Bioengineering cellulose-hemicellulose networks in plants

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Contents

To my wife, Adefunke,

My son, Oluwanifemi, and

My daughter, Imuseoluwa

GENERAL INTRODUCTION

IMPORTANCE OF PLANT CELL WALLS

The plant cell wall is a dynamic, highly organised extracytoplasmic matrix consisting of various polysaccharides, structural proteins, and aromatic substances, which are constantly remodelled and restructured during growth and development. These complex matrices define the shape and size of individual cells within the plant body and ultimately plant morphology (O'Neill and York, 2003). Other divergent and vital functions provided by the cell wall include tensile strength, defending the plant against pathogens and mediating the communication with symbionts and neighbouring cells (Carpita and Gibeaut, 1993; John et al., 1997; Dhugga, 2001).

Plant cell wall polysaccharides receive a lot of attention at the moment, because they constitute an important part of food and feed, and consequently they can be important in food processing. Wall polysaccharides are used commercially as gums, gelling agents and stabilizers (Morris and Wilde, 1997). Also, digestibility of feed products depends to a large extent on cell wall composition. Forage maize (*Zea mays*) is excellent roughage for ruminants because of its high energy content. The maize forage digestibility is however lowered by the presence of a considerable level of lignin and hemicelluloses, which create a physical and chemical barrier to digestion (Fontaine et al., 2003). Moreover, plant cell wall polysaccharides are the predominant components of fibres. Natural fibre is a thread-like material from plants, which can be used for making products such as cloth and rope. Fibre crops include cotton, hemp, flax, agave and tree species. Natural fibres have a wide range of industrial applications. Wood fibres have found application in pulp and paper industries whereas cotton, flax, hemp and agave fibres are used in the textile industries. Environmental considerations are giving room for the use of natural fibres as an alternative for synthetic polymers in industrial composites (Gustavsson et al., 2005). For the various applications, it is important to gain control over fibre characteristics, which in turn are determined by cell wall composition and interactions of wall components.

POLYMER NETWORKS IN THE PLANT CELL WALLS

A number of models have been proposed for the structure and architecture of the primary cell wall (Carpita and Gibeaut, 1993; McCann and Roberts, 1994; Ha et al., 1997). These models emphasise three independent but interacting networks based on cellulose-hemicellulose (>50% dry weight), pectin (25-40% dry weight) and structural glycoproteins (1-10% dry weight). Cellulose is a linear polymer of β-1,4-linked glucose, with each glucose residue oriented 180° to its neighbour such that the polymeric repeating unit is cellobiose (Brown et al., 1996). This allows the glucan chain to adopt a flat, ribbon-like structure. Hemicelluloses are wall polysaccharides that are not solubilized by hot water, but are solubilized by aqueous alkali (O'Neill and York, 2003). They are usually branched polysaccharides, which are structurally

homologous to cellulose, in that they have a backbone composed of β-1,4-linked pyranosyl residues, such as glucose, mannose, and xylose. This structural similarity facilitates a strong, non-covalent association of the hemicellulose with cellulose microfibrils. Xyloglucan is the most abundant hemicellulosic polysaccharide in the primary cell walls of non-graminaceous plants (about 20% dry weight). The cellulose-xyloglucan network is the principal load-bearing element in the primary cell wall. Two principal xyloglucan domains are identified in the models describing the cellulose-hemicellulose network in primary wall. One is bound to the paracrystalline periphery of the microfibrils preventing lateral association of the microfibrils and another fraction of xyloglucan, that is longer and more flexible, connects different cellulose microfibrils. A third possible domain comprises xyloglucan, which is trapped within microfibrils (O'Neill and York, 2003). Xyloglucan has a 'cellulosic' backbone of β-1,4-linked glucosyl (Glc) residues. Unlike the linear cellulose, however, the backbone residues bear α-linked xylopyranose branches attached to the O6 position of glucose, which may be further substituted by galactopyranosyl residues and other monosaccharides. Xylans including arabinoxylans, glucuronoxylans, and glucuronoarabinoxylans are the major hemicellulosic polysaccharide in the secondary cell wall (Ebringerova and Heinze, 2000). They are composed of a β-1,4-D-xylosyl backbone, which is substituted to varying extent at the O2 or O3 position of the xylosyl residues with glucuronyl (GlcA), acetyl and arabinosyl groups (O'Neill and York, 2003). Mannose-containing hemicelluloses, including (galacto)mannans and (galacto)glucomannans, are found in considerable amounts in a variety of plant species as carbohydrate reserves. (Galacto)mannans have a β-1,4-linked D-mannosyl (Man) backbone that is substituted at the O6 position of certain Man residues (Stephen, 1982). Glucomannans are abundant in secondary cell walls of woody species; they have a backbone that contains both 1,4-linked Man and 1,4-linked Glc residues. Galactoglucomannans are particularly abundant in the primary cell walls of Solanaceous species (O'Neill and York, 2003). They have a similar backbone as the glucomannans, but some of the backbone Man residues bear single-unit galactosyl side chains at the O6 position (Stephen, 1982).

Cross-linking pectic polysaccharides predominate the middle lamella, which is located between two contiguous cells (Raven et al., 1992) and constitute the embedding matrix for the cellulose-hemicellulose network of the primary cell wall (Carpita and Gibeaut, 1993). Extensin, the main structural glycoprotein in the primary cell wall, adds rigidity and strength to the wall by cross-linking with themselves (Brady et al., 1996) or with pectins (Brady et al., 1996; MacDougall et al., 2001). It is worth mentioning that these two structural components of the wall are of less importance, with respect to this thesis.

The secondary cell wall is added at the inner face after cessation of growth and it contains cellulose and non-cellulosic cross-linking glycans e.g. xylan (Awano et al., 2002) and lignin, together with a variety of proteins and other minor components (Turner et al., 2001). The lignin presumably binds wall polysaccharides through both

covalent and non-covalent interactions to form a lignin-polysaccharide complex, reviewed in O'Neil and York (2003).

FACTORS THAT CAN INFLUENCE CELLULOSE-HEMICELLULOSE NETWORKS

There are three main approaches that can be used to modify the cellulose-hemicellulose networks in the plant: (i) by interfering with the biosynthesis of polysaccharide, (ii) by directly interfering with the cellulose-hemicellulose interactions, and (iii) by degradation of wall polysaccharides. In order to use the first approach, an understanding of the (hemi)cellulose biosynthesis is imperative. An overview of literature on (hemi)cellulose biosynthesis is given below, starting with cellulose biosynthesis. Similarly, overviews on the other two approaches are subsequently given.

(i) Interference with polysaccharide biosynthesis

Cellulose biosynthesis. The crystalline cellulose microfibril is formed by the spontaneous association of about 36 β-D-glucan chains, which are simultaneously synthesised by a large membrane-localised complex that has been visualised by microscopy (Tsekos and Reiss, 1992). The association of the membrane complex with cellulose microfibrils as revealed by freeze-fracture electron microscopy suggested that the complexes are the sites of cellulose synthesis (Kimura et al., 1999). In vascular plants, these complexes are rosette structures with six-fold symmetry and a diameter of 24-30 nm (Mueller and Brown, 1980). The rosettes have been proposed to consist of six subunits, each of which has six catalytic subunits molded in the rosette structure (Delmer and Amor, 1995; Brown and Saxena, 2000). In addition to freeze-fracture evidence, mutation studies also showed positive correlation between cellulose content and numbers of rosettes in the mutants (Kokubo et al., 1991; Arioli et al., 1998), confirming that they are the biosynthetic machinery of cellulose. However, the underlying mechanisms of rosette assembly, the precise nature of cellulose biosynthesis, as well as the full identity of the components of the cellulose synthase complex are still not well understood.

Cellulose synthase (CesA). CesA proteins are thought to catalyse the polymerisation of glucose into a glucan chains, using UDP-glucose as the donor substrate. The first two plant genes for cellulose biosynthesis, GhCesA-1 and GhCesA-2, were identified by random sequencing of cDNA libraries from developing cotton fibres (Pear et al., 1996). Antibody labelling of the membrane-bound rosette with an antibody raised against the cotton CesAs demonstrated that CesA proteins are indeed members of the multiple-enzyme cellulose synthesising complex (Kimura et al., 1999; Itoh and Kimura, 2001).

The GhCesA proteins had conserved regions surrounding the D, DxD, D and QxxRW motifs, (x stands for any amino acid) (Figure 1), previously identified in *Acetobacter xylinum* CesA (Saxena et al., 1995). This motif is presumably involved in substrate binding and catalysis, and is characteristic of processive glycosyltransferases (GTs).
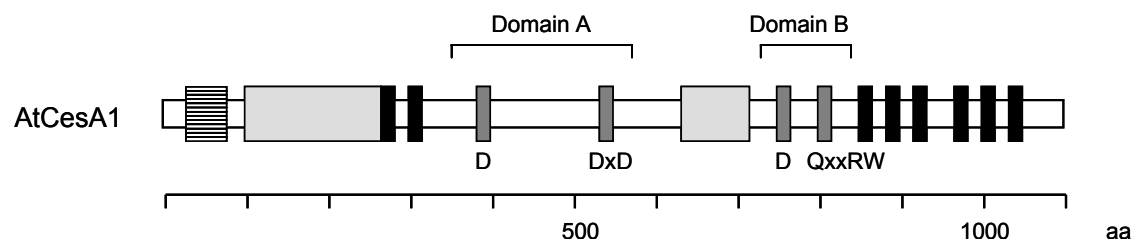
Figure 1. Schematic representation of the plant CesA proteins using the *Arabidopsis* CesA1 protein as a model. The striped box at the amino acid terminus represents the zinc finger domain. The grey boxes represent the two class specific regions. The black boxes represent the eight putative transmembrane regions. Regions labelled domain A and domain B represent the catalytic domain with the four conserved regions containing the D, DxD, D, QxxRW motif. The conserved amino acids in domain A are thought to be responsible for binding UDP-Glucose, while domain B is presumed to be responsible for polymerization.

From hydropathy analysis plots, it was predicted that the deduced GhCesA proteins have two trans-membrane helices in the N-terminal region and six in the C-terminal region (Delmer, 1999). The central region of the protein, comprising the D, DxD, D and QxxRW motifs plus two plant-specific insertions, is predicted to be within the cytoplasm. This is consistent with the notion that these motifs are involved in binding the substrate UDP-glc and carry out catalysis on the cytoplasmic face of the enzyme. A zinc-binding domain at the N-terminal of GhCesA was found as another plant CesA-specific motif. Mutant studies have since demonstrated the involvement of CesA proteins in cellulose biosynthesis. Earliest evidence came from the studies on two cellulose-deficient *Arabidopsis* mutants, *rsw1* (Arioli et al., 1998), and *irx3* (Turner and Somerville, 1997; Taylor et al., 1999). There is a body of evidence that indicates that at least three CesA proteins are involved in cellulose synthesis within the same cell at the same developmental stage. Three *Arabidopsis* CesA isoforms (AtCesA4, 7 and 8) have been shown to interact and constitute the subunits of the same complex that synthesises cellulose during secondary wall deposition in xylem cells (Gardiner et al., 2003; Taylor et al., 2003). Similarly, three CesA isoforms (AtCesA1, 3 and 6) have been reported to be required for cellulose synthesis in primary-walled cells of *Arabidopsis* (Fagard et al., 2000; Scheible et al., 2001; Desprez et al., 2002; Robert et al., 2004).

CesA genes are members of a large multi-gene family. In *Arabidopsis* there are up to 40 genes that bear similarity to the original CesA genes. This super-family has been divided into one family of ten 'true' CesA genes (CesA) and six families of about 30 cellulose synthase-like genes (Csl) that are less closely related (Richmond and Somerville, 2000; Richmond and Somerville, 2001). A web site maintained by Richmond and Somerville (http://cellwall.stanford.edu) documents sequence data for cellulose synthase and cellulose synthase-like genes in several different plant species.

Other proteins involved in cellulose biosynthesis. Apart from the CesA proteins, other proteins whose functions have been linked to cellulose biosynthesis have been identified. For instance, the role of a membrane-anchored endo-1,4-β-glucanase (KORRIGAN) in deposition of cellulose in the cell walls has been reported (Nicol et

al., 1998; Lane et al., 2001). The gene of this glucanase was isolated from the *kor* mutant that causes a decrease in the content of crystalline cellulose and abnormal cell wall formation. It was demonstrated that the catalytic domain of the enzyme is located on the outside of the plasma membrane (cell wall side) (Molhoj et al., 2001).

In addition to *korrigan*, a novel membrane-associated form of sucrose synthase has been proposed to be involved in cellulose synthesis, by channeling UDP-glucose to cellulose synthase in a closely coupled reaction (Amor et al., 1995). Immunolocalisation of sucrose synthase at the site of cellulose deposition in tracheary elements was demonstrated by electron microscopy (Salnikov et al., 2001). Furthermore, the involvement of an enzyme, UDP-Glc:sterol glucosyltransferase (SGT), in cellulose biosynthesis was suggested recently (Peng et al., 2002). The authors observed that digestion of noncrystalline cellulose with cellulase released not only CesA proteins, but also small amounts of a sitosterol linked to glucose (Peng et al., 2001; Peng et al., 2002). Further metabolic studies led the authors to propose a biosynthetic pathway for cellulose that starts with a SGT-mediated transfer of a glucosyl residue from the soluble cytoplasmic substrate UDP-glucose onto sitosterol to form sitosterol-β-glucoside (SG) on the inner surface of the plasma membrane. The idea is that SG, being a hydrophobic glucoside, may serve as a primer for glucan chain elongation. A link between sterol biosynthesis and cellulose synthesis has indeed been established recently, through the analysis of sterol biosynthesis mutants (Schrick, 2004) .

Hemicellulose biosynthesis. Unlike the cellulose, which is synthesised at the plasma membrane by CesA proteins, the non-cellulosic matrix polysaccharides (hemicelluloses) are produced within the Golgi by glycan synthases and glycosyltransferase (Keegstra and Raikhel, 2001). Research on hemicellulose biosynthesis is trailing behind that on cellulose. At the start of this research, there was no single gene available, which encodes a hemicellulose synthase polymerising the backbone of the polysaccharides. Efforts to identify enzymes mediating the biosynthesis by using biochemical purification strategies were successful for *Arabidopsis* xyloglucan fucosyltranferase (Perrin et al., 1999), galactomannan galactosyltransferase from fenugreek (Edwards et al., 1999) and xyloglucan xylosyl transferases from Arabidopsis (Faik et al., 2002). Mutant screens and reverse genetics strategies have also led to the identification of *Arabidopsis* xyloglucan galactosyltransferase (Madson et al., 2003). The sequence similarity between the CesA genes and the Csl genes, especially with respect to the conserved D, DxD, D and QXXRW motifs, originally suggested that they also encode processive glycosyltransferases (Cutler and Somerville, 1997). Based on this similarity, it was suggested that the backbone of hemicelluloses may be biosynthesised by Golgi-resident Csl proteins (Richmond and Somerville, 2001; Hazen et al., 2002). This hypothesis has indeed been supported by recent biochemical evidence that a CslA gene from guar encodes β-mannan synthase, which is involved in the formation of the β-1,4-mannan backbone of galactomannan (Dhugga et al., 2004). This biological

function of the CslA gene has also been confirmed by heterologous expression of the *Arabidopsis* CslA in *Drosophila* (Liepman et al., 2005).

Table 1. Influence of hemicellulose on cellulose-hemicellulose composite

Cellulose sample	Cellulose/hemicellulose ratio	Cellulose molecular composition	
		Crystalline (Iα+Iβ)	non-crystalline
Acetobacter cellulose		82%	18%
Xyloglucan	1:0.38	53%	47%
Konjac glucomannan*	1:0.59	25%	75%
Locust bean gum** galactomannan	1:0.54	50%	50%

*man: glc = 1.63:1
**man: gal = 3.57:1

Interference with hemicellulose biosynthesis might have implications on cellulose-hemicellulose interactions. Indications for this were provided by the following observations made with different cellulose-hemicellulose composites produced in the *Acetobacter* model system (Whitney et al., 1995; Whitney et al., 1998; Whitney et al., 2000). It was shown with the *Acetobacter* model system that the interactions of hemicelluloses with cellulose microfibrils during cellulose biosynthesis cause cellulose to lose its crystallinity (Whitney et al., 1995; Whitney et al., 1999). Table 1 shows percent crystalline cellulose and non-crystalline cellulose of different cellulose-hemicellulose composites produced with the *Acetobacter* system. Varying levels of reduction in cellulose crystallinity of the native bacterial cellulose were observed in the composites. For instance in the cellulose-xyloglucan composite, a 29% reduction was observed, whereas in cellulose-glucomannan composite, a 57% reduction was observed (Whitney et al., 1998; Whitney et al., 1999). It was also demonstrated that the composites have lower stiffness, leading to dramatic reduction in mechanical strength of cellulose, for example 80% reduction in composites with xyloglucan (Whitney et al., 1999; Whitney et al., 2000). In addition to modulating hemicellulose content, there are reports, which indicated that fibre properties can be modified by interfering with the side chain substitution of the cross-linking polysaccharide. Studies on *Arabidopsis* mutants with mutations of the MUR2 and MUR3 genes, which encode xyloglucan-specific fucosyl and galactosyl transferases, respectively, revealed that tensile strength of the fibre was enhanced by increased galactosylation of the xyloglucan (Ryden et al., 2003; Pena et al., 2004).

(ii) Interference with cellulose-hemicellulose interactions

Carbohydrate-binding modules (CBMs). Cellulose-hemicellulose networks might also be modified by polysaccharide-binding proteins. Of particular interest are proteins or parts thereof, which specifically bind polysaccharides without exerting an activity towards them. In nature, numerous organisms express a repertoire of glycoside hydrolases, esterases, and polysaccharide lyases. Cell wall polysaccharide hydrolases from aerobic micro-organisms are generally modular in structure comprising a catalytic module appended to one or more non-catalytic carbohydrate-binding modules (CBMs) (Freelove et al., 2001). However, in anaerobic bacteria the plant cell wall degradative enzymes assemble into large multi-protein complexes that bind tightly to cellulose (Bayer et al., 1998). The main function of CBMs is to attach the enzyme to the polymeric surface and thereby increase the local concentration of the enzyme, leading to more effective catalysis (Bolam et al., 1998; Gill et al., 1999). In addition to binding, some CBMs may also display functions such as substrate disruption or the sequestering and feeding of single glycan chains into the active site of the adjacent catalytic module (Din et al., 1994; Southall et al., 1999).

Family classification of CBMs. CBMs are divided into 43 families based on amino acid sequence similarities, details of which can be found in the regularly updated web site for carbohydrate active enzymes (CAZY), http://afmb.cnrs-mrs.fr/~cazy/CAZY/index.html. The classification has predictive value for binding specificity and structure. A list of CBMs with relevance to cell wall polysaccharide-binding is presented in Table 2. The CBMs exist in different sizes, ranging from 40-60 to 200 amino acids. Families 1, 5 and 10 are examples of the small CBMs while families 11 and 17 represent the large CBMs. CBMs can accommodate the heterogeneity of the plant cell wall polysaccharides (Boraston et al., 2004). For example, most CBMs that recognise cellulose, bind to both crystalline and amorphous cellulose but with differing binding affinities. In addition to that, some of them can accommodate backbone heterogeneity through selective flexibility, as exhibited by those of the family 29, which recognises the β-1,4-linked backbone of mannose and glucose and to a lesser extent, those of xylan and xyloglucan (Table 2) (Freelove et al., 2001; Charnock et al., 2002).

Relationship between structure and function of the CBMs. NMR and X-ray crystal structures have revealed that the CBMs that bind soluble polysaccharides are grooved and that the depth of the clefts varies from very shallow to being able to accommodate the entire width of a pyranose ring (Boraston et al., 2004). Examples of these CBMs include family 29 (Freelove et al., 2001), family 2b (Bolam et al., 2001) and family 22 (Charnock et al., 2000). These are the CBMs we have used in our investigations. The 3-D structures of many of these CBMs show a characteristic groove, except CBM2b (Figure 2). Aromatic residues (Trp, Tyr) play a pivotal role in ligand binding and the orientation of these amino acids are key determinants of specificity of these CBMs (Simpson et al., 2000). Alternatively, CBMs binding insoluble crystalline cellulose

Table 2. Summary of carbohydrate-binding modules with relevance for binding cellulosic- and hemicellulosic polysaccharides.

Module	Approx. size (aa)	Occurrence	Specificity					
			CC	AC	X	XG	M	MLG
CBM1	~40	fungi	X	X				
CBM2a	~100	bacteria	X					
CBM2b	~100	bacteria		X	X			
CBM3	~150	bacteria	X					
CBM4	~150	bacteria		X	X			
CBM5	~60	bacteria	X	X				
CBM6	~120	bacteria		X	X			
CBM9	~170	bacteria	X	X	X	X		
CBM10	~50	bacteria	X	X				
CBM11	180-200	bacteria		X				
CBM13	~150	plant			X			
CBM15	~150	bacteria			X			
CBM17	~200	bacteria		X				
CBM22	~160	plants			X			X
CBM27	~122	bacteria					X	
CBM28	~178	bacteria		X				X
CBM29	~124	fungi	X	X	X	X	X	
CBM30	~174	bacteria	X					X
CBM31	~124	bacteria			X			
CBM35	~130	bacteria			X		X	
CBM36	~120-130	bacteria			X			
CBM37	~100	bacteria	X	X	X			
CBM43	~90-100	plants		X				X

Legend: shaded background = three-dimensional structure available; MLG = mixed linkage β-glucan; CC = crystalline cellulose; AC = amorphous cellulose; X = xylan; M = mannan; XG = xyloglucan.

have a flat surface, which enables them to attach to cellulose. CBM1 and CBM3 are typical flat-surface-binding CBMs (Figures 3). Their 3-D structures show that the residues, which are involved in binding, are oriented in a geometry that is complementary to the flat surface of cellulose. It is worth mentioning that CBM2b (Fig. 2A), which binds xylan also, has a flat side on the protein. CBM2b is closely related to the CBM2a (not shown), which binds cellulose. The major difference between the two is that in CBM2a, the three tryptophan residues, which are involved in binding, are coplanar, as has been indicated for CBM1 and CBM3 (Tormo et al., 1996). In the CBM2b, the two surface tryptophan residues are perpendicular to each other, forming a twisted binding site (Fig. 2D), which is complementary to the structure of xylan. Generally, at least two aromats are required for interaction with the target ligand, the binding of which is often reinforced by hydrogen bonding interactions between the CBM and the carbohydrate. The number of glycosyl residues

bound by the CBM can be different, i.e. six for CBM2b (Simpson et al. 2000) and CBM29 (Charnock et al. 2002), and four for CBM22 (Charnock et al. 2000). It has been suggested that short and shallow grooves might better accommodate polysaccharides with side chains, which is important with respect to hemicelluloses, because they are often heavily branched.

Binding affinity of CBMs. Some polysaccharide-degrading enzymes may possess more than one CBM, in order to facilitate increased affinity of the enzymes for the polysaccharide. This idea is supported by many binding affinity studies involving one versus two CBMs. For example, an artificial protein construct, consisting of two covalently linked family 1 CBMs, had 6-to-10-fold higher affinity for insoluble cellulose, as compared to the individual modules (Linder et al., 1996). Also, two CBMs of the family 29, CBM29-1 and CBM29-2, exist naturally in tandem as component of the *Piromyces equi* cellulase-hemicellulase complex (Freelove et al., 2001). The tandem CBM29-1-2 was shown to possess much higher binding affinity than the single CBM29-1 and CBM29-2 modules, indicating a synergy between the two single modules. Another possible role for the multiple CBMs is that they increase the diversity of polysaccharides that the parent enzyme can interact with (Gill et al., 1999). Prime example of such enzymes is the *Cellulomonas fimi* xylanase 11A, which contains two family 2b CBMs, CBM2b-1 and CBM2b-2. CBM2b-1 specifically binds to xylan while CBM2b-2 additionally binds to cellulose (Bolam et al., 2001). As for the tandem CBM29-1-2, the two family 2b CBMs were also shown to have higher affinity when incorporated into a single protein species, than when expressed as discrete entities. Another interesting CBM, CBM22 from *Clostridium thermocellum* (Fig. 2C) (Charnock et al., 2000), which has affinity for xylan also exist in *Arabidopsis* xylanases in multiple copies (Henrissat et al., 2001; Suzuki et al., 2002).

CBMs can modify properties of composites. In *Acetobacter xylinum*, which has long been regarded as the model system for cellulose biosynthesis, polymerisation and crystallisation of cellulose are coupled processes. It was observed that interference with crystallisation in the model system results in acceleration of polymerisation (Benziman et al., 1980). A number of cellulose-binding, organic substances like carboxymethyl cellulose (CMC) and fluorescent brightening agents (FBAs, e.g. calcofluor white) prevent microfibril crystallisation in the *Acetobacter* model system, thereby enhancing polymerisation (Haigler, 1991). These molecules bind to the polysaccharide chains immediately after their extrusion from the cell surface, preventing normal assembly of microfibrils and cell walls (Haigler, 1991). It was also demonstrated that microbial CBMs could modulate cellulose biosynthesis, by achieving an up to 5-fold increase in the rate of biosynthesis as compared with the controls (Shpigel et al., 1998). A hypothetical model of the physico-mechanical mechanism of action has been proposed, whereby a flat-surface, cellulose-recognising CBM slides between cellulose fibres and separates them in a wedge-like action (Fig. 4) (Levy and Shoseyov, 2002). The authors speculated that when the interaction occurs during the initial stages of crystallisation, the result is increased rate of synthesis and splayed fibrils. Post-synthesis interaction results in non-hydolytic fibre disruption (Levy and Shoseyov, 2002).
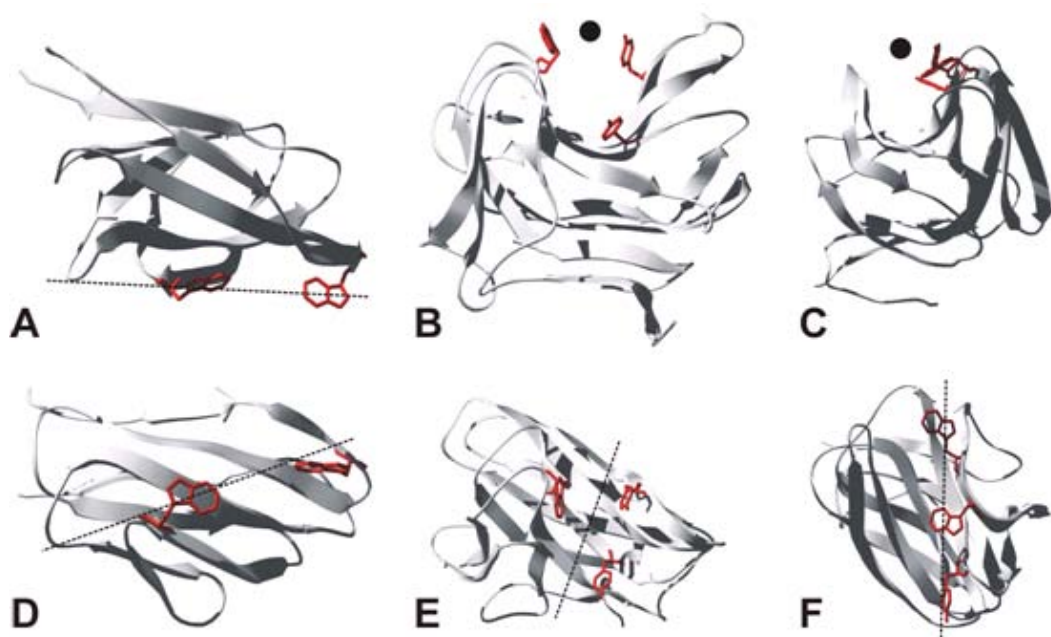
Figure 2. 3-D structures of selected CBMs that bind non-crystalline polysaccharides, showing topology and residues involved in binding. D, E, and F represent the same CBMs as A, B, and C, respectively, but the structures have been rotated 90° with front view facing down (E,F) or up (D). Aromatic amino acid residues, which have been implicated in binding glycosyl residues, are indicated in red. CBM2b (2XBD) with aromatic residues Trp259 and Trp291 has no binding groove (A, front view) and perpendicular orientation of the two tryptophan residues (D, bottom view). CBM22-2 (1DYO) with aromats Trp53, Tyr103 and Tyr134, has a polysaccharide binding groove (B, front view); the top view shows the position and orientation of the aromats (E). The CBM29-2 (1GWK) with aromats Trp24, Trp26 and Tyr46 has a shallower groove (C) than CBM22. It can be seen that the aromats in CBM29-2 are positioned differently in the groove as compared to those in CBM22. Closed black circles (B and C) indicate the binding groove. Dotted lines (A, D, E, and F) indicate the path of binding of the polysaccharides. 2XBD, 1DYO, and 1GWK represent the codes for the files containing the atomic coordinates for building the structural models.

Figure 3. 3-D structures of selected CBMs that bind crystalline cellulose. Amino acid residues, which have been implicated in binding glycosyl residues, are indicated in red. CBM1 (1CBH) with aromats Tyr 5, Tyr 31 and Tyr 32 (A), and CBM3 (1NBC) with residues His57 Tyr 67, Trp 118 (B), reveal the coplanar orientation of the residues involved in binding, which is characteristic of CBMs that bind the flat surface of cellulose.
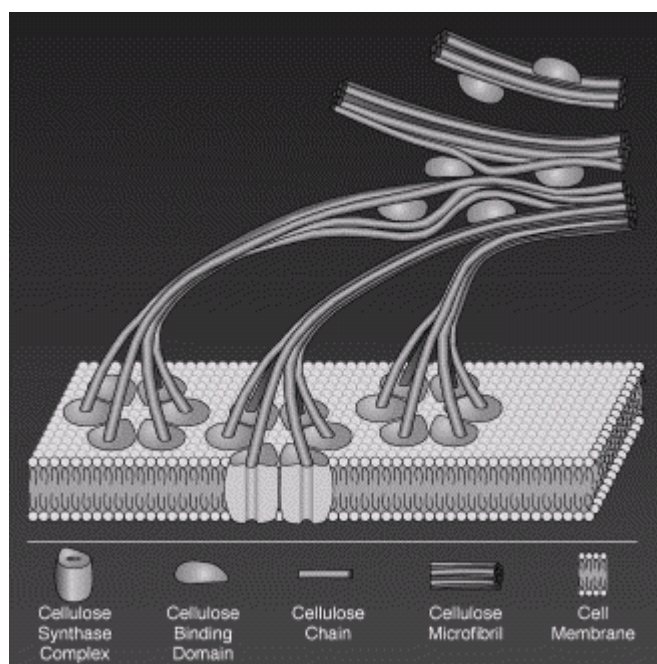
Figure 4. A model describing the interactions between cellulose-binding domain (CBD) and cellulose fibres. Two types of interactions have been proposed. (i) interaction during the initial stages of crystallization, which may result in an increased rate of synthesis and (ii) post-synthesis interaction, which result in non hydrolytic fibre disruption. "Reprinted from Molecular Bioengineering, Vol. 19, Ilan Levy, Ziv Shani and Oded Shoseyov, Modification of polysaccharides and plant cell wall by endo-1,4-β-glucanase and cellulose-binding domains, 17-30, © (2002), with permission from Elsevier".

Furthermore, it was indicated that CBMs can interfere with the attachment of hemicellulose to cellulose, as reported for CBM3, which competed with xyloglucan for binding sites when it was added first to *Acetobacter* cellulose (Shpigel et al., 1998). The competition decreased the amount of the xyloglucan that bound to the cellulose in the absence of CBM by about 12%. This indicates that the CBMs can be used to prevent cellulose-hemicellulose interactions in plants, leading to the production of cellulose fibres with higher crystallinity, as discussed earlier under hemicellulose biosynthesis.

In plant systems, it was shown that the elongation growth of *Arabidopsis* seedlings and peach (*Prunus persica* L.) pollen tubes can be affected *in vitro* by exogenous supply of a recombinant bacterial cellulose-recognising CBM3 (Levy et al., 2002b). Furthermore, it has been indicated that CBMs can modulate cell wall structure and growth of transgenic plants (Kilburn et al., 2000; Shoseyov, 2001; Quentin, 2003). Introduction of the CBM3 gene from *Clostridium cellulovorans* into poplar tree plants was reported to enhance the growth rate of the transgenic tree plants (Shoseyov, 2001). A similar claim of altered plant growth rate was reported for transgenic plants expressing a mannan-recognising CBM27 (Kilburn et al., 2000). The foregoing thus provides preliminary evidence that CBM can influence developmental processes in plants, as they were either presented as unpublished data or as patent claims.

Expansins. Expansins are cell-wall-loosening proteins that have been proposed to be involved in the control of cell enlargement (Cosgrove, 2003a). Extracted expansin

proteins have been shown to induce stress relaxation and extension of isolated cell walls (McQueen-Mason et al., 1992; McQueen-Mason and Cosgrove, 1995). A hypothetical model for the putative domain structure of expansins (Cosgrove, 2000a) depicts them as a double domain structure with a signal peptide at the N-terminus. They are proposed to have an N-terminal domain I that resembles family-45 glycosyl hydrolases (glucanase) and a C-terminal domain II with resemblance to cellulose-specific CBMs. Expansin's CBM have not been formally classified and are therefore not included in Table 2. Expansins have not been characterised for their binding specificity to date, because of difficulty in expressing active recombinant protein in heterologous expression systems. However, their expression has been modulated in transgenic plants, leading to altered morphology and plant development (Brummell et al., 1999; Cho and Cosgrove, 2000; Pien et al., 2001; Choi et al., 2003). It remains to be determined whether the putative CBM of the expansins can be independently expressed *in planta*, to modulate cell wall structure like the complete expansin is able to do.

(iii) Degradation of wall polysaccharides

Another approach that can be employed to modify the cellulose-hemicellulose network is *in planta* polysaccharide degradation. Cell wall disassembly is a common feature of many developmental processes such as, fruit softening, organ abscission and dehiscence, and seed germination. These processes are characterised by marked irreversible changes in wall structure and wall strength (Rose et al., 2003). Owing to the heterogeneous nature of the plant cell wall, many wall degrading proteins act in synergy, where one class of protein enhances the activity of the other. For instance, exo-acting glycoside hydrolases removing polysaccharide side chains might expose the polymer backbone and enhance its rapid depolymerization by endo-acting glycoside hydrolases (Rose et al., 2003). Similarly, the non-hydrolytic disruption of non-covalent polysaccharide interactions by proteins such as expansins, may also facilitate easy accessibility of a range of substrates to their enzymes (Rose et al., 2003).

OBJECTIVES AND OUTLINE OF THE THESIS

This thesis will focus mainly on strategies, highlighted above, aimed at modifying cellulose-hemicellulose networks through altered content of cellulose and its association with other wall polysaccharides. This was with a view to developing a biological tool-box for cell wall modification. It is envisaged that the tools, when developed, can be adapted for modifying fibre properties in more economically important species like flax and hemp. The specific aims of the research include (i) modulation of cellulose content through genetic crossing of two antisense potato lines in order to obtain offspring with greater reduction in cellulose content and over-expression of a CesA gene, in order to increase cellulose content, (ii) heterologous expression of different microbial CBM genes in tobacco, to obtain transgenic lines with altered cell wall structures, and (iii) heterologous expression of a putative

expansin CBM in tobacco, with a view to unravelling the function of the expansin's CBM in modifying cell walls.

Our choice of interfering with cellulose biosynthesis in potato plants was for one part directed by the availability of materials. There were a few antisense transgenic potato plants exhibiting down to 60% down-regulation of the cellulose content in potato tubers and a sense plant exhibiting 200% up-regulation from previous work on potato CesAs in our laboratory (Oomen et al., 2004). Hence, our drive to further analyse those transformants by (i) investigating the threshold limit for cellulose reduction the potato tuber can tolerate, and (ii) repeating the sense expression with a different CesA. The work on the CBMs was performed in tobacco. Our choice for tobacco was based on the fact that it is used as a model species in fundamental cell wall research, mainly with respect to fibre modification (Chabannes et al., 2001; Pincon et al., 2001; Boudet et al., 2003; Compier, 2005) and also because it is very amenable to genetic transformation. Most of the earlier research activities, relating to the potential use of CBMs for plant cell wall modification have been on the cellulose-specific CBMs (Shpigel et al., 1998; Kilburn et al., 2000; Shoseyov, 2001; Levy et al., 2002b). In this work, however, we were interested in CBMs that interact with individual glycan chains rather than with crystalline surfaces. The results from this work may provide valuable information for modification of polysaccharides and plant cell walls for various industrial applications, such as for instance the production of cellulose fibre with high tensile strength for textile manufacturing and the production of fibres with less attachment of lignin for paper manufacturing. Other specific objectives are discussed below under experimental chapters of this thesis.

Chapter 2 describes the results obtained from the genetic crossing of two antisense CesA transgenic potato lines, csr2-1 and csr4-8 (Oomen et al., 2004). The investigation was carried out in order to achieve a greater reduction in cellulose content than with one antisense gene fragment and to investigate the effect of such reduction on tuber development. Molecular analyses of gene integration, segregation and expression are discussed. Macroscopic and microscopic examinations of the offspring were carried out in order to identify organ and cellular phenotypes. Subsequently biochemical investigations were carried out to confirm the phenotypes.

Chapters 3 and 4 describe the results obtained on the heterologous expression of microbial CBMs in tobacco. Promiscuous CBM29 modules from *Piromyces equi* were used in Chapter 3, while a less promiscuous CBM2b-1-2 from *Clostridium thermocellum* was used in Chapter 4. The investigations were carried out to compare and contrast the effects of the two types of CBMs in transgenic tobacco plants. Constructs containing gene fragments encoding the tandem CBM29-1-2 and its single derivative CBM29-2 were transformed into tobacco. Similarly, a construct containing gene fragments encoding the tandem CBM2b-1-2 was transformed into tobacco. Macroscopic and microscopic observations were made to identify transformants with altered phenotypes. Additionally, effects of the CBM29 transgenes on the seed germination rate were investigated to test whether the mannan-recognising CBM29-1-2 can enhance germination, presumably by mobilising the mannan reserve of the seed endosperm cell walls or not.

In Chapter 5, we investigated the ability of a potato expansin CBM to modulate cell wall structure in transgenic tobacco. The investigation was premised on reports that modulation of the complete expansin altered morphology and development of transgenic plants. Sequence alignment and homology modeling, in relation to phenotypic observations are discussed. Finally, the chapter compares and contrasts the effects of the putative expansin CBM with those of the complete expansin proteins.

In Chapter 6, the overall results of the various investigations are discussed with regard to the feasibility of modifying cellulose-hemicellulose networks in plants. Possible future directions are discussed in relation to the applicability of the findings to fibre crops like hemp and flax, with a view to achieving higher quality fibre for use in the various applications.

References

Alonso-Simon A, Encina AE, Garcia-Angulo P, Alvarez JM, Acebes JL (2004) FTIR spectroscopy monitoring of cell wall modifications during the habituation of bean (Phaseolus vulgaris L.) callus cultures to dichlobenil. Plant Science **167:** 1273-1281

Amor Y, Haigler CH, Johnson S, Wainscott M, Delmer DP (1995) A membrane-associated form of sucrose synthase and its potential role in synthesis of cellulose and callose in plants. Proceedings of The National Academy of Sciences of The United States of America **92:** 9353-9357

Arioli T, Peng LC, Betzner AS, Burn J, Wittke W, Herth W, Camilleri C, Hofte H, Plazinski J, Birch R, Cork A, Glover J, Redmond J, Williamson RE (1998) Molecular analysis of cellulose biosynthesis in *Arabidopsis*. Science **279:** 717-720

Awano T, Takabe K, Fujita M (2002) Xylan deposition on secondary wall of *Fagus crenata* fibre. Protoplasma **219:** 106-115

Banka RR, Mishra S, Ghose TK (1998) Fibril formation from cellulose by a novel protein from Trichoderma reesei: A non-hydrolytic cellulolytic component? World Journal of Microbiology & Biotechnology **14:** 551-558

Barral P, Suarez C, Batanero E, Alfonso C, Alche JD, Rodriguez-Garcia MI, Villalba M, Rivas G, Rodriguez R (2005) An olive pollen protein with allergenic activity, Ole e 10, defines a novel family of carbohydrate-binding modules and is potentially implicated in pollen germination. Biochemical Journal **390:** 77-84

Barre A, Rouge P (2002) Homology modeling of the cellulose-binding domain of a pollen allergen from rye grass: structural basis for the cellulose recognition and associated allergenic properties. Biochemical And Biophysical Research Communications **296:** 1346-1351

Baskin TI, Busby CH, Fowke LC, Sammut M, Gubler F (1992) Improvements in immunostaining samples embedded in methacrylate: Localization of microtubules and other antigens throughout developing organs of diverse taxa. Planta **187:** 405-413

Baucher M, Halpin C, Petit-Conil M, Boerjan W (2003) Lignin: Genetic engineering and impact on pulping. Critical Reviews In Biochemistry And Molecular Biology **38:** 305-350

Bayer EA, Chanzy H, Lamed R, Shoham Y (1998) Cellulose, cellulases and cellulosomes. Current Opinion In Structural Biology **8:** 548-557

Benziman M, Haigler Candace H, Brown RM, R. WA, Cooper KM (1980) Polymerization and crystallization are coupled processes in *Acetobacter xylinum*. Proceedings of the National Academy of Sciences of the United States of America **77, 6678-82**

Bolam DN, Ciruela A, McQueen-Mason S, Simpson P, Williamson MP, Rixon JE, Boraston A, Hazlewood GP, Gilbert HJ (1998) Pseudomonas cellulose-binding domains mediate their effects by increasing enzyme substrate proximity. Biochemical Journal **331:** 775-781

Bolam DN, Xie HF, White P, Simpson PJ, Hancock SM, Williamson MP, Gilbert HJ (2001) Evidence for synergy between family 2b carbohydrate binding modules in Cellulomonas fimi xylanase 11A. Biochemistry **40:** 2468-2477

Bonke M, Thitamadee S, Mahonen AP, Hauser MT, Helariutta Y (2003) APL regulates vascular tissue identity in Arabidopsis. Nature **426:** 181-186

Boraston AB, Bolam DN, Gilbert HJ, Davies GJ (2004) Carbohydrate-binding modules: fine-tuning polysaccharide recognition. Biochemical Journal **382:** 769-781

Boudet AM, Kajita S, Grima-Pettenati J, Goffner D (2003) Lignins and lignocellulosics: a better control of synthesis for new and improved uses. Trends In Plant Science **8:** 576-581

Brady JD, Sadler IH, Fry SC (1996) Di-isodityrosine, a novel tetrameric derivative of tyrosine in plant cell wall proteins: A new potential cross-link. Biochemical Journal **315:** 323-327

Brands A, Ho THD (2002) Function of a plant stress-induced gene, HVA22. Synthetic enhancement screen with its yeast homolog reveals its role in vesicular traffic. Plant Physiology **130:** 1121-1131

Brett CTW, K. (1996) Physiology and Biochemistry of Plant Cell walls. Chapman & Hall, London

Brown RM, Saxena IM (2000) Cellulose biosynthesis: A model for understanding the assembly of biopolymers. Plant Physiology And Biochemistry **38:** 57-67

Brown RM, Saxena IM, Kudlicka K (1996) Cellulose biosynthesis in higher plants. Trends In Plant Science **1:** 149-156

Brummell DA, Harpster MH, Civello PM, Palys JM, Bennett AB, Dunsmuir P (1999) Modification of expansin protein abundance in tomato fruit alters softening and cell wall polymer metabolism during ripening. Plant Cell **11:** 2203-2216

Burk DH, Liu B, Zhong RQ, Morrison WH, Ye ZH (2001) A katanin-like protein regulates normal cell wall biosynthesis and cell elongation. Plant Cell **13:** 807-827

Burton RA, Gibeaut DM, Bacic A, Findlay K, Roberts K, Hamilton A, Baulcombe DC, Fincher GB (2000) Virus-induced silencing of a plant cellulose synthase gene. Plant Cell **12:** 691-705

Cano-Delgado AI, Metzlaff K, Bevan MW (2000) The eli1 mutation reveals a link between cell expansion and secondary cell wall formation in Arabidopsis thaliana. Development **127:** 3395-3405

Cano Delgado A, Penfield S, Smith C, Catley M, Bevan M (2003) Reduced cellulose synthesis invokes lignification and defense responses in Arabidopsis thaliana. Plant Journal. **34:** 351-362.

Carpita NC, Defernez M, Findlay K, Wells B, Shoue DA, Catchpole G, Wilson RH, McCann MC (2001) Cell wall architecture of the elongating maize coleoptile. Plant Physiology **127:** 551-565

Carpita NC, Gibeaut DM (1993) Structural models of primary-cell walls in flowering plants - consistency of molecular-structure with the physical-properties of the walls during growth. Plant Journal **3:** 1-30

Ceccardi TL, Barthe GA, Derrick KS (1998) A novel protein associated with citrus blight has sequence similarities to expansin. Plant Molecular Biology **38:** 775-783

Chabannes M, Barakate A, Lapierre C, Marita JM, Ralph J, Pean M, Danoun S, Halpin C, Grima-Pettenati J, Boudet AM (2001) Strong decrease in lignin content without significant alteration of plant development is induced by simultaneous down-regulation of cinnamoyl CoA reductase (CCR) and cinnamyl alcohol dehydrogenase (CAD) in tobacco plants. Plant Journal **28:** 257-270

Charnock SJ, Bolam DN, Nurizzo D, Szabo L, McKie VA, Gilbert HJ, Davies GJ (2002) Promiscuity in ligand-binding: The three-dimensional structure of a Piromyces carbohydrate-binding module, CBM29-2, in complex with cello-and mannohexaose. Proceedings of The National Academy of Sciences of The United States of America **99:** 14077-14082

Charnock SJ, Bolam DN, Turkenburg JP, Gilbert HJ, Ferreira LMA, Davies GJ, Fontes C (2000) The X6 "thermostabilizing" domains of xylanases are carbohydrate-binding modules: Structure and biochemistry of the Clostridium thermocellum X6b domain. Biochemistry **39:** 5013-5021

Charnock SJ, Henrissat B, Davies GJ (2001) Three-dimensional structures of UDP-sugar glycosyltransferases illuminate the biosynthesis of plant polysaccharides. Plant Physiology **125:** 527-531

Cho HT, Cosgrove DJ (2000) Altered expression of expansin modulates leaf growth and pedicel abscission in Arabidopsis thaliana. Proceedings of The National Academy of Sciences of The United States of America **97:** 9783-9788

Choi DS, Lee Y, Cho HT, Kende H (2003) Regulation of expansin gene expression affects growth and development in transgenic rice plants. Plant Cell **15:** 1386-1398

Church GM, Gilbert W (1984) Genomic sequencing. Proceedings of the National Academy of Sciences of the United States of America **81:** 1991-1995

Compier M (2005) Hemicellulose biosynthesis and degradation in tobacco cell walls (PhD Thesis). PhD thesis. Wageningen University, Wageningen

Cosgrove DJ (2000a) Loosening of plant cell walls by expansins. Nature **407:** 321-326

Cosgrove DJ (2000b) New genes and new biological roles for expansins. Current Opinion In Plant Biology **3:** 73-78

Cosgrove DJ (2003a) Expansion of plant cell wall, in The Plant Cell Wall, (ed. Jocelyn K. C. Rose), Vol 8. CRC Press LLC, Boca Raton, FL, USA, pp pp. 237-258

Cosgrove DJ (2003b) Expansion of plant cell wall, in The Plant Cell Wall, (ed. Jocelyn K. C. Rose), Vol 8. CRC Press LLC, Boca Raton, FL, USA, pp 237-258

Cutler S, Somerville C (1997) Cellulose synthesis: Cloning in silico. Current Biology **7:** R108-R111

Darley CP, Forrester AM, McQueen-Mason SJ (2001) The molecular basis of plant cell wall extension. Plant Molecular Biology **47:** 179-195

De Marino S, Morelli MAC, Fraternali F, Tamborini E, Musco G, Vrtala S, Dolecek C, Arosio P, Valenta R, Pastore A (1999) An immunoglobulin-like fold in a major plant allergen: the solution structure of Phl p 2 from timothy grass pollen. Structure **7:** 943-952

Delmer DP, Amor Y (1995) Cellulose Biosynthesis. Plant Cell **7:** 987-1000

Desprez T, Vernhettes S, Fagard M, Refregier G, Desnos T, Aletti E, Py N, Pelletier S, Hofte H (2002) Resistance against herbicide isoxaben and cellulose deficiency caused by distinct mutations in same cellulose synthase isoform CESA6. Plant Physiology **128:** 482-490

Dhugga KS (2001) Building the wall: genes and enzyme complexes for polysaccharide synthases. Current Opinion In Plant Biology **4:** 488-493

Dhugga KS, Barreiro R, Whitten B, Stecca K, Hazebroek J, Randhawa GS, Dolan M, Kinney AJ, Tomes D, Nichols S, Anderson P (2004) Guar seed beta-mannan synthase is a member of the cellulose synthase super gene family. Science **303:** 363-366

Din N, Damude HG, Gilkes NR, Miller RC, Warren RAJ, Kilburn DG (1994) C-1-C-X revisited - intramolecular synergism in a cellulase. Proceedings of The National Academy of Sciences of The United States of America **91:** 11383-11387

Doblin MS, Kurek I, Jacob Wilk D, Delmer Deborah P (2002) Cellulose biosynthesis in plants: From genes to rosettes. Plant and Cell Physiology. **43:** 1407-1420.

