Cellulose and Cell Elongation

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Cellulose and Cell Elongation

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Abstract The cell wall (CW) is a strong but dynamic exoskeleton, which determines the wide variety of cell shapes (for review see Martin et al. 2001) and provides a mechanical barrier against pathogens (Vorwerk et al. 2004). CW structure and composition vary according to cell type and growth stage: the primary CW is a thin and constantly modified structure allowing cell growth driven by turgor pressure (see Verbelen and Vissenberg, in this volume), whereas secondary CWs are thick and rigid structures, which are laid down as soon as the cell has reached its final size.

Cellulose microfibrils (CMFs) constitute the fibre component of the composite material that makes up the plant CW. Cellulose represents 10–14% of the dry weight of primary CWs, 40–60% of secondary CWs and up to 98% in specialized cells, such as cotton fibres. CMFs are highly oriented and in this way influence the mechanical properties and viscoplasticity of the wall (see Burgert and Fratzl, in this volume). Recent data suggest that, besides controlling cell shape, cellulose synthesis also plays a critical role in the transition between cellular growth stages.

In this chapter we focus on the question of how the synthesis and deposition of cell wall material is coordinated with cell expansion in different cell types. Although several actors involved in cellulose synthesis have been identified, the mechanism and regulation of deposition remain largely unknown. Also, how cellulose associates with other CW polymers and how the cells monitor the status of the cell wall is not understood. We will first describe how genetic screens allowed the isolation of key components of the cellulose synthesis machinery in primary and secondary CWs. Next we will discuss recent findings on the role of CW synthesis in the control of cell expansion.

Abbreviations

CESA	Cellulose synthase catalytic subunit
CSC	Cellulose synthase complex
CMFs	Cellulose microfibrils
CW	Cell wall

1
Cellulose Biosynthesis

1.1
Cellulose: A Simple Structure Made by Complex Machinery

The remarkable tensile strength of cellulose micofibrils (CMFs), which is comparable to that of steel, reflects their unique structure. They are com-

posed of multiple β-1,4-linked glucan chains with a parallel orientation, which are linked to each other by hydrogen bonds and van der Waals forces, thus forming a paracrystalline structure referred to as cellulose I. The parallel orientation is thermodynamically metastable : upon denaturation of the microfibrils, the glucan chains reassemble with an antiparallel orientation, yielding cellulose II. This metastable structure can only be understood in the context of the biosynthesis mode: glucans associate upon extrusion from high molecular weight cellulose synthase complexes (CSC) in the plasma membrane (for a review see Doblin et al. 2002). This stands in contrast to chitin, which is a polymer of β-1,4-linked *N*-acetylglucosamine (GlcNAc) that forms, in most organisms, type II crystals with antiparallel chains that assemble after bulk release into the extracellular space (Carlstrom 1957). In higher plants, CSCs consist of six distinguishable globules that assemble in 25 nm hexameric rosettes (Brown and Montezinos 1976). The dimensions of the microfibril in primary cell walls are compatible with the presence of 36 glucan chains, in which case each globule may produce six glucan chains (Doblin et al. 2002). Immunolabelling has confirmed the presence of the catalytic subunit of cellulose synthase in the rosettes (Kimura et al. 1999). Attempts to purify the CSC have remained unsuccessful so far and their exact composition is unknown.

Cellulose synthase catalytic subunits (CESAs) are members of the glycosyltransferase (GT) family 2 based on the CAZy classification (Coutinho et al. 2003). They consist of eight predicted transmembrane domains (TM) and, between TM 2 and 3, a large catalytic domain exposed to the cytosol with the characteristic D,D,D,QXXRW motif of processive glycosyl transferases. The N-terminal cytosolic region contains a ring-finger domain, which is involved in the homo- or heterodimerization of CESAs through disulfide bonds, and may act as a ubiquitin E3 ligase and mediate the targeted degradation of the monomeric form of CESA (Kurek et al. 2002).

The *Arabidopsis* genome contains ten *CESA* genes and over 30 more distantly related cellulose synthase-like (*CSL*) genes (Richmond and Somerville 2000, 2001). Among the ten *CESAs*, two functional groups can be distinguished based on expression patterns and mutant phenotypes. For *CESA1*, 3 and 6, transcripts are present in most tissues, mutants are dwarfed or seedling-lethal and show cellulose defects in primary cell walls. *CESA4*, 7 and 8 are expressed in developing xylem and interfascicular fibre cells. Mutants lack the characteristic secondary thickenings in xylem cells, which causes the collapse of the xylem. Interestingly, orthologues for each of the six isoforms can be found in other species including the monocots rice, barley and maize and the Gymnosperm *Pinus taeda* (Table 1). This indicates that the six isoforms are evolutionary conserved and that their origin preceded the divergence between dicots and monocots (Fig. 1) and, at least for *CESA4*, 7 and 8, the divergence between Angiosperms and Gymnosperms. The requirement for CESA triplets in the same cell types suggests that they are part of the same complex. This has been confirmed in co-immunoprecipitation experi-

