíz en respuesta a una reducción en el contenido en celulosa.

4. Mediante análisis bioinformáticos se identificaron trece genes de maíz pertenecientes a la familia Pfam PF02458. Todas las secuencias proteicas compartían el dominio transferasa HXXXDG característico de las proteínas BAHD relacionadas con la acilación de arabinoxilanos. De los 13 genes identificados, seis de ellos se expresaron en ambas líneas celulares, no habituadas y habituadas a DCB. El patrón de expresión de tres de ellos (*ZmBAHD1*, *ZmBAHD2* y *ZmBAHD12*) junto con los perfiles de co-expresión con otros genes relacionados con la pared celular sugieren que éstos son candidatos a ser responsables de la feruloilación/cumaroilación en cultivos celulares de maíz con un contenido reducido en celulosa.

5. Las suspensiones celulares de maíz tratadas con cobtorina 10 μ M no mostraron lignificación ectópica, mientras que aquellas expuestas a DCB 6 o 10 μ M depositaron un material tipo lignina que se teñía con floroglucinol, acompañado por una progresiva acumulación de fenoles en las paredes celulares. Además, el tratamiento con DCB se asoció a la sobreexpresión de los genes *ZmBAHD5*, 6, 9 y 12 y a un incremento de la actividad de peroxidasas de clase III. Por lo tanto, la acumulación de lignina ectópica inducida por DCB parece ser un proceso espacio-temporal mediado por la expresión de varios genes BAHD y un incremento de la actividad peroxidasa, que contribuye a reforzar la estructura debilitada de la pared celular como consecuencia de una deficiencia en celulosa.

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