Ebringerova A, Heinze T (2000) Xylan and xylan derivatives - biopolymers with valuable properties, 1 - Naturally occurring xylans structures, procedures and properties. Macromolecular Rapid Communications **21:** 542-556

Edwards ME, Dickson CA, Chengappa S, Sidebottom C, Gidley MJ, Reid JSG (1999) Molecular characterisation of a membrane-bound galactosyltransferase of plant cell wall matrix polysaccharide biosynthesis. Plant Journal **19:** 691-697

Emons AMC, Mulder BM (1998) The making of the architecture of the plant cell wall: How cells exploit geometry. Proceedings of The National Academy of Sciences of The United States of America **95:** 7215-7219

Fagard M, Desnos T, Desprez T, Goubet F, Refregier G, Mouille G, McCann M, Rayon C, Vernhettes S, Hofte H (2000) PROCUSTE1 encodes a cellulose synthase required for normal cell elongation specifically in roots and dark-grown hypocotyls of *Arabidopsis*. Plant Cell **12:** 2409-2423

Faik A, Price NJ, Raikhel NV, Keegstra K (2002) An Arabidopsis gene encoding an alpha-xylosyltransferase involved in xyloglucan biosynthesis. Proceedings of The National Academy of Sciences of The United States of America **99:** 7797-7802

Fontaine AS, Bout S, Barriere Y, Vermerris W (2003) Variation in cell wall composition among forage maize (Zea mays L.) inbred lines and its impact on digestibility: Analysis of neutral detergent fibre composition by pyrolysis-gas chromatography-mass spectrometry. Journal of Agricultural And Food Chemistry **51:** 8080-8087

Freelove ACJ, Bolam DN, White P, Hazlewood GP, Gilbert HJ (2001) A novel carbohydrate-binding protein is a component of the plant cell wall-degrading complex of Piromyces equi. Journal of Biological Chemistry **276:** 43010-43017

Gao PJ, Chen GJ, Wang TH, Zhang YS, Liu J (2001) Non-hydrolytic disruption of crystalline structure of cellulose by cellulose binding domain and linker sequence of cellobiohydrolase I from Penicillium janthinellum. Acta Biochimica Et Biophysica Sinica **33:** 13-18

Gardiner JC, Taylor NG, Turner SR (2003) Control of cellulose synthase complex localization in developing xylem. Plant Cell **15:** 1740-1748

Gehring CA, Irving HR (2003) Natriuretic peptides - a class of heterologous molecules in plants. International Journal of Biochemistry & Cell Biology **35:** 1318-1322

Giardina T, Gunning AP, Juge N, Faulds CB, Furniss CSM, Svensson B, Morris VJ, Williamson G (2001) Both binding sites of the starch-binding domain of Aspergillus niger glucoamylase are essential for inducing a conformational change in amylose. Journal of Molecular Biology **313:** 1149-1159

Gill J, Rixon JE, Bolam DN, McQueen-Mason S, Simpson PJ, Williamson MP, Hazlewood GP, Gilbert HJ (1999) The type II and X cellulose-binding domains of Pseudomonas xylanase A potentiate catalytic activity against complex substrates by a common mechanism. Biochemical Journal **342:** 473-480

Gubler F (1989) Immunofluoresence localization of microtubules in plant root tips embedded in Butyl-methyl methacrylate. Cell Biology International reports **13:** 137-145

Guex N, Peitsch MC (1997) SWISS-MODEL and the Swiss-PdbViewer: An environment for comparative protein modeling. Electrophoresis **18:** 2714-2723

Gustavsson MT, Persson PV, Iversen T, Hult K, Martinelle M (2004) Polyester coating of cellulose fibre surfaces catalyzed by a cellulose-binding module-Candida antarctica lipase B fusion protein. Biomacromolecules **5:** 106-112

Gustavsson MT, Persson PV, Iversen T, Martinelle M, Hult K, Teeri TT, Brumer H (2005) Modification of cellulose fibre surfaces by use of a lipase and a xyloglucan endotransglycosylase. Biomacromolecules **6:** 196-203

Ha MA, Apperley DC, Jarvis MC (1997) Molecular rigidity in dry and hydrated onion cell walls. Plant Physiology **115:** 593-598

Hackney JM, Atalla RH, Vanderhart DL (1994) Modification of Crystallinity And Crystalline-Structure of Acetobacter-Xylinum Cellulose In The Presence of Water-Soluble Beta-1,4-Linked Polysaccharides - C-13-Nmr Evidence. International Journal of Biological Macromolecules **16:** 215-218

Haigler CH (1991) Relationship between polymerization and crystallization in microfibril biogenesis, in Biosynthesis and biodegradation of cellulose (eds. Haigler, C. H., Weimer, P.J.). Marcel Dekker, New York

Handford MG, Baldwin TC, Goubet F, Prime TA, Miles J, Yu XL, Dupree P (2003) Localisation and characterisation of cell wall mannan polysaccharides in Arabidopsis thaliana. Planta **218:** 27-36

Hazen SP, Scott-Craig JS, Walton JD (2002) Cellulose synthase-like genes of rice. Plant Physiology **128:** 336-340

Hellens RP, Edwards EA, Leyland NR, Bean S, Mullineaux PM (2000) pGreen: a versatile and flexible binary Ti vector for Agrobacterium-mediated plant transformation. Plant Molecular Biology **42:** 819-832

Henrissat B, Coutinho PM, Davies GJ (2001) A census of carbohydrate-active enzymes in the genome of *Arabidopsis thaliana*. Plant Molecular Biology **47:** 55-72

His I, Driouich A, Nicol F, Jauneau A, Hofte H (2001) Altered pectin composition in primary cell walls of korrigan, a dwarf mutant of Arabidopsis deficient in a membrane-bound endo-1,4-beta-glucanase. Planta **212:** 348-358

Hosoo Y, Yoshida M, Imai T, Okuyama T (2002) Diurnal difference in the amount of immunogold-labeled glucomannans detected with field emission scanning electron microscopy at the innermost surface of developing secondary walls of differentiating conifer tracheids. Planta **215:** 1006-1012

Itoh T, Kimura S (2001) Immunogold labeling of terminal cellulose-synthesizing complexes. Journal of Plant Research. **114:** 483-489.

Ji Q, Oomen RJF, Vincken JP, Bolam DN, Gilbert HJ, Suurs L, Visser RGF (2004) Reduction of starch granule size by expression of an engineered tandem starch-binding domain in potato plants. Plant Biotechnology Journal **2:** 251-260

John M, Rohrig H, Schmidt J, Walden R, Schell J (1997) Cell signalling by oligosaccharides. Trends In Plant Science **2:** 111-115

Kataeva IA, Seidel RD, Shah A, West LT, Li XL, Ljungdahl LG (2002) The fibronectin type 3-like repeat from the Clostridium thermocellum cellobiohydrolase CbhA promotes hydrolysis of cellulose by modifying its surface. Applied And Environmental Microbiology **68:** 4292-4300

Keegstra K, Raikhel N (2001) Plant glycosyltransferases. Current Opinion In Plant Biology **4:** 219-224

Kende H, Bradford KJ, Brummell DA, Cho HT, Cosgrove DJ, Fleming AJ, Gehring C, Lee Y, McQueen-Mason S, Rose JKC, Voesenek L (2004) Nomenclature for members of the expansin superfamily of genes and proteins. Plant Molecular Biology **55:** 311-314

Kilburn DG, Warren AJ, Stoll D, Gilkes NR, Shoseyov O, Shani Z (2000) Expression of a mannan binding domain to alter plant morphology. WO 2000/068391

Kimura S, Laosinchai W, Itoh T, Cui XJ, Linder CR, Brown RM (1999) Immunogold labeling of rosette terminal cellulose-synthesizing complexes in the vascular plant Vigna angularis. Plant Cell **11:** 2075-2085

Kokubo A, Sakurai N, Kuraishi S, Takeda K (1991) Culm brittleness of barley (Hordeum-Vulgare L) mutants is caused by smaller number of cellulose molecules in cell-wall. Plant Physiology **97:** 509-514

Kuipers AGJ, Jacobsen E, Visser RGF (1994) Formation and deposition of amylose in the potato-tuber starch granule are affected by the reduction of granule-bound starch synthase gene-expression. Plant Cell **6:** 43-52

Kuipers AGJ, Soppe WJJ, Jacobsen E, Visser RGF (1995) Factors affecting the inhibition by antisense RNA of granule-bound starch synthase gene-expression in potato. Molecular & General Genetics **246:** 745-755

Kurek I, Kawagoe Y, Jacob-Wilk D, Doblin M, Delmer D (2002) Dimerization of cotton fibre cellulose synthase catalytic subunits occurs via oxidation of the zinc-binding domains. Proceedings of The National Academy of Sciences of The United States of America **99:** 11109-11114

Kuroiwa T, Kuroiwa H, Mita T, Fujie M (1990) Fluorescence Microscopic Study of the Formation of Giant Mitochondrial Nuclei in the Young Ovules of Pelargonium-Zonale. Protoplasma **158:** 191-194

Kutschera U (1990) Cell-Wall Synthesis And Elongation Growth In Hypocotyls of Helianthus-Annuus L. Planta **181:** 316-323

Kutschera U (2001) Stem elongation and cell wall proteins in flowering plants. Plant Biology **3:** 466-480

Lane DR, Wiedemeier A, Peng LC, Hofte H, Vernhettes S, Desprez T, Hocart CH, Birch RJ, Baskin TI, Burn JE, Arioli T, Betzner AS, Williamson RE (2001) Temperature-sensitive alleles of RSW2 link the KORRIGAN endo-1,4-beta-glucanase to cellulose synthesis and cytokinesis in arabidopsis. Plant Physiology **126:** 278-288

Lee S, Roberts AW (2004) Tracheary element differentiation is correlated with inhibition of cell expansion in xylogenic mesophyll suspension cultures. Plant Physiology and Biochemistry **42:** 43-48

Levy I, Nussinovitch A, Shpigel E, Shoseyov O (2002a) Recombinant cellulose crosslinking protein: a novel paper-modification biomaterial. Cellulose **9:** 91-98

Levy I, Shani Z, Shoseyov O (2002b) Modification of polysaccharides and plant cell wall by endo-1,4-beta-glucanase and cellulose-binding domains. Biomolecular Engineering **19:** 17-30

Levy I, Shoseyov O (2002) Cellulose-binding domains biotechnological applications. Biotechnology Advances **20:** 191-213

Li Y, Jones L, McQueen-Mason S (2003) Expansins and cell growth. Current Opinion In Plant Biology **6:** 603-610

Liepman AH, Wilkerson CG, Keegstra K (2005) Expression of cellulose synthase-like (Csl) genes in insect cells reveals that CslA family members encode mannan synthases. Proceedings of The National Academy of Sciences of The United States of America **102:** 2221-2226

Linder M, Nevanen T, Teeri TT (1999) Design of a pH-dependent cellulose-binding domain. Febs Letters **447:** 13-16

Linder M, Salovuori I, Ruohonen L, Teeri TT (1996) Characterization of a double cellulose-binding domain - Synergistic high affinity binding to crystalline cellulose. Journal of Biological Chemistry **271:** 21268-21272

Ludidi NN, Heazlewood JL, Seoighe C, Irving HR, Gehring CA (2002) Expansin-like molecules: Novel functions derived from common domains. Journal of Molecular Evolution **54:** 587-594

MacDougall AJ, Brett GM, Morris VJ, Rigby NM, Ridout MJ, Ring SG (2001) The effect of peptide behaviour-pectin interactions on the gelation of a plant cell wall pectin. Carbohydrate Research **335:** 115-126

Madson M, Dunand C, Li XM, Verma R, Vanzin GF, Calplan J, Shoue DA, Carpita NC, Reiter WD (2003) The MUR3 gene of *Arabidopsis* encodes a xyloglucan galactosyltransferase that is evolutionarily related to animal exostosins. Plant Cell **15:** 1662-1670

Maryani MM, Bradley G, Cahill DM, Gehring CA (2001) Natriuretic peptides and immunoreactants modify osmoticum-dependent volume changes in Solanum tuberosum L. mesophyll cell protoplasts. Plant Science **161:** 443-452

McCann MC, Roberts K (1994) Changes In Cell-Wall Architecture During Cell Elongation. Journal of Experimental Botany **45:** 1683-1691

McCartney L, Gilbert HJ, Bolam DN, Boraston AB, Knox JP (2004) Glycoside hydrolase carbohydrate-binding modules as molecular probes for the analysis of plant cell wall polymers. Analytical Biochemistry **326:** 49-54

McQueen-Mason S, Cosgrove DJ (1994) Disruption of hydrogen-bonding between plant-cell wall polymers by proteins that induce wall extension. Proceedings of The National Academy of Sciences of The United States of America **91:** 6574-6578

McQueen-Mason SJ, Cosgrove DJ (1995) Expansin mode of action on cell-walls - Analysis of wall hydrolysis, stress-relaxation, and binding. Plant Physiology **107:** 87-100

McQueen-Mason SJ, Durachko D.M., Cosgrove DJ (1992) Two endogenous proteins that induce cell-wall extension in plants. Plant Cell **4:** 1425-1433

Mohanty AK, Misra M, Drzal LT (2001) Surface modifications of natural fibres and performance of the resulting biocomposites: An overview. Composite Interfaces **8:** 313-343

Molhoj M, Pagant S, Hofte H (2002) Towards understanding the role of membrane-bound endo-beta-1,4-glucanases in cellulose biosynthesis. Plant and Cell Physiology. **43:** 1399-1406.

Molhoj M, Ulvskov P, Dal Degan F (2001) Characterization of a functional soluble form of a Brassica napus membrane-anchored endo-1,4-beta-glucanase heterologously expressed in Pichia pastoris. Plant Physiology **127:** 674-684

Morris VJ, Wilde PJ (1997) Interactions of food biopolymers. Current Opinion In Colloid & Interface Science **2:** 567-572

Mueller SC, Brown RM (1980) Evidence For An Intramembrane Component Associated With A Cellulose Microfibril-Synthesizing Complex In Higher-Plants. Journal Of Cell Biology **84:** 315-326

Mulder BM, Emons AMC (2001) A dynamical model for plant cell wall architecture formation. Journal of Mathematical Biology **42:** 261-289

Nicol F, His I, Jauneau A, Vernhettes S, Canut H, Hofte H (1998) A plasma membrane-bound putative endo-1,4-beta-D-glucanase is required for normal wall assembly and cell elongation in *Arabidopsis*. Embo Journal **17:** 5563-5576

O'Neill MA, York WS (2003) The composostion and structure of plant primary cell wall. In The Plant Cell Wall. (ed. J.K.C. Rose). Blackwell Publishing, Oxford, UK

Obembe OO, Cifuentes C, Huigen D, Kieft H, Jacobsen E, Emons AMC, Visser RGF, Vincken J-P (2006a) Bioengineering cellulose-hemicelluose networks in plants (Chapter 2, This Thesis). Wageningen University, Wageningen

Obembe OO, Jacobsen E, Gilbert H, Blake AW, Knox JP, Visser RGF, Vincken J-P (2006b) Bioengineering cellulose-hemicelluose networks in plants (Chapter 4, this Thesis). Wageningen University, Wageningen

Obembe OO, Jacobsen E, Timmers J, Gilbert H, Blake AW, Knox JP, Visser RGF, Vincken J-P (2006c) Bioengineering cellulose-hemicelluose networks in plants (Chapter 3, This Thesis). PhD thesis. Wageningen University, Wageningen

Oomen R, Doeswijk-Voragen CHL, Bush MS, Vincken JP, Borkhardt B, van den Broek LAM, Corsar J, Ulvskov P, Voragen AGJ, McCann MC, Visser RGF (2002) In muro fragmentation of the rhamnogalacturonan I backbone in potato (Solanum tuberosum L.) results in a reduction and altered location of the galactan and arabinan side-chains and abnormal periderm development. Plant Journal **30:** 403-413

Oomen R, Tzitzikas EN, Bakx EJ, Straatman-Engelen I, Bush MS, McCann MC, Schols HA, Visser RGF, Vincken JP (2004) Modulation of the cellulose content of tuber cell walls by antisense expression of different potato (Solanum tuberosum L.) CesA clones. Phytochemistry **65:** 535-546

Pauly M, Andersen LN, Kauppinen S, Kofod LV, York WS, Albersheim P, Darvill A (1999) A xyloglucan-specific endo-beta-1,4-glucanase from Aspergillus aculeatus: expression cloning in yeast, purification and characterization of the recombinant enzyme. Glycobiology **9:** 93-100

Pear JR, Kawagoe Y, Schreckengost WE, Delmer DP, Stalker DM (1996) Higher plants contain homologs of the bacterial celA genes encoding the catalytic subunit of cellulose synthase. Proceedings of The National Academy of Sciences of The United States of America **93:** 12637-12642

Peitsch MC (1995) Protein Modeling By E-Mail. Bio-Technology **13:** 658-660

Pena MJ, Ryden P, Madson M, Smith AC, Carpita NC (2004) The galactose residues of xyloglucan are essential to maintain mechanical strength of the primary cell walls in *Arabidopsis* during growth. Plant Physiology **134:** 443-451

Peng L, Xiang F, Roberts E, Kawagoe Y, Greve LC, Kreuz K, Delmer DP (2001) The experimental herbicide CGA 325'615 inhibits synthesis of crystalline cellulose and causes accumulation of non-crystalline beta-1,4-glucan associated with CesA protein. Plant Physiology **126:** 981-992.

Peng LC, Kawagoe Y, Hogan P, Delmer D (2002) Sitosterol-beta-glucoside as primer for cellulose synthesis in plants. Science **295:** 147-150

Perrin RM, DeRocher AE, Bar-Peled M, Zeng WQ, Norambuena L, Orellana A, Raikhel NV, Keegstra K (1999) Xyloglucan fucosyltransferase, an enzyme involved in plant cell wall biosynthesis. Science **284:** 1976-1979

Peters WS, Tomos AD (1996) The history of tissue tension. Annals of Botany **77:** 657-665

Peters WS, Tomos AD (2000) The mechanic state of "Inner tissue" in the growing zone of sunflower hypocotyls and the regulation of its growth rate following excision. Plant Physiology **123:** 605-612

Pharmawati M, Maryani MM, Nikolakopoulos T, Gehring CA, Irving HR (2001) Cyclic GMP modulates stomatal opening induced by natriuretic peptides and immunoreactive analogues. Plant Physiology And Biochemistry **39:** 385-394

Pien S, Wyrzykowska J, McQueen-Mason S, Smart C, Fleming A (2001) Local expression of expansin induces the entire process of leaf development and modifies leaf shape. Proceedings of The National Academy of Sciences of The United States of America **98:** 11812-11817

Pincon G, Chabannes M, Lapierre C, Pollet B, Ruel K, Joseleau JP, Boudet AM, Legrand M (2001) Simultaneous down-regulation of caffeic/5-hydroxy ferulic acid-O-methyltransferase I and cinnamoyl-coenzyme a reductase in the progeny from a cross between tobacco lines homozygous for

each transgene. Consequences for plant development and lignin synthesis. Plant Physiology **126:** 145-155

Quentin MGE (2003) Cloning, characterisation and assessment of the wall modifying ability of a fungal cellulose-binding domain. (PhD Thesis). Wageningen University, Wageningen

Rabinovich ML, Melnick MS, Bolobova AV (2002) The structure and mechanism of action of cellulolytic enzymes. Biochemistry-Moscow **67:** 850-871

Raven PH, Evert RF, Eichhorn SE (1992) Biology of plants. Fifth edition. Worth Publishers, New York, USA

Reid JSG, Edwards ME, Dickson CA, Scott C, Gidley MJ (2003) Tobacco transgenic lines that express fenugreek galactomannan galactosyltransferase constitutively have structurally altered galactomannans in their seed endosperm cell walls. Plant Physiology **131:** 1487-1495

Richmond TA, Somerville CR (2001) Integrative approaches to determining Csl function. Plant Molecular Biology **47:** 131-143

Robert S, Mouille G, Hofte H (2004) The mechanism and regulation of cellulose synthesis in primary walls: lessons from cellulose-deficient Arabidopsis mutants. Cellulose **11:** 351-364

Rochange SF, Wenzel CL, McQueen-Mason SJ (2001) Impaired growth in transgenic plants over-expressing an expansin isoform. Plant Molecular Biology **46:** 581-589

Rose JKC, Catalá C, Gonzalez-Carranza ZH, Roberts JA (2003) Cell wall disassembly. In The Plant Cell Wall. (ed. J.K.C. Rose). Blackwell Publishing, Oxford, UK.

Ryden P, Sugimoto-Shirasu K, Smith AC, Findlay K, Reiter WD, McCann MC (2003) Tensile properties of Arabidopsis cell walls depend on both a xyloglucan cross-linked microfibrillar network and rhamnogalacturonan II-borate complexes. Plant Physiology **132:** 1033-1040

Salnikov VV, Grimson MJ, Delmer DP, Haigler CH (2001) Sucrose synthase localizes to cellulose synthesis sites in tracheary elements. Phytochemistry **57:** 823-833

Saloheimo M, Paloheimo M, Hakola S, Pere J, Swanson B, Nyyssonen E, Bhatia A, Ward M, Penttila M (2002) Swollenin, a Trichoderma reesei protein with sequence similarity to the plant expansins, exhibits disruption activity on cellulosic materials. European Journal of Biochemistry **269:** 4202-4211

Saxena IM, Brown RM (2005) Cellulose biosynthesis: Current views and evolving concepts. Annals of Botany **96:** 9-21

Saxena IM, Brown RM, Dandekar T (2001) Structure-function characterization of cellulose synthase: relationship to other glycosyltransferases. Phytochemistry **57:** 1135-1148

Saxena IM, Brown RM, Fevre M, Geremia RA, Henrissat B (1995) Multidomain architecture of beta-glycosyl transferases - implications for mechanism of action. Journal of Bacteriology **177:** 1419-1424

Scheible WR, Eshed R, Richmond T, Delmer D, Somerville C (2001) Modifications of cellulose synthase confer resistance to isoxaben and thiazolidinone herbicides in *Arabidopsis* Ixr1 mutants. Proceedings of the National Academy of Sciences of the United States of America **98:** 10079-10084

Schrick K (2004) A link between sterol biosynthesis, the cell-wall, and cellulose in Arabidopsis (vol 38, pg 227, 2004). Plant Journal **38:** 562-562

Schroder R, Wegrzyn TF, Bolitho KM, Redgwell RJ (2004) Mannan transglycosylase: a novel enzyme activity in cell walls of higher plants. Planta **219:** 590-600

Schwede T, Kopp J, Guex N, Peitsch MC (2003) SWISS-MODEL: an automated protein homology-modeling server. Nucleic Acids Research **31:** 3381-3385

Shcherban TY, Shi J, Durachko DM, Guiltinan MJ, McQueen-Mason SJ, Shieh M, Cosgrove DJ (1995) Molecular-Cloning And Sequence-Analysis of Expansins - A Highly Conserved, Multigene Family of Proteins That Mediate Cell-Wall Extension In Plants. Proceedings of The National Academy of Sciences of The United States of America **92:** 9245-9249

Shoseyov O, Yosef K, Shani Z, Rehovoth, Shpigel E, Medigo K (2001) Transgenic Plants of Altered Morphology. US patent 6,184,440.

Shoseyov OY, K; Shani, Z; Rehovoth; Shpigel, E; Medigo, K (2001) Transgenic Plants of Altered Morphology. US patent 6,184,440, 2001.

Shpigel E, Roiz L, Goren R, Shoseyov O (1998) Bacterial cellulose-binding domain modulates in vitro elongation of different plant cells. Plant Physiology **117:** 1185-1194

Simpson PJ, Xie HF, Bolam DN, Gilbert HJ, Williamson MP (2000) The structural basis for the ligand specificity of family 2 carbohydrate-binding modules. Journal of Biological Chemistry **275:** 41137-41142

Southall SM, Simpson PJ, Gilbert HJ, Williamson G, Williamson MP (1999) The starch-binding domain from glucoamylase disrupts the structure of starch. Febs Letters **447:** 58-60

Stephen AM (1982) Other plant polysaccharides. In the polysaccharides, Vol 2 (ed. G.O. Aspinall). Academic Press, New York, USA.

Suzuki M, Kato A, Nagata N, Komeda Y (2002) A xylanase, AtXyn1, is predominantly expressed in vascular bundles, and four putative xylanase genes were identified in the *Arabidopsis* thaliana genome. Plant And Cell Physiology **43:** 759-767

Szyjanowicz PMJ, McKinnon I, Taylor NG, Gardiner J, Jarvis MC, Turner SR (2004) The irregular xylem 2 mutant is an allele of korrigan that affects the secondary cell wall of Arabidopsis thaliana. Plant Journal **37:** 730-740

Tanaka K, Murata K, Yamazaki M, Onosato K, Miyao A, Hirochika H (2003) Three distinct rice cellulose synthase catalytic subunit genes required for cellulose synthesis in the secondary wall. Plant Physiology **133:** 73-83

Taylor NG, Gardiner JC, Whiteman R, Turner SR (2004) Cellulose synthesis in the Arabidopsis secondary cell wall. Cellulose **11:** 329-338

Taylor NG, Howells RM, Huttly AK, Vickers K, Turner SR (2003) Interactions among three distinct CesA proteins essential for cellulose synthesis. Proceedings of the National Academy of Sciences of the United States of America **100:** 1450-1455

Taylor NG, Laurie S, Turner SR (2000) Multiple cellulose synthase catalytic subunits are required for cellulose synthesis in Arabidopsis. Plant Cell **12:** 2529-2539

Taylor NG, Scheible WR, Cutler S, Somerville CR, Turner SR (1999) The irregular xylem3 locus of *Arabidopsis* encodes a cellulose synthase required for secondary cell wall synthesis. Plant Cell **11:** 769-780

Timell TE (1967) Recent progress in the chemistry of wood hemicellulose. Wood Science and Technology **1:** 45-70

Toone EJ (1994) Structure And Energetics of Protein Carbohydrate Complexes. Current Opinion In Structural Biology **4:** 719-728

Tormo J, Lamed R, Chirino AJ, Morag E, Bayer EA, Shoham Y, Steitz TA (1996) Crystal structure of a bacterial family-III cellulose-binding domain: A general mechanism for attachment to cellulose. Embo Journal **15:** 5739-5751

Tsabary G, Shani Z, Roiz L, Levy I, Riov J, Shoseyov O (2003) Abnormal 'wrinkled' cell walls and retarded development of transgenic Arabidopsis thaliana plants expressing endo-1,4-beta-glucanase (cel1) antisense. Plant Molecular Biology **51:** 213-224

Tsekos I, Reiss HD (1992) Occurrence of the putative microfibril-synthesizing complexes (linear terminal complexes) in the plasma-membrane of the epiphytic marine red alga Erythrocladia-Subintegra Rosenv. Protoplasma **169:** 57-67

Turner SR, Somerville CR (1997) Collapsed xylem phenotype of Arabidopsis identifies mutants deficient in cellulose deposition in the secondary cell wall. Plant Cell **9:** 689-701

Updegraff DM (1969) Semi-microdetermination of cellulose in biological materials. Analytical Biochemistry **32:** 420-424

Whitney SEC, Brigham JE, Darke AH, Reid JSG, Gidley MJ (1995) In-Vitro Assembly of Cellulose/Xyloglucan Networks - Ultrastructural And Molecular Aspects. Plant Journal **8:** 491-504

Whitney SEC, Brigham JE, Darke AH, Reid JSG, Gidley MJ (1998) Structural aspects of the interaction of mannan-based polysaccharides with bacterial cellulose. Carbohydrate Research **307:** 299-309

Whitney SEC, Gidley MJ, McQueen-Mason SJ (2000) Probing expansin action using cellulose/hemicellulose composites. Plant Journal **22:** 327-334

Whitney SEC, Gothard MGE, Mitchell JT, Gidley MJ (1999) Roles of cellulose and xyloglucan in determining the mechanical properties of primary plant cell walls. Plant Physiology **121:** 657-663

Wittink FRA, Knuiman B, Derksen J, Capkova V, Twell D, Schrauwen JAM, Wullems GJ (2000) The pollen-specific gene Ntp303 encodes a 69-kDa glycoprotein associated with the vegetative membranes and the cell wall. Sexual Plant Reproduction **12:** 276-284

Wood PJ (1980) Specificity in the interaction of direct dyes with polysaccharides. Carbohydrate Research **85:** 271-287

Xiao ZZ, Gao PJ, Qu YB, Wang TH (2001) Cellulose-binding domain of endoglucanase III from Trichoderma reesei disrupting the structure of cellulose. Biotechnology Letters **23:** 711-715

Zenoni S, Reale L, Tornielli GB, Lanfaloni L, Porceddu A, Ferrarini A, Moretti C, Zamboni A, Speghini S, Ferranti F, Pezzotti M (2004) Downregulation of the Petunia hybrida alpha-expansin gene PhEXP1 reduces the amount of crystalline cellulose in cell walls and leads to phenotypic changes in petal limbs. Plant Cell **16:** 295-308

Zhong RQ, Burk DH, Morrison WH, Ye ZH (2004) FRAGILE FIBRE3, an Arabidopsis gene encoding a type II inositol polyphosphate 5-phosphatase, is required for secondary wall synthesis and actin organization in fibre cells. Plant Cell **16:** 3242-3259

Zhong RQ, Morrison WH, Freshour GD, Hahn MG, Ye ZH (2003) Expression of a mutant form of cellulose synthase AtCesA7 causes dominant negative effect on cellulose biosynthesis. Plant Physiology **132:** 786-795

Irregular deposition of cell wall polymers resulting from defective cellulose synthase complexes

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Abstract

Down-regulation of cellulose synthase catalytic subunits (CesAs) by antisense strategy has been previously shown to reduce cellulose content in the potato tuber cell walls. The antisense constructs were based on the class specific region (csr) of CesA proteins. Two transgenic potato lines csr2-1 and csr4-8, containing two different antisense constructs, csr2 and csr4, respectively, were crossed to investigate the possibility of achieving double transformants with combined effects of the two antisense transgenes on cellulose deposition in the potato tuber cell walls. Offspring containing either one or both transgenes produced more tubers than the control plants but individual tubers were mostly smaller and had lesser weight than the control tubers. These altered phenotypes were more pronounced in the csr2 tubers than in csr4 and double transformant tubers. Fluorescence microscopy with calcofluor white revealed an unusually strong fluorescence in the cell corners and less prominent and uneven fluorescence around the cells of the csr2 tubers as compared to others. Despite its lowest level of cellulose content, csr2/csr4 double transformants showed far less unusual deposition in cell corners than the single csr2 tranformants. Additionally in the csr2 tubers, we observed ectopic proliferation of xylem cells, which we speculate to have transdifferentiated from parenchyma cells of perimedullary tissue. Light microscopy of csr2 tubers revealed that the proliferation of xylem cells was associated with lignification of their cell walls. This paper provides the first report of a non-*Arabidopsis* primary cell wall variant exhibiting both altered cellulose deposition and ectopic xylem proliferation. These results may help to understand the dynamics of cellulose deposition better.

Introduction

Cellulose deposition in the cell wall plays a vital role in controlling cell growth which in turn influences the final morphology of plant organs. Crystalline cellulose microfibrils are formed in the cell wall by spontaneous association of, probably 36, β-D-glucan (cellulose) polymers synthesized by plasma membrane-bound cellulose synthase (CesA) complexes (rosettes), which are believed to comprise six subunits (Delmer and Amor, 1995). Each of the complex subunits is conceived to have at least six CesA proteins to be able to synthesise 36 glucan chains. (Brown and Saxena, 2000). There is a body of evidence that indicates that each subunit of the cellulose synthase complex comprises at least three distinct CesA isoforms. Three *Arabidopsis* CesA isoforms (AtCesA4, 7 and 8) have been shown, through immunoprecipation and co-localisation studies, to interact and constitute the subunits of the same complex that synthesises cellulose during secondary wall deposition in xylem cells (Gardiner et al., 2003; Taylor et al., 2003). Mutation studies in rice tissue depositing the secondary cell wall have also identified three distinct rice CesA genes, Os CesA4, Os CesA7 and Os CesA9 to be responsible for brittle culm mutations of rice (Tanaka et al., 2003). The authors observed that expression profiles of the three genes were almost identical, suggesting that the CesA proteins may interact to form a complex. Similarly, the analysis of *isoxaben-resistance* mutants and expression studies with promoter-GUS fusions and *in situ* hybridisation have indicated that at least three CesAs isoforms (AtCesA1, 3 and 6) are required for cellulose synthesis in primary-walled cells of *Arabidopsis* (Fagard et al., 2000; Scheible et al., 2001; Desprez et al., 2002; Robert et al., 2004), and has led to the idea that the complexes involved in primary wall synthesis also contain at least three CesA isoforms. With the experimental evidence that CesA proteins can form homodimers through their N-terminal ring-finger domain (Kurek et al., 2002), it has been suggested that each complex subunit would contain three homodimers of distinct CesA isoforms. It is therefore not surprising to observe that impairment of a CesA isoform in the *rsw1* (radial swelling) mutant of *Arabidopsis* (AtCesA1, Arioli *et al.*, 1998) affects the normal assembly of the CesA complex and consequently, cellulose deposition. Impairment of the CesA complex also alters cellulose synthesis and deposition in two other primary cell wall mutants *prc1* (procuste, AtCesA6) and *eli1* (ectopic lignification, AtCesA3) (Arioli et al., 1998; Cano-Delgado et al., 2000; Fagard et al., 2000).

The ordering of cellulose microfibrils during their deposition has been explained with a theory, which takes into consideration the stiffness of the microfibrils and the density of active CesA complexes (Emons and Mulder, 1998; Mulder and Emons, 2001). The theory proposes that once activated in the plasma membrane, the complexes move forward by the propelling forces generated in the microfibril's deposition and crystallisation process. The direction of the complexes at any given moment, and hence the orientation of the microfibrils deposited, is determined by the total number of active complexes along their path. Agreeably, impairment to the complexes would have severe implication on cellulose deposition and crystallisation as well as the processes they influence.

Oomen *et al.* (2004) reported varying degrees of up- and down-regulation of cellulose content in the potato tuber cell walls. These transgenic potato tuber clones were obtained by transforming the potato plant with antisense/sense constructs of a complete cDNA of potato cellulose synthase, (AS-CesA3/SE-CesA3) and antisense constructs of the class-specific regions (CSR) of four corresponding potato cellulose synthase genes (CSR1, 2, 3 and 4). Two transgenic lines csr2-1 and csr4-8, which showed considerable cellulose reduction (40% and 60%, respectively) in their tuber cell walls were identified by the anthrone colorimetric assay (Oomen *et al,* 2004). These two lines also grouped together in the same cluster following Fourier Transform Infra Red spectroscopy and Principal Component Analysis. These observations have led us to speculate that CesA2 and CesA4 proteins are probably part of the same complex. Hence our interest in exploring the possibility of achieving double transformants with combined effects of the two transgenes on cellulose synthesis and deposition.

In this study, we have crossed the transgenic potato lines csr2-1 and csr4-8, whose tuber cell walls exhibit low levels of cellulose as compared to the wild type control. The offspring was screened and microscopically analysed for tuber cell wall phenotypes. We report the expected cellulose reduction in csr2/csr4 double transformants and new remarkable observations on the offspring plants containing the csr2 construct. Probable mechanisms underlying these observations are presented.

Materials and Methods

Plant Material and Growth Conditions
Potato (*Solanum tuberosum*) plants used for the cross carried, in antisense orientation, csr2 sequence of the potato CesA2 gene (accession number AY221089) or the csr4 sequence of the CesA4 gene (accession number AY221088) (Oomen et al., 2004). Expression of the antisense constructs was targeted to the tuber by using a granule bound starch synthase (gbss) promoter to drive its expression. Pollen of plant line csr4-8 was used to fertilise plant line csr2-1 to produce berries. A total of 488 seeds were removed from the berries, dried and prepared for germination. 100 seedlings, representing the different genotypes, were grown in soil in the greenhouse under 3,000lux lighting and in a light / dark period of 16/8h.

Southern Analysis
Copy number of transgene integration of the parents and segregation pattern in the offspring was determined by Southern analysis. A total of 84 F1 plants were used for segregation analysis. Genomic DNA was isolated from young leaves by using GenElute[TM] Plant Genomic DNA Miniprep Kit (Sigma). 8 μg of the prepared DNA was digested with restriction enzyme HindIII and electrophoresed on 0.8% (w/v) agarose gels. Electrophoresed DNA samples were transferred onto Nylon N- membrane (Amersham). Hybridization was performed at 65°C with modified Church buffer (Church and Gilbert, 1984) containing [^{32}P] UTP-labelled NptII probe.

Isolation of cell wall material and analysis of the cellulose content
One tuber from three representatives of the four clones from the offspring were pooled and ground to a fine powder in liquid nitrogen. For each isolation, 10 g of this tuber material was extracted in a 50 mM Tris[HCl], pH 7.2 solution containing 1% SDS for 3 h at room temperature with continuous shaking. The CWM was spun down by centrifugation at 4600 rpm for 30 min. Subsequently, the residue was washed with water, ethanol, acetone and air-dried. 50 mg of this CWM was then incubated for 90 min

at 120°C in 5 ml 2 M triflouroacetic acid (TFA). The remaining cellulose was spun down and obtained as pellet, which was washed with water and ethanol. The pellet was solubilized in 67% (v/v) H_2SO_4 and diluted appropriately to determine the cellulose content colorimetrically using anthrone as a colouring agent according to Updegraff, 1969. Acid hydrolysis of cell wall materials and colorimetric assays were performed in quadruplicate and standard deviations were calculated accordingly.

Quantitative SYBR-Green RT-PCR Analysis for four potato CesA genes

Gene-specific primers were developed for four CesA genes (CesA 1, 2, 3 and 4) and for ubiquitin (as internal control) using the Primer Express software (version 1.5, PE Applied BioSystems, CA, USA). Sequences of all the primers used are shown in Table 1. The following tissues were used for the analysis: tubers, swollen tips, stolons, roots, stem, nodes, midribs/petioles, leaves, developing flowers and berries. Total RNA was isolated from 3 g (fresh weight) of the different tissues as described elsewhere (Kuipers et al., 1994). Reverse transcription reaction and SYBR-Green PCR were performed as described below. For first strand cDNA synthesis, 1µg of total RNA was treated with 0.5 µl DNAse I RNase free (10 U/µl; Invitrogen) and incubated with 5 µl of 10 x Taqman RT buffer, 11 µl of 25 mM $MgCl_2$, 10 µl of 10 mM dNTP mix, 2.5 µl of 50 µM random hexamer primers, 1.0µl RNAse inhibitor (20U/µl) and H_2O until a final volume of 39 µl for 30 min at $37^{\circ}C$ and 5 min at $75^{\circ}C$. The mixture was then incubated for 10 min at $25^{\circ}C$ and 30 min at $48^{\circ}C$ with 1 µl of MultiScribe reverse transcriptase (50U/µl; Applied Biosystems). The reaction was then terminated by heating the sample for 5 min at $95^{\circ}C$. Aliquots of 50ng of cDNA were used in SYBR-Green PCR Analysis according to the manufacturer's protocol on the ABI PRISM7700 sequence detection system (Perkin-Elmer Applied Biosystems) with the primers listed in Table 1. Relative quantification of the CesAs RNA expressions was performed using the comparative C_T method according to the User Bulletin #2 (ABI PRISM7700 sequence detection system; Perkin-Elmer Applied Biosystems). The differences in Ct values, called ΔC_t, between the CesA mRNA and endogenous ubiquitin control mRNA were calculated in order to normalize the differences in the cDNA concentrations for each reaction. RNA expression level was expressed as percentage of the control RNA expression level using the equation $2^{-\Delta Ct}$ x 100%.

Table 1. Overview of SYBR-Green Primers used. Primers for the CesA genes were based on the class specific regions of the corresponding CesA.

Primer	Gene	Sequences (5´ to 3´)
CSR1-F	CesA1	CAGCCCTCATGCCTCAGATAA
CSR1-R	CesA1	AAATACCGGTGATTGGCCAA
CSR2-F	CesA2	TGAGGCAGATTTGGAGCCA
CSR2-R	CesA2	GACCCACCACAACAGCTCTTC
CSR3-F	CesA3	CGGCTGTTTTTGTTGCTTCA
CSR3-R	CesA3	CGATTGAGGAACACCACCATT
CSR4-F	CesA4	TCGAGGAAGGAATCGAAGGA
CSR4-R	CesA4	GCGGCATGAGGGAAGCTT
UBI3-F	Ubiquitin	TTCCGACACCATCGACAATGT
UBI3-F	Ubiquitin	CGACCATCCTCAAGCTGCTT

Quantitative SYBR-Green RT-PCR Analysis for CesA2 and CesA4 in the tubers of the offspring plants

The same procedure as above was used for RNA isolation and the SYBR-Green RT-PCR analysis. The gene-specific primers of CesA2 and CesA4 genes as well as that of the ubiquitin were used to analyse their expression in the tubers of the four clones; single csr2 transformant, single csr4 transformant, double csr2/4 transformant and the control plant. The analysis was done with young, freshly harvested tubers of about the same developmental stage.

Lignin and Starch staining

Potato tuber slices perpendicular to stolon axis were hand cut with a razor blade. Slices were stained for lignin with 1% (w/v) phloroglucinol in 96% ethyl alcohol, and for starch with 0.01% iodine potassium iodide. Each histochemical staining was performed in triplicates. Photograph was taken with a Nikon digital Camera.

Light microscopy

1 mm-thick potato tuber sections were fixed in 3% glutardialdehyde and 3% paraformaldehyde in 0.1 M phosphate buffer containing 0.1% Triton x100 for 2 hours. The samples were then washed and dehydrated in ethanol series. After dehydration, they were embedded in Technovit 7100 resin (Kuroiwa et al., 1990). 4 micron-thick tissue sections were stained with calcofluorwhite (0.04%) and toluidine Blue (0.1%) and examined by light microscope. Each microscopic examination was done in triplicates. To verify our observation for the calcofluorwhite-stained csr2 tuber sections, we made fresh staining of csr2 and the control tuber sections with 0.1% aniline blue in 0.1 M K_3PO_4 and examined under fluorescence microscope. We next did calcofluorwhite staining of the aniline blue-stained tuber sections and examined under fluorescence microscope for differential staining patterns. Lignin staining was also repeated for fresh csr2 and the control sections to verify our earlier observations on tuber slices stained with phloroglucinol.

BMM-embedding and xyloglucanase treatment

In order to perform enzyme treatment on stem sections, a different method of fixation and embedding in comparison with the one described before was necessary. Tuber samples were fixed in 5% glutardialdehyde in 0.1M phosphate buffer, containing 0.1% Triton x100 for 2 hours. The samples were then washed with the buffer without glutardialdehyde, and dehydrated in an ethanol series (0, 10, 30, 50% ethanol containing dithiothreitol (DDT)) and embedded in butyl-methyl methacrylat (BMM) as described elsewhere (Gubler, 1989; Baskin et al., 1992). 4 micron-thick tissue sections were made from the BMM-embedded stem samples and mounted on glass slides. The embedding material was removed from the sections with acetone, and then the sections were immediately washed with 25 mM citrate buffer pH 3.5. Incubation of glass-mounted sections was done at room temperature in 20 ml 25 mM citrate buffer pH 3.5 containing 0.1 U/mL of an endo-beta-1,4-glucanase from *Aspergillus aculeatus*, which is specific for xyloglucan (Pauly et al., 1999). It can be calculated that a stem section of approximately 1 cm^2 area and 4 μm in thickness contains approximately 10^{-2} μmol of linkages (as part of xyloglucan) that can be cleaved. Further assumptions were that 5% of the section consists of cell wall material, 20% of which is xyloglucan, and that the average molecular mass of a xyloglucan oligosaccharide is about 1000 Da. After treatment, the sections were stained and observed under the microscope, and the incubation buffers were subjected to malditof-ms and HPAEC analyses, to check for the release of xyloglucan oligosaccharides. In parallel, 15 mg of potato xyloglucan (extracted with 6 M KOH) was incubated in 1.5 mL 25 mM citrate buffer pH 3.5 containing 20 U/mL of an endo-beta-1,4-glucanase, as the control sample. The incubation of the control and the section were performed for 2 hours at room temperature.

Results

Segregation Analysis and Phenotype of the offspring

Southern analysis of the parent plants (Fig. 1) revealed that parent csr2-1 contained 2 copies of the transgene in tandem while parent csr4-8 contained 3 copies (1 single insertion and 1 tandem repeat). Based on the transgene insertion number in the parents, we determined the segregation pattern of the offspring, using Southern analysis.
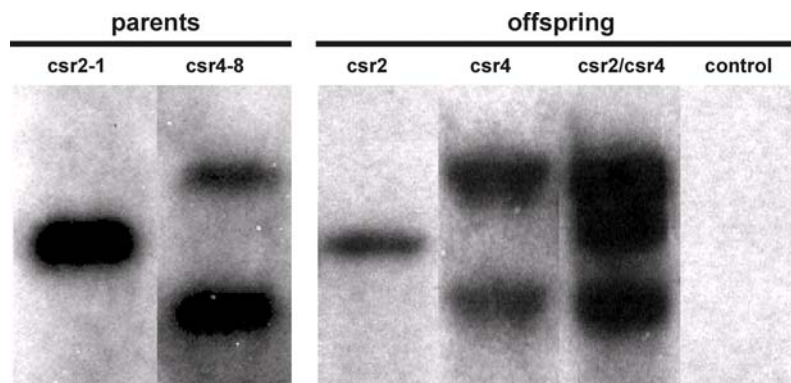
Figure 1. Southern blots showing transgene integration in the parents and the observed segregation pattern in the offspring. Double insertions of the csr2 construct in the csr2-1 parent and triple insertions of the csr4 construct in the csr4-8 parent.

