JIPB Journal of Integrative Plant Biology

Ectopic lignification in primary cellulose-deficient cell walls of maize cell suspension cultures

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Abstract Maize (*Zea mays* L.) suspension-cultured cells with up to 70% less cellulose[Q1] were obtained by stepwise habituation to dichlobenil (DCB), a cellulose biosynthesis inhibitor. Cellulose deficiency was accompanied by marked changes in cell wall matrix polysaccharides and phenolics as revealed by Fourier transform infrared (FTIR) spectroscopy. Cell wall compositional analysis indicated that the cellulose-deficient cell walls showed an enhancement of highly branched and cross-linked arabinoxylans, as well as an increased content in ferulic acid, diferulates and *p*-coumaric acid, and the presence of a polymer that stained positive for phloroglucinol. In accordance with this, cellulose-deficient cell walls showed a fivefold increase in Klason-type lignin. Thioacidolysis/GC-MS analysis of cellulose-deficient cell walls indicated the presence of a lignin-like polymer with a Syringyl/Guaiacyl ratio of 1.45, which differed from the *sensu stricto* stress-related lignin that arose in response to short-term DCB-treatments. Gene expression analysis of these cells indicated an overexpression of genes specific for the biosynthesis of monolignol units of lignin. A study of stress signaling pathways revealed an overexpression of some of the jasmonate signaling pathway genes, which might trigger ectopic lignification in response to cell wall integrity disruptions. In summary, the structural plasticity of primary cell walls is proven, since a lignification process is possible in response to cellulose impoverishment.

Keywords: Cellulose; dichlobenil; dichlobenil; ectopic lignin; maize
Citation: Hugo M Hugo M, Asier L-G Asier L-G, Esther N-U Esther N-U, Rogelio S Rogelio S, Federico P Federico P, Pedro G Pedro G, Penélope G-a Penélope G-a, José Luis A José Luis A, Jesús Á Jesús Á, Antonio E Antonio E (2015) Ectopic lignification in primary cellulose-deficient cell walls of maize cell suspension cultures. **J Integr Plant Biol** XX:XX–XX doi: 10.1111/jipb.12346
Edited by: Kurt Fagerstedt, Helsinki University, Finland
Received Nov. 28, 2014; **Accepted** Feb. 25, 2015
Available online on Mar. 4, 2015 at www.wileyonlinelibrary.com/journal/jipb

INTRODUCTION

The primary cell wall is a complex structure surrounding the protoplasm of elongating plant cells and it is crucial for shape maintenance and directional growth during cell development (Carpita 1996). Moreover, as the outermost layer of the plant cell, it is an active component in response to biotic and abiotic stresses with the capacity to monitor and maintain its integrity by means of structural and compositional changes (Hamann 2014). As with other grasses, the primary cell wall in maize (type II) is mainly composed of a framework of cellulose microfibrils embedded in a matrix of arabinoxylans. Smaller amounts of xyloglucan, mixed-linked glucans, pectins and glycoproteins can also be found as cell wall matrix components (Carpita 1996).

Cellulose, the main load-bearing structure of plant cell walls, is a polymer of β-1,4 linked glucan chains synthesized by transmembrane protein complexes (Guerriero et al. 2010). Cellulose is deposited in the cell wall in the form of microfibrils probably composed of 18 or 24 chains (Jarvis 2013). Arabinoxylans, the second major component of maize primary cell walls, play a pivotal role since different populations function by tethering adjacent cellulose microfibrils and forming the matrix phase of cell walls (Scheller and Ulvskov 2010). The arabinoxylan backbone is composed of β-1,4-linked xylose residues commonly substituted at C(O) 3 and/or C(O) 2 with arabinose or (4-O-methyl) glucuronic acid (Fincher 2009). One of the unique features of arabinoxylans from grasses is that the arabinose residues are often esterified at C(O) 5 with the hydroxycinnamates, ferulic and *p*-coumaric acid. Due to their high reactivity, polysaccharide-esterified hydroxycinnamates promote arabinoxylan cross-linking, playing a major role in maintaining the integrity of grass cell walls (Buanafina 2009).

Lignin is a complex phenolic heteropolymer predominantly deposited in the secondarily thickened cell walls of

specialized plant cell types. Lignin drastically modifies cell wall structure and functions, since after its deposition cell walls acquire hydrophobicity and increase their resistance to mechanical and chemical degradation (Vanholme et al. 2010; Liu 2012) being a key factor in the evolution of tracheophytes vascular system (Lucas et al. 2013). The main building blocks of lignin are the 4-hydroxycinnamyl alcohols (or monolignols): coniferyl and sinapyl alcohols with lesser amounts of *p*-coumaryl alcohol (Boerjan et al. 2003). Monolignols are synthesized in the cytosol from phenylalanine by the phenylpropanoid pathway and transported into the cell wall where they are subjected to oxidative cross-linking by cell wall peroxidases, laccases or otherphenol oxidases using hydrogen peroxide or oxygen as oxidants (Passardi et al. 2004; Fagerstedt et al. 2010; Kärkönen and Kuchitsu 2014). Once polymerized into lignin, *p*-coumaryl, coniferyl and sinapyl alcohol give rise to *p*-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) units, respectively (Vanholme et al. 2010; Liu 2012).

Lignification is a tightly developmentally regulated process commonly associated with the formation of a secondarily thickened cell wall during cell specialization. Besides, the developmentally regulated lignin, biotic and abiotic stresses can induce unexpected lignification known as ectopic lignification (Caño-Delgado et al. 2000; Moura et al. 2010; Sattler and Funnell-Harris 2013; Miedes et al. 2014). Although there are very few reports in the literature of this phenomenon in exclusively primary-walled cell cultures, lignin-like polymers have been shown to be produced by *in vitro* model systems under certain conditions (Novo-Uzal et al. 2009; Kärkönen and Koutaniemi 2010; Shen et al. 2013). In some systems, hormonally triggered cells can differentiate into tracheary elements, in which lignin is deposited in the newly formed secondary cell wall (Fukuda and Komamine 1980; Oda et al. 2005). In other cases, triggered cell cultures (normally by sucrose or elicitor treatments) release extracellular lignin into the culture medium (Simola et al. 1992; Lange et al. 1995; Nose et al. 1995; Kärkönen et al. 2009).

In the last few decades, a series of different approaches using cellulose biosynthesis inhibitors, mutants or transgenic plants have revealed compensatory effects between cellulose and non-cellulosic components of both primary and secondary cell walls. A reduction in cellulose content or an altered pattern of cellulose deposition has been demonstrated to cause changes in matrix polysaccharides and cell wall ectopic lignification (Caño-Delgado et al. 2000, 2003; Desprez et al. 2002; Ellis et al. 2002; Hernández-Blanco et al. 2007; Bischoff et al. 2009; Hamann et al. 2009; Denness et al. 2011; Brabham et al. 2014). Furthermore, lignin-defective transgenic plants respond with qualitative and quantitative changes in the polysaccharide counterpart[Q2] (Sonbol et al. 2009; Ambavaram et al. 2011; Fornalé et al. 2012).

In previous studies, maize cell lines habituated to otherwise lethal concentrations of DCB (2,6-dichlorobenzonitrile, dichlobenil), a well-known cellulose biosynthesis inhibitor, were obtained by means of incremental exposure over many culturing cycles (Mélida et al. 2009; de Castro et al. 2014). These cell cultures had the capacity to cope with DCB through the acquisition of a modified cell wall in which the cellulosic scaffold was completely or partially replaced by a more extensive network of highly cross-linked arabinoxylans (Mélida et al. 2009 2010a, 2010b, 2011). Our preliminary data indicated that DCB habituation could also induce ectopic lignification (as cellulose-deficient walls from habituated cells resulted positively for phloroglucinol staining) as a consequence of a reduction in cellulose in maize cells. An in-depth characterization of this phenomenon could further our understanding of the chemical composition of ectopic lignin and the relationship between ectopic lignification and stress responses. In this study, we characterized cell walls from maize suspension-cultured cells habituated to low (1 μM) and high (6 μM) DCB concentrations and from DCB short-term treated cell suspensions (I_{50} value for maize suspension cultured cells is 0.5 μM DCB; de Castro et al. 2014), paying special attention to the putative ectopic lignin/lignin-like component as well as the expression levels of genes specific for the biosynthesis of monolignol units of lignin and others involved in common stress signaling pathways.

RESULTS

Cell wall fingerprinting indicated increased phenolics-to-polysaccharides ratios due to DCB exposure

FTIR spectra of non-habituated (SNH), DCB short-term treated (SNH+DCB) and habituated to (SH1) 1 and (SH6) 6 μM DCB maize suspension-cultured cells were obtained, normalized and baseline corrected. Averaged difference spectra were obtained by digital subtraction of SNH spectra from each of the DCB-treated/habituated cell lines (Figure 1). Compared with SNH spectra, those from both short-term treated and habituated cell walls showed negative peaks in the region ranging from 900 to 1,200 cm^{-1} where most of the cell wall polysaccharides, including cellulose, absorb (Alonso-Simón et al. 2011; Largo-Gosens et al. 2014). In addition, positive peaks were detected associated with wave numbers indicative of aromatic rings (1,515, 1,600 and 1,630 cm^{-1}), phenolic rings (1,500 cm^{-1}) and phenolic esters (1,720 cm^{-1}) (Kačuráková et al. 2000), indicating that both DCB-treated and DCB-habituated cells were enriched in phenolics. In accordance with this, wave number ratios 1,540/1,160; 1,540/1,425 and 1,540/1,740 cm^{-1} normally associated with increased lignin-to-polysaccharides ratios raised in both DCB-treated and DCB-habituated cells (Table 1).

Highly branched and cross-linked arabinoxylans increased in parallel to the DCB habituation process

Cell wall fractionation showed that in both DCB-treated and DCB-habituated cells, most of the non-cellulosic cell wall polysaccharides (70-80%) corresponded to KOH-extractable hemicelluloses, namely KI and KII fractions (Figure S1). Moreover, differences were observed in cell wall fractionation among cell lines. Of particular note was the increase in strong alkali-extracted hemicelluloses (KII fraction: 29% in SNH vs. 42% in SH6) exclusively associated with habituation to high DCB concentrations (Figure S1).

