Characterising the Cellulose Synthase Complexes of Cell Walls

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Thesis

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Contents

To my beloved parents

and much-loved twin sister Sahar

Chapter 1

General Introduction

Plant cell walls

The presence of a structural cell wall distinguishes the plant from the animal kingdom. This semi rigid, dynamic interface that surrounds the plant cell constitutes the raw material used to manufacture textiles, paper, lumber and other products. The cell wall is the fundamental determinant of the size, shape and distinct morphology of plant cells (Somerville et al., 2004). It naturally has to allow for cell expansion, growth and differentiation while providing structural support to the plant cells and counterbalancing the internal turgor pressure. It offers protection to the cells from the adverse surrounding changes such as pathogen attacks and mechanical abrasion (Gaffe et al., 1997; Willats et al., 2006). Moreover, plants cell walls control cell adhesion (Cosgrove, 2005), determine porosity and are important reservoirs for biological molecules such as storage polysaccharides (Santos et al., 2004).

The cell walls are generally categorized into two types, primary cell walls and secondary cell walls. All plant cells possess a primary cell wall; a thin, flexible and extensible layer which is laid down during cell division and is responsible for cell expansion and subsequent growth of the plant wall (McNeil et al., 1984; Sánchez-Rodríguez et al., 2010). After cessation of cell expansion, the thicker secondary cell wall which is the most abundant source of renewable biomass produced by plants is deposited inside the scaffold formed by the primary cell wall. The rigidity and strength of the plant is dependent on the secondary cell wall and constitutes the major part of treachery elements and fibres in wood (Carroll and Somerville, 2009; Pauly and Keegstra, 2010).

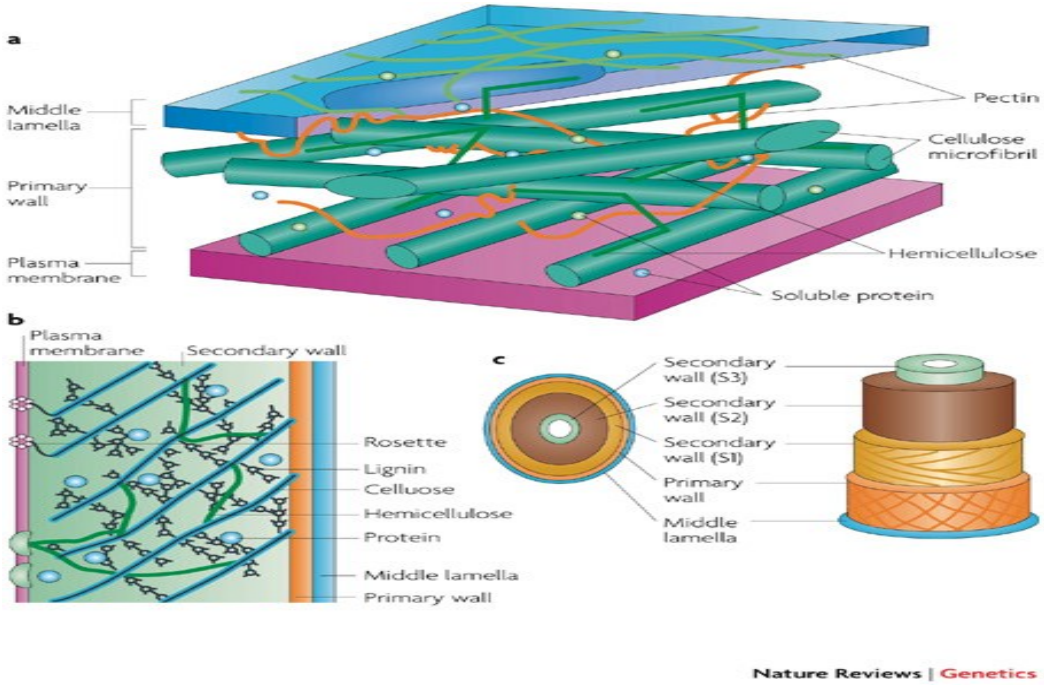

Figure 1: Plant cell wall structure. a, Cell wall containing cellulose microfibrils, hemicellulose, pectin and proteins. b, Cell wall containing cellulose microfibrils, hemicellulose, lignin and proteins with the cellulose synthase enzymes floating in the plasma membrane and in the form of rosette complexes. c, Lignification occurs in the S1, S2 and S3 layers of the cell wall. With permission from Nature Reviews journal, Sticklen, 2008.

The cell wall matrix

Both primary and secondary cell walls of higher plants are comprised of various polysaccharides, enzymes and proteins (Figure 1). The different composition of the primary and secondary cell wall components account for their difference in function (Carpita and Gibeaut, 1993). The primary cell wall polysaccharides are comprised mainly of cellulose, whose microfibrils constitute the rigid framework of the cell wall, hemicellulose mainly consisting of xyloglucan (XG) and pectins all alongside the presence of proteins (structural or enzymatic). The secondary cell wall consists of cellulose, hemicellulose (mostly xylans) and lignin (Somerville et al., 2004; Turner et al., 2007; Mellerowicz and Sundberg, 2008). Additionally, primary cell walls contain more pectins and proteins compared to secondary cell walls (Cosgrove, 2005).

Cellulose is the most abundant biopolymer on earth and accounts for more than 50% of the carbon in the biosphere with an estimated 180 billion tons produced annually and is used in paper, textiles, cellophane, plastics and photographic films (Somerville, 2006). Cellulose is the major structural component of both the primary and secondary cell walls of plants. In the primary cell wall, it is a vital component of the load-bearing network and an important determinant of the orientation of cell expansion (Carpita and McCann, 2000). In the secondary cell wall, it provides the plants with the mechanical strength it requires. This highly crystalline linear polymer of beta 1,4-linked glucose residues forms a chain in which every other glucose residue rotates 180 degrees with respect to its neighbour and forms cellobiose, the repeating structural unit of cellulose (Zugenaier, 2001). Cellulose biosynthesis requires the use of its substrate UDP-glucose (UDP-Glc) to polymerize the glucan chains and generate microcrystalline cellulose (Saxena and Brown, 2005). The process of chain elongation in cotton has been suggested to require the primer sitosterol-beta-glucoside (SG) as the initial acceptor for chain elongation to which glucose units are added (Peng et al., 2002). The elongated cellulose chains lie parallel to each other linked via hydrogen bonds and form strong, mostly insoluble and chemically stable crystalline microfibrils (Delmer and Amor, 1995; Cosgrove 1997; Brown and Saxena, 2000; Saxena and Brown, 2005). The microfibrils represent the load bearing polymer and permit cellulose to play its structural role in the plant by controlling the direction for the extension of the cell through its orientation.

Hemicelluloses are heteropolymeric polysaccharides that are structurally homologous to cellulose because they have a backbone composed of (1,4)-β-D-hexosyl residues. They form cross-linking networks with cellulose microfibrils (Cosgrove, 2005), allowing the cell walls to be modified during cell development (Cosgrove, 2005; Scheller and Ulvskov, 2010) while providing the structural support for the cell wall (Persson et al., 2007). They also give lateral strength across the matrix, control cell shape and growth. The branched nature of hemi-cellulose gives it the necessary points from which the cross-links can be formed (Somerville et al., 2004). Hemicelluloses can be grouped into four main classes according to the main type of sugar residues present: xylans, xyloglucans, mannans and mixed linkage β-glucans (Mellerowicz and Sundberg, 2008). Xylan (glucuronoarabinoxylan) is present in the primary cell wall however it is the main hemicellulose of the secondary cell wall (Scheller and Ulvskov, 2010). It is a linear β-(1→4)-linked D-xylose backbone substituted to varying

extents with side chains of α-1,2-arabinans as well as galactosyl and glucuronyl groups (Carpita and McCann 2000). Xyloglucan is the dominant hemicellulose in the primary cell walls composed of linear chains of β-(1→4)-D-glucan backbone with numerous α-D-xylose residues or xylose-galactose-fucose moieties (Reiter, 2002; Liepman *et al.*, 2010). Mannans including galactomannans and galactoglucomannans, have a similar three-dimensional structure to cellulose, and are important structural components of the cell wall. They are also a source of storage polysaccharides and may play a role in the growth of pollen tubes and roots (Goubet et al., 2003). Mixed linkage β-glucans consist of an unbranched backbone of glycosyl residues containing both β-1,3- and β-1,4-linkages (Lerouxel et al., 2006).

Pectins are a group of polysaccharides that are rich in glucuronic acid and 1-4 linked α-D-galacturonic acid residues. They are present in the cell wall structure interweaving in the cellulose-hemicellulose network. The four main types of pectins are: homogalacturonan, rhamnogalacturonan I, rhamnogalacturonan II, and xylogalacturonan (Somerville et al., 2004; Mohnen, 2008). Homogalacturonan is composed of linear chains of galacturonic acid residues. The rhamnogalacturonan I backbone consists of alternating α-1-2-linked rhamnose and α-1-4- linked galacturonic acid residues with the rhamnose residues branched with side chains of other pectins domains such as arabinose, galactose and arabinogalactan molecules. Rhamnogalacturonan II has a backbone of α-1-4 linked galacturonic acid, which is substituted with four complex and structurally different side chains at different locations (Ridley et al. 2001; Willats et al., 2001; McCartney et al. 2001; Somerville et al., 2004; O'Neill et al., 2004; Mohnen, 2008). Finally, xylogalacturonan is a homogalacturonan in which the α-1-4 linked galacturonic acid backbone is substituted with a xylose (Nakamura et al., 2002).

Lignin is, after cellulose, the second most abundant terrestrial biopolymer and accounts for approximately 30% of the organic carbon in the biosphere (Boerjan et al., 2003). Lignin consists of a group of polymers composed of highly cross-linked phenolic molecules deposited in the final stages of xylem cell differentiation in the secondary walls. These aromatic alcohols impart the stiffness and considerable strength of the the woody tissue found in plants (Chabannes et al., 2001; Jones et al., 2001) and prevents further alterations in the cell also making the plant less vulnerable to pathogen attack. In addition, lignin waterproofs the cell wall allowing the transport of water through the vascular system (Wardrop 1971, Baucher et al. 1998, Boerjan et al. 2003).

The cell wall polysaccharides are synthesised in various subcellular localizations. Cellulose is thought to be synthesised at the plasma membrane whereas the other non-cellulosic polysaccharides are assumed to be assembled in the Golgi apparatus and then exported from the Golgi to the cell wall (Ridley et al., 2001; Reiter, 2002).

These cell wall polysaccharides and lignin form interpenetrating networks which together form the complex cell walls of the plant. The interacting networks consist of cellulose-hemicellulose with pectin or lignin as well as structural and enzymatic proteins. The cellulose-hemicellulose network is thought to be the major load bearing component in the cell walls (Carpita and Gibeut, 1983; McCann and Roberts, 1992) with the branched hemicelluloses binding to the surface of cellulose microfibrils and linking them together

(Cosgrove, 1997). Xyloglucans are believed to coat cellulose and form cross links between the microfibrils to hold them in place (Whitney 2000). During growth, the side chains of xyloglucans cause the strong cellulose network to weaken, thus allowing for the expansion of the cell (Cosgrove, 2001; Wilson et al. 2006). The pectin network occupies the space in the cellulose-hemicellulose network and provides an environment for the deposition, extension, and slippage of the mentioned network to allow cell growth. The lignin network is the last to be deposited and adds additional stiffness by forming a rigid impermeable (Jeffries, 1990). Recently by using NMR technology the intermolecular relationships and dynamics of cellulose, hemicelluloses, and pectins was determined and was suggested that the load bearing in plant cell walls is accomplished by a single network of all three types of polysaccharides and not by just a cellulose−xyloglucan, cellulose-pectin or pectin-xyloglucan network (Dick-Pérez et al., 2011).

In addition to the interacting polysaccharide networks, a small amount of protein can be found in the plant cell walls, some of which are thought to increase mechanical strength and some consist of enzymes, which form, remodel, or break down the structural networks of the wall. So far, only a limited number of cell wall proteins and enzymes have been identified and characterized. Expansins, extensins, xyloglucan endotransglucosylase/hydrolases and peroxidases are some of the proteins detected in the cell walls (Jamet et al., 2006).

Cellulose biosynthesis

In plants the formation of cellulose involves the synthesis of cellulose microfibrils through membrane bound protein particles known as `terminal complexes' (Cosgrove, 2005). Terminal complexes were first identified by their association with the ends of cellulose microfibrils and were first observed in the bacterium *Acetobacter xylinum* (Brown et al. 1976), the plasma membrane of algae (Brown and Montezinos, 1976) and later on in higher plants (Muller and Brown 1980). Freeze fracture experiments also showed hexagonally arranged particles embedded in the plasma membrane of a vascular plant (Figure 2a) thus naming it a rosette terminal complex (Mueller and Brown, 1980; Herth, 1985). The rosettes have been detected in all higher plants, mosses, ferns, liverworts and certain green alga (Brown, 1996). These complexes were suggested to be responsible for cellulose synthesis as they disappeared after treatment with cellulose synthase inhibitors (Heim et al., 1989). Using a freeze-fracture replica labelling technique with antibodies raised against the catalytic region of a cotton cellulose synthase (GhCESA) clearly showed the hexagonal rosette terminal complex of vascular plants present at the plasma membrane of Azuki beans and proved that cellulose synthase is a component of the rosette terminal complex in vascular plants (Kimura et al., 1999).

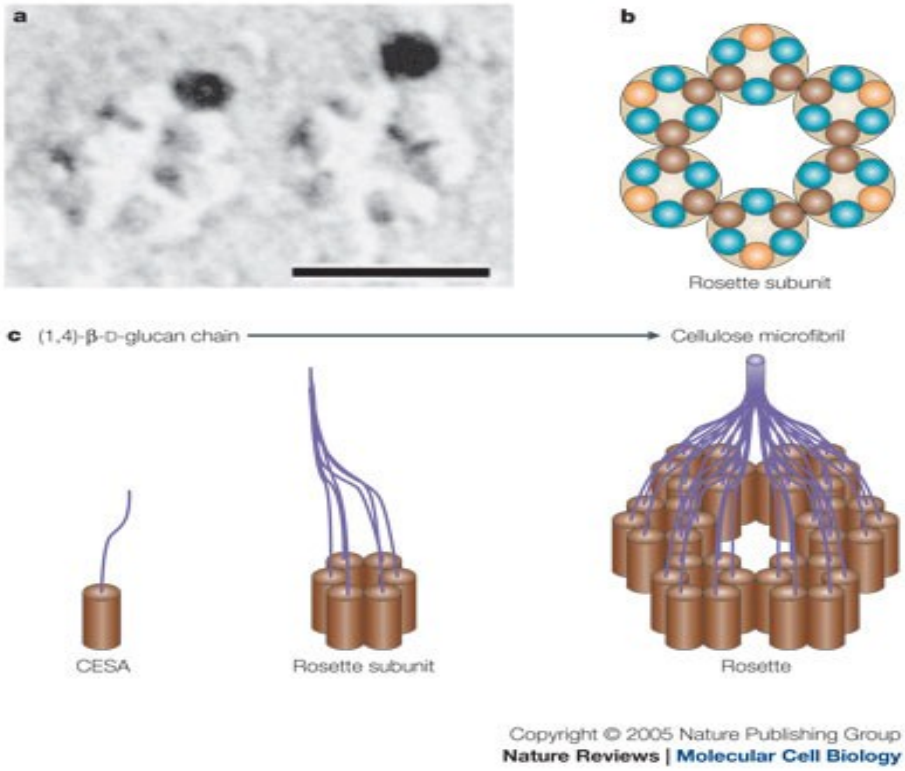

Figure 2: The cellulose synthesizing machinery of the cell wall. a. Rosette terminal complex in the plasma membrane of a vascular plant. b. Model for the hexameric particle showing how the CESAs may be organized in the rosette subunits and c. Model for the structure of the rosette. Six subunits, containing six CESA proteins interact to form a rosette each synthesizing a single (1,4)-β-linked D- glucan chain. With permission from Nature reviews Cosgrove, 2005.