This analysis revealed an expected co-segregation of both the single copy and the tandem repeat of csr4 transgenes, which implies that they were integrated in the same locus, thus leading to an expected segregation ratio of 1:1:1:1 of the four classes (χ^2_3 = 3.3; P<< 0.05). The observed segregation pattern of a total of 84 plants analysed was: twenty five plants contained the csr4 construct, twenty four the csr2 construct, fifteen both the csr2 and csr4 constructs, and 18 plants contained none of the constructs. Although, the number of plants containing the double constructs or no construct was lower than that of plants containing either of the two transgenes, statistically it is well within the expected segregation ratio.

There were differences in tuber production whereas there was no visible difference in plant morphology, phyllotaxis, growth and development of the offspring. It was observed that 100% and 90% of the control and csr4 plants produced tubers respectively, whereas for the csr2 and csr2/csr4 plants, only 70% of them produced tubers.
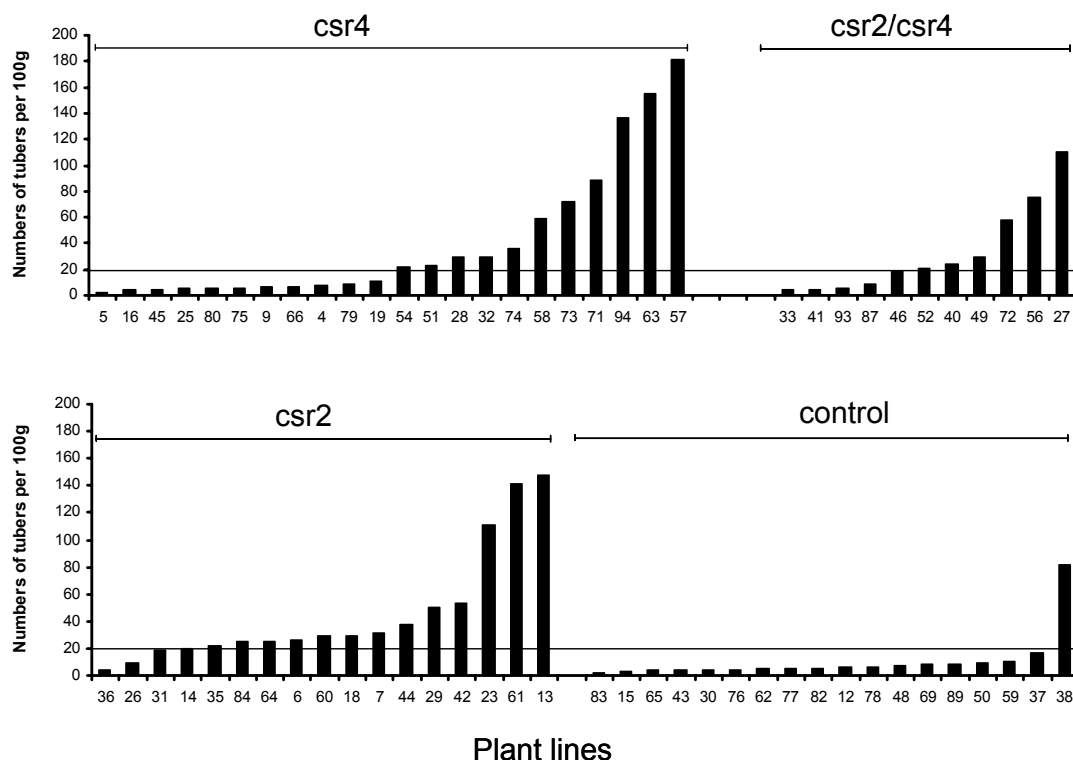

Plant lines

Figure 2. Number of tubers of offspring plants corresponding to normalized 100g weight. Average weight of tuber for a particular F1 plant is deduced by dividing the normalized 100g weight by the corresponding number of tubers.

It was also clear that offspring plants containing either one or both antisense transgenes produced more tubers than the control plants, but individual tubers were mostly smaller and had lesser weight than the control tubers. By deduction from Figure 2, 88% of the csr2 plants and 64% of the csr2/csr4 plants produced tubers with an average weight of equal or less than 5 g, whereas 50% of the csr4 plants and 6% of the control plants produced tubers with an average weight of equal or less than 5 g. Our observations showed that the antisense phenotypes leading to small sized tubers in the offspring were most pronounced in the csr2 tubers.

Relative expression of four potato CesA genes in the potato plant
Relative expression of four potato CesA mRNAs in various tissues of the potato plant was examined using quantitative SYBR-Green Reverse Transcriptase-mediated (RT) PCR. The relative abundance of CesA2 and CesA3 mRNAs was high in most tissues used for the expression analysis, whereas the relative abundance of CesA1 and CesA4 mRNAs was intermediate and low, respectively, in most tissues (Fig. 3A). Of particular interest to us was the relative abundance of CesA2 and CesA4 mRNA levels in the potato tuber. We observed that CesA2 mRNA level was relatively more abundant in the tuber than that of CesA4 mRNA. This probably explains the observed more severe phenotype in the csr2 tuber.

Expression analysis for CesA2 and CesA4 in the tubers of the offspring plants
The relative expression of CesA2 and CesA4 mRNAs in the tubers of the four clones of the offspring plants was examined using quantitative SYBR-Green RT PCR. The analysis confirms our previous expression analysis that the relative abundance of the CesA2 mRNA was higher than that of CesA4, irrespective of the genetic background. Figure 3B shows similar expression of CesA2 in the csr2 and the double csr2/csr4 clones but strangely a higher expression in the csr4 clone. The analysis indicates general up-regulation of CesA genes in the presence of the csr4 construct. This notion was corroborated by similar up-regulation of CesA4, in the csr4 and the double csr2/csr4 clones. It may be that the down-regulation of the CesA4 has triggered up-regulation of other CesA genes. Though this event can compensate for the down-regulated gene, it does not guarantee that the products of the newly triggered CesA genes will assemble into cellulose synthase complex.
An argument can be raised that the level of down-regulation of the CesA2 mRNA was not strong enough to justify the phenotype in the csr2 tubers. This might be explained by the possibility of cross-amplification of other CesA sequences in the potato genome, which may have influenced the result. To verify this possibility, we have used the two sets of primers for csr2 and csr4, in standard PCR, using plasmid DNAs of the four csr sequences as templates. We observed that both sets could still amplify the other three CesAs sequences to a minor extent (data not shown). We could not

have guarded against the problem of cross-amplification better than we did. The primers were designed such that they are as specific to their respective gene sequences as they can possibly be.
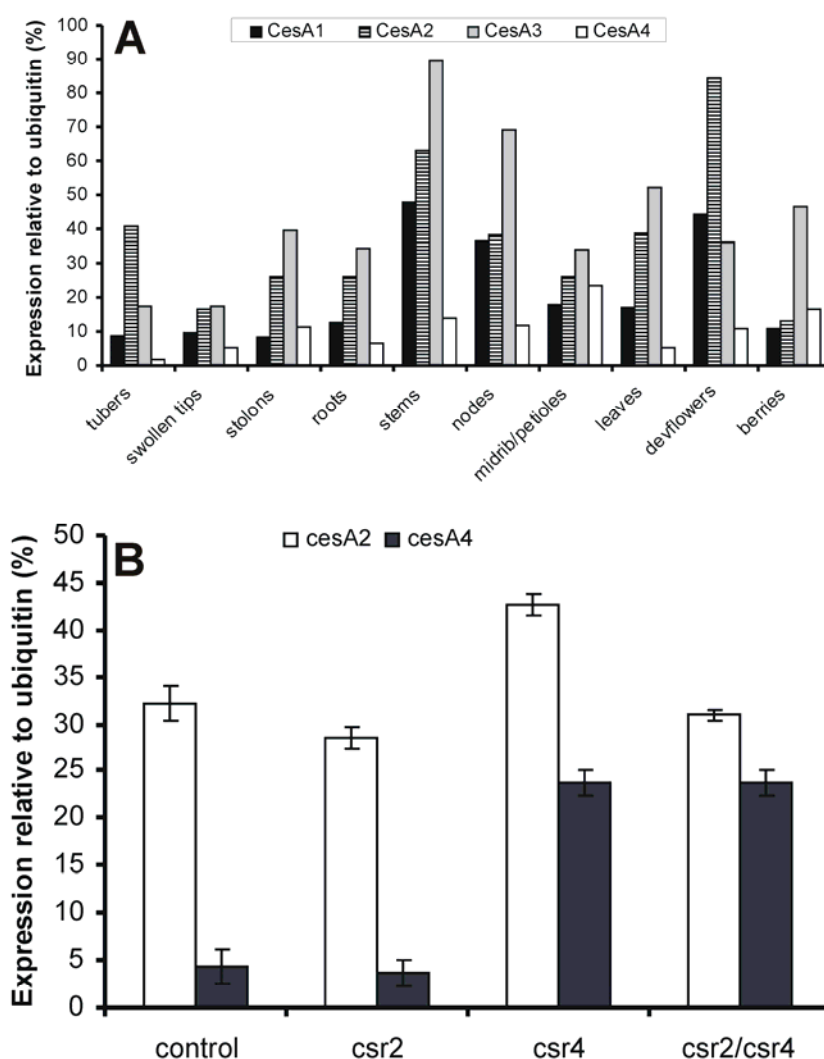

Figure 3. SYBR-Green Real-time RT-PCR analysis of CesA genes. Transcript expressionanalysis of CesA genes in various tissues of the wild type potato plant (A). Transcript expression analysis of CesA2 and CesA4 genes in the offspring (B). RNA levels for each were expressed relative to the amount of ubiquitin RNA and multiplied by 100.

Transformants show reduced cellulose content

Cell wall material (CWM) was isolated from the potato tubers and cellulose content in the cell walls was determined by a colorimetric assay, and expressed as percentage (w/w) of cellulose per total 'crude' CWM (without removal of the starch). Relative to the control, Figure 4 infered a 60% cellulose reduction in the csr2/csr4 double transformant, whereas the single transformants csr2 and csr4 had a 40% and 20% reduction in cellulose content, respectively.
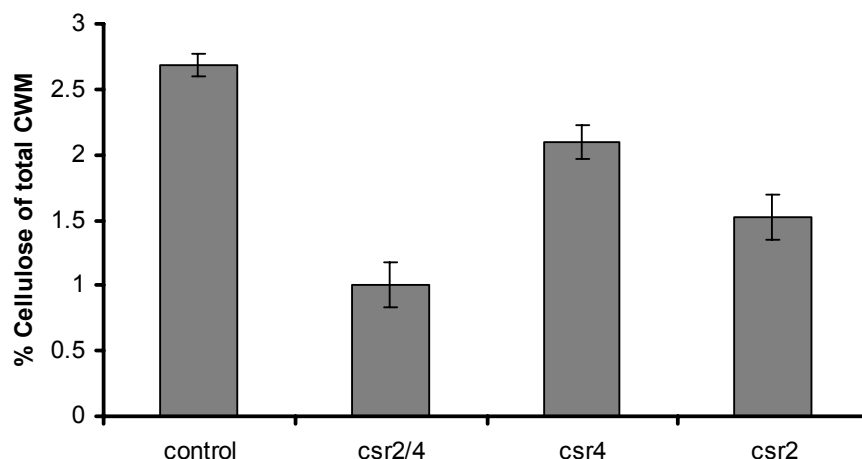
Figure 4. Cellulose content in the offspring tubers. Cellulose content of tuber material was determined using a colorimetric assay. The cellulose levels are represented as the percentage of cellulose in total cell wall material (including starch). All measurements were performed as quadruplicate and standard deviations are represented by the bars.

Lignin and Starch staining

For fast screening for changes in cellular morphology and physiology, we stained tuber slices for lignin. The idea of this fast screening step was informed by our prior observation of a prominent vascular ring on the transverse surface of csr2 tuber slices. After staining with phloroglucinol and HCl, we found that only csr2 tuber slices stained intense red for lignin as shown in Figure 5 (upper panel). This is indicative of considerable lignification of the tuber cell walls. Similarly, for starch staining with iodine-potassium iodide, there appeared to be differential starch staining patterns between the csr2 tuber slices and others (Fig. 5, lower panel). The csr2 tuber slices stained less intensely for starch than others, which is suggestive of a lower starch content.

Ectopic proliferation of xylem cells and lignification in csr2 tubers

In order to observe cellular morphology, 4μm transverse sections of technovit-embedded tubers were stained with toluidine blue and examined under a light microscope. Figures 6A and 7C show a large region of xylem cells that formed a well defined ring in the csr2 tuber section. For the double csr2/csr4 transformant, the single csr4 transformant, and the control tubers, lesser numbers of xylem cells, with no distinct vascular ring, were spotted in their sections (Fig. 6B-6D). Additionally, there is an indication that the csr2 tubers exhibit a smaller cell size than the other tubers (Fig. 6). This reduction in cell size may have impacted the overall size of the tubers, thus leading to the production of smaller tubers. Several cellulose-deficient mutants of *Arabidopsis* have been shown to exhibit a reduced cellulose content leading to a reduced cell size (Arioli et al., 1998). We next verified the ectopic proliferation and lignification of xylem cells of the csr2 tubers by staining fresh tuber sections with phloroglucinol-HCl. Figure 7A revealed an ectopic proliferation of xylem cells and a consequent lignification in csr2 tubers as compared to the control tuber. A closer look
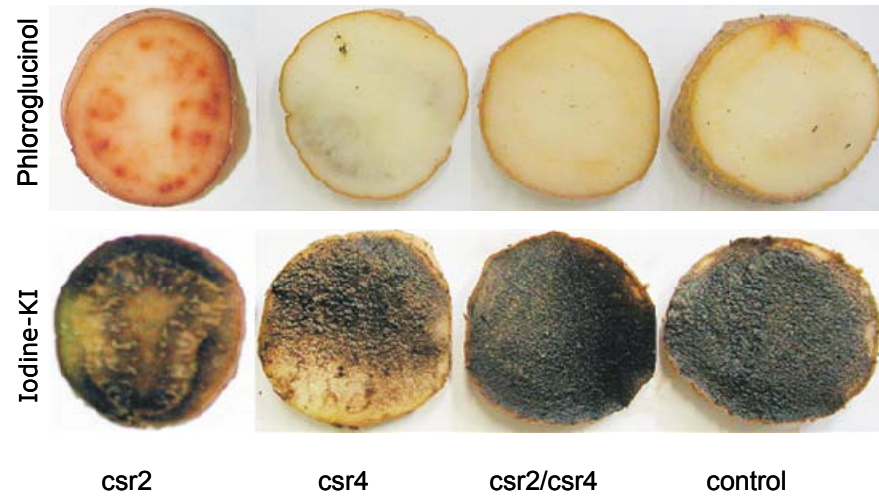
Figure 5. Histochemical staining for lignin and starch. Tuber slices in the upper panel were stained with phloroglucinol, while those in the lower panel were stained with 0.01% iodine-potassium iodide.
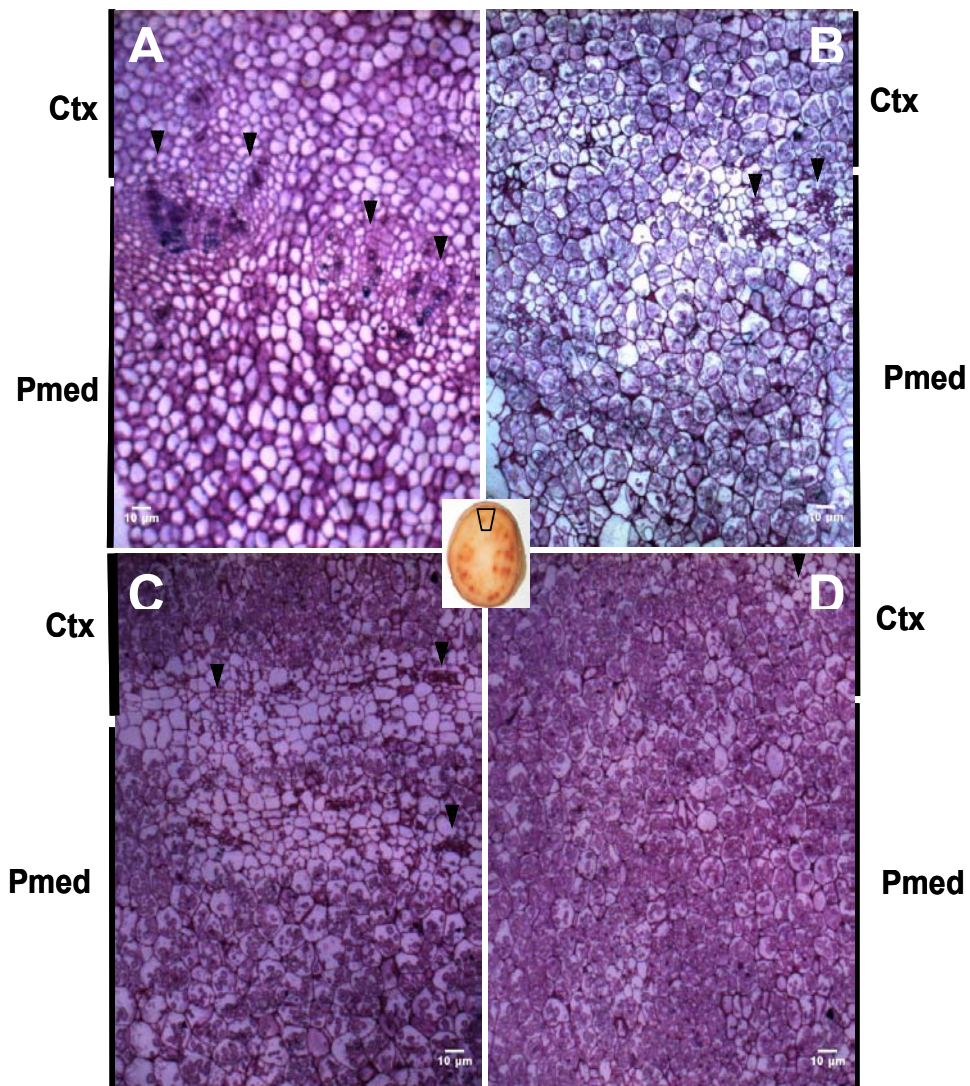

Figure 6. Toluidine staining of technovit-embedded tuber sections. Bright field micrographs of 5 μm transverse sections of cortex and perimedullary tissues of (A) csr2, (B) csr4, (C) csr2/csr4 and (D) the control tubers. Ct, cortex; Pmed, perimedullary. Arrow head indicates proliferation of xylem cells.

at the sections revealed milder staining of the ectopic xylem cells as compared to the control, which is indicative of recent differentiation and development. It was also remarkable to observe that the ectopic xylem cells of csr2 tubers were laid down perpendicular to the stolon axis (Fig. 7C) whereas those of the control tubers ran parallel to the stolon axis (Fig. 7D).
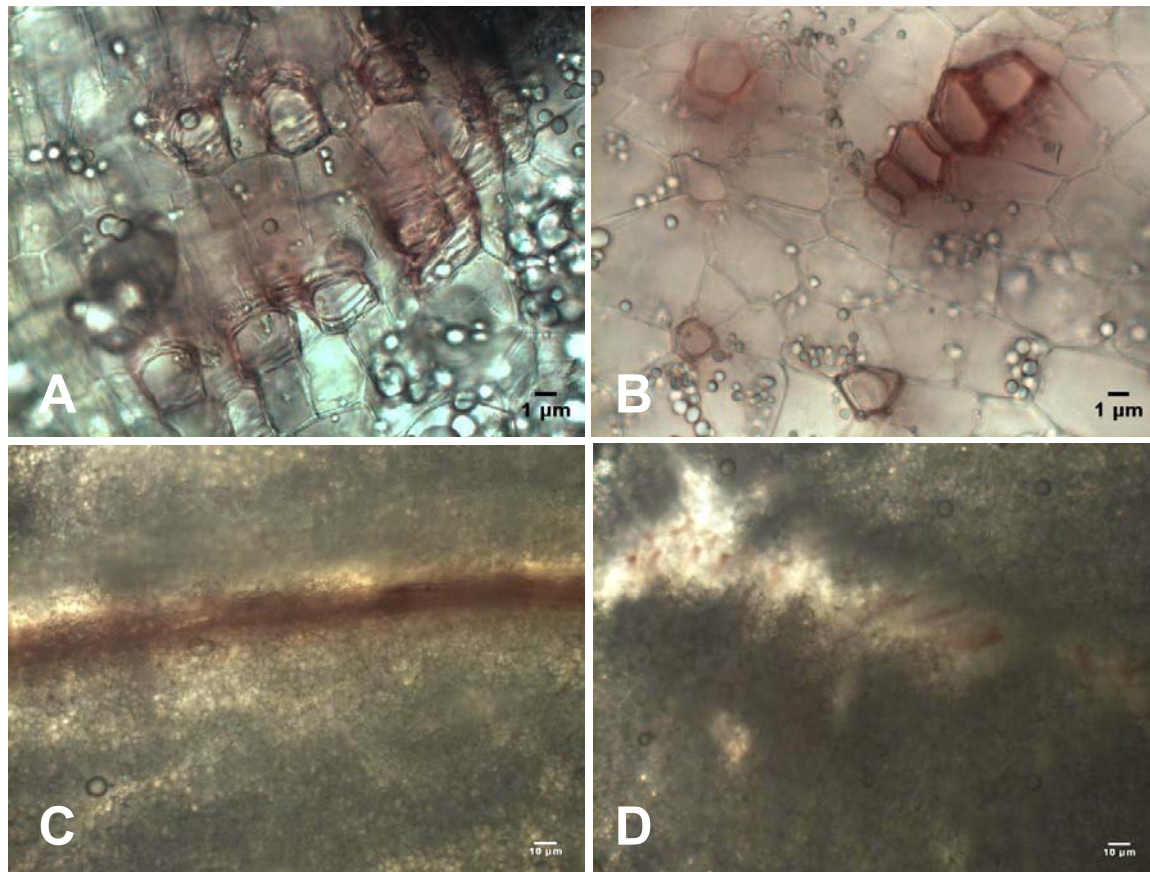

Figure 7. Phloroglucinol staining of fresh tuber sections. The upper panel shows bright field micrographs of hand-cut thin sections of the vascular regions of csr2 tubers (A) and of the control (B). The lower panel shows bright field micrographs of hand-cut thick sections showing the vascular rings of csr2 tubers (C) and the control (D).

Preferential deposition of polymers in cell wall corners

Fluorescence microscopy with calcofluor white staining revealed an unusually strong fluorescence in cell wall corners of the csr2 tubers (Fig. 8A). This irregular deposition of wall materials was observed in all tissues except for the epidermis. Additionally, we observed that the fluorescence in the cell walls of csr2 tubers is much less prominent and mostly uneven as compared to the control, the csr4 and the double csr2/csr4 transformants. We however needed to verify that the observed fluorescence in cell corners was actually from cellulose deposit since calcofluor white stains both cellulose and other β-glucan chain polysaccharides, such as callose and xyloglucan (Wood, 1980). In order to discriminate between cellulose and callose deposit, a step-

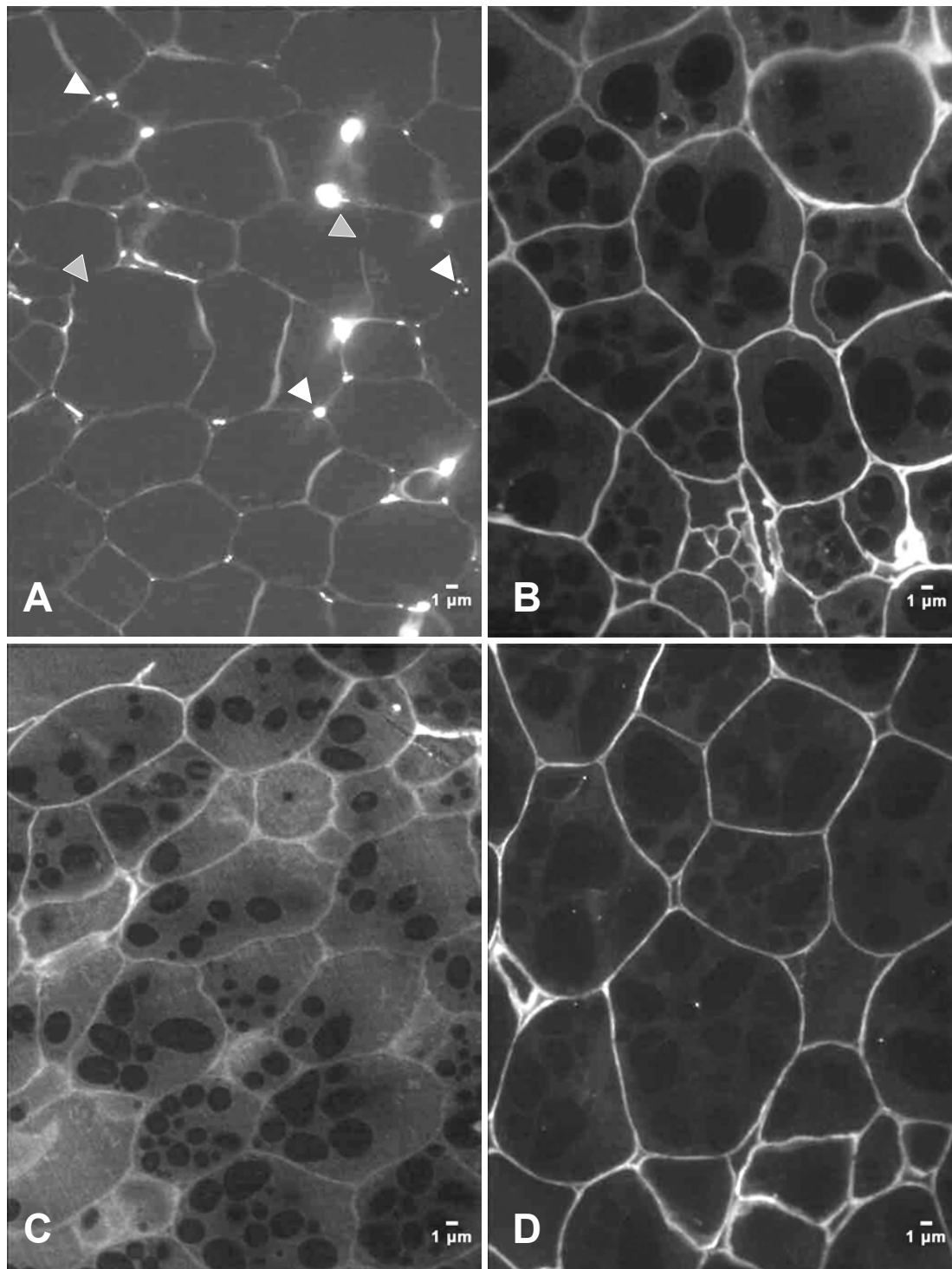wise staining, with aniline blue and calcofluor white was made, which indicated that

Figure 8. Calcofluor white staining of technovit-embedded tuber sections. Fluorescence micrographs of perimedullary tissues of (A) csr2, (B) csr4, (C) csr2/csr4 and (D) the control tubers. White arrow head indicates cell corners fluorescence. Grey arrow head indicates uneven fluorescence around the cell.

The deposit was not callose (Fig. 9). This confirmation was based on the observation that aniline blue, which stains callose specifically, did not give fluorescence in the cell

wall corners. Selective degradation of xyloglucan by a xyloglucan-specific endoglucanase (xyloglucanase) (Pauly et al., 1999) was used for discriminating between cellulose and xyloglucan. After the enzyme treatment, light micrographs of stained sections were similar to Figures 8A and 9B, which indicates that either the deposit is not xyloglucan, or that the enzyme treatment was not effective. Appropriate precautions were taken to ensure that embedding and de-waxing would not influence enzyme activity; similar procedures were used by others for antibody labelling of sections (Gubler, 1989; Baskin et al., 1992). We verified that the xyloglucanase was
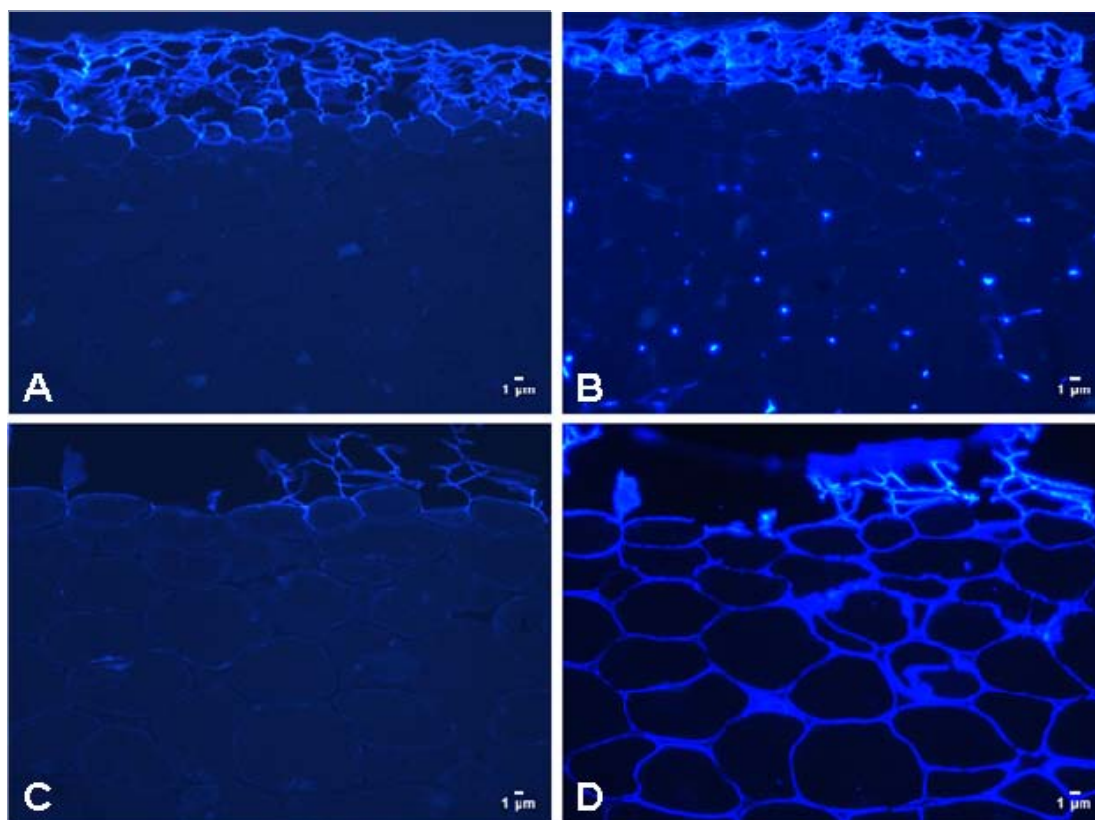

Figure 9. Stepwise staining with aniline blue and calcofluor white to confirm that wall corner deposit is not callose. Micrograph (A) is csr2 embedded section stained with aniline blue. Micrograph (B) is csr2 embedded section stained with aniline blue and calcofluor. Micrographs (C) and (D) are control embedded sections stained with aniline blue, and aniline blue and calcofluor, respectively.

active by incubating a potato xyloglucan with the enzyme. The release of the xyloglucan oligosaccharides was shown with high-pH anion-exchange chromatography (HPAEC) and matrix-assisted Laser desorption/ionisation time-of-flight mass spectrometry (MALDI TOF MS) (data not shown), indicating that the enzyme was active. However, we have been unable to detect xyloglucan oligosaccharides released from the sections into the incubation buffer upon xyloglucanase treatment, even after 100x concentration of the incubation buffer by SepPak C_{18} solid-phase extraction. For incubation of the section, a higher xyloglucanase-to-substrate ratio was used than for the potato xyloglucan, which indicates that the amount of the enzyme added to the section should have been

sufficient. It is possible that the amount of oligosaccharides released from the section is simply too low to be detected. It is also possible that the xyloglucanase could not access its substrate, because pectin might hinder the access of the xyloglucanase to xyloglucan. A pectinase was not included in the incubation mixture. Thus, our experiments hint that the corner deposit is cellulose, but the possibility of xyloglucan can not be excluded.

Discussion

We have crossed two cellulose-deficient transgenic potato lines csr2-1 and csr4-8, containing transgenes csr2 and csr4, respectively, with the aim of achieving double transformants with combined antisense effects of two transgenes on cellulose synthesis and deposition. Our observations suggest an additive effect of the presence of the two transgenes in the double transformant only with respect to cellulose content. Three major observations made on tubers obtained from the four classes of the offspring plants, with respect to cellulose content, deposition and the consequent cellular events, are discussed.

Cellulose reduction was observed in transformed tuber cell walls
Our results showed the expected reduction in the cellulose content of the offspring containing the transgenes as compared to the control, with the double csr2/csr4 transformant exhibiting an additive reduction of 60% in cellulose content. This is in agreement with the report of Lane et al. (2001) that showed additional reduction in cellulose production in the double mutant rsw2-1 rsw1 of the *Arabidopsis* relative to both single mutants rsw2-1 and rsw1. Our observations of a lower cellulose content (40%) in csr2 offspring as compared to 20% in the csr4 offspring is in agreement with our results on transcript expression analysis of the different CesAs in various tissues of the wild type potato plant as well as the analysis of CesA2 and CesA4 in the four offspring clones. The expression studies showed that the CesA2 mRNA level, which was relatively more abundant in the potato tuber than that of the CesA4, was down-regulated in the csr2 background, whereas there was no indication of CesA4 down-regulation in the csr4 background. Furthermore, the 40% reduction in cellulose in the csr2 offspring tubers confirms previous observation made by Oomen et al. (2004) in the single csr2-1 parent. However, our result of 20% reduction in the csr4 tubers contradicts the 60% reduction in the single csr4-8 (parent) transformants reported by the authors. The authors indeed remarked that the cellulose content of the csr4 transformant series was not correlated to the Principal Component Analysis cluster plot, a discrepancy, which might explain our conflicting results. We had expected that the double transformants would display the most severe phenotypes owing to their lowest level of cellulose content. The question as to why the double csr2/csr4 transformant tubers are not exhibiting similar features as the single csr2 transformant might be explained with the reasoning that the presence of the two antisense constructs in one background may have hindered complex assembly. Even though, this implies that less cellulose microfibrils are produced in the double transformants,

the ones that are produced might be normal, and as such there may be no impact on morphology. One is left to speculate therefore that factors other than reduction in crystalline cellulose content might be important in bringing about structural and morphological changes. The cell wall defects in the *Arabidopsis* primary cell wall mutant rsw1 was caused by the reduction in crystalline cellulose with concomitant accumulation of non-crystalline cellulose (Arioli et al., 1998). It is conceivable that absolute reduction in cellulose content alone may trigger compensatory mechanisms, leading to forced synthesis of other wall polysaccharides, such as pectin (Burton et al., 2000; Fagard et al., 2000; His et al., 2001; Alonso-Simon et al., 2004), to maintain the integrity of the cell wall. This might explain the seemingly normal phenotypes of the double transformant tubers, when compared to the csr2 single transformant tubers. In contrast, where reduction in crystalline cellulose is associated with accumulation of non-crystalline cellulose, there may not be a need for supplementary synthesis of other polysaccharides. This may compromise the structural integrity of the cell wall leading to severe phenotypes. It is suggested that this may have been the case for the csr2 single transformant tubers. Moreover, there are reports on tobacco lignin modification that suggest that individual single transformants tend to show more severe phenotypes and specific features than the double transformants (Chabannes et al., 2001; Pincon et al., 2001)

Deposition of wall polymers in cell wall corners
Fluorescence microscopy with calcofluor white stain revealed an unusually strong fluorescence in cell wall corners of the csr2 potato tuber. This is indicative of preferential deposition of cellulosic polymers, although we cannot exclude the possibility that it might be xyloglucan. As a result of antisense suppression of CesA2, a principal CesA protein in the tuber, not enough of it is available for interaction with the other protein partners in the cellulose synthase complex. Based on the evidence of homodimerisation of CesA proteins (Kurek *et al.,* 2002), we expect that the other two CesA proteins will interact but will assemble a defective complex of 24 subunits. The implication of this would thus be a mixture of normal and defective complexes in the csr2 tubers since we did not observe a complete knockout of the CesA2 gene (Fig. 3B). One consequence of the defective complex could be the synthesis of cellulose microfibrils having less glucan chains, being 24 instead of 36, which would presumably be weaker and more flexible. Based on the theory of ordering of cellulose microfibrils (Emons, 1994), we propose an hypothesis for cell wall deposition of cellulose in cell corners as schematically presented in Figure 10. It is conceivable that the abnormal cellulose deposition in cell wall corners results from the inability of the weak and flexible microfibrils to steer complexes around the cell wall corners, as a result of which cellulose is not being spun round the cell wall but is deposited mainly in the corners. The same explanation can be given for the uneven fluorescence around the walls of the csr2 tubers. Since the density of the active intact complex is presumably low, the ordering theory also implies that microfibrils will be deposited in a less ordered fashion in several different directions. The mixture of stiff but disordered microfibrils from intact complexes and flexible microfibrils from defective

complexes may have been responsible for the unevenness. This is especially possible if the defective microfibrils have considerable amorphous regions, which, are more susceptible to the action of cellulolytic enzymes, such as endo-1,4-β-glucanases (Rabinovich et al., 2002). Preferential cellulose deposition in cell wall corners was also observed in cellulose-deficient *Arabidopsis* primary cell wall mutants *rsw1* (AtCesA1), *eli1* (AtCesA3) and *kor1* (Nicol et al., 1998; Cano-Delgado et al., 2000), but no explanation was given by these groups for this phenomenon. It is intriguing that the cellulose reduction in the double csr2/csr4 transformant tuber cell walls does not show the phenotypes displayed by the csr2 cell walls. The observed even deposition of cellulose in the double csr2/csr4 transformant tuber cell walls might be
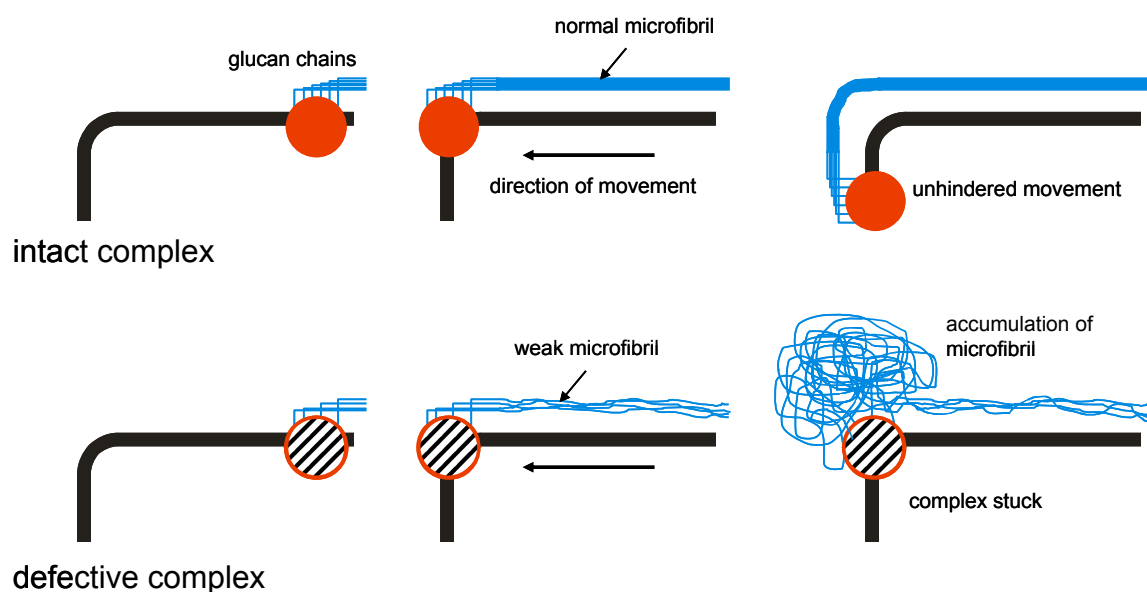

Figure 10. A model for cellulose deposition in the cell wall corners. The model is based on the geometrical model for cellulose deposition (Emons, 1994). It presents two scenarios of an intact complex and a defective complex. The intact complex produces strong and stiff microfibril, which is needed for propelling the complexes forward. Thus when the complex comes against a corner, it is steered pass the corner by the propelling force of microfibril deposition and crystallisation. On the contrary, the defective complex, which presumably is an assemblage of homodimers of two CesA proteins, produces weak and flexible microfibrils of 24 chains. Thus not enough propelling force is generated to steer the complex pass the corners. So the complex gets stuck and keeps on producing at the spot.

explained by the biosynthetic activity of remnant intact complexes. These complexes would produce normal but less cellulose microfibrils, which are evenly deposited round the cell wall.

We cannot rule out the possibility that the cellulose synthase complexes in the double transformant assemble into a different geometry involving only one isoform, which can produce a sort of crystalline cellulose. Alternatively, if the deposit is xyloglucan, it might be explained as follows. Due to a reduced amount of cellulose, xyloglucan cannot be kept in the wall, and diffuses to the cell corners. It has been observed before

(Oomen et al., 2002), that galactan can move to the cell corner of potato tissue when it is released from rhamnogalacturonan by rhamnogalacturonan lyase. We are thus left with open arguments on the most appropriate explanation for our observation on the double csr2/csr4 transformant tuber cell walls.

Ectopic xylem differentiation and lignification

Phloroglucinol staining revealed extensive xylem proliferation coupled to considerable lignification in the csr2 tubers. It is possible that the ectopic xylem differentiation may have resulted from inhibition of cell expansion (Lee and Roberts, 2004), which in turn, may be the consequence of altered cellulose deposition (Cano-Delgado *et al.*, 2000). The products of such forced differentiation have always been disrupted xylem strands and many tracheids as also observed in the csr2 tubers (data not shown) in addition to the abnormal perpendicular orientation of the xylem strands to the stolon axis of the tuber. The milder phloroglucinol staining intensity that was observed for the csr2 fresh tuber slices as compared to the control indicates that the xylem differentiation in the csr2 was recent and as such the xylem cells were not as well developed as in the control tuber. To a varying extent, similar observations of ectopic xylem formation and ectopic lignification have been reported for several cellulose-deficient *Arabidopsis* mutants. Eli1 (ectopic lignification), a mutant of AtCesA3 (Cano Delgado et al., 2003), exhibited ectopic production of heavily lignified xylem cells (Cano-Delgado *et al.*, 2000). Ectopic xylem differentiation and lignification were also observed in *apl* (altered phloem development) and *mux* (multiple xylem) (Bonke et al., 2003). Apart from the xylem cells, ectopic lignification was also reported in cells that are not normally lignified as in *rsw1* mutants of AtCesA1 (Arioli et al., 1998), kor1 (Nicol *et al.* 1998), a mutant of KORRIGAN (a membrane-bound endo-β-1,4-glucanase) that has been implicated in cellulose biosynthesis and deposition (Molhoj et al., 2002) and *lit* (Lion's tail) mutants (Cano-Delgado *et al.*, 2000).

Our results attest to the fact that impairment to a primary cell wall complex could have significant influence on both cellulose synthesis and deposition as well as on the development of secondary-walled tissues. We reckon that these results may help to understand the dynamics of cellulose deposition better.

Acknowledgements

References

Alonso-Simon A, Encina AE, Garcia-Angulo P, Alvarez JM, Acebes JL (2004) FTIR spectroscopy monitoring of cell wall modifications during the habituation of bean (Phaseolus vulgaris L.) callus cultures to dichlobenil. Plant Science **167:** 1273-1281

Arioli T, Peng LC, Betzner AS, Burn J, Wittke W, Herth W, Camilleri C, Hofte H, Plazinski J, Birch R, Cork A, Glover J, Redmond J, Williamson RE (1998) Molecular analysis of cellulose biosynthesis in *Arabidopsis*. Science **279:** 717-720

Baskin TI, Busby CH, Fowke LC, Sammut M, Gubler F (1992) Improvements in immunostaining samples embedded in methacrylate: Localization of microtubules and other antigens throughout developing organs of diverse taxa. Planta **187:** 405-413

Bonke M, Thitamadee S, Mahonen AP, Hauser MT, Helariutta Y (2003) APL regulates vascular tissue identity in Arabidopsis. Nature **426:** 181-186

Brown RM, Saxena IM (2000) Cellulose biosynthesis: A model for understanding the assembly of biopolymers. Plant Physiology And Biochemistry **38:** 57-67

Burton RA, Gibeaut DM, Bacic A, Findlay K, Roberts K, Hamilton A, Baulcombe DC, Fincher GB (2000) Virus-induced silencing of a plant cellulose synthase gene. Plant Cell **12:** 691-705

Cano-Delgado AI, Metzlaff K, Bevan MW (2000) The eli1 mutation reveals a link between cell expansion and secondary cell wall formation in Arabidopsis thaliana. Development **127:** 3395-3405

Cano Delgado A, Penfield S, Smith C, Catley M, Bevan M (2003) Reduced cellulose synthesis invokes lignification and defense responses in Arabidopsis thaliana. Plant Journal. [print] May 2003 2003; **34:** 351-362.

Chabannes M, Barakate A, Lapierre C, Marita JM, Ralph J, Pean M, Danoun S, Halpin C, Grima-Pettenati J, Boudet AM (2001) Strong decrease in lignin content without significant alteration of plant development is induced by simultaneous down-regulation of cinnamoyl CoA reductase (CCR) and cinnamyl alcohol dehydrogenase (CAD) in tobacco plants. Plant Journal **28:** 257-270

Church GM, Gilbert W (1984) Genomic sequencing. Proceedings of the National Academy of Sciences of the United States of America **81:** 1991-1995

Delmer DP, Amor Y (1995) Cellulose Biosynthesis. Plant Cell **7:** 987-1000

Desprez T, Vernhettes S, Fagard M, Refregier G, Desnos T, Aletti E, Py N, Pelletier S, Hofte H (2002) Resistance against herbicide isoxaben and cellulose deficiency caused by distinct mutations in same cellulose synthase isoform CESA6. Plant Physiology **128:** 482-490.