The monosaccharide composition of each of the fractions was determined by gas chromatography and spectrophotometric methods (Figure 2). The CDTA-pectic fraction was enriched in uronic acids, and minor amounts of the neutral sugars Ara, Xyl, Gal and Glc were also detected (Figure 2A). The abundance of uronic acids compared to neutral sugars indicated the presence in the CDTA fraction of homopolymers

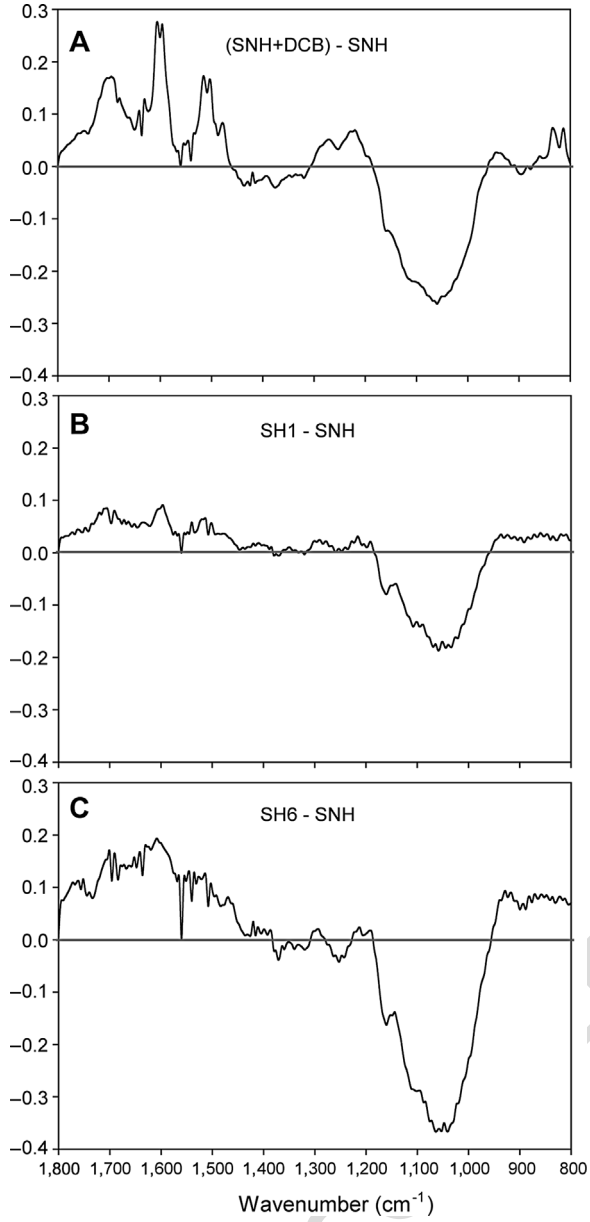

Figure 1. Fourier transform infrared (FTIR) analysis of cell walls

Averaged FTIR difference spectra obtained after digital subtraction of the spectra of non-habituated (SNH) cell wall FTIR spectra from SNH + dichlobenil (DCB), SH1 or SH6 cell wall FTIR spectra. Maize cell lines were annotated as follow: non-habituated (SNH); DCB short-term treated (SNH + DCB), habituated to 1 μM DCB (SH1) or habituated to 6 μM DCB (SH6) maize cell suspension-cultured cells.

based on the acidic sugars (i.e. homogalacturonan). However, only minor differences in the CDTA fraction were found between SNH and the rest of the lines. KOH-extracted hemicelluloses were mainly composed of Ara, Xyl and uronic acids, indicative of (glucurono-) arabinoxylans and/

Table 1. Fourier transform infrared (FTIR) wave number ratios characteristic of lignin and cell wall polysaccharides

FTIR peak height ratio	SNH	SNH + DCB	SH1	SH6
1540/1160 cm^{-1}	0.09	0.14	0.13	0.21
1540/1425 cm^{-1}	0.16	0.21	0.19	0.28
1540/1740 cm^{-1}	0.16	0.18	0.18	0.25

Peak assignations[Q3], 1,160 cm^{-1}, C–O–C vibration of the glycosidic link in cellulose, xyloglucan or pectic polysaccharides; 1,425 cm^{-1}, C–H stretching in CH_2 groups of cellulose; 1,540 cm^{-1}, aromatic ring stretching in lignin; 1,740 cm^{-1}, C–O stretch in ester groups.

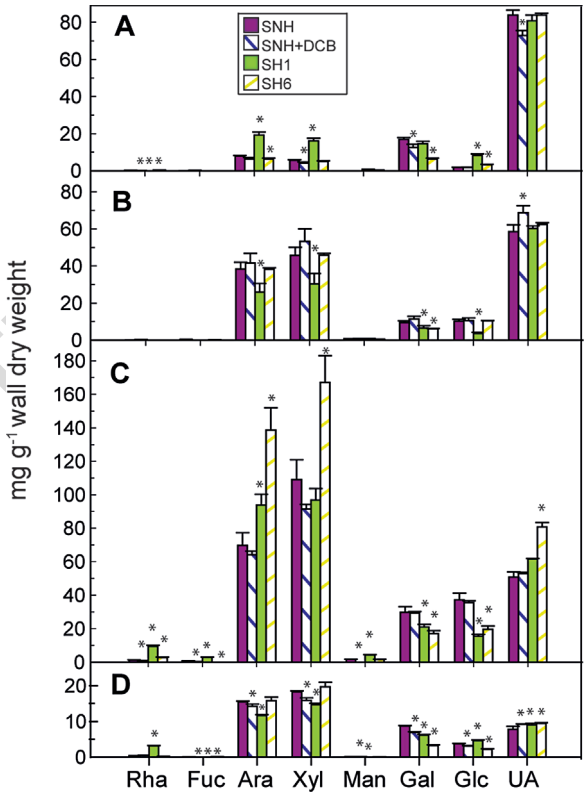

Figure 2. Cell wall sugar analysis

Sugar composition of **(A)** CDTA, **(B)** KI, **(C)** KII and **(D)** trifluoroacetic acid (TFA) cell wall fractions obtained from (open square) spectra of non-habituated (SNH), (light grey square) SNH + dichlobenil (DCB), (dark grey square) SH1 and (black square) SH6 cell lines. For maize cell line annotations see Figure 1 legend. Ara (arabinose), Fuc (fucose), Gal (galactose), Glc (glucose), Man (mannose), Rha (rhamnose), UA (uronic acids), Xyl (xylose). Data represents the means values ± standard deviation (SD) of three technical replicates. Asterisks indicate values that are significantly different from SNH after a Student's *t*-test ($P < 0.05$).

Table 2. Arabinose and xylose content in the KII fractions

	(%) Ara + Xyl		
	Cell wall	KII-extracted sugars	Ara:Xyl ratio
SNH	17.9 ± 0.9	59.5 ± 1.7	0.64
SNH+DCB	15.6 ± 0.5	56.7 ± 0.3	0.70
SH1	19.1 ± 1.3	62.4 ± 0.8	0.97
SH6	**30.6 ± 2.9**	**71.5 ± 1.4**	0.83

Mean values ± standard deviation (SD) of three technical replicates per line. For cell line annotation see Figure 1 legend.

or acidic pectins associated to arabinoxylans (de Castro et al. 2014), followed by minor amounts of Gal and Glc (Figure 2B, C). Quantitatively, KII represented the main fraction, and a monosaccharide analysis revealed an increase in the Ara and Xyl proportions associated with habituation to DCB (especially with high concentrations), but not with short-term exposures (Figure 2C; Table 2). The observed increase in the Ara-to-Xyl ratio detected in SH1 and SH6 when compared with SNH, indicated not only a quantitative increase in heteroxylans but also the presence of highly substituted xylan populations (Table 2). The final residues after CDTA and alkali extractions were TFA-hydrolyzed. Gas chromatography analysis of the TFA fraction resembled that from KI fraction with lesser proportions of the acidic sugars, suggestive of alkali-resistant heteroxylans (Figure 2D). No differences among cell lines were found for this fraction.

Cellulose reduction is a consequence of habituation to DCB

Cellulose averaged approximately 25% of the cell wall dry weight when assayed in SNH cells (Figure 3). Cellulose content decreased along DCB-habituated cells in a dose-dependent manner, up to the 50 and 70% reduction found, respectively, in SH1 and SH6 cells when compared with SNH cells (Figure 3). However, short-term incubations with the cellulose biosynthesis inhibitor did not induce significant reductions in cellulose content. On the other hand, these short-term incubations did increase the amount of a Klason-resistant residue, which could be associated with lignin or a lignin-like phenolic-rich material (Figure 3). This lignin-like material was also found to be increased in the cell wall of the DCB-habituated cells. Indeed, SH1 and SH6 cells contained approximately 2 to 5 times more of this residue, respectively, when compared with the SNH counterpart (Figure 3).

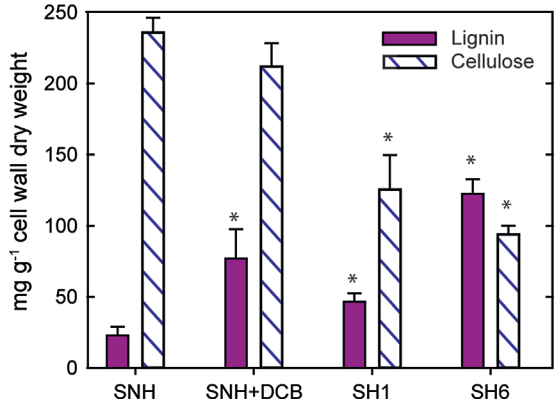

Figure 3. Comparison of cellulose and lignin content in spectra of non-habituated (SNH), SNH + dichlobenil (DCB), SH1 and SH6 cell lines

For maize cell line annotations see Figure 1 legend. Data represents means ± standard deviation (SD) of at least four replicates. Asterisks indicate values that are significantly different from SNH after a Student's *t*-test ($P < 0.05$).

