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RESEARCH PAPER

Thaxtomin A affects CESA-complex density, expression of cell wall genes, cell wall composition, and causes ectopic lignification in *Arabidopsis thaliana* seedlings

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Abstract

Thaxtomin A, a phytotoxin produced by *Streptomyces* eubacteria, is suspected to act as a natural cellulose synthesis inhibitor. This view is confirmed by the results obtained from new chemical, molecular, and microscopic analyses of *Arabidopsis thaliana* seedlings treated with thaxtomin A. Cell wall analysis shows that thaxtomin A reduces crystalline cellulose, and increases pectins and hemicellulose in the cell wall. Treatment with thaxtomin A also changes the expression of genes involved in primary and secondary cellulose synthesis as well as genes associated with pectin metabolism and cell wall remodelling, in a manner nearly identical to isoxaben. In addition, it induces the expression of several defence-related genes and leads to callose deposition. Defects in cellulose synthesis cause ectopic lignification phenotypes in *A. thaliana*, and it is shown that lignification is also triggered by thaxtomin A, although in a pattern different from isoxaben. Spinning disc confocal microscopy further reveals that thaxtomin A depletes cellulose synthase complexes from the plasma membrane and results in the accumulation of these particles in a small microtubule-associated compartment. The results provide new and clear evidence for thaxtomin A having a strong impact on cellulose synthesis, thus suggesting that this is its primary mode of action.

Key words: *Arabidopsis thaliana*, callose, cellulose, defence response, isoxaben, lignin, microtubule, phytotoxin, *Streptomyces*, thaxtomin.

Introduction

Cellulose is the major component of the plant cell wall (CW). The CW provides general plant strength (Shedletzky *et al.*, 1992) and is important for the protection of cells against pathogens, dehydration, and other environmental factors (Braam, 1999; Vorwerk *et al.*, 2004). In addition, the primary CW contributes to the determination of plant development, cell shape (Delmer and Amor, 1995), and cell–cell interactions. Once expansion growth has ceased, some cell types deposit a thick secondary CW (Turner *et al.*, 2001), which is mainly composed of highly aligned, crystalline cellulose and xylans (Ebringerova and Heinze, 2000; Brown *et al.*, 2005), and which can also be lignified in order to provide further strength. Defects or irregularities in cellulose deposition and CW integrity have been shown to trigger defence pathways in both primary and secondary CW mutants (Caño-Delgado *et al.*, 2000; Ellis *et al.*, 2002; Hernandez-Blanco *et al.*, 2007).

Synthetic chemical compounds, for example, isoxaben or dichlobenil (DCB), that specifically inhibit CW synthesis have been identified previously (Hogetsu *et al.*, 1974; Heim *et al.*, 1990). Pinpointing such a compound's molecular and cellular effects on CW synthesis and identification of the

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Abbreviations: 4CL, 4-coumarate:CoA ligase; C4H, cinnamate-4-hydroxylase; CAD, cinnamyl alcohol reductase; CCR, cinnamoyl CoA reductase; CESA, cellulose synthase; CESA-C, cellulose synthase complex; DCB, dichlobenil; FT-IR, Fourier-transform infrared; GFP, green fluorescent protein; HG, homogalacturonan; MASC, microtubule-associated cellulose synthase compartment; PM, plasma membrane; XTH, xyloglucan endotransglucosylase/hydrolases.

genetic modifications in mutants resistant or hypersensitive to such compounds can advance our understanding of CW biology. Genetic studies revealed that the herbicide isoxaben has a direct effect on cellulose synthases (CESA), i.e. CESA3 and CESA6 (Scheible *et al.*, 2001; Desprez *et al.*, 2002) as well as CESA2 and CESA5 (Desprez *et al.*, 2007). Isoxaben has also been reported to prevent incorporation of glucose into the CW and to inhibit the assembly of hemicelluloses (Heim *et al.*, 1990, 1998). More recently, isoxaben and DCB were also shown to cause aberrant CESA complex (CESA-C) patterns in the plasma membrane (PM) (Paredez *et al.*, 2006; DeBolt *et al.*, 2007) by changing CESA motility and to affect cortical microtubule arrays (Lazzaro *et al.*, 2003; Paredez *et al.*, 2006, 2008; DeBolt *et al.*, 2007).

The cyclic dipeptide thaxtomin A is the only somewhat familiar natural compound for which inhibition of cellulose synthesis has been suggested (King *et al.*, 2001; Fry and Loria, 2002; Scheible *et al.*, 2003). Thaxtomin A is produced by plant-pathogenic soil bacteria of the genus *Streptomyces* (King and Lawrence, 1996) implying that cellulose synthesis is a natural target in plant–pathogen interactions. The molecular mode of action of thaxtomin A remains unknown. It was noted that nanomolar concentrations of thaxtomin A cause swelling of hypocotyls and reduced seedling growth in *A. thaliana* (Scheible *et al.*, 2003). In tobacco suspension cultures, it causes radial cell swelling in dividing or expanding cells, but does not affect mitotic and cortical microtubules (Fry and Loria, 2002). Thaxtomin A also inhibits normal cell elongation of tobacco suspension cells in a manner that suggests an effect on primary CW development (Fry and Loria, 2002), and the incorporation of [^{14}C]-glucose into the acid-insoluble CW fraction (i.e. cellulose) in *A. thaliana* hypocotyls (Scheible *et al.*, 2003). Furthermore, etiolated *A. thaliana* seedlings treated with nanomolar concentrations of thaxtomin A display a Fourier-transform infrared (FT-IR) spectral phenotype that is most related to those of *A. thaliana rsw1/cesa1* mutant seedlings or *A. thaliana* seedlings treated with cellulose-synthesis inhibitors like DCB, isoxaben, or flupoxam (Scheible *et al.*, 2003; Robert *et al.*, 2004).