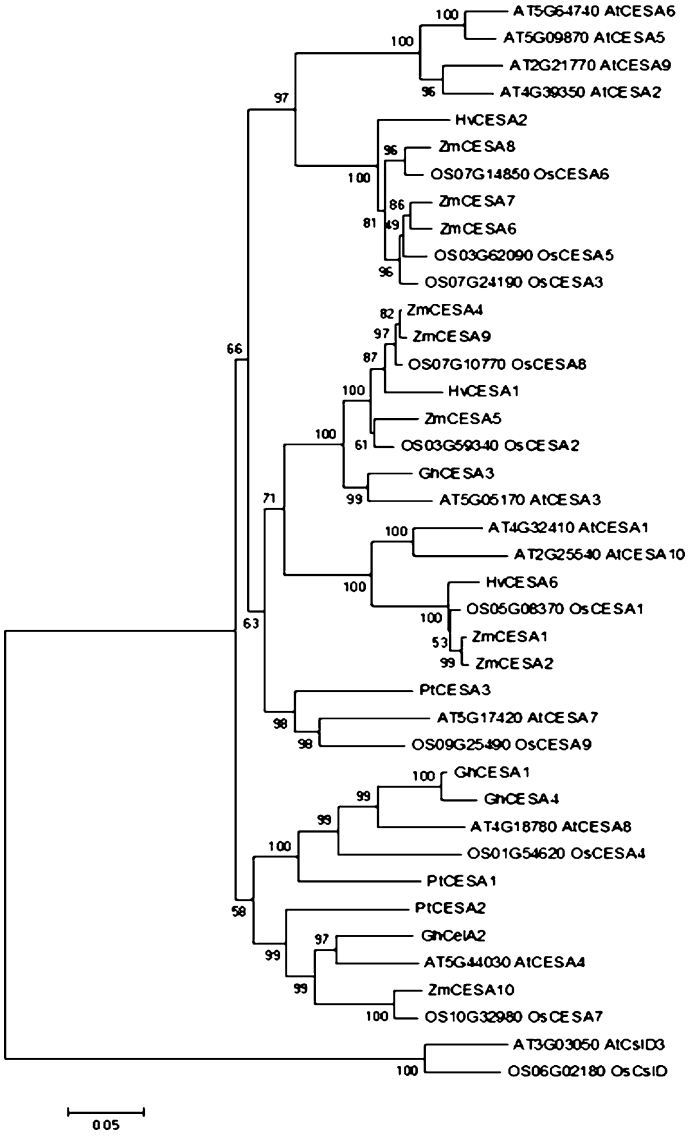

Fig. 1 Neighbor-joining dendrogram of relationship among CESAs of different plant species, rooted with Arabidopsis and Rice CslD/KJK (most closely related CSL family to CESA). The phylogenetic tree is based on ClustalW 1.83 alignment of the full-length protein sequence of *Arabidopsis thaliana* At, *Oryza sativa* Os, *Gossypium hirsutum* Gh, *Hordeum vulgare* Hv, and *Pinus taeda* Pt. The GhCesA2 sequence was assembled from two partial EST covering > 95% of the coding sequence. Sequences have been retrieved from http://cellwall.stanford.edu/, The bootstrapped phylogenetic tree (sampling 1000 times) was generated using MEGA 3.1 (Kumar et al. 2004) based on a ClustalW alignment. The bootstrap value is indicated. Gene numbers for Arabidopsis and Rice are from FLAGdb (http://urgv.evry.inra.fr/projects/FLAGdb++/HTML/index.html/)

Table 1 Orthologous CESA triplets in different species

	Arabidopsis thaliana	*Oryza sativa*	*Hordeum vulgare*	*Zea mays*	*Gossypium hirsutum*	*Pinus taeda*
Primary CW	AtCESA1,10	OsCESA1	HvCESA6	ZmCESA1,2	N.A.	N.A.
	AtCESA3	OsCESA2,8	HvCESA1	ZmCESA4,5,9	GhCESA3	N.A.
	AtCESA6,5,2,9	OsCESA3,5,6	HvCESA2	ZmCESA6,7,8	N.A.	N.A.
Secondary CW	AtCESA4	OsCESA7	N.A.	ZmCESA10	GhCESA2	PtCESA2
	AtCESA7	OsCESA9	N.A.	ZmCESA3?	N.A.	PtCESA3
	AtCESA8	OsCESA4	N.A.	ZmCESA3?	GhCESA1,4	PtCESA1

N.A. full length sequence not available

ments for CESA4, 7 and 8 (Gardiner et al. 2003; Taylor et al. 2003) and *CESA1*, 3 and 6 (Gonneau M and Desprez T, unpublished data). Additional genetic evidence corroborates the direct in vivo interaction, at least between CESA3 and CESA6 subunits. Indeed, mutations in either CESA3 (*ixr1-1* and *2*; Scheible et al. 2001) or CESA6 (*ixr2-1*; Desprez et al. 2002) confer increased resistance to the cellulose inhibitor isoxaben. The simplest explanation for the existence of two non-redundant resistance loci is that isoxaben recognizes an epitope associated with the CESA3- and CESA6-containing complex.

The sequence comparisons suggest that *CESA2*, *5*, *9* and *10* correspond to more recent gene duplications (Fig. 1, Table 1). CESA2, 5 and 9 are most closely related to CESA6, whereas CESA10 is most similar to CESA1. The transcript profiles show that *CESA2*, *5*, *9* and *10* are expressed during embryogenesis. They are partially redundant, as shown by the absence of an observable phenotype for loss of function mutations (Juraniec M and Gonneau M, unpublished results). Interestingly, *cesA6* mutants show a short hypocotyl phenotype only when grown in the dark. Light, through the activation of phytochrome, rescues the cellulose defect and the growth phenotype (Desnos et al. 1996). The expression of *CESA5* is redundant in the light with *CESA6* (Fig. 2, AtGE_7: green part 7 days). CESA5 may therefore replace CESA6 in light-grown tissues, which would explain the absence of a light-grown phenotype in *cesA6* mutants.

1.2 Other Proteins Involved in Cellulose Synthesis

1.2.1 Other Proteins Required for Cellulose Synthesis in Primary Walls

A common phenotype of mutants with cellulose defects in primary cell walls is the reduced cell elongation in dark-grown hypocotyls associated with an