Cell wall phenolic profile

Maize primary cell wall typically contains high levels of wall-esterified phenolics, which appear as side-chain decorations of arabinoxylans. *p*-Coumarate, ferulate and their oxidative coupling products, diferulates, increased steeply over the course of the DCB habituation process (Table 3). In comparison with SNH, SH1 and SH6 cell walls were enriched in the 5,5′, 8,5′ and specially the 8-*O*-4′ form of diferulates. In all cases, enrichment was more noticeable in SH6 cells.

In comparison to SNH cells, trends similar to those for the DCB-habituated cells were observed when the phenolic profile of DCB short-term treated cells was analyzed (Table 3). Most notably, there was a marked increase in cell wall esterified *p*-coumarate, with SNH+DCB cells being 132- and 15-fold enriched in *p*-coumarate when compared with SNH and SH6 cells, respectively.

DCB induced the deposition of lignin-like polymers in maize cultured cells

Phloroglucinol-HCl, which specifically stains 4-*O*-linked hydroxycinnamyl aldehyde residues of lignin (Pomar et al. 2002), was used to preliminarily confirm the presence of a lignin-like phenolic-rich material in the cell walls of DCB short-term treated and habituated cells. This strategy demonstrated that lignin accumulation depended on the presence of DCB in the culture medium (Figure 4); maize suspension-cultured cells stained negative for phloroglucinol when cultivated in a medium lacking DCB (Figure 4A). In the case of maize cells were incubated short-term in 6 μM DCB

Table 3. Cell wall esterified phenolics composition

			Diferulates			
	p-Coumarate	Ferulate	Total	5,5′-	8-O-4′-	8,5′-[a]
	mg g^{-1} cell wall					
SNH	0.21	0.82	1.83	0.48	0.76	0.59
SNH+DCB	27.80	6.60	2.33	0.56	1.20	0.57
SH1	0.52	11.55	2.29	0.54	1.13	0.62
SH6	1.83	17.71	3.58	1.07	1.58	0.93

Mean values from two independent experiments per cell line. For cell line annotation see Figure 1 legend. [a] 8,5′-diferulate was calculated as the sum of 8-5-open and 8-5 benzofurans forms.

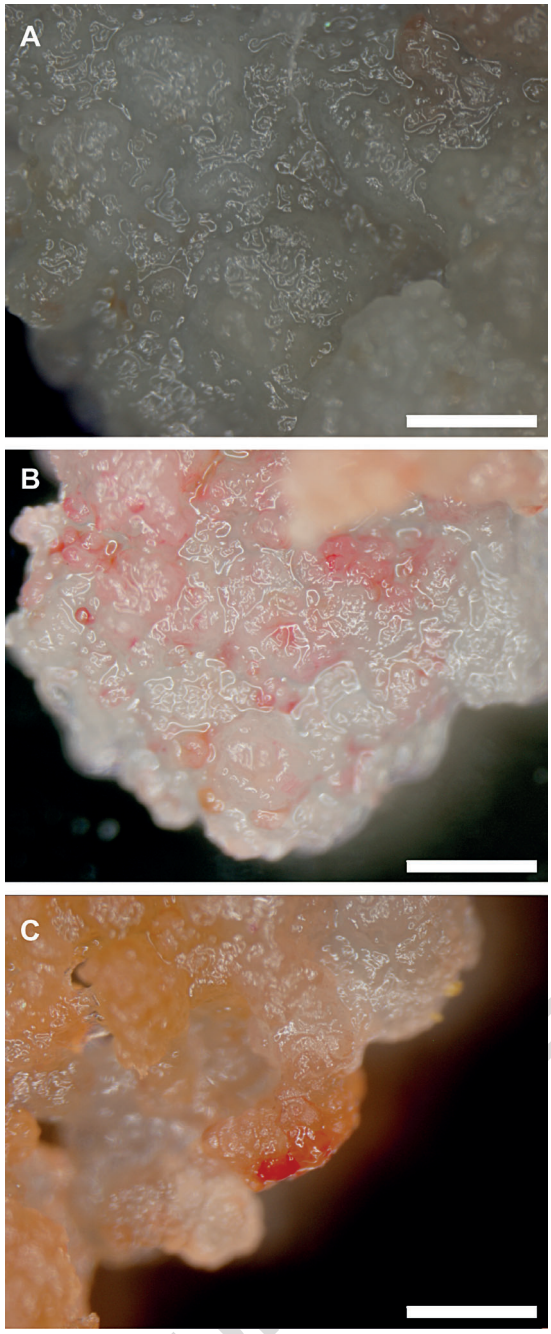

Figure 4. Phloroglucinol/HCl staining of spectra of non-habituated (SNH), SNH + dichlobenil (DCB), and SH6 cells
For maize cell line annotations see Figure 1 legend. Bar = 0.5 mm.

(Figure 4B) and DCB-habituated cells (Figure 4C), positive phloroglucinol staining was observed on the surface of cell-aggregates. No evidence of differentiation into tracheary elements were observed in any case (data not shown).

The presence of lignin-like polymers was further confirmed by thioacidolysis followed by gas chromatography coupled to mass spectrometry (GC-MS) of the cleavage products. This analysis also confirmed the presence of trace amounts of sinapyl alcohol (S units) in SNH, SNH+DCB and SH1 cell walls (Figure 5B, D and data not shown). In addition to the S units, measurable amounts of coniferyl alcohol (G units) were detected in SH6 cell walls, but not in the other cell lines (Figure 5E, F). Indeed, S units were (semi-quantitatively) estimated to be more abundant in SH6 than in any other cell line. Based on thioacidolysis results, an S/G ratio of 1.45 was estimated for the SH6 lignin-like material (Table 4).

Lignin biosynthesis-specific genes are overexpressed in DCB-habituated cells

In a previous study by our group, we demonstrated that the genes functioning in the initial steps of the phenylpropanoid pathway (*Phenylalanine Ammonia-Lyase*, *Cinnamate 4-Hydroxylase*, *4-Coumarate CoA Ligase*, *Hydroxycinnamoyl-CoA Shikimate/quinate hydroxycinnamoyl Transferase* and *Caffeic acid O-Methyltransferase*) are overexpressed in DCB-habituated cells (Mélida et al. 2010a). The corresponding proteins from such genes are involved in the production of *p*-coumaroyl-CoA and feruloyl-CoA, the substrates for hydroxycinnamate esterification of arabinoxylans (Lindsay and Fry 2008). Given the evidence of the presence of lignin-like polymers in DCB-habituated cells, quantitative RT-PCR was used to monitor the transcript abundance of *cinnamoyl-CoA reductase* (*CCR*), *ferulate 5-hydroxylase* (*F5H*) and *cinnamyl alcohol dehydrogenase* (*CAD*), involved in the last steps of monolignol synthesis (Figures 6, 7).

A general overexpression of the two *ZmCCR* genes was observed in DCB-habituated cell lines (Figure 6A, B), whereas short-term treatment with DCB induced only minor changes in *ZmCCR1* and *ZmCCR2* mRNA levels. The expression of *ZmF5H2* was significantly increased in all cell lines when compared with SNH cells, and this enhancement was especially noticeable in DCB-habituated lines (Figure 6D). In the case of *ZmF5H1*, only SH1 cells showed higher transcript abundance, and indeed this gene was repressed in SNH+DCB and SH6 (Figure 6C). Both DCB short-term treatment and DCB habituation induced an overexpression of *ZmCAD1*, *ZmCAD5* and *ZmCAD7* genes in comparison with SNH cells (Figure 7A, D, F). This enhancement was especially marked in the case of *ZmCAD7* transcript levels in SH1 cells, whereas the abundance of *ZmCAD6* transcripts was only significantly increased in habituated cells (Figure 7E). Moreover, there was a high overexpression (12-fold) of *ZmCAD2* in SH6 cells; however, the transcript levels of this gene were significantly reduced in SH1 cells (Figure 7B). Surprisingly, there was a significant repression of the transcript levels coding for ZmCAD3 in DCB-habituated cell lines (Figure 7C). The *ZmCAD4* transcription levels were too low to be accurately quantified by this procedure. Given the general overexpression of the genes coding for CAD proteins in response to DCB, we measured CAD activity in the different cell lines (Figure 7G). CAD activity assayed from cell extracts was significantly increased in SNH+DCB and SH6 when compared with SNH cell lines, but unchanged in the case of SH1 cells.

Apoplastic hydrogen peroxide accumulation

Both DCB short-term treated and DCB-habituated cells accumulated significantly more H_2O_2 in the spent medium