In addition to thaxtomin A's putative role as a cellulose synthesis inhibitor, its application to plants was proposed to trigger an early Ca^{2+} signalling cascade that might be crucial for plant-pathogen interactions (Tegg *et al.*, 2005). However, although thaxtomin A leads to programmed cell death, no evidence for an activation of defence responses like oxidative burst, rapid medium alkalization or an activation of defence genes has been reported (Duval *et al.*, 2005).

In this work, further effects of thaxtomin A on *A. thaliana* are described, confirming its role as a natural cellulose synthesis inhibitor. These include a more detailed CW fractionation study to reveal further insights into the nature of non-cellulosic CW fractions, something that was not addressed in previous reports. Thaxtomin A's impact on the expression of CW and defence response-related genes was also investigated and it is shown that thaxtomin A triggers lignification in *A. thaliana* seedlings, in a pattern that differs from the one of isoxaben. Finally, it is demonstrated that thaxtomin A has clear impact on the motility of CESA-Cs as well as their distribution in the PM.

Materials and methods

Seedling growth conditions and thaxtomin A treatment

A. thaliana wild-type Col-0 seeds from an in-house collection, *cesa3^{je5}*::GFP-CESA3 seeds (Desprez *et al.*, 2007) and *cesa6^{prc}*::GFP-TUA6 seeds (Ueda *et al.*, 1999) were surface-sterilized and stratified at 4 °C for 3 d, before they were exposed to white light for 3 h to induce and synchronize germination, and then grown in sterile liquid cultures as previously described by Scheible *et al.* (2004). Thaxtomin A was purified from 4–7-d-old oatmeal broth cultures of *Streptomyces scabies* by ethyl acetate extraction and reverse phase thin-layer chromatography, essentially as described by King and Lawrence (1996). Purified thaxtomin A (or isoxaben) was dissolved in methanol and added to the *A. thaliana* seedling cultures from a 20 μM (or 0.5 μM) stock solution to a final concentration of 200 nM (or 5 nM). Seedlings were treated 4 d after transfer to liquid media and were harvested for analyses 2 d after the treatment. Etiolated seedlings cultures were dark-grown in flasks wrapped with aluminium foil, and treated with thaxtomin A (or isoxaben) as above.

Cellulose [^{14}C]-sucrose incorporation assay and CW fractionation

The radiolabel incorporation assay was performed according to Scheible *et al.* (2003) and Fagard *et al.* (2000), however, [U-^{14}C]-sucrose (Amersham) was used instead of [^{14}C]-glucose. CW fractionation was performed according to Peng *et al.* (2000). Fractions analysed for their ^{14}C incorporation were the (i) chloroform fraction, (ii) ammonium-oxalate fraction, (iii) 0.1 M KOH fraction, (iv) 4 M KOH fraction, (v) the acid-soluble fraction, and (vi) the acid-insoluble cellulosic fraction (Peng *et al.*, 2000).

RNA isolation, DNase I treatment, cDNA synthesis, qRT-PCR and primers

All experiments were performed as described by Czechowski *et al.* (2005) and Udvardi *et al.* (2008). RNA from whole seedlings was isolated using the Qiagen Plant RNeasy Mini Kit. After RNA quantification, 5 μg total RNA was digested with DNase I (Sigma) according to the manufacturer's instructions. After checking for the absence of genomic DNA, cDNA was synthesized from DNA-free RNA using Supercript III reverse transcriptase (Invitrogen) according to the manufacturer's instructions. QRT–PCR was carried out in 10 μl reaction volumes using an ABI Prism® HT-7900 Sequence detection system (Applied Biosystems). Primer sequences are presented in Supplementary

Table S1 at *JXB* online. Results were normalized with the internal reference gene *At4g05320* (*UBQ10*).

Lignin and callose staining

Seedlings were put on a microscope slide and were treated with two drops of freshly prepared phloroglucinol solution, consisting of 25 mg phloroglucinol dissolved in 25 ml 100% methanol and 25 ml 37% hydrochloric acid. After incubation for 2 min, seedlings were observed using a Leica MZ 12.5 stereo microscope. Callose staining with aniline blue was performed on 6-d-old etiolated seedlings as described previously (http://commonweb.unifr.ch/biol/pub/mauchgroup/staining.html; Stone *et al.*, 1985). Aniline blue fluorescence was observed with an Olympus BX51 epifluorescence stereo microscope.

Spinning disc microscopy

Seedlings expressing GFP::CESA3 and GFP::TUA6 fusion proteins were cultivated as described by Chan *et al.* (2005). Imaging of GFP-CESA3 and GFP-TUA6 was conducted as follows. Hypocotyls of 3-d-old etiolated seedlings were analysed on an Axiovert 200M microscope (Zeiss, Thornwood, NY), equipped with a Yokogawa CSU22 spinning disc, Zeiss 100/1.4 N.A. oil objective and Andor EMCCD iXon DU 895 camera (Plateforme d'Imagerie Dynamique, Institut Pasteur, Paris, France). A 488 nm diode pumped solid-state laser was used for excitation, and emission collected using a BP 488/25 filter (Semrock, Rochester, NY). Particle velocities were calculated from kymographs created in Image J (W Rasband, National Institutes of Health, Besthesda, MD, USA). Three-day-old, chamber-cultivated *A. thaliana* seedlings were treated with or without 200 nM thaxtomin A.