The hexameric rosette terminal complex is one of the largest protein complexes known with a diameter of approximately 25-30 nm (Kimura et al., 1999a) and is composed of six subunits each of which in turn contain six cellulose synthases (CESA proteins) providing a total of thirty-six CESA proteins per rosette (Mueller and Brown, 1980; Kimura et al., 1999; Saxena and Brown, 2005). Considering that each cellulose synthase was assumed to form a glucan chain, then the microfibril which is composed of glucan chains would consist of 36 glucan chains (Herth, 1983; Delmer, 1999; Kimura et al., 1999; Perrin et al., 2001; Doblin et al., 2002; Somerville et al., 2004). It must be mentioned that no direct evidence is available for this assumption, however it has become commonly accepted (Figure 2b, c).

Immunolabeling of freeze-fracture replicas of the rosette terminal complex have demonstrated the cellulose synthase enzyme to be part of the complex at the plasma membrane (Kimura et al., 1999). The cellulose synthases (CESAs) are believed to be the catalytic subunits of the rosette complex (Kimura et al., 1999). They are integral membrane proteins (Figure 3) with a conserved structure and contain a zinc-binding domain, eight transmembrane domains. A 600 amino acid cytosolic loop situated between the second and third transmembrane domain which encompasses the catalytic site and includes the conserved D, D, D QxxRW motif found in the Glycosyl transferase family 2 proteins (Richmond, 2000; Vergara and Carpita, 2001; Krauskopf et al., 2005).

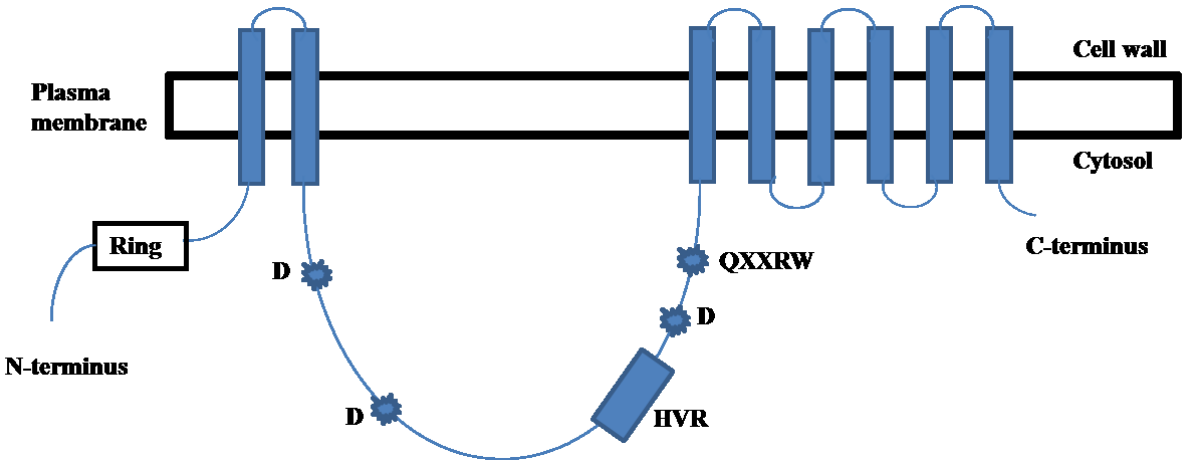

Figure 3: Model for the topology of a CESA protein. The CESA protein contains eight transmembrane domains and a large cytosolic domain, an N terminal zinc finger and a cytosolic C terminal.

CESA genes which encode the CESA proteins are members of multigene families. No less than ten *CESAs* in Arabidopsis have been identified which are expressed in different tissue and cell types (Richmond 2000; Richmond and Somerville, 2000; Brett, 2000; Somerville, 2006). The large number of Arabidopsis *CESA* genes is interesting to note. However, as the function of the individual CESAs is unknown, it is not clear whether the *CESA* genes have identical roles in cellulose biosynthesis, suggesting functional redundancy between them or distinct and none overlapping roles. If there were to be an overlap in function, mutational analysis in any one of the genes would result in a disruption in cellulose biosynthesis and reduced cellulose content. Mutational analysis of the Arabidopsis *CESA* genes along with gene expression analysis have shown that there is indeed some functional redundancy between the CESAs (Persson et al., 2007).

Tissue specific expression and mutational analysis in the *CESA* genes have resulted in the CESAs being divided into two groups according to their involvement in cellulose synthesis in the primary or secondary cell walls. Genetic complementation and co-immunoprecipitation experiments have shown that cellulose biosynthesis in Arabidopsis cells involves the function of up to three distinct CESAs (Taylor et al. 2000; Scheible et al. 2001; Taylor et al. 2003; Desprez et al. 2007; Persson et al., 2007). Out of the 10 *CESA* genes in Arabidopsis, mutations in *CESA4*, *CESA7* and *CESA8* revealed that these three CESA proteins are required for cellulose biosynthesis in the secondary cell wall by showing decreased cellulose content due to a collapsed xylem (Turner and Somerville 1997; Taylor et al. 2003). Mutations in the remaining genes, *CESA1*, *2*, *3*, *5*, *6* and *9* caused defects in primary cell wall synthesis and reduced cellulose content (Arioli et al., 1998). Mutations in two or more of the primary CESAs revealed partial redundancy between CESA2, 5, 9 and CESA6 (Desprez et al., 2007; Somerville, 2006; Lerouxel et al., 2006; Persson et al., 2007). A single mutation in CESA1 identified as the *rsw1* mutant showed stunted growth, reduced cell elongation and hypocotyl length and radially swollen root phenotype in restrictive temperature by causing the disassembly of plasma membrane rosette complex (Arioli et al., 1998). Null mutations in both CESA1 and CESA3 were gametophytic lethal, however CESA3 missense mutants (*ixr1*) showed to confer resistance to the cellulose synthase inhibitor isoxaben and resulted in a

retarded growth in roots and shoots and reduced cellulose content implying that in the absence of a functional CESA, the cellulose synthase complex and rosette structures don't function properly. Despite showing decreased cell elongation, stunted roots and increased radial swelling specifically in dark grown Arabidopsis hypocotyls, the *Procuste1* mutant of CESA6 in Arabidopsis (Fagard et al., 2000) doesn't die during pollen development suggesting that other CESA subunits may act redundantly with CESA6 i.e. CESA2, CESA5 and CESA9, thus concealing more severe phenotypes (Desprez et al. 2007; Persson et al., 2007; Paredez et al. 2007).

The rosette composition

The CESA proteins which are localized in the rosette have been shown to interact with each other in the primary and secondary cellulose synthase complex by forming homodimers and heterodimers both *in vivo* and *in vitro* (Taylor et al, 2003; Desprez et al., 2007; Atanassov et al, 2009; Timmers et al., 2009). Understanding the interaction between the CESAs in order to form a functional rosette complex is essential for a proper comprehension of the formation of cellulose synthase complex and the organization of glucan chains synthesized by each CESA into crystalline microfibrils. It is important to know if there are specific positions assigned to the CESAs in the rosette or whether they are placed at random. In the primary cell wall, bimolecular fluorescence complementation assay and pull down experiments on the relative mutants of the primary CESAs showed that the primary Arabidopsis CESAs all interact with one another and form both homodimers and heterodimers *in vivo* (Desprez et al., 2007; Wang et al., 2006; 2008) thus confirming the presence of the three CESAs in the primary cellulose synthase complex. Co-immunoprecipitation, co-localization experiments and mutational analysis showed all the three secondary cell wall CESAs; CESA4, CESA7 and CESA8 interacting with each other and being expressed in exactly the same cells at the same time, all containing approximately a third of wild-type cellulose levels, thus being equally important in cellulose biosynthesis (Taylor et al. 2003). The protein levels of the three interacting secondary cell wall CESAs in the CESA8 weak mutant were similar to the wild type suggesting possible differences in interaction patterns between the secondary CESAs (Taylor et al., 2003). Confirming differences in interaction patterns between the secondary CESAs, it was shown both *in vivo* and *in planta* that only CESA4 was able to form a homodimer and the rest all formed heterodimers (Timmers et al., 2009) with specific positions suggested for each of the secondary CESAs in the complex. Again highlighting the requirement of three CESAs for the formation of the cellulose synthase complex.

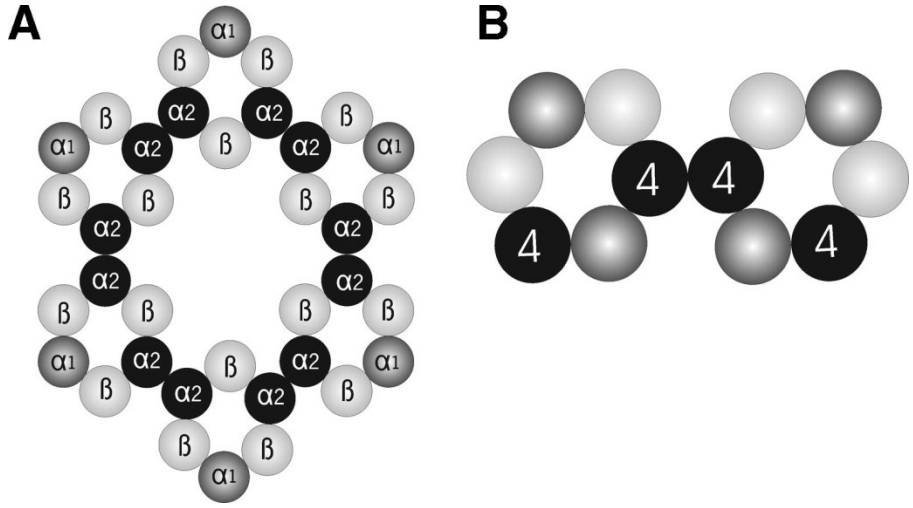

Figure 4: Proposed models for the structure of the rosette. A, Six subunits containing six polypeptides interact to form a rosette as suggested by Doblin and co-workers. B, the modified model based upon the interactions between the secondary CESA isoforms. CESA4 interacts with all isoforms, the homodimerization links the subunits together, and the two other positions are filled by CESA7 and CESA8. With permission from FEBS Letters, Timmers et al., 2009.

The transport of the cellulose synthase complex

The presence of the rosette terminal complex at the plasma membrane is vital for cellulose to be made at the cell surface. Cellulose synthases which constitute the catalytic subunits of the rosette complex are initially made in the ER and move to the Golgi. Once assembled, the complex is then transported to the plasma membrane for cellulose synthesis by moving and pausing at various sites along the microtubules to be delivered to the plasma membrane (Crowell et al., 2009; Guitierrez et al., 2009), Cortical microtubules interacting with the Golgi vesicles guide the cellulose synthase complex through the plasma membrane (Paradez et al., 2006; Crowell et al., 2009). In this delivery, small CESA containing particles have been detected below the plasma membrane named MASCs (microtubule-associated cellulose synthase compartments, Crowell et al., 2009) or SMaCCs (small CesA compartments, Gutierrez et al., 2009). Disruption of the cellulose synthase complex with drugs such as isoxaben, which deplete the CESAs from the membrane (Paredez et al., 2006), shows increased distribution of the MASC particles with CESAs accumulating inside suggesting that by delivering the complex to the plasma membrane they help regulate cellulose biosynthesis (Crowell et al., 2009).

Other components involved in cellulose biosynthesis

While it is widely assumed that a minimum of three CESA proteins are required for cellulose synthase and are involved in its assembly, there is currently little information on whether proteins other than CESAs are present in the complex. Genetic evidence has suggested other proteins to participate in cellulose biosynthesis, although direct involvement with the complex has not been demonstrated before (Nicol et al., 1998; Sato et al., 2001; Ruan et al., 2003; Szyjanowicz et al., 2004; Somerville, 2006; Coleman et al., 2009). Using a blue native

polyacrylamide gel electrophoresis (BN-PAGE) of solubilized Arabidopsis cell extracts, a complex of 840 kDa in size was detected containing the primary CESAs (Wang et al., 2008). Apart from identifying the CESAs in the complex, no further purification of the complex was carried out to ensure the presence of proteins other than the CESAs. Affinity purification of an intact epitope-tagged complex resulted in the detection of only CESA-CESA interactions and no intact complex purification (Atanassov et al. 2009), the same sized complex was also detected in *Populus* using co-immunoprecipitation with antibodies for specific CESAs to pull down the cellulose synthase complex (Song et al., 2010). Since the complexes detected were larger than the expected size of the cellulose synthase complex with six CESA subunits, the presence of other proteins in the complex is possible. Mutational analysis of various candidates showing defects in cellulose biosynthesis has also led to the conclusion that proteins other than CESAs are also part of the complex. Based on analysis of different mutants in Arabidopsis, proteins such as sucrose synthases, KORRIGAN, COBRA, KOBITO and CTL1 have all been suggested to be associated with the complex (Somerville, 2006). However the direct association of most of these proteins with the CESAs has not been demonstrated. It must be mentioned that these proteins have been identified by co-immunoprecipitation with the CESAs in poplar further supporting a direct role in cellulose biosynthesis (Song et al., 2010).

One of the proteins deemed important in cellulose biosynthesis is sucrose synthase. UDP-glucose, a substrate for the synthesis of glucan chains in plants, can be made from sucrose through a reaction catalysed by sucrose synthase (SUS; Amor et al., 1995; Haigler et al., 2001). Studies in cotton cells have shown that the down regulation of the cotton susy (*GhSUS1*) gene in cotton plants can repress the initiation and elongation of cotton fibre cells (Ruan et al., 2003), suggesting that SUS proteins may channel UDP-glucose to the cellulose synthase complex for cellulose formation. Overexpression of the *Gossypium hirsutum SUS* gene in poplar lead to increased cellulose formation (Coleman et al., 2009). It was also shown that SUS is actually an integral component of the cellulose synthase complex according to the immunogold labelling with anti-SUS antibodies under an electron microscope (Fujii et al., 2010) supporting an association between these proteins. In Arabidopsis, six isoforms of sucrose synthase are encoded in the genome and extensive studies have been made of the tissue specific expression patterns of all six isoforms (Baud et al., 2004; Bieniawska et al., 2007). Comparison of the deduced amino acid sequences shows that Arabidopsis SUS proteins are divided in three classes (Baud et al., 2004), the first comprises of SUS1 and SUS4, the second SUS2 and SUS3 and the third include SUS5 and SUS6. To date the exact role of the Arabidopsis sucrose synthase isoforms are unknown, however it has been suggested that SUS5 and SUS6 have a specific function in callose synthesis which uses the same UDP-glucose substrate as cellulose synthases (Barratt et al., 2009). Surprisingly however, single, double and quadruple knockouts of Arabidopsis sucrose synthase genes have shown no obvious cellulose deficient phenotype (Baud et al., 2004, Bieniauwska et al., 2007; Barrat et al., 2009) which may be due to the overlap in function between SUS isoforms rather than a lack of association between sucrose synthase and cellulose biosynthesis.