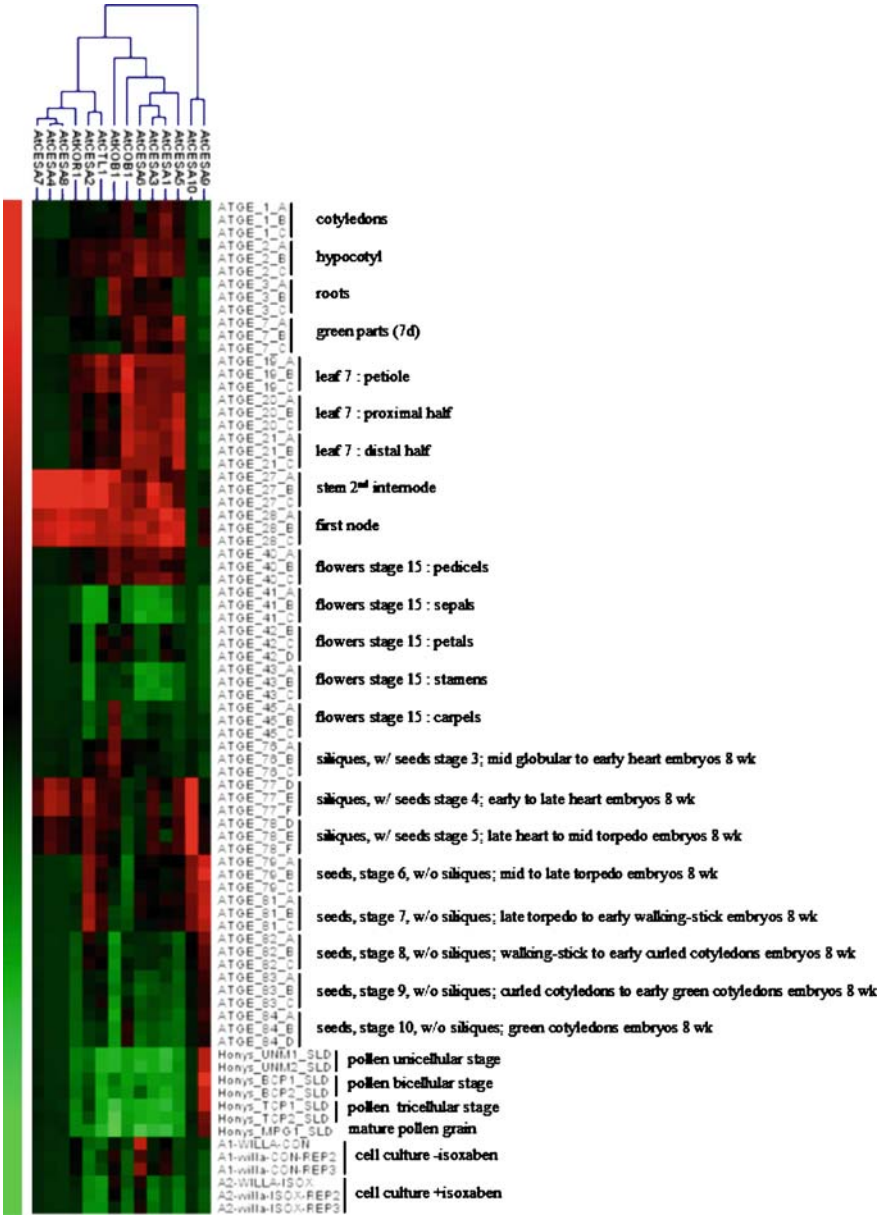

Fig. 2 Hierarchical clustering of several actors in cellulose synthesis (see Table 2 for gene numbers). Genes were clustered based on their expression levels across 79 selected ATH1 microarray data sets from (Honys and Twell 2004; Manfield et al. 2004; Schmid et al. 2005) hosted by NASC http://affymetrix.arabidopsis.info/narrays/experimentbrowse.pl. Clustering was performed using Genesis 1.5.0. software (Sturn et al. 2002). Gene expression levels have been normalized per gene prior to hierarchical clustering. *Green* and *red colour scales* represent low and high expression levels, respectively

Table 2 Arabidopsis cellulose-deficient mutants

AGI number	Function	Name alleles	Refs.
At4g32410	Cellulose synthase catalytic subunit, CESA1	*rsw1*	Arioli et al. 1998
At5g05170	Cellulose synthase catalytic subunit, CESA3	*eli1*	Cano-Delgado et al. 2000, 2003
		cev1	Ellis and Turner 2001; Ellis et al. 2002
		ixr1	Scheible et al. 2001
At5g64740	Cellulose synthase catalytic subunit, CESA6	*prc1*	Desnos et al. 1996; Fagard et al. 2000
		ixr2	Desprez et al. 2002
		YFP-CESA6	Paredez et al. 2006
At5g60920	GPI anchored protein	*cob1*	Schindelman et al. 2001 Roudier et al. 2005
At3g08550	Novel type II intrinsic membrane protein or secreted serine rich protein	*kob1*	Pagant et al. 2002
		eld1	Lertpiriyapong and Sung 2003
		abi8	Brocard-Gifford et al. 2004
At1g05850	Putative secreted basic chitinase-like protein (AtCTL1), GH19	*pom1*	Hauser et al. 1995
		elp1	Zhong et al. 2002b
At5g49720	Membrane-bound endo-β-1,4-glucanase, GH9 activity against CMC and non-crystalline cellulose, not against xyloglucan (Molhoj et al. 2002)	*kor1*	Nicol et al. 1998
		rsw2	Lane et al. 2001
		acw1	Sato et al. 2001
		tsd1	Frank et al. 2002
		irx2	Szyjanowicz et al. 2004
		GFP-KOR1	Robert et al. 2005
At5g44030	Cellulose synthase catalytic subunit, CESA4	*irx5*	Taylor et al. 2003

Table 2 (continued)

AGI number	Function	Name alleles	Refs.
At5g17420	Cellulose synthase catalytic subunit, CESA7	*irx3* *fra5*	Taylor et al. 1999 Zhong et al. 2003
At4g18780	Cellulose synthase catalytic subunit, CESA8	*irx1* *fra6*	Taylor et al. 2000 Zhong et al. 2003
At5g47820	Kinesin	*fra1*	Zhong et al. 2002a
At2g28110	Xylan glucuronyltransferase, GT47	*fra8* *irx7*	Zhong et al. 2005 Brown et al. 2005
At5g15630	COBRA-Like4	*Cobl4/irx6*	Brown et al. 2005
At5g22130	Mannosyltransferase GPI-anchor synthesis	*pnt1*	Gillmor et al. 2005
At2g39770	Mannose-1-phosphate guanylyltransferase	*Cyt1*	Lukowitz et al. 2001
At1g67490	Glucosidase I	*gcs1* *knf*	Boisson et al. 2001 Gillmor et al. 2002
At5g63840	Glucosidase II	*rsw3*	Burn et al. 2002
At5g13710	C-24 methyltransferase	*smt1/cph*	Reviewed in Schrick et al. 2004
At3g52940	C-14 reductase	*fk*	
At1g20050	C-7,8 isomerase	*hyd1*	
At1g12840	Vacuolar ATP-ase subunit	*det3*	Schumacher et al. 1999