Results

Thaxtomin A alters cell wall [^{14}C]-isotope partitioning

It has previously been shown that thaxtomin A treatment results in a reduced level of [^{14}C]-isotope incorporation into the cellulosic fraction (Scheible *et al.*, 2003). This reduction of radiolabel incorporated into cellulose was shown to be compensated for by an increase in the non-cellulosic fraction, while the uptake of radiolabel *per se* was unchanged by thaxtomin A. Because *A. thaliana* CWs contain three major classes of polysaccharides (cellulose, hemicelluloses, and pectins), thaxtomin A's effect on the *de novo* synthesis of these polysaccharides was analysed in more detail. The incorporation of [^{14}C]-isotope, provided as [U-^{14}C]-sucrose, into several CW fractions was determined, as obtained by sequential extractions with (i) chloroform, (ii) ammonium-oxalate, (iii) 0.1 M KOH, (iv) 4 M KOH, and (v) acetic/nitric acid (acid-soluble fraction). In addition, the acid-insoluble fraction was analysed (vi). These CW fractions are considered to contain (i) lipids, (ii) neutral polysaccharides and pectins, (iii, iv) hemicelluloses/pectins, (v) residual pectins, non-crystalline cellulose, and hemicelluloses, and (vi) pure, crystalline cellulose, respectively (Peng *et al.*, 2000). Thaxtomin A application induced significant changes in [^{14}C]-isotope incorporation within several CW fractions (Fig. 1). In agreement with Scheible *et al.* (2003), etiolated *A. thaliana* seedlings treated with 200 nM thaxtomin A showed a strongly reduced level of incorporated isotope label into the cellulosic fraction and FTIR analyses confirmed that thaxtomin A-treated seedlings clustered with cellulose-deficient mutants (Scheible *et al.*, 2003). Compared with the untreated control, the ammonium-oxalate fraction, representing mainly pectins, showed a significant increase in [^{14}C]-isotope. The [^{14}C]-isotope in the chloroform fraction (lipids), the 0.1 M KOH and 4 M KOH fractions, respectively (hemicelluloses/pectins) and the acid-soluble fraction (hemicelluloses) were slightly increased.

Expression analysis of CW-related genes

It was intended to investigate whether the observed changes in CW polysaccharide composition induced by thaxtomin A application to *A. thaliana* seedlings are also accompanied by transcriptional changes. Therefore a number of CW genes were chosen (Fig. 2A; see Supplementary Table S1 at *JXB* online) that are either involved in cellulose or pectin synthesis or CW remodelling or were previously shown to respond to isoxaben treatment in *A. thaliana* suspension-cultured cells (Manfield *et al.*, 2004), and their expression levels were determined by quantitative real-time RT-PCR. Thaxtomin A application resulted in a clear repression of 15 genes and the induction of three genes (see Supplementary Table S1 at *JXB* online; Fig. 2A), whereas 25 CW genes were less or not affected, based on a $\Delta\Delta C_T$ value <2 or >–2

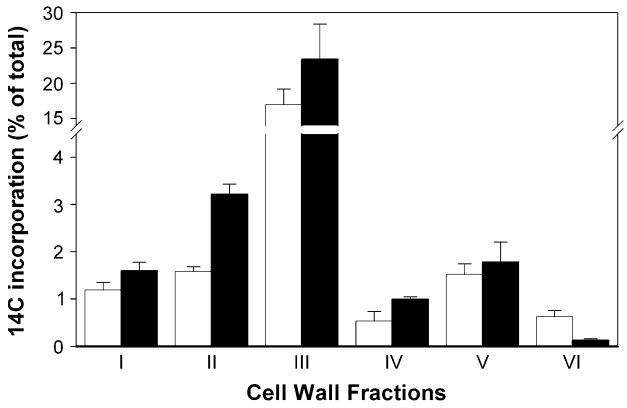

Fig. 1. Thaxtomin A alters ^{14}C incorporation in cell wall fractions. ^{14}C incorporation in 6-d-old, liquid culture-grown, etiolated *Arabidopsis* seedlings treated with 200 nM thaxtomin A for 2 d (black bars) and control seedlings (white bars) is shown for different cell wall fractions as follows: (i) chloroform fraction, (ii) ammonium-oxalate fraction, (iii) 0.1 M KOH fraction, (iv) 4 M KOH fraction, (v) acetic/nitric acid soluble fraction, and (vi) acid insoluble fraction. ^{14}C incorporation in each fraction is expressed as a percentage of total ^{14}C uptake. Each bar represents the mean ±SE from four biological replicates.