Emons AMC, Mulder BM (1998) The making of the architecture of the plant cell wall: How cells exploit geometry. Proceedings of The National Academy of Sciences of The United States of America **95:** 7215-7219

Fagard M, Desnos T, Desprez T, Goubet F, Refregier G, Mouille G, McCann M, Rayon C, Vernhettes S, Hofte H (2000) PROCUSTE1 encodes a cellulose synthase required for normal cell elongation specifically in roots and dark-grown hypocotyls of *Arabidopsis*. Plant Cell **12:** 2409-2423

Gardiner JC, Taylor NG, Turner SR (2003) Control of cellulose synthase complex localization in developing xylem. Plant Cell **15:** 1740-1748

Gubler F (1989) Immunofluoresence localization of microtubules in plant root tips embedded in Butyl-methyl methacrylate. Cell Biology International reports **13:** 137-145

His I, Driouich A, Nicol F, Jauneau A, Hofte H (2001) Altered pectin composition in primary cell walls of korrigan, a dwarf mutant of Arabidopsis deficient in a membrane-bound endo-1,4-beta-glucanase. Planta **212:** 348-358

Kuipers AGJ, Jacobsen E, Visser RGF (1994) Formation and deposition of amylose in the potato-tuber starch granule are affected by the reduction of granule-bound starch synthase gene-expression. Plant Cell **6:** 43-52

Kurek I, Kawagoe Y, Jacob-Wilk D, Doblin M, Delmer D (2002) Dimerization of cotton fibre cellulose synthase catalytic subunits occurs via oxidation of the zinc-binding domains. Proceedings of The National Academy of Sciences of The United States of America **99:** 11109-11114

Kuroiwa T, Kuroiwa H, Mita T, Fujie M (1990) Fluorescence Microscopic Study of the Formation of Giant Mitochondrial Nuclei in the Young Ovules of Pelargonium-Zonale. Protoplasma **158:** 191-194

Lane DR, Wiedemeier A, Peng LC, Hofte H, Vernhettes S, Desprez T, Hocart CH, Birch RJ, Baskin TI, Burn JE, Arioli T, Betzner AS, Williamson RE (2001) Temperature-sensitive alleles of RSW2 link the KORRIGAN endo-1,4-beta-glucanase to cellulose synthesis and cytokinesis in arabidopsis. Plant Physiology **126:** 278-288

Lee S, Roberts AW (2004) Tracheary element differentiation is correlated with inhibition of cell expansion in xylogenic mesophyll suspension cultures. Plant Physiology and Biochemistry **42:** 43-48

Molhoj M, Pagant S, Hofte H (2002) Towards understanding the role of membrane-bound endo-beta-1,4-glucanases in cellulose biosynthesis. Plant and Cell Physiology. **43:** 1399-1406

Mulder BM, Emons AMC (2001) A dynamical model for plant cell wall architecture formation. Journal of Mathematical Biology **42:** 261-289

Nicol F, His I, Jauneau A, Vernhettes S, Canut H, Hofte H (1998) A plasma membrane-bound putative endo-1,4-beta-D-glucanase is required for normal wall assembly and cell elongation in *Arabidopsis*. Embo Journal **17:** 5563-5576

Oomen R, Doeswijk-Voragen CHL, Bush MS, Vincken JP, Borkhardt B, van den Broek LAM, Corsar J, Ulvskov P, Voragen AGJ, McCann MC, Visser RGF (2002) In muro fragmentation of the rhamnogalacturonan I backbone in potato (Solanum tuberosum L.) results in a reduction and altered location of the galactan and arabinan side-chains and abnormal periderm development. Plant Journal **30:** 403-413

Oomen R, Tzitzikas EN, Bakx EJ, Straatman-Engelen I, Bush MS, McCann MC, Schols HA, Visser RGF, Vincken JP (2004) Modulation of the cellulose content of tuber cell walls by antisense expression of different potato (Solanum tuberosum L.) CesA clones. Phytochemistry **65:** 535-546

Pauly M, Andersen LN, Kauppinen S, Kofod LV, York WS, Albersheim P, Darvill A (1999) A xyloglucan-specific endo-beta-1,4-glucanase from Aspergillus aculeatus: expression cloning in yeast, purification and characterization of the recombinant enzyme. Glycobiology **9:** 93-100

Pincon G, Chabannes M, Lapierre C, Pollet B, Ruel K, Joseleau JP, Boudet AM, Legrand M (2001) Simultaneous down-regulation of caffeic/5-hydroxy ferulic acid-O-methyltransferase I and cinnamoyl-coenzyme a reductase in the progeny from a cross between tobacco lines homozygous for each transgene. Consequences for plant development and lignin synthesis. Plant Physiology **126:** 145-155

Rabinovich ML, Melnick MS, Bolobova AV (2002) The structure and mechanism of action of cellulolytic enzymes. Biochemistry-Moscow **67:** 850-871

Robert S, Mouille G, Hofte H (2004) The mechanism and regulation of cellulose synthesis in primary walls: lessons from cellulose-deficient Arabidopsis mutants. Cellulose **11:** 351-364

Scheible WR, Eshed R, Richmond T, Delmer D, Somerville C (2001) Modifications of cellulose synthase confer resistance to isoxaben and thiazolidinone herbicides in *Arabidopsis* Ixr1 mutants. Proceedings of the National Academy of Sciences of the United States of America **98:** 10079-10084

Tanaka K, Murata K, Yamazaki M, Onosato K, Miyao A, Hirochika H (2003) Three distinct rice cellulose synthase catalytic subunit genes required for cellulose synthesis in the secondary wall. Plant Physiology **133:** 73-83

Taylor NG, Howells RM, Huttly AK, Vickers K, Turner SR (2003) Interactions among three distinct CesA proteins essential for cellulose synthesis. Proceedings of the National Academy of Sciences of the United States of America **100:** 1450-1455

Updegraff DM (1969) Semi-microdetermination of cellulose in biological materials. Analytical Biochemistry **32:** 420-424

Wood PJ (1980) Specificity in the interaction of direct dyes with polysaccharides. Carbohydrate Research **85:** 271-287

Promiscuous, non-catalytic, tandem carbohydrate-binding modules alter development of tobacco plants

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Abstract

Plant cell expansion and consequently plant growth are largely controlled by the extent of loosening of the cell walls, which might be catalyzed by non-hydrolytic proteins. We hypothesized that the growth restraining cellulose-hemicellulose network of the cell wall can be influenced by proteins that exhibit promiscuous polysaccharide recognition, such as the promiscuous carbohydrate-binding modules (CBMs) of family 29. CBM29 modules are derived from a non-catalytic protein1, NCP1 of the *Piromyces equi* cellulase/hemicellulase complex and are promiscuous to cellulose, mannan-based polysaccharides and, to a lesser extent, to xylan and xyloglucan. To investigate this hypothesis, we used heterologous expression of two *Piromyces* CBM29 modules (a tandem CBM29-1-2 and its single derivative CBM29-2) in tobacco cell walls. Transgenic plants expressing the tandem CBM29-1-2 displayed reduced stem elongation and prolonged juvenility resulting in delayed flower development. Histological examinations revealed layers of collapsed cortical cells of the tandem CBM29-1-2-expressing stems as the cellular basis for the reduced stem elongation. Cryo-scanning electron microscopic examinations unravelled the underlying cause of the collapsed cells of the cortex as altered cell expansion in the xylem and the cortex, which in turn might be a consequence of loosened cell walls. Transgenic plants neither exhibit altered cellulose content nor do the seeds exhibit altered germination rate. Nevertheless, the results support the hypothesis that promiscuous CBMs can modulate wall loosening, cell enlargement and plant growth.

Introduction

Plant cell expansion depends on interplay between internal driving forces and the controlled yielding of the cell wall. The ability of the wall to extend under this internal pressure is largely determined by wall loosening processes that modify the interaction of cellulose chains with one another, and/or with hemicelluloses (Darley et al., 2001; Brett, 1996). These processes engage the activities of wall-loosening agents, such as expansins that enable a kind of turgor-driven polymer creep that results in stress relaxation and expansion of the wall (Cosgrove, 2003b). Likewise, organ growth is brought about by differential rates of wall loosening, which causes differential tissue growth and consequently tissue tension within the organ (Peters and Tomos, 1996; Peters and Tomos, 2000). Thus the whole plant development consists of progressive modification of the cellulose-hemicellulose (mostly cellulose-xyloglucan) network. There are an increasing number of reports on the interactions between mannan-based polysaccharides and cellulose that influence the structural properties of the cell wall (Hackney et al., 1994; Whitney et al., 1998; Whitney et al., 2000; Carpita et al., 2001; Hosoo et al., 2002). An immunolocalisation study in Arabidopsis revealed that mannan polysaccharides were present in all thickened cell walls of stem and leaves including those of the xylem parenchyma and epidermis (Handford et al., 2003). Recently, mannan transglycosylase was identified and characterized as a cell wall enzyme activity acting on mannan-based plant polysaccharides in primary cell walls of higher plants (Schroder et al., 2004). This body of evidence thus supports an emerging idea that mannan-based polysaccharides in the cell walls of these cell types may have a role analogous to that of xyloglucans, introducing flexibility and forming a growth restraining network with cellulose. There is evidence that indicates that carbohydrate-binding modules (CBMs) could play a role in modification of these interactions (Shpigel et al., 1998; Levy et al., 2002b). Furthermore, there exists a patent (Shoseyov et al., 2001) that claims that the expression of a cellulose-specific bacterial CBM in plants results in transgenic plants having altered structural morphology. Finally, an altered cell expansion and stem elongation have been demonstrated with transgenic *Arabidopsis* plants expressing a cellulose-binding module from *Aspergillus japonicus* (Quentin, 2003).

CBMs are non-catalytic polysaccharide-recognizing modules, appended to glycoside hydrolases that degrade insoluble polysaccharides, which concentrate the enzymes on to the polysaccharide substrates. CBMs are grouped into families based on amino acid sequence similarities and there are currently 43 families of CBMs in the database (http://afmb.cnrs-mrs.fr/CAZY/). Biochemical characterizations of members from most of the CBM families have been established, including those that target non-cell wall polysaccharides like starch and glycogen; for review see Boraston et al. (2004). Most CBMs are linked to the catalytic modules of carbohydrate active enzymes; however, there exist some non-catalytic carbohydrate binding proteins that are not appended to any catalytic module. Examples of such proteins include putative CBMs of the *Arabidopsis* former X8 family, now CBM43 [GenBank accession no. AL161503] (Henrissat et al., 2001), with olive pollen allergen Ole e 10 (GenBank

accession no. AY082335) representing the first characterized member of this family (Barral et al., 2005). Another example is the non-catalytic protein1, NCP1 (CBM29, GenBank accession no. AY026754), which is a component of the anaerobic fungus *Piromyces equi* cellulase/hemicellulose complex (Freelove et al., 2001).

Due to the heterogeneous nature of the cell wall structures, several CBMs exist that have evolved structures, which enable them to recognize more than one wall polysaccharide (Boraston et al., 2004). This promiscuous recognition of the CBMs enables the associating enzyme (complex) to be more efficient in cell wall degradation. We hypothesize that the interactions between cellulose chains to one another and to the cross-linking glycans might be modified by expressing CBMs that exhibit promiscuous recognition in plants, with a view to modulating cell wall loosening and ultimately the whole plant development. The CBM29 modules present an excellent model for promiscuity in protein-carbohydrate recognition and, as such, could be used to test our hypothesis. The modules bind soluble glucomannan, galactomannan, β-glucan, and hydroxyethylcellulose (HEC), as well as insoluble forms of cellulose and mannan (Freelove et al., 2001; Charnock et al., 2002). For all ligands tested, tandem CBM29-1-2 had an affinity considerably higher than the additive value of the individual CBM29s. This demonstrates that the two CBMs act in synergy to bind all their target ligands with CBM29-2 displaying higher affinity than CBM29-1. Additionally, the tandem CBM29-1-2 was able to interact with xyloglucan and different forms of xylans, whereas the single showed little affinity for these polysaccharides (Freelove et al., 2001; McCartney et al., 2004). An *ex-situ* labeling study has revealed that the tandem CBM29-1-2 modules bind strongly to the cell walls of the maize coleoptile sheath and the enclosed developing leaves (McCartney et al., 2004). This indicates considerable abundance of their interacting ligands in the primary cell walls of grasses. We have expressed two constructs derived from the *Piromyces* CBM29 modules, the tandem CBM29-1-2 and the single derivative CBM29-2 in tobacco cell walls under the control of 35S cauliflower mosaic virus (CaMV) promoter. Our results support the hypothesis that promiscuous carbohydrate binding modules can influence biological processes of cell wall loosening, cell enlargement and plant growth.

Materials and Methods

Preparation of constructs

Two constructs were prepared for expression in tobacco plants, one for single CBM29-2 and one for tandem CBM29-1-2. The constructs were prepared by amplifying their respective gene fragments from pET22b, a recombinant plasmid vector used for cloning and expression in *Escherichia coli* (Freelove et al., 2001). The polymerase chain reaction (PCR) was performed using primers that included BamHI and SmaI recognition sites (5'-cgggatccgttagtgctacttactctgttgtttat-3' and 5'-tccccgggccttttaatttattgggtcaacgaaa-3'; the BamHI and SmaI sites are underlined, respectively). The three bases highlighted in bold type represent the stop codon. The amplified 914 base pairs fragment of the tandem CBM29-1-2 was digested with BamHI and SmaI (Invitrogen, The Netherlands) and cloned into a similarly digested binary vector pGreen 7k (Hellens et al., 2000). Similarly amplification of the single CBM29-2 fragment was performed using primers with the same restriction sites as for the

tandem CBM29-1-2 (5'-cg<u>ggatcc</u>cgtaatgtcagagccacttacactgt-3' and 5'-tcc<u>cccggg</u>cctt**tta**atttattgggtcaacgaaa-3'; the BamHI and SmaI sites are underlined respectively). The amplified 559 base pairs fragment was digested with BamHI and SmaI (Invitrogen, The Netherlands) and cloned into a pGreen7k binary vector. The cloning of the two fragments was in-frame with fusion peptides of two sequences, which were cloned upstream in the binary vector (Fig. 1).
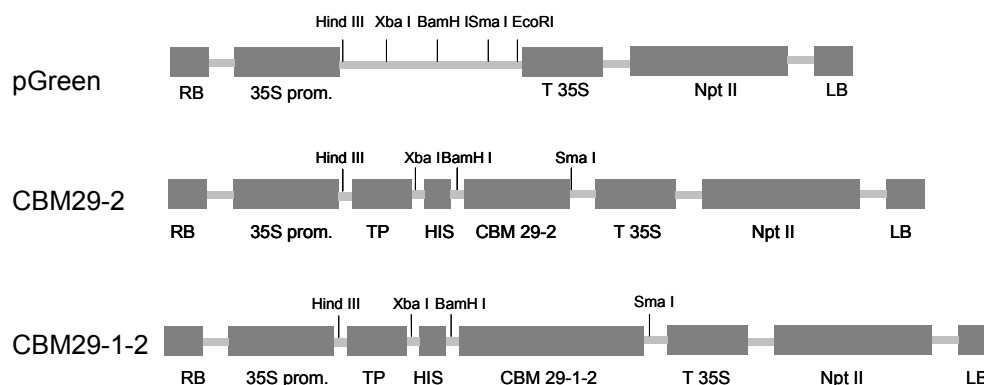

Figure 1. Transgene constructs of the CBM29 modules and the control for tobacco transformation. pGreen, empty vector control with no CBM29 insert but the selectable marker gene nptII; CBM29-2, construct for the single derivative CBM29-2; CBM29-1-2, construct for the tandem CBM29-1-2. Gene expression is driven by 35S CaMV promoter (35S prom.). Flanking squares on each line represent right border (RB) and left border (LB) of the T-DNA in *Agrobacterium* Ti (tumor-inducing) plasmid

The first sequence codes for a tobacco transit peptide for transporting a cellular glycoprotein NTP303 across the plasma membrane into the cell wall (Wittink et al., 2000), while the second sequence encodes a hexa-histidine tag. The sequence of the transit peptide was obtained as a product of annealing two oligonucleotide primers, TP1 (5'-<u>agctt</u>atgggaagtggtaaagtaacatttgtggctttgctactttgcctctccgtaggggtgatagctt-3') and TP2 (5'-<u>ctaga</u>agctatcacccctacggagaggcaaagtagcaaagccacaaatgttactttaccacttcccata-3'). The underlined nucleotides produced overhangs of HindIII and XbaI, respectively. This fragment was then cloned into the HindIII and XbaI cloning sites of the vector. Similarly, the sequence of hexa-histidine tag was obtained and ligated into XbaI and BamHI sites as annealing product of His1 (5'-<u>ctaga</u>agaggatcgcatcaccatcaccatcacg-3') and His2 (5'-<u>gatcc</u>gtgatggtgatggtgatgcgatcctctt-3'), with the underlined producing overhangs of XbaI and BamHI, respectively. The purpose of using the hexa-histidine tag was to facilitate affinity purification of the CBM29 proteins. The control construct did not contain any of the CBMs, the transit peptide and the hexa-histidine epitope tag. All constructs were sequenced to verify their integrity.

Immunofluorescence detection of CBM29-1-2 binding and microscopy

Wild type tobacco plants (*Nicotina tabacum* L.) were grown at 24°C under a regime of 16 h light and 8 h dark for 6-7 weeks. Regions of stem were excised and immediately fixed in PEM buffer (50 mM PIPES (piperazine-N-N'-bis[2-ethane-sulfonic acid])), 5 mM EGTA (ethylene glycol bis(β-aminoethyether)-N,N,N'N'-tetraacetic acid), and 5 mM MgSO$_4$, pH 6.9) containing 4% paraformaldehyde. Samples were then dehydrated in an ethanol series (30%, 50%, 70%, 90%, 97%, each for 30 min at 4°C, ethanol and wax 1:1, 37°C, overnight) and embedded in Steedman's wax. Sections were cut to a thickness of 12 μm and collected on poly-lysine coated microscope slides (BDH Laboratory Supplies, Dorset, UK), de-waxed and re-hydrated through a reverse ethanol series into phosphate-buffered saline (PBS) (97% for 3 x 10 min, 90%, 50% and water for 10 min each, and a final step of water 90 min). For CBM labeling, sections were incubated in milk protein/PBS (to reduce any non-specific binding of proteins) and 5 μg/mL CBM29-1-2 for 1.5 h. Immunofluorescence detection of

CBM29-1-2 binding to sections and microscopy was carried out as described elsewhere (McCartney et al., 2004).

Tobacco transformation and regeneration

In vitro leaf explants of *Nicotiana tabacum* cv. Samsun NN were used for *Agrobacterium tumefaciens*-mediated transformation. Cloned binary vector pGreen 7k was co-transformed with the helper plasmid, pSoup (Hellens et al., 2000), into *Agrobacterium tumefaciens* strain LBA4404 by electroporation. This was plated out on LB-agar plates containing kanamycin (100 μg/mL) and rifampicin (30 μg/mL), and incubated for three days at 28°C, to obtain single colonies. The integrity of the binary plasmids was tested by restriction analysis of plasmids isolated from *A. tumefaciens* cultures used for plant transformation. 20 mL LB medium without selection was inoculated with a single colony and incubated overnight at 28°C. 100 μL of the grown culture was added to a petridish containing 10 mL Murashige-Skoog liquid medium supplemented with 30 g of sucrose (MS30). Leaf explants, without major veins and edges, from young seedlings were transferred upside down in to the MS30 medium. *Agrobacterium* infection was done in the dark for 2 days at 24-25°C. Two controls were used for the procedure, untransformed wild type control and empty pGreen 7k control without an insert. Leaf explants were washed in three changes of liquid washing medium of MS30 with 250 mg/L carbenicillin. The washed explants were transferred upside-up to MS30-phytagel plate containing 0.1 mg/L α-naphtalene acetic acid (NAA), 1 mg/L 6-benzylaminopurine (BAP), 200 mg/L kanamycin and 250 mg/L carbenicillin, and incubated overnight in the dark at 28°C. Plants were transferred to a growth chamber (25°C), where they were gradually adapted to light and incubated for callus induction. After two weeks in culture, calli generated were transferred to shoot-inducing medium, MS20-phytagel, containing 0.2 mg/L NAA, 2 mg/L BAP, 200 mg/L kanamycin and 250 mg/L carbenicillin. Well-formed shoots were harvested and transferred to root-inducing medium, MS15-phytagel plate containing 100 mg/L kanamycin, 250 mg/L carbenicillin and 200 mg/L vancomycin. Transformed plantlets were transferred to the greenhouse to generate mature plants.

RNA gel blot analysis of transgenic plants

Total RNA was isolated from 3-5 g of transformed *in vitro* shoots as described elsewhere (Kuipers et al., 1995). Aliquots of 20 μg/lane were separated on a 1% formaldehyde agarose gel and blotted onto a Hybond-N nylon membrane (Amersham) by vacuum transfer in 0.4 N sodium hydroxide. The membranes were hybridized with probes consisting of 60 ng of $[\alpha^{32}P]dCTP$ labeled BamHI-SmaI fragments of CBM29-1-2 or CBM29-2. The radioactive labeled blots were exposed to X-OMAT S and AR scientific imaging films (Kodak) at -80°C. The blots were re-hybridized, after stripping, with a 489 bp $[\alpha^{32}P]dCTP$ labeled fragment of a tobacco 18S ribosomal RNA gene (GenBank accession no. AJ236016), as a control. The ribosomal probe was amplified from tobacco genomic DNA using oligonucleotides 5'-gaaactgcgaatggctcatt-3'and 5'-attaccgcggctgctggc-3' for PCR amplification.

Growth curves of the CBM29s transformed plants

To monitor stem elongation of the transformed plants, three replicates per line were grown in the greenhouse. Height measurements of the stems were taken weekly for nine weeks, starting from week two after transplant. Average height of six individual high expressers, each from transgenic lines containing CBM29-1-2 and CBM29-2 constructs, eight transformed control and four untransformed controls were used to plot a stem elongation curve over the monitoring period.

Light microscopy

Three individual plants per transgenic tobacco line and the wild type control were used for microscopic examination. Stem samples were taken from the second internode from the top of the plant. 1 mm-thick stem sections were fixed in 3% glutardialdehyde (Merck) and 3% paraformaldehyde (Merck) in 0.1 M

phosphate buffer containing 0.1% Triton x100 for 2 hours. The samples were then washed and dehydrated in an ethanol series. After dehydration, they were embedded in Technovit 7100 resin (USA) (Kuroiwa et al., 1990). 4 μm-thick sections were stained with 0.1% toluidine blue (Aldrich) and examined under a bright field microscope.

Cryo-scanning electron microscopy

One high expresser from each of the CBM29s transgenic lines and one individual plant each from the controls were used for cryo-SEM examination. Stem sampling was same as for the light microscopy. 6 mm-thick stem samples were mounted in a brass cylindrical sample holder with TBS (Tissue Freezing Medium EMS, Washington, PA, USA). The frozen samples were placed in a sample holder in a cryo-ultra microtome (Reichert Ultracut E/FC4D) and cut at specimen temperature of 100°C. These samples were first planed with a glass knife, after which the surface was planed with a diamond knife (Histo no trough, 8 mm 45°C, Drukker International, The Netherlands). After planing, the samples were placed in a dedicated cryo-preparation chamber (CT 1500 HF, Oxford instruments, UK). All the samples in the cryo-preparation chamber were freeze dried for 3 min at -90°C and 8×10^{-4} Pa, and subsequently sputtered with a layer of 10 nm Pt. The samples were cryo-transferred into the field emission scanning microscope (JEOL 6300F, Japan) on the sample stage at -190°C. All images were recorded digitally (Orion, 6 E.L.I. sprl, Belgium) at a scan rate of 100 s (full frame) at the size of 2528 x 2030, 8 bit. The images were optimized and resized for publication by Adobe Photoshop CS.

Quantification of cell size and cell wall diameter

To quantify the micrographic images, files were opened with Image J software developed at the National Institute of Health, USA (http://rsb.info.nih.gov/ij/).The surface area of three hundred cortical and xylem cells was measured as shown by the inset in Figure 8A and 8B. One hundred cells per field of view were measured in three replicates. Similarly, cell wall thickness of one hundred and fifty cells was quantified as shown by the inset in Figure 8C. Fifty cells per field of view were measured in three replicates. Standard deviation of three measurements was determined.

Isolation of cell wall material and analysis of the cellulose content

Stem samples from one high expresser of the CBM29-1-2 and CBM29-2 series, one pGreen 7k vector control, and one wild type control plant were ground to a fine powder in liquid nitrogen. For each isolation, 1 g of this stem material was extracted in a 50 mM Tris[HCl], pH 7.2 solution containing 1% SDS, for 3 h at room temperature (RT) with continuous shaking. The cell wall materialCWM was spun down by centrifugation at 1300 rpm for 15 min. Subsequently, the residue was washed with water, ethanol, acetone and air-dried. 10 mg of the different cell wall materials was hydrolyzed in 1 ml 2 M TFA. The TFA-insoluble cellulose was spun down at 1300 rpm for 15 min, and pellet was dissolved in 67% sulphuric acid. The acid hydrolysis was performed in quadruplicate. Cellulose content was determined as previously described (Obembe et al., 2006a).

Germination studies on transgenic seeds

To investigate transgenic seeds containing the mannan-recognizing CBM29 modules for altered germination rates, twenty-five seeds each, from one high and one low expresser of the tandem CBM29-1-2, one high expresser of the single CBM29-2 and a wild type plant were sterilized and sown on MS30 medium. The seeds were germinated at 28°C and early stages of germination were monitored from seed coat rupture till the emergence of two foliage leaves. The experiment was repeated three times and the results were consistent.

Results

Expression of *Piromyces* CBM29 module genes in Tobacco
To detect expression of the introduced CBM29 genes in the tobacco plant, we carried out Northern blot analysis with total RNA. Figure 2 shows representatives of three classes of transcript expression (high, low and none) of the two CBM29 modules as revealed by the Northern analysis.
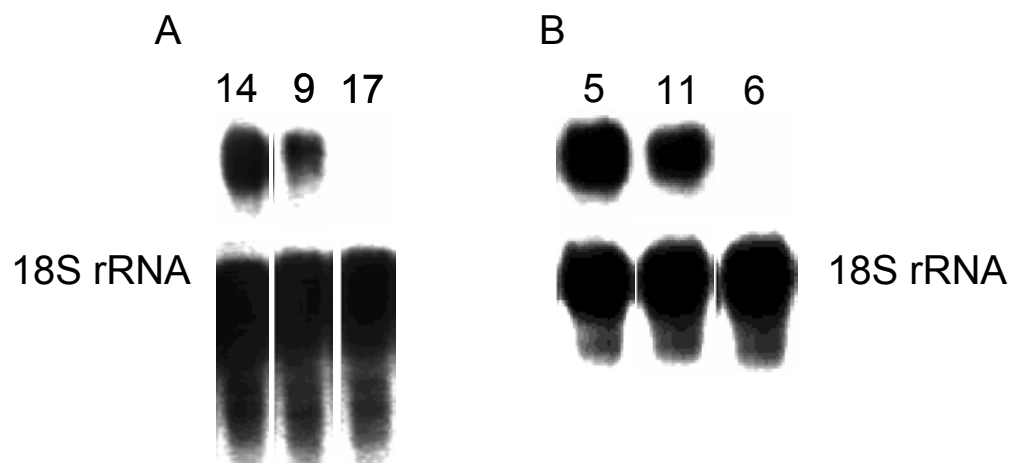

Figure 2. Transcript expression analysis of CBM29 genes in transgenic tobacco leaves. A differential transcript expression pattern is shown in the upper panel with the representative of each class in the two transgenic lines CBM29-2 (A) and CBM29-1-2 (B). Lines 14 and 5 represent high expressers, lines 9 and 11 represent intermediate expressers, and lines 17 and 6 represent low/none expressers. Lower panel shows RNA blots for the ribosomal RNA internal control with comparable intensities.

Ten plants of the CBM29-1-2 plants were classified as high expressers, five as low expressers and two as none expressers. Similarly for the CBM29-2 plants, ten lines were classified as high expressers, seven as low expressers and two as none expressers (note that none expressers may include plants with very low RNA expression, which could not be detected). Preliminary attempts at purifying CBM29 proteins using affinity purification with the hexa-histidine tag were not successful (data not shown). Similarly, western detection of a hexa-histidine epitope-tagged fungal elicitor protein ECP2 that was infiltrated into the apoplast of leaves did not succeed in the tomato plants but succeeded in *Arabidopsis* (Peter van Esse, Laboratory of Phytopathology WU, personal communication). The results indicated that the histidine tag was cleaved off in the tomato plants, but not in *Arabidopsis*. These observations have now led to the idea that the hexa-histidine epitope tags have cleavage sites that are being recognized by proteases, which are specific for the cell walls of the solanaceous species.

Transgenic plants display reduced stem elongation and delayed flower development
Plant height was monitored weekly for nine weeks, starting from the second week after transfer to the green house. At week eight, all plants except those expressing the

CBM29 modules had reached their final height at maturity (Figs. 3A-D, 4). It was also observed that flower development was delayed in the tandem CBM29-1-2-expressing plants. We observed that at week eight, when most plants had already flowered and even set fruit (Fig. 3E), the tandem CBM29-1-2- expressing plants were just developing flower buds (Fig. 3H). The flower buds eventually developed into normal flowers and also set fruits. Additionally, at week eight, most plants, except the CBM29-1-2 plants, had lost considerable number of leaves, due to faster ageing than the latter (Figs. 3A-D). Figure 4 shows the differential stem elongation between transgenic tobacco plants and the wild type control as a function of time. Note that the transgenic plants being referred to constitute the high expressers of the CBM29

Figure 3. Phenotypic alteration and delayed flower development of CBM 29-1-2 transgenic tobacco plants. Non-transformed control (A) and (E), empty pGreen7k control (B) and (F), single CBM 29-2 (C) and (G), tandem CBM29-1-2 (D) and (H).

modules and plants expressing the pGreen7k control. An indication of reduced stem elongation in the transgenic plants expressing the tandem CBM29-1-2 was first given after the third week of transfer. The differential stem elongation between the tandem CBM29-1-2 expressing plants and others gradually became more pronounced until the ninth week when other plants had reached their final height. The tandem CBM29-1-2-expressing plants still continued to increase in height until the eleventh week when they reached their final height, average of 58 cm, at maturity. There was however no obvious differential stem elongation between transgenic plants expressing the single CBM29-2 and the empty vector control. It is important to mention that the reduced stem elongation phenotype displayed by the tandem CBM29-1-2-expresing plants was more pronounced in the high expressers, which is why we have used only the high expressers of both modules to plot the stem elongation curves.

CBM29-1-2 indiscriminately binds to every tissue of the stem
Immunofluorescence detection of CBM2b-1-2 binding to stems of the wild type tobacco plant was done in order to know *a priori* the particular tissues of the stem, where the introduced CBM29-1-2 module would bind to. We expect to see the most pronounced alteration in these tissues. Indirect immunofluorescence micrographs (Fig. 5) revealed that CBM29-1-2 indiscriminately binds to every tissue of the stem, from the epidermis through the cortex and the vascular tissue to the pith parenchyma in the innermost part (Fig. 5A). The control (no CBM) however showed some weak autofluorescence in the xylem vessel cell walls (Fig. 5B). This *ex situ* evidence for unselective binding of the CBM29-1-2 to both the primary and the secondary cell walls of the mature stem of the tobacco plant thus complements an earlier evidence for its binding to a predominantly primary-walled maize coleoptile (McCartney et al., 2004). Additionally, it should be noted that tobacco is a dicotyledonous plant while maize is monocotyledonous, and that there are considerable differences in the polysaccharide composition of the walls of these species. Both results thus confirm the binding promiscuity of the tandem CBM29-1-2 as previously characterized (Freelove et al., 2001; Charnock et al., 2002).

Transgenic plants have collapsed cortical cell layers
The cellular basis for the plant phenotypes was examined by histological examinations on the transgenic tobacco stems. The examination revealed prominent layers of collapsed cells in the cortex of the stems of the tandem CBM29-1-2-expressing plants (Fig. 6A and 6E). We also observed that this morphological alteration is more severe in the high expressers than in the low expressers. This alteration was also observed in the high expressers of the single CBM29-2, with much less severity though (Fig. 6C). No such alteration was observed in the two controls (Fig. 6B and 6D). Another observation we made was altered cell enlargement across the tissues in the CBM29s-expressing stems. This was particularly true for the tandem CBM29-1-2-expressing plants (for example comparing Figure 6A, 6E and 6G to 6B/D, 6F and 6H, respectively). The altered cell size phenotype is widespread in the

high and the low CBM29-1-2-expressing plants. This is not the case, however, in the
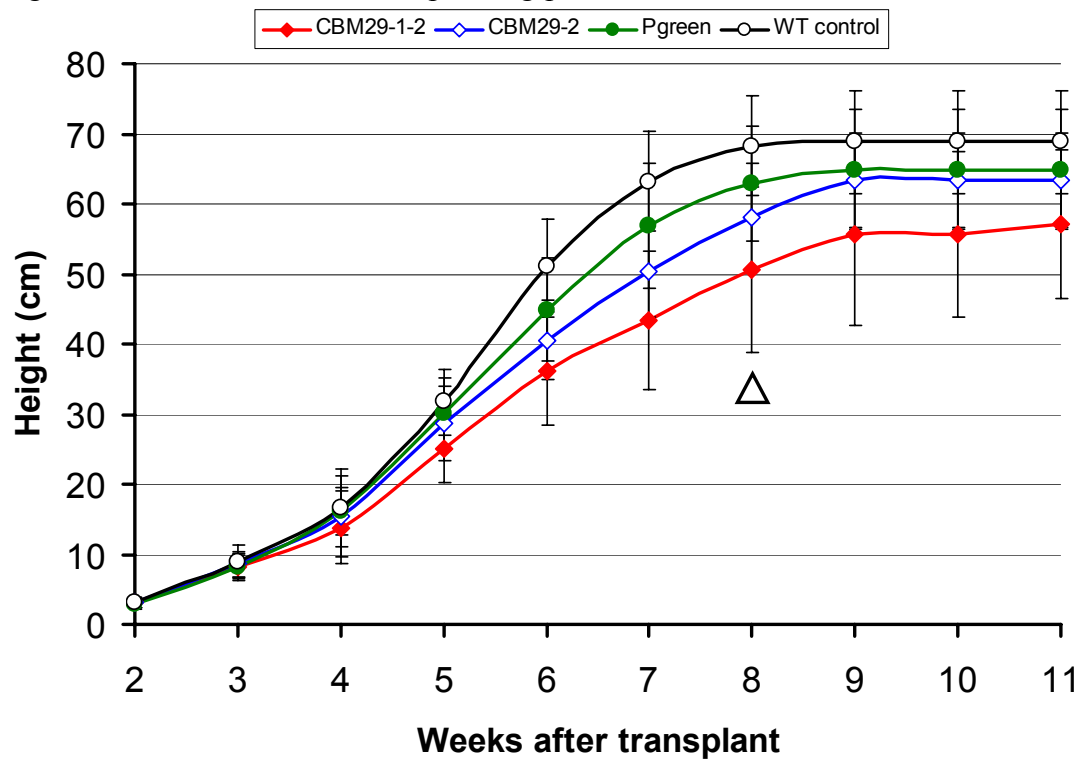

Figure 4. Effect of CBM29 modules on stem elongation of the transgenic plants. The curves represent CBM29-1-2 and CBM 29-2 lines, the empty pGreen7k control and the wild type control. Each data point is the average of 4 to 6 plants (higher expressers for both CBM29 lines). The triangle indicates the time when the picture presented in Figure 3 was taken.
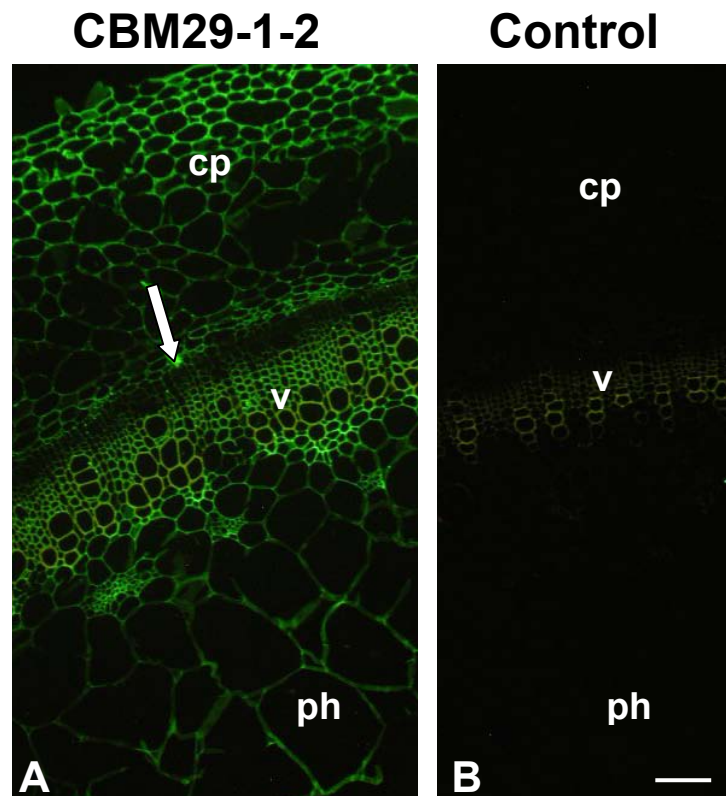
Figure 5. Micrographs showing the binding of CBM29-1-2 to transverse sections of wild type tobacco stem using indirect immunofluorescence microscopy. The CBM29-1-2 (A) binds indiscriminately to every tissue of the stem, from the epidermis through the cortex and the vascular tissue to the pith parenchyma in the innermost part., cp = cortical parenchyma, v = vascular tissue, ph = pith. Arrow head indicates the epidermis. Arrow indicates the phloem fibres. Scale bar = 100 μm.

single CBM29-2-expressing stems, in which only the high expressers exhibited noticeable larger cells in the epidermal, cortex and xylem tissues than those of the controls did. Furthermore, we observed that the phloem fibre cells in the control stems stained more intensely for lignin than those in the CBM29-expressing stems (comparing Fig. 6F to 6E). There was however no marked difference between the staining intensities of the phloem fibre cells of the stems of the two CBM29s-expressing transgenic tobacco lines. There was no clear indication of altered enlargement of the stem pith parenchyma cells of the transgenic plants.
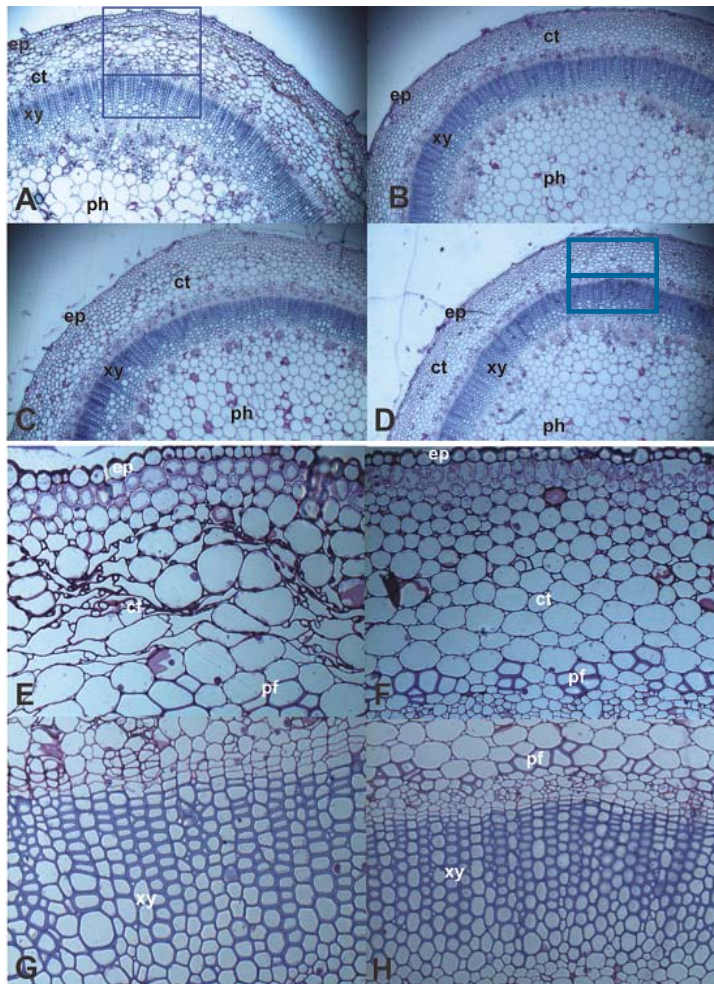

Figure 6. Cross sections of transgenic and control stems, showing collapsed cortical cells and enlarged cortical and xylem cells of the CBM29-1-2 stems. Sections of CBM29-1-2 (A, E and G), empty pGreen7k control (B), CBM29-2 stem (C), and wild type (D, F and H) were stained with toluidine blue. Boxed areas indicate tissues shown with higher magnification in the lower panel. ct = cortex, ph = pith, ep = epidermis, xy = xylem, pf = phloem fibre.

Cryo-scanning electron microscopy of tobacco stems

To establish that the transgenic stems indeed had larger cortical and xylary cells than those of the controls, we made cryo-scanning electron microscopic examinations of stem samples. Figure 7A showed the collapsed as well as the enlarged cells of the cortex in the sections of transgenic line 5 of CBM29-1-2, as previously observed with light microscopic examination. The cortical cells of the control plant were smaller and
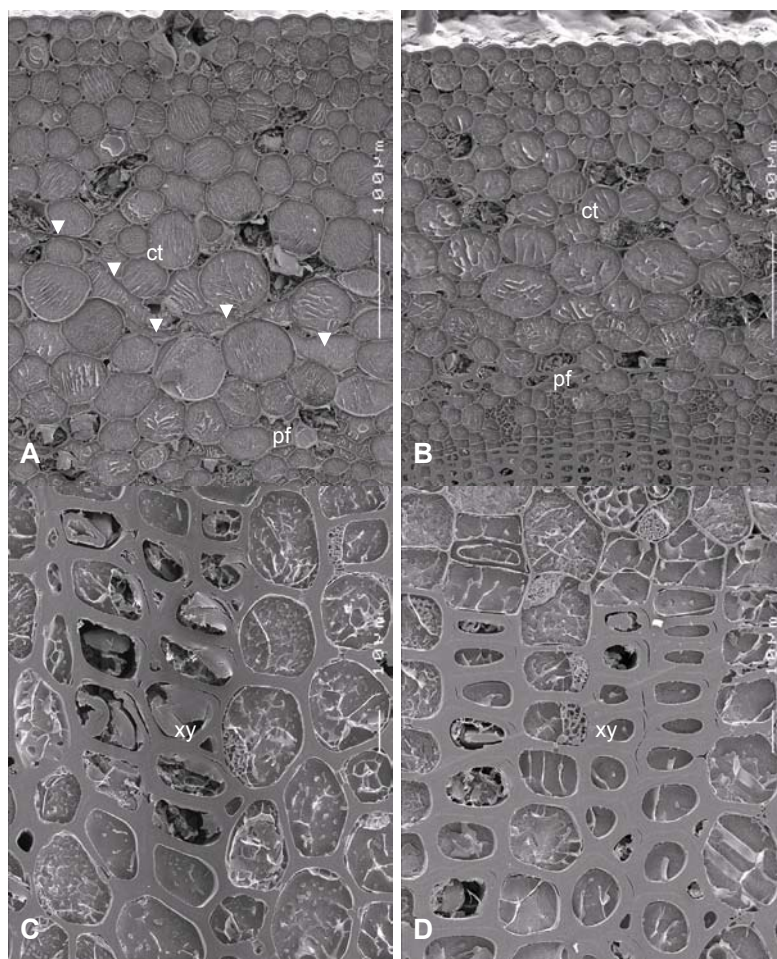

Figure 7. Scanning electron micrographs of transgenic tobacco stems. CBM 29-1-2 (A) and (C) and Empty pGreen7k control (B) and (D). 7A and 7B show the cortex tissue; details of the collapsed layer of cortical cells of the CBM 29-1-2 are shown in 7A. Figure 7B and 7D show the xylem tissue; details of larger cells as well as thinner cell walls of the CBM 29-1-2 stems compared to the pGreen7k control are shown. pf = phloem fibre, xy = xylem, ct = cortex. Arrow heads indicate collapsed layer of cortical cells.

did not exhibit the collapsed phenotype (Figure 7B). The micrographs also showed that xylem cells were indeed larger in the CBM29s-expressing stems than in the controls (comparing transgenic line 5, Figure 7C and the empty vector control, Figure 7D). The micrographs also gave indication that the cell walls of the xylem cells were thinner in the CBM29s-expressing stems (Fig. 7C) than in the controls (Fig. 7D). These notable observations were later substantiated by quantification.
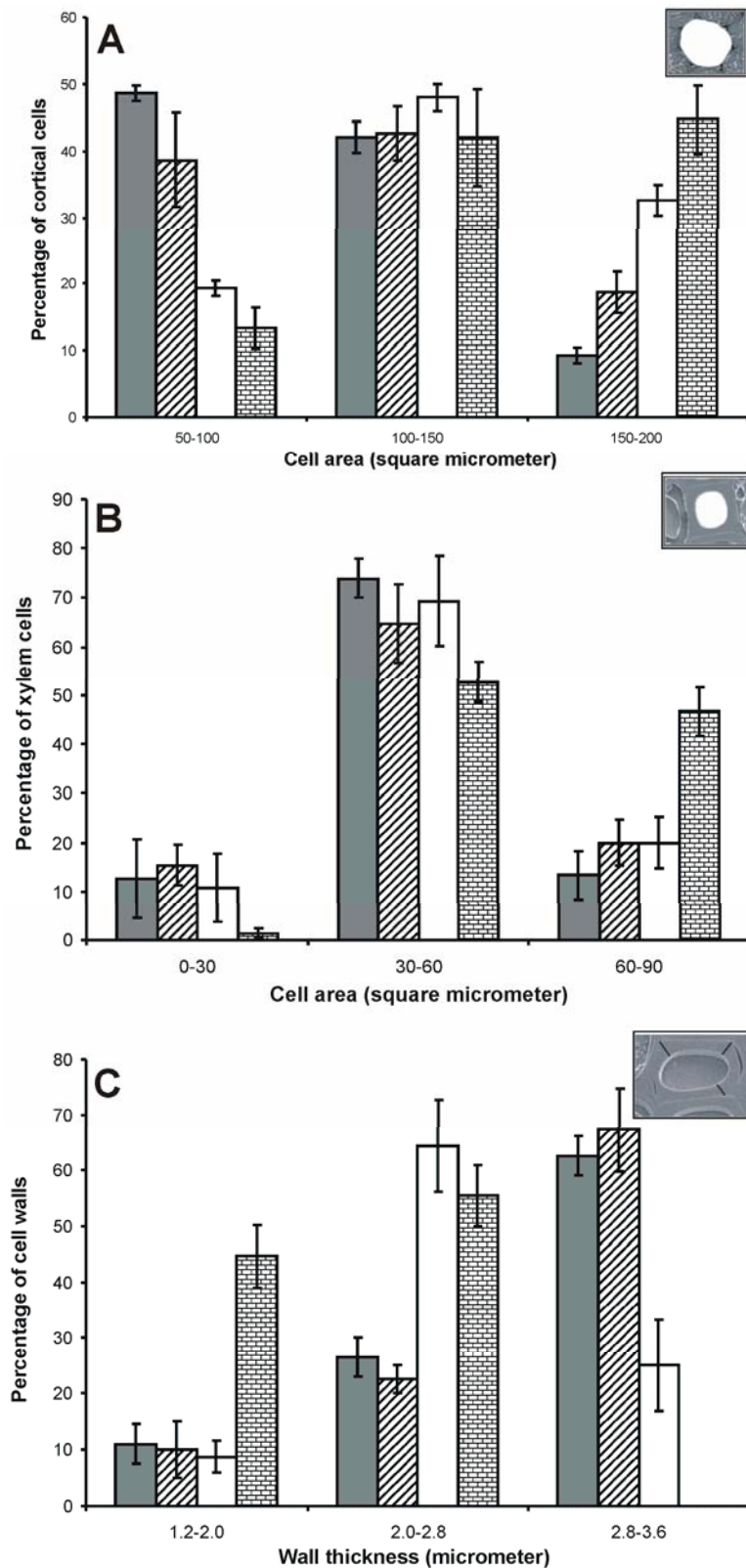
Figure 8. Cell size distribution in the cortex (A), cell size distribution in the xylem (B) and cell wall thickness of xylem cells (C) in the stems of transgenic and the wild type tobacco plants. Insets show how the surface area and the wall thickness measurements were taken. Surface area of 300 cells were measured, 100 cells per field of view. Cell wall diameter of 150 cells were measured, 50 cells per field of view. Error bars represent standard deviation. Gray bar represents the wild type plants. Wide downward diagonal bar represents empty pGreen7k transformants. Open bar represents single CBM29-2 transformants. Horizontal brick bar represents double CBM29-1-2 transformants.