The other protein suggested to be involved in cellulose biosynthesis is KORRIGAN. The *korrigan* mutant is deficient in an endo-β(1,4)-D-glucanase (Nicol et al., 1998) and displays lateral organ swelling, reduced cellulose production and altered pectin composition and has been hypothesized to play a role in relieving the tensional stress generated during the assembly of multiple glucan chains into microfibrils (Somerville, 2006; Sato et al., 2001). The idea of a cellulase enzyme being present in the cellulose synthase complex for the correction of wrongly assembled cellulose chains highlights the importance of KORRIGAN in the orderly production of cellulose biosynthesis.

A novel CESA interactive protein 1 (CSI1) has been detected and suggested to be associated with the cellulose synthase complex by bridging the CESA complexes and cortical microtubules. The *csi1* knockout mutant plants showed a reduced cellulose phenotype (Gu et al.; 2010, Li et al., 2011). Mutations in the *COBRA* (*COB*) and *KOBITO (KOB)* genes, thought to be involved in the deposition of cellulose microfibrils, resulted in cellulose-deficient mutants and changes in the orientation of cell expansion (Schindelman et al., 2001; Pagant et al., 2002). *POM1*, also known as *CHITINASE-like 1* (*CTL1*), may be involved in the production of chitin-like polymers but its function remains unclear (Zhong et al., 2002). Mutations in the endo chitinase-like gene (*CTL1*) cause ectopic deposition of lignin and cell deformation in pith cells due to a decrease in cellulose (Zhong *et al*., 2002) and has recently been suggested to be the link between cellulose microfibrils and hemicelluloses (Sanchez-Rodriguez et al., 2012). Although these mutants have an effect on the cellulose synthesis, it is unknown whether all are directly linked to the rosette structure and the cellulose machinery.

Scope and outline of the thesis

Since the identification of the CESA genes and the completion of the sequencing of the Arabidopsis genome, extensive research has been carried out in various areas of cellulose biosynthesis and significant progress has been made in elucidating the process of cellulose synthesis. However, many questions still remain unanswered regarding the structure, function and assembly of CESAs in the rosette complex and further work is required in order to completely understand the synthesis of this important polymer.

Taking both structure and function into consideration, it's not known if the CESA isoforms can be interchangeable in the rosette complex. Mutant analysis showed that some primary CESAs can partially rescue the phenotype of other primary CESA mutants; however it is not clear if this is just restricted to the primary CESA complex or it can also be extended to the secondary CESAs and possibly to functional mixed complexes. It is also unclear how the CESAs are positioned in the rosette, are they randomly distributed in the rosette or specific positions are allocated to certain CESAs. It has been shown that in some cases one CESA isoform cannot effectively compensate for the loss of another which strongly suggests that the presence of some of the isoforms are critical for cellulose synthesis, and argues a case for non-random incorporation of CESA proteins into rosettes.

The function of other proteins thought to be associated with the cellulose synthase complex which may have a role in cellulose biosynthesis remains unknown as well. It is unknown if

they are part of the cellulose rosette terminal complex or whether they are chaperones for cellulose biosynthesis interacting with the complex only at specific times and for specific reasons. Some of these questions have been addressed in this dissertation.

In Chapter 2, the parallels between the cellulose synthase complex of the primary and secondary cell wall are analysed. In order to investigate whether there is any overlap in the structure or function of the CESAs in the primary and secondary cell wall, we carried out experiments to assess the possible presence of primary CESA proteins in the secondary cell wall rosette and *vice versa*. By using the split-ubiquitin membrane based yeast-two-hybrid system and the bimolecular fluorescence methodology we demonstrate that the primary and secondary CESAs are able to interact and form both homo and heterodimers both *in vivo* and *in planta*. Through a series of promoter swaps between the primary and secondary CESAs, limited interchangeability between the two complexes was demonstrated. The partial rescue of certain primary mutants with certain secondary CESA constructs and certain secondary mutants with primary CESA constructs demonstrates that additional selectivity exists. The incompleteness of the rescue suggests the development of some specialization in the function or regulation of CESA families.

In Chapter 3, the direct association of KORRIGAN with the CESAs proteins in the primary and secondary cell wall was assessed to confirm its presence in the rosette structure. Using various methods, both *in vitro* and *in planta*, we have shown that KOR1 specifically interacts with the primary cell wall CESA proteins and with two secondary cell wall CESA proteins. This interaction was studied in more detail to identify the domain responsible for the interaction. Moreover, the localization and dynamics of KOR1 fused to green fluorescent protein (GFP), expressed under its endogenous KOR1 promoter in the *kor1-1* mutant background was analysed showing that, GFP-KOR1 is expressed at the plasma membrane in the epidermis of etiolated hypocotyls further supporting a model in which KOR1 participates in the cellulose synthase complex (CSC).

In Chapter 4 the interactions between each of the CESA proteins in the primary and secondary cell walls with the sucrose synthase isoforms in Arabidopsis were tested to confirm its role as the UDP-glucose substrate provider for cellulose biosynthesis. Using both *in vitro* and *in planta* protein interaction assays it is shown that not all the sucrose synthase isoforms are able to interact with the rosette complex. In order to further verify the direct association of the CESAs with the sucrose synthase isoforms the localization of SUS6 fused to green fluorescent protein (GFP), expressed under its endogenous (*SUS6*) promoter in a fluorescently tagged CESA6 mutant background was analysed which showed co-localization of SUS6 and CESA6 probably at the plasma membrane and the Golgi apparatus supporting a role for sucrose synthase in providing UDP-glucose for the Cellulose Synthase Complex.

In Chapter 5, new candidates involved in cellulose biosynthesis were identified by screening a library with the membrane-based yeast two-hybrid system using each of the three primary CESA proteins as bait (CESA 1, 3 and 6). Several criteria were used to discriminate between the proteins found which resulted in a list of candidates appearing more relevant in having a role in cellulose biosynthesis. Some of the candidates showed interaction with at least two out

of three CESA proteins with the list consisting of some proteins known to be involved in cell wall metabolism, such as the endo-chitinase-like gene *ctl1* alongside other proteins for which no previous links to cellulose biosynthesis have been made and are promising targets for future research.

In chapter 6, the results obtained in the previous experimental chapters and insights into the biosynthesis of cellulose are discussed.

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Chapter 2

Complexes with Mixed Primary and Secondary Cellulose Synthases are Functional in *Arabidopsis thaliana* Plants

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Abstract

In higher plants cellulose is synthesized by so called rosette protein complexes with cellulose synthases (CESAs) as catalytic subunits of the complex. The CESAs are divided into two distinct families, three of which are thought to be specialized for the primary cell wall and three for the secondary cell wall. In this article the potential of the primary and secondary CESAs being able to form a functional rosette complex has been investigated. The membrane-based yeast two hybrid (MbYTH) and biomolecular fluorescence (BiFC) systems were used to assess the interactions between three primary (CESA1, CESA3, CESA6) and three secondary (CESA4, CESA7, CESA8) *Arabidopsis thaliana* CESA's. The results showed that all primary CESAs can physically interact both *in vitro* and *in planta* with all secondary CESAs. Although the CESAs are broadly capable of interacting in pairwise combinations, they are not all able to form functional complexes *in planta*. Analysis of transgenic lines showed that CESA7 can partially rescue defects in primary cell wall biosynthesis in a *cesa3* mutant (*je5*). GFP-CESA protein fusions revealed that when CESA3 was replaced by CESA7 in the primary rosette the velocity of the mixed complexes was slightly faster than the native primary complexes. Isoxaben treatment gave insights in the final assembly of rosette complexes in the small microtubule associated compartments (SMaCCs). CESA1 in turn can partly rescue defects in secondary cell wall biosynthesis in the *cesa8* knock out mutantresulting in an increase of cellulose content relative to the *cesA8ko*. These results demonstrate that sufficient parallels exist between the primary and secondary complexes for cross-functionality and open the possibility that mixed complexes of primary and secondary CESAs may occur at particular times.

Introduction

Cellulose is the most abundant component of the biosphere, with more than 10^{11} tons estimated to be synthesized each year (Brown, 2004). This linear β-1,4 glucan polymer, is synthesized by the membrane-embedded cellulose synthase (CESA) which is represented by 10 isoforms in Arabidopsis (Doblin et al., 2002; Somerville 2006). In higher plants, CESA proteins form a rosette complex of 25nm in diameter at the plasma membrane and are proposed to consist of 36 CESA subunits (Taylor et al., 2003; Scheibleet al., 2001; Muller et al., 1980; Gidding et al., 1980; Kimura et al., 1995). Genetic evidence shows that at least three isoforms are involved in the synthesis of primary walls in growing cells, CESA1, 3, 6, while three other isoforms are involved in the deposition of secondary walls in xylem cells, CESA4, 7, 8 (Fagard et al., 2000; Scheible et al., 2001; Desprez et al., 2002; Ellis et al., 2002; Taylor et al.2000; Persson et al., 2007; Desprez et al., 2007). Double and triple mutants analysis in Arabidopsis demonstrates that the remaining CESA proteins namely CESA2, 5 and 9 are partially redundant with CESA6 (Persson et al., 2007; Desprez et al., 2007), suggesting specialized functions for CESAs in certain developmental or environmental conditions (Mutwil et al., 2008).

Phylogenetic analysis reveals six distinct *CESA* clades found in seed plants, each corresponding to one of the six required genetic components in Arabidopsis (Carroll and Specht 2011). The interaction between the different CESA proteins in the primary and secondary rosette has been characterized previously by co-immunoprecipitation and yeast two hybrid methods, showing parallels in interaction patterns between primary and secondary CESAs. These results suggest that despite the ancient divergence of the families, the complexes may have retained similar architectures (Timmers et al., 2009; Desprez et al., 2007, Attanasov et al., 2009).

The primary and secondary cell walls are formed at different developmental stages. The primary cell wall is synthesized during cell division, and expansion, while the secondary cell wall is deposited in specialized cells after the expansion phase. Primary *CESAs* do not appear to be coordinately expressed with secondary *CESAs* (Persson et al., 2005). The primary *CESA*s are thought to be expressed from the initial stages of cell formation till soon after the end of cell expansion, while the secondary *CESA* genes are assumed to be expressed from the last stages of cell expansion till cell death. Thus there may be a limited period of time when both primary and secondary *CESA* genes are co-expressed. It is largely unknown how cellulose is synthesized in the transition between the primary and secondary cell wall synthesis.

GFP-labeled CESA complexes are seen by confocal microscopy as particles in the plasma membrane which move in linear tracks (Paredez et al., 2006). Fluorescently labeled CESAs are also seen in Golgi bodies and in small microtubule associated compartments called SMaCCs (or MASCs), which are implicated in trafficking CESA from the Golgi to the plasma membrane (Gutierrez et al., 2009; Crowell et al., 2009). However, the nature of the association of CESA complexes with microtubules remains an open question, as well as the mechanism of CESA complex assembly.

Here we demonstrate limited interchangeability between primary and secondary CESAs, which suggests retention of CESA positioning in the rosette complex and similarities in function across primary and secondary CESA complexes. The parallels between the primary and secondary CESA complexes were investigated by introducing primary CESA proteins in the secondary rosette complex and vice versa. The interactions between both primary and secondary CESA proteins in Arabidopsis were probed using the split-ubiquitin membrane based yeast-two-hybrid and bimolecular fluorescence and revealed they are able to interact and form both homo and heterodimers. Through a series of promoter exchanges, we demonstrate specific interchangeability between the CESAs as certain secondary CESA constructs are able to partially rescue mutants of certain primary CESAs, and be incorporated into the complex at the plasma membrane in these mutants. The functional incorporation of specific primary CESAs into the secondary walls are also shown. The incompleteness of the rescue suggests the development of some specialization in the function or regulation of CESA families. These results may also suggest that the synthesis of cellulose during the transition between the primary and secondary cell wall may involve the action of mixed primary-secondary complexes.

Materials and Methods

Constructs for the split ubiquitin membrane based yeast two-hybrid

The full-length cDNAs were obtained from the Riken Bioresource center (Seki et al., 1998; Seki *et al*., 2002) *AtCESA1* (RAFL09-89-G08), *AtCESA3* (RAFL05-19-M03), and *AtCESA6* (RAFL05-02-P19), *AtCESA4* (RAFL15-30-K05), *AtCESA7* (RAFL09-35-F05), and *AtCESA8* (RAFL09-65-M12) (Timmers et al., 2009).The cDNA's of the *CESA* genes were amplified by PCR using the Phusion DNA Polymerase (Finnzymes, Helsinki, Finland) with the primers in Table 1. The resulting PCR-products were digested and ligated into the pTFB1 vector (Bait) and the pADSL-Nx vector (Prey) (Dualsystems Biotech AG). Bait and prey expression was regulated by the TEF1 and ADH1 promoter, respectively. The sequences of the inserts were confirmed by Sanger sequence analysis. Both the bait and prey protein were fused N-terminally to the Cub-TF reporter cassette of the vector pTFB1 and NubG cassette of the vector pADSL-Nx respectively.

The split-ubiquitin membrane-based yeast two hybrid screen (MbYTH)

The interactions between the CESA proteins were assayed using the split-ubiquitin membrane-based yeast two-hybrid system (Johnsson and Varshavsky, 1994; Reinders et al., 2002) with the yeast strain NYM51 in the Split Ubiquitin System kit (Dualsystems Biotech AG). The assays were performed according to supplier instructions (DUAL membrane Kit 1). This system (Stagljar et al., 1998; Stagljar and Heesen , 2000) was used to detect interaction between the CESAs, in which each CESA was fused to the *Cub*-coding sequence of vector p TFB1 (bait), the Cub-Transcription factor (TF) and the *NubG*-coding sequence of vector pADSL-Nx (prey, Fetchko and Stagljar, 2004). Interactions were quantified by 100 colonies spotted on SD medium (lacking Leucine, Tryptophan, Histidine and Adenine) containing the appropriate concentration of 3-ammonium-triazole (3-AT) as shown in Table 2 (Timmers *et*

al., 2009) and grown at 30 C for five days, the number of spots grown was scored. The bait was also screened using the inhibitor (3-AT) in the selection medium to rule out auto activation. Detection of β-galactosidase activity was performed with the filter-lift assay (Breeden and Nasmyth, 1985). All experiments have been performed in quadruplicate. Having two different auxotrophic markers for selection increased the reliability of the system in that the prey had to circumvent two different pathways to auto-activate the system, as well as a colorimetric marker.

Constructs for split-YFP

The full-length cDNA of the *CESA* genes were generated through Phusion DNA Polymerase (Finnzymes, Helsinki, Finland) with suitable primers (Desprez 2007; Timmers , 2009; Table 1). Coding sequences of the CESAs were cloned into the gateway-compatible destination vectors pBIFc-2 and pBIFc-3 plasmids regulated by the constitutive 35S promoter (Hu *et al*., 2002). The N-terminal and C-Terminal fragments of YFP were both fused to the N-terminus of the coding sequences of the *CESAs*. As a positive control, the aquaporin PIP2-1 (Boursiac et al., 2005; Desprez et al., 2007) was used, as aquaporins are known to form homotetramers in the plasma membrane (Murata et al., 2000). As a negative control, PIP2-1 chimera was co-expressed with the corresponding CESA constructs.