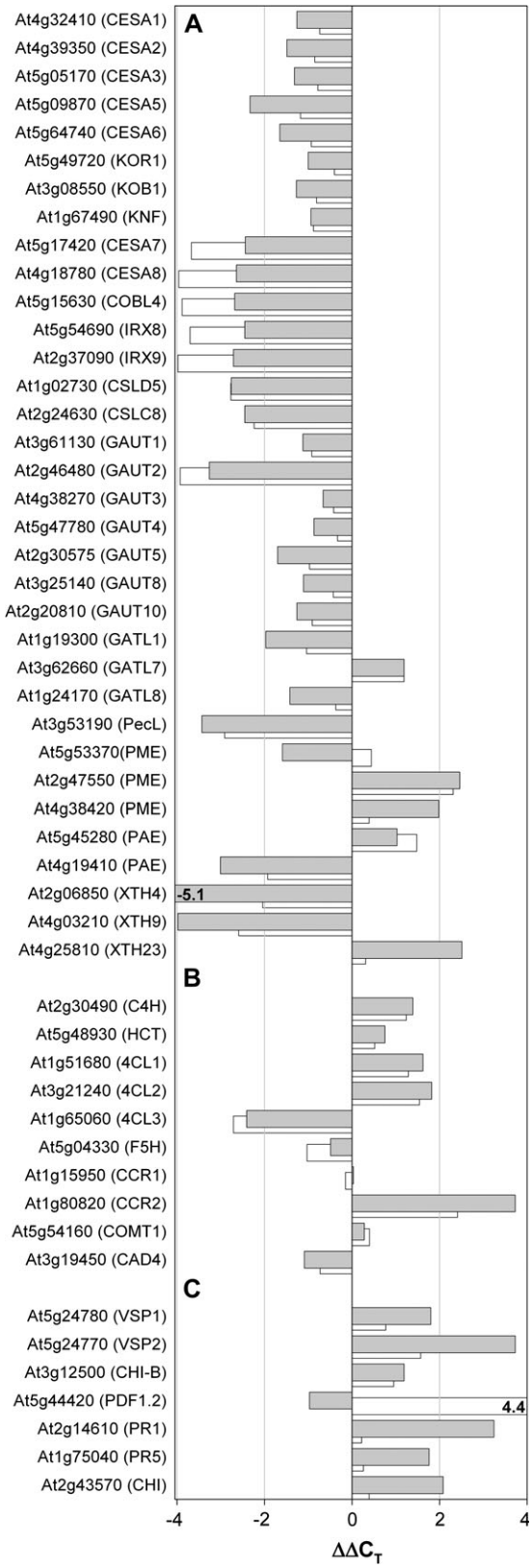

Fig. 2. Changes in gene expression in thaxtomin A and isoxaben-treated seedlings. (A) Cell wall genes, (B) genes involved in lignin synthesis, and (C) genes involved in defence responses. The expression levels are given expressed as $\Delta\Delta C_T$ (i.e. a logarithmic scale), where $\Delta\Delta C_T$ is the difference in normalized qRT-PCR threshold cycle number of the respective gene between thaxtomin- or isoxaben-treated seedlings and control seedlings. Negative and positive numbers thus represent repression and induction in thaxtomin-treated seedlings, respectively. Normalization was performed using the *UBQ10* (*At4g05320*) as a reference. Data for thaxtomin A-treated seedlings are shown as grey bars, data for seedlings treated with isoxaben as slightly displaced white bars at the back. Each value represents the mean of four biological replicas from two independent experiments. Standard errors of C_T values are small and not shown.

(i.e. less than 3–4-fold induction/repression). Thaxtomin A treatment resulted in a consistent but mild repression of genes involved in primary cellulose biosynthesis such as *CESA1*, *CESA2*, *CESA3*, *CESA5*, *CESA6* or *KORRIGAN*, *KOBITO1*, and *KNOPF* (Fig. 2A), which could be due to the fact that elongation/expansion growth of the primary CW was advanced in 6-d-old etiolated seedlings. It is known that *KORRIGAN*, *KOBITO1*, and *KNOPF* affect primary wall deposition during cell elongation/expansion and seed development (Nicol *et al.*, 1998; Pagant *et al.*, 2002; Gillmor *et al.*, 2002). Genes involved in secondary CW synthesis such as *CESA7* and *CESA8* consistently showed a stronger repression ($\Delta\Delta C_T$ value <–2; Fig. 2A; see Supplementary Table S1 at *JXB* online). Inhibition of secondary wall synthesis is also reflected in repression of *COBRA-like 4 (COBL4)* (Brown *et al.*, 2005), *IRX8* and *IRX9* (Fig. 2A). *IRX8* (*GAUT12*) and *IRX9* are important in glucuronoxylan- and homogalacturonan biosynthesis during secondary CW formation (Persson *et al.*, 2005, 2007; Brown *et al.*, 2005; Pena *et al.*, 2007). Our set of surveyed genes also contained two *CESA*-like genes, *CslC8* and *CslD5*. The latter was recently found to be important for xylan synthesis (Bernal *et al.*, 2007), while for the former a biosynthetic role in CW polymer formation is so far only predicted (Richmond and Somerville, 2000) although a close homologue, *CslC4*, was recently shown to encode a β-1,4 glucan synthase (Cocuron *et al.*, 2007). Both these genes were also found to be strongly repressed in thaxtomin A-treated seedlings (Fig. 2A).

Transcript levels for a number of genes encoding pectin synthetic/modifying enzymes were also changed after thaxtomin A application. Several members of the family of galacturonosyltransferase genes (Sterling *et al.*, 2006) and especially *GAUT2* displayed reduced transcript levels, whereas the transcript for *GATL7* was increased (Fig. 2A; see discussion for more information). In our set of examined CW genes, strong repression of a pectate lyase-like gene (*At3g53190*) and of a pectin acetylesterase (*At4g19410*) was also found, while the expression of two pectin methylesterases (*At2g47550*, *At4g38420*) was increased and one (*At5g53370*) was slightly decreased (Fig. 2A; see Supplementary Table S1 at *JXB* online). Strong transcript changes were also observed for three genes from the family of xyloglucan endotransglucosylase/hydrolases (XTH) (Fig. 2A; see Supplementary Table S1 at *JXB* online), which are presumably involved in wall loosening, resulting in CW extension (Smith and Fry, 1991; Vissenberg

et al., 2005). Expression of two *XTH* genes (*XTH4* and *XTH19*) was repressed in thaxtomin A treated seedlings, whereas *XTH23* was induced. Interestingly, the transcript changes shown in Fig. 2A and others, that are only shown in Supplementary Table S1 at *JXB* online, were also recapitulated in a similar or nearly identical manner in isoxaben-treated seedlings (cf. white bars in Fig. 2A), suggesting that thaxtomin A and isoxaben affect *A. thaliana* seedlings in the same or a very similar manner and, possibly, also have a similar mode of action at the molecular level.