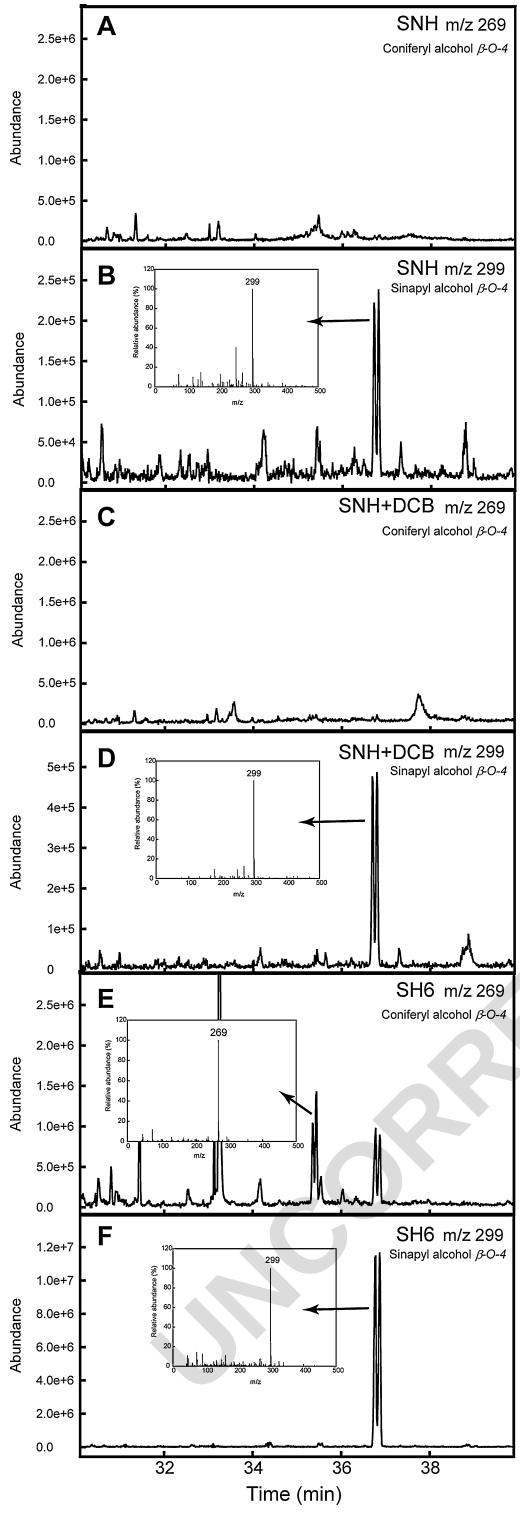

Figure 5. Lignin monomer composition
Gas chromatography (GC) profiles of the thioethylated monomers (erythro and threo isomers) arising from aryl-glycerol-b-aryl ether (β-*O*-4) structures derived from **(A**, **C** and **E)** coniferyl and **(B**, **D** and **F)** sinapyl alcohols from cell walls of **(A**, **B)** SNH; **(C**, **D)** SNH+DCB; and **(E**, **F)** SH6 cell lines**.** For maize cell line annotations see Figure 1 legend.

Table 4. Lignin monomeric composition as revealed by thioacidolysis

	Total ionic current ($\times 10^6$) mg^{-1} cell wall		
	Coniferyl alcohol (G units)	Sinapyl alcohol (S units)	S/G ratio
SNH	0	7.1 ± 1.2	–
SNH+DCB	0	**9.7 ± 0.7**	–
SH1	0	**9.5 ± 0.2**	–
SH6	**20.6 ± 1.0**	**29.9 ± 2.5**	1.45

Mean values ± standard deviation (SD) of three replicates per cell line. For cell line annotation see Figure 1 legend. Values

than SNH cells (Table 5), although there is no clear relationship between H_2O_2 accumulation and the presence of lignin-like polymers. SH6 cells, which showed the strongest ectopic lignification, did not peak in H_2O_2 content when compared with SH1 or SNH+DCB cells. In fact, SH6 cells accumulated less H_2O_2 during the lag and exponential phases than SH1 or SNH+DCB cells.

JA synthetic and JA signalling pathways overexpressed

To determine whether the accumulation of lignin-like material formed part of an abiotic stress response mechanism, RT-PCR was used to monitor the expression levels of several genes from the jasmonic acid (JA) and salicylic acid (SA) stress signaling pathways (Figure 8). Three *12-oxophytodienoate reductase* (*OPR*) genes, coding for proteins involved in the synthesis of JA, were analyzed. Two of them (*ZmOPR1* and *ZmOPR2*) were always overexpressed in the presence of DCB, but *ZmOPR7* was only overexpressed in DCB-habituated cell lines, and was slightly repressed by the short-term exposure of SNH cells to DCB (Figure 8). *NADPH oxidase* (*NADPHOX*) and *maize protease inhibitor* (*MPI*) genes are reported to be JA-induced in response to abiotic stresses (Shivaji et al. 2010). The results showed that both genes were overexpressed in the presence of DCB.

For the SA stress signaling pathway, *pathogenesis related protein 1* (*PR1*) and *non-expressor of PR1* (*NPR1*) genes were studied. The *ZmNPR1* gene was detected, but there were no differences in the expression pattern induced by either DCB exposure or DCB habituation. *ZmPR1* transcripts were not detected in any cell line.

DISCUSSION

In their natural habitats, plant cells must continuously remodel their cell walls in order to grow and to interact with the environment. In order to understand the limits of these interactions, plant cells can be cultivated in fully controlled experimental systems where their capacity to cope with different situations can be better studied. The habituation of plant cell cultures to cellulose biosynthesis inhibitors such as DCB represents a valuable tool to improve our knowledge of the mechanisms involved in plant cell wall structural plasticity (Shedletzky et al. 1992; Encina et al. 2002;

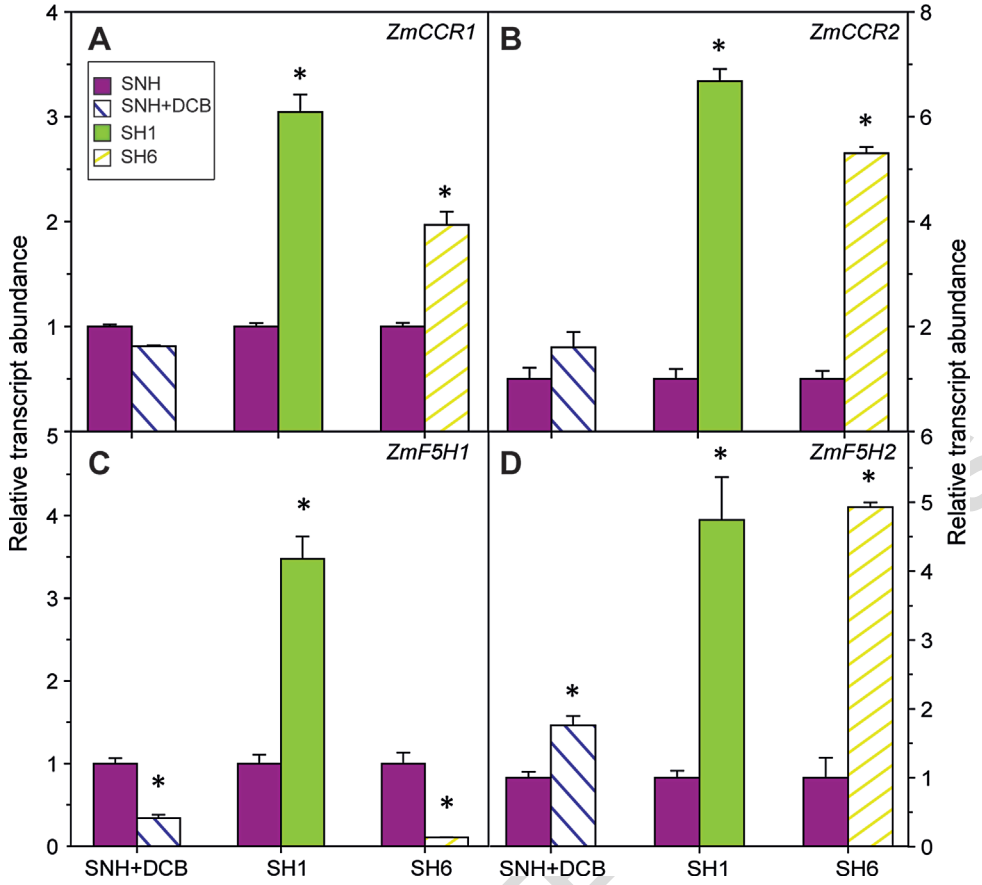

Figure 6. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) characterization of (A, B) *ZmCCR* and (C, D) *ZmF5H* genes of spectra of non-habituated (SNH), SNH + dichlobenil (DCB), SH1 and SH6 cell lines
The gene expression levels of SNH+DCB, SH1 and SH6 cell lines were always compared against the SNH ones, which are represented as left-sided bars. For maize cell line annotations see Figure 1 legend. Data represent relative fold change relative to SNH genes ± standard deviation (SD) of three replicates. Asterisks indicate values that are significantly different from SNH after a Student's *t*-test ($P < 0.05$).

Manfield et al. 2004; García-Angulo et al. 2009; Mélida et al. 2009; Brochu et al. 2010; de Castro et al. 2014, 2015).

In previous studies, we have shown that the habituation of maize cells to DCB involves several metabolic modifications (Mélida et al. 2010a; de Castro et al. 2014, 2015). Maize cells habituated to high DCB levels (≥ 30 times higher than DCB I_{50} value) display strong reduction in cellulose and altered expression of several *Cellulose Synthase* genes (Mélida et al. 2009; 2010a). Although DCB induces oxidative damage (based on lipid peroxidation levels in maize cultured cells; unpublished results), given the level of detoxifying/antioxidant activities measured, it seems that DCB-habituated maize cells do not rely on an antioxidant strategy to cope with this herbicide, which contrasts with the strategy observed in cells of other species, such as bean, in which antioxidant capacity is enhanced when habituated to DCB (García-Angulo et al. 2009; Mélida et al. 2010a). Indeed, the ability of maize cells to grow under high DCB concentrations resides mainly in their capacity to reorganize their cell wall architecture. Through compositional analysis and structural characterization of DCB-habituated cell walls, it has been possible to demonstrate that these cells compensate for cellulose impoverishment with other cell wall components. The mechanism for this accommodation consists of producing a more extensive, cross-linked network of arabinoxylans (Mélida et al. 2009; 2010a, 2010b, 2011). More recently, we have found that some of the cell wall modifications differ according to DCB habituation level (de Castro et al. 2014).

In this study, we used maize cell suspension cultures habituated to low (1 μM DCB, SH1) and high (6 μM DCB, SH6) levels of DCB as well as non-habituated cells treated for a short time with lethal doses of the herbicide (SNH+6 μM DCB). In agreement with previous studies, we have shown that habituated cell lines display dose-dependent reductions in their cellulose content. These cellulose reductions (up to 70% less than in SNH) were compensated by a more extensive network of arabinoxylans, which could only be extracted with strong alkali.

In line with previous results obtained for maize callus cultures habituated to high DCB concentrations (Mélida et al.