Figure 8A showed that almost 50% and 40% of the stem cortical cells of the wild type and pGreen7k control plants were respectively grouped in the small class, whereas less than 20% of the stem cortical cells of the CBM29s-expressing plants grouped in the small class. An opposite trend was observed in the large class wherein less than 20% of the cortical cells of the two control stems were grouped compared to the more than 30% and 40% of the stems expressing the single CBM29-2 and the tandem CBM29-1-2, respectively. We thus infer that the cortical cells of the CBM29-2-expressing stems are larger than those of the control stems. Similar quantification data were obtained with the xylem cells as presented in Figure 8B, which showed that only the xylem cells of the CBM29-1-2-expressing stems are larger than those of the control stems. There was no difference in the size of the xylem cells of the single CBM29-2 stems and those of the controls. Similarly, we quantified the thickness of the cell walls of the xylem cells of the CBM29-2-expressing stems and the pGreen7k control stems as presented in Figure 8C. Approximately 45% of the cell walls of the xylem cells of the CBM29-1-2-expressing stem grouped in the thin class, whereas less than 10% of the stem xylem cell walls of the single CBM29-2, the pGreen7k control and the wild type control plants grouped in this class. More than 60% of the xylem cell walls of the two control stems were grouped in the thick class, whereas about 22% and 0% of the stem xylem cell walls of the single CBM29-2 and the tandem CBM29-1-2 grouped in this class, respectively. We can also infer from this result that the thickness of the cell walls of the xylem cells of the CBM29-2-expressing stems (particularly of the tandem CBM29-1-2) are thinner than those of the pGreen7k and the wild type control stems.

Analysis of cellulose content

A colorimetric assay was performed on samples of crystalline cellulose obtained from four separate acid hydrolyses to determine the cellulose content in the stem cell walls of the CBM29s transformants and the control plants. Figure 9 showed the levels of cellulose in the stem cell walls of the transgenic tobacco lines expressing the single CBM29-2 and the tandem CBM29-1-2, the empty vector control and a wild type control. There was no considerable difference in the levels of cellulose in the stem cell walls of the transformants and the two control plants. Hence, it can be concluded that the use of these CBM29 modules for cell wall modification did not interfere with the extent of cellulose biosynthesis.
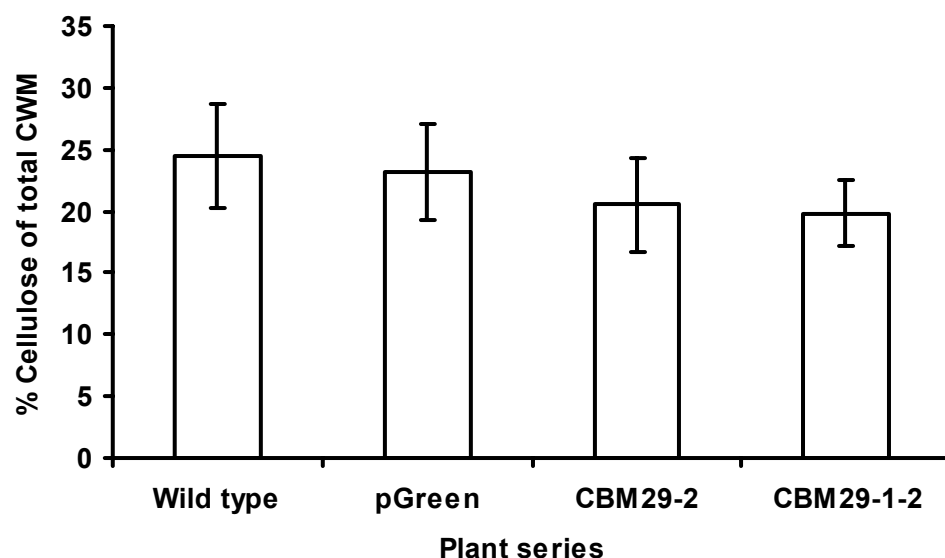
Figure 9. Cellulose content in transgenic tobacco plants. Cellulose content of stem cell wall materials of high expressers of the tandem CBM29-1-2, the single CBM29-2, the tandem CBM2b-1-2 and the two control plants was determined using a colorimetric assay. The cellulose levels are represented as the percentage of cellulose in total cell wall material. All measurements were performed as quadruplicate and standard deviation of the means are represented by the bars.

Transgenic seeds did not exhibit altered germination rate

There are several reports, which demonstrated that CBMs can disrupt the surface of their substrates, thereby making the substrates more accessible to enzyme degradation. The cellulose-binding domains (CBDs) of many cellulose degrading enzymes have been shown to disrupt the surfaces of cotton cellulose fibres and releases small particles from them (Gao et al., 2001; Xiao et al., 2001). It was also reported that starch-binding domains caused similar effects of structural disruption to starch (Southall et al., 1999; Giardina et al., 2001). Proteins other than the well classified CBMs such as the expansins (McQueen-Mason and Cosgrove, 1994; Cosgrove, 2000a), swollenin (Saloheimo et al., 2002) and fibronectin (Kataeva et al., 2002) and a novel protein called fibril-forming protein (FFP) from *Trichoderma reesei* (Banka et al., 1998) have all been shown, through paper assay, to exhibit the non-hydrolytic disruption of cellulose fibres. Furthermore, a patent report has claimed that transgenic seeds with altered germination rates were generated by targeting a mannan-recognizing CBM preferentially to the cell walls of the seed coat (Kilburn et al., 2000). The notion here, in line with the foregoing, is that the mannan-recognizing CBMs may also exhibit non-hydrolytic disruption of the seed mannan polysaccharides, causing rapid weakening of the cell wall, and thus a faster germination rate. The authors suggested that altered germination rate could also be achieved by modifying the mannan reserve in the cell wall of the seed endosperm, with mannan-recognizing CBM. Meanwhile it has been shown that the endosperm

cell walls of the tobacco seed are rich in galactomannan (Reid et al., 2003). Since a
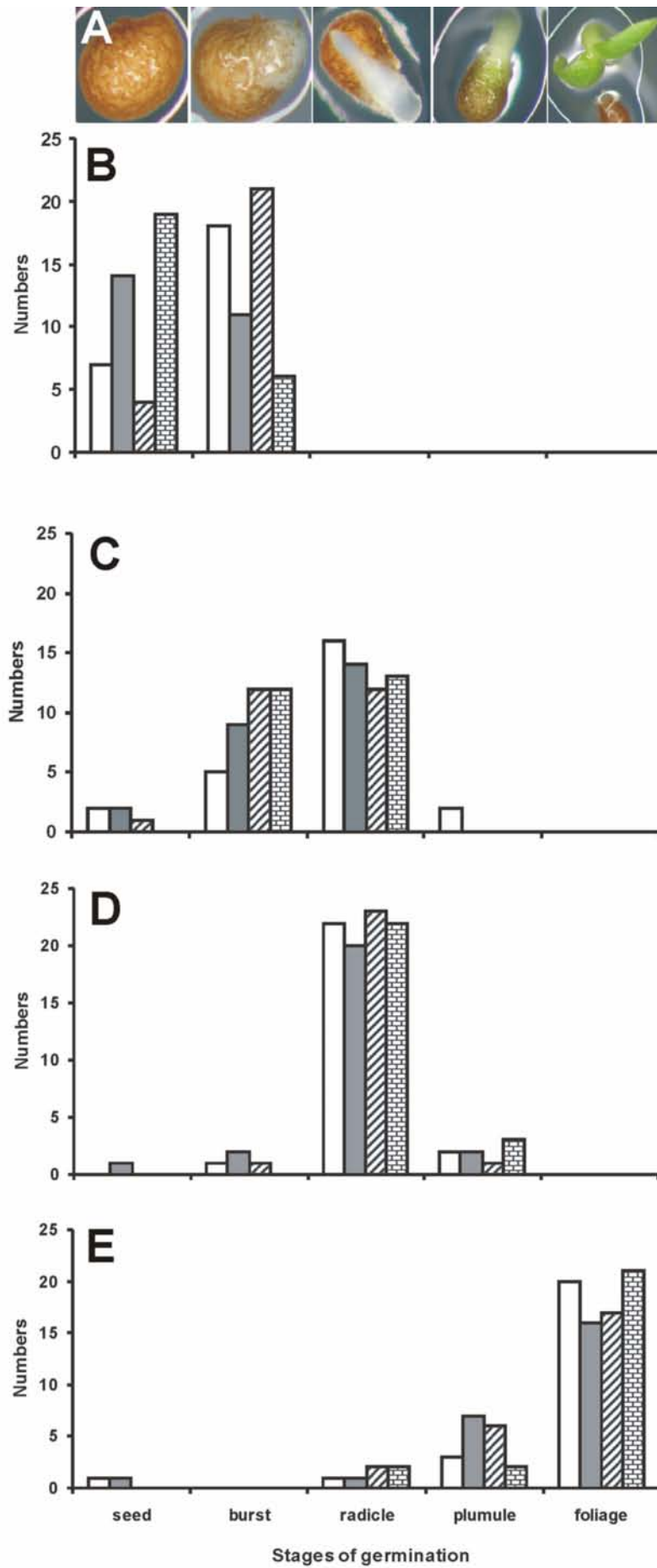
Figure 10. Germination rate in various transgenic seeds compared with the wild type seeds. Figure 10A shows the different stages of germination that were used for scoring the germination rates of the different seeds at different time points. Figures 10B, C, D, and E, show the extent of seed germination in each plant line at time points 46, 62, 78 and 93 h after sowing *in vitro*, respectively. Open bar represents high expressers of double CBM29-1-2 transformants. Gray bar represents low expressers of double CBM29-1-2 transformants. Wide upward diagonal bar represents high expressers of single CBM29-2 transformants. Horizontal brick bar represents wild type.

constitutive promoter has been used to drive the expression of the two CBM29 constructs in tobacco, transgenic seeds containing the CBMs might exhibit an altered germination rate. To investigate this possibility, we have carried out three independent seed germination experiments with twenty five seeds each of one high- and one low expresser of the tandem CBM29-1-2, one high expresser of the single CBM29-2 and one wild type control. Our results in each case showed that the transgenic seeds containing the CBM29 constructs did not exhibit altered germination rate. A representative result of the three experiments is presented in Figure 10, which showed no particular trend in seed germination of the different transgenic seeds through all the stages of germination i.e. from seed coat rupture (burst) to the emergence of the two foliage leaves. The results thus give an indication that constitutively expressed CBM29s might not be effective in generating transgenic seeds with altered seed germination.

Discussion

Plant cell wall polysaccharide networks play a crucial role in plant development. Organ growth is largely based on progressive remodeling of the cellulose-hemicellulose networks in the different tissues within the growing organ. Promiscuous CBMs are hypothesized to have the capabilities of modifying these networks with envisaged consequence on plant development. We have targeted the expression of two promiscuous *Piromyces* CBM29-containing proteins to tobacco cell walls, and investigated their ability to influence these networks and the effect on plant development. Our results demonstrate that the tandem CBM29-1-2 modified the tobacco cell wall structure with consequent altered cellular and organ morphology. Additionally, the tandem CBM29-1-2 modules show more effect than the single, without pronounced concomitant changes in cellulose content. Moreover, the more pronounced, widespread cellular alteration across the stem tissues of the CBM29-1-2-expressing plants is consistent with the result of the immunolabeling study with the protein, which detected its ligands in every tissue of the stem.

The expression of the tandem CBM29-1-2 modules in the tobacco cell wall affected plant development as reflected by reduced stem elongation leading to a reduction in plant height throughout the entire course of development, as well as enlarged xylem and collapsed cortical cells. The stem phenotype of the CBM29-1-2-expressing plants was more of delayed development of the whole plant rather than mere elongation defects of the stem. We suggest that the delayed development (prolonged juvenility) was a result of reduced rate of anabolism in the tandem CBM29-1-2-expressing plant.

It could be that the structural remodeling of the cell wall has affected the normal developmental cues that signal the cells to synthesize new cell wall materials. It is conceivable that when the cell walls are held loosely continuously as a result of the continuous presence of CBM29-1-2, the rate of wall extension will out-pace biosynthetic rate. Consequently the cell walls may show excessive thinning (Kutschera, 1990), which may have led to the eventual collapse of the cells. Additionally, it is widely known that the driving force for growth is provided by the thin-walled, turgid inner tissues of an organ, whereas the rate of stem growth is regulated by loosening and stiffening events that are restricted to the peripheral walls (Peters and Tomos, 1996; Peters and Tomos, 2000; Kutschera, 2001). Thus, due to the extensive loosening of their cell walls and consequent altered expansion, the cortical cells may have yielded to the tissue pressure within the stem. The altered cell enlargement, which led to the collapse of the cortical cells of the tandem CBM29-1-2-expressing stems, is being proposed as the cellular basis for the reduced stem elongation. Our observation on reduced growth is in line with a report, which demonstrated that transgenic *Arabidopsis thaliana* plants expressing a fungal cellulose-binding module exhibited a dwarfish phenotype due to a defect in cell elongation (Quentin, 2003). We have not noticed that the increased size of the xylem cells led to thicker stems. Posssibly, the gain in cell size of the xylem cells is neutralized by the collapse of the cortical cells.

There are reports, which attributed cell wall phenotypes in mutant or transgenic plants to abnormal wall assembly in general, or depletion in cellulose content in particular. Prime examples of such plants include the cellulose-deficient, irregular xylem (*irx*) mutants of *Arabidopsis* (Turner and Somerville, 1997; Taylor et al., 1999; Taylor et al., 2000; Szyjanowicz et al., 2004), which displayed abnormal cellulose deposition and abnormal, collapsed secondary walls of the xylem cells (Turner and Somerville, 1997). Also, transgenic *Arabidopsis* plants expressing the antisense gene of endo-1,4-β-glucanase (cel1) have been shown to exhibit reduced cellulose content that was associated with a 'wrinkled' cell wall phenotype in the vascular tissues (Tsabary et al., 2003). Finally, the *Arabidopsis* fragile fibre (*fra*) mutants exhibited thinner cell walls with resultant shortening and widening of cells (mostly vascular tissues) and a concomitant reduction in cellulose content (Burk et al., 2001; Brands and Ho, 2002; Zhong et al., 2003; Zhong et al., 2004). In all the cases cited above, the plants exhibited deformed walls in vascular cells and reduced growth as a result of cellulose depletion, whereas our results demonstrated that CBM29-1-2-expressing tobacco plants showed deformed walls in cortical cells and reduced growth, without a marked reduction in cellulose content. The abnormal radial widening of particularly the vascular cells of the transgenic tobacco plants is probably a result of excessive wall-loosening. The deformation of the stem cortical cells in the CBM29-1-2 transformants is an indirect effect of the more pronounced cell expansion. The cortical parenchyma has no secondary walls, suggesting that the walls are considerably weaker here; consequently, this tissue will collapse, when challenged by excessive expansion of flanking tissues.

On the whole, it seems that the promiscuous tandem CBM29-1-2 has been used successfully to remodel the wall structure in the expressing stem, leading to a sort of continuous loosening process with consequent cellular and organ morphological alterations.

Acknowledgements

References

Banka RR, Mishra S, Ghose TK (1998) Fibril formation from cellulose by a novel protein from Trichoderma reesei: A non-hydrolytic cellulolytic component? World Journal of Microbiology & Biotechnology **14:** 551-558

Barral P, Suarez C, Batanero E, Alfonso C, Alche JD, Rodriguez-Garcia MI, Villalba M, Rivas G, Rodriguez R (2005) An olive pollen protein with allergenic activity, Ole e 10, defines a novel family of carbohydrate-binding modules and is potentially implicated in pollen germination. Biochemical Journal **390:** 77-84

Boraston AB, Bolam DN, Gilbert HJ, Davies GJ (2004) Carbohydrate-binding modules: fine-tuning polysaccharide recognition. Biochemical Journal **382:** 769-781

Brands A, Ho THD (2002) Function of a plant stress-induced gene, HVA22. Synthetic enhancement screen with its yeast homolog reveals its role in vesicular traffic. Plant Physiology **130:** 1121-1131

Brett CTW, K. (1996) Physiology and Biochemistry of Plant Cell walls. Chapman & Hall, London

Burk DH, Liu B, Zhong RQ, Morrison WH, Ye ZH (2001) A katanin-like protein regulates normal cell wall biosynthesis and cell elongation. Plant Cell **13:** 807-827

Carpita NC, Defernez M, Findlay K, Wells B, Shoue DA, Catchpole G, Wilson RH, McCann MC (2001) Cell wall architecture of the elongating maize coleoptile. Plant Physiology **127:** 551-565

Charnock SJ, Bolam DN, Nurizzo D, Szabo L, McKie VA, Gilbert HJ, Davies GJ (2002) Promiscuity in ligand-binding: The three-dimensional structure of a Piromyces carbohydrate-binding module, CBM29-2, in complex with cello-and mannohexaose. Proceedings of The National Academy of Sciences of The United States of America **99:** 14077-14082

Cosgrove DJ (2000) Loosening of plant cell walls by expansins. Nature **407:** 321-326

Cosgrove DJ (2003) Expansion of plant cell wall, in The Plant Cell Wall, (ed. Jocelyn K. C. Rose), Vol 8. CRC Press LLC, Boca Raton, FL, USA, pp 237-258

Darley CP, Forrester AM, McQueen-Mason SJ (2001) The molecular basis of plant cell wall extension. Plant Molecular Biology **47:** 179-195

Freelove ACJ, Bolam DN, White P, Hazlewood GP, Gilbert HJ (2001) A novel carbohydrate-binding protein is a component of the plant cell wall-degrading complex of Piromyces equi. Journal of Biological Chemistry **276:** 43010-43017

Gao PJ, Chen GJ, Wang TH, Zhang YS, Liu J (2001) Non-hydrolytic disruption of crystalline structure of cellulose by cellulose binding domain and linker sequence of cellobiohydrolase I from Penicillium janthinellum. Acta Biochimica Et Biophysica Sinica **33:** 13-18

Giardina T, Gunning AP, Juge N, Faulds CB, Furniss CSM, Svensson B, Morris VJ, Williamson G (2001) Both binding sites of the starch-binding domain of Aspergillus niger glucoamylase are essential for inducing a conformational change in amylose. Journal of Molecular Biology **313:** 1149-1159

Hackney JM, Atalla RH, Vanderhart DL (1994) Modification of Crystallinity And Crystalline-Structure of Acetobacter-Xylinum Cellulose In The Presence of Water-Soluble Beta-1,4-Linked Polysaccharides - C-13-Nmr Evidence. International Journal of Biological Macromolecules **16:** 215-218

Handford MG, Baldwin TC, Goubet F, Prime TA, Miles J, Yu XL, Dupree P (2003) Localisation and characterisation of cell wall mannan polysaccharides in Arabidopsis thaliana. Planta **218:** 27-36

Hellens RP, Edwards EA, Leyland NR, Bean S, Mullineaux PM (2000) pGreen: a versatile and flexible binary Ti vector for Agrobacterium-mediated plant transformation. Plant Molecular Biology **42:** 819-832

Henrissat B, Coutinho PM, Davies GJ (2001) A census of carbohydrate-active enzymes in the genome of *Arabidopsis thaliana*. Plant Molecular Biology **47:** 55-72

Hosoo Y, Yoshida M, Imai T, Okuyama T (2002) Diurnal difference in the amount of immunogold-labeled glucomannans detected with field emission scanning electron microscopy at the innermost surface of developing secondary walls of differentiating conifer tracheids. Planta **215:** 1006-1012

Kataeva IA, Seidel RD, Shah A, West LT, Li XL, Ljungdahl LG (2002) The fibronectin type 3-like repeat from the Clostridium thermocellum cellobiohydrolase CbhA promotes hydrolysis of cellulose by modifying its surface. Applied And Environmental Microbiology **68:** 4292-4300

Kilburn DG, Warren AJ, Stoll D, Gilkes NR, Shoseyov O, Shani Z (2000) Expression of a mannan binding domain to alter plant morphology. WO 2000/068391

Kuipers AGJ, Soppe WJJ, Jacobsen E, Visser RGF (1995) Factors affecting the inhibition by antisense RNA of granule-bound starch synthase gene-expression in potato. Molecular & General Genetics **246:** 745-755

Kuroiwa T, Kuroiwa H, Mita T, Fujie M (1990) Fluorescence Microscopic Study of the Formation of Giant Mitochondrial Nuclei in the Young Ovules of Pelargonium-Zonale. Protoplasma **158:** 191-194

Kutschera U (1990) Cell-Wall Synthesis And Elongation Growth In Hypocotyls of Helianthus-Annuus L. Planta **181:** 316-323

Kutschera U (2001) Stem elongation and cell wall proteins in flowering plants. Plant Biology **3:** 466-480

Levy I, Shani Z, Shoseyov O (2002) Modification of polysaccharides and plant cell wall by endo-1,4-beta-glucanase and cellulose-binding domains. Biomolecular Engineering **19:** 17-30

McCartney L, Gilbert HJ, Bolam DN, Boraston AB, Knox JP (2004) Glycoside hydrolase carbohydrate-binding modules as molecular probes for the analysis of plant cell wall polymers. Analytical Biochemistry **326:** 49-54

McQueen-Mason S, Cosgrove DJ (1994) Disruption of hydrogen-bonding between plant-cell wall polymers by proteins phat induce wall extension. Proceedings of The National Academy of Sciences of The United States of America **91:** 6574-6578

Obembe OO, Cifuentes C, Huigen D, Kieft H, Jacobsen E, Emons AMC, Visser RGF, Vincken J-P (2006) Bioengineering cellulose-hemicelluose networks in plants (Chapter 2, This Thesis). PhD thesis. Wageningen University, Wageningen

Peters WS, Tomos AD (1996) The history of tissue tension. Annals of Botany **77:** 657-665

Peters WS, Tomos AD (2000) The mechanic state of "Inner tissue" in the growing zone of sunflower hypocotyls and the regulation of its growth rate following excision. Plant Physiology **123:** 605-612

Quentin MGE (2003) Cloning, characterisation and assessment of the wall modifying ability of a fungal cellulose-binding domain (PhD Thesis). PhD thesis. Wageningen University, Wageningen

Reid JSG, Edwards ME, Dickson CA, Scott C, Gidley MJ (2003) Tobacco transgenic lines that express fenugreek galactomannan galactosyltransferase constitutively have structurally altered galactomannans in their seed endosperm cell walls. Plant Physiology **131:** 1487-1495

Saloheimo M, Paloheimo M, Hakola S, Pere J, Swanson B, Nyyssonen E, Bhatia A, Ward M, Penttila M (2002) Swollenin, a Trichoderma reesei protein with sequence similarity to the plant expansins, exhibits disruption activity on cellulosic materials. European Journal of Biochemistry **269:** 4202-4211

Schroder R, Wegrzyn TF, Bolitho KM, Redgwell RJ (2004) Mannan transglycosylase: a novel enzyme activity in cell walls of higher plants. Planta **219:** 590-600

Shoseyov O, Yosef K, Shani Z, Rehovoth, Shpigel E, Medigo K (2001) Transgenic Plants of Altered Morphology. US patent 6,184,440

Shpigel E, Roiz L, Goren R, Shoseyov O (1998) Bacterial cellulose-binding domain modulates in vitro elongation of different plant cells. Plant Physiology **117:** 1185-1194

Southall SM, Simpson PJ, Gilbert HJ, Williamson G, Williamson MP (1999) The starch-binding domain from glucoamylase disrupts the structure of starch. Febs Letters **447:** 58-60

Szyjanowicz PMJ, McKinnon I, Taylor NG, Gardiner J, Jarvis MC, Turner SR (2004) The irregular xylem 2 mutant is an allele of korrigan that affects the secondary cell wall of Arabidopsis thaliana. Plant Journal **37:** 730-740

Taylor NG, Laurie S, Turner SR (2000) Multiple cellulose synthase catalytic subunits are required for cellulose synthesis in Arabidopsis. Plant Cell **12:** 2529-2539

Taylor NG, Scheible WR, Cutler S, Somerville CR, Turner SR (1999) The irregular xylem3 locus of *Arabidopsis* encodes a cellulose synthase required for secondary cell wall synthesis. Plant Cell **11:** 769-780

Tsabary G, Shani Z, Roiz L, Levy I, Riov J, Shoseyov O (2003) Abnormal 'wrinkled' cell walls and retarded development of transgenic Arabidopsis thaliana plants expressing endo-1,4-beta-glucanase (cel1) antisense. Plant Molecular Biology **51:** 213-224

Turner SR, Somerville CR (1997) Collapsed xylem phenotype of Arabidopsis identifies mutants deficient in cellulose deposition in the secondary cell wall. Plant Cell **9:** 689-701

Whitney SEC, Brigham JE, Darke AH, Reid JSG, Gidley MJ (1998) Structural aspects of the interaction of mannan-based polysaccharides with bacterial cellulose. Carbohydrate Research **307:** 299-309

Whitney SEC, Gidley MJ, McQueen-Mason SJ (2000) Probing expansin action using cellulose/hemicellulose composites. Plant Journal **22:** 327-334

Wittink FRA, Knuiman B, Derksen J, Capkova V, Twell D, Schrauwen JAM, Wullems GJ (2000) The pollen-specific gene Ntp303 encodes a 69-kDa glycoprotein associated with the vegetative membranes and the cell wall. Sexual Plant Reproduction **12:** 276-284

Xiao ZZ, Gao PJ, Qu YB, Wang TH (2001) Cellulose-binding domain of endoglucanase III from Trichoderma reesei disrupting the structure of cellulose. Biotechnology Letters **23:** 711-715

Zhong RQ, Burk DH, Morrison WH, Ye ZH (2004) FRAGILE FIBRE3, an Arabidopsis gene encoding a type II inositol polyphosphate 5-phosphatase, is required for secondary wall synthesis and actin organization in fibre cells. Plant Cell **16:** 3242-3259

Zhong RQ, Morrison WH, Freshour GD, Hahn MG, Ye ZH (2003) Expression of a mutant form of cellulose synthase AtCesA7 causes dominant negative effect on cellulose biosynthesis. Plant Physiology **132:** 786-795

Constitutive expression of a *Cellulomonas fimi* tandem xylan-binding module in tobacco

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Abstract

Xylan is the major hemicellulosic component in the secondary cell wall of plants. It interacts with both cellulose and lignin producing a cellulose-xylan-lignin complex, which gives the cell wall mechanical strength. Carbohydrate-binding modules have been proposed to be able to alter plant cell wall structural architecture. We have expressed a tandem xylan-binding module, CBM2b-1-2 of *Cellulomonas fimi* xylanase 11A in tobacco plants. Histological examinations of the transgenic stems expressing the tandem CBM2b-1-2 with a light microscope revealed that their cortical cells were irregularly shaped, whereas this phenotype was not observed in the control stems. Cryo-scanning electron microscopic examinations and image quantification indicated that 40% of the xylem cells of the transgenic stems expressing the tandem CBM2b-1-2 are larger in size (radial surface area) than those of the controls. Our results demonstrate that the effect of the introduced CBM2b-1-2 is not restricted only to the vascular tissue, where the protein binds to, as detected by an immuno-labeling study on wild type tobacco stems. There were no observable changes in the height or the appearance of the transgenic plants expressing the tandem CBM2b-1-2 module.

Introduction

The interaction of cellulose and hemicellulose (xyloglucan, xylan and mannan) no doubt plays a central role in the diverse functions of plant cell walls, especially in giving mechanical support and shape to the cell. Xylan is the major component of the hemicellulose in the secondary cell wall (Timell, 1967; Ebringerova and Heinze, 2000), which is laid down after cell expansion. Xylan can connect to both cellulose and lignin (Awano et al., 2002). This cellulose-xylan-lignin complex is thought to be responsible for the mechanical strength of the cell wall.

We have demonstrated previously that promiscuous carbohydrate-binding modules can modify plant cell walls with consequent cellular and whole plant phenotypes (Obembe et al., 2006c). Our aim in this work was to investigate the effect of targeting a less promiscuous CBM to the plant cell wall of tobacco. C*ellulomonas fimi* xylanase 11A has been shown to contain two consecutive family 2b CBMs (CBM2b-1 and CBM2b-2. The tandem CBM2b-1-2 exhibits very strong affinity for xylan (K_a = ~ 1.0 x 10^6 M^{-1}) and much lower affinity for insoluble acid-swollen cellulose (K_a = ~ 6.0 x 10^4 M^{-1}) (Bolam et al., 2001). Furthermore, it has a 18-20-fold higher affinity for xylan as compared to the individual modules. The CBMs did not interact with soluble forms of other plant cell wall polysaccharides.

Previously, we observed that the expression of the tandem CBM29-1-2 modules in transgenic tobacco plants resulted in more pronounced phenotypes than the expression of the single CBM29-2 (Obembe et al., 2006c). Similar observations were made for tandem starch-binding domains, which were shown to have 10-fold higher affinity for starch than the single starch-binding domain (SBD) (Ji et al., 2004). These tandem SBDs were also shown to have more impact in the plant than the single. Premised on these observations, we have used heterologous expression of the xylan-specific, tandem CBM2b-1-2 of *C. fimi* xylanase 11A in the tobacco plants. The expression of the gene encoding this CBM was driven by the 35S cauliflower mosaic virus (CaMV) promoter and the protein was targeted to the cell walls. Our observations indicate that structural alterations in the cell walls of the transgenic plants expressing the tandem CBM2b-1-2 is less severe as compared with those of the more promiscuous tandem CBM29-1-2.

Materials and methods

Preparation of constructs

The tandem CBM2b-1-2 construct was prepared by amplifying its gene fragment from pET28a, a recombinant plasmid vector used for cloning and expression in *Escherichia coli* (Bolam et al., 2001). The polymerase chain reaction (PCR) was performed using primers that included SmaI and EcoRI recognition sites at either end (5'-<u>cccggg</u>tgacacgggcggaggcggcggt-3' and 5'-<u>ggaattc</u>**tca**gcccgtggcgca-3'; the SmaI and EcoRI sites are underlined, respectively). The three bases highlighted in bold type encode a stop codon. The amplified 635 base pairs fragment of the tandem CBM2b-1-2 was digested with SmaI and EcoRI (Invitrogen, The Netherlands) and cloned into a similarly digested binary vector pGreen 7k (Hellens et al., 2000). The cloning of the fragment was done such that the protein was in-

frame with the fused sequences encoding a transit peptide and a hexa-histidine tag as presented in Figure 1. The cloning was done as previously described (Obembe et al., 2006c). The control construct did not contain the CBM as it was just an empty vector without the insert. The construct was sequenced to verify its integrity.
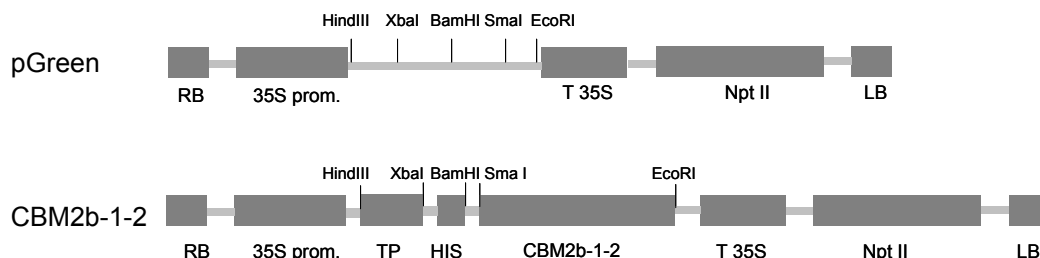

Figure 1. Transgene construct of the CBM2b-1-2 modules and the control for tobacco transformation. pGreen, the empty vector control with no CBM2b-1-2 insert, contained the selectable marker gene nptII, CBM2b-1-2, the construct for the expression of tandem CBM2b-1-2. Gene expression is driven by the 35S CaMV promoter (35S prom.). Flanking squares in the schematic constructs represent right border (RB) and left border (LB) of the T-DNA in *Agrobacterium* Ti (tumor-inducing) plasmid.

Tobacco transformation and regeneration

In vitro leaf explants of *Nicotiana tabacum* cv. Samsun NN were used for *Agrobacterium tumefaciens*-mediated transformation and were regenerated as previously described (Obembe et al., 2006c). Sixteen transformed plantlets were transferred to the greenhouse to generate mature plants.

RNA Gel Blot Analysis of Transgenic Plants

Total RNA was isolated from 3-5 g of transformed *in vitro* shoots as described elsewhere (Kuipers et al., 1995). Aliquots of 20 μg/lane were separated on a 1% formaldehyde agarose gel and blotted onto a Hybond-N nylon membrane (Amersham) by vacuum transfer in 0.4 N sodium hydroxide. The membrane was hybridized with a probe consisting of 60 ng of a $[\alpha^{32}P]$dCTP labeled SmaI-EcoRI fragment of CBM2b-1-2. Exposure of hybridization blot to imaging films was done as described previously (Obembe et al., 2006c).

Immunofluorescence detection of CBM2b-1-2 binding and Microscopy
The procedures for immunofluorescence detection of CBM2b-1-2 binding and microscopy were carried out as previously described (Obembe et al., 2006c).

Light microscopy
Three transgenic lines each from three expression classes, i.e. high, intermediate and low/none expressers of the CBM2b-1-2 (as determined by northern blot), were used for microscopic examination. Similarly, three independent transformants of the pGreen7k vector were examined as control. Stem sampling, fixation, dehydration, infiltration of embedded material, sectioning and microscopic examination were carried out as previously described (Obembe et al., 2006c).

Cryo-scanning electron microscopy (cryo-SEM)
Transgenic line 5 with high expression of the CBM2b-1-2 and one pGreen7k control plant were used for cryo-SEM examination. Stem sampling was the same as for the light microscopy. 6 mm-thick stem samples were mounted in a brass cylindrical sample holder with TBS (Tissue Freezing Medium EMS,

Washington, PA, USA). Cryo-sectioning and cryo-SEM examination were carried out as previously described (Obembe et al., 2006c).

Quantification of cell size and cell wall thickness
To quantify the micrographic images, files were opened with Image J software developed at the National Institute of Health, USA (http://rsb.info.nih.gov/ij/). The surface area of three hundred xylem cells was measured as shown by the inset in Figure 6A. Hundred cells per field of view were measured in three replicates. Similarly, cell wall thickness of one hundred and fifty cells was quantified as shown by the inset in Figure 6B. Fifty cells per field of view were measured in three replicates. Standard deviation of three measurements was determined.

Isolation of cell wall material and analysis of the cellulose content
Stem samples from transgenic line 5, a pGreen 7k vector control plant, and a wild type control plant were ground to a fine powder in liquid nitrogen. For each isolation, 1 g of this stem material was extracted in a 1 ml 50 mM Tris[HCl] pH 7.2 solution, containing 1% SDS, for 3 h at room temperature (RT) with continuous shaking. 10 mg of the different cell wall materials was hydrolyzed in 1 ml 2 M TFA. The TFA- insoluble cellulose was spun down and dissolved in 67 % sulphuric acid. The hydrolysis was performed in quadruplicate. Cellulose content was determined as previously described (Obembe et al., 2006a).

Results

CBM2b-1-2 binding is restricted to the vascular tissue of the stem
Immunofluorescence detection with CBM2b-1-2 binding to stems of the wild type tobacco plant was done in order to know *a priori* the particular tissues of the stem, where our introduced CBM2b-1-2 module would bind to. Indirect immunofluorescence micrographs (Fig. 2) revealed that CBM2b-1-2 binding is restricted to the secondary cell walls of stem vascular tissue: xylem cells and phloem fibres. There was no detectable fluorescence in the epidermis as well as in the cortical
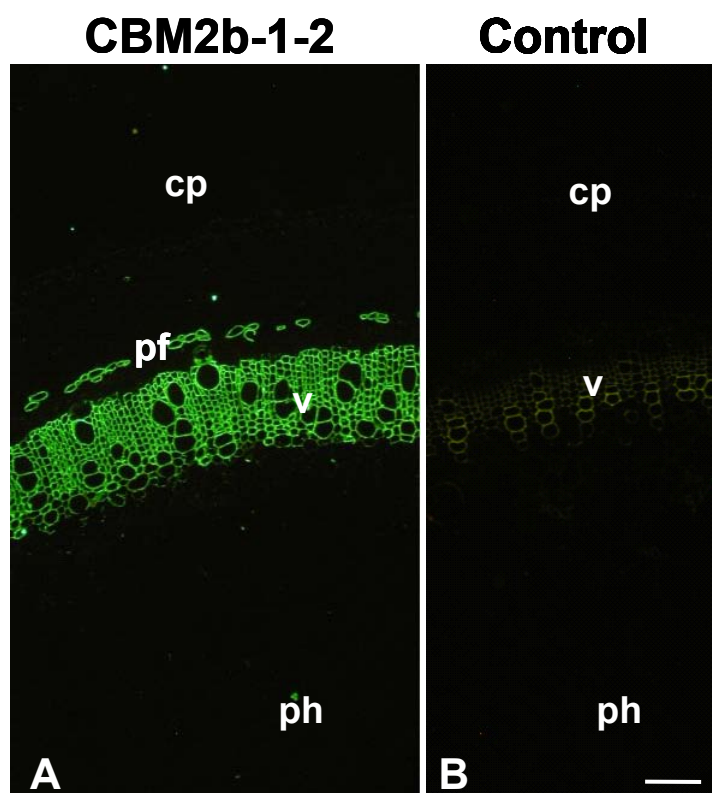
Figure 2. Micrographs showing the binding of CBM2b-1-2 to transverse sections of wild type tobacco stem using indirect immunofluorescence microscopy. The binding of CBM2b-1-2 is restricted to the secondary cell walls of stem vascular tissue. Pf = phloem fibres, cp = cortical parenchyma, v = vascular tissue, ph = pith parenchyma. Scale bar = 100 μm.

and pith parenchyma. The control (no CBM) however shows some weak autofluorescence in the xylem vessel cell walls. We therefore conclude, based on this observation, that the CBM2b-1-2 ligands are present in the vascular tissue of the stem.

Transgenic tobacco plants with tandem CBM2b-1-2 expression

To express the *C. fimi*-derived xylan-binding module in tobacco plants, we cloned the tandem CBM2b-1-2 gene into a binary plasmid pGreen 7k, the expression of which was driven by the CaMV 35S promoter. The plasmid pGreen 7k without the CBM gene was used as a vector control. Sixteen antibiotic-resistant tobacco transformants carrying the tandem CBM2b-1-2 transgene were regenerated and transferred to the greenhouse. Total RNA from leaves of the transgenic plants was isolated to analyze the expression level of the *C. fimi* tandem CBM2b-1-2 in each transgenic line by Northern blot analysis using the tobacco 18S ribosomal RNA gene as a control. Varying hybridization intensities with the transgenic plants revealed differential expression of the tandem CBM2b-1-2 gene in the individual plants. Based on the levels of expression, transgenic plants have been categorized into three classes as high, low and none expressers; the respective representatives for the classes are shown in Figure 3. Six plants each were classified as high and low expressers, whereas four
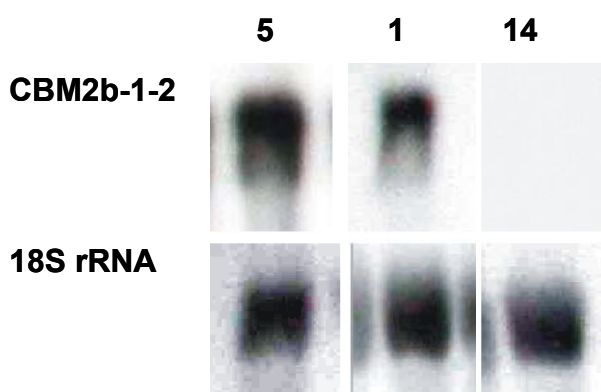

Figure 3. Transcript expression analysis of the CBM2b-1-2 gene in transgenic tobacco leaves. A differential transcript expression pattern is shown in the upper panel with the representative of each class in the transgenic line CBM2b-1-2. Lines 5, 1 and 14 represent high, low and none expressers, respectively. Lower panel shows RNA blots for the ribosomal RNA internal control with comparable intensities.

plants were classified as none expressers. It should be noted that these none expressers may include plants with very low RNA expression, which could not be detected. Attempts at purifying hexa-histidine-tagged proteins of the CBM29 modules using affinity purification with the hexa-histidine tag were not successful for reasons

previously discussed (Obembe et al., 2006c). Hence we did not make further attempts to purify the protein.

There was no morphological or developmental change in the transgenic plants when compared to the pGreen 7k vector control. The average plant height of the transgenic plants expressing the tandem CBM2b-1-2 gene at maturity was 71 cm, which was comparable to 75 cm average height for the pGreen 7k vector control plants. We also did not observe a particular trend in plant development with respect to stem elongation and flower formation. Slight differences in stem elongation, which were observed within individual plants expressing CBM2b-1-2, did not correlate with RNA expression levels.

Transgenic stems exhibit irregularly shaped cortical cells and enlarged xylem cells

To determine whether there were cellular events resulting from the expression of CBM2b-1-2 in the tobacco plant, we made light microscopic examinations of stem sections stained with toluidine blue. Three representatives of each expression class were examined. Figure 4B shows a micrograph revealing irregularly shaped cells of the cortex in the high CBM2b-1-2 expresser, whereas no such phenotype was observed for the pGreen 7k control (Fig. 4A). We did not observe the phenotype of
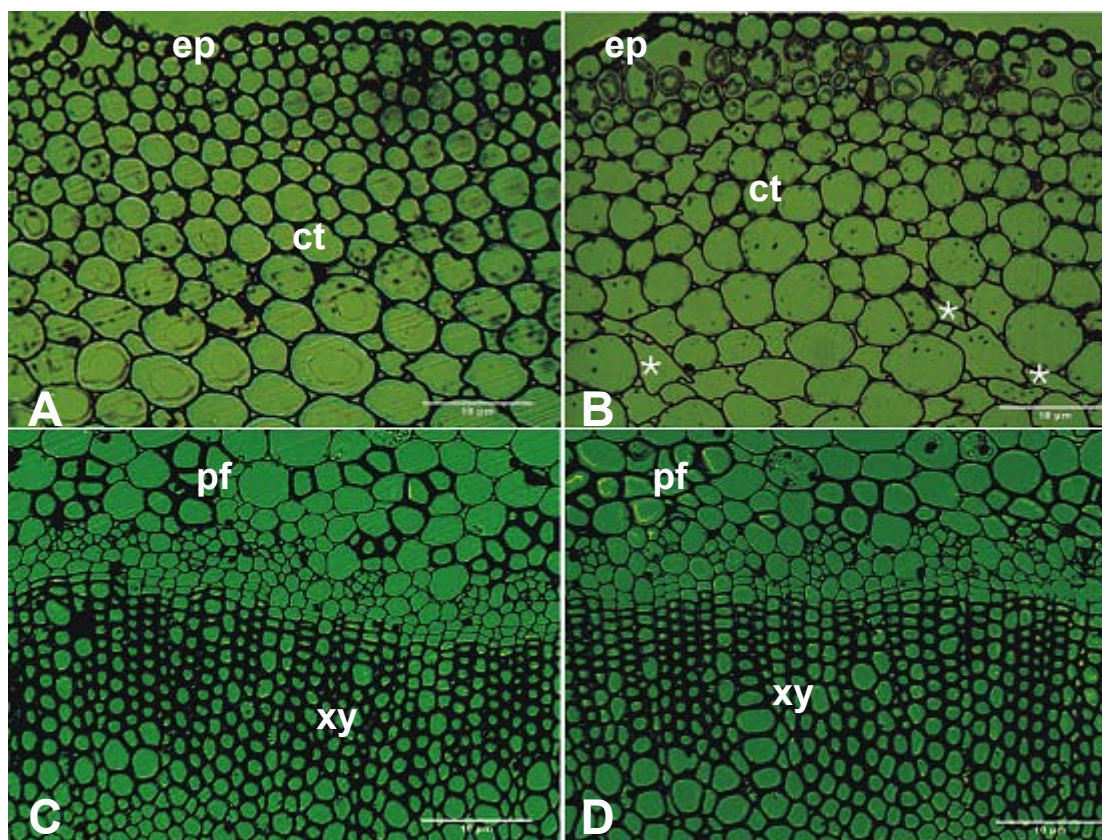

Figure 4. Cross sections of representative transgenic and control stems. Sections of transgenic line 5, high expresser of CBM2b-1-2 module (B) and (D) and empty pGreen7k control (A) and (C) were stained with toluidine blue. Figures 4 A and B show the cortex of the sections of the control and

CBM2b-1-2, respectively. Figures 4 C and D show the xylem of the stem sections of the control and CBM2b-1-2, respectively. ct = cortex, ep = epidermis, xy = xylem, pf = phloem fibre. Asterisks indicate irregularly shaped cortical cells. Scale bar = 10 μm

irregular cortical cells in the low and the none expressers. Apart from the irregular shape of the cells of the cortex, we observed an altered cell enlargement in the stem xylem tissues of the CBM2b-1-2 -expressing plants (Fig. 4D). The xylem cells are generally larger than those of the control plants (comparing 4C and 4D). Furthermore, it seemed that the phloem fibre cells in the control stems stained more intensely for lignin than those in the CBM2b-1-2-expressing plants. This is however not widespread as there were parts of the sections of the transgenic stems where the phloem fibre cell walls were considerably stained as well.