Thaxtomin A invokes ectopic lignification and induction of defence-response genes

A previous study showed that *A. thaliana eli1/cesa3* mutant seedlings and seedlings treated with the cellulose-synthesis inhibitor isoxaben exhibit strong ectopic lignification (Caño-Delgado *et al.*, 2000) and up-regulation of defence response genes. To analyse whether thaxtomin A phenocopies isoxaben in this respect, lignification was investigated in 6-d-old seedlings. In contrast to methanol-treated control seedlings, which displayed no ectopic lignin (Fig. 3B, E), seedlings treated with 200 nM thaxtomin A showed strong lignification in the hypocotyls (Fig. 3A), whereas, unlike isoxaben-treated seedlings (Caño-Delgado *et al.*, 2003; Fig. 3F), they had no ectopic lignin in the root (Fig. 3D). The lignification pattern in light-grown seedlings was similar to the pattern found in etiolated seedlings (data not shown).

Ectopic lignification in thaxtomin A-treated seedlings is in agreement with the altered expression of genes involved in the lignin biosynthetic pathway (Raes *et al.*, 2003) as depicted in Fig. 2B. Thaxtomin A treatment resulted in strong up-regulation of the cinnamoyl CoA reductase gene *CCR2*. Likewise, a cinnamate-4-hydroxylase gene (*C4H*), two genes encoding isoforms of 4-coumarate:CoA ligase (*4CL1*, *4CL2*), and a hydroxycinnamoyltransferase gene (*HCT*) were induced, while a third 4-coumarate:CoA ligase gene (*4CL3*) was repressed. *AtCCR2* is known to be expressed during pathogenic attacks and stress and, together with cinnamyl alcohol reductase, leads to monolignol precursors of lignin (Humphreys and Chapple, 2002) via a reduction of phenylpropanoid cinnamoyl CoA-ester intermediates. The expression of other genes like *CCR1*, *COMT*, *F5H*, or *CAD4* did not differ much from the one in control seedlings. Very similar results were also obtained with isoxaben-treated *A. thaliana* plantlets (Fig. 2B; Caño-Delgado *et al.*, 2003).

As ectopic lignification and induction of defence genes are a consequence of perturbations in cellulose synthesis or

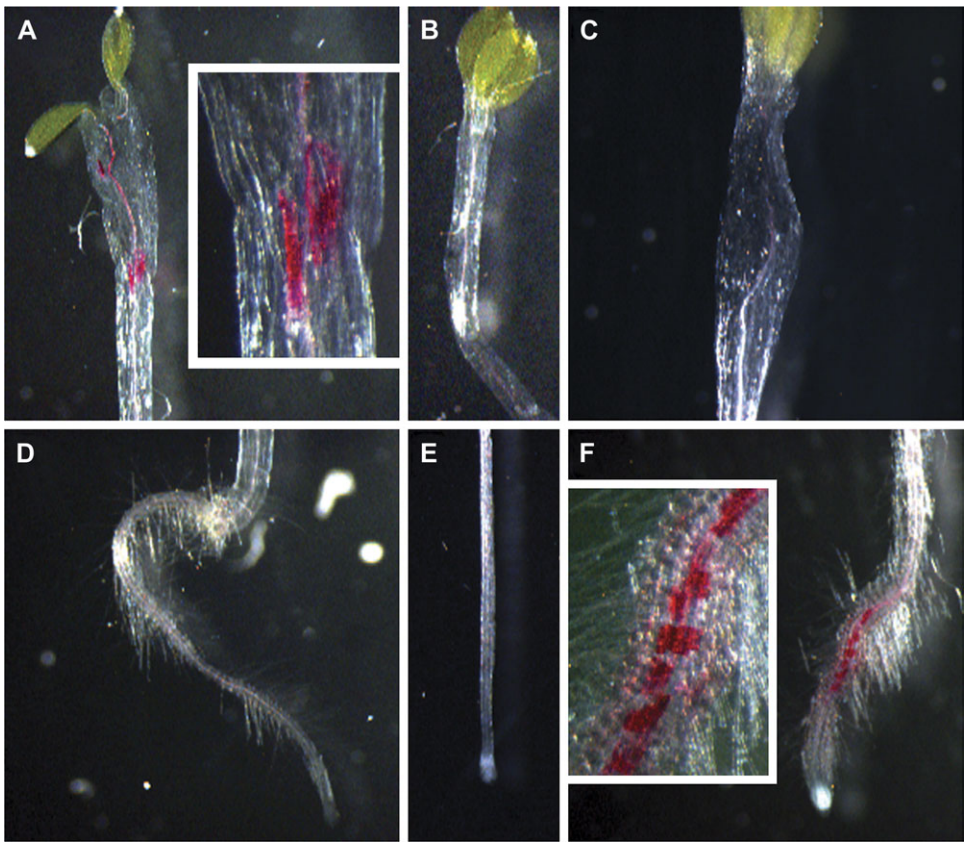

Fig. 3. Thaxtomin A causes ectopic lignification in etiolated *Arabidopsis* seedlings. Lignification was analysed in 6-d-old, liquid culture-grown, etiolated *Arabidopsis* seedlings treated with 200 nM thaxtomin A for 2 d (A, D), methanol-treated control seedlings (B, E) or seedlings treated with 5 nM isoxaben (C, F). Typical staining patterns are shown. Insets in (A) and (F) show magnifications of strongly lignified regions.

reduced CW integrity (Ellis *et al.*, 2002; Caño-Delgado *et al.*, 2003), the expression of several candidate genes involved in plant defence responses were also analysed (Fig. 2C; see Supplementary Table S1 at *JXB* online). In this respect, and in addition to *CCR2*, thaxtomin A was found to induce significantly the expression of *VSP1*, *VSP2*, and *CHI-B*, three marker genes of the jasmonic acid-dependent defence (Turner *et al.*, 2002), *PR1* and *PR5*, which are markers for the salicylic acid-dependent defence (Turner *et al.*, 2002), and also the chitinase *At2g43570* (Pastori *et al.*, 2003). Interestingly *PDF1.2* (*At5g44420*) was unaffected in thaxtomin A-treated seedlings, whereas this ethylene and jasmonate-responsive plant defensin gene is strongly induced in isoxaben-treated etiolated seedlings (Fig. 2C) or isoxaben-treated, light-grown *A. thaliana* plantlets (Caño-Delgado *et al.*, 2003).