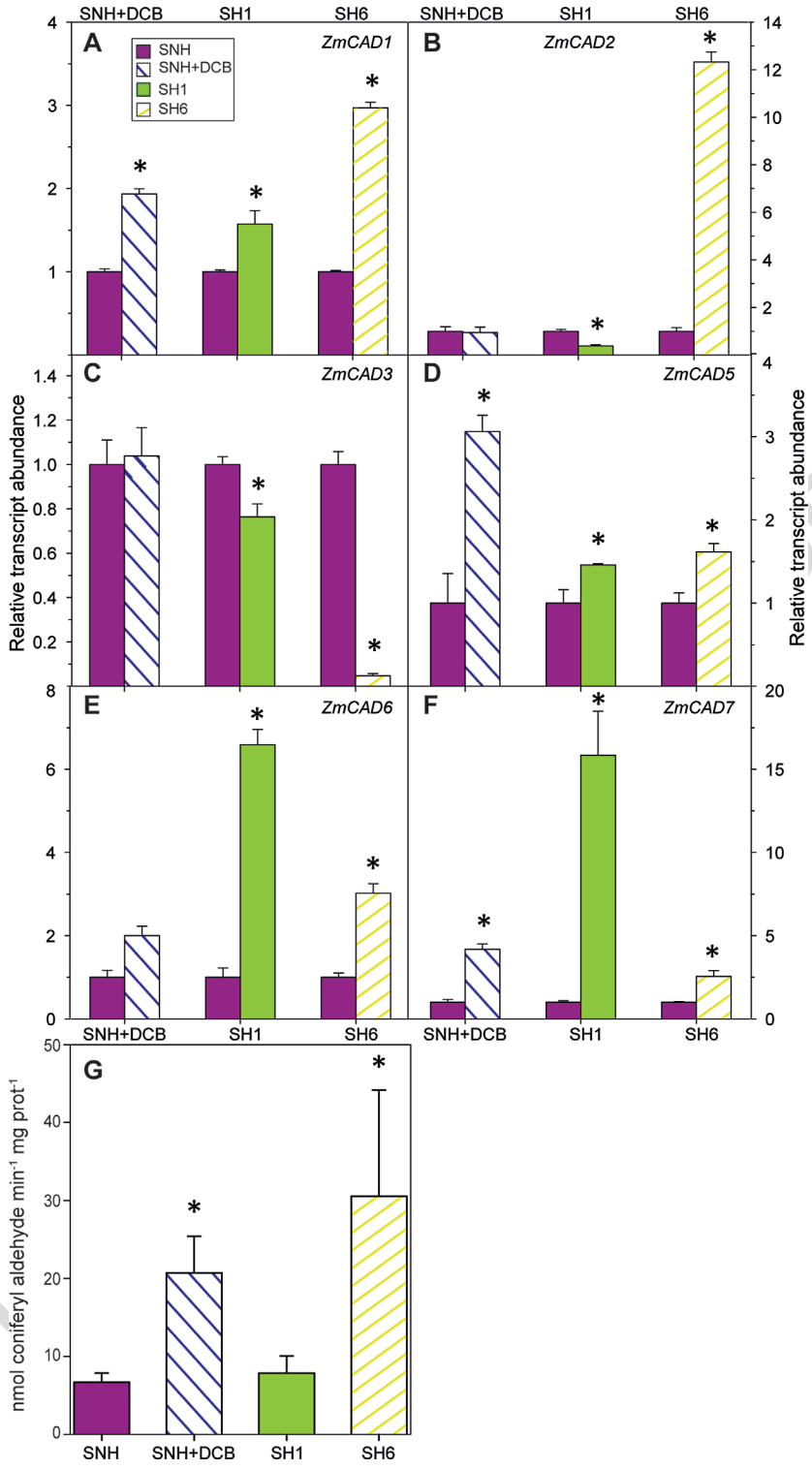

Figure 7. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) characterization of (A–F) *ZmCAD* genes and (G) CAD enzyme activity assay of spectra of non-habituated (SNH), SNH + dichlobenil (DCB), SH1 and SH6 cell lines

The gene expression levels of SNH+DCB, SH1 and SH6 cell lines were always compared against the SNH ones, which are represented as left-sided bars. For maize cell line annotations see Figure 1 legend. For A–F, data represent relative fold change relative to SNH genes ± standard deviation (SD) of three replicates. For G, data represents means ± SD of at least nine replicates. Asterisks indicate values that are significantly different from SNH after a Student's *t*-test ($P < 0.05$). *ZmCAD4* mRNA transcripts were not detected.

Table 5. Apoplastic H_2O_2 concentration measured in the spent medium of the different cell lines

	Lag phase	Exponential phase	Stationary phase
		H_2O_2 (μM)	
SNH	0.4 ± 0.3	0.8 ± 0.1	0.6 ± 0.1
SH1	**1.9 ± 0.3**	**2.4 ± 0.2**	**2.3 ± 0.2**
SH6	**0.8 ± 0.1**	**1.7 ± 0.5**	**2.4 ± 0.2**
Incubation time	1 day	6 days	
SNH+DCB	**1.0 ± 0.1**	**2.0 ± 0.1**	

Mean values ± standard deviation (SD) of three replicates per line. Values were obtained at the different growth phases for each line. Short-term treated cells (SNH + DCB) were measured 1 day and 6 days after the addition of DCB. Values

2010b; 2011), we found that hydroxycinnamates, the arabinoxylan cross-linkers, experienced quantitative changes that indicated a prominent role of these compounds in a cellulose-deficient cell wall. This is actually one of the singularities of this model system. Most of the cell lines habituated to cellulose biosynthesis inhibitors (or other cell wall stresses) have had type I primary cell walls (i.e. *Arabidopsis*, poplar, bean, tomato), where cellulose reductions were compensated by pectins (Shedletzky et al. 1990; Encina et al. 2002; Manfield et al. 2004; Brochu et al. 2010). In contrast to type I, type II primary cell walls are characterized by the presence of phenylpropanoids (mainly ferulic and *p*-coumaric acids), which have an important role in cross-linking hemicelluloses (Wallace and Fry 1994). Ferulate and its dimers increased steeply over the course of the DCB habituation process, but it was the changes in the proportions of esterified *p*-coumarate, which indicated that something else was happening. Indeed, in this case the changes observed for the short-term treatments were quite striking, as SNH+DCB cells were 132- and 15-fold enriched in *p*-coumarate when compared with SNH and SH6 cells, respectively. In the case of maize plants, small amounts of *p*-coumaric acid are esterified to arabinoxylans in primary walls, but later on in wall development, it is found more extensively esterified to lignin (Iiyama et al. 1994; Ralph et al. 1994a). Indeed *p*-coumarate incorporation into the cell wall has been positively correlated with lignification (Hatfield and Marita 2010).

These findings suggest the presence of ectopic lignin or lignin-like polymers at least in the case of short-term treated cells, where the induced stress would explain their presence. Surprisingly, not only the short-term treated cells but also the DCB-habituated ones displayed a pink to brownish colour after phloroglucinol staining, indicative of lignin or lignin-like polymers (Pomar et al. 2002).

Our results clearly show the presence of ectopic lignin in maize primary cell walls of both DCB-habituated and short-

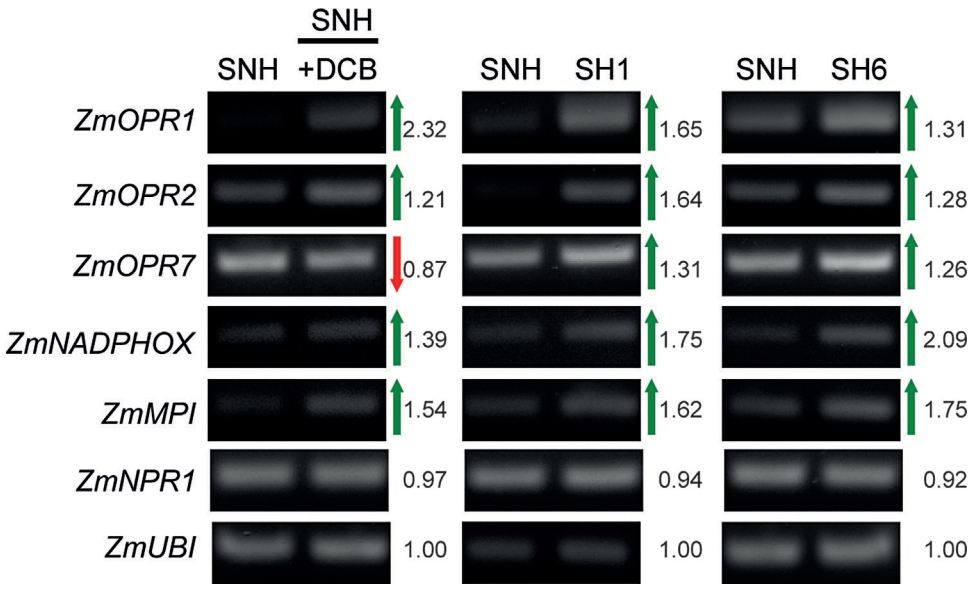

Figure 8. Relative expression levels of jasmonic acid (JA) and salicylic acid (SA) signaling pathways genes analyzed by reverse transcription-polymerase chain reaction (RT-PCR) of spectra of non-habituated (SNH), SNH + dichlobenil (DCB), SH1 and SH6 cell lines

For maize cell line annotations see Figure 1 legend. á and â indicate less and more mRNA accumulation than SNH cells, respectively. 12-oxophytodienoate reductase (*ZmOPR1*, *ZmOPR2* and *ZmOPR7*), NADPH oxidase (*ZmNADPHOX*), maize protease inhibitor (*ZmMPI*), and nonexpressor of pathogenesis related protein 1 (*ZmNPR1*). Pathogenesis related protein 1 (*ZmPR1*) was not detected. Primers can be found in Supplemental Table S1. Numerals indicate the normalised ratios of RT-PCR band intensities calculated by dividing the band intensity of SNH+DCB, SH1 or SH6 by SNH for each gene.

term treated cells. Therefore, phenolics not only act as hemicellulose cross-linking units in this system, but also constitute monolignol-based polymers similar to lignin that might contribute to stiffening of a cellulose-impoverished wall. Although ectopic lignification has been observed in *Arabidopsis* mutants with reduced cellulose synthesis and in seedlings treated with cellulose biosynthesis inhibitors (Caño-Delgado et al. 2003; Bischoff et al. 2009; Denness et al. 2011), there are few reports of this phenomenon in exclusively primary-walled cell cultures (Ros Barceló 1997). Moreover, transcriptomic approaches using *Arabidopsis* and poplar cell cultures habituated to cellulose biosynthesis inhibitors have shown that several genes specifically involved in lignin synthesis are downregulated (Manfield et al. 2004; Brochu et al. 2010). Lignin-like polymers have been shown to be produced by other *in vitro* model systems under certain conditions (Kärkönen and Koutaniemi 2010). However, although these systems achieve lignin production in plant cultured cells, ectopic lignin deposition in primary cell wall, the feature of cell suspension cultures presented in this study, has rarely been reported (Christiernin et al. 2005; Novo-Uzal et al. 2009; Shen et al. 2013).