Grey and *black bars* represent genes involved in cellulose synthesis in the primary and secondary CW, respectively. *Light grey bar* represents "housekeeping genes" required for normal cellulose synthesis

exaggerated radial expansion. Genetic screens for mutations affecting dark-grown hypocotyl elongation yielded numerous cellulose-deficient mutants (Mouille et al. 2003; Robert et al. 2004). Other screens for temperature-sensitive *radial swelling* (*rsw*) mutants (Williamson et al. 2001) or sugar-sensitive *COnditional Root Expansion* (*CORE*) mutants (Hauser et al. 1995), provided other cellulose-deficient mutants and showed the importance of cellulose for cell expansion. Other cellulose–deficient mutants were found in screens for embryo-defective phenotypes (Gillmor et al. 2002, 2005). All these screens identified main actors in cellulose synthesis in the primary CW, like CESAs but also several non-GT proteins (Table 2).

KORRIGAN1 (KOR1). Mutant alleles have been identified with phenotypes ranging from seedling-lethal with cytokinesis defects; uncontrolled cell proliferation in the meristem; temperature-sensitive root-swelling; and dwarf to normal size with collapsed xylem. These phenotypes show that KOR1 is required for cellulose synthesis at all growth stages. KOR1 encodes a membrane-bound cellulase (Nicol et al. 1998). The protein produced in *Pichia pastoris* shows Ca^{2+}-dependent activity on non-crystalline cellulose and not on xyloglucan (Molhoj et al. 2001, Master et al. 2004). KOR1 cycles through the Golgi apparatus, endosomal compartments and the plasma membrane (Robert et al. 2005). All cellulose-producing organisms investigated also express cellulases. In *Agrobacterium* and *Rhizobium*, a cellulase is part of the celllulose synthase operon and mutant analysis showed that the protein has an essential role in cellulose synthesis (Hayashi et al. 2005). The exact role in cellulose synthesis is not known. It has been proposed that KOR1 may play a role in the recycling of the sitosterol-glucoside primer (Peng et al. 2002), remove uncorrectly assembled glucan chains or release the cellulose synthase complex from the growing glucan chain.

COBRA (COB1). Strong mutants are seedling-lethal, a weaker allele shows radial expansion of the root in the presence of high concentrations of sucrose or at high temperatures. COB1 is a GPI-anchored extracellular protein, which in the root is localized above the cortical microtubules in elongating cells. It may play a role in linking CESA complexes to cortical microtubules (Schindelman et al. 2001; Roudier et al. 2005).

POMPOM1(POM1)/ECTOPIC LIGNIN IN PITH (ELP1). Mutants are dwarfed, cellulose-deficient (Mouille et al. 2003) and produce ectopic lignin (Hauser et al. 1995; Zhong et al. 2000). POM1 shows sequence similarity to basic chitinases, but lacks critical catalytic residues, which suggests that the protein does not have chitinase activity (Zhong et al. 2002b). Plants do not contain chitin. N-linked glycans and arabinogalactan proteins, and perhaps NOD-related oligosaccharides, contain instead the chitin building block GlcNAc. It is conceivable that POM1 interacts with one of those and thus regulates cellulose synthesis in an unknown way.

KOBITO1 (KOB1)/ELONGATION DEFECTIVE (ELD1)/ABSCISSIC ACID INSENSITIVE 8 (ABI8). This locus was identified in screens for dwarf mu-

tants (*eld1*), cellulose-deficient dwarf mutants (*kob1*), or mutants that show abscisic acid-insensitive germination (*abi8*). In mutant roots at early growth stages, the cell division zone appears normal and the elongation zone is strongly reduced. Field emission SEM of the innermost cell wall layers in root cells showed the presence of transversely oriented microfibrils in the cell division zone of both wild type and mutant. In the rudimentary elongation zone in *kob1*, only an amorphous matrix was observed. This matrix consisted of pectins since treatment with pectate lyase removed the layer and uncovered an underlying network of randomly oriented microfibrils. The randomization of the remaining microfibrils may be the result of their reorientation during cell expansion or the perturbation of the oriented microfibril deposition. At later growth stages, the mutant phenotype also propagated apically with the disappearance of the root meristem (Brocard-Gifford et al. 2004). Interestingly, the wild type phenotype is rescued in the elongation zone in the presence of 1% glucose.

KOB1 is a type II membrane protein of unknown function. GFP-KOB1 expressed from the 35S promoter was observed in intracellular compartments in the dividing cells and in the plasma membrane in elongating cells. A translational GUS-fusion from its own promoter showed an intracellular punctate staining. An ELD1-GFP fusion instead accumulated in the cell wall. These differences may be related to differences in the fusion proteins: GFP fused to the N-terminus (KOB1) or the C-terminus (ELD1), or GUS fused to the C-terminus (ABI8). Alternatively, they could be due to the promoter used (35S vs endogenous promoter). Resolving this discrepancy will require immunolocalization of the endogenous protein. The relation between the growth defect, the cellulose defect, the ABA-insensitivity and the effect of glucose on the phenotype is not understood, but reflects cross-talk between cellulose metabolism, soluble sugars and hormone signalling.

KOR1, POM1, COB and KOB1 are all members of multigene families with 3, 2, 12 and 3 members, respectively. The function of the other family members remains to be determined.