As with lignification and the expression of defence genes, callose deposition in places other than the nascent cell plate or pollen tube cell walls, is regarded as a marker of the plant defence response (Carpita and McCann, 2000). Hence the deposition of callose in thaxtomin A and isoxaben-treated etiolated *Arabidopsis* seedlings was analysed by aniline blue staining (Stone *et al.*, 1985). Treatment with one or the other inhibitor again led to similar results (see Supplementary Fig. S1 at *JXB* online). Both inhibitors led to ectopic callose in the elongating root zone just above the root tip, and thaxtomin A also resulted in callose staining in the upper, swollen part of the etiolated hypocotyl, which is reminiscent of the observed lignification patterns (Fig. 3). In addition, thaxtomin A was found to induce callose deposition in cotelydons of light-grown seedlings, which again recapitulates the lignification pattern observed in such growth conditions (results not shown). The fact that thaxtomin A or isoxaben-treated seedlings are able to produce callose also suggests that the inhibitors do not affect the synthesis of UDP-glucose, which is the substrate for the synthesis of cellulose and callose at the PM surface thus suggesting that the inhibitory effect is specific for cellulose.

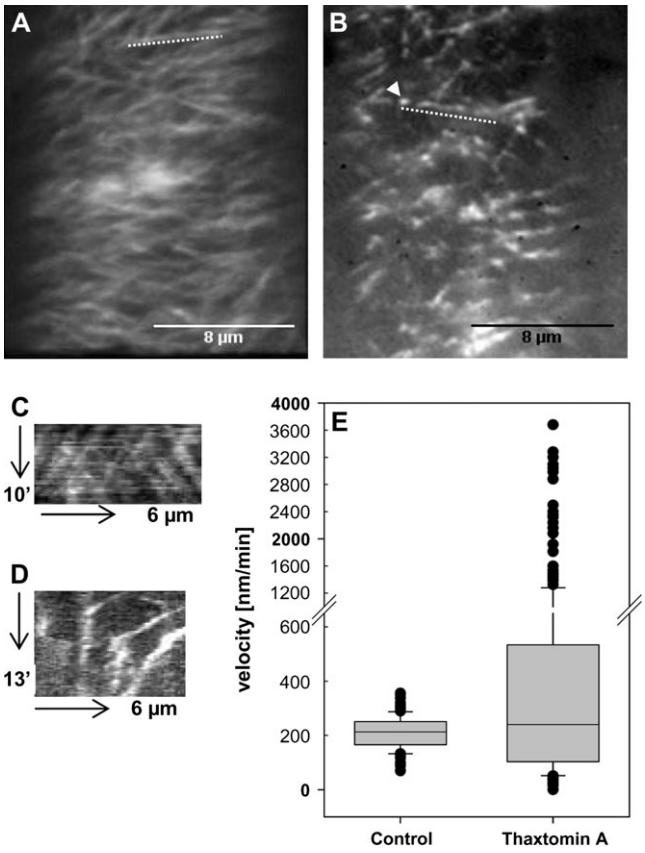

Fig. 4. Thaxtomin A affects CESA-C abundance and motility. (A, B) Optical sections of plasma membrane in upper hypocotyl cells of 3-d-old etiolated *cesa3*^{ie5}:GFP-CESA3 seedlings. Images were acquired by spinning disc confocal microscopy. Average projections of 39 and 54 frames acquired in 15 s intervals in the plane of the cell membrane of control (A) and thaxtomin A-treated seedlings (B), respectively are shown. Average projections illustrate the movement of labelled particles along linear tracks. (C) Kymograph of the region marked by the dotted line in (A) displaying steady and bidirectional particle translocation. (D) Kymograph of region marked by the dotted line in (B) displaying steady, slow, accelerated or very fast particle translocation. (E) Box plot diagram of particle velocities calculated from 140 or 300 particles of control or thaxtomin A-treated seedlings, respectively. Particle velocity was measured in six cells from four seedlings and was determined by manual tracking.

Thaxtomin A causes depletion of CESA complexes from the PM

Short-term thaxtomin A treatments (2.5 h) of seedlings expressing GFP-CESA3 (Desprez *et al.*, 2007) were performed to study the effect of the phytotoxin on the density and velocity of PM-localized CESA-Cs and on cortical microtubule organization. Thaxtomin A strongly compromised CESA-C density and -motility (Fig. 4). Thaxtomin A treatment largely decreased the intensity of GFP-CESA3 signals at the PM in cells of the upper hypocotyl (Fig. 4B) as compared to hypocotyls of untreated control seedlings (Fig. 4A). From this it is concluded that thaxtomin A, like isoxaben (Paredez *et al.*, 2006), also clears CESA-Cs from the PM. CESA-Cs then accumulated in a novel intracellular small microtubule-associated compartment termed MASC (Fig. 4B, arrow) (EF Crowell, V Bischoff, T Desprez, A Rolland, JD Stierhof, K Schumacher, M Gonneau, H Höfte, S Vernhettes; unpublished results). It was observed that the particles display erratic movement; particles show steady and fast, sometimes accelerating and in some cases slowing down movement, generally along linear tracks (Fig. 4D; see Video S1 at *JXB* online). The velocity pattern of the MASCs is different from the normal GFP-CESA3 particles of untreated control seedlings that usually follow linear and bidirectional trajectories with constant velocities (Paredez *et al.*, 2006; Fig. 4C; see Video S2 at *JXB* online) and had similar velocities to those reported in the literature (Desprez *et al.*, 2007) (207±66 nm min^{-1}; range 69–351 nm min^{-1}) (Fig. 4E). The average velocity of MASC particles in thaxtomin A-treated upper hypocotyl cells was as high as