Split-YFP screen

The Bimolecular Fluorescence Complementation screen (BiFC) was used to analyze *in planta* the interaction between the different CESA proteins. All possible combinations between the three primary and three secondary CESAs were analysed with this method: YFP/N-CESA1/YFP/C-CESA4, YFP/N-CESA1/YFP/C-CESA7, YFP/N-CESA1/YFP/C-CESA8, YFP/N-CESA3/YFP/C-CESA4, YFP/N-CESA3/YFP/C-CESA7, YFP/N-CESA3/YFP/C-CESA8, YFP/N-CESA6/YFP/C-CESA4, YFP/N-CESA6/YFP/C-CESA7, YFP/N-CESA6/YFP/C-CESA8. These interaction were also tested in the reverse combination, thus with both C- and N-terminus of the YFP. Leaves of 3-week-old tobacco (*Nicotiana benthamiana*) plants were infiltrated following transformation with *Agrobacterium tumefaciens* strain GV3101pMP90 (Koncz and Schell, 1986) by transient co-expression of the desired protein pairs (Desprez et al., 2007). YFP fluorescence was detected 3 days after infiltration using the 514-nm laser line of a SP2 AOBS CLSM (Confocal Laser Scanning microscope, Leica, Solms, Germany) equipped with an argon laser. To check the YFP reconstitution, spectral analysis was performed with the 496-nm laser line. All experiments were carried out in triplicates.

Promoter swap constructs

Using the same full length cDNA genes previously indicated, the coding sequence for each of *CESAs* (*CESA4*, *7*, and *8*) was amplified using Phusion DNA Polymerase (Finnzymes, Helsinki, Finland) with primers suitable for the gateway BP cloning reaction. These were inserted into pDONR207 through a BP reaction. *CesA7* was amplified with Phusion DNA Polymerase, an adenine overhang added through 30 minute incubation with Taq-polymerase

at 72°C, and inserted into the PCR8 TOPO vector from Invitrogen. All three DONR vectors were inserted into destination vectors carrying the 2kb upstream promoter region of each of the primary *CESAs* and the coding sequence from GFP immediately prior to the attR recombination sites (Desprez et al. 2007). The final vectors were sequenced over the entire length of their *CESA* coding region to confirm that no point mutations were present and to confirm that the GFP-CESA fusion was in-frame. These constructs were made with N-terminal GFP fusions as well as untagged versions of the constructs. This resulted in two sets of 9 constructs which were termed PX-G-CY for the fusion of the promoter for CesA X to the GFP-fused coding sequence of CesA Y (P1-G-C4, P1-G-C7, P1-G-C8, P3-G-C3, P3-G-C4, P3-G-C7, P3-G-C8, P6-G-C4, P6-G-C7, and P6-G-C8) and PX-CY for the untagged construct (P1C4, P1C7, P1C8, P3C4, P3C7, P3C8, P6C4, P6C7, and P6C8) to designate the promoter (P) driving the coding sequence (C) in each construct. *CESA1* promoter constructs were transformed into the temperature sensitive CESA1 mutant *rsw1-1* (lines P1-G-CY(C1ts), *CESA3* promoter constructs were transformed into the weak CESA3 mutant *je5 (*P3-G-CY(C3w), and *CESA6* promoter constructs were transformed into the CESA6 null line *prc* P6-G-CY(C6KO) through the floral dip method (Clough and Bent 1998). These constructs and lines are illustrated in Figure 1.

The *CESA7* promoter was amplified using primers indicated in Table 1. The amplified *CESA7* promoter was inserted into PCR8 TOPO (Invitrogen). Sequence confirmed PCR8-*pCESA7* was digested using Sma I/Xba I and inserted into pGW2 vector (Nakagawa et al. 2007) to replace the 35S promoter. The full-length cDNAs of *CESA1* and *CESA3* were PCR-amplified and cloned into pDONR-zeo using primers in Table 1. *CESA1* and *CESA3* were then inserted to destination vectors containing the 2kb *CESA7* promoter using LR clonase II (Invitrogen).

Cellulose measurement

Rosette leaves or stems were harvested and grounded in liquid nitrogen. After overnight extraction in 80% ethanol at 65°C in water bath, tissues were exchanged with acetone. Dry cell wall materials were ball-milled to fine powder. Cellulose was measured as described by Updegraff (1969). Data were collected from five technical replicates for each tissue sample.

Xylem staining

Stems from Arabidopsis were hand cut by a razor blade and stained in 0.02% toluidine blue O as previously described (Persson et al. 2005). Stem sections were rinsed, mounted in water, and viewed with a compound microscope (Leitz DMRB, Leica, Deerfield, IL). Around 5 individual plants were examined for each line.

Isolation of T-DNA insertion line

The identification of secondary *cesa* knockout lines from the SIGNAL collection (http://signal.salk.edu/cgi-bin/tdnaexpress) was based on a combination of database searches and PCR amplification of T-DNA flanking regions. For T-DNA lines identified from the SIGNAL collection, seeds were obtained from the Arabidopsis Biological Resource Center (ABRC, Ohio State University, Columbus, OH). PCR reactions were carried out to identify

single plants for the T-DNA insertion. Primers used for T-DNA genotyping of *cesa* alleles were listed in Table 1.

Confocal microscopy

For analyses of GFP-CESA proteins expressed in the promoter swap lines, seeds were germinated on MS agar plates and grown vertically in darkness for 3 d at 22°C. Seedlings were mounted between two cover-slips in water. Imaging was performed on a Yokogawa CSUX1 spinning disk system featuring the DMI6000 Leica motorized microscope and a Leica 100×/1.4 NA oil objective. GFP was excited at 488 nm, and a band-pass filter (520/50 nm) was used for emission filtering. Image analysis was performed using Metamorph (Molecular Devices) and Imaris (Bitplane) software.

Image analysis was performed with ImageJ (Magelhaes et al. 2004) and Imaris software. Movies were first contrast enhanced in ImageJ and a walking average of 4 frames taken using the kymograph plugin for ImageJ. These steps were performed to improve the accuracy of automated particle recognition performed in subsequent steps. These images were then opened in Imaris 6.2.1 then switched from Z-series to time series. The voxel size was set to 135 nm/voxel based on measurements from the scope and the time interval to 5 seconds. The particle recognition algorithm in Imaris was performed with a spot size of 250 nm. High intensity signal was filtered to eliminate Golgi signal. Following this the connected components program was run, which determines particle identity over several frames and converts a particles' movement into tracks. All tracks present for less than 60 seconds (12 frames) were discarded. The displacement and duration of the remaining tracks were exported to a spread sheet and their average velocity, distribution of velocities, and any directional bias were calculated.

Results

Primary and secondary CESAs can interact with each other

All possible combinations of one-to-one interactions between the primary CESAs (CESA1, CESA3 and CESA6) and secondary CESAs (CESA4, CESA7 and CESA8) were assessed using the spit-ubiquitin yeast two hybrid system (MbYTH, Dualsystems Biotech AG). Upon testing the interactions between the three primary CESA isoforms the results show that all the primary CESAs were able to form both homodimers and heterodimers with all the other primary CESA isoforms (Figure 1). These protein interactions were carried out with each of the primary CESAs as bait and as prey and both sets of experiments showed the same results (Figure 1). The lack of growth in the negative controls indicated that the interactions were specific as an unrelated protein expressed as prey and an empty prey vector (pADSL-Nx) were not able to activate the system.

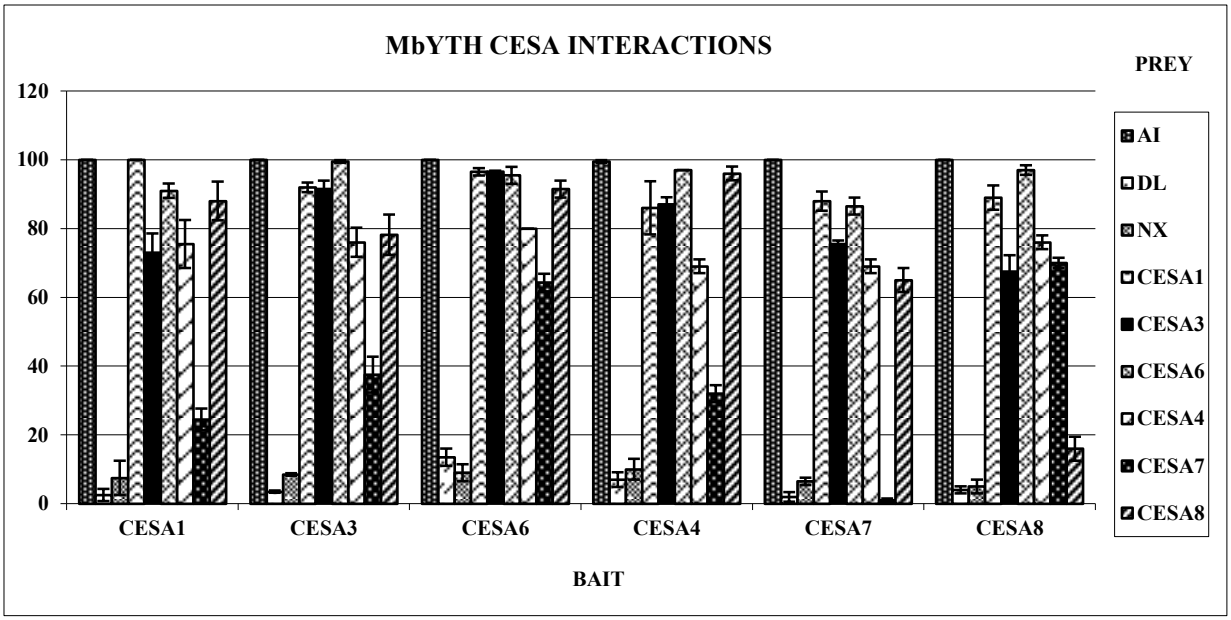


Figure 1: Interactions between the secondary CESA visualized by yeast growth. Yeast expressing CESA1, CESA3, CESA6, CESA4, CESA7 and CESA8 as bait with N-terminal fusions of Nub and Cub to a CESA and with the ALG5 protein fused to NubI as positive control (AI) and NubG as negative control (DL) and an empty prey vector as another negativecontrol (Nx) and different CESA proteins fused to NubG, as prey. The percentage of colonies that show visible growth after 5 days at 30°C on selective medium is shown. Standard deviation is indicated by the error bar.

In a second step the interactions were determined between three members of the primary CESAs (CESA1, CESA3, CESA6) and the secondary CESAs (CESA4, CESA7, CESA8) using the same MbYTH system. Though with different interaction strength, the six primary and secondary CESAs all had the ability to form homodimers and heterodimers in all combinations tested except CESA7 and CESA8, which as previously reported (Timmers et al, 2009), are not able to form homodimers (Figure 1).

Primary and secondary CESAs can be part of the same complex in planta

The bimolecular fluorescence (BiFC) technique offers the possibility of analysing protein interactions in living plant cells (Walter *et al*., 2004). To analyse the interaction between the three primary CESAs (CESA1, CESA3 and CESA6) and the secondary CESAs *in planta* the BiFC assays were used (Figure 2). We observed that YFP fluorescence was reconstituted for all of the combinations, indicating that all isoforms from the primary CESAs (CESA1, CESA3, CESA6) can interact with that of the secondary CESAs (CESA4, CESA7, CESA8). The intensity of the YFP signals was not the same for all combinations. Upon interaction of CESA3 and CESA7 a weaker signal was observed which may indicate that dimerization is less stable. All the pair-wise CESA combinations were carried out with each of the CESA's fused with the N- and C- terminus of the YFP and both sets of experiments showed the same results.

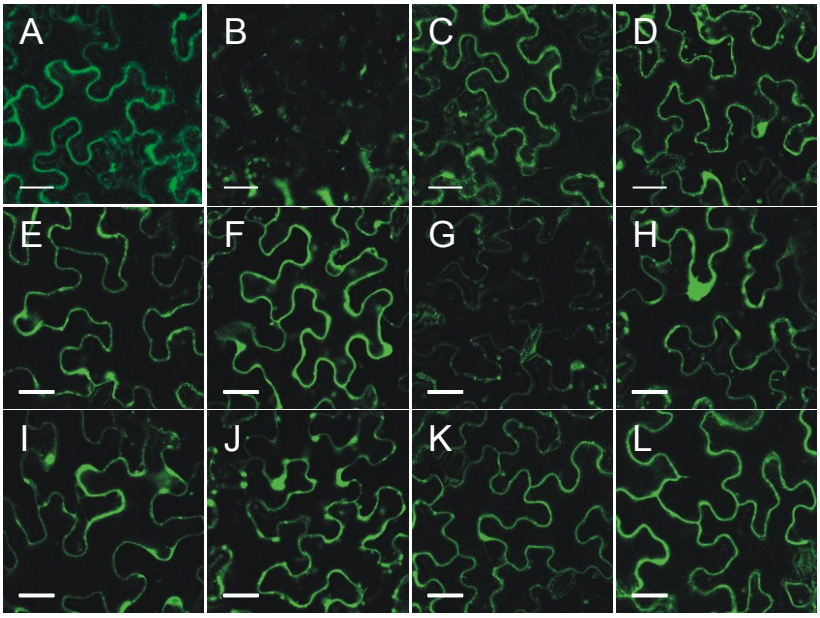


Figure 2: Bimolecular fluorescence (BiFC) analysis of the one-to-one interactions between the different primary and secondary CESA's proteins. The proteins were transiently expressed in *N.benthamiana* leaf epidermal cells. (A) Positive Control YN-PIP/YC-PIP, (B)Negative Control YN-PIP/YC-CESA7, (C)YFP/N-CESA1/YFP/C-CESA4, (D)YFP/N-CESA1/YFP/C-CESA7, (E)YFP/N-CESA1/YFP/C-CESA8, (F)YFP/N-CESA3/YFP/C-CESA4, (G)YFP/N-CESA3/YFP/C-CESA7, (H)YFP/N-CESA3/YFP/C-CESA8, (I)YFP/N-CESA6/YFP/C-CESA4, (J)YFP/N-CESA6/YFP/C-CESA7, (K)YFP/N-CESA6/YFP/C-CESA8. Scale bar =100μm

CESA7 can partially rescue the defects in the *cesa3* mutant *je5*

To determine whether CESAs from the secondary complex could enter and function in the primary complex, a series of promoter-swap constructs was generated. Combinations of each of the primary promoters placed upstream of each of the secondary CESAs, both with N-terminal GFP and without. We name these constructs PX-CY based on the promoter and coding sequence used. A construct containing the *CESA1* promoter is P1, while one containing the coding sequence of *CESA4* is C4, giving the combination of the two the name P1-C4. If GFP is N-terminally fused, we place the character "G" before the coding sequence. The fusions with GFP (P1-G-C4, P1-G-C7, P1-G-C8, P3-G-C4, P3-G-C7, P3-G-C8, P6-G-C4, P6-G-C7, and P6GC8) and without GFP (P1C4, P1C7, P1C8, P3C4, P3C7, P3C8, P6C4, P6C7, and P6C8) were transformed into the mutant lines corresponding to the promoter used. The *CESA1* promoter constructs were transformed into the temperature sensitive (ts) CESA1 mutant *rsw1-1* (lines P1-G-CY(C1ts), *CESA3* promoter constructs were transformed into the weak CESA3 mutant *je5 (*P3-G-CY(C3w), and *CESA6* promoter constructs were transformed into the CESA6 null line *prc* P6-G-CY(C6KO). In addition, the weak CESA3 mutant *je5* was transformed with the P3-G-C3 construct (Figure 3)