A number of cellulose-deficient mutants show embryo-defective phenotypes. The corresponding genes encode enzymes involved in *N*-glycan modification or sterol synthesis (Table 2). The cell walls of these mutants show a reduced cellulose content without important changes in other polysaccharides. This suggests that, at least during embryogenesis, cellulose synthesis is particularly sensitive to *N*-glycan-mediated quality control in the ER (Boisson et al. 2001; Lukowitz et al. 2001; Gillmor et al. 2002) and to changes in sterol content, in contrast to enzymes involved in matrix polysaccharide synthesis. The sensitivity of cellulose synthesis to variations in sterol composition may be related to the requirement for sterol-glucoside as a primer for cellulose synthesis, or the requirement of sterol-containing lipid rafts for the correct targeting of cellulose synthase complexes to the plasma membrane (Peng et al. 2002).

1.2.2
Other Proteins Required for Cellulose Synthesis in Secondary Walls

Genes involved in the synthesis of secondary cell walls have been identified in screens for *irregular xylem (irx)*, (Turner and Somerville 1997) and *fragile fibre (fra)*, (Zhong et al. 2001) mutants. These mutants in general do not show growth defects but lack the secondary cell wall thickenings that line the lumen of xylem elements and interfascicular fibres. As for the primary CW, a triplet of CESAs (CESA4, 7 and 8) is essential for cellulose synthesis. Interestingly, KOR1 and POM1 seem to be involved in both primary and secondary CWs whereas a COB1 paralogue (COBL4/IRX6) instead is involved in cellulose synthesis in secondary CWs only (Brown et al. 2005).

1.2.3
The Function of CSL Genes

The CSL family has been classified into eight subfamilies according to sequence similarities. Typically, monocots possess CSLA, C, D, F, H and dicots CSLA, B, C, D and G. The function of most of the CSL proteins is unknown except for the subclass CSLA and CSLF, members of which have been shown to be involved in the synthesis of (gluco)mannans (Dhugga et al. 2004; Liepman et al. 2005) and of mixed-linked β1,3;1,4 glucan (Burton et al. 2006).

At least one member of the CSLD family (CSLD3/KOJAK) is required for cell elongation, specifically in tip growing cells. Mutants in this gene have a normal phenotype except for the root hairs, which are initiated normally but subsequently swell and lyse (Favery et al. 2001). Another family member NaCSLD1 is strongly expressed in pollen tubes of *Nicotiana alata* (Doblin et al. 2001). Both pollen tubes and root hairs are unaffected in *cesA* mutants and express CESA isoforms at basal levels whereas they strongly express CSLD isoforms. Given the high sequence similarity between CSLD and CESA and the presence of the N-terminal ring-finger domain, which is absent in other CSL isoforms, they also might multimerize to form rosettes and synthesize cellulose.

1.3
Transcriptional Co-Regulation Identifies Novel Genes Potentially Involved in Cellulose Synthesis

As expected, the transcripts of the three *CESA* isoforms that are part of the same complex are co-regulated, as shown by the analysis of public transcriptome data sets (Manfield et al. 2004; Persson et al. 2005; Jen et al. 2006, see groups clustered in Fig. 2). Given this co-regulation, it is reasonable to assume that other potential protein partners can be identified by searching for co-regulated genes using the available microarray data for *Arabidopsis*. Fig-

ure 2 shows expression profiles for the above-mentioned genes extracted from 79 Affymetrix chip experiments. *CESA1*, 3 and 6 are indeed co-regulated and form a cluster with *CESA5* and *COB1*. *CESA2*, *POM1/CTL1* and *KOB1* form a distinct cluster. The profiles of *CESA4*, 7 and 8 form a cluster with *KOR1*, confirming its involvement also in secondary wall synthesis. Finally *CESA10* is exclusively expressed during embryogenesis and *CESA9* in the developing embryo and in microspores. Using a similar approach, Brown et al. (2005) identified a series of genes with expression patterns that are correlated with *CESA4*, 7 and 8 using *CESA7* as bait. Interestingly, among 19 co-regulated genes tested, ten showed a collapsed xylem phenotype when mutated, confirming their role in secondary cell wall synthesis.

1.4 Regulation of Cellulose Synthesis

The synthesis of cellulose microfibrils involves a large number of steps, which are all potential regulatory targets. As shown above, CESAs are regulated at the transcript level during cellular differentiation by redox-dependent dimerization of CESA monomers and perhaps through glycosylation and quality control in the ER. Other potential levels of regulation are the assembly of dimers into globules and into hexameric complexes, regulated intracellular trafficking from the Golgi apparatus to the cell surface, supply of the substrate UDP-glucose, priming of glycan synthesis, movement of the complexes in the plasma membrane, extrusion and assembly of glucan chains into microfibrils, termination of polymerization, endocytosis and turnover of the complex.

1.4.1 Intracellular Trafficking

Recently, evidence was obtained showing an important role for intracellular trafficking of CESA6 and KOR1 in the regulation of cellulose synthesis. Using functional YFP-CESA6 fusions expressed from the endogenous promoter, Paredez et al. (2006) studied epidermal cells of dark-grown hypocotyls and showed that YFP-CESA6 was present in subcellular compartments, including the Golgi apparatus and the plasma membrane. Interestingly, whereas plasma membranes were strongly labelled in smaller cells at the top of the hypocotyl, larger cells towards the hypocotyl basis showed a much stronger labelling of intracellular compartments. Treatment of seedlings with isoxaben also led to the rapid disappearance of YFP-CESA6 from the cell surface. Together, these observations show that the insertion into and the retrieval from the plasma membrane of the complexes is highly regulated. The cellulase KOR1 also accumulated in intracellular compartments (Robert et al. 2005), including Golgi apparatus and early endosomes and tonoplast, but could not be detected at

the cell surface. Several observations suggest, however, that KOR1 cycles between intracellular compartments and the plasma membrane. Interestingly, intracellular KOR1-containing compartments were motile and this motility required intact microtubules, in contrast to the motility of the Golgi apparatus, which depends on actin and not on microtubules. One role for KOR1 could be in the removal of the complexes from the glucan chains prior to their endocytosis.