638±558 nm min^{-1} (range 16–3679 nm min^{-1}), or sometimes even reached 10,500 nm min^{-1} (data not shown). Besides the effects on CESA-C density and movement, treatment of seedlings with thaxtomin A did not cause any significant effects on cortical microtubule orientation (see Supplementary Fig. S2 at *JXB* online).

Discussion

Thaxtomin A has previously been described as a putative cellulose synthesis inhibitor (King *et al.*, 2001; Fry and Loria, 2002; Scheible *et al.*, 2003). Additional effects of thaxtomin A on *A. thaliana* seedlings were studied to determine its role in this regard. By analysis of radiolabelled CW fractions it was possible to show that, in particular, the synthesis of pectins, and hemicelluloses (non-cellulosic carbohydrate fractions) was stimulated after thaxtomin A treatment, whereas the synthesis of cellulose was strongly reduced. This study provides more detailed evidence of thaxtomin A's effects on CW composition than has previously been known (Scheible *et al.*, 2003). Based on these results, it is suggested that thaxtomin A-treated plants compensate for their decreased cellulose synthesis by producing other wall polysaccharides. Similar changes have been reported in CW mutants (Burton *et al.*, 2000; Fagard *et al*, 2000; Zhong *et al.*, 2003) and in plants treated with cellulose biosynthesis inhibitors such as isoxaben (Heim *et al.*, 1990, 1998).

The biochemical changes in CW composition invoked by thaxtomin A were accompanied by changes in CW gene expression. Primary *CESA* genes and additional primary CW synthesis genes (*KORRIGAN* and *KOBITO1*) were repressed. The repression was even more pronounced for genes involved in secondary CW synthesis (*CESA7*, *CESA8*, *COBRA-like 4*, *IRX8*, and *IRX9*). From this, it is concluded that, besides its known effects on primary cellulose synthesis (Fagard *et al.*, 2000; Fry and Loria, 2002), thaxtomin A also affects secondary CW synthesis, possibly due to the inability of thaxtomin A-treated seedlings to properly initiate this developmental program when primary wall deposition is altered or incomplete. This interpretation is also mirrored in the strongly reduced expression in thaxtomin A-treated seedlings of a gene named *CAD9* (cf. Table S1 at *JXB* online), which, according to Kim *et al.* (2007), encodes a dehydrogenase of unknown function with predominant expression in xylem-forming vascular tissue.

Thaxtomin A also led to changes in the expression of additional CW-related genes. Some putative galacturonosyltransferases genes, proposed to be necessary for pectin synthesis (Sterling *et al.*, 2006), were slightly repressed, and one was more strongly repressed. This seems contradictory to the increase in radiolabelled pectins, but might be explained by the fact that only a few genes suspected of being involved in pectin synthesis have been proved to be, for example *Qua1* (Bouton *et al.*, 2002) or *Qua2* (Mouille *et al.*, 2007), and others were not investigated in this study. Also transcriptional regulation of pectin synthetic/modifying genes might be overcompensated for by post-transcriptional/post-translational levels of control. The transcriptional regulation of these genes could be more important during development when co-expression of large sets of CW genes is required for proper and largely simultaneous synthesis of CW polymers.

De-esterification of pectins is a prerequisite for the formation of extensive Ca^{2+}-bridged junction zones (Powell *et al.*, 1982; Brett and Waldron, 1990) and of cross-links between free carboxyl groups, for example, between pectins and hemicelluloses or pectins and lignin (Carpita and McCann, 2000). A pectin acetylesterase and two pectin methylesterases were found to be up-regulated by thaxtomin A treatment. Together with the observed strong decrease of a pectate lyase transcript this could signify a higher degree of crosslinking of the pectic network and hence result in the strengthening of the CW as a compensatory response to inhibition of cellulose synthesis by thaxtomin A. This interpretation is supported by previous data on compensation of the loss of cellulose by an increased amount of pectin with a lower degree of esterification (Burton *et al.*, 2000).

Thaxtomin A treatment also resulted in the strong repression of two xyloglucan endotransglucosylase/hydrolases (*XTH*), and the weaker induction of a *XTH*-related gene. The role of individual XTHs is often unknown. Some XTHs have been proposed to catalyse a type of wall-loosening that leads to CW extension both by cutting and restructuring the existing wall-bound xyloglucans (Rose *et al.*, 2002). Repression of such *XTH* genes might thus be a strategy to prevent CW loosening when cellulose synthesis is inhibited. Some XTHs also function in the incorporation of newly secreted xyloglucan chains into the CW (Smith and Fry, 1991; Vissenberg *et al.*, 2005), and the induction of genes encoding these XTHs might strengthen the CW.

While the observed changes in CW gene expression are (i) not always easily understood due to the lack of knowledge of the precise biological functions of the encoded proteins and (ii) not necessarily transferable to the observed changes in CW composition (Fig. 1), the qRT-PCR results support the general idea of extensive CW remodelling to compensate for reduced cellulose in thaxtomin A-treated seedlings. More importantly, the comparison of thaxtomin A and isoxaben clearly reveals that both agents lead to identical qualitative and very similar quantitative effects on CW gene expression, making a strong case for thaxtomin A acting as a cellulose synthesis inhibitor in a manner comparable to isoxaben, namely the inhibition of cellulose synthase *per se* (Scheible *et al.*, 2001; Desprez *et al.*, 2002).