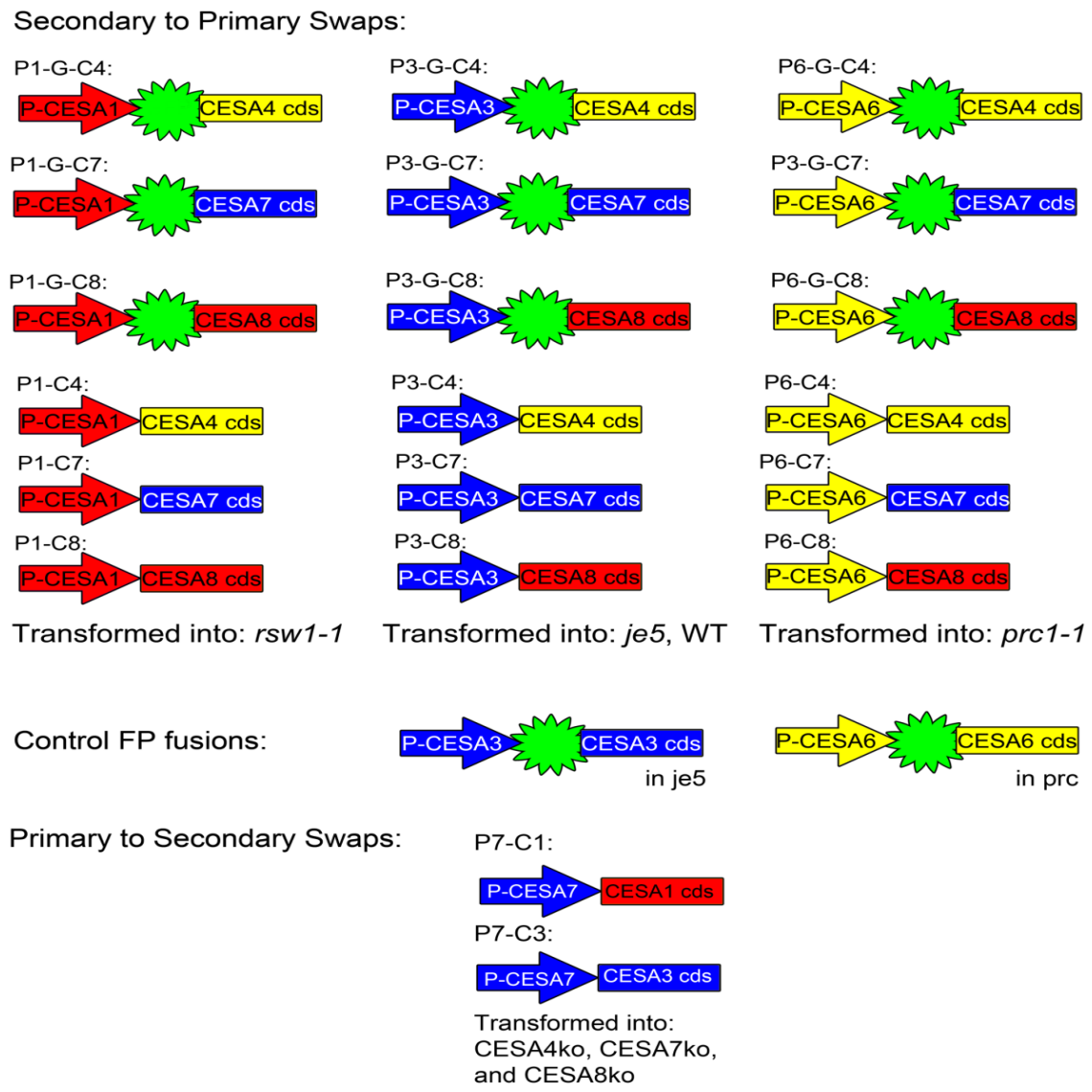


Figure 3: Promoter swap constructs generated and transformed into plants. Arrows indicate promoter regions; the presence of the green star-like symbol indicates that the coding sequence of GFP is N-terminally fused in frame to the coding sequence of one of the secondary CESAs, indicated as a labelled rectangular box. Primary and secondary promoter and coding sequences are coloured based on grouping of their sequence similarity at the C-terminus.

Etiolated seedlings of P3C7 in *je5* were not significantly different in hypocotyl length from Colombia plants or from P3-G-C3(C3w) plants up to 2.5 days of growth. After 2.5 days, however, P3-G-C7(C3w) does not elongate as rapidly as Colombia or P3-G-C3(C3w; Figure 4A). A partial rescue in the P3-G-C7(C3w) lines was observed (Figure 4B). No rescue was apparent for either the P6-G-C7(C6KO) or P1-G-C7(C1ts). The CESA4 and CESA8 constructs did not rescue any of the primary cell wall mutants, either with (data not shown) or without the N-terminal GFP.

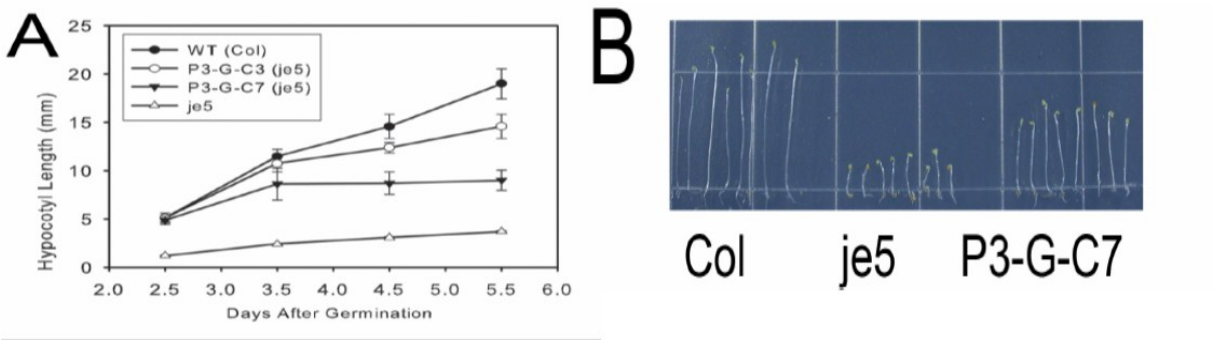


Figure 4: GFP-CESA7 is able to partially rescue the *cesa3* mutant je5. **(A)** A growth curve of hypocotyl elongation after various periods of etiolation revealed that early in growth the rescue of GFP-CESA7 is more complete, with elongation slowing after 3.5 days. Error bars represent standard deviation; asterisks indicate significant difference from WT control at $p < 0.001$. **(B)** At 5.5 days after germination in dark grown conditions, the GFP-CESA7 containing line P3C7 in the *je5* background is able to partially rescue the *je5* phenotype of reduced hypocotyl elongation.

Mixed rosette complexes behave differently from primary rosettes

Spinning disk confocal microscopy analysis in 2.5 day old P3-G-C7(C3w) and P3-G-C3(C3w) etiolated seedlings revealed GFP signal in Golgi bodies and in membrane particles (Figure 5A,B Movie S1, S2). The velocity distributions of both P3-G-C7(C3w) and P3-G-C3(C3w) were calculated by tracking individual particles in a number of time-lapse movies. This revealed that membrane particles were approximately half as abundant in P3-G-C7(C3w) compared to P3-G-C3(C3w) (Table 3). Interestingly, complexes in the P3-G-C7 (C3w) line migrated about 30% faster than P3-G-C3(C3w) complexes, a difference that is significant at $p < 0.001$ in a two-tailed T-test (Figure 5C). The number of plasma-membrane localized particles decreased for both P3-G-C3(C3w) and P3-G-C7(C3w) lines after 3.5 days of etiolation, but the decrease in particle number was far more pronounced in P3-G-C7(C3w), making it difficult to track enough particles for an adequate characterization of particle velocity in P3-G-C7(C3w) after 2.5 days of growth.

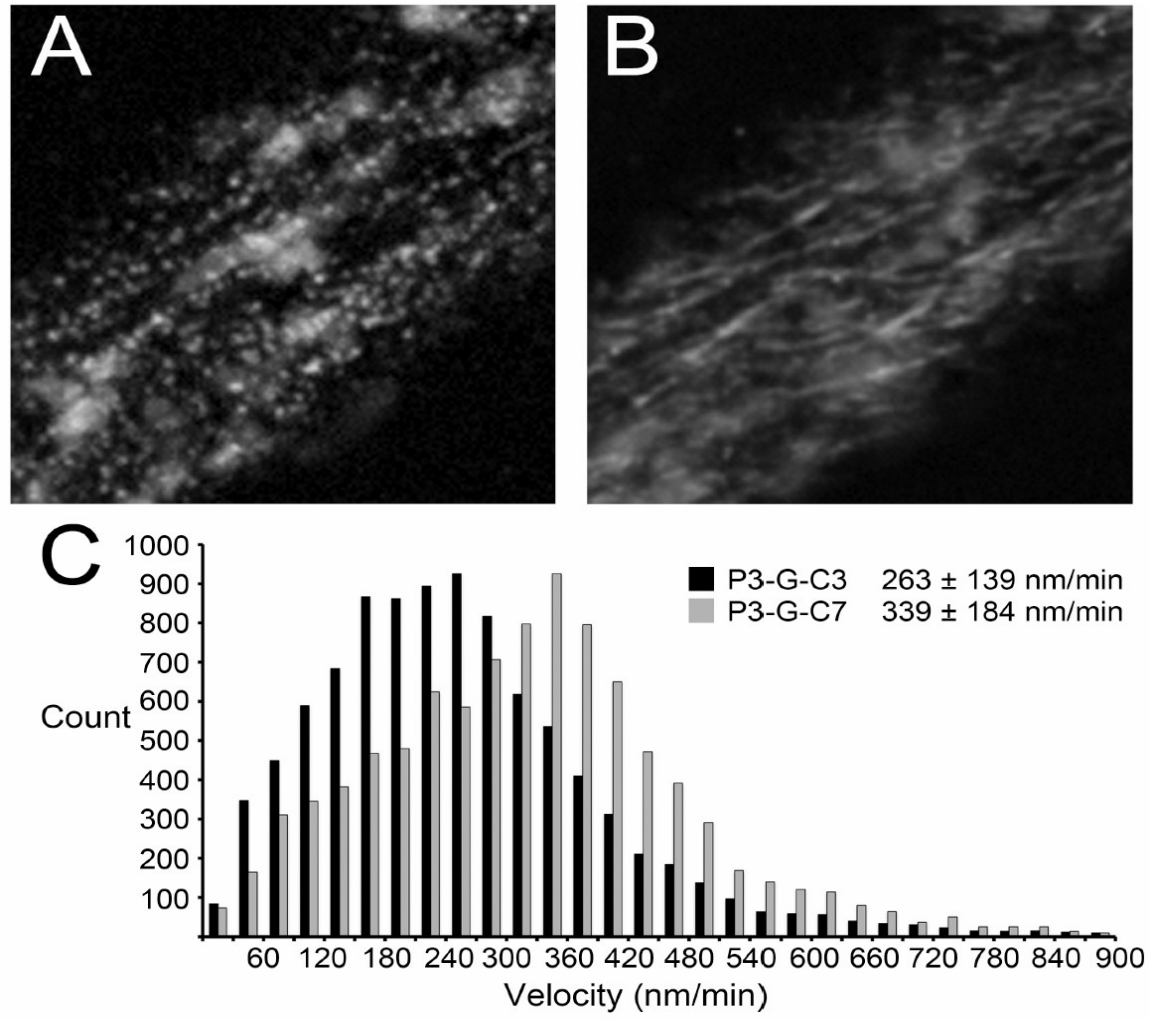


Figure 5: GFP-CESA7 incorporates into CESA complexes in the *je5* background. (A) GFP-CESA7 containing puncta are visible at the plasma membrane focal plane, and are arranged in linear tracks. Slightly out of focus Golgi bodies containing GFP-CESA7 near the membrane are also visible as large, circular areas of fluorescence. (B) A time projection of a 3 minute movie shows the motion of individual CESAs along tracks in the membrane. (C) The distribution of particle velocity indicates that GFP-CESA7 containing complexes have a faster average velocity than those observed in GFP-CESA3 containing complexes.

In P1-G-C4 (C1ts) and P1-G-C8 (C1ts) plants, confocal microscopy revealed strong fluorescence in the Golgi bodies, but no membrane complexes were detected (Figure S2). Additionally, small fluorescent bodies were faintly visible in focal planes near the membrane whose behaviour resembled previously reported sub-population of SMaCCs. In P1-G-C7 (C1ts), the GFP-CESA7 signal in SMaCCs was more apparent when plants were grown at the restrictive temperature of 30°C (Movie S3). To determine whether the failure of GFP-CESA7 to reach membrane complexes was due to the compromised CESA6 and CESA1 proteins in these mutant lines, or due to competition from the WT CESA3, the P3-G-C7 construct was transformed into WT, generating the line P3-G-C7 (WT). These lines did not have any noticeable phenotype (Figure S1), indicating that the incompleteness of the rescue in P3-G-C7 (C3w) was not due to a dominant negative effect of CESA7 expression. P3-G-C7 (WT) plants had strong GFP-CESA7 fluorescence in Golgi bodies but no signal from membrane

complexes, just as in the *rsw1-1* and *prc* transformants, indicating that exclusion of GFP-CESA7 from the CESA complex occurs due to competition from WT-CESA3 protein (Supplementary Material, Movie S4).

Isoxaben increases the accumulation of CESA7 in SMaCC's

In P3-G-C7 (WT), the GFP-CESA7 signal could be seen in particles whose size and behaviour resembled SMaCCs. The drug isoxaben, as well as osmotic and physical stress, has been shown to increase SMaCC abundance. The mechanism could involve either a block in CESA secretion to the membrane, or through internalization of CESA proteins from the membrane (Gutierrez et al. 2009). By demonstrating that relocation of CESA under osmotic stress is complete within 6 minutes, Crowell et al., (2009) concluded that the effect was due to recycling from the membrane as opposed to a block on secretion. Since the P3-G-C7 (WT) line showed identifiable SMaCCs, but no linear moving membrane complexes, this line was used to test the hypothesis that isoxaben enriches CESAs in SMaCCs through recycling from the membrane.

Upon isoxaben treatment of the P3-G-C7 (C3w) line, a substantial increase in the accumulation of GFP-CESA7 was observed in the SMaCCs. The behaviour of the Golgi bodies in P3-G-C7 (C3w) was thus altered by isoxaben treatment in a similar manner to the shift in Golgi behavior that occurs in isoxaben-treated plants expressing GFP-CESA3 (Supplementary Material, Figure S2C Movie S5, S6). This indicated that abundance of GFP-CESA in SMaCCs could be increased, even when GFP-CESA signal was absent from plasma membrane complexes. This suggests that isoxaben can act to block secretion of CESA complexes, possibly in addition to a role in triggering complex recycling.