Cryo-Scanning Electron Microscopy and Quantification
Cryo-SEM microscopy was done with the stem samples in order to quantify the cellular observations made with the light microscopy. The micrographs revealed that the xylem cells of the CBM2b-1-2-expressing plants are larger than those of the control plants (Fig. 5). There was indication that the thickness of the cell walls of
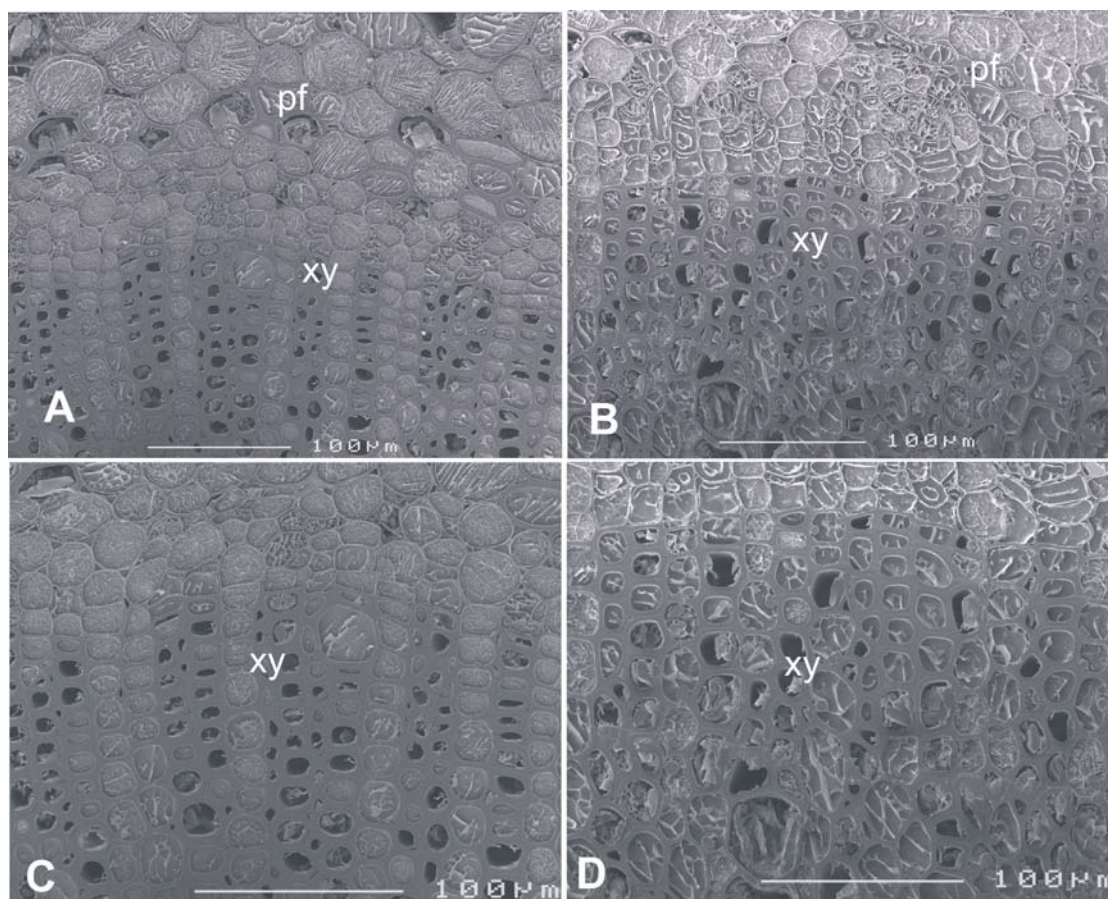

Figure 5. Scanning electron micrographs of stem xylem cells of the transgenic tobacco line 5 and the empty pGreen7k control, at x300 magnification. Micrographs are representatives of several fields of view. Figures 4C and 4D are enlarged by zooming in, in order to show the details of cell enlargement

and cell wall diameter. Empty pGreen7k control (A) and (C) and CBM2b-1-2 (B) and (D). xy = xylem, pf = phloem fibre. Scale bar = 100 μm

the xylem cells was thinner in the CBM2b-1-2-expressing plants than in the controls (comparing Fig. 5C to 5D). Both the cell size and the cell wall thickness were substantiated by quantification. Figure 6A shows that most of the xylem cells in the pGreen7k control stem (93%) grouped in the two small classes, whereas only 53% of the xylem cells of the tandem CBM2b-1-2 module-expressing stems grouped in these
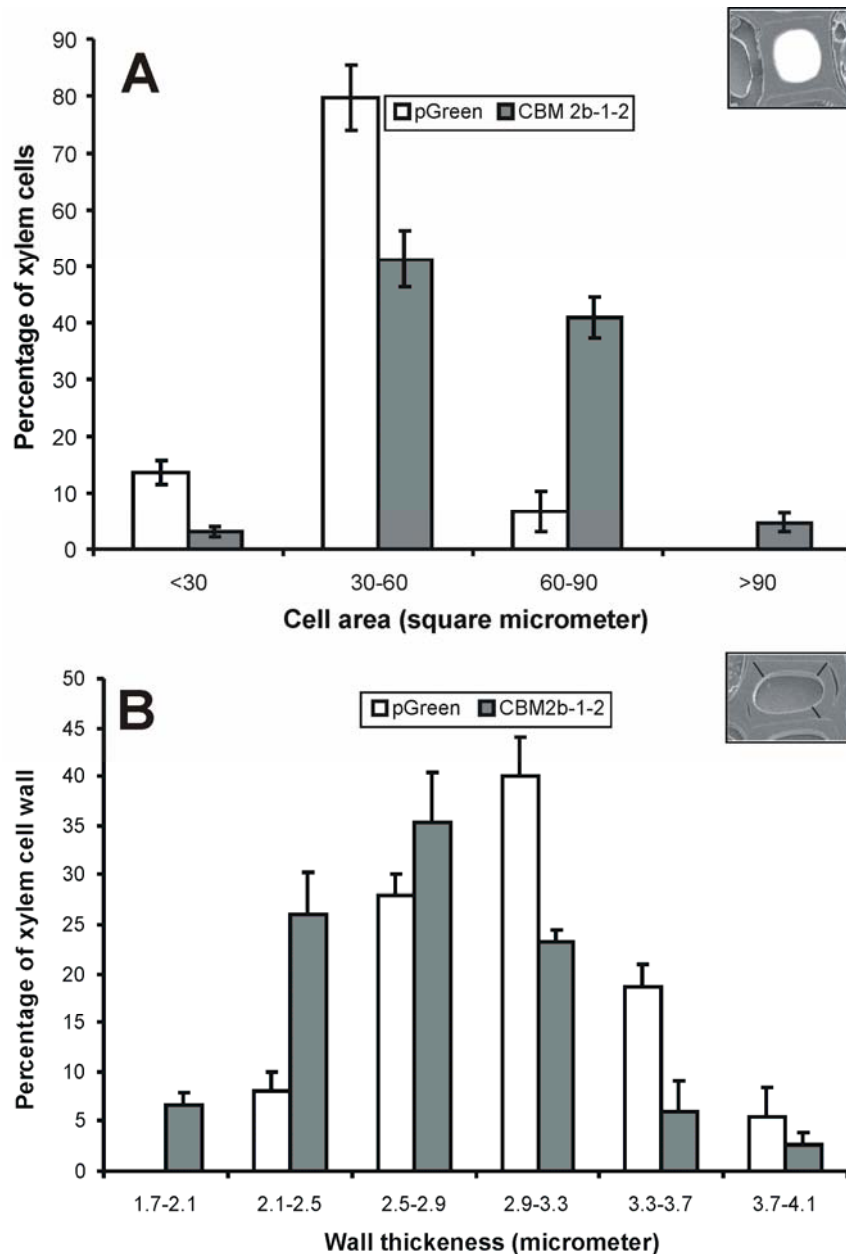

Figure 6. Cell size distribution (A) and cell wall thickness (B) of xylem cells in the stems of transgenic tobacco plants. Inset shows how the surface area measurement was taken. Surface areas of 300 cells were measured, 100 cells per field of view. Cell wall diameters of 150 cells were measured, 50 cells per field of view. Inset shows how the diameter of the cell walls was measured.

80

classes. It also shows that 47% of the stem xylem cells of the CBM2b-1-2-expressing plants grouped in the two large classes, whereas only 7% of the cells of the pGreen7k control stems grouped in these classes. We thus infer that the stems xylem cells of the CBM2b-1-2-expressing plants are larger than those of the pGreen7k control plants. Similarly, we quantified the thickness of the cell walls of the stem xylem cells of the CBM2b-1-2-expressing plants and the pGreen7k control plants as presented in Figure 6B. It was observed that 33% of the cell walls of the xylem cells of the CBM2b-1-2 module-expressing plants grouped in the two thinnest classes, whereas only 8% of the cell walls of the pGreen7k control stems grouped in these classes. Figure 6B also shows that only 10% of the cell walls of the stem xylem cells of the CBM2b-1-2-expressing plants grouped in the two thickest classes, whereas 25% of the cell walls of the xylem cells of the pGreen7k control stems grouped in these classes. Similarly as for the cell size, it can be concluded that the cell walls of the xylem cells of the CBM2b-1-2-expressing stems are thinner than those of the pGreen7k control stems.

Analysis of cellulose content
We have determined the cellulose content of the stem cell walls of transgenic tobacco line 5, the empty vector control and a wild type control (Figure 7). Four cell wall samples were independently hydrolyzed and assayed to determine the amount of crystalline cellulose in the stem cell walls. The analysis shows that there was no significant difference in the levels of cellulose in the stem cell walls of the transformant and the two control plants.

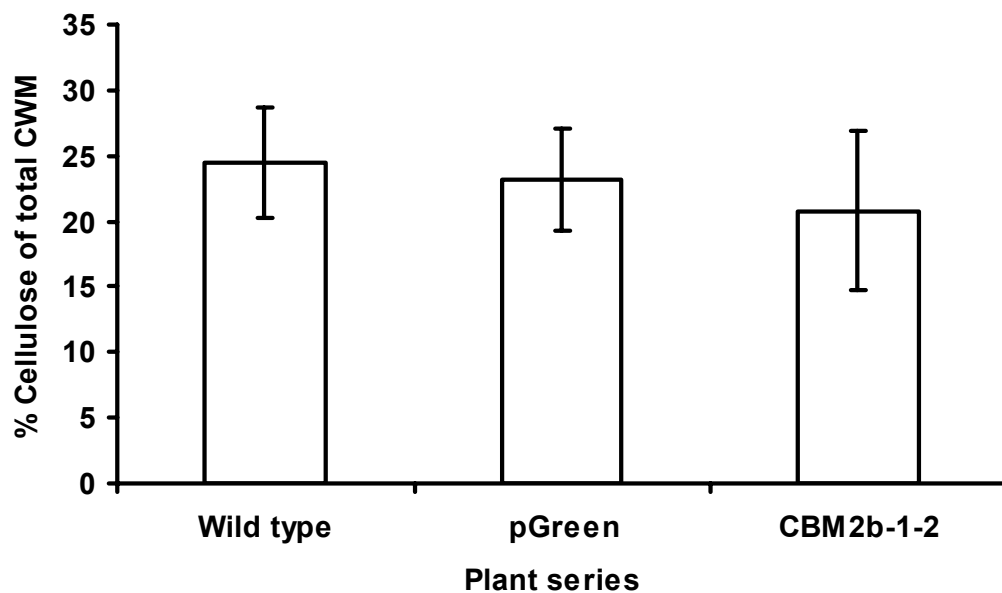**Figure 7.** Cellulose content in transgenic and control tobacco plants. Cellulose content of stem wall materials of transgenic line 5 and the two control plants was determined using a colorimetric assay. The cellulose levels are represented as the percentage of cellulose in total cell wall material. All measurements were performed as quadruplicate and standard deviation of the means are represented by the bars.

Discussion

It has been shown previously that tandem CBM29-1-2 has the capabilities of altering cellulose-hemicellulose networks of the cell walls *in planta* (Obembe et al., 2006c). Carbohydrate-binding modules can differ in the kinds of polysaccharides that they recognize (Boraston et al., 2004). The CBM29-1-2 has a broad range carbohydrate-binding specificity. It is promiscuous towards soluble glucomannan, galactomannan, β-glucan, and hydroxyethylcellulose (HEC), as well as insoluble forms of cellulose and mannan (Freelove et al., 2001; Charnock et al., 2002). The CBM29-1-2 was also able to interact with xyloglucan and different forms of xylans, but to a lesser extent (McCartney et al., 2004). In contrast, the CBM2b-1-2 protein is specific for xylan and, to a lesser extent, for cellulose (Bolam et al., 2001).

Ex-situ immunolabeling studies with CBM2b-1-2 revealed that the protein bound preferentially to the vascular tissue of wild type tobacco stem, which was consistent with its biochemical characterization. It is generally believed that xylan is deposited in the secondary cell wall (Timell, 1967; Awano et al., 2002), which predominates the vascular tissue. We have therefore targeted this less promiscuous tandem CBM2b-1-2 of *C. fimi* xylanase 11A to tobacco cell walls with a view to comparing its effect with that of the more promiscuous CBM29-1-2. Our results demonstrate that the tandem CBM2b-1-2 can also modify the tobacco cellular morphology, but to a lesser extent.

The expression of the CBM2b-1-2 modules in the tobacco cell wall altered cellular morphology of the transgenic plants, as reflected by the irregular shape of the cortical cells and altered cell enlargement of the xylem cells of the stems. The largely irregularly shaped cells of the cortex may have resulted from a combination of their mechanically weak cell walls (they have no secondary cell wall deposition) and altered cell expansion of the xylem cells. However, the deformation of the cortical cells of the CBM2b-1-2 expressing plants is less pronounced than that of the cortical cells of the CBM29-1-2 transformants. Additionally, the expression of the tandem CBM29-1-2 in tobacco led to altered structural morphology and altered development of the transgenic plants, whereas the expression of the tandem CBM2b-1-2 only had effect on cellular morphology and not on plant morphology. However, the structural alterations in both cases appeared not to interfere with cell wall biosynthesis to any large extent, in that there were no remarkable changes in cellulose contents.

Premised on the results of CBM2b-1-2-labelling studies on the wild type tobacco stem, we expected that the effect of targeting the CBM2b-1-2 protein to the cell walls would be more pronounced in the vascular tissue than in any other stem tissue. This turned out to be true with the altered cell expansion, which was restricted to the stem xylem cells of the high expressers. Owing to the fact that cell expansion precedes secondary cell wall formation (Cano-Delgado et al., 2000), it is unlikely that the interaction of CBM2b-1-2 with xylan alone is responsible for the observed altered expansion of xylem cells of the transgenic stem, because xylan is mainly deposited in the secondary wall. Possibly, the low affinity of CBM2b-1-2 to cellulose (Bolam et al., 2001) may contribute to the altered cell expansion.

The finding that CBM2b-1-2 did not label the cortex (Fig. 3) suggests that the deformation of the cortical cells is an indirect effect of CBM2b-1-2 expression. The weak walls of the cortical parenchyma cells may not have possessed the required tensile strength to resist the internal tension within the stem, as previously explained (Obembe et al., 2006c), and consequently they are compressed out of shape. Extensive discussion on altered cellular morphology upon expression of CBMs in plants has been previously provided (Obembe et al., 2006c).

Our results, taken together, indicate that the structural integrity of the cell walls of transgenic plants expressing the tandem CBM2b-1-2 has been compromised. The structural alterations caused by this somewhat promiscuous CBM were, however, much less severe than those caused by the more promiscuous tandem CBM29-1-2.

Acknowledgements

References

Awano T, Takabe K, Fujita M (2002) Xylan deposition on secondary wall of *Fagus crenata* fibre. Protoplasma **219:** 106-115

Bolam DN, Xie HF, White P, Simpson PJ, Hancock SM, Williamson MP, Gilbert HJ (2001) Evidence for synergy between family 2b carbohydrate binding modules in Cellulomonas fimi xylanase 11A. Biochemistry **40:** 2468-2477

Boraston AB, Bolam DN, Gilbert HJ, Davies GJ (2004) Carbohydrate-binding modules: fine-tuning polysaccharide recognition. Biochemical Journal **382:** 769-781

Cano-Delgado AI, Metzlaff K, Bevan MW (2000) The eli1 mutation reveals a link between cell expansion and secondary cell wall formation in Arabidopsis thaliana. Development **127:** 3395-3405

Charnock SJ, Bolam DN, Nurizzo D, Szabo L, McKie VA, Gilbert HJ, Davies GJ (2002) Promiscuity in ligand-binding: The three-dimensional structure of a Piromyces carbohydrate-binding module, CBM29-2, in complex with cello-and mannohexaose. Proceedings of The National Academy of Sciences of The United States of America **99:** 14077-14082

Ebringerova A, Heinze T (2000) Xylan and xylan derivatives - biopolymers with valuable properties, 1 - Naturally occurring xylans structures, procedures and properties. Macromolecular Rapid Communications **21:** 542-556

Freelove ACJ, Bolam DN, White P, Hazlewood GP, Gilbert HJ (2001) A novel carbohydrate-binding protein is a component of the plant cell wall-degrading complex of Piromyces equi. Journal of Biological Chemistry **276:** 43010-43017

Hellens RP, Edwards EA, Leyland NR, Bean S, Mullineaux PM (2000) pGreen: a versatile and flexible binary Ti vector for Agrobacterium-mediated plant transformation. Plant Molecular Biology **42:** 819-832

Ji Q, Oomen RJF, Vincken JP, Bolam DN, Gilbert HJ, Suurs L, Visser RGF (2004) Reduction of starch granule size by expression of an engineered tandem starch-binding domain in potato plants. Plant Biotechnology Journal **2:** 251-260

Kuipers AGJ, Soppe WJJ, Jacobsen E, Visser RGF (1995) Factors affecting the inhibition by antisense RNA of granule-bound starch synthase gene-expression in potato. Molecular & General Genetics **246:** 745-755

McCartney L, Gilbert HJ, Bolam DN, Boraston AB, Knox JP (2004) Glycoside hydrolase carbohydrate-binding modules as molecular probes for the analysis of plant cell wall polymers. Analytical Biochemistry **326:** 49-54

Obembe OO, Cifuentes C, Huigen D, Kieft H, Jacobsen E, Emons AMC, Visser RGF, Vincken J-P (2006a) Bioengineering cellulose-hemicelluose networks in plants (Chapter 2, This Thesis). PhD thesis. Wageningen University, Wageningen.

Obembe OO, Jacobsen E, Timmers J, Gilbert H, Blake AW, Knox JP, Visser RGF, Vincken J-P (2006b) Bioengineering cellulose-hemicelluose networks in plants (Chapter 3, This Thesis). PhD thesis. Wageningen University, Wageningen.

Timell TE (1967) Recent progress in the chemistry of wood hemicellulose. Wood Science and Technology **1:** 45-70

Expression of a putative carbohydrate-binding module from potato expansin modulates cell enlargement in transgenic tobacco plants

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Abstract

Expansins are believed to be involved in disrupting the non-covalent adhesion of cellulose to matrix polysaccharides, thereby promoting wall creep. We have targeted a putative potato expansin (EXPA) carbohydrate-binding module (CBM) to the cell walls of tobacco plants. Histological examinations and electron microscopy indicated that 30% of the xylem cells of the transgenic stems with high expression of the expansin CBM are wider (radial surface area) than those of the controls. Similarly, 37% of the xylem cells of the stems of the high expressers have thinner cell walls than those of the controls. There were no such phenotypes in the low and none expressers, as well as in the control plants. The transgenic tobacco plants expressing the potato expansin CBM did not exhibit marked changes in plant morphology. Analysis of cellulose content in the stem cell walls was similar for the high expresser of the transgene and the control plants. Nonetheless, our results taken together demonstrate that expansin CBM alone can bring about changes in the plant cell walls.

Introduction

Expansins are plant proteins discovered over a decade ago, which have the capacity to induce extensibility and stress relaxation of plant cell walls *in vitro* (McQueen-Mason et al., 1992; McQueen-Mason and Cosgrove, 1995). The proteins are thought to bind the interface between cellulose microfibrils and matrix polysaccharides, and facilitate wall expansion by catalyzing the disruption of hydrogen bonds between the two, thereby causing turgor-driven slippage between microfibrils and other components of the wall (McQueen-Mason and Cosgrove, 1995). Evidence for this is seen in its weakening effect on pure cellulose paper (a hydrogen-bonded network of glucans) without evidence of hydrolytic activity (McQueen-Mason and Cosgrove, 1994). Furthermore, a cucumber expansin (EXPA) caused rapid weakening of artificial composites made of bacterial cellulose and xyloglucan (Whitney et al., 2000). The authors found that weakening was stronger in the cellulose-xyloglucan composite material than in cellulose-only material. Studies with transgenic plants indicate that endogenous expansins are involved in regulating growth and developmental processes in which rearrangements in the cell wall are thought to be important (Cho and Cosgrove, 2000; Pien et al., 2001; Choi et al., 2003; Zenoni et al., 2004).

Based on their phylogenetic relationship, expansins and expansin-like proteins can be classified into four families (web site http://www.bio.psu.edu/expansins/arabidopsis.htm and (Kende et al., 2004)). The four protein families currently recognized are EXPA, EXPB, EXLA and EXLB. Members of the EXPA and EXPB families have been shown to possess *in vitro* weakening effect on cellulose paper. The amino acid sequences of the two expansin-like families EXLA and EXLB are divergent from EXPA and EXPB and no biological or biochemical function has been established for any member of these families. All members of the expansin superfamily consist of two domains: an N-terminal domain I that is distantly related to the catalytic domain of glycoside hydrolase family-45 (GH-45) and a C-terminal domain II that is distantly related to group-2 grass pollen allergens (Cosgrove, 2000a). Plants also have single-domain proteins known as immunoreactant plant natriuretic peptides (irPNPs), which have marked sequence similarity with domain I of the expansins (Ceccardi et al., 1998; Ludidi et al., 2002; Gehring and Irving, 2003). The irPNP-like molecules can enhance osmoticum-dependent water transport across plasma membranes (Maryani et al., 2001; Pharmawati et al., 2001) and hence increases cell turgor (Ludidi *et al.*, 2002). This has led to the idea that the irPNP-like molecules function cooperatively with expansins in bringing about cellular expansion. Similarly, rice possesses a group of truncated proteins that have high similarity to the carboxy-terminal domain II of expansins but lack the domain I part of these proteins (Li et al., 2003). However unlike the irPNP-like molecules, the function of these domain II-like proteins is unknown.

In theory, each module of a particular modular protein such as expansins is capable of folding independently into a functioning protein. Therefore one would expect to gain more insight into the function of the C-terminal domain II of the expansins by over-

expressing it in a suitable host. It has been proposed that expansin's domain II share similarity with cellulose-binding domains (Shcherban et al., 1995), leading to the speculation that it is probably a carbohydrate-binding module (CBM) (Cosgrove, 2000a; Barre and Rouge, 2002). However, such binding properties have not been demonstrated experimentally till date, because of the difficulty in producing active recombinant expansin, let alone its putative CBM. This explains why the protein has not been formally classified as a carbohydrate-binding module (web site http://www.bio.psu.edu/expansins/arabidopsis.htm). Hence, an over-expression study *in planta* remains a credible alternative means of verifying its speculated role in polysaccharide binding.

We have previously shown that separate CBMs derived from microbial proteins can bring about changes in tobacco cell walls (Obembe et al., 2006b; Obembe et al., 2006c). Our previous results demonstrated that the fungal tandem CBM29-1-2 with broad range specificity (promiscuous) produced more severe phenotypes in the transgenic plants than a less promiscuous tandem CBM2b-1-2 from a bacterial xylanase. While the CBM29-1-2 is promiscuous towards most cellulosic- and hemicellulosic-polysaccharides, the CBM2b-1-2 has affinity just for xylan and cellulose. Plants also contain CBMs, of which CBM22 and CBM43 are examples (http://afmb.cnrs-mrs.fr/CAZY/). Members of the CBM22 family have xylan-binding function (Charnock et al., 2000), while a callose-binding function has been demonstrated for a member of the CBM43 family (Barral et al., 2005).

In this study, we have targeted the expression of a putative potato expansin CBM to tobacco cell walls. The expression of the gene encoding this CBM was driven by 35S cauliflower mosaic virus (CaMV) promoter. In this paper, we describe the cellular events that are associated with the heterologous over-expression of a potato expansin CBM in transgenic tobacco plants. Our observations indicate that the expansin CBM alone can alter cell wall structure.

Materials and methods

Preparation of constructs

The EST clone (EST accession number BG097738) of the putative expansin CBM was obtained from an EST library of potato leaves and petioles. Standard polymerase chain reaction (PCR) was performed on the EST fragment encoding the expansin CBM, using primers that included BamHI and EcoRI restriction sites (5'- cgcggatccggtggaataaggtttacaattaacgg-3' and 5'- cttcttttcgaattcactatttaaactctg -3'; the BamHI and EcoRI sites are underlined, respectively). The three bases highlighted in bold type represent the stop codon. The amplified 319 base pairs fragment of the potato expansin CBM was digested with BamHI and EcoRI (Invitrogen, The Netherlands) and cloned into a similarly digested binary vector pGreen 7k (Hellens et al., 2000). The cloning of the fragment was done as described previously (Obembe et al., 2006b; Obembe et al., 2006c). The construct assembly as shown in Figure 1 contains a sequence encoding a transit peptide (for targeting to the cell wall) and a hexa-histidine epitope tag (Obembe et al., 2006c). The control construct did not contain the CBM gene, the transit peptide and the epitope tag. The construct was sequenced to verify its integrity.
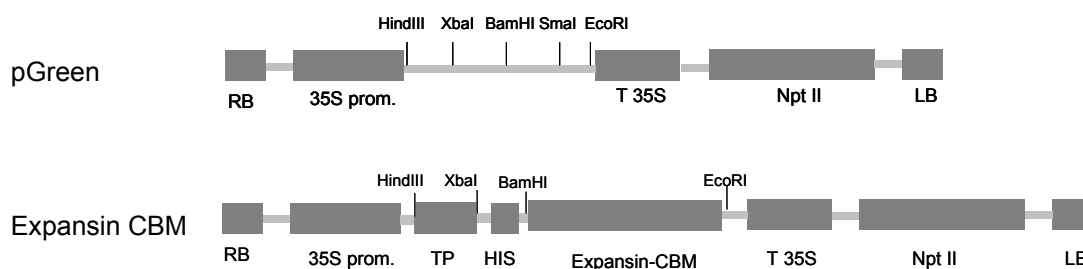
Figure 1. Transgene constructs of the potato expansin CBM and the pGreen 7k empty vector control for tobacco transformation. The control has only the selectable marker gene nptII but no expansin CBM insert. Gene expression is driven by the 35S CaMV promoter (35S prom.). Flanking squares on each line represent right border (RB) and left border (LB) of the T-DNA in *Agrobacterium* Ti (tumor-inducing) plasmid.

Sequence alignment and homology modeling

The program DNA-Star was used to compare the amino acid sequences of a pollen allergen Phl P2 of timothy grass (1WHO) and domain II sequences of *Arabidopsis* expansins EXPA1 (AAG60095), EXPB1 (AY084478), EXLA1 (AY058142), EXLB1 (TAIR accession 2130444), and the potato expansin CBM. Multiple amino acid sequence alignment was based on Clustal W, with gap penalties of 10 and a PAM250 matrix. The amino acid sequences of the representatives of the four expansin families above were obtained from the expansins web site (http://www.bio.psu.edu/expansins/arabidopsis.htm). This site was linked to the ExPASy (Expert Protein Analysis System) proteomic server (http://us.expasy.org/), designed for detecting protein modular domains. The alignment was manually edited for structurally homologous (according to Risler's matrix) residues occurring in the CBM.

An alignment, based on Clustal W, of the amino acid sequence of the potato expansin CBM and the Phl P2, together with SWISS-MODEL Version 36.0003 program (available from http://swissmodel.expasy.org//SWISS-MODEL.html) (Peitsch, 1995; Guex and Peitsch, 1997; Schwede et al., 2003) were used for homology modeling, in which the atomic coordinates of Phl P2 (1WHO) served as a template.

Tobacco transformation and regeneration

The expansin CBM construct and the empty vector control construct were used for *Agrobacterium tumefaciens*-mediated transformation of *in vitro* leaf explants of *Nicotiana tabacum* cv. Samsun NN, which were regenerated as previously described (Obembe et al., 2006b; Obembe et al., 2006c). Twelve transformed plantlets were transferred to the greenhouse to generate mature plants.

Semi-quantitative RT-PCR Analysis of Transgenic Plants

Total RNA isolation and first strand cDNA synthesis were performed as described previously (Obembe et al., 2006a). The first strand cDNA was then used as template in standard PCR using construct-specific primers. The forward primer, SPFOR 5'- agcttatgggaagtggta -3', was specific for the signal peptide while the reverse primer, HISREV 5'- tccgtgatggtgatggt- 3', was specific for the hexa-histidine tag. Rubisco-specific primers, RUBFOR 5'- cagaaatcatcaggaaaggaaaca - 3' and RUBREV 5'- tctcttcacgttttcttgctcttg - 3' were used to amplify rubisco cDNA, as internal control.

Light Microscopy
Three individual plants from the high expressing and two plants from the low/none expressing class were used for microscopic examination. The empty pGreen7k transformants were used as the control.

Stem sampling, fixation, sectioning and microscopic examination were done as described previously (Obembe et al., 2006b; Obembe et al., 2006c).

Cryo-scanning electron microscopy (cryo-SEM)
Transgenic line 16 with high expression of the expansin CBM and one pGreen7k control plant were used for cryo-SEM examination. The procedure was as described previously (Obembe et al., 2006b; Obembe et al., 2006c). To quantify the micrographic images, files were opened with Image J software developed at the National Institute of Health, USA (http://rsb.info.nih.gov/ij/). Measurements of cell surface area and cell wall thickness of xylem cells were taken as previously described (Obembe et al., 2006b; Obembe et al., 2006c).

Isolation of cell wall material and analysis of the cellulose content
Stem samples from transgenic line 16, pGreen 7k vector control and a wild type control plant were separately ground to a fine powder in liquid nitrogen. Cell wall isolation and cellulose content determination were performed as previously described (Obembe et al., 2006a; Obembe et al., 2006c).