In addition to its roles in cell wall stiffening, lignin deposition has long been implicated as an important defense mechanism against pests and pathogens[Q4] (Vance 1980; Barros-Rios et al. 2011). Lignin or lignin-like polymers are induced and rapidly deposited in cell walls in response to both biotic and abiotic stresses (Moura et al. 2010; Sattler and Funnell-Harris 2013; Miedes et al. 2014). Two types of lignin can be distinguished: (i) the one normally present in secondarily thickened cell walls with a purely structural role, and (ii) ectopic lignin, unexpectedly deposited in response to biotic and abiotic stresses. Lignin composition is highly heterogeneous and phylogenetically dependent, but also depends on the role the lignin is expected to play. 'Defense' lignin is often associated with elevated levels of H subunits compared with structural lignin (Ride 1975; Lange et al. 1995; Sattler and Funnell-Harris 2013). Although H units were not present in our system (minor component in monocot lignin; Boerjan et al. 2003), given the phloroglucinol-tonality and compositional differences between SNH+DCB (pink/indicative of a predominance of S units) and the SH6 (brown/S+G units) lignin-like polymers, we propose that these polymers could arise from different stimuli. While short-term DCB-treated cells might produce a *sensu stricto* stress-related lignin, habituated cells might accumulate a structural-related lignin. In accordance with this, S to G ratio estimated for the lignin-like polymer found in DCB-habituated cells (1.5) is close to that of lignin from maize stems (1.4) (Fornalé et al. 2012).

By catalyzing the final hydroxyl-cinnamaldehyde reduction to the corresponding alcohols, CAD is a key enzyme in determining lignin content and composition (Mansell et al. 1974; Fornalé et al. 2012). Although several *CAD* isoforms (*1*, *5*, *6* and *7*) were overexpressed in SH1 cells, CAD activity was found unchanged. Therefore, it could be assumed for this cell line that higher proportions of the cinnamaldehyde moieties are incorporated into the phenolic polymers, as occurs in CAD-transgenic and mutant plants (Ralph et al. 2001; Dauwe et al. 2007; Fornalé et al. 2012). However, CAD activity was found to be approximately three to four times enhanced for SNH+DCB and SH6 cells, respectively, compared to SNH. The increased CAD activity in SNH+DCB cells correlated with the overexpression of several *CAD* isoforms (*1*, *5*, *6* and *7*), and since only S units were found in measurable amounts in their cell walls, these proteins are most probably involved in the sinapaldehyde conversion to sinapyl alcohol. All of these isoforms were also overexpressed in SH6 cells. As a differential result, habituated cells showed a high overexpression of *CAD2*, which could be responsible for the synthesis of coniferyl alcohol from coniferaldehyde. In view of these results, we propose ZmCAD2 as a candidate for the production of G units, at least in the case of maize cell cultures, as well as a key player in the production of lignin-like polymers in SH6 cells. Interestingly, *CAD2* has been specifically associated with the synthesis of structural lignin in maize plants (Fornalé et al. 2012), which would agree with the synthesis of a structural related lignin in SH6 cells.

Concerning the two steps prior to CAD, different expression patterns were found in each case. CCR isoforms are responsible for the reduction of *p*-coumaroyl-CoA and feruloyl-CoA to their respective aldehydes. Downregulation of *CCR* in transgenic poplar has been associated with an up to 50% reduction in lignin content and an increased proportion of cellulose (Leplé et al. 2007). Interestingly, in contrast to these poplar trees, DCB-habituated cells with the opposite situation for the load-bearing polymers (less cellulose and more lignin) showed a significant overexpression of both *CCR* isoforms. On the other hand, and also in poplar, upregulation of *F5H* increased the proportion of S units, yielding an S/G ratio of greater than 35 versus approximately 2 for wild type poplar lignin (Stewart et al. 2009). Both *F5H* isoforms were overexpressed in SH1 cells, where only S units could be detected, while one of them was highly downregulated for SH6 cells. In summary, rather than a general stress response, a tight regulation of the monolignol biosynthetic pathway was observed in DCB-habituated cells.

Lignin polymerization is preceded by the peroxidase+H_2O_2 (and/or lacasse+O_2) dependent activation of monolignols to free radicals (Fagerstedt et al. 2010). The spent cell culture medium can be regarded as an extension of the apoplast and it can therefore be used as a compartment to monitor changes in the level of cell wall H_2O_2 (Kärkönen and Kuchitsu 2014). The H_2O_2 over-production of SNH+DCB and SH cells may be explained in the context of a reactive oxygen species over-production following cellulose inhibition, as has been previously reported for *Arabidopsis* plants (Dennes et al. 2011) and maize cultured cells habituated to low DCB concentrations (A Largo unpublished data). Given the steep increase in lignin over the course of DCB habituation, a relationship between lignin accumulation and increased apoplastic H_2O_2 contents may be expected (Nose et al. 1995; Kärkönen et al. 2002). However, no differences in apoplastic H_2O_2 were found when SH1 and SH6 cells were compared, indicating that H_2O_2 is not a limiting factor in the ectopic lignification reported in this system. An alternative explanation would be that the lignification is consuming apoplastic H_2O_2 explaining the lower level of apoplastic H_2O_2 measured in SH6 cells when compared with SH1 or SNH+DCB ones. Moreover, a study of class III peroxidase activity did not show differences due to DCB habituation in maize cultured cells (data not shown).

There are several lines of evidence that link ectopic lignification in response to cellulose deficiency with JA signaling. *Constitutive expression of vegetative storage protein 1* (*cev1*) and *ectopic lignin 1* (*eli1-1*) *Arabidopsis* mutants, are defective in the cellulose synthase gene *CESA3* involved in cellulose biosynthesis during primary cell wall formation (Ellis and Turner 2001; Ellis et al. 2002; Caño-Delgado et al. 2003). In these mutants, cellulose biosynthesis impairment was compensated by mechanisms such as ectopic lignification, constitutive activation of the JA signaling pathway, and increases in JA and ethylene proportions. In addition, treatments with the cellulose biosynthesis inhibitor isoxaben have been found to phenocopy *eli1-1* lignification in *Arabidopsis* wild type seedlings (Caño-Delgado et al. 2003; Hamann et al. 2009). In JA-insensitive plants, ectopic lignification by isoxaben is reduced, indicating that JA signaling is necessary (Caño-Delgado et al. 2003), a deduction which is further confirmed by the finding that external addition of methyl jasmonate to *Arabidopsis* cell cultures led to increased expression of phenylpropanoid, particularly monolignol biosynthesis (Pauwels et al. 2008). Our results confirm a JA-dependent signaling process in response to cellulose biosynthesis impairment, which led to ectopic lignification. However, according to our RT-PCR results and previous data from proteomic approaches (Mélida et al. 2010a; M de Castro unpublished data), stimulation of the lignification mechanism seems to be SA- and ethylene-independent.

In summary, maize suspension-cultured cells with up to 70% less cellulose produced a more extensive and cross-linked network of arabinoxylans together with a polymeric lignin-like material. This modified cell wall architecture is the result of the high structural plasticity of plant primary cell walls in response to a disruption of cell wall integrity. We propose that a JA signaling program might be triggering the observed ectopic lignification, and this model system will be used in future research in order to study the complex networks involved in cell wall integrity maintenance mechanisms.

MATERIALS AND METHODS

Plant material and DCB habituation process

Maize callus-cultured cells (*Zea mays* L. Black Mexican sweetcorn) were obtained from immature embryos and maintained in Murashige and Skoog media (Murashige and Skoog 1962) supplemented with 9 μM 2,4-D, 20 g L^{-1} sucrose and 8% agar at 25 °C under photoperiodic conditions (16:8; 3,000 lux ≈ 41 μmol m^{-2} s^{-1}). Callus-cultured cells were habituated to grow under originally lethal DCB concentrations, by stepwise transfers to higher DCB levels up to a 12 μM concentration (Mélida et al. 2009). Those cells growing on solid medium were disaggregated and transferred to a liquid medium containing 6 μM DCB (SH6) (Mélida et al. 2011). SH6 cells were maintained at 25 °C under light, rotary shaken and routinely subcultured every 15 days. Control cells were designated as non-habituated maize suspension-cultured cells (SNH). Cell lines habituated to grow under 1 μM DCB (SH1) were obtained from SNH (de Castro et al. 2014).

In order to distinguish toxic DCB effects from those owing to the habituation, short-term treatments with high (lethal) DCB concentrations were performed. Maize control cells were grown in a liquid medium containing 6 μM DCB for 6 days, ensuring a toxic effect but not giving sufficient time to kill the cells (H Mélida unpublished data). These cells were referred to as SNH+DCB.