Primary CESA1 substitutes CESA8 in secondary walls

The expression profile comparison between primary *CESAs* and secondary *CESAs* indicates that secondary *CESAs* are more stringently controlled (Figure S3), therefore the promoter of *CESA7* was chosen to be used in the promoter swap constructs. The null mutants of CESA4, CESA7, and CESA8 (*cesa4ko*, *cesa7ko*, *cesa8ko*) were identified by PCR identification of the T-DNA flanking regions (Table 1). All the secondary promoter swap constructs (P7C1, and P7C3) were transformed into *cesa4ko*, *cesa7ko*, *cesa8ko*. Among all the possible combinations, only is it P7C1 partially complemented cesa8ko phenotype. The leaf morphology of *cesa4ko*, *cesa7ko*, and *cesa8ko* is indistinguishable from each other, all displaying dark green and reduced leaf size (Figure 6A, B). The leaf of P7C3 (*cesa8ko*) is reverted almost to its wild type size. However, the margin of leaf is not as even as those of wild type. The adult plants of *cesa4ko*, *cesa7ko*, and *cesa8ko* were dwarfed, mainly due to the reduced elongation of internodes. In addition, *cesa4ko*, *cesa7ko*, and *cesa8ko* were almost completely sterile. P7C3 (*cesa8ko*) partially recovered the elongation defect in internodes and these recoveries were more obvious in the main stem. In addition, P7C3 plants were fully fertile, though the silique length was not fully recovered as compared with wild type plants. Lesions in either *IRX1, IRX3*, or *IRX5* plants result in a decrease in cellulose of more than 70% in stems (Taylor, 2000). We sought to analyse the cellulose content in these null alleles. In stems and leaves, the cellulose content in *cesa4ko*, *cesa7ko*, and *cesa8ko* were reduced.

Correlated with the morphological recovery of the leaves and stems, the cellulose content of P7C3 (cesa8ko) was increased in both stems and leaves (Figure 6C) and the xylem cell wall thickness of the P7C3 stems was decreased (Figure 6D) both indicating that P7C3 was functionally incorporated into the secondary CESA complexes.

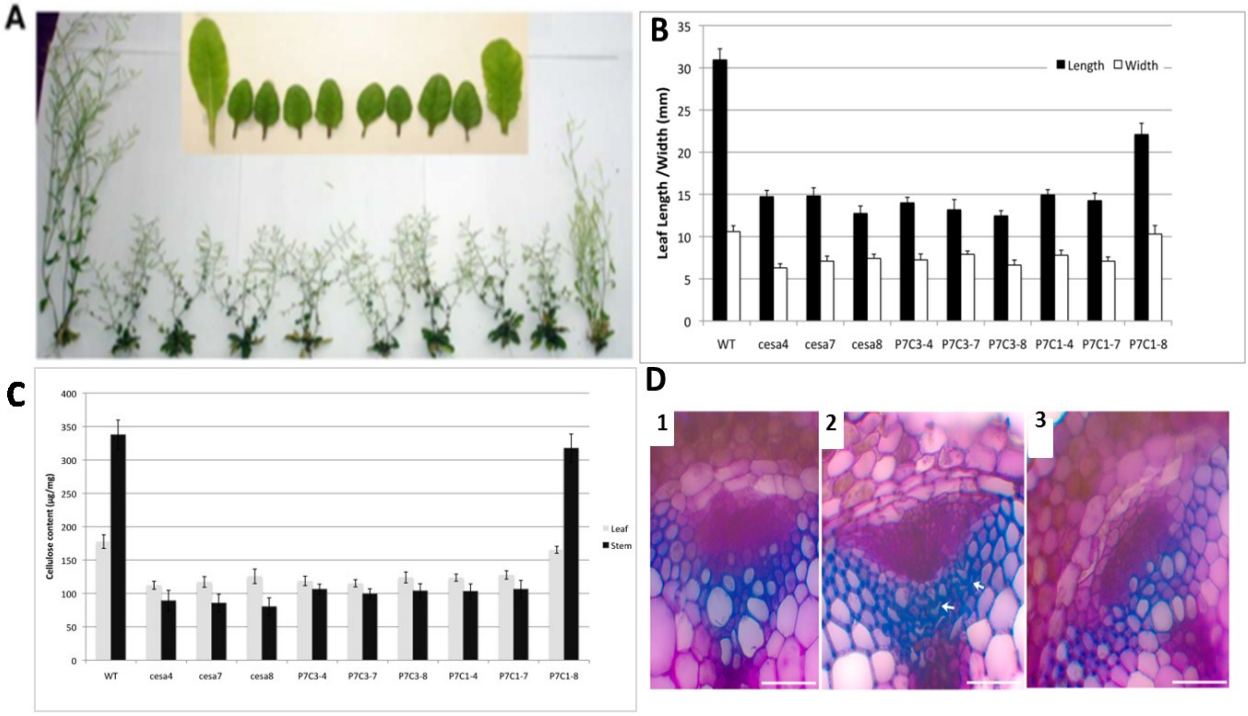


Figure 6: P7C1 is able to complement morphological and molecular defect in *cesa8ko*. Whole-plant morphology of various transformants in secondary *cesa* mutants. Insert shows leaf morphology. From the left to right, wild type (WT), *cesa4ko*, c*esa7ko*, *cesa8ko*, P7C3 in *cesa4ko* (P7C3-4), P7C3 in *cesa7ko* (P7C3-7), P7C3 in *cesa8ko* (P7C3-8), P7C1 in *cesa4ko* (P7C1-4), P7C1 in *cesa7ko* (P7C1-7), P7C1 in *cesa8ko* (P7C1-8)**. (B)** Measurement of leaf length. From the left to right, wild type (WT), *cesa4ko*, c*esa7ko*, *cesa8ko*, P7C3 in *cesa4ko* (P7C3-4), P7C3 in *cesa7ko* (P7C3-7), P7C3 in *cesa8ko* (P7C3-8), P7C1 in *cesa4ko* (P7C1-4), P7C1 in *cesa7ko* (P7C1-7), P7C1 in *cesa8ko* (P7C1-8)**. (C)** Cellulose content in leaf or stem from wild type and various transformants in secondary *cesa* mutants. Error bars represent SE, n = 5. **(D)** Cross sections of stem vascular bundles. Stem sections were stained with toluidine blue O. (1) WT. (2) *cesa8ko*. (3) P7C1 in *cesa8ko* (P7C1-8)**.** Arrows indicate collapsed xylem vessels. Bar = 50 □M.

Discussion

Several studies have shown absolute requirements for three unique CesA proteins, AtCESA1, AtCESA3 and AtCESA6, which form the primary cellulose synthase complexes (Desperez et al., 2002; 2007), and AtCesA4, AtCesA7 and AtCesA8 which form the secondary cellulose synthase complexes (Taylor et al., 2008, Timmers et al., 2009). Phylogenetic analysis reveals that these unique components represent distinct gene families which diverged early in the evolution of land plants (Carroll and Specht, 2011).

The yeast-two hybrid and bimolecular fluorescence results indicated that the CESAs can broadly interact with each other and that this interaction can be observed both *in vitro* and *in planta*. The primary CESA interaction results demonstrated here confirm previous studies carried out with various other methods such as co-immunoprecipitation, BiFC and pull down assays (Desprez et al., 2007; Wang et al., 2008) which all demonstrate direct association of three distinct primary CESA proteins. In contrast to the secondary cell wall, all primary wall CESAs are able to homodimerize suggesting that in the primary cellulose synthase complex there is more flexibility in the positioning of the individual CESAs than in the secondary cellulose synthase complex, where only CESA4 is able to form homodimers (Timmers et al., 2009).

The primary and secondary wall CESAs are thought to be expressed at different time intervals in plants (Persson et al., 2005). The exact time frame for the start and finish of the primary wall CESAs transcription and translation as well as that for the secondary wall CESAs (CESA4, CESA7, and CESA8) is unclear. It is uncertain how long the overlap in transcription and translation of primary and secondary wall CESAs is, and the presence of the primary cellulose synthase complexes and the secondary cellulose synthase complexes in the cell membrane between the end of the primary cell wall deposition and the start of the secondary cell wall formation.

The protein-protein interaction results strongly support the hypothesis that the mentioned primary and secondary Arabidopsis CESAs are able to physically interact with each other *in vitro and in planta* at least during the transition stage from primary to secondary cell wall formation further speculating that the CESAs in the primary and secondary cellulose synthase complex may both be expressed at a certain time point in intact plants and may have the potential to form mixed primary/secondary complexes. In other words, if incorporation of CESAs into the cellulose synthase complex is mediated through CESA-CESA interactions, it could be possible for secondary cell wall CESA proteins to enter the primary cellulose synthase complex and *vice versa*.

The ability of primary and secondary CESAs to interact in all combination indicates that these CESAs have the potential to be part of the same rosette complex providing they are co-localizing. There are several reports supporting the idea that primary and secondary wall formation are interrelated. Over expression of a mutant allele of the Arabidopsis *CESA7* gene, named *fra5,* resulted in changes in cellulose synthesis during primary wall formation (reduced thickness of the cell wall and cell elongation) as well as causing a dominant negative effect on cellulose synthesis during secondary wall formation (Zhong et al., 2003) as was also suggested in the case of the widely recognized secondary wall-specific *AtCesA*7 (*MUR*10) being required for normal primary cell wall carbohydrate composition in mature leaves, normal plant growth, hypocotyl strength and fertility (Bosca et al., 2010). Another study shows that despite CESA9 having already been classified as a primary cell wall CESA (Persson et al., 2007, Desprez et al., 2007) a non-redundant role was shown in secondary cell wall thickening in seed coat (Stork, 2010). The rice *brittle culm* mutant *bc11* has shown both altered primary (increased callose, pecticn, arabinan and xylan) and secondary (brittleness of the culm, abnormal secondary structure, decreased wall thickness and reduced cellulose

content) wall composition (Zhang et al 2009). In addition, the ability of putatively primary and secondary CESAs to change roles through evolution appears more dynamic than was once believed, as recent results have shown that the secondary cellulose synthase complexes produce secondary thickenings of cotton fibers, while the primary cellulose synthase complexes have acquired this role in the analogous Arabidopsis structure of trichomes (Betancur2010).

In order to further analyse the similarities and differences between the primary and secondary CESAs comprising the complex, the site of the C-terminus *rsw5* mutation implicated in disrupting the incorporation of CESA3 into the primary cellulose synthase complex were compared (Wang et al. 2006; Carroll and Specht, 2011). This site was absolutely conserved in CESA families 3, 4, 6, and 7, but not in CESA families 1 and 8, with CESA families 3 and 7 showing more similarity to each other than with the other CESAs. Although the divergence of the CESA families is ancient, CESA7 is still partially able to rescue defects in primary cell wall biosynthesis in a cesa3 mutant. At the same time, CESA7 could not rescue mutants of CESA1 or CESA6, indicating that the rescue occurs due to CESA7's ability to substitute for CESA3. The exclusion of GFP-CESA7 from the plasma membrane of WT-CESA3 plants suggests that WT-CESA3 out competes CESA7 for inclusion in the cellulose synthase complex, indicating that there has been a small degree of shift in the interactions required to place a protein into the complex at the CESA3 position. Shifts in the affinity of CESA-CESA interactions over time could also explain the inability of CESA4 and CESA8 to rescue any of the primary CESA mutants. One interpretation of these results is that individual isoforms within the CESA-complex can be thought of as having assigned "positions." These positions could be consistent with distinct spatial locations assigned in the structure of the complex, or they could instead arise more loosely from stronger interaction affinities between CESA classes during assembly of the complex. From these results, it appears the CESA3 and CESA7 can gain access to the same position in the complex.

The similarity of sequences at the C-terminus of the CESAs also predicts the partial rescue of *cesa8ko* by CESA1, where this region is also relatively less conserved than the other families. The C-terminus is a putatively cytosolic region of approximately 20 amino acids which follows the 8^{th} transmembrane domain. The C-terminal region contains two strongly conserved cysteines. We speculate that the formation of disulfide bonds between the C-terminus of one CESA with one of the other cysteine rich regions in another CESA might help mediate complex assembly. Chimeric CESA and CESA/CSLD proteins exchanging the N-terminal region (Wang et al. 2006) and catalytic domain (Park et al. 2011) have both retained the identity of the genetic position or localization of the C-terminal domain.

The behavior of GFP-CESA7 in WT plants may provide interesting insights into how the assembly of the cellulose synthase complex relates to its trafficking. If GFP-CESA7 in WT plants is outcompeted from incorporating into membrane complexes by WT CESA3 protein, the fact that GFP-CESA7 can reach all of the localizations that GFP-CESA3 can, except the plasma membrane, suggests that the final assembly of the cellulose synthase complex occurs within the SMaCCs, and that successful assembly of the complex is required for transfer of CESA proteins from SMaCCs to the plasma membrane. It is also possible that delivery of

complexes containing CESA7 is impaired relative to those containing only CESA3, which is supported by the weaker interaction between CESA7 and CESA3 in the transient BiFC experiments. In either case, the process must involve competition from the WT CESA3 protein, since in the absence of WT CESA3; GFP-CESA7 can reach the membrane.

Additionally, the response of GFP-CESA7 protein expressed in WT plants provides information about the effects of isoxaben on CESA trafficking. It has been previously suggested that isoxaben triggers the accumulation of GFP-CESA protein in SMaCCs through the recycling of cellulose synthase complexes in the plasma membrane (Crowell 2009). However, GFP-CESA7 signal is able to accumulate in SMaCCs of isoxaben-treated WT plants even though there are no GFP-CESA7 labeled complexes in the plasma membrane. This would argue against recycling as the sole cause of CESA accumulation in SMaCCs of isoxaben-treated plants. It is also possible that isoxaben treatment interferes with reverse delivery of CESA proteins back to Golgi bodies (although there is no evidence that such retrograge transport occurs at this time). None of these possibilities are mutually exclusive, and the enrichment of CESAs in SMaCCs could potentially involve a block of secretion, an increase in recycling, and a block of retrograde transport. However, the results indicate that recycling from the membrane cannot be the only mechanism for the effect of isoxaben treatment on SMaCC abundance.

The faster movement of GFP-CESA7 compared to GFP-CESA3 could reflect higher specific activity in CESA7, which proceeds to drive the complex at a higher rate. Substantially higher rates of CESA compartment movement have been reported previously (Wightman et al. 2009). The faster rate may also reflect a compensatory mechanism for the lower particle number in the GFP-CESA7 containing line: the plant is able to respond to a CESA complex deficit by driving the assembled particles at a higher rate. It could also occur because regulation of CESA3 helps modulate the activity of the complex. CESA7 is missing the phosphorylation sites known for CESA3 and may have different interacting partners. The removal of this regulation could release a regulatory break on the rate of cellulose synthesis. The decrease in complex number in P3-G-C7 could be due to a reduced ability of GFP-CESA7 to incorporate into complexes. This is supported by the observation that CESA3 is able to exclude CESA7 from the complex when both are present.

Although CESAs are broadly capable of interacting in pairwise combinations *in planta*, only CESA7 is able to enter the primary CESA complexes and rescue the CESA3 *je5* weak mutant and CESA1 is able to substitute CESA8 in secondary walls. These results demonstrate that additional selectivity exists within the plant cell, either through directed assembly or competition for interacting partners.