Results

Characteristics of the putative potato expansin CBM

The size of a complete expansin protein is about 25-28 kDa. The potato CBM that was targeted to tobacco cell wall was about 15 kDa (133 amino acids) in size, including the transit peptide and the hexa-histidine epitope tag. A blast search with the amino acid sequence of the EST clone of the potato expansin CBM revealed that it has high homology with the *Arabidopsis* expansin 18, which belongs to the EXPA family. We were faced with the question of which part of the sequence should be used for engineering a construct expressing a functional domain II of the potato expansin. Meanwhile, the putative CBM of an EXPB family Lol P2 from rye grass was found to have about 50% sequence identity with a pollen allergen, Phl P2 from timothy grass (Cosgrove, 2000b), whose structure was solved (De Marino et al., 1999). An amino acid alignment with the potato expansin EST clone and Phl P2 was made, in order to deduce the start of the potato expansin CBM more precisely. The 3-D structure of Phl P2 starts with Val27 (Val1 in the alignment presented in Figure 2), which is five
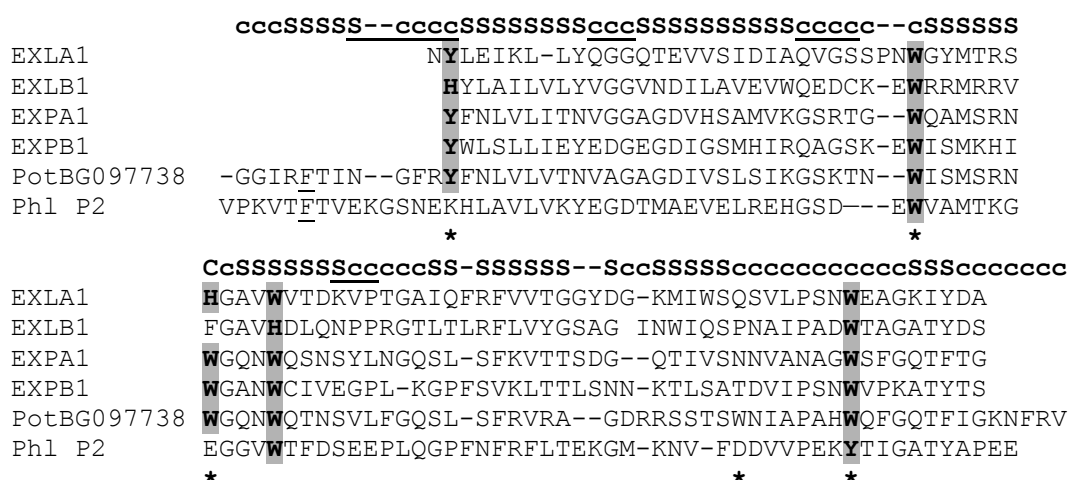
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            cccSSSSS--ccccSSSSSSSSScccSSSSSSSSSSSccccc--cSSSSSS
EXLA1               NYLEIKL-LYQGGQTEVVSIDIAQVGSSPNWGYMTRS
EXLB1               HYLAILVLYVGGVNDILAVEVWQEDCK-EWRRMRRV
EXPA1               YFNLVLITNVGGAGDVHSAMVKGSRTG--WQAMSRN
EXPB1               YWLSLLIEYEDGEGDIGSMHIRQAGSK-EWISMKHI
PotBG097738  -GGIRFTIN--GFRYFNLVLVTNVAGAGDIVSLSIKGSKTN--WISMSRN
Phl P2       VPKVTFTVEKGSNEKHLAVLVKYEGDTMAEVELREHGSD—--EWVAMTKG
                         *                                   *
            CcSSSSSSSccccccSS-SSSSS--SccSSSSSccccccccccccSSSccccccc
EXLA1       HGAVWVTDKVPTGAIQFRFVVTGGYDG-KMIWSQSVLPSNWEAGKIYDA
EXLB1       FGAVHDLQNPPRGTLTLRFLVYGSAG INWIQSPNAIPADWTAGATYDS
EXPA1       WGQNWQSNSYLNGQSL-SFKVTTSDG--QTIVSNNVANAGWSFGQTFTG
EXPB1       WGANWCIVEGPL-KGPFSVKLTTLSNN-KTLSATDVIPSNWVPKATYTS
PotBG097738 WGQNWQTNSVLFGQSL-SFRVRA--GDRRSSTSWNIAPAHWQFGQTFIGKNFRV
Phl P2      EGGVWTFDSEEPLQGPFNFRFLTEKGM-KNV-FDDVVPEKYTIGATYAPEE
                *                          *     *
```

Figure 2. Amino acid sequence alignment of At-EXPA1, At-EXPB1, At-EXLA1, At-EXLB1 (all four from *Arabidopsis thaliana*), Phl p2 (pollen allergen from *Phleum pratense*) and the putative potato

expansin CBM (EST accession number BG097738). Conserved aromatic residues are shaded and shown in bold letters. Secondary structure elements of Phl P2 and of the putative potato cellulose-binding domain are indicated with letter 'S' and 'c' above the sequences. Asterisk denotes exposed aromatic residues and histidine on the modeled structure. Underlining of secondary structure elements indicates that this region does not have a good match with the 3D structure of the template used for modeling.

amino acids before the conserved Phe6 (underlined). We deduced an amino acid sequence (96 amino acids) for a potentially functional putative CBM of the potato expansin, starting with Gly1, four positions before the conserved Phe5 (underlined). We particularly singled out Gly1 as the starting point because it is directly preceded by an exposed two-basic residue RR sequence (data not shown), which is a conserved cleavage signal between domain I and domain II of expansins (Barre and Rouge, 2002). An amino acid sequence alignment of the potato expansin CBM, with four Arabidopsis expansin CBMs (representing each of the four classes of the expansins family), and a pollen allergen Phl P2 from Timothy grass, was made (Figure 2). The amino acid sequences of the four representatives above were obtained from a web site (http://www.bio.psu.edu/expansins/arabidopsis.htm), and correspond to the domain II or CBM as determined by the ExPASy proteomic server (http://us.expasy.org/). These four sequences are shorter than the sequence we deduced for potato CBM, which explains the gaps left in the alignment at the beginning of these sequences. We have incorporated a number of extra amino acids at the N-terminal side to reduce the risk of an incorrectly folding domain, when expressing the construct in tobacco. The alignment information revealed that the potato CBM shared varying homologies with the CBMs of the four Arabidopsis expansin families; 64% identity with the CBM of EXPA1, 24% with EXPB1, 28% with EXLA1 and 26% with EXLB1. Furthermore, the alignment result revealed that aromatic amino acids (tryptophan and tyrosine) are mostly conserved in the different sequences particularly among the four expansins families. Our particular interest in the aromatic residues was premised on their involvement in carbohydrate binding (Toone, 1994 and reviewed in Boraston et al., 2004).

We have constructed a putative 3D-structure for the potato expansin CBM by homology modeling using the structure of Phl P2 as a template. This was with a view to revealing the location of its conserved aromatic residues. The modeled 3-D structure of the potato expansin CBM consisted of β strands and coils (Fig. 3). Accompanied information on the modeled CBM revealed that the putative structure had 85% identity to the template Phl P2. The identical regions include all regions containing the eight β strands (indicated in Figure 2 with letter 'S'). Only four coil-constituting regions of the model are structurally divergent to Phl P2 as indicated in Figure 2 by underlining of the secondary structure elements. All aromatic amino acid residues (tyrosine, tryptophan) of the sequence except one are exposed. The histidine residue was also visualized because of its ability to mediate pH-dependent binding of proteins to carbohydrates (Linder et al., 1999). One side of the modeled CBM
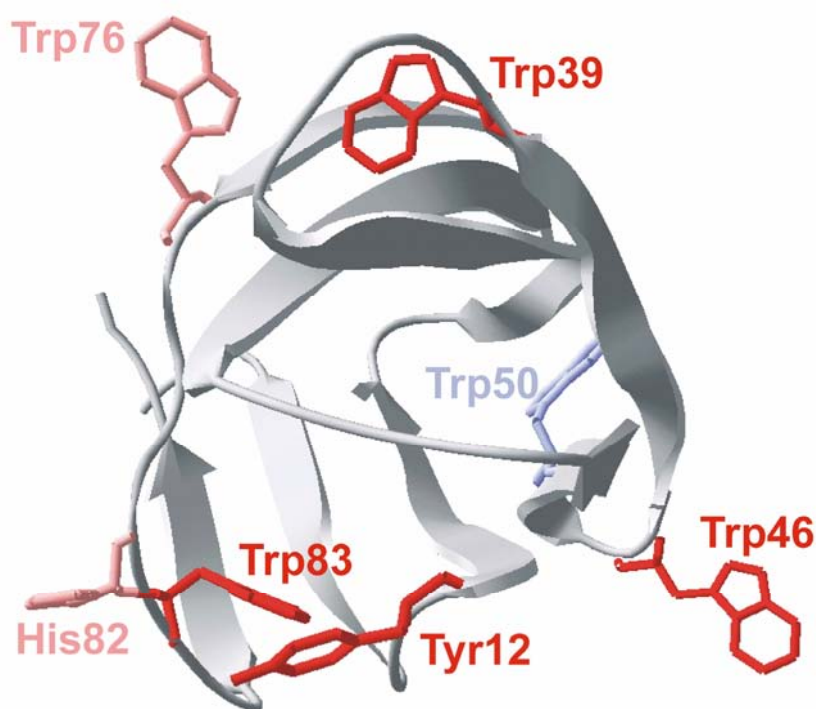
Figure 3. Putative 3-D structure of potato expansin CBM, obtained by homology modeling, using the 3-D structure of pollen allergen Phl P2 as template. Conserved aromatic residues, which are exposed and might be involved in polysaccharide binding, are: Trp39, Trp46, Trp76, Trp83, Tyr12 and His82. Trp50 is mostly buried; hence it is indicated with light colour. Side chains of none conserved residues His82 and Trp76 are also indicated in light colour. Conserved residues are presented in dark colours.

revealed a patch of three exposed residues, Tyr12, His82, and Trp83. It should be noted from the alignment result, however, that the His82 is not conserved. This therefore raises the question as to which extent of it is involved in binding. At the opposite side of the protein is a straight line of exposed aromatic residues Trp39, Trp46, and Trp76. It should be noted as well that, even though, Trp76 is not conserved across the four families, it is quite conserved within the EXPA family (data not shown). The well-conserved Trp50 is mostly buried, and therefore its involvement in binding is not very likely. It may have a role in maintaining protein structure through hydrophobic interactions.

Characterization of tobacco transformants expressing the putative potato expansin CBM

Twelve antibiotic-resistant tobacco transformants carrying the expansin CBM transgene were regenerated and transferred to the green house. RNA was isolated from leaves of the transgenic plants to analyze the expression level of the expansin CBM in each transgenic line by semi-quantitative RT-PCR analysis, using the constitutively expressed rubisco gene as an internal control. Varying amplification

intensities of the different transgenic plants revealed differential expression of the
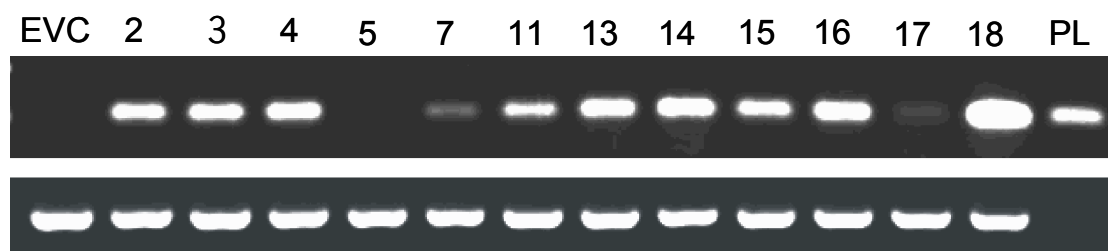
| EVC | 2 | 3 | 4 | 5 | 7 | 11 | 13 | 14 | 15 | 16 | 17 | 18 | PL |

Figure 4. Semi quantitative RT-PCR analysis of the selected transformants and the wild type leaf RNA. The upper panel shows the product amplified using the hexa-histidine tag-specific forward primer and pGreen construct-specific reverse primer. The lower panel shows the product amplified using specific primers for rubisco, as internal control. Plant lines 5, 7, and 17 are classified as low/none expresser, because of low or none visible intensities. The rest are classified as high expressers. EVC = empty vector negative control, PL = CBM-containing plasmid, as positive control.

potato expansin CBM gene in the individual tobacco transformants, thus making it possible to categorize them into two classes, as high and low/none expressers (Figure 4). Transgenic lines 5, 7 and 17 represent the low/none expressing transformants, while the remaining nine lines represent the high expressers. The detection of the introduced CBM using affinity purification with the hexa-histidine tag was not considered feasible for reasons previously discussed (Obembe et al., 2006c). There was no obvious phenotype in the transgenic plants when compared to the pGreen 7k vector control. The average plant height of the transgenic plants expressing the potato expansin CBM gene at maturity was 70 cm, which was comparable to 75 cm average height for the pGreen 7k vector control plants. There was no particular trend in plant development with respect to stem elongation and flower formation, as observed previously (Obembe et al., 2006c).

Transgenic stems exhibit enlarged xylem cells and thin cell walls
To examine cellular events resulting from the expression of the potato expansin CBM in the tobacco plant, we made light microscopic examinations of stem sections from three representatives from the high expressers and two from the low/none expressers stained with toluidine blue. Figures 5A and 5B show micrographs revealing relatively larger xylem cells in a representative high expresser of the potato CBM than those of the pGreen7k control. Furthermore, the light microscopic examinations of the stained sections indicated that the cell walls of the phloem fibres of the transgenic plants were thinner than those of the control. Other cell types of the transgenic stems were the same as those of the control. It is also worth mentioning that there was no intermediate effect with the two low/none expressers, as they were comparable with the control plants.
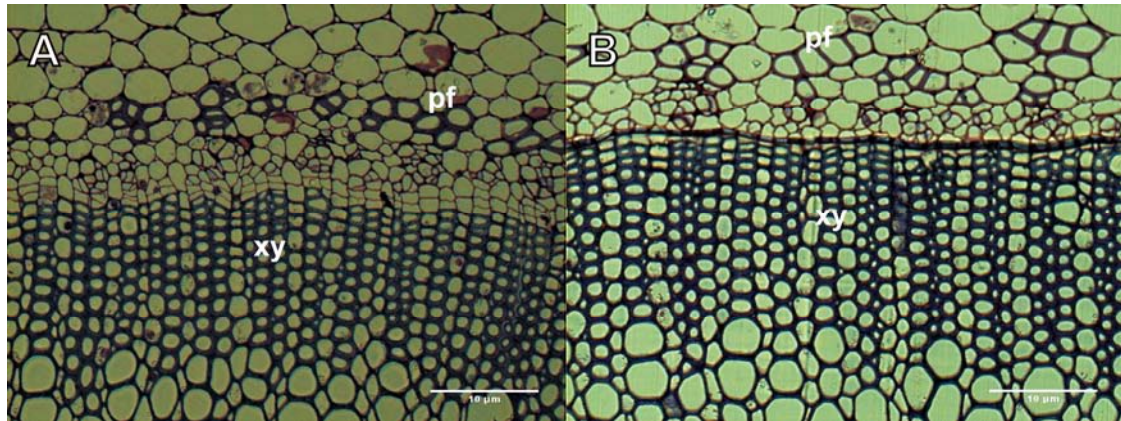
Figure 5. Cross sections of representative transgenic and control stems. Sections of empty pGreen7k control (A) and transgenic line 16, high expresser of potato expansin CBM (B) were stained with toluidine blue. A and B show the vascular tissue, phloem fibres and xylem cells of the stem sections of the control and the expansin CBM high expresser, respectively. Pf = phloem fibre, xy = xylem.

Cryo-scanning electron microscopy and quantification

Cryo-SEM was done with the stem samples in order to quantify the cellular observations made with the light microscopy. The micrographs revealed that the xylem cells of the transgenic stem of line 16 (Fig. 6B) were larger than those of the control stems (Fig. 6A).
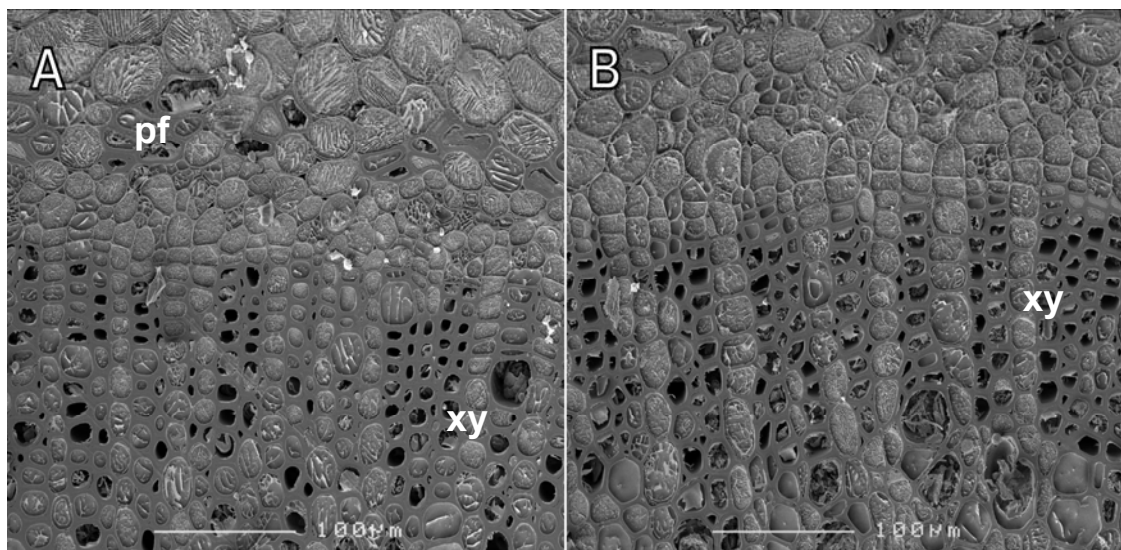

Figure 6. Scanning electron micrographs of stem vascular tissue of the empty pGreen7k control (A) and the transgenic tobacco line 16 (B), at x300 magnification. Micrographs are representatives of several fields of view. Pf = phloem fibre, xy = xylem.

To validate our SEM observations, we subjected the images to quantitative measurements. Figure 7A shows that 40% of the xylem cells in the pGreen7k control stem were grouped in the small class, whereas only 10% of the xylem cells of the transgenic stems grouped in this class. It also shows that 25% of the xylem cells in the transgenic stems grouped in the large class, whereas only 5% of the cells of the

pGreen7k control stems grouped in this class. There was no large difference between
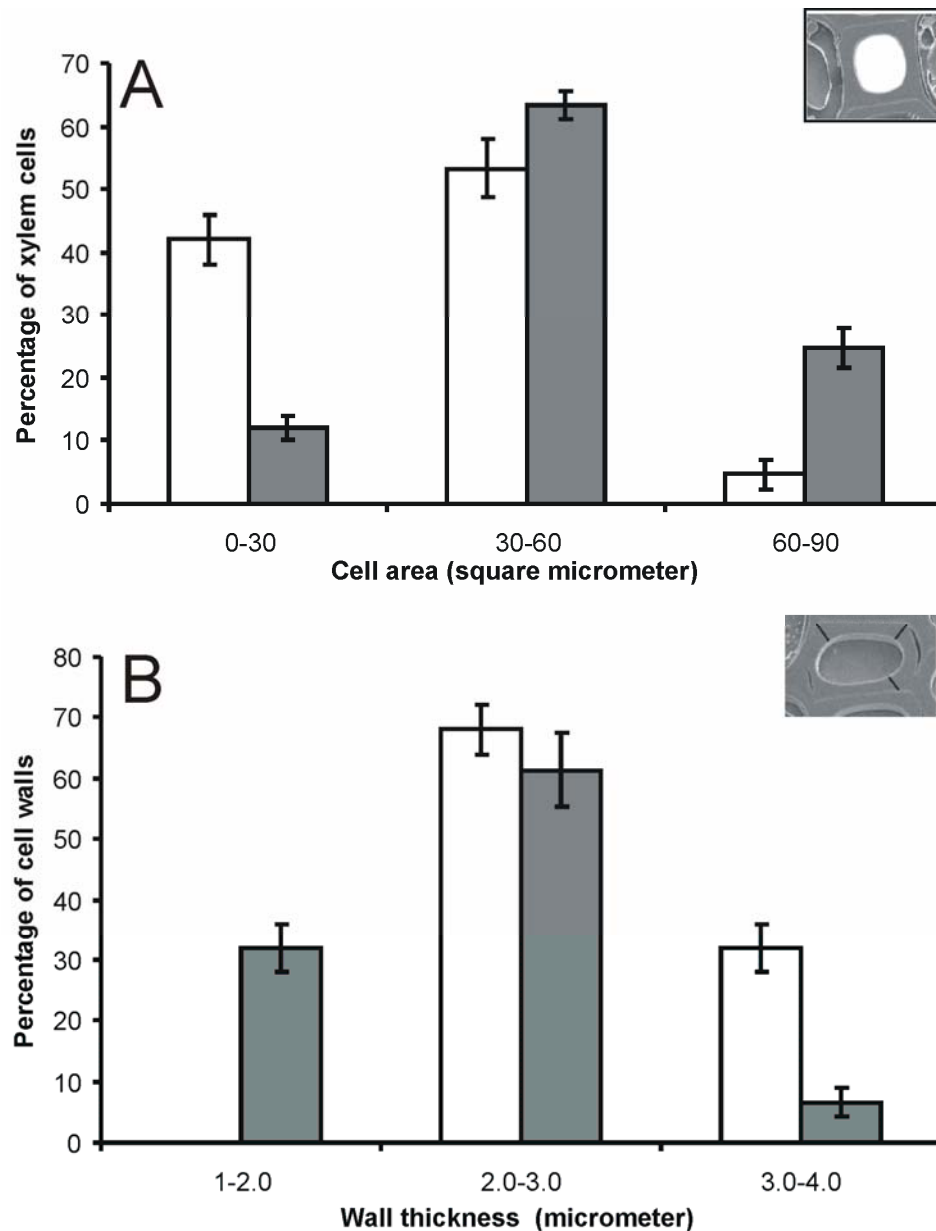

Figure 7. Cell size distribution (A) and cell wall thickness (B) of xylem cells in the stems of transgenic tobacco plants. Inset shows how the surface area measurement was taken. Surface areas of 300 cells were measured, 100 cells per field of view. Cell wall diameters of 150 cells were measured, 50 cells per field of view. Inset shows how the cell wall thickness was measured.

transgenic and the control plants, with respect to the percentage of xylem cells that were grouped in the medium class. We thus can infer that the stem xylem cells of the potato expansin CBM-expressing plants were larger than those of the pGreen7k control plants. Similarly, for cell wall quantification, we measured the thickness of the cell walls of the xylem cells of the transgenic and the pGreen7k control plants as presented in Figure 7B. We observed that 32% of the cell walls of the stem xylem cells of the transgenic plants expressing the expansin CBM grouped in the thin class, whereas 0% of the cell walls of the pGreen7k control stem xylem cells grouped in this

class. Figure 7B also shows that only 5% of the cell walls of the stem xylem cells of the transgenic plant grouped in the thick classes, whereas 32% of the cell walls of the pGreen7k control stem xylem cells grouped in same class. Similarly as for the cell size, there was no difference between the transgenic and the control, with respect to the distribution of cell wall thickness in the medium class. We can also infer from this result that the thickness of the cell walls of the stem xylem cells of the transgenic tobacco plants expressing the potato expansin CBM are thinner than those of the pGreen7k control plants.

Cellulose content of transgenic stems is unaltered
We have determined the cellulose content of the stem cell walls of the expansin CBM-expressing plant and the control plants. Colorimetric assays were performed on four independently hydrolyzed cell wall samples from transgenic tobacco line 16, the empty vector control, and a wild type control (Figure 8). It seems that the cellulose content of the transgenic plant is lower than that of the control plants, but given the large variation in the measurements (due to heterogeneity of the fibrous cell wall material of the stem) the differences are not significant. Meanwhile, it has been suggested that expansins may play a role in the assembly of the cell wall by affecting cellulose synthesis or deposition (Zenoni et al., 2004).
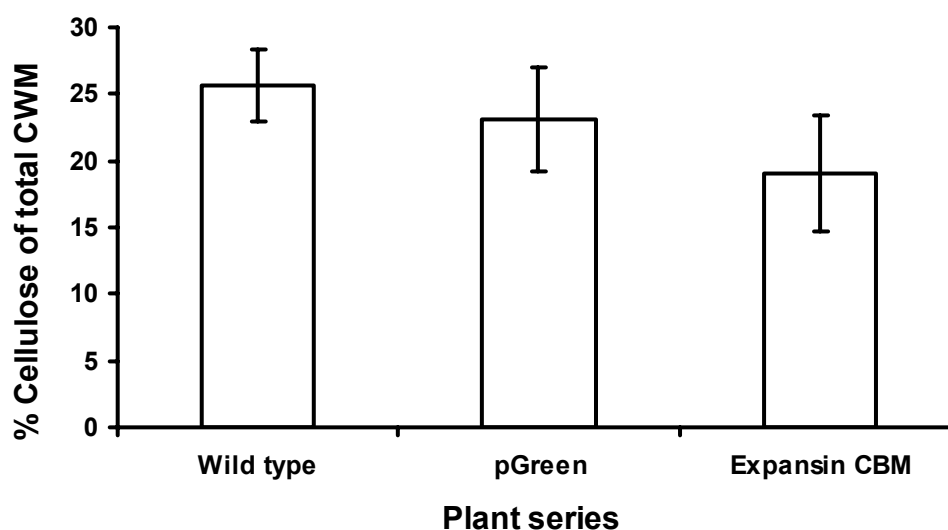

Figure 8. Cellulose content in transgenic tobacco plants. Cellulose content of stem wall material of transgenic line 16 was determined using a colorimetric assay. The cellulose levels are represented as the percentage of cellulose in total cell wall material. All measurements were performed as quadruplicate and standard deviation of the means are represented by the bars.

Discussion

We have over-expressed a putative CBM of a potato expansin in tobacco, to investigate whether expansin's domain II can bring about changes in the cell wall on its own, with a view to understanding the mode of action of the complete protein

better. Results of the investigation are discussed with respect to its binding specificity and its ability to alter cell wall structure.

The modeled three-dimensional structure of the putative potato expansin CBM showed that most of its conserved aromats were located on the surface. The patch of three aromatic residues on one side and the linear strip of another three aromatic residues on the opposite side of the protein are indicative of polysaccharide binding property. Homology modeling of the 3-D structure of the C-terminal domain II of the expansins has been possible with the resolution of the 3-D structure of Phl P2 (De Marino et al., 1999). The domain structure of C-terminal domain II of Lol PI (EXPB) had been modeled by others using the Phl P2 as template (Cosgrove, 2000a; Barre and Rouge, 2002). That model also revealed that most of the conserved aromatic residues (tryptophan and tyrosine) were exposed around a linear strip and a groove (Barre and Rouge, 2002).

Even though our *in planta* characterization of the putative CBM is not conclusive, with respect to its binding specificity, we observed the effect of over-expression of the protein in the cellulose- and xylan-rich vascular tissue. This is consistent with the speculation that it binds at the interface between cellulose microfibrils and hemicellulosic polysaccharides (Whitney et al., 2000), including xylan (McQueen-Mason and Cosgrove, 1995).

We observed an abnormal enlargement (radial expansion) of cells of the xylem and phloem fibre cells in the high expressing transgenic tobacco plants. Unlike the microbial CBMs, the effect of the expansin CBM seemed to be restricted to the vascular tissue. These observations are consistent in the three independent high-expressing lines examined, suggesting that they are related to the expression of the transgene rather than to other factors. Apart from cell enlargement, the cell walls of the vascular tissue were observed to be thinner in the transgenics as compared to the control plants. As discussed previously (Obembe et al., 2006c), cell wall thinning can cause cell wall weakening, as a consequence of uncontrolled disruption of cellulose-hemicellulose networks and the associated abnormal cell expansion. Despite these cellular phenotypes, transgenic plants exhibit normal morphology and development. Altered expansion of the cortical and epidermal cells was reported for transgenic tomato stems with high levels of a recombinant cucumber expansin (Rochange et al., 2001). However, in contrast to our observation of normal growth phenotype of the transgenic plants, high levels of over-expression or heterologous expression of expansin genes in transgenic plants was shown to lead to impaired growth phenotypes (Rochange et al., 2001; Choi et al., 2003). A sense expression of AtEXP10 was also shown to enhance leaf growth in the transgenic *Arabidopsis* plants (Cho and Cosgrove, 2000). Similarly, local expansin expression within the meristem was shown to induce the process of leaf formation (Pien et al., 2001). It is worth noting, however, that all these reports had used the complete expansin protein, while we have used only the CBM in this work. Thus one might conclude that both domains of the expansins are needed to bring about developmental changes in the plant.

The less pronounced cellular phenotypes of the transgenic tobacco plants expressing the potato expansin CBM as compared to the transgenic tobacco expressing the

microbial CBMs may well be the result of transforming with a single module of the expansin CBM. Our earlier investigation involving the single and the double CBM29 modules showed that transgenic tobacco plants expressing the double CBM29-1-2 exhibited more severe phenotypes than those expressing the single CBM29-2 (Obembe et al., 2006c). It seems as if the effect of the expansin CBM on cell walls of the phloem fibres of the stem were more pronounced than that of the single CBM29-2. There were no marked differences in the effects of the single CBM29-2 compared to the control, with respect to this. This might indicate that expression of a double expansin CBM might be worthwhile, if modulation of the cell wall were an objective. Taken together, our results tend to support the idea that plant expansin CBM is involved in polysaccharide binding. Furthermore, our results indicate that expansin CBM alone can bring about cellular morphological changes in plant cell walls.

Acknowledgements

References

Barral P, Suarez C, Batanero E, Alfonso C, Alche JD, Rodriguez-Garcia MI, Villalba M, Rivas G, Rodriguez R (2005) An olive pollen protein with allergenic activity, Ole e 10, defines a novel family of carbohydrate-binding modules and is potentially implicated in pollen germination. Biochemical Journal **390:** 77-84

Barre A, Rouge P (2002) Homology modeling of the cellulose-binding domain of a pollen allergen from rye grass: structural basis for the cellulose recognition and associated allergenic properties. Biochemical And Biophysical Research Communications **296:** 1346-1351

Boraston AB, Bolam DN, Gilbert HJ, Davies GJ (2004) Carbohydrate-binding modules: fine-tuning polysaccharide recognition. Biochemical Journal **382:** 769-781

Ceccardi TL, Barthe GA, Derrick KS (1998) A novel protein associated with citrus blight has sequence similarities to expansin. Plant Molecular Biology **38:** 775-783

Charnock SJ, Bolam DN, Turkenburg JP, Gilbert HJ, Ferreira LMA, Davies GJ, Fontes C (2000) The X6 "thermostabilizing" domains of xylanases are carbohydrate-binding modules: Structure and biochemistry of the Clostridium thermocellum X6b domain. Biochemistry **39:** 5013-5021

Cho HT, Cosgrove DJ (2000) Altered expression of expansin modulates leaf growth and pedicel abscission in Arabidopsis thaliana. Proceedings of The National Academy of Sciences of The United States of America **97:** 9783-9788

Choi DS, Lee Y, Cho HT, Kende H (2003) Regulation of expansin gene expression affects growth and development in transgenic rice plants. Plant Cell **15:** 1386-1398

Cosgrove DJ (2000a) Loosening of plant cell walls by expansins. Nature **407:** 321-326

Cosgrove DJ (2000b) New genes and new biological roles for expansins. Current Opinion In Plant Biology **3:** 73-78

De Marino S, Morelli MAC, Fraternali F, Tamborini E, Musco G, Vrtala S, Dolecek C, Arosio P, Valenta R, Pastore A (1999) An immunoglobulin-like fold in a major plant allergen: the solution structure of Phl p 2 from timothy grass pollen. Structure **7:** 943-952

Gehring CA, Irving HR (2003) Natriuretic peptides - a class of heterologous molecules in plants. International Journal of Biochemistry & Cell Biology **35:** 1318-1322

Guex N, Peitsch MC (1997) SWISS-MODEL and the Swiss-PdbViewer: An environment for comparative protein modeling. Electrophoresis **18:** 2714-2723

Hellens RP, Edwards EA, Leyland NR, Bean S, Mullineaux PM (2000) pGreen: a versatile and flexible binary Ti vector for Agrobacterium-mediated plant transformation. Plant Molecular Biology **42:** 819-832

Kende H, Bradford KJ, Brummell DA, Cho HT, Cosgrove DJ, Fleming AJ, Gehring C, Lee Y, McQueen-Mason S, Rose JKC, Voesenek L (2004) Nomenclature for members of the expansin superfamily of genes and proteins. Plant Molecular Biology **55:** 311-314

Li Y, Jones L, McQueen-Mason S (2003) Expansins and cell growth. Current Opinion In Plant Biology **6:** 603-610

Linder M, Nevanen T, Teeri TT (1999) Design of a pH-dependent cellulose-binding domain. Febs Letters **447:** 13-16

Ludidi NN, Heazlewood JL, Seoighe C, Irving HR, Gehring CA (2002) Expansin-like molecules: Novel functions derived from common domains. Journal of Molecular Evolution **54:** 587-594

Maryani MM, Bradley G, Cahill DM, Gehring CA (2001) Natriuretic peptides and immunoreactants modify osmoticum-dependent volume changes in Solanum tuberosum L. mesophyll cell protoplasts. Plant Science **161:** 443-452

McQueen-Mason S, Cosgrove DJ (1994) Disruption of hydrogen-bonding between plant-cell wall polymers by proteins that induce wall extension. Proceedings of The National Academy of Sciences of The United States of America **91:** 6574-6578

McQueen-Mason SJ, Cosgrove DJ (1995) Expansin mode of action on cell-walls - Analysis of wall hydrolysis, stress-relaxation, and binding. Plant Physiology **107:** 87-100

McQueen-Mason SJ, Durachko D.M., Cosgrove DJ (1992) Two endogenous proteins that induce cell-wall extension in plants. Plant Cell **4:** 1425-1433

Obembe OO, Cifuentes C, Huigen D, Kieft H, Jacobsen E, Emons AMC, Visser RGF, Vincken J-P (2006a) Bioengineering cellulose-hemicelluose networks in plants (Chapter 2, This Thesis). Wageningen University, Wageningen

Obembe OO, Jacobsen E, Gilbert H, Blake AW, Knox JP, Visser RGF, Vincken J-P (2006b) Bioengineering cellulose-hemicelluose networks in plants (Chapter 4, this Thesis). Wageningen University, Wageningen

Obembe OO, Jacobsen E, Timmers J, Gilbert H, Blake AW, Knox JP, Visser RGF, Vincken J-P (2006c) Bioengineering cellulose-hemicelluose networks in plants (Chapter 3, This Thesis). Wageningen University, Wageningen

Peitsch MC (1995) Protein Modeling By E-Mail. Bio-Technology **13:** 658-660

Pharmawati M, Maryani MM, Nikolakopoulos T, Gehring CA, Irving HR (2001) Cyclic GMP modulates stomatal opening induced by natriuretic peptides and immunoreactive analogues. Plant Physiology And Biochemistry **39:** 385-394

Pien S, Wyrzykowska J, McQueen-Mason S, Smart C, Fleming A (2001) Local expression of expansin induces the entire process of leaf development and modifies leaf shape. Proceedings of The National Academy of Sciences of The United States of America **98:** 11812-11817

Rochange SF, Wenzel CL, McQueen-Mason SJ (2001) Impaired growth in transgenic plants over-expressing an expansin isoform. Plant Molecular Biology **46:** 581-589

Schwede T, Kopp J, Guex N, Peitsch MC (2003) SWISS-MODEL: an automated protein homology-modeling server. Nucleic Acids Research **31:** 3381-3385

Shcherban TY, Shi J, Durachko DM, Guiltinan MJ, McQueen-Mason SJ, Shieh M, Cosgrove DJ (1995) Molecular-Cloning And Sequence-Analysis of Expansins - A highly conserved, multigene family of Proteins that mediate cell-wall extension in plants. Proceedings of The National Academy of Sciences of The United States of America **92:** 9245-9249

Toone EJ (1994) Structure and energetics of protein carbohydrate complexes. Current Opinion In Structural Biology **4:** 719-728

Whitney SEC, Gidley MJ, McQueen-Mason SJ (2000) Probing expansin action using cellulose/hemicellulose composites. Plant Journal **22:** 327-334

Zenoni S, Reale L, Tornielli GB, Lanfaloni L, Porceddu A, Ferrarini A, Moretti C, Zamboni A, Speghini S, Ferranti F, Pezzotti M (2004) Downregulation of the Petunia hybrida alpha-expansin gene PhEXP1 reduces the amount of crystalline cellulose in cell walls and leads to phenotypic changes in petal limbs. Plant Cell **16:** 295-308

General discussion

The research described in this thesis was performed to investigate the possibilities of altering cellulose-hemicellulose networks in plant cell walls. This is with a view to generating a biological tool-box, which can be adapted for commercially important fibre species with respect to modifying the fibre properties. The three major aims of this work were (i) modulation of cellulose content through genetic crossing of two antisense potato lines in order to obtain offspring with greater reduction in cellulose content and over-expression of a CesA gene, in order to increase cellulose content, (ii) heterologous expression of different microbial CBM genes in tobacco, in order to investigate whether it is possible to use CBMs to modify cell wall structures, and (iii) heterologous expression of a putative expansin CBM in tobacco, with a view to unravelling the function of expansin's CBM in cell wall loosening. The results described in the previous chapters are herewith discussed, with respect to the above goals.

(i) Modulation of cellulose content
Several reports indicate that the cellulose-hemicellulose network can be influenced by modulating the content of interacting polysaccharides (see overview in Chapter 1, under hemicellulose biosynthesis section). It should be noted that this level of interaction and influence on cellulose fibre properties are only achievable during biosynthesis but not *ex vivo*. A gain of control over hemicellulose deposition would have been an interesting investigation to alter cellulose-hemicellulose networks. However, at the start of the research, there were no genes identified for the synthesis of the backbone of any hemicellulosic polysaccharide. Unlike hemicellulose synthases, enzymes involved in cellulose biosynthesis were identified, and genes encoding these enzymes had been isolated from several plant species, for review see Doblin et al (2002) and Saxena and Brown (2005). This, and the availability of transgenic CesA potato lines has led us to focus on cellulose biosynthesis in our investigation.

There were reports, which indicated that modulation of cellulose content, through altered expression of the CesA proteins, could alter the cellulose-hemicellulose network. Analyses of the various primary and secondary cell wall mutants of *Arabidopsis* revealed that some of them had severe reduction in cellulose synthesis, with consequential abnormal cell wall assembly (Turner and Somerville, 1997; Arioli et al., 1998; Fagard et al., 2000; Taylor et al., 2004). Probably a lower cellulose/hemicellulose ratio yields more amorphous cellulose fibres, whereas a higher cellulose/hemicellulose ratio might favour cellulose crystallinity.

Reduction of cellulose content. To explore the possibility of influencing cellulose-hemicellulose networks in potato, with respect to cellulose reduction, we have carried out genetic crossing of two antisense potato lines, csr2-1 and csr4-8 (Chapter 2), which were previously identified to exhibit reduced cellulose content as compared to the wild type (Oomen et al., 2004). Our working hypothesis was that greater reduction

in crystalline cellulose content in the double transformants would have stronger effect on cellulose-hemicellulose networks, as would be revealed by more severe cell wall phenotypes. The main aim was to investigate the threshold limit for cellulose reduction that the potato tuber could tolerate. Alternatively, it was hypothesised that lower cellulose content might lead to higher pectin content. It was observed previously that the tubers of the two antisense lines that were to be crossed had about 5% and 10% higher uronic acid content than those of the control plants (Oomen et al., 2004). Hence it was thought that a much-reduced cellulose content in the double transformant tubers might lead to an even higher pectin content than in either of the parents. Supplementary synthesis of pectin in the cellulose-depleted potato cell wall might give added value to it and thus enhance its potential use in food industrial applications.

Analyses of the offspring from the genetic crossing led to a number of interesting observations (Chapter 2). These include (i) abnormal cellulosic deposition in cell wall corners of single transformant tubers containing the csr2 antisense construct, (ii) proliferation of xylem cells and abnormal lignin formation in the single csr2 tubers, (iii) 60% reduction in cellulose content of the csr2/csr4 double transformant tubers, as compared to 40% and 20% reduction in the csr2 and csr4 tubers, respectively, and (iv) widespread reduction in size and weight of the transgenic tubers containing either one or both antisense constructs.

The investigation was successful in achieving the first goal, which was to generate double transformant tubers with further reduction in cellulose content. However, the second goal, which was to investigate the induction of higher pectin content, was not pursued any further, as we did not see it as a viable option based on the following reasons. First, the extent of further reduction in cellulose content in the double transformant tubers was not as large as we had anticipated. Second, the general reduction in the weight of transgenic tubers would neutralize any increase in pectin content, or worse still, may lead to a net loss in pectin content when compared to the control tuber. From a scientific point of view, the cell corner deposit was intriguing; hence priority was given to the analysis of this phenomenon. Preliminary analyses indicated that the deposit is cellulose while we could not exclude the possibility that it might be xyloglucan. The revelation of the identity of the deposit might give new insight into the process of cellulose biosynthesis (deposition) and/or its interaction with xyloglucan. Hence, more in-depth analyses of the deposit are being considered.

Up-regulation of cellulose content. Alternatively, levels of cellulose can be up-regulated as a means to alter the cellulose-hemicellulose network. Higher cellulose content might imply higher yield for paper and pulp industries. 200% up-regulation of cellulose content in the potato tuber was demonstrated by targeting the expression of potato CesA3 gene to the tuber (Oomen et al., 2004). However, this level of up-regulation was only observed in one plant. In order to investigate this further, we considered to repeat the experiment with a modification. The modification was to place a hexa-histidine epitope tag at the N-terminus of the protein. However, our cloning strategy to make a fusion protein of the tag and the CesA3 protein did not

succeed. This problem did not exist in the cloning of CesA4, which we intended to investigate alongside CesA3, in order to compare their effects. Originally, the investigation was to serve three purposes (i) to figure out whether over-expression of a different CesA would also increase cellulose content, (ii) to use the tagged CesA4 as a bait to 'fish out' the CelS complex(es), into which it has been incorporated, with a view to revealing the identities of other constituent proteins, and (iii) to repeat the previous experiment with a 2-fold increase in cellulose. Transgenic plants with constitutive and tuber-specific expression were generated and analyzed for RNA expression of the CesA4. There was no notable abnormality in the morphology and development of the transgenic plants and their tubers. The investigation, however, did not go further largely because affinity purification of active CelS complex might be difficult if not impossible. This concern was based on the indication that the hexa-histidine tag might be cleaved off as was discussed in Chapter 3. It should be stressed, however, that the tagged proteins discussed in Chapter 3 were targeted to the cell wall whereas in this case, the tag was in the cytosolic part of the cell. It is possible that the tag might not be cleaved off in the cytoplasm. We did not investigate this further because downstream procedures to be followed to identify the interacting protein partners in the complex were laborious and risky.

Other approaches for modification of cellulose fibre properties. In view of its vast industrial applications, a great deal of research effort has been made to modify cellulose fibre properties. For example, advances have been made to improve the quality of tree cellulosic raw material by reducing its lignin content through genetic modification. However, this approach falls outside the scope of our work; hence it will not be discussed in detail, but it is exhaustively reviewed in Baucher et al. (2003). Cellulose fibres are also receiving attention these days for use in other applications ranging from food packaging to their use in production of fibres, plastic films and drugs (Gustavsson et al., 2005). Particular attention has been given to the preparation of water-repellent cellulosic composites for packaging liquid and food products (Mohanty et al., 2001). Two environment-friendly *in vitro* approaches involving enzymatic modification of cellulose fibre surface are being explored lately. First, direct coating of the cellulose fibre surface with hydrophobic polyester through the use of a CBM1-*Candida Antarctica* Lipase B (CBM1-CALB) fusion protein (Gustavsson et al., 2004). The enzyme is immobilized on the fibre surface by the CBM to ensure that polymerization of lactones was done in close proximity to the cellulose surface. This novel use of CBM underlines the potential of CBMs in cellulose modification. A second and indirect approach involves the production of enzyme-mediated derivatized xyloglucan (Gustavsson et al., 2005). The CALB enzyme is used to acylate xyloglucan oligosaccharides, which then act as glycosyl acceptors in a transglycosylation reaction catalysed by xyloglucan endotransglycosylase. The acylated oligosaccharides are then incorporated into high molecular weight xyloglucan chains. The high affinity of xyloglucan for cellulose then allows the functional groups to be attached to the fibre surface. Furthermore, recombinant fusion protein of two cellulose-specific CBM3s, cellulose cross-linking

protein (CCP), has been indicated to give water-repellent properties to filter paper, when added in saturating amounts (Levy et al., 2002a). These *in vitro* modifications have an advantage over direct interference with biosynthesis, in that they do not interfere with plant development, and as such are more promising at this stage than generating transgenic plants. An alternative *in planta* approach, which would have minimal effect on plant development, would be of great potential for the bioengineering of cellulose fibre surface properties.

(ii) Heterologous expression of CBM genes in tobacco
It has been shown that the interactions of hemicelluloses with cellulose can influence fibre properties (Whitney et al., 1995; Whitney et al., 1998; Whitney et al., 2000). Thus, it is conceivable that *in planta* expression of CBMs, which bind soluble cellulosic and hemicellulosic polysaccharides, as we have used in this research, can prevent these interactions. Consequently, the polymers fail to interact adequately, leading to more crystallinity of the cellulose fibre. In addition to this speculation were the indications that the CBMs can actually enhance plant development (Shpigel et al., 1998; Kilburn et al., 2000; Shoseyov et al., 2001). Hence, we investigated the effects of introducing promiscuous CBM29s and a xylan-specific CBM2b-1-2 in tobacco (Chapters 3 and 4). Specifically, we also investigated whether double CBMs (CBM29-1-2) in tandem would have greater effect in plants than a single CBM29-2 (Chapter 3). It is worth mentioning that a third CBM of the family 22, which is specific for xylan was also investigated alongside the two mentioned above. However, the transgenic plants expressing the single CBM22 gene were not investigated further, because microscopic examinations of their stem sections did not reveal any marked phenotype, and also because of the fact that we had used a single CBM for the transformation, instead of double CBMs as for the other two CBMs.

Our observations support the speculation that CBMs can interfere with the interactions between cellulose and hemicelluloses. Our results, however, did not agree with the previous indications that CBMs (CBM3 and 27) can enhance plant growth (Shpigel et al., 1998; Kilburn et al., 2000; Shoseyov et al., 2001). On the one hand, the use of CBM29-1-2 actually hindered plant development (Chapter 3) and on the other hand the use of CBM2b-1-2 did not affect plant development (Chapter 4). It should be noted that in studies of others different CBMs were used for expression in plants. CBM1 and CBM3 bind crystalline cellulose while CBM27 binds mannan. However, the difference in specificity cannot explain the contrasting effects of their and our CBMs on plant development. (i) The enhanced root elongation that was reported for *in vitro* cultured *Arabidopsis* seedlings was only observed in the presence of a low concentration of CBM3. Root growth inhibition was actually observed at high concentrations of the CBM3 (Shpigel et al., 1998). (ii) There was a report that *in planta* expression of CBM1 caused reduced growth in all transgenic *Arabidopsis* plants (Quentin, 2003). This observation indicates that growth enhancement in transgenic plants is not a common feature of CBMs specific for crystalline cellulose (Shoseyov et al., 2001). It is also possible that the apparent inconsistencies are related

to the use of different plant species (poplar, *Arabidopsis*), which might respond differently to the presence of CBMs.

On the whole, our investigation has provided a proof of concept that CBMs can interfere with cellulose-hemicellulose networks in the plant cell wall but the actual effect on the cell wall is still unpredictable. We would like to examine stem longitudinal sections of the high expressers of the CBM29-1-2, to verify longitudinal collapse of cells. This is to establish the actual basis for the growth and developmental defect. The use of CBMs for *in planta* modification still needs further investigation. It is possible to prevent the interaction between cellulose and hemicellulose with CBMs, which are specific for a particular hemicellulose e.g. xylan. Promiscuous CBMs can interact with both cellulose and xylan, and, in principle might interfere with the process of crystallisation of cellulose to microfibrils, whereas a hemicellulose-specific CBM does not. Hence, we can explore the possibility of using specific CBMs to prevent the binding of a particular hemicellulose to cellulose during biosynthesis. Examples of such would include CBM13, 15, 22 31 and 36 (Table 2, introduction). These CBMs bind exclusively to xylan, and as such, they might prevent xylan from interacting with the cellulose, and by implication prevent the lignin deposition on cellulose. Such *in planta* production of quality fibre with less lignin attachment is desirable in paper manufacturing. We recommend that such constructs should contain two modules of the particular CBM in tandem, in order to enhance affinity. Also, it might be advantageous to target specific cell types, such as the phloem fibre cells, for the expression of these CBMs, with the use of an appropriate promoter.

(iii) Heterologous expression of expansin's CBM in tobacco

In Chapter 5 of this thesis we investigated the possibility of expansin's CBM to modify the cell wall. The investigation was premised on the observations that altered expression of the complete expansins modulated plant development (Brummell et al., 1999; Cho and Cosgrove, 2000; Pien et al., 2001; Choi et al., 2003). In addition to that was the inspiration for using CBM to bring about structural changes in the cell wall by interfering with the cellulose-hemicellulose network. Data obtained from the investigation has made us to conclude that the expansin CBM alone can modify the cell wall, but unlike the complete expansin, it does not affect normal development of the plant. Additionally, one can conclude, to a certain extent, that the effect of expansin CBM on cellulose-rich cells of the vascular tissues compares to those of the classified CBMs used previously in this research.

Concluding remarks

Besides chemical derivatization, *in vitro* enzyme-mediated modification of fibres is the trend nowadays for tailoring cellulose fibres with enhanced properties for specific industrial applications. This approach has the advantage that plant development is not compromised. However, it has its own limitation as well, in that it cannot modify inherent fibre properties. This is only possible during cell wall polysaccharide biosynthesis, and as such, with an *in planta* approach. Hence, both approaches might complement each other. At the moment, the tools to do this in a deliberate manner are

not yet in our hands, but might be in the future. More so, with the identification, in the last 5 years, of MUR genes which encode xyloglucan-specific fucosyl (Perrin et al., 1999) and galactosyl transferases (Madson et al., 2003), xyloglucan xylosyl transferases (Faik et al., 2002) and mannan synthase (Dhugga et al., 2004) we can now explore the possibility of improving fibre properties with these enzymes, *in planta*.

References

Alonso-Simon A, Encina AE, Garcia-Angulo P, Alvarez JM, Acebes JL (2004) FTIR spectroscopy monitoring of cell wall modifications during the habituation of bean (Phaseolus vulgaris L.) callus cultures to dichlobenil. Plant Science **167:** 1273-1281

Amor Y, Haigler CH, Johnson S, Wainscott M, Delmer DP (1995) A membrane-associated form of sucrose synthase and its potential role in synthesis of cellulose and callose in plants. Proceedings of The National Academy of Sciences of The United States of America **92:** 9353-9357

Arioli T, Peng LC, Betzner AS, Burn J, Wittke W, Herth W, Camilleri C, Hofte H, Plazinski J, Birch R, Cork A, Glover J, Redmond J, Williamson RE (1998) Molecular analysis of cellulose biosynthesis in *Arabidopsis*. Science **279:** 717-720

Awano T, Takabe K, Fujita M (2002) Xylan deposition on secondary wall of *Fagus crenata* fiber. Protoplasma **219:** 106-115

Banka RR, Mishra S, Ghose TK (1998) Fibril formation from cellulose by a novel protein from Trichoderma reesei: A non-hydrolytic cellulolytic component? World Journal of Microbiology & Biotechnology **14:** 551-558

Barral P, Suarez C, Batanero E, Alfonso C, Alche JD, Rodriguez-Garcia MI, Villalba M, Rivas G, Rodriguez R (2005) An olive pollen protein with allergenic activity, Ole e 10, defines a novel family of carbohydrate-binding modules and is potentially implicated in pollen germination. Biochemical Journal **390:** 77-84

Barre A, Rouge P (2002) Homology modeling of the cellulose-binding domain of a pollen allergen from rye grass: structural basis for the cellulose recognition and associated allergenic properties. Biochemical And Biophysical Research Communications **296:** 1346-1351

Baskin TI, Busby CH, Fowke LC, Sammut M, Gubler F (1992) Improvements in immunostaining samples embedded in methacrylate: Localization of microtubules and other antigens throughout developing organs of diverse taxa. Planta **187:** 405-413

Baucher M, Halpin C, Petit-Conil M, Boerjan W (2003) Lignin: Genetic engineering and impact on pulping. Critical Reviews In Biochemistry And Molecular Biology **38:** 305-350

Bayer EA, Chanzy H, Lamed R, Shoham Y (1998) Cellulose, cellulases and cellulosomes. Current Opinion In Structural Biology **8:** 548-557

Benziman M, Haigler Candace H, Brown RM, R. WA, Cooper KM (1980) Polymerization and crystallization are coupled processes in *Acetobacter xylinum*. Proceedings of the National Academy of Sciences of the United States of America **77, 6678-82**

Bolam DN, Ciruela A, McQueen-Mason S, Simpson P, Williamson MP, Rixon JE, Boraston A, Hazlewood GP, Gilbert HJ (1998) Pseudomonas cellulose-binding domains mediate their effects by increasing enzyme substrate proximity. Biochemical Journal **331:** 775-781

Bolam DN, Xie HF, White P, Simpson PJ, Hancock SM, Williamson MP, Gilbert HJ (2001) Evidence for synergy between family 2b carbohydrate binding modules in Cellulomonas fimi xylanase 11A. Biochemistry **40:** 2468-2477

Bonke M, Thitamadee S, Mahonen AP, Hauser MT, Helariutta Y (2003) APL regulates vascular tissue identity in Arabidopsis. Nature **426:** 181-186

Boraston AB, Bolam DN, Gilbert HJ, Davies GJ (2004) Carbohydrate-binding modules: fine-tuning polysaccharide recognition. Biochemical Journal **382:** 769-781

Boudet AM, Kajita S, Grima-Pettenati J, Goffner D (2003) Lignins and lignocellulosics: a better control of synthesis for new and improved uses. Trends In Plant Science **8:** 576-581

Brady JD, Sadler IH, Fry SC (1996) Di-isodityrosine, a novel tetrameric derivative of tyrosine in plant cell wall proteins: A new potential cross-link. Biochemical Journal **315:** 323-327

Brands A, Ho THD (2002) Function of a plant stress-induced gene, HVA22. Synthetic enhancement screen with its yeast homolog reveals its role in vesicular traffic. Plant Physiology **130:** 1121-1131

Brett CTW, K. (1996) Physiology and Biochemistry of Plant Cell walls. Chapman & Hall, London

Brown RM, Saxena IM (2000) Cellulose biosynthesis: A model for understanding the assembly of biopolymers. Plant Physiology And Biochemistry **38:** 57-67

Brown RM, Saxena IM, Kudlicka K (1996) Cellulose biosynthesis in higher plants. Trends In Plant Science **1:** 149-156

Brummell DA, Harpster MH, Civello PM, Palys JM, Bennett AB, Dunsmuir P (1999) Modification of expansin protein abundance in tomato fruit alters softening and cell wall polymer metabolism during ripening. Plant Cell **11:** 2203-2216

Burk DH, Liu B, Zhong RQ, Morrison WH, Ye ZH (2001) A katanin-like protein regulates normal cell wall biosynthesis and cell elongation. Plant Cell **13:** 807-827

Burton RA, Gibeaut DM, Bacic A, Findlay K, Roberts K, Hamilton A, Baulcombe DC, Fincher GB (2000) Virus-induced silencing of a plant cellulose synthase gene. Plant Cell **12:** 691-705

Cano-Delgado AI, Metzlaff K, Bevan MW (2000) The eli1 mutation reveals a link between cell expansion and secondary cell wall formation in Arabidopsis thaliana. Development **127:** 3395-3405

Cano Delgado A, Penfield S, Smith C, Catley M, Bevan M (2003) Reduced cellulose synthesis invokes lignification and defense responses in Arabidopsis thaliana. Plant Journal. [print] May 2003 2003; **34:** 351-362.

Carpita NC, Defernez M, Findlay K, Wells B, Shoue DA, Catchpole G, Wilson RH, McCann MC (2001) Cell wall architecture of the elongating maize coleoptile. Plant Physiology **127:** 551-565

Carpita NC, Gibeaut DM (1993) Structural models of primary-cell walls in flowering plants - consistency of molecular-structure with the physical-properties of the walls during growth. Plant Journal **3:** 1-30

Ceccardi TL, Barthe GA, Derrick KS (1998) A novel protein associated with citrus blight has sequence similarities to expansin. Plant Molecular Biology **38:** 775-783