Cell wall preparation and fractionation

Cell walls were prepared according to Mélida et al. (2009). Briefly, cells were collected during their exponential growth phase, washed extensively with distilled water and immediately frozen. The cells were disrupted in liquid nitrogen using a mortar and pestle. The resulting fine powders were subjected to extraction in 70% (v/v) ethanol for 5 days. The suspensions were filtered through glass-fiber filters (GF/A, Whatman[Q5]), and the pellets were washed six times with 70% ethanol and six times with acetone and were subsequently air dried, to obtain the alcohol insoluble residue. These were then resuspended in 90% dimethylsulphoxide for 8 h three times, filtered as above, washed twice with 0.01 M phosphate buffer pH 7.0 and incubated with 2.5 U mL^{-1} of α-amylase type VI-A dissolved in the same buffer for 24 h at 37 °C. The suspensions were filtered again and washed with ethanol and acetone as indicated above. The dry pellets were treated with phenol:acetic acid:water (2:1:1, v/v/v) for two periods of 8 h, then washed and air dried. The final dry pellets were considered the cell wall extracts.

Cell wall fractions were obtained by consecutively treating the cell wall residues with KOH solutions according to Mélida et al. (2009). Cell walls were extracted at room temperature with 50 mM *trans*-1,2-Diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid (CDTA) at pH 6.5 for 8 h and washed with distilled water. The residue was then incubated with 0.1 M KOH + 20 mM $NaBH_4$ for 2 h (×2) and washed with distilled water. Then 4 M KOH + 20 mM $NaBH_4$ was added to the residue for 4 h (×2), and washed again with distilled water. The extracts were acidified to pH 5.0 with acetic acid, dialyzed and freeze-dried, representing CDTA, KI and KII fractions, respectively. The residue after 4 M KOH extraction was hydrolyzed with 2 M trifluoroacetic acid (TFA) for 2.5 h at 120 °C, and after centrifugation, the supernatant was lyophilized and referred to as the TFA fraction.

Cell wall analysis

Tablets for Fourier transform infrared (FTIR) spectroscopy were prepared in a GrasebySpecac press from small samples (2 mg) of cell walls mixed with KBr (1:100, w/w). Spectra were obtained on a Perkin Elmer Spectrum 2000 instrument at a resolution of 1 cm^{-1}. A window between 800 and 1,800 cm^{-1}, which contains information of characteristic polysaccharides, was selected in order to monitor cell wall structure modifications. All spectra were normalized and baseline corrected with Spectrum software (v5.3.1). Then, data were exported to Microsoft Excel 2010 and all spectra were area-normalized.

Cellulose was quantified in crude cell walls by the Updegraff method as described by Encina et al. (2002). Total sugar quantification of cell wall fractions was performed by the phenol-sulphuric acid method[Q6] (Dubois et al. 1956) and results were expressed as glucose equivalents. The uronic acid

sugars were quantified by the *m*-hydroxydiphenyl method described by Blumenkrantz and Asboe-Hansen (1973) using galacturonic acid as reference standard.

For the analysis of neutral sugars, freeze-dried cell wall fractions were hydrolyzed with 2 M TFA at 121b °C for 1 h. *Myo*-inositol was used as an internal standard. The resulting monosaccharides were converted to alditol acetates as described previously [Q7](Albersheim et al. 1967) and analyzed by gas chromatography (GC) on a SP-2380 capillary column (30 m × 0.25 mm i.d.; Supelco) using a Perkin Elmer Autosystem.

Ferulate and *p*-coumarate monomers and ester-bound diferulates were extracted at room temperature from 50 mg of the alcohol-insoluble residues (AIR) using 2 M NaOH for 4 h and analyzed by high performance liquid chromatography (HPLC) based on a method previously described by Santiago et al. (2006). Retention time and UV spectrum of 5,5′-DFA were compared with freshly prepared external standard solutions of 5,5′-DFA, kindly provided by Dr. John Ralph's group (Department of Biochemistry, University of Wisconsin, Madison, USA). The UV absorption spectra of other DFAs were compared with previously published spectra (Waldron et al. 1996) and absorbance at 325 nm was used for quantification. Total ester-linked-DFAs concentration was calculated as the sum of three isomers of DFA identified and quantified by this analytical procedure: 8,5′-DFA, 8–*O*–4′-DFA, and 5,5′-DFA. The 8,5′-DFA concentration was calculated as the sum of the 8,5′-non-cyclic (or open)-DFA and 8,5′-cyclic (or benzofuran)-DFA because the non-cyclic form is most likely formed during alkaline hydrolysis from the native cyclic form (Ralph et al. 1994b).

Lignin-like material was quantified by the Klason gravimetric method with minor modifications. Cell wall extracts were hydrolyzed with 72% (w/v) sulfuric acid for 1 h at 30 °C. Then, the sulfuric acid concentration was diluted to 2.5% (w/v) with water and further incubated at 115 °C for 1 h. The residues were filtrated through Durapore polyvinylidene fluoride (PVDF) filters (Millipore[Q8], 0.45 μm), dried and weighed.

Thioacidolysis of cell walls, which solubilizes the β-*O*-4lignin core, and GC-MS analyses were performed (Novo-Uzal et al. 2009) using a Thermo Finnigan[Q9] Trace GC gas chromatograph, a Thermo Finnigan Polaris Q mass spectrometer, and a DB-XLB, J&W (60 m × 0.25 mm I.D.) column.

Histochemical staining of cinnamyl-aldehydes

Intact filtered maize cells were incubated with 1% (w/v) 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. Stained cells were observed under Nikon SMZ1500 magnifier and photographed using a Nikon Digital Camera DXM1200F.

Relative gene expression analysis

DCB-habituated cells (SH1 and SH6) were collected during their respective exponential growth phases and a set of SNH cells were collected at the same time for comparison. In the case of short-term DCB treatments, SNH+DCB cells and a set of SNH cells were collected on the third day of culture. Total RNA was extracted from homogenized cells of all lines following the procedures established for Trizol reagent (Invitrogen[Q10]). The purity and integrity of the extracted RNA was evaluated spectrophotometrically using a Nanodrop 1000 and running the RNA in 1% agarose gels. RNA (2 μg) was reverse-transcribed with Super Script III First strand retro-transcriptase (Invitrogen) using oligo $(dT)_{20}$ as primer. The synthesized cDNA was used to perform the gene expression analyses by standard and quantitative PCR methods.

Semiquantitative expression analysis by RT-PCR was performed for jasmonic (JA) and salicylic acid (SA) signaling pathway genes: *12-oxophytodienoatereductase* (*ZmOPR1*, AY921638; *ZmOPR2*, AY921639 and *ZmOPR7*, AY921644), *NADPH oxidase* (*ZmNADPHOX*, CK849936), *maize protease inhibitor* (*ZmMPI*, X78988), *pathogenesis related protein 1* (*ZmPR1*, UB2200) and *non-expressor of PR1* (*ZmNPR1*, EU95584). Primers can be found in Supplemental Table S1. 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 SYBR Safe DNA gel stain (Invitrogen) and gel images acquired with an Alphaimager HP system (ProteinSimple[Q11]). The quantification of the bands was performed by using the Alpha view v3.4.0.0. software (ProteinSimple). Band intensity was expressed as relative intensity units. For each individual gene, the band intensity was normalized in relation to ubiquitin and then, the normalized intensity ratios for SNH+DCB/SNH; SH1/SNH and SH6/SNH were calculated.

Relative gene expression was determined by qRT-PCR using specific primers for the following genes: *ferulate 5-hydroxylase* [*ZmF5H1* (AC210173.4) and *ZmF5H2* (GRMZM2G100158)], *cinnamoyl-CoA reductase* [*ZmCCR1* (GRMZM2G131205) and *ZmCCR2* (GRMZM2G131836)] and *cinnamyl alcohol dehydrogenase* [*ZmCAD1* (Y13733; GRMZM5G844562), *ZmCAD2* (GRMZM2G118610), *ZmCAD3* (GRMZM2G046070), *ZmCAD4* (GRMZM2G700188), *ZmCAD5* (GRMZM2G443445), *ZmCAD6* (GRMZM2G090980) and *ZmCAD7* (GRMZM167613)] as described by Guillaumie et al. (2007). *Folylpolyglutamate synthase* (*ZmFPGS*; GRMZM2G393334) and the *Ubiquitin carrier protein* (*ZmUBCP*; GRMZM2G102471) genes were used as reference genes (Manoli et al. 2012). Primers can be found in Supplemental Table S1.

The qPCR was carried out in a StepOnePlus platform (Applied Biosystems[Q12]) using Power SYBR green PCR master mix (Applied Biosystems), 2 μL of each cDNA concentration (50 and 100 ng μL^{-1}) and a mix of both primers at 10 μM. All samples were run in triplicate with the following temperature profile: initial denaturation at 95 °C for 10 min, followed by 40 cycles of 95 °C for 10 s and 60 °C for 1 min (annealing and elongation). The relative gene expression was calculated by 'δ–δ' method (Livak and Schmittgen 2001) implemented in the StepOne Software v2.2.2. A no-template negative control and a melting curve were performed in each sample set to control the primer dimers and contaminants in the reactions.

CAD enzyme activity assay

CAD enzyme activity was measured by following the method described by Chabannes et al. (2001) modified by Fornalé et al. (2012). Fresh cells were homogenized under liquid nitrogen with a mortar and pestle until a fine powder was obtained, and 5 mL of extraction buffer (100 mM Tris-HCl pH 7.5, 2% (w/v) PEG 6000, 5 mM DTT and 2% (w/v) PPVP) were added. The

suspension was centrifuged at 10,000 *g* for 10 min at 4 °C and the supernatant was collected. The centrifugation process was repeated until the supernatant was clear.

CAD activity assays were carried out by measuring the absorbance increment at 400 nm when coniferyl alcohol was oxidized to coniferyl aldehyde. The reactions were performed in 96-well plates containing 140 μL of 140 mM Tris-HCl pH 8.8, 20 μL of 1 mM coniferyl alcohol, 20 μL of 200 μM $NADP^+$ and 20 μL of sample. The mixtures were mixed and incubated at 30 °C for 10 min, and the reactions were measured over the following 10 min in a plate reader Synergy HT (Bio-Teck[Q13]) at 30 °C. Reaction and sample blanks were routinely used.