Supplementary Material

Suplementary Table 1: DNA primers used in the study**.**

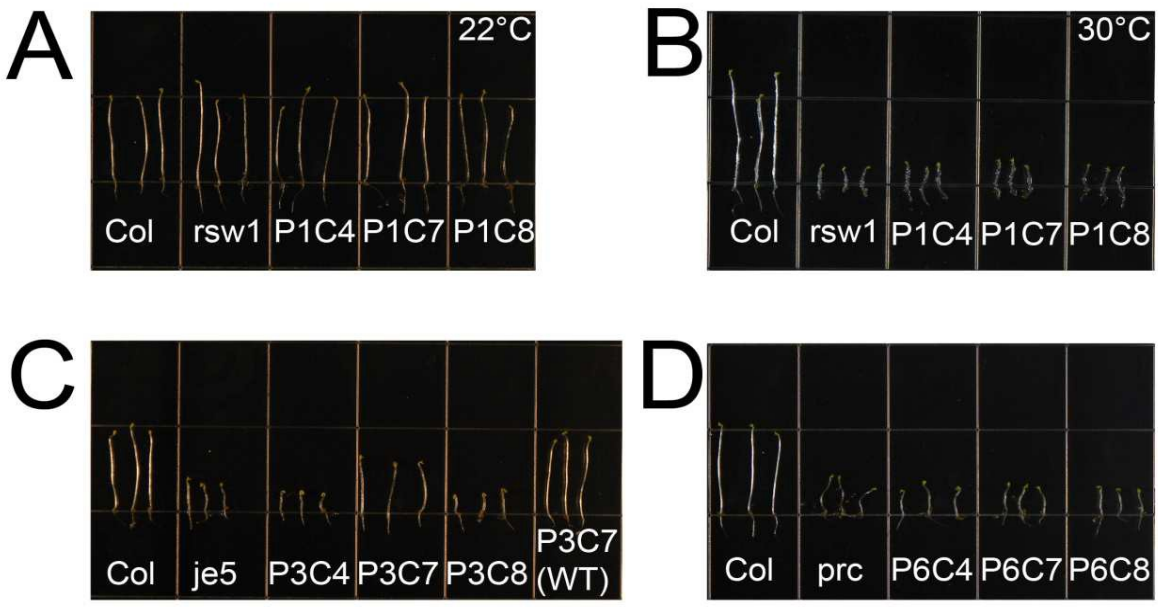
		MbYTH-Bait		MbYTH-Prey
CESA1	F	aagactgcagaatggaggccagtgccggc	F	aagaggccattacggccatggaggccagtgccggc
	R	aacaggcgccctaaaagacacctcctttgcc	R	aagaggccgaggcggccaagtaaaagacacctcctttgccat
CESA3	F	agaaccatggaatggaatccgaggagaaacc	F	agaacggccattacggccatggaatccgaaggagaaacc
	R	aagaactagttcaacagttgattccacttcc	R	gaggccgaggcggccgtcaacagttgattccacattccagaat
CESA6	F	agaaccatggaatgaacaccggtggtcgg	F	agaacggccattacggccatgaacaccggtggtcggttaatcgc
	R	aagaactagttcacaagcagtctaaacca	R	gaggccgaggcggccgtcacaagcagtctaaaccacagatctcgagaat
CESA4	F	aaaccgcggatggaaccaaacacc	F	aaactcgagatggaaccaaacaccatg
	R	aaactagttaacagtcgacgccaca	R	aaactcgagttaacagtcgacgcca
CESA7	F	aagaccgcggatggaagctagcgccggtcttgt	F	aagaggccattacggccatggaagctagcgccggtcttgt
	R	aagaagcgcttcagcagttgatgccacacttg	R	aagaggccgaggcggccatcagcagttgatgccacacttg
CESA8	F	aagactgcagaatgatggagtctaggtctccc	F	aagaggccattacggccatgatggagtctaggtctccc
	R	agaaccatggcattagcaatcgatcaaaagacagttc	R	aagaggccgaggcggccattagcaatcgatcaaaagacagttc
		p BiFC2 and p BiFC3		
CESA1	F	ggggacaagtttgtacaaaaaagcaggctccatggaggccagtgccggc		
	R	ggggaccactttgtacaagaaagctgggtctaaaagacacctcctttgcc		
CESA3	F	ggggacaagtttgtacaaaaaagcaggctccatggaatccgaggagaaacc		
	R	ggggaccactttgtacaagaaagctgggt tcaacagttgattccacttcc		
CESA6	F	ggggacaagtttgtacaaaaaagcaggctccatgaacaccggtggtcgg		
	R	ggggaccactttgtacaagaaagctgggt tcacaagcagtctaaacca		
CESA4	F	ggggacaagtttgtacaaaaaagcaggctccatggaaccaaacaccatggcc		
	R	ggggaccactttgtacaagaaagctgggttcaacttaacagtcgacgccacattgc		
CESA7	F	ggggacaagtttgtacaaaaaagcaggctccatggaagctagcgccggtct		
	R	ggggaccactttgtacaagaaagctgggttcaactcagcagttgatgccacac		
CESA8	F	ggggacaagtttgtacaaaaaagcaggctccatgatggagtctaggtctccc		
	R	ggggaccactttgtacaagaaagctgggttagcaatcgatcaaaag		
		T-DNA lines		
Salk_084627 (cesa4ko)	F	tcttccaccaaatcttgttgc		
	R	gcttcaaagtctttccccaac		
Salk_029940 (cesa7ko)	F	agagaagcttaaggaaaccgc		
	R	gaacaacacaagagcagaggg		
Salk_026812 (cesa8ko)	F	gaacaacacaagagcagaggg		
	R	ttccgatttttcacaatccac		
		Swap constructs		
CESA1 promoter	F	ggggacaactttgtatagaaaagttgaagtatcagcaggagtcctg		
	R	ggggacatgctttttttgtacaaacttgtctgtgtgtcggtggctgcg		
CESA4	F	ggggacaagtttgtacaaaaaagcaggctccatggaaccaaacaccatggcc		
	R	ggggaccactttgtacaagaaagctgggttcaacttaacagtcgacgccacattgc		
CESA7	F	atggaagctagcgccggtcttg		
	R	tcagcagttgatgccacacttg		
CESA8	F	ggggacaagtttgtacaaaaaagcaggctccatgatggagtctaggtctccc		
	R	ggggaccactttgtacaagaaagctgggttagcaatcgatcaaaag		
CESA7 promoter	F	ccgggggtggcaagctaggatcgga		
	R	tctagaagggacggccggagattagc		
CESA1	F	gcgatggaggccagtgccggc		
	R	gcaaaagacacctcctttgcca		
CESA3	F	gaaatggaatccgaaggggaaa		
	R	ggaacagttgattccacattc		

Suplementary Table 2: The optimized 3-AT (mM) concentration for the bait to rule out auto activation.

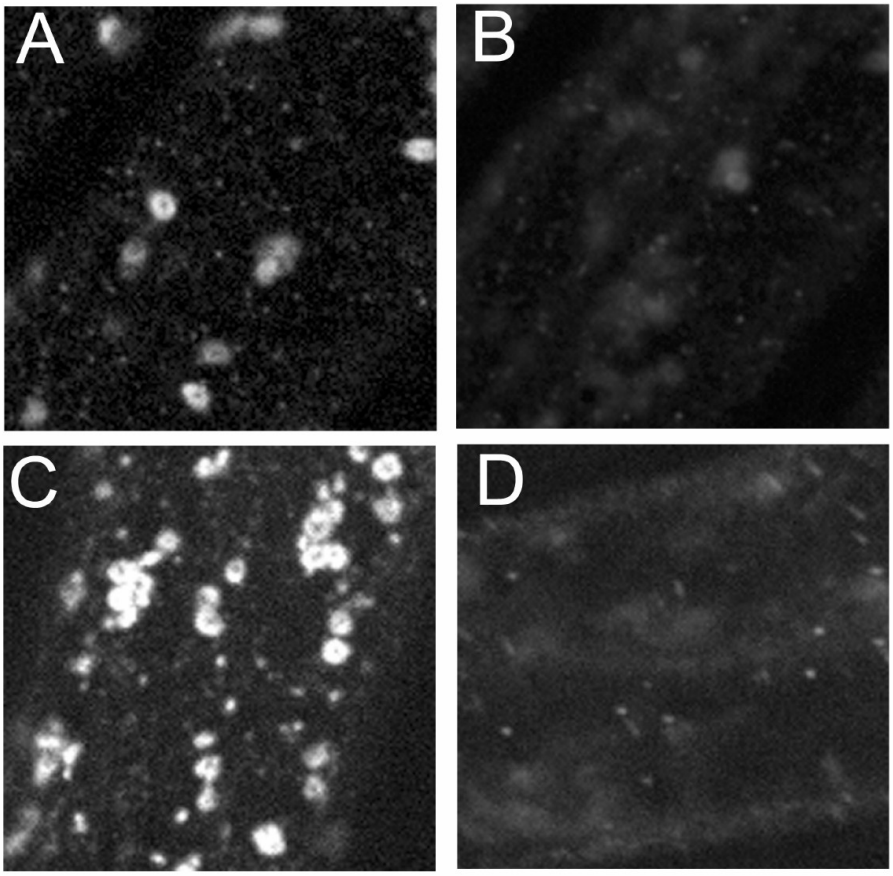
CESA	mM 3 AT concentration
CESA1	20
CESA3	80
CESA6	100
CESA4	140
CESA7	160
CESA8	75

Suplementary Table 3: CESA complexes containing GFP-CESA7 are less abundant than complexes containing GFP-CESA3 in the *je5* background. Movies were selected in which the membrane of a single cell spans the field of view. 3 minute movies were taken with frames captured at 2 second time intervals. Particles tracked for longer than 30 seconds were counted. The difference between P3C7 and GFP-CESA3 is significant at $p < 0.003$.

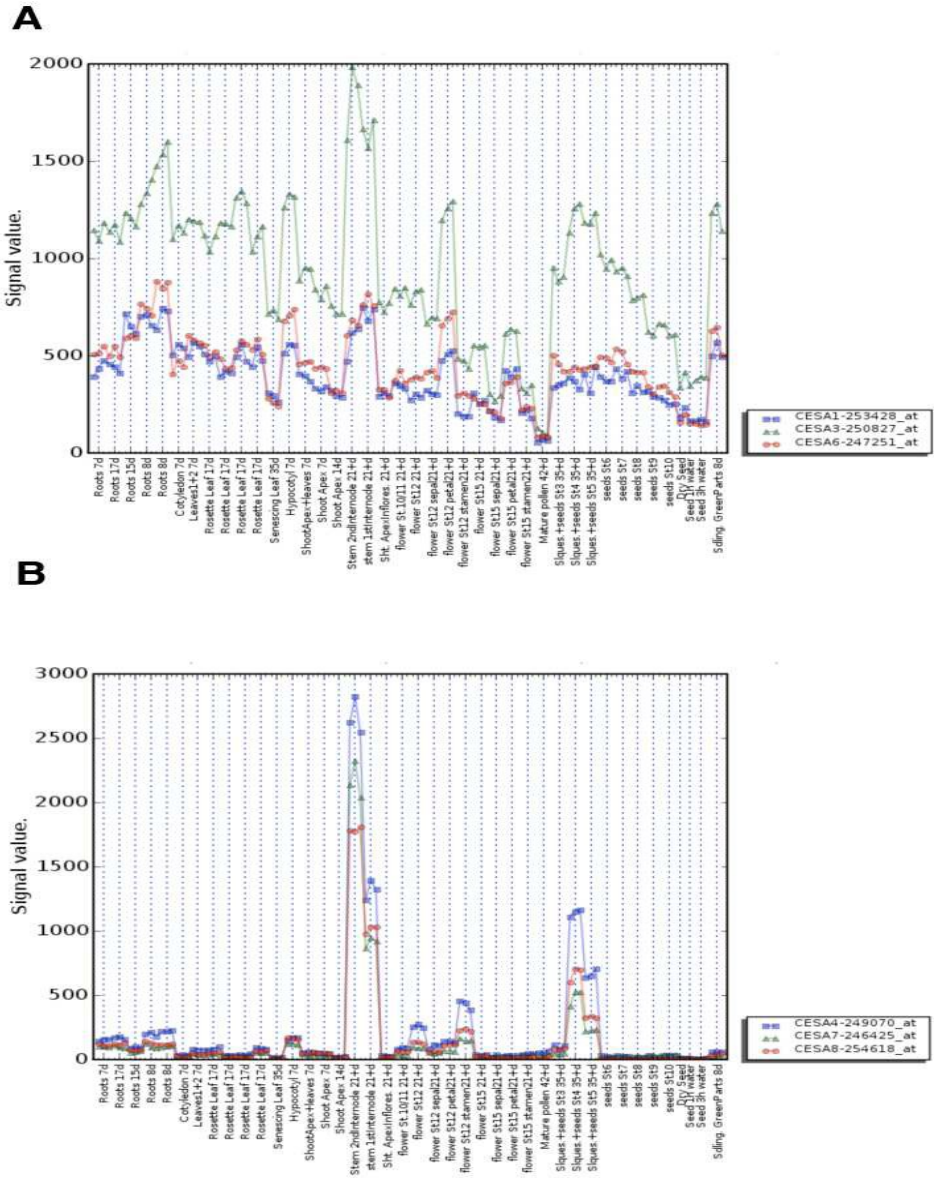
Line	Number of movies	Particles per Cell, S.D
GFP-CESA3	34	2255 ± 1145
P3C7	15	1294 ± 629



Suplementary Figure 1: Rescues from secondary swap constructs without N-terminal GFP fusion. (A) The temperature sensitive CESA1 mutant *rsw1-1* was transformed with the swap constructs. No difference from WT (Col) was seen at the permissive temperature. (B) No rescue was observed at the restrictive temperature. (C) The weak CESA3 mutant, *je5* is partially rescued by the CESA7 construct, but neither CESA4 nor CESA8 constructs can rescue *je5*. There is no dominant negative effect seen in the plants with a WT copy of CESA3. (D) The CESA6 null mutant *prc1-1* is not rescued by any of the swap constructs. All pictures were taken 3.5 days after germination



Suplementary Figure 2: Visualization of GFP-CESA's with the confocal microscope. (A) GFP-CESA7 is constrained to the Golgi bodies (large, circular objects) and to SMaCCs (small, trafficking vesicles) when transformed into WT lines. (B) A time projection of a three minute movie shows that the SmaCCs do not move in a constant velocity in linear tracks, they are mostly stationary as indicated by their appearance as dots instead of lines in a time projection. (C) Treatment with isoxaben for 30 minutes increases the number and brightness of GFPCESA7 containing SmaCCs in a WT background. (D) A time projection of GFP-CESA8 in the CESA6 mutant *prc* shows weak signal that is mostly localized to SmaCCs and Golgi bodies**.**



Supplementary Figure 3: Expression profile of primary vs. secondary CESAs. (A) Expression profile for primary CESAs. (B) Expression profile for secondary CESAs

Movie S1: GFP-CESA3 particles observed at the plasma membrane of a P3-G-C3 (C3w) plant. Complexes containing GFP-CESA3 are visible as small, distinct puncta moving in linear trajectories at the plasma membrane. Large, bright Golgi bodies containing GFP-CESA3 are seen moving rapidly from cytoplasmic streaming and coming into and out of the focal plane near the membrane. Movie is 5 minutes in length, with one frame taken every 5 seconds.

Movie S2: GFP-CESA7 particles observed at the plasma membrane of a P3-G-C7 (C3w) plant. Complexes containing GFP-CESA7 are visible as small, distinct puncta moving in linear trajectories at the plasma membrane. Large, bright Golgi bodies containing GFP-CESA7 are seen moving rapidly from cytoplasmic streaming and coming into and out of the focal plane near the membrane. Movie is 5 minutes in length, with one frame taken every 5 seconds.

Movie S3: GFP-CESA7 is observed in Golgi bodies and SmaCCs of a P1-G-C7 (C1ts) plant imaged at the restrictive temperature of 30° **C.** GFP-CESA7 signal is observed in small, distinct puncta near the plasma membrane, showing behaviors of being stationary at the membrane, moving rapidly in linear tracks, or random, erratic motion characteristic of SMaCCs. Signal is also visible in Golgi bodies. Movie is 5 minutes in length, with one frame taken every 5 seconds.