1.4.2 Surface Movement of CESA6

In YFP-CESA6 transformants, one can observe linear arrays of fluorescent dots in epidermal cells of the hypocotyl. These are presumably the cellulose synthase complexes, which are aligned above the microtubules in the plasma membrane. These objects display linear bidirectional trajectories, which follow microtubules, also when they are reoriented after illumination of the seedling or upon treatment for short periods with the microtubule depolymerizing drug oryzalin. Interestingly, upon complete removal of microtubules by longer treatments with oryzalin, YFP-CESA6 re-adopted transverse trajectories (Paredez et al. 2006). This shows that microtubules are not required for the motility of YFP-CESA6, which must be propelled by the polymerization of glucan chains. The rigidity of the microfibrils most likely ensures the maintenance of linear trajectories. In addition, these results show that, at least for the trajectories of CESA6-containing complexes, two levels of control exist: on one hand a default self-organizing behaviour, which leads to defined trajectories and on the other hand, the coupling to cortical microtubules, which in certain conditions overrides the default organization and imposes a direction, presumably via direct interaction between the cellulose synthase complex and cortical microtubules. Evidence for such a default organization exists for instance during secondary cell wall deposition in *Equisetum* root hairs, in which microfibrils are deposited in a helicoidal pattern, whereas microtubules adopt an axial orientation (Traas et al. 1985). It will be interesting to see whether such uncoupling occurs every time microtubules reorient towards an axial orientation after cessation of elongation of other cell types. A model has been proposed that predicts the orientation of the microfibrils based on the geometry of the cell and the density of the complexes (Emons and Mulder 1998).

1.4.3 Phosphorylation

Recent phosphoproteomics studies on cytoplasmic domains of plasma membrane proteins from *Arabidopsis* cell suspensions identified phosphopeptides that correspond to CESA3, CESA5 and KOR1 (Nuhse et al. 2004). The Ser/Thr

phophorylation sites in CESA3 and CESA5 are located in conserved residues in the isoform-specific "hypervariable region" within the N-terminal cytoplasmic domain. Also in KOR1, the three phosphorylation sites in the cytoplasmic tail are highly conserved among different species. It will be interesting to see at what level (activity, assembly, targeting or turnover) phosphorylation plays a regulatory role.

2
Coordination Between Cell Wall Synthesis and Cell Elongation

As explained extensively in the chapter by Fricke and Chaumont in this volume, plants have a hydrostatic skeleton, consisting of cells filled with water and solutes under pressure, which are surrounded by a highly resistant cell wall. Burgert and Fratzl (in this volume) explained that the oriented microfibrils with their high tensile strength play a key role in the resistance of the thin cell wall (0.1–0.5 μm) to the extreme tensile forces imposed by the cell's turgor pressure (0.5–1 MPa). The drawback of this building plan is that the tough cell wall imposes important constraints on all aspects of growth and development.

A long-standing question in plant biology in this respect is how plant cells manage to expand despite the presence of the wall. Early models proposed that cells could grow through intussusception of new wall material within the existing cell wall (Nageli 1858). With such a mode of cell expansion, one expects a perfect correlation between wall incorporation and cell elongation. Subsequent studies on excised oat coleoptile segments (Heyn and Van Overbeek 1931) and on Avena coleoptiles (Bonner 1934) showed that the addition of "growth substance" (later christened auxin) could in certain conditions stimulate cell elongation without a change in the wall synthesis rate. Heyn and Van Overbeek (1931) showed instead that auxin stimulated the plastic extensibility of oat coleoptile walls. These findings were confirmed in intact plants by Roland et al. (1982), who observed a thick helicoïdal wall at early growth stages of epidermal cells of mungbean hypocotyls and a much thinner wall in more elongated cells. Kutschera and Briggs (1987) compared cell wall dynamics in light and dark-grown sunflower hypocotyls. Both light- and dark-grown hypocotyls produced the same amount of wall material despite the dramatic differences in growth rate. They also observed substantial thinning of the wall in more extensively growing cells of dark-grown seedlings. Finally, consistent with previous findings, the extensibility of the wall was greatly reduced in the light compared to the dark. Together, these and other observations led to the conclusion that cell expansion is regulated by the plastic extensibility of the wall rather than by the synthesis rate of wall material. This explains why wall extensibility has been and still is more intensively studied than wall synthesis.

In this context, the acid growth theory plays a central role. This theory states that auxin causes acidification of the apoplast, which in turn stimulates viscoplastic deformation of the wall through the regulation of pH-sensitive wall-modifying agents (for a review see Rayle and Cleland 1992). This theory was corroborated with the discovery of expansins as pH-dependent cell wall-lubricating proteins (see McQueen-Mason et al, in this volume). The acid growth theory requires a mechanism for acidification, via the activation of H^+-ATPases in the plasma membrane, as well as the presence of expansins and perhaps other wall-modifying agents in the cell wall. This theory does not require changes in the composition of the cell wall polymers. Indeed, Kutschera and Briggs (1987) did not observe differences in the relative proportion of cellulose, hemicellulose and pectin between walls of sunflower seedlings grown in the dark or in the light. Brummell and Hall (1985) suggested instead a critical role for the incorporation of matrix polysaccharides in auxin-promoted cell elongation in growing etiolated pea hypocotyl segments. Interestingly, the ionophore monensin, which inhibits secretion of proteins and matrix polysaccharides, inhibited auxin-stimulated elongation, whereas the cellulose synthesis inhibitor DCB did not influence this process.

Along the same lines, Takeda et al. (2002) showed that the incorporation into the cell wall of xyloglucan oligosaccharides by xyloglucan endotransglycosylase/hydrolase (XTH; see Nishitani and Vissenberg, in this volume) promoted cell expansion in pea stem segments, whereas grafting larger XG fragments inhibited cell expansion. The specific action of XTH observed in the root elongation zone of *Arabidopsis* (Vissenberg et al. 2000) is consistent with a role for XG grafting in growth control. Interestingly, expression of a fungal XGase in transgenic poplar caused increased growth and cellulose content, suggesting that the incorporation of XG oligomers in the cell wall may not only control cell elongation but also may feed back to cellulose synthesis (Park et al. 2004). Finally, the pectin composition also appears to change during cell elongation (see Verhertbruggen and Knox, in this volume). The anti-β-1,4-galactan monoclonal antibody LM5 specifically labels elongating cells (McCartney et al. 2003). It is not clear whether this transient galactan accumulation plays a critical role in the control of cell elongation.