A similar conclusion is reached by the study of ectopic lignification and the analysis of genes involved in lignin biosynthesis. Our data reveal that, when applied at low inhibiting concentrations, thaxtomin A, like isoxaben (Caño-Delgado *et al.*, 2003), invokes ectopic lignification. However, thaxtomin A results in lignification exclusively in the hypocotyl, whereas isoxaben treatment results in lignification in the roots of etiolated *A. thaliana* seedlings

(Fig. 3; Desprez *et al.*, 2002). This suggests that thaxtomin A either affects different tissues and/or molecular targets than isoxaben, or that the uptake of the two agents happens in different tissues. Thaxtomin A-induced lignification might represent another avenue that plant cells can choose to stabilize the compromised CW. Lignin molecules can form entire new CW networks or can be bound to de-esterified carboxyl groups of pectins (see above). The analysis of genes involved in lignin biosynthesis revealed that *CCR2* was highly up-regulated. CCR (cinnamoyl-CoA reductase) proteins play a major role in all proposed lignin pathways (Boerjan *et al.*, 2003; Fan *et al.*, 2006) as they convert CoA intermediates into their aldehydes. In particular, *CCR2* is active during lignification in response to pathogenic attacks (Lauvergeat *et al.*, 2001) and also seems to have a key role in thaxtomin A- and isoxaben-induced ectopic lignification. Up-regulation of *4CL1* and *4CL2* which encode the isoforms involved in lignin formation (Ehlting *et al.*, 1999) and *C4H* further support the induction of lignin biosynthesis at the molecular level. Repression of *4CL3* is irrelevant in this regard as it participates in the biosynthetic pathway leading to flavonoids.

Thaxtomin A and isoxaben-treatments also resulted in the strong up-regulation of several well-known defence genes (Caño-Delgado *et al.*, 2003). However, the overlap between the two agents was not nearly as good as for the CW- or lignin biosynthesis genes (Fig. 2C). It seems likely that induction of defence genes by both agents is the consequence of perturbed cellulose synthesis, but that the spectrum of genes and their quantitative response is related to the place of action (root versus hypocotyl; cf. Fig. 3). It also cannot be ruled out that parts of the defence response represent a more direct response of plants to thaxtomin A in a function similar to that of a bacterial elicitor.

Cellulose synthesis inhibitors like isoxaben and DCB also cause altered CESA accumulation patterns and affect cortical microtubule arrays (DeBolt *et al.*, 2007; Paredez *et al.*, 2006, 2008; Lazzaro *et al.*, 2003). Isoxaben treatments cause loosening of the radial microtubule array in pollen tubes (Lazzaro *et al.*, 2003), a shift of cortical microtubule alignment from transverse to oblique (Paredez *et al.*, 2008) and a release of CESA-C from the PM. Seedlings treated with DCB revealed a reduced CESA-C motility and an accumulation of CESA-C at the PM (DeBolt *et al.*, 2007). No drastic DCB effects on microtubule organization have been demonstrated so far. This study revealed that thaxtomin A, like isoxaben, cleared the CESA3 proteins off the PM. The loss of CESA3 from the PM was accompanied by an accumulation of CESA3-containing particles, which were identified as a novel microtubule-dependent intracellular compartment described previously (EF Crowell, V Bischoff, T Desprez, A Rolland, JD Stierhof, K Schumacher, M Gonneau, H Höfte, S Vernhettes; unpublished results). These particles, named MASC, accumulate CESA proteins and are of a much higher velocity than regular GFP-CESA3 proteins but their function remains unclear. Because of the lack of significant effects on the cortical microtubule array, we propose that thaxtomin A directly affects the CESA-C stability and this reduces CESA-C density in the PM. Similar effects have been found following treatments with other CW inhibitors (isoxaben, DCB, CGA615′325 (Paredez *et al.*, 2006; DeBolt *et al.*, 2007; EF Crowell, V Bischoff, T Desprez, A Rolland, JD Stierhof, K Schumacher, M Gonneau, H Höfte, S Vernhettes; unpublished results), but direct molecular targets have only been proposed for isoxaben (Scheible *et al.*, 2001, Desprez *et al.*, 2002). It seems unlikely that thaxtomin A targets a cellular component that interacts with both CESAs and the cortical microtubule array as proposed for morlin (DeBolt *et al.*, 2007). We propose that thaxtomin A's effect on CESA-C density in the PM directly contributes to the reported reduction in crystalline cellulose biosynthesis (Scheible *et al.*, 2003).

In conclusion, it is suggested that the loss of crystalline cellulose following thaxtomin A treatment is directly linked to the release of CESA-C from the PM. As seen from the transcript profiling data and the [^{14}C]-sucrose incorporation assay, thaxtomin A is very comparable to isoxaben and leads to major changes in CW composition towards the production of pectins and hemicelluloses, and results in additional CW reinforcement by triggering ectopic lignification. In summary, this work provides new and, so far, the most advanced insights into the mode of action of the phytotoxin thaxtomin A, and its cellular and molecular effects on CW biosynthesis.

Supplementary data

The following supplementary data are available at *JXB* online.

Table S1. Quantitative real-time RT-PCR results and primer sequences.

Fig. S1. Aniline blue staining of *Arabidopsis* seedlings treated with thaxtomin A or isoxaben.

Fig. S2. Effect of thaxtomin A on GFP-TUA6-labelled cortical microtubules.

Video S1. Distribution and motility of MASC in the cell membrane.

Video S2. Distribution and motility of GFP-CESA3 in the cell membrane.

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