Chabannes M, Barakate A, Lapierre C, Marita JM, Ralph J, Pean M, Danoun S, Halpin C, Grima-Pettenati J, Boudet AM (2001) Strong decrease in lignin content without significant alteration of plant development is induced by simultaneous down-regulation of cinnamoyl CoA reductase (CCR) and cinnamyl alcohol dehydrogenase (CAD) in tobacco plants. Plant Journal **28:** 257-270

Charnock SJ, Bolam DN, Nurizzo D, Szabo L, McKie VA, Gilbert HJ, Davies GJ (2002) Promiscuity in ligand-binding: The three-dimensional structure of a Piromyces carbohydrate-binding module, CBM29-2, in complex with cello-and mannohexaose. Proceedings of The National Academy of Sciences of The United States of America **99:** 14077-14082

Charnock SJ, Bolam DN, Turkenburg JP, Gilbert HJ, Ferreira LMA, Davies GJ, Fontes C (2000) The X6 "thermostabilizing" domains of xylanases are carbohydrate-binding modules: Structure and biochemistry of the Clostridium thermocellum X6b domain. Biochemistry **39:** 5013-5021

Charnock SJ, Henrissat B, Davies GJ (2001) Three-dimensional structures of UDP-sugar glycosyltransferases illuminate the biosynthesis of plant polysaccharides. Plant Physiology **125:** 527-531

Cho HT, Cosgrove DJ (2000) Altered expression of expansin modulates leaf growth and pedicel abscission in Arabidopsis thaliana. Proceedings of The National Academy of Sciences of The United States of America **97:** 9783-9788

Choi DS, Lee Y, Cho HT, Kende H (2003) Regulation of expansin gene expression affects growth and development in transgenic rice plants. Plant Cell **15:** 1386-1398

Church GM, Gilbert W (1984) Genomic sequencing. Proceedings of the National Academy of Sciences of the United States of America **81:** 1991-1995

Compier M (2005) Hemicellulose biosynthesis and degradation in tobacco cell walls (PhD Thesis). PhD thesis. Wageningen University, Wageningen,

Cosgrove DJ (2000a) Loosening of plant cell walls by expansins. Nature **407:** 321-326

Cosgrove DJ (2000b) New genes and new biological roles for expansins. Current Opinion In Plant Biology **3:** 73-78

Cosgrove DJ (2003a) Expansion of plant cell wall, in The Plant Cell Wall, (ed. Jocelyn K. C. Rose), Vol 8. CRC Press LLC, Boca Raton, FL, USA, pp pp. 237-258

Cosgrove DJ (2003b) Expansion of plant cell wall, in The Plant Cell Wall, (ed. Jocelyn K. C. Rose), Vol 8. CRC Press LLC, Boca Raton, FL, USA, pp 237-258

Cutler S, Somerville C (1997) Cellulose synthesis: Cloning in silico. Current Biology **7:** R108-R111

Darley CP, Forrester AM, McQueen-Mason SJ (2001) The molecular basis of plant cell wall extension. Plant Molecular Biology **47:** 179-195

De Marino S, Morelli MAC, Fraternali F, Tamborini E, Musco G, Vrtala S, Dolecek C, Arosio P, Valenta R, Pastore A (1999) An immunoglobulin-like fold in a major plant allergen: the solution structure of Phl p 2 from timothy grass pollen. Structure **7:** 943-952

Delmer DP, Amor Y (1995) Cellulose Biosynthesis. Plant Cell **7:** 987-1000

Desprez T, Vernhettes S, Fagard M, Refregier G, Desnos T, Aletti E, Py N, Pelletier S, Hofte H (2002) Resistance against herbicide isoxaben and cellulose deficiency caused by distinct mutations in same cellulose synthase isoform CESA6. Plant Physiology **128:** 482-490.

Dhugga KS (2001) Building the wall: genes and enzyme complexes for polysaccharide synthases. Current Opinion In Plant Biology **4:** 488-493

Dhugga KS, Barreiro R, Whitten B, Stecca K, Hazebroek J, Randhawa GS, Dolan M, Kinney AJ, Tomes D, Nichols S, Anderson P (2004) Guar seed beta-mannan synthase is a member of the cellulose synthase super gene family. Science **303:** 363-366

Din N, Damude HG, Gilkes NR, Miller RC, Warren RAJ, Kilburn DG (1994) C-1-C-X revisited - intramolecular synergism in a cellulase. Proceedings of The National Academy of Sciences of The United States of America **91:** 11383-11387

Doblin MS, Kurek I, Jacob Wilk D, Delmer Deborah P (2002) Cellulose biosynthesis in plants: From genes to rosettes. Plant and Cell Physiology. [print] December 2002 2002; **43:** 1407-1420.

Ebringerova A, Heinze T (2000) Xylan and xylan derivatives - biopolymers with valuable properties, 1 - Naturally occurring xylans structures, procedures and properties. Macromolecular Rapid Communications **21:** 542-556

Edwards ME, Dickson CA, Chengappa S, Sidebottom C, Gidley MJ, Reid JSG (1999) Molecular characterisation of a membrane-bound galactosyltransferase of plant cell wall matrix polysaccharide biosynthesis. Plant Journal **19:** 691-697

Emons AMC, Mulder BM (1998) The making of the architecture of the plant cell wall: How cells exploit geometry. Proceedings of The National Academy of Sciences of The United States of America **95:** 7215-7219

Fagard M, Desnos T, Desprez T, Goubet F, Refregier G, Mouille G, McCann M, Rayon C, Vernhettes S, Hofte H (2000) PROCUSTE1 encodes a cellulose synthase required for normal cell elongation specifically in roots and dark-grown hypocotyls of *Arabidopsis*. Plant Cell **12:** 2409-2423

Faik A, Price NJ, Raikhel NV, Keegstra K (2002) An Arabidopsis gene encoding an alpha-xylosyltransferase involved in xyloglucan biosynthesis. Proceedings of The National Academy of Sciences of The United States of America **99:** 7797-7802

Fontaine AS, Bout S, Barriere Y, Vermerris W (2003) Variation in cell wall composition among forage maize (Zea mays L.) inbred lines and its impact on digestibility: Analysis of neutral detergent fiber composition by pyrolysis-gas chromatography-mass spectrometry. Journal of Agricultural And Food Chemistry **51:** 8080-8087

Freelove ACJ, Bolam DN, White P, Hazlewood GP, Gilbert HJ (2001) A novel carbohydrate-binding protein is a component of the plant cell wall-degrading complex of Piromyces equi. Journal of Biological Chemistry **276:** 43010-43017

Gao PJ, Chen GJ, Wang TH, Zhang YS, Liu J (2001) Non-hydrolytic disruption of crystalline structure of cellulose by cellulose binding domain and linker sequence of cellobiohydrolase I from Penicillium janthinellum. Acta Biochimica Et Biophysica Sinica **33:** 13-18

Gardiner JC, Taylor NG, Turner SR (2003) Control of cellulose synthase complex localization in developing xylem. Plant Cell **15:** 1740-1748

Gehring CA, Irving HR (2003) Natriuretic peptides - a class of heterologous molecules in plants. International Journal of Biochemistry & Cell Biology **35:** 1318-1322

Giardina T, Gunning AP, Juge N, Faulds CB, Furniss CSM, Svensson B, Morris VJ, Williamson G (2001) Both binding sites of the starch-binding domain of Aspergillus niger glucoamylase are essential for inducing a conformational change in amylose. Journal of Molecular Biology **313:** 1149-1159

Gill J, Rixon JE, Bolam DN, McQueen-Mason S, Simpson PJ, Williamson MP, Hazlewood GP, Gilbert HJ (1999) The type II and X cellulose-binding domains of Pseudomonas xylanase A potentiate catalytic activity against complex substrates by a common mechanism. Biochemical Journal **342:** 473-480

Gubler F (1989) Immunofluoresence localization of microtubules in plant root tips embedded in Butyl-methyl methacrylate. Cell Biology International reports **13:** 137-145

Guex N, Peitsch MC (1997) SWISS-MODEL and the Swiss-PdbViewer: An environment for comparative protein modeling. Electrophoresis **18:** 2714-2723

Gustavsson MT, Persson PV, Iversen T, Hult K, Martinelle M (2004) Polyester coating of cellulose fiber surfaces catalyzed by a cellulose-binding module-Candida antarctica lipase B fusion protein. Biomacromolecules **5:** 106-112

Gustavsson MT, Persson PV, Iversen T, Martinelle M, Hult K, Teeri TT, Brumer H (2005) Modification of cellulose fiber surfaces by use of a lipase and a xyloglucan endotransglycosylase. Biomacromolecules **6:** 196-203

Ha MA, Apperley DC, Jarvis MC (1997) Molecular rigidity in dry and hydrated onion cell walls. Plant Physiology **115:** 593-598

Hackney JM, Atalla RH, Vanderhart DL (1994) Modification of Crystallinity And Crystalline-Structure of Acetobacter-Xylinum Cellulose In The Presence of Water-Soluble Beta-1,4-Linked Polysaccharides - C-13-Nmr Evidence. International Journal of Biological Macromolecules **16:** 215-218

Haigler CH (1991) Relationship between polymerization and crystallization in microfibril biogenesis, in Biosynthesis and biodegradation of cellulose (eds. Haigler, C. H., Weimer, P.J.). Marcel Dekker, New York.

Handford MG, Baldwin TC, Goubet F, Prime TA, Miles J, Yu XL, Dupree P (2003) Localisation and characterisation of cell wall mannan polysaccharides in Arabidopsis thaliana. Planta **218:** 27-36

Hazen SP, Scott-Craig JS, Walton JD (2002) Cellulose synthase-like genes of rice. Plant Physiology **128:** 336-340

Hellens RP, Edwards EA, Leyland NR, Bean S, Mullineaux PM (2000) pGreen: a versatile and flexible binary Ti vector for Agrobacterium-mediated plant transformation. Plant Molecular Biology **42:** 819-832

Henrissat B, Coutinho PM, Davies GJ (2001) A census of carbohydrate-active enzymes in the genome of *Arabidopsis thaliana*. Plant Molecular Biology **47:** 55-72

His I, Driouich A, Nicol F, Jauneau A, Hofte H (2001) Altered pectin composition in primary cell walls of korrigan, a dwarf mutant of Arabidopsis deficient in a membrane-bound endo-1,4-beta-glucanase. Planta **212:** 348-358

Hosoo Y, Yoshida M, Imai T, Okuyama T (2002) Diurnal difference in the amount of immunogold-labeled glucomannans detected with field emission scanning electron microscopy at the innermost surface of developing secondary walls of differentiating conifer tracheids. Planta **215:** 1006-1012

Itoh T, Kimura S (2001) Immunogold labeling of terminal cellulose-synthesizing complexes. Journal of Plant Research. **114:** 483-489.

Ji Q, Oomen RJF, Vincken JP, Bolam DN, Gilbert HJ, Suurs L, Visser RGF (2004) Reduction of starch granule size by expression of an engineered tandem starch-binding domain in potato plants. Plant Biotechnology Journal **2:** 251-260

John M, Rohrig H, Schmidt J, Walden R, Schell J (1997) Cell signalling by oligosaccharides. Trends In Plant Science **2:** 111-115

Kataeva IA, Seidel RD, Shah A, West LT, Li XL, Ljungdahl LG (2002) The fibronectin type 3-like repeat from the Clostridium thermocellum cellobiohydrolase CbhA promotes hydrolysis of cellulose by modifying its surface. Applied And Environmental Microbiology **68:** 4292-4300

Keegstra K, Raikhel N (2001) Plant glycosyltransferases. Current Opinion In Plant Biology **4:** 219-224

Kende H, Bradford KJ, Brummell DA, Cho HT, Cosgrove DJ, Fleming AJ, Gehring C, Lee Y, McQueen-Mason S, Rose JKC, Voesenek L (2004) Nomenclature for members of the expansin superfamily of genes and proteins. Plant Molecular Biology **55:** 311-314

Kilburn DG, Warren AJ, Stoll D, Gilkes NR, Shoseyov O, Shani Z (2000) Expression of a mannan binding domain to alter plant morphology. WO 2000/068391 A1., Ed C12N 15/31, 15/63, 15/82, C07K, 14/195, A01H 1/00

Kimura S, Laosinchai W, Itoh T, Cui XJ, Linder CR, Brown RM (1999) Immunogold labeling of rosette terminal cellulose-synthesizing complexes in the vascular plant Vigna angularis. Plant Cell **11:** 2075-2085

Kokubo A, Sakurai N, Kuraishi S, Takeda K (1991) Culm brittleness of barley (Hordeum-Vulgare L) mutants is caused by smaller number of cellulose molecules in cell-wall. Plant Physiology **97:** 509-514

Kuipers AGJ, Jacobsen E, Visser RGF (1994) Formation and deposition of amylose in the potato-tuber starch granule are affected by the reduction of granule-bound starch synthase gene-expression. Plant Cell **6:** 43-52

Kuipers AGJ, Soppe WJJ, Jacobsen E, Visser RGF (1995) Factors affecting the inhibition by antisense RNA of granule-bound starch synthase gene-expression in potato. Molecular & General Genetics **246:** 745-755

Kurek I, Kawagoe Y, Jacob-Wilk D, Doblin M, Delmer D (2002) Dimerization of cotton fiber cellulose synthase catalytic subunits occurs via oxidation of the zinc-binding domains. Proceedings of The National Academy of Sciences of The United States of America **99:** 11109-11114

Kuroiwa T, Kuroiwa H, Mita T, Fujie M (1990) Fluorescence Microscopic Study of the Formation of Giant Mitochondrial Nuclei in the Young Ovules of Pelargonium-Zonale. Protoplasma **158:** 191-194

Kutschera U (1990) Cell-Wall Synthesis And Elongation Growth In Hypocotyls of Helianthus-Annuus L. Planta **181:** 316-323

Kutschera U (2001) Stem elongation and cell wall proteins in flowering plants. Plant Biology **3:** 466-480

Lane DR, Wiedemeier A, Peng LC, Hofte H, Vernhettes S, Desprez T, Hocart CH, Birch RJ, Baskin TI, Burn JE, Arioli T, Betzner AS, Williamson RE (2001) Temperature-sensitive alleles of RSW2 link the KORRIGAN endo-1,4-beta-glucanase to cellulose synthesis and cytokinesis in arabidopsis. Plant Physiology **126:** 278-288

Lee S, Roberts AW (2004) Tracheary element differentiation is correlated with inhibition of cell expansion in xylogenic mesophyll suspension cultures. Plant Physiology and Biochemistry **42:** 43-48

Levy I, Nussinovitch A, Shpigel E, Shoseyov O (2002a) Recombinant cellulose crosslinking protein: a novel paper-modification biomaterial. Cellulose **9:** 91-98

Levy I, Shani Z, Shoseyov O (2002b) Modification of polysaccharides and plant cell wall by endo-1,4-beta-glucanase and cellulose-binding domains. Biomolecular Engineering **19:** 17-30

Levy I, Shoseyov O (2002) Cellulose-binding domains biotechnological applications. Biotechnology Advances **20:** 191-213

Li Y, Jones L, McQueen-Mason S (2003) Expansins and cell growth. Current Opinion In Plant Biology **6:** 603-610

Liepman AH, Wilkerson CG, Keegstra K (2005) Expression of cellulose synthase-like (Csl) genes in insect cells reveals that CslA family members encode mannan synthases. Proceedings of The National Academy of Sciences of The United States of America **102:** 2221-2226

Linder M, Nevanen T, Teeri TT (1999) Design of a pH-dependent cellulose-binding domain. Febs Letters **447:** 13-16

Linder M, Salovuori I, Ruohonen L, Teeri TT (1996) Characterization of a double cellulose-binding domain - Synergistic high affinity binding to crystalline cellulose. Journal of Biological Chemistry **271:** 21268-21272

Ludidi NN, Heazlewood JL, Seoighe C, Irving HR, Gehring CA (2002) Expansin-like molecules: Novel functions derived from common domains. Journal of Molecular Evolution **54:** 587-594

MacDougall AJ, Brett GM, Morris VJ, Rigby NM, Ridout MJ, Ring SG (2001) The effect of peptide behaviour-pectin interactions on the gelation of a plant cell wall pectin. Carbohydrate Research **335:** 115-126

Madson M, Dunand C, Li XM, Verma R, Vanzin GF, Calplan J, Shoue DA, Carpita NC, Reiter WD (2003) The MUR3 gene of *Arabidopsis* encodes a xyloglucan galactosyltransferase that is evolutionarily related to animal exostosins. Plant Cell **15:** 1662-1670

Maryani MM, Bradley G, Cahill DM, Gehring CA (2001) Natriuretic peptides and immunoreactants modify osmoticum-dependent volume changes in Solanum tuberosum L. mesophyll cell protoplasts. Plant Science **161:** 443-452

McCann MC, Roberts K (1994) Changes In Cell-Wall Architecture During Cell Elongation. Journal of Experimental Botany **45:** 1683-1691

McCartney L, Gilbert HJ, Bolam DN, Boraston AB, Knox JP (2004) Glycoside hydrolase carbohydrate-binding modules as molecular probes for the analysis of plant cell wall polymers. Analytical Biochemistry **326:** 49-54

McQueen-Mason S, Cosgrove DJ (1994) Disruption of hydrogen-bonding between plant-cell wall polymers by proteins that induce wall extension. Proceedings of The National Academy of Sciences of The United States of America **91:** 6574-6578

McQueen-Mason SJ, Cosgrove DJ (1995) Expansin mode of action on cell-walls - Analysis of wall hydrolysis, stress-relaxation, and binding. Plant Physiology **107:** 87-100

McQueen-Mason SJ, Durachko D.M., Cosgrove DJ (1992) Two endogenous proteins that induce cell-wall extension in plants. Plant Cell **4:** 1425-1433

Mohanty AK, Misra M, Drzal LT (2001) Surface modifications of natural fibers and performance of the resulting biocomposites: An overview. Composite Interfaces **8:** 313-343

Molhoj M, Pagant S, Hofte H (2002) Towards understanding the role of membrane-bound endo-beta-1,4-glucanases in cellulose biosynthesis. Plant and Cell Physiology. [print] December 2002 2002; **43:** 1399-1406

Molhoj M, Ulvskov P, Dal Degan F (2001) Characterization of a functional soluble form of a Brassica napus membrane-anchored endo-1,4-beta-glucanase heterologously expressed in Pichia pastoris. Plant Physiology **127:** 674-684

Morris VJ, Wilde PJ (1997) Interactions of food biopolymers. Current Opinion In Colloid & Interface Science **2:** 567-572

Mueller SC, Brown RM (1980) Evidence For An Intramembrane Component Associated With A Cellulose Microfibril-Synthesizing Complex In Higher-Plants. Journal of Cell Biology **84:** 315-326

Mulder BM, Emons AMC (2001) A dynamical model for plant cell wall architecture formation. Journal of Mathematical Biology **42:** 261-289

Nicol F, His I, Jauneau A, Vernhettes S, Canut H, Hofte H (1998) A plasma membrane-bound putative endo-1,4-beta-D-glucanase is required for normal wall assembly and cell elongation in *Arabidopsis*. Embo Journal **17:** 5563-5576

O'Neill MA, York WS (2003) The compossotion and structure of plant primary cell wall. In The Plant Cell Wall. (ed. J.K.C. Rose). Blackwell Publishing, Oxford, UK.

Obembe OO, Cifuentes C, Huigen D, Kieft H, Jacobsen E, Emons AMC, Visser RGF, Vincken J-P (2006a) Bioengineering cellulose-hemicelluose networks in plants (Chapter 2, This Thesis). PhD thesis. Wageningen University, Wageningen

Obembe OO, Jacobsen E, Gilbert H, Blake AW, Knox JP, Visser RGF, Vincken J-P (2006b) Bioengineering cellulose-hemicelluose networks in plants (Chapter 4, this Thesis). PhD thesis. Wageningen University, Wageningen

Obembe OO, Jacobsen E, Timmers J, Gilbert H, Blake AW, Knox JP, Visser RGF, Vincken J-P (2006c) Bioengineering cellulose-hemicelluose networks in plants (Chapter 3, This Thesis). PhD thesis. Wageningen University, Wageningen

Oomen R, Doeswijk-Voragen CHL, Bush MS, Vincken JP, Borkhardt B, van den Broek LAM, Corsar J, Ulvskov P, Voragen AGJ, McCann MC, Visser RGF (2002) In muro fragmentation of the rhamnogalacturonan I backbone in potato (Solanum tuberosum L.) results in a reduction and altered location of the galactan and arabinan side-chains and abnormal periderm development. Plant Journal **30:** 403-413

Oomen R, Tzitzikas EN, Bakx EJ, Straatman-Engelen I, Bush MS, McCann MC, Schols HA, Visser RGF, Vincken JP (2004) Modulation of the cellulose content of tuber cell walls by antisense expression of different potato (Solanum tuberosum L.) CesA clones. Phytochemistry **65:** 535-546

Pauly M, Andersen LN, Kauppinen S, Kofod LV, York WS, Albersheim P, Darvill A (1999) A xyloglucan-specific endo-beta-1,4-glucanase from Aspergillus aculeatus: expression cloning in yeast, purification and characterization of the recombinant enzyme. Glycobiology **9:** 93-100

Pear JR, Kawagoe Y, Schreckengost WE, Delmer DP, Stalker DM (1996) Higher plants contain homologs of the bacterial celA genes encoding the catalytic subunit of cellulose synthase. Proceedings of The National Academy of Sciences of The United States of America **93:** 12637-12642

Peitsch MC (1995) Protein Modeling By E-Mail. Bio-Technology **13:** 658-660

Pena MJ, Ryden P, Madson M, Smith AC, Carpita NC (2004) The galactose residues of xyloglucan are essential to maintain mechanical strength of the primary cell walls in *Arabidopsis* during growth. Plant Physiology **134:** 443-451

Peng L, Xiang F, Roberts E, Kawagoe Y, Greve LC, Kreuz K, Delmer DP (2001) The experimental herbicide CGA 325'615 inhibits synthesis of crystalline cellulose and causes accumulation of non-crystalline beta-1,4-glucan associated with CesA protein. Plant Physiology **126:** 981-992

Peng LC, Kawagoe Y, Hogan P, Delmer D (2002) Sitosterol-beta-glucoside as primer for cellulose synthesis in plants. Science **295:** 147-150

Perrin RM, DeRocher AE, Bar-Peled M, Zeng WQ, Norambuena L, Orellana A, Raikhel NV, Keegstra K (1999) Xyloglucan fucosyltransferase, an enzyme involved in plant cell wall biosynthesis. Science **284:** 1976-1979

Peters WS, Tomos AD (1996) The history of tissue tension. Annals of Botany **77:** 657-665

Peters WS, Tomos AD (2000) The mechanic state of "Inner tissue" in the growing zone of sunflower hypocotyls and the regulation of its growth rate following excision. Plant Physiology **123:** 605-612

Pharmawati M, Maryani MM, Nikolakopoulos T, Gehring CA, Irving HR (2001) Cyclic GMP modulates stomatal opening induced by natriuretic peptides and immunoreactive analogues. Plant Physiology And Biochemistry **39:** 385-394

Pien S, Wyrzykowska J, McQueen-Mason S, Smart C, Fleming A (2001) Local expression of expansin induces the entire process of leaf development and modifies leaf shape. Proceedings of The National Academy of Sciences of The United States of America **98:** 11812-11817

Pincon G, Chabannes M, Lapierre C, Pollet B, Ruel K, Joseleau JP, Boudet AM, Legrand M (2001) Simultaneous down-regulation of caffeic/5-hydroxy ferulic acid-O-methyltransferase I and cinnamoyl-coenzyme a reductase in the progeny from a cross between tobacco lines homozygous for each transgene. Consequences for plant development and lignin synthesis. Plant Physiology **126:** 145-155

Quentin MGE (2003) Cloning, characterisation and assessment of the wall modifying ability of a fungal cellulose-binding domain. PhD thesis. Wageningen University, Wageningen

Rabinovich ML, Melnick MS, Bolobova AV (2002) The structure and mechanism of action of cellulolytic enzymes. Biochemistry-Moscow **67:** 850-871

Raven PH, Evert RF, Eichhorn SE (1992) Biology of plants. Fifth edition. Worth Publishers, New York, USA

Reid JSG, Edwards ME, Dickson CA, Scott C, Gidley MJ (2003) Tobacco transgenic lines that express fenugreek galactomannan galactosyltransferase constitutively have structurally altered galactomannans in their seed endosperm cell walls. Plant Physiology **131:** 1487-1495

Richmond TA, Somerville CR (2000) The cellulose synthase superfamily. Plant Physiology **124:** 495-498

Richmond TA, Somerville CR (2001) Integrative approaches to determining Csl function. Plant Molecular Biology **47:** 131-143

Robert S, Mouille G, Hofte H (2004) The mechanism and regulation of cellulose synthesis in primary walls: lessons from cellulose-deficient Arabidopsis mutants. Cellulose **11:** 351-364

Rochange SF, Wenzel CL, McQueen-Mason SJ (2001) Impaired growth in transgenic plants over-expressing an expansin isoform. Plant Molecular Biology **46:** 581-589

Rose JKC, Catalá C, Gonzalez-Carranza ZH, Roberts JA (2003) Cell wall disassembly. In The Plant Cell Wall. (ed. J.K.C. Rose). Blackwell Publishing, Oxford, UK.

Ryden P, Sugimoto-Shirasu K, Smith AC, Findlay K, Reiter WD, McCann MC (2003) Tensile properties of Arabidopsis cell walls depend on both a xyloglucan cross-linked microfibrillar network and rhamnogalacturonan II-borate complexes. Plant Physiology **132:** 1033-1040

Salnikov VV, Grimson MJ, Delmer DP, Haigler CH (2001) Sucrose synthase localizes to cellulose synthesis sites in tracheary elements. Phytochemistry **57:** 823-833

Saloheimo M, Paloheimo M, Hakola S, Pere J, Swanson B, Nyyssonen E, Bhatia A, Ward M, Penttila M (2002) Swollenin, a Trichoderma reesei protein with sequence similarity to the plant expansins, exhibits disruption activity on cellulosic materials. European Journal of Biochemistry **269:** 4202-4211

Saxena IM, Brown RM (2005) Cellulose biosynthesis: Current views and evolving concepts. Annals of Botany **96:** 9-21

Saxena IM, Brown RM, Dandekar T (2001) Structure-function characterization of cellulose synthase: relationship to other glycosyltransferases. Phytochemistry **57:** 1135-1148

Saxena IM, Brown RM, Fevre M, Geremia RA, Henrissat B (1995) Multidomain architecture of beta-glycosyl transferases - implications for mechanism of action. Journal of Bacteriology **177:** 1419-1424

Scheible WR, Eshed R, Richmond T, Delmer D, Somerville C (2001) Modifications of cellulose synthase confer resistance to isoxaben and thiazolidinone herbicides in *Arabidopsis* Ixr1 mutants. Proceedings of the National Academy of Sciences of the United States of America **98:** 10079-10084

Schrick K (2004) A link between sterol biosynthesis, the cell-wall, and cellulose in Arabidopsis (vol 38, pg 227, 2004). Plant Journal **38:** 562-562

Schroder R, Wegrzyn TF, Bolitho KM, Redgwell RJ (2004) Mannan transglycosylase: a novel enzyme activity in cell walls of higher plants. Planta **219:** 590-600

Schwede T, Kopp J, Guex N, Peitsch MC (2003) SWISS-MODEL: an automated protein homology-modeling server. Nucleic Acids Research **31:** 3381-3385

Shcherban TY, Shi J, Durachko DM, Guiltinan MJ, McQueen-Mason SJ, Shieh M, Cosgrove DJ (1995) Molecular-cloning and sequence-analysis of expansins - A highly conserved, multigene family of proteins that mediate cell-wall extension in plants. Proceedings of The National Academy of Sciences of The United States of America **92:** 9245-9249

Shoseyov O, Yosef K, Shani Z, Rehovoth, Shpigel E, Medigo K (2001) Transgenic Plants of Altered Morphology. US patent 6,184,440.

Shpigel E, Roiz L, Goren R, Shoseyov O (1998) Bacterial cellulose-binding domain modulates in vitro elongation of different plant cells. Plant Physiology **117:** 1185-1194

Simpson PJ, Xie HF, Bolam DN, Gilbert HJ, Williamson MP (2000) The structural basis for the ligand specificity of family 2 carbohydrate-binding modules. Journal of Biological Chemistry **275:** 41137-41142

Southall SM, Simpson PJ, Gilbert HJ, Williamson G, Williamson MP (1999) The starch-binding domain from glucoamylase disrupts the structure of starch. Febs Letters **447:** 58-60

Stephen AM (1982) Other plant polysaccharides. In the polysaccharides, Vol 2 (ed. G.O. Aspinall). Academic Press, New York, USA.

Suzuki M, Kato A, Nagata N, Komeda Y (2002) A xylanase, AtXyn1, is predominantly expressed in vascular bundles, and four putative xylanase genes were identified in the *Arabidopsis* thaliana genome. Plant And Cell Physiology **43:** 759-767

Szyjanowicz PMJ, McKinnon I, Taylor NG, Gardiner J, Jarvis MC, Turner SR (2004) The irregular xylem 2 mutant is an allele of korrigan that affects the secondary cell wall of Arabidopsis thaliana. Plant Journal **37:** 730-740

Tanaka K, Murata K, Yamazaki M, Onosato K, Miyao A, Hirochika H (2003) Three distinct rice cellulose synthase catalytic subunit genes required for cellulose synthesis in the secondary wall. Plant Physiology **133:** 73-83

Taylor NG, Gardiner JC, Whiteman R, Turner SR (2004) Cellulose synthesis in the Arabidopsis secondary cell wall. Cellulose **11:** 329-338

Taylor NG, Howells RM, Huttly AK, Vickers K, Turner SR (2003) Interactions among three distinct CesA proteins essential for cellulose synthesis. Proceedings of the National Academy of Sciences of the United States of America **100:** 1450-1455

Taylor NG, Laurie S, Turner SR (2000) Multiple cellulose synthase catalytic subunits are required for cellulose synthesis in Arabidopsis. Plant Cell **12:** 2529-2539

Taylor NG, Scheible WR, Cutler S, Somerville CR, Turner SR (1999) The irregular xylem3 locus of *Arabidopsis* encodes a cellulose synthase required for secondary cell wall synthesis. Plant Cell **11:** 769-780

Timell TE (1967) Recent progress in the chemistry of wood hemicellulose. Wood Science and Technology **1:** 45-70

Toone EJ (1994) Structure And Energetics of Protein Carbohydrate Complexes. Current Opinion In Structural Biology **4:** 719-728

Tormo J, Lamed R, Chirino AJ, Morag E, Bayer EA, Shoham Y, Steitz TA (1996) Crystal structure of a bacterial family-III cellulose-binding domain: A general mechanism for attachment to cellulose. Embo Journal **15:** 5739-5751

Tsabary G, Shani Z, Roiz L, Levy I, Riov J, Shoseyov O (2003) Abnormal 'wrinkled' cell walls and retarded development of transgenic Arabidopsis thaliana plants expressing endo-1,4-beta-glucanase (cel1) antisense. Plant Molecular Biology **51:** 213-224

Tsekos I, Reiss HD (1992) Occurrence of the putative microfibril-synthesizing complexes (linear terminal complexes) in the plasma-membrane of the epiphytic marine red alga Erythrocladia-Subintegra Rosenv. Protoplasma **169:** 57-67

Turner SR, Somerville CR (1997) Collapsed xylem phenotype of Arabidopsis identifies mutants deficient in cellulose deposition in the secondary cell wall. Plant Cell **9:** 689-701

Updegraff DM (1969) Semi-microdetermination of cellulose in biological materials. Analytical Biochemistry **32:** 420-424

Whitney SEC, Brigham JE, Darke AH, Reid JSG, Gidley MJ (1995) In-Vitro Assembly of Cellulose/Xyloglucan Networks - Ultrastructural And Molecular Aspects. Plant Journal **8:** 491-504

Whitney SEC, Brigham JE, Darke AH, Reid JSG, Gidley MJ (1998) Structural aspects of the interaction of mannan-based polysaccharides with bacterial cellulose. Carbohydrate Research **307:** 299-309

Whitney SEC, Gidley MJ, McQueen-Mason SJ (2000) Probing expansin action using cellulose/hemicellulose composites. Plant Journal **22:** 327-334

Whitney SEC, Gothard MGE, Mitchell JT, Gidley MJ (1999) Roles of cellulose and xyloglucan in determining the mechanical properties of primary plant cell walls. Plant Physiology **121:** 657-663

Wittink FRA, Knuiman B, Derksen J, Capkova V, Twell D, Schrauwen JAM, Wullems GJ (2000) The pollen-specific gene Ntp303 encodes a 69-kDa glycoprotein associated with the vegetative membranes and the cell wall. Sexual Plant Reproduction **12:** 276-284

Wood PJ (1980) Specificity in the interaction of direct dyes with polysaccharides. Carbohydrate Research **85:** 271-287

Xiao ZZ, Gao PJ, Qu YB, Wang TH (2001) Cellulose-binding domain of endoglucanase III from Trichoderma reesei disrupting the structure of cellulose. Biotechnology Letters **23:** 711-715

Zenoni S, Reale L, Tornielli GB, Lanfaloni L, Porceddu A, Ferrarini A, Moretti C, Zamboni A, Speghini S, Ferranti F, Pezzotti M (2004) Downregulation of the Petunia hybrida alpha-expansin gene PhEXP1 reduces the amount of crystalline cellulose in cell walls and leads to phenotypic changes in petal limbs. Plant Cell **16:** 295-308

Zhong RQ, Burk DH, Morrison WH, Ye ZH (2004) FRAGILE FIBER3, an Arabidopsis gene encoding a type II inositol polyphosphate 5-phosphatase, is required for secondary wall synthesis and actin organization in fiber cells. Plant Cell **16:** 3242-3259

Zhong RQ, Morrison WH, Freshour GD, Hahn MG, Ye ZH (2003) Expression of a mutant form of cellulose synthase AtCesA7 causes dominant negative effect on cellulose biosynthesis. Plant Physiology **132:** 786-795

Summary

Plant cell wall polysaccharides are used commercially as gums, gelling agents and stabilizers. They are also the predominant components of fibres. Natural fibres have a wide range of industrial applications, such as in paper and textile industries. Furthermore, their demand for use as bio-composites in building and automotive applications is also increasing. For the various applications, a gain of control over fibre characteristics is important. Inherent fibre characteristics are largely determined by interactions between cellulose and hemicelluloses. The ratio of these groups of wall polysaccharides is important in the determination of these characteristics. This thesis explores the possibility of bioengineering these interactions, with a view to obtaining baseline information on *in planta* modification of fibre properties. Two strategies, interference with polysaccharide biosynthesis and direct interference with the cellulose-hemicellulose interactions, were employed. These strategies culminated in the goals of the thesis, (i) modulation of cellulose content through genetic crossing of two antisense potato lines in order to obtain offspring with greater reduction in cellulose content, (ii) heterologous expression of different microbial CBM genes in tobacco, in order to investigate whether it is possible to use CBMs to modify cell wall structures, and (iii) heterologous expression of a putative expansin CBM in tobacco, with a view to unravelling the function of expansin's CBM in cell wall loosening. We have investigated the first goal with potato because we had ready-to-use plant materials at the start of the work. For the second and third goals, we have used tobacco because it is widely used in cell wall research, with respect to fibre modification. The expectation is that the project would provide useful information as to how to improve the inherent fibre properties *in planta*, with a view to transferring the technology to more economically important species, such as flax, hemp and poplar.

At the start of the research, there was no gene identified for the synthesis of the backbone of any hemicellulosic polysaccharide. This was not the case for cellulose biosynthesis. In many crops, including potato, CesAs were identified and genes encoding these enzymes were isolated. Previous work in our laboratory on CesAs in potato, culminated in the generation of antisense and sense transgenic potato lines with varying levels of down- and up-regulation of cellulose content. We have carried out genetic crossing of two of these antisense lines (Chapter 2), which exhibited reduced cellulose content as compared to the wild type. This was with a view to producing double transformant tubers with lesser cellulose content than the parents. It was thought that even lower crystalline cellulose content in the double transformants might have stronger effects on tuber development. The investigation was successful in generating double transformant tubers with much reduced cellulose content (60%), as compared to the two single transformants. However, this reduction did not lead to severe phenotypes in the double transformant tubers. The single csr2 transformant tubers exhibited more interesting phenotypes than the double csr2/csr4 transformant tubers. The most striking observation was the cell corner deposition of cellulosic material in the csr2 tubers. Preliminary analysis of this material indicated that it is cellulose, presumably non-crystalline. Also, it was observed that offspring containing

either one or both transgenes produced more tubers than the control plants, but individual tubers were mostly smaller and had lesser weight than the control tubers. These phenotypes were more pronounced in the csr2 tubers than in csr4 and double transformant tubers. Furthermore, ectopic proliferation of xylem cells with associated lignification of their cell walls was also observed in the csr2 tubers.

In addition to modulating cellulose biosynthesis, we have also investigated direct interference of the cellulose-hemicellulose interactions through the use of carbohydrate binding modules (CBMs). There were indications that CBMs can enhance plant growth and we hypothesized that by binding to hemicellulose, they may decrease the incorporation of hemicellulose in cellulose microfibrils. We investigated the effects of introducing promiscuous CBM29s and a less promiscuous CBM2b-1-2 in tobacco (Chapters 3 and 4). The CBM29 modules bind to several cellulosic and hemicellulosic polysaccharides, whereas the CbM2b-1-2 binds to xylan and, to a lesser extent, to cellulose. We have shown that the promiscuous, tandem CBM29-1-2 gave more severe phenotypes than the less promiscuous CBM2b-1-2. There were widespread effects of the promiscuous CBM29-1-2 in most parts of the stem of the transgenic lines (Chapter 3), whereas the effects of CBM2b-1-2 were restricted mainly to the vascular tissues (Chapter 4). Specifically, larger cells were observed in the stem epidermal, cortex and xylem tissues of the CBM29-1-2 plants, whereas this altered cell expansion was only observed in the stem xylem tissue of the CBM2b-1-2 plants. Additionally, collapsed cortical cells were observed in the stem sections of the CBM29-1-2 plants. It was striking to observe that CBM29-1-2 did not only alter stem elongation in the transgenic plants, but it also influenced plant development in general. The notion that mannan-recognizing CBMs may exhibit non-hydrolytic disruption of the seed mannan polysaccharides, causing rapid weakening of the cell wall, and thus a faster germination rate was not supported by our investigation with CBM29-1-2. To the best of our knowledge, this is the first report, which demonstrates the use of a CBM to modulate whole plant development.

Expansins are plant proteins believed to be involved in disrupting the non-covalent adhesion of cellulose to matrix polysaccharides, thereby promoting wall creep. They consist of two domains: an N-terminal domain I and a C-terminal domain II. The latter is suggested to have carbohydrate binding ability based on its similarity to cellulose-binding domains. We investigated the capability of a putative CBM from potato expansin to modify tobacco cell walls independent of the domain I (Chapter 5). Cell wall phenotypes (mainly expansion of xylem cells and reduction in wall thickness) were observed in vascular tissues, which are rich in cellulose and xylan, suggesting that the putative CBM may have bound to either or both polysaccharides, and consequently interfered with their interactions. However, this investigation did not link its binding definitively to a particular wall polysaccharide.

The thesis is concluded with a general discussion (Chapter 6) on the results of the different approaches used to generate plants with modified cellulose-hemicellulose networks. On the whole, the work has generated a proof of concept for the use of CBMs to influence cell wall architecture. Improvements in the strategies employed are required before they can be transferred to the relevant fibre crops.

Samenvatting

Plantencelwandpolysacchariden worden commercieel gebruikt als verdikkings- en geleermiddel, en stabilisator. Daarnaast zijn ze ook de hoofdbestanddelen van vezels. Natuurlijke vezels hebben uiteenlopende toepassingen in de industrie, zoals onder andere in de papier- en textielindustrie. Bovendien stijgt de vraag naar vezels (of biocomposieten) voor toepassingen in de bouw en autoindustrie. Het is belangrijk om voor de verschillende toepassingen controle over de vezeleigenschappen te verkrijgen. Intrinsieke vezeleigenschappen worden grotendeels bepaald door de interacties tussen cellulose en hemicellulose. De verhouding tussen deze groepen van celwandpolysacchariden ligt ten grondslag aan vezelkwaliteit. Dit proefschrift onderzoekt de mogelijkheid om deze interacties in de plant te beïnvloeden, met het uiteindelijke doel om betere vezelgewassen te verkrijgen. In dit onderzoek werden twee strategieën gevolgd om cellulose-hemicellulose netwerken te modificeren; enerzijds werd getracht om de hoeveelheid van de verschillende polysacchariden te moduleren, anderzijds werd geprobeerd om de interactie tussen cellulose en hemicellulose te beïnvloeden. Deze strategieën leidden tot de volgende doelstellingen van dit proefschrift: (i) verandering van de hoeveelheid cellulose door kruising van twee antisense cellulose synthase (CesA) aardappellijnen ter verkrijging van nakomelingen met een grotere afname in cellulose hoeveelheid dan de enkelvoudige transformanten, (ii) heterologe expressie van verschillende microbiële "carbohydrate-binding modules" (CBM) genen in tabak, om te onderzoeken of het mogelijk is om de celwandstructuur op deze wijze te veranderen, en (iii) heterologe expressie in tabak van een expansinedomein, waarvan verondersteld wordt dat het een CBM is, met als doel de functie van dit deel van het eiwit in celwand-gerelateerde processen te bepalen. Een verdere reductie in de hoeveelheid cellulose werd onderzocht in aardappel, omdat geschikt plant materiaal reeds aan het begin van het onderzoek in het laboratorium aanwezig was. Voor de overige experimenten werd gebruik gemaakt van tabak, omdat het een veel gebruikte plantensoort met betrekking tot onderzoek naar vezelmodificatie is. Het was de verwachting dat het project bruikbare informatie zou opleveren met betrekking tot het *in planta* modificeren van vezeleigenschappen, om uiteindelijk deze kennis te vertalen naar economisch belangrijkere soorten zoals vlas, hennep of populier.

Aan het begin van dit onderzoek was nog geen enkel gen betrokken bij de biosynthese van hemicellulose hoofdketens geïdentificeerd. Dit was niet het geval voor de cellulose biosynthese. In veel gewassen, inclusief aardappel, zijn CesAs geïdentificeerd, en genen die hiervoor coderen geïsoleerd. Voorgaand onderzoek met betrekking tot CesAs van aardappel in ons laboratorium, heeft een aantal antisense en sense CesA transgene aardappellijnen opgeleverd, met zowel lagere als hogere cellulosegehalten in de knollen. Twee van deze antisense lijnen (csr2 en csr4), met een lager cellulosegehalte dan wildtype planten, werden gekruist (Hoofdstuk 2). Het doel van de kruising was het produceren van dubbel-transformanten met nog minder cellulose dan de ouders. Het onderzoek was succesvol in het genereren van dubbel-transformanten, waarvan de knollen een lager cellulosegehalte (60%) hadden dan de

twee ouders (enkelvoudige transformanten). Ondanks deze afname werden geen opvallende fenotypen aan de dubbel-transgene knollen waargenomen. Het was opmerkelijk dat de knollen van de csr2 enkelvoudige transformant een interessanter fenotype lieten zien dan de knollen van de csr2/csr4 dubbel transformant. Het meest opvallend was de ophoping van cellulose-achtig materiaal in de celhoeken van de csr2 knollen, hetgeen waargenomen werd met behulp van microscopie na kleuring met "calcofluor white". De voorlopige analyse van dit materiaal wijst er op dat de ophoping waarschijnlijk amorf cellulose is. Verder werd gevonden dat de nakomelingen met een of beide transgenen meer knollen produceerden dan de controle planten, en dat de individuele knollen vaak kleiner en lichter in gewicht waren. Deze fenotypen waren meer uitgesproken bij de csr2 knollen dan in die uit de csr4 en csr2/csr4 serie. Bovendien werd in de knollen van csr2 transformanten ongebreidelde proliferatie van xyleemcellen waargenomen, gepaard gaande met lignificatie van de celwanden.

Naast modulatie van cellulose biosynthese, werd ook gekeken of de interactie tussen cellulose en hemicellulose verstoord kon worden door middel van suiker-bindende eiwitten, CBMs, zonder catalytische activiteit. Er waren aanwijzingen in de (patent) literatuur dat CBMs plantengroei konden versnellen. Onze hypothese was dat door binding van CBMs aan hemicellulose, inbouw van hemicellulose in cellulose microfibrillen kon worden verhinderd. Hiertoe hebben we aspecifieke CBM29s (CBM29 alleen, en de tandem CBM29-1-2) en de meer-specifieke CBM2b-1-2 geïntroduceerd in tabak (Hoofdstuk 3 en 4). CBM29 kan binden aan cellulose en verschillende hemicelluloses (mannaan, galactomannaan, glucomannaan, xyloglucaan, xylaan), terwijl de CBM2b-1-2 bindt aan xylaan en in mindere mate aan cellulose. De aspecifieke tandem CBM29-1-2 liet een sterker fenotype zien dan de meer-specifieke CBM2b-1-2. De cellen in de meeste delen van de stengel van de transgene lijnen met de aspecifieke CBM29-1-2 waren anders van vorm en grootte (Hoofdstuk 3), terwijl de effecten van de meer-specifieke CBM2b-1-2 voornamelijk beperkt bleven tot het vaatweefsel (Hoofdstuk 4). Het meest in-het-oog-springend was dat de cortex cellen in stengelsecties van de CBM29-1-2 planten zeer onregelmatig van vorm waren. Het was opvallend dat CBM29-1-2 expressie niet alleen van invloed was op stengelgroei maar ook de ontwikkeling van de tabaksplant in het algemeen beïnvloedde. Het concept dat mannaan-herkennende CBMs het in zaad opgeslagen galactomannaan toegankelijk kunnen maken, en deze reserves sneller kunnen mobiliseren werd niet ondersteund door ons onderzoek, aangezien zaden van CBM29-1-2 tabakslijnen geen verhoogde ontkiemingssnelheid lieten zien. Voor zover wij weten, is dit het eerste rapport dat aantoont dat plantontwikkeling met behulp van CBMs kan worden veranderd.

Expansinen zijn planteneiwitten waarvan gedacht wordt dat ze de non-covalente interactie van cellulose en matrixpolysacchariden kunnen verstoren, waardoor de celwand zich gemakkelijker aan kan passen gedurende processen als b.v. celstrekking. Ze bestaan uit twee domeinen: een N-terminaal domein I en een C-terminaal domein II. Van de laatste wordt verondersteld dat het een CBM is, op basis van haar homologie met cellulose-bindende domeinen. Het vermogen van een hypothetische

aardappel expansine CBM om celwanden te veranderen, onafhankelijk van domein I, werd bestudeerd door het corresponderende gen in tabak tot expressie te brengen (Hoofdstuk 5). Een vergroting van xyleemcellen (en een daarmee gepaard gaande reductie in wanddikte) werd waargenomen in stengelsecties. Aangezien het vaatweefsel rijk in cellulose en xylaan is, suggereert dit resultaat dat de expansine CBM mogelijk een van deze twee, of beide, polysacchariden bindt. Vergelijking van onze resultaten met de literatuur suggereert dat beide domeinen van expansine belangrijk zijn voor de werking van het eiwit in de plant.

In de algemene discussie van dit proefschrift (Hoofdstuk 6) worden de resultaten van onze verschillende aanpakken, gebruikt om het cellulose-hemicellulose netwerk *in planta* te veranderen, besproken. In zijn algemeenheid kan gesteld worden dat dit onderzoek heeft laten zien dat het mogelijk is om met behulp van CBMs de celwandarchitectuur te beïnvloeden. Optimalisering van de gebruikte strategieën is nodig voordat ze gebruikt kunnen worden ter verbetering van vezelkwaliteit in relevante vezelgewassen.

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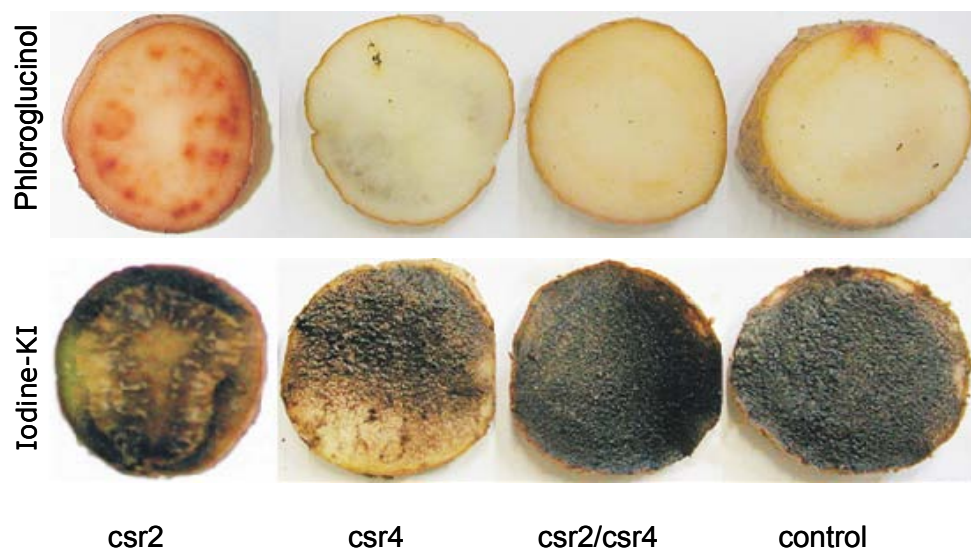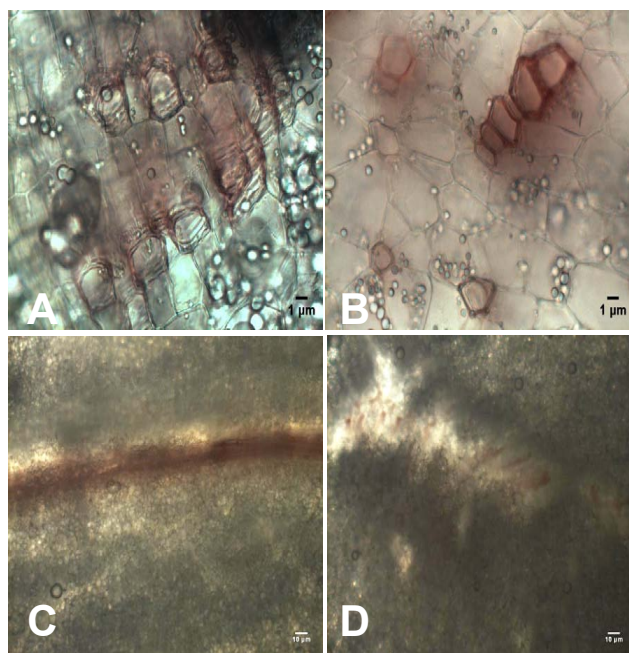
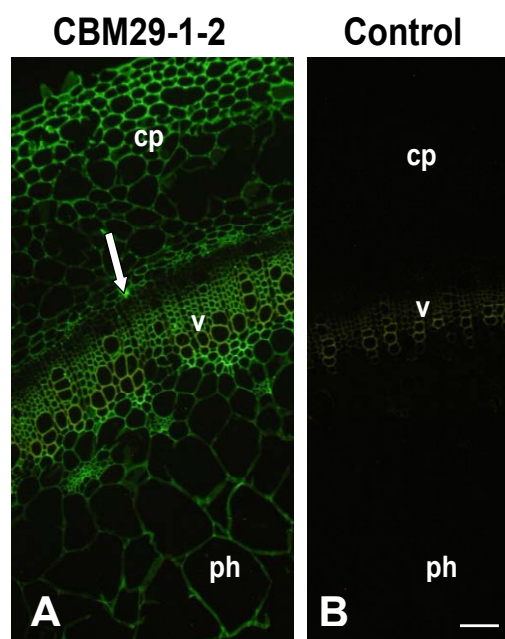**Appendix**
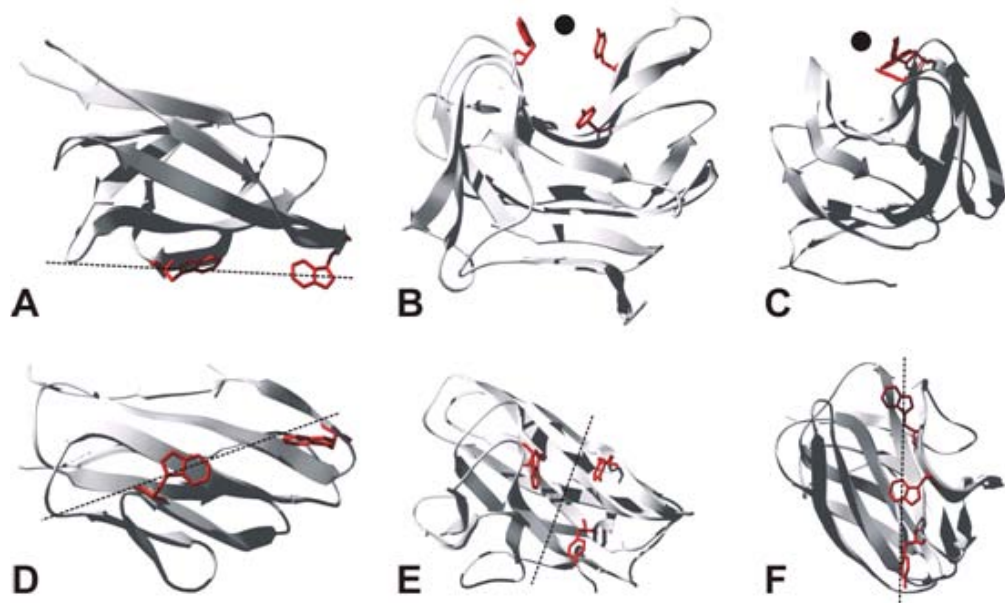
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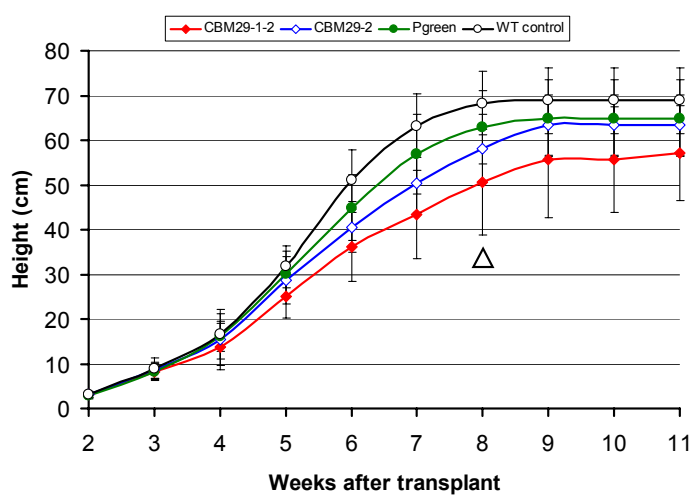

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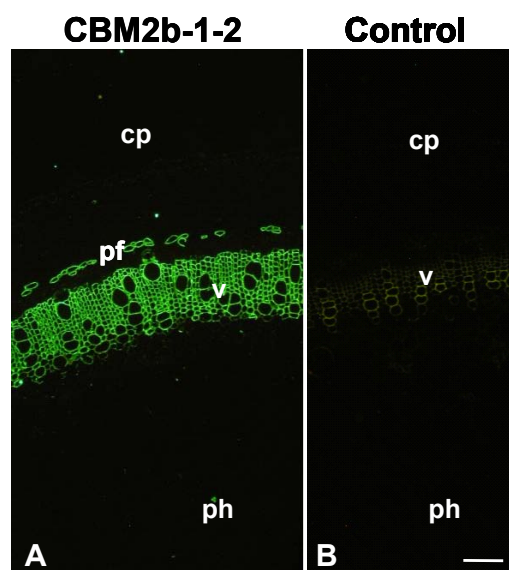

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Chapter 2 Fig. 7 Page 41
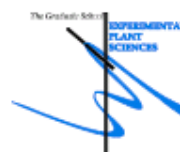
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Chapter 3 Fig. 4 Page 60

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Education Statement of the Graduate School

Experimental Plant Sciences

Issued to: Olawole Odun Obembe
Date: January 30, 2006
Group: Laboratory of Plant Breeding, Wageningen University

1) Start-up phase	_date_
► First presentation of your project	
Bioengineering cell wall crystallinity	January 2002
► Writing or rewriting a project proposal	
► Writing thesis introduction : introduction to bioengineering cellulose-hemicellulose networks in plants	2002-2005
► MSc courses (1) recombinant DNA en genetische manipulatie (2) moleculaire plantcelbiologie	
(3) genetische variatie en modificatie	May - Aug 2001
► Laboratory use of isotopes	
Course Radiation Hygiene, level 5B	26 June 2002
Subtotal Start-up Phase	15 credits*

2) Scientific Exposure	_date_
► EPS PhD student days	
PhD student day 2002, Wageningen	January 24, 2002
PhD student day 2003, Utrecht	March 27, 2003
PhD student day 2004, Amsterdam	June 3, 2004
► EPS theme symposia	
Theme symposium IV, 2001, Nijmegen	December 13, 2001
Theme symposium III, 2001, Utrecht	December 20, 2001
Theme symposium III, 2002, Utrecht	October 11, 2003
Theme symposium III, 2003, Amsterdam	December 11, 2003
► NWO Lunteren days and other National Platforms	
ALW Meeting Lunteren 2003, Plant Sciences	April 7-8 2003
ALW Meeting Lunteren 2004, Plant Sciences	April 5-6 2004
ALW Meeting Lunteren 2005, Plant Sciences	April 4-5 2005
► Seminars (series), workshops and symposia	
Plant Breeding Seminar Series	2001-2005
Frontiers in Plant Development Seminar Series	2002-2004
► Seminar plus	
► International symposia and congresses	
Gordon Conference, Italy	May 4-9 2003
2nd Cell wall Biosynthesis Meeting, USA	August 4-7 2005
► Presentations	
Oral Presentation at Plant Breeding Seminar Series	2001-2005
Oral Presentation at the 2nd Cell wall Biosynthesis Meeting, USA	August 4-7 2005
► IAB interview	March 28, 2003
► Excursions	
Subtotal Scientific Exposure	10.7 credits*

3) In-Depth Studies	_date_
► EPS courses or other PhD courses	
Summer course Glycosciences	23-27 June 2002
Summer course Protein Engineering	8-10 March 2004
► Journal club	
Weekly discussion at Plant Breeding	2001 - 2005
► Individual research training	
Subtotal In-Depth Studies	5.4 credits*

4) Personal development	_date_
► Skill training courses	
Scientific Writing	June 29 - July 2, 2004
Presentation skills	April 19 - May 17, 2004
Project and Time Management	March - May, 2004
► Organisation of PhD students day, course or conference	
► Membership of Board, Committee or PhD council	
Subtotal Personal Development	4.6 credits*

TOTAL NUMBER OF CREDIT POINTS*	35.7

* A credit represents a normative study load of 28 hours of study