2.1
Coupling Cellulose Synthesis and Cell Elongation in the Arabidopsis Hypocotyl

We are using the dark-grown *Arabidopsis* hypocotyl as a model to study the control of cell elongation (Gendreau et al. 1997). Post-embryonic growth occurs in the absence of cell divisions in the epidermis, except for those involved in stomatal guard cell differentiation. The 20 epidermal cells in each cell file of the hypocotyl epidermis measure 10 μm in the embryo and can reach up to 1 mm at the end of elongation. In our standard conditions, seeds germinate 24 h post-imbibition (pi). Between 24 h pi and 48 h pi, all hypocotyl cells

grow slowly and synchronously (Refregier et al. 2004), but between 48 h and 52 h pi, cells at the hypocotyl basis show an abrupt fourfold increase in relative elemental growth rate (REGR, a measure for the growth rate per unit length). The REGR diminishes in these cells between 52 and 55 h. To investigate the molecular events in the CW that underly this transient growth acceleration, we used FT-IR microspectroscopy to follow changes in the cell wall architecture before, during and after the growth acceleration (see inset for details; Pelletier S, Renou J-P and Höfte H, unpublished data). This technique provides a molecular fingerprint of the cell walls, reflecting the relative proportions of molecules, but does not provide information on their absolute amounts.

Between 45 h pi and 48 h pi prior to the growth acceleration, we observed major changes in the ratio between cell wall polymers at the hypocotyl basis. As a negative control during the same period we did not observe significant changes in cells at the top of the hypocotyl, in which growth acceleration takes place only at 80 h pi or beyond. Observed changes involved an increase in the relative cellulose content and a reduced protein and pectin content, with no apparent changes in the degree of methylesterification (DM). These changes can be explained by an increased cellulose synthesis without an increase in pectin deposition. During the growth acceleration between 48 h pi and 52 h pi, we observed a further increase in relative cellulose content and a decrease in a specific ester peak (at 1751 cm^{-1}), without an increase in carboxylic acids. This suggests the concomitant de-methyl-esterification and turnover of pectic polysaccharides.

Between 52 h pi and 55 h pi, we did not observe significant changes in relative cell composition, suggesting that the cell walls had reached a new steady state equilibrium. No significant changes were observed at the top of the hypocotyl throughout the 45–55 h pi period. These findings suggest that the ratio between cell wall polymers is stable in cells with a constant REGR (before but also after the growth acceleration) and that the growth acceleration is preceded and accompanied by major changes in the cell wall composition, including an increased relative cellulose content.

Using isoform-specific antisera, we showed that the increase in relative cellulose content coincided with the accumulation of CESA3 and CESA6 cellulose synthase isoforms (Desprez T, Gonneau M and Höfte H, unpublished data). CESA1 has not been analysed yet in this context. An essential role for CESA6 in this process was shown using the loss of function mutant *cesA6*$^{prc1\text{-}1}$. Hypocotyls of this mutant failed to accumulate cellulose and maintained a low relative cellulose/pectin ratio. Interestingly, *cesA6*$^{prc1\text{-}1}$ hypocotyl cells showed a slow elongation phase indistinguishable from that of the wild type, but failed to accelerate their growth and maintained the slow elongation rate instead throughout post-embryonic development (Refregier et al. 2004). An essential role for the increase in relative cellulose content in growth acceleration was further confirmed using isoxaben treatments be-

fore and after the growth acceleration. Administration of 4 nM of isoxaben to seedlings at 30 h pi (Refregier et al. 2004) or even at 45 h pi (unpublished data) inhibited subsequent growth acceleration. Interestingly, adding 4 nM isoxaben after 52 h pi did not inhibit further cell elongation. In conclusion, the results show that an increase in the cellulose/pectin ratio occurs prior to and during growth acceleration in cells at the hypocotyl basis. The activity of CESA6 plays a key role in the relative increase in cellulose content and is also required for the growth acceleration to occur, as shown by the *cesA6* mutant phenotype and isoxaben treatments. Once growth has accelerated, cell elongation is not further inhibited by isoxaben. The increase in relative cellulose content may act as a check-point, controlling the transition from cytoplasmic growth to vacuolar cell expansion.

The dwarf phenotype of cellulose-deficient mutants is generally interpreted as being the result of the loss of growth anisotropy as a result of the fragilization of the cellulose-deficient cell wall. Our observations show a more complex picture. The inhibition of the growth acceleration occurs rapidly, within 3 h and before changes in microfibril orientation or radial swelling can be observed. This growth inhibition presumably involves an active process since second-site mutations, corresponding to at least three loci, were found that partially restored the growth acceleration of *cesA6* hypocotyls, without restoring the cellulose defect. One of these genes was cloned and encodes a plasma membrane receptor-like ser/thr kinase (Hématy K and Höfte H, unpublished data). This kinase is a good candidate for a cell wall integrity sensor.

3 Conclusions

Genetic studies and life cell imaging have shed new light on the complexity of the cellulose synthesis machinery. New molecular actors have been identified and an important regulatory role for intracellular trafficking has been demonstrated. Finally, recent evidence shows that cell wall synthesis plays a critical role in the control of early stages of cell elongation.