Apoplastic H_2O_2 content determination

Apoplastic H_2O_2 content was determined with the xylenol orange method as described by Bindschedler et al. (2001). For the reactions, 150 μL of culture media was mixed with 1 mL of reaction mixture (125 μM xylenol orange, 100 mM D-sorbitol, 25 μM $FeSO_4$, 25 μM $(NH_4)_2SO_4$ and 25 μM H_2SO_4), and absorbance (560 nm) was measured after 40 min of incubation.

ACKNOWLEDGEMENTS

This work was supported by grants from the Ministry of Economy and Competitiveness (Spain) (AGL2011-30545-C02-2 to AE; JdC to ENU; Ramón y Cajal to RS). HM was supported by funds from the European Commission (IEF-SignWALLINg-624721). ALG hold a predoctoral grant from the University of León. We gratefully acknowledge Professor Stephen C. Fry (University of Edinburgh) for his kind provision of maize cell cultures and Denise Phelps for the English revision of the manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article.

Figure S1. Cell wall fractionation Total sugars in (A) CDTA, (B) KI, (C) KII and (D) TFA cell wall fraction obtained from SNH, SNH + DCB, SH1 and SH6 cell lines. For maize cell line annotation see Figure 1 legend. Data represents the means values ± s.d. of 3 technical replicates. Asterisks indicate values that are significantly different from SNH after a Student's t-test ($P < 0.05$).

Table S1. Primers used in RT-PCR and RT-qPCR experiments

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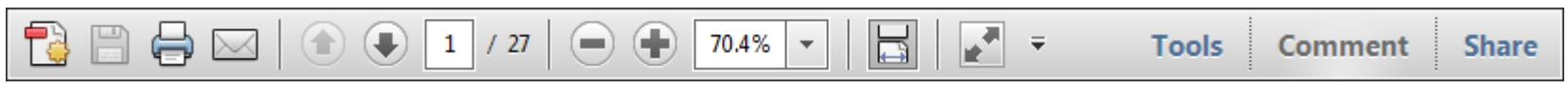

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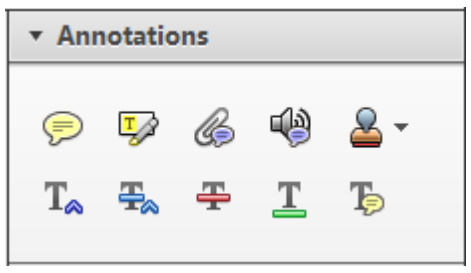

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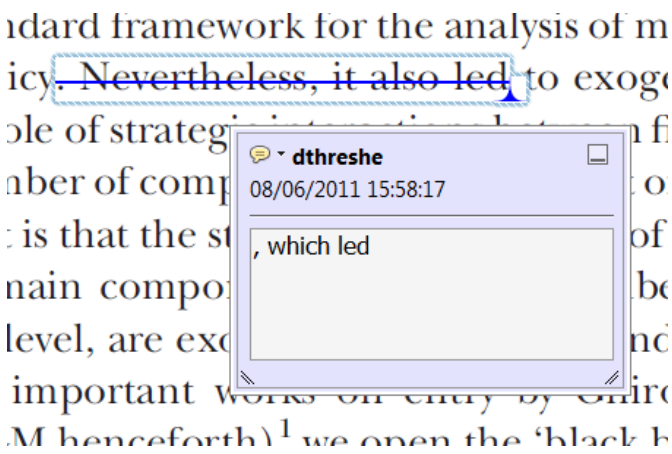

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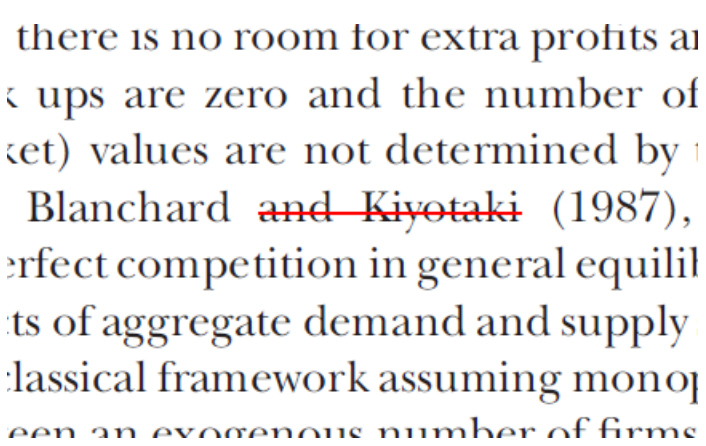

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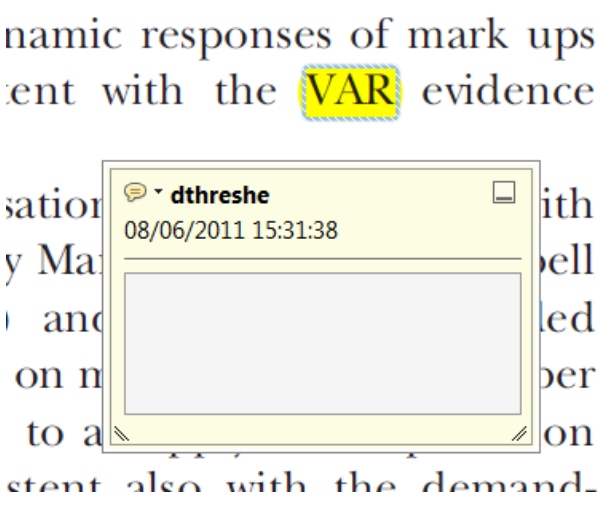

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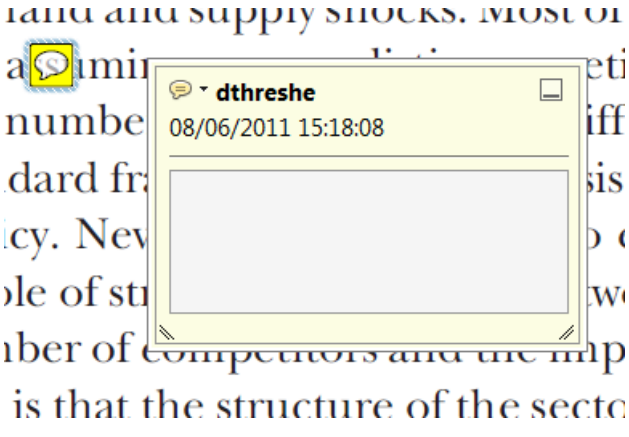

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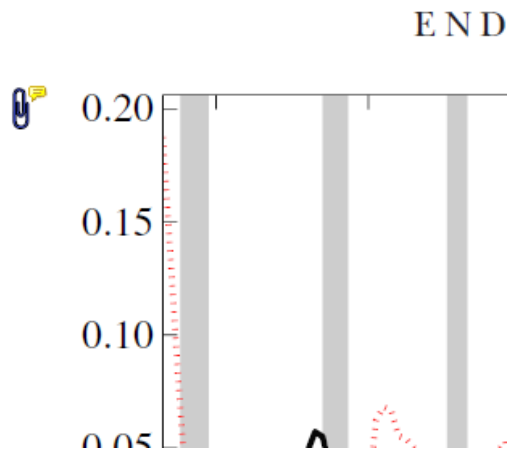

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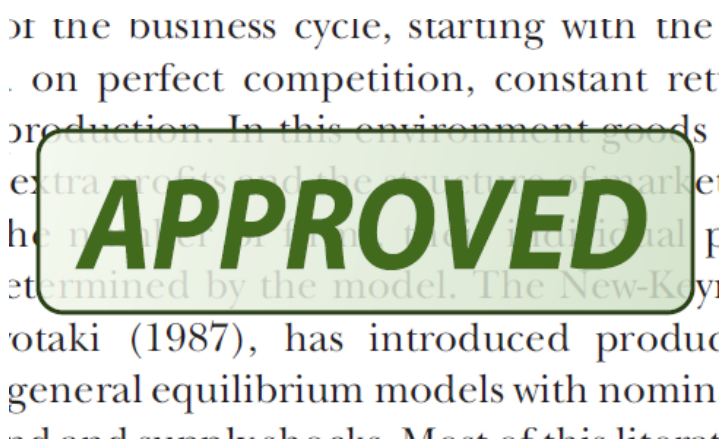

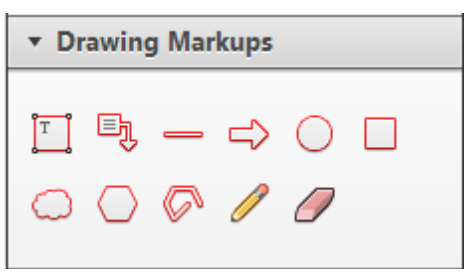

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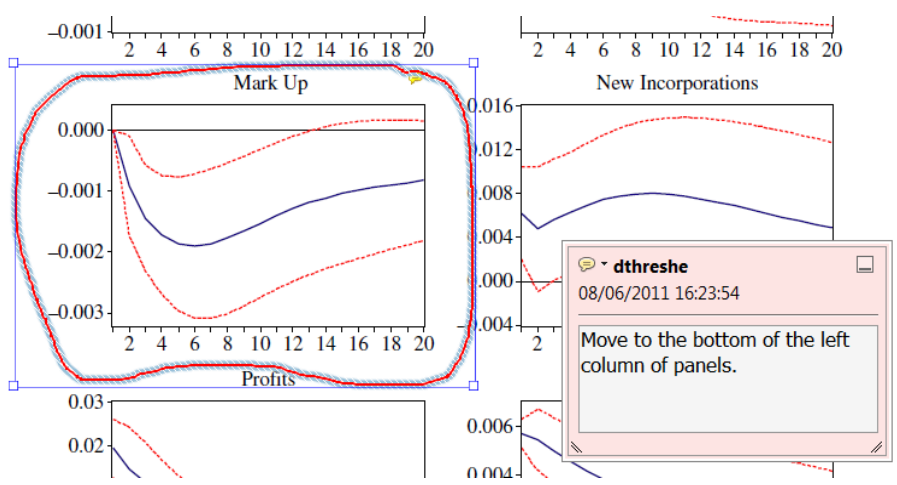

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