FT-IR BOX

Chemical Imaging of Plant Cell Walls Using FT-IR Microspectroscopy

Principle

The chemical analysis of cell walls is notoriously difficult due to the complexity and heterogeneity of cell wall polymers and the insolubility in aqueous

solutions of many components, such as cellulose or lignin. Spectroscopic techniques combined with a microscope can provide relevant information on the cell wall composition at a cellular level. Fourier transform infra red (FT-IR) spectroscopy is based on the absorption of IR frequencies (10 000 to 100 cm^{-1}) by the vibration of asymmetric chemical bonds, with each

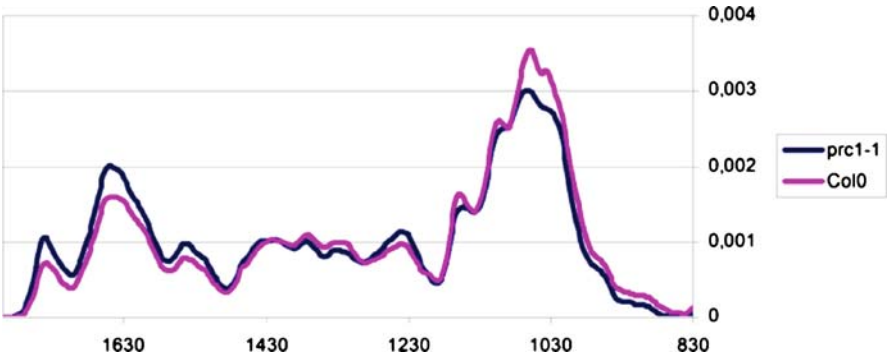

Fig. 3 FT-IR microspectroscopy can be used to distinguish cell wall mutant from wild type. Absorption spectra were obtained in transmission mode from a 50 × 50 μm surface of 4-day-old dark-grown *Arabidopsis* hypocotyls. Comparison between average spectra from cellulose-deficient seedlings *cesa6*$^{prc1-1}$ and the wild type controls; axis is wavenumber and ordinate is relative intensity (courtesy of G. Mouille)

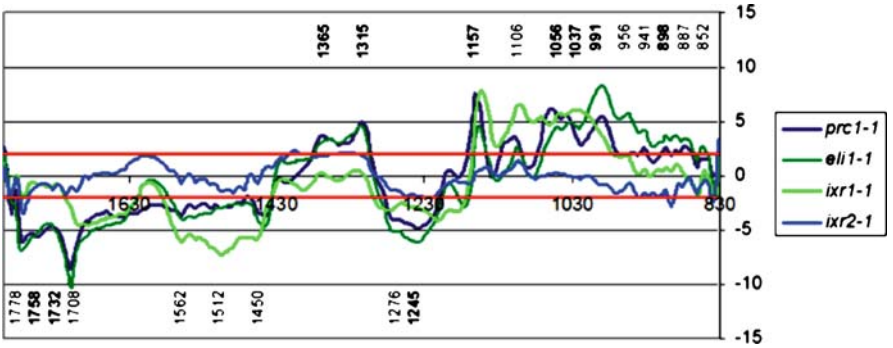

Fig. 4 Student's *t* test on comparison between average spectra from wild type against *cesa3*$^{eli1-1}$, and *cesa3*$^{ixr1-1}$, *cesa6*$^{prc1-1}$, *cesa6*$^{ixr2-1}$. Ordinate is *t*; a value of 2 is significant at $p = 0.01$ (*red line*). Highly significant positive peaks can be assigned to absorption maxima of cellulose (1365, 1315, 1157, 1056, 1037, 991 and 898 cm^{-1} *in bold*). Negative peaks correspond to carboxylic ester (1758, 1732 cm^{-1}) or carboxylic acid (1708 cm^{-1}) bonds and a C – O stretch (1245 cm^{-1}), presumably of pectic polysaccharides. *prc1-1* and *eli1-1* are cellulose-deficient but show increased pectic content. *ixr1-1* and *ixr2-1* are isoxaben-resistant alleles of CESA3 and CESA6, respectively, which do not show a growth phenotype. Note that the cell wall composition of *ixr1-1* is significantly different from the wild type even in the absence of isoxaben, in contrast to the cell walls of *ixr2-1* (courtesy of G. Mouille)

structural feature of a molecule absorbing at characteristic wavenumbers (expressed in cm^{-1}). Digitalization of the interferogram, followed by fast Fourier transformation of the data, quickly provide (within minutes) an absorption spectrum at different wavenumbers (Fig. 3). FT-IR spectroscopy can contribute to the identification of chemical groups, the structure of the molecular backbone and the interaction of the molecule with its environment. Simple unknown compounds can be identified by comparison with a reference database. Complex mixtures like cell walls contain too many chemical bonds with overlapping absorption wavenumbers to allow precise analysis. We use the spectral data between 1830 and 830 cm^{-1} of the mid-IR (4000 to 700 cm^{-1}), which contains information on C = O stretches in carboxylic esters and carboxylic acids, amides and the polysaccharide fingerprint region.

Methodology

Intact organs or homogenized material can be used. Studying intact material preserves the spatial information of the tissue but may generate artefactual information related to differences in cell shapes or in the reflectance of cell surfaces. Analysing homogenized material may provide more easily interpretable data, however, all spatial information is lost. Aiming specific cell types by microspectroscopy allows the separate analysis of primary and secondary cell walls (Sibout et al. 2005). We will focus here on the primary CW analysis developed in our laboratory (Mouille et al. 2003). Four-day-old dark-grown seedlings are squashed, rinsed to eliminate cytoplasmic contents and dried on a BaF_2 slide prior to scanning. Absorption spectra are sampled in transmission mode from a 50×50 μm area on the side of the central cylinder. Given the simple anatomy of this organ, this area corresponds to only two cell types: epidermis and cortex. After base line correction and area normalization (Fig. 3), significantly different wavenumbers are identified by the Student's *t* test (Fig. 4), Mutants with related cell wall changes also can be clustered, based on their FT-IR spectra. To this end, discrimant wavenumbers are first selected and used to calculate the Mahalanobis distance between the mutants. These distances are used in a hierarchical clustering procedure. This method is validated by the fact that independently isolated alleles of comparable strength cluster together (Mouille et al. 2003; Robert et al. 2004).

Applications

- High throughput screening for altered CW composition: The method so far has allowed the distinction between mutants with cellulose, pectin, xyloglucan or microtubule defects.
- Chemical screens: The effect of chemicals on the CW can be rapidly assessed.

- In situ analysis with enough precision to allow the visualization of different cell types: In this way we have studied cell walls of the cortex + epidermis, root hairs, mature or germinated pollen grains, parenchyma, xylem and phloem cells on stem sections, envelope and septum in siliques, etc.
- Analysis of CW evolution during growth: We have studied cell wall changes during hypocotyl etiolation in darkness or during cell division, elongation and differentiation in the root.

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