Movie S4: GFP-CESA7 is observed in Golgi bodies and SmaCCs of a P3-G-C7 (WT) plant. GFP-CESA7 signal is observed in small, distinct puncta near the plasma membrane, showing behaviors of being stationary at the membrane, moving briefly and rapidly in linear tracks, or random, erratic motion. These behaviors are characteristic of SmaCCs. Puncta moving in linear tracks with a regular velocity are not observed, indicating that the presence of a WT CesA3 allele causes GFP-CESA7 to be excluded from membrane particles. Movie is 5 minutes in length, with one frame taken every 5 seconds

Movie S5: The behavior of GFP-CESA3 shifts under isoxaben treatment. After treatment with isoxaben, GFP-CESA3 behavior shifts from complexes mostly moving in linear tracks at constant velocity to puncta showing the behaviors of being stationary at the membrane, moving briefly and rapidly along a linear track, or random, erratic motion. These behaviors are characteristic of SmaCCs. The enrichment of CesA signal in SmaCCs is characteristic of isoxaben treatment. Movie is 5 minutes in length, with one frame taken every 5 seconds.

Movie S6: GFP-CESA7 signal is also enriched in P3-G-C7 (WT) plants under isoxaben treatment. After treatment of isoxaben, GFP-CESA7 signal is more easily identified in SmaCCs. This increase occurs even though untreated P3-G-C7 (WT) plants do not have GFP-CESA7 signal localized to the plasma membrane. This indicates that enrichment of GFP-CESA in SmaCCs under isoxaben treatment cannot be a result of recycling alone. Movie is 5 minutes in length, with one frame taken every 5 seconds.

Movie S7: Incorporation of CesA4 and CesA8 protein into complexes is minimal. GFP-CESA8 signal is observed weakly in Golgi bodies and SmaCCs in P1-G-C8 (C1ts) plants grown at the restrictive temperature of 30° C. Movie is 5 minutes in length, with one frame taken every 5 seconds

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Chapter 3

KORRIGAN Interacts Specifically with Components of the Cellulose Production Machinery

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To be submitted

Abstract

Cellulose is synthesized by the so called rosette protein complex. The catalytic subunits of this complex are the cellulose synthases (CESAs). It is thought that the rosette complexes in the primary and secondary cell walls contains each at least three different non-redundant cellulose synthases. In addition to the CESA proteins, cellulose biosynthesis almost certainly requires the action of other proteins, although few have been identified and little is known about the biochemical role of those that have been identified. One of these proteins is KORRIGAN (KOR1), a membrane-bound endo-1,4-β-D-glucanase with a single trans membrane domain and two putative polarized targeting signals in the cytosolic tail. Mutant analysis of this protein in Arabidopsis showed altered cellulose content in both the primary and secondary cell wall. KORRIGAN is thought to be required for cellulose synthesis acting as a cellulase at the plasma membrane–cell wall interface, however direct interaction with the complex has never been demonstrated. Using various methods, both *in vitro* and *in planta*, it was shown that KOR1 interacts with the three primary cell wall CESA proteins and with two of the secondary CESA proteins. The KOR1 protein domain(s) involved in the interaction with the CESA proteins were identified by analyzing the interaction of truncated forms of KOR1 with CESA proteins. The localization and dynamics of KOR1 fused to green fluorescent protein (GFP) was also investigated in the *kor1-1* mutant background and observed that, GFP-KOR1 localizes to the plasma membrane and to intracellular compartments in the epidermis of etiolated hypocotyls, GFP-KOR1 is expressed in the same cells at the same time as GFP-CESA3. Surprisingly, GFP-KOR1 and GFP-CESA3 migrate with comparable velocities along linear trajectories at the cell surface. The data presented supports a model in which KOR1 participates in the Cellulose Synthase Complex at the plasma membrane.

Introduction

Cellulose is synthesized by a large rosette terminal complex, which comprises at least three different cellulose synthases (CESAs) On the basis of *Arabidopsis* mutant analysis, CESAs are not assumed to work alone, some proteins were predicted to be associated with the complex, although their direct interaction with the complex has never been demonstrated. One of these proteins is KORRIGAN (KOR1), a membrane-bound endo-1,4-β-D-glucanase with a single transmembrane domain and two putative polarized targeting signals in the cytosolic tail (Nicol, 1998; Zuo, 2000). KOR1 is thought to be required for cellulose synthesis acting at the plasma membrane–cell wall interface in plants and bacteria (Molhoj et al., 2001; Matthysse et al., 1995). However the exact role is unknown, KORRIGAN is thought to be required for the assembly of glucan chains in cellulose microfibrils by having a kind of proof-reading activity involved in hydrolyzing disordered amorphous cellulose to relieve stress generated during the assembly of glucan chains in cellulose microfibrils (Molhoj et al., 2002). The enzyme produced in *Pichia pastoris* cleaves non substituted but non-crystalline 1,4-β–linked glucan chains and shows no activity against xyloglucans (Molhoj et al., 2001; Master et al., 2004; Robert et al., 2005). It has also been proposed that a member of the endo-1, 4-β-glucanase family mediates the transfer of a glucose residue to a growing β-glucan chain during cellulose synthesis in *Agrobacterium tumefaciens* and *Acetobacter xylinum* (Matthysse et al. 1995; Standal et al., 1994).

As well as *KOR1*, two additional genes encoding membrane-anchored endoglucanases (EGase) have also been characterized in Arabidopsis (Molhoj 2001). The *KOR1* gene is the most often expressed membrane-anchored EGase throughout the plant while *KOR2* and *KOR3* are active in specific cell types. Expression of a GUS reporter gene driven by the *endogenous* promoters of *KOR2* and *KOR3* have shown that *KOR2* is active in trichomes and floral organs while *KOR3* is active in developing root hairs.. Micrososmal fractioning demonstrated that KOR1 is present in the tonoplast, the Golgi apparatus (Brummel et al., 1997), the cell plate (Zuo et al. 2000) and importantly in the plasma membrane (Nicol et al., 1998) where cellulose biosynthesis occurs (Delmer, 1999).

The dwarf mutant *korrigan* (*kor1-1*) showed defects in some aspect of cell wall loosening in primary cell wall biosynthesis (Nicol et al., 1998). Other *KOR* mutations (*kor1-2*) have shown aberrant cell plates and incomplete cell walls (Zuo et al., 2000). Morphological and chemical analysis of KOR temperature sensitive, single base pair mutants (*acw1* altered cell wall and *rsw2* root swelling) showed abnormal plant morphology, defects in primary cell wall formation, reduced cellulose content, increased pectin synthesis, and aberrant cell division similar to that found in the *CESA1* mutant *rsw1* (Nicol et al., 1998; Peng et al., 2000; Zuo et al., 2000; Sato et al., 2001; Lane et al., 2001). Cellulose synthesis, which occurs concomitantly with cell wall loosening during cell elongation, is impaired in the temperature sensitive elongation deficient *acw1* mutant grown at 31ºC and shows a 40% reduction in crystalline cellulose content compared to the wild type (Sato et al., 2001). Significant reduction in cellulose production was seen in seedlings of double mutants of cellulose synthase (*rsw1*) and *KOR* (*rsw2*) in comparison to either of the single mutants which demonstrates that cellulose biosynthesis in the primary cell walls of plants requires both a

glycosyl transferase and glycosyl hydrolase providing further evidence that KOR has a key role in cellulose deposition in the primary cell wall (Lane et al., 2001).

Reports have indicated that KOR also plays a role in secondary cell wall development. An accumulation of a *KOR* homolog was observed during secondary cell wall deposition in cotton (Peng et al. 2002). Two independent mutations (*irx2-1* and *irx2-2*) in the *KOR1* genes showed similar phenotypes as the *irx* mutants, i.e. collapsed xylem cell walls due to reduced cellulose synthesis in the secondary cell wall at the base of mature stems (Szyjanowicz et al., 2004). It must also be mentioned that the *kor1-1* mutant that was reported to be a primary cell wall mutant also demonstrated severely collapsed xylem cells in the stems similar to the *irx2* mutants (Szyjanowicz et al., 2004). Furthermore, targeted down-regulation of the *KORRIGAN* gene from hybrid poplar led to moderate to severe defects in plant growth, an irregular xylem (*irx*) phenotype commonly associated with other secondary cellulose-specific mutants in *Arabidopsis* (Maloney and Mansfield, 2010a).

Since KOR appears to be associated with cellulose synthesis, it is important to determine whether or not there is direct interaction. In this study, Using the membrane-based yeast two hybrid system (MbYTH; Fetchko and Stagljar, 2004; Timmers et al., 2009), and the bimolecular fluorescence complementation method (BiFC), it is shown that KOR1 interacts specifically with CESAs involved in the deposition of both primary and secondary cell walls. The data demonstrates that the interaction between KOR1 and the CESA proteins is specific, and takes place in the membrane. The dynamics of GFP-KOR1 expressed under the control of its endogenous promoter in the *kor1-1* mutant background in living cells also demonstrates that GFP-KOR1 is found in discrete particles at the plasma membrane in the same cells as GFP-CESAs (Crowell et al., 2009). We also show that GFP-KOR1 plasma membrane particles migrate along linear trajectories with comparable velocities as those observed for GFP-CESAs. The new information provided here will help to shed light on the role of KOR1 in cellulose synthesis.

Materials and Methods

Constructs for the MbYTH system

The constructs for the MbYTH system concerning the secondary CESAs were described previously (Timmers et al., 2009). The full-length cDNAs were obtained from the Riken Bioresource center (Seki et al., 1998; Seki *et al*., 2002) At*CESA1* (RAFL09-89-G08), At*CESA3* (RAFL05-19-M03), and At*CESA6* (RAFL05-02-P19) and *AtKOR1* (RAFL05-02-G06). Several truncated forms of KOR1, KORRIGAN Transmembrane Domain (KORTMD;AA 70 to 94, Figure 3D). The TMD with the C-terminal portion of the protein (KORC; AA 70 to 621, Figure 3C). The N-terminal part of the protein together with the TMD (KORN; Amino Acids 1 to 94, Fig. 3B) and only the cytosolic part (N-terminal and C-terminal) of the protein (KORSOL AA 1 to 69 + 95 to 621, Fig. 3E) were also made (Figure 3). The cDNA's of the mentioned genes were amplified by PCR using the Phusion DNA Polymerase (Finnzymes, Helsinki, Finland) with the primers (Supplementary table 1). The resulting PCR-products were digested and ligated into the pTFB1 vector (Bait) and the

pADSL-Nx vector (Prey; Dualsystems Biotech AG). Bait and prey expression is regulated by the TEF1 and ADH1 promoter, respectively. The sequences of the inserts were confirmed by Sanger sequence analysis. Both the bait and prey protein were fused N-terminally to the Cub-TF reporter cassette of the vector p TFB1 and NubG cassette of the vector p ADSL-Nx respectively.

Membrane based yeast two hybrid screen

The interactions between the CESAs and KOR1 were assayed with the split-ubiquitin membrane-based yeast two-hybrid (Johnsson and Varshavsky, 1994; Reinders et al., 2002) using yeast NYM51 strain in the Split Ubiquitin System kit (Dualsystems Biotech AG). Interactions were performed according to supplier instructions (DUAL membrane Kit 1) and were tested with KOR1 fused to the C-terminal part of the ubiquitin (Cub) and the transcription factor (bait), whereas the CESA1, 3 and 6 proteins were fused to the N-terminal part of the ubiquitin (Nub; preys). The bait and prey constructs were co-transformed into the yeast strain NMY51 (Dualsystems Biotech AG) according to the provided transformation procedure (DUAL membrane Kit 1). Upon interaction between the bait and the prey the transcription factor (TF) is released into the nucleus where it activates reporter genes allowing the yeast to grow on selective medium lacking leucine and tryptophan (SD med.-L-T), and subsequently grown at 30ºC for three days. To quantify the interactions between different preys 100 colonies of each combination were spotted onto selection medium containing the appropriate amount of 3-ammonium-triazole (3-AT) and grown at 30ºC for three days. The number of spots grown was then counted. Detection of β-galactosidase activity was performed with the filter-lift assay (Breeden and Nasmyth, 1985).

Constructs for Split-YFP

The full-length cDNA of the primary and secondary *CESA* genes, *KORRIGAN* as well as the truncated forms of *KOR1* were generated through Phusion DNA Polymerase (Finnzymes, Helsinki, Finland) with suitable primers (Desprez et al., 2007; Timmers et al., 2009; Table 1). Coding sequences of the genes were cloned into the Gateway-compatible destination vectors pBIFc-2 and pBIFc-3 plasmids regulated by the constitutive 35S promoter (Hu *et al*.,2002). The N-terminal and the C-Terminal fragments of YFP were both fused to the N terminus of the coding sequences of the CESAs. As a positive control, the aquaporin PIP2-1 (Boursiac et al., 2005; Desprez et al., 2007) was used, as Aquaporins are known to form homotetramers in the plasma membrane (Murata et al., 2000). As a negative control PIP2-1 chimera was co-expressed with the corresponding CESA constructs

Bimolecular Fluorescence Complementation screen

Using this system, the interaction between the primary CESAs and KOR1 were tested in leaves of 3-week-old *Nicotiana benthamiana* plants. Two YFP fragments, either YFP/N or YPF/C, each linked to the N-terminus of the proteins, were transiently expressed by infiltration as described (Voinnet et al., 2003)in the plant. Upon interaction between the two proteins, the fragments restore fluorescence, which can be detected. YFP fluorescence was detected 3 days after infiltration by using the 514-nm laser line of a SP2 AOBS confocal laser

scanning microscope (Leica, Solms, Germany) equipped with an argon laser. To check the YFP reconstitution, spectral analysis was performed with the 496-nm laser line. The fluorescence with all constructs was detected at the same photo-multiplier tube (PMT) settings (760), except for the negative interactions for which the PMT was increased up to 880.

Plant expression vectors

Standard molecular cloning techniques were performed essentially as described (Sambrook and Russell, 2001). Constructs were made by using Gateway cloning technology (Invitrogen). A 1.3-kb fragment of the *KOR1* gene was amplified by PCR with specific primers (Supplementary Table 1) and cloned into the *HindIII- XbaI* site of the pGWB6 vector after the removal of the *35S* promoter. An LR reaction was performed to obtain the promoter KOR1-GFP-KOR1 construct (GFP-KOR1). The final expression vector was electroporated in *Agrobacterium tumefaciens*. GFP-KOR1 construct was introduced into *kor1-1* mutant. Primary transformants were selected on hygromycin and F2 progenies were used for the visualization of the fluorescent protein.

Spinning disk analysis and image analysis

Spinning disk analysis and image analysis were performed as described (Crowell et al., 2009). Hypocotyls of 3-day-old etiolated seedlings were analyzed on an Axiovert 200M microscope (Zeiss) equipped with a Yokogawa CSU22 spinning disk Zeisss 100/1.4 numerical aperture oil objective and Andor EMCDD iXon DU 895 camera (Plateforme d'Imagerie Dynamique, Institut Pasteur, Paris, France). GFP was excited at 488 nm by a diode pumped solid-state laser, and fluorescence emission was collected through a 505/555-nm band-pass filter (Semrock, Rochester, NY). GFP-KOR1 and GFP-CESA3 velocities were quantified by using the manual tracking plugin (Fabrice Cordelières) in ImageJ (W. Rasban, National Institutes of Health, Bethesda, MD).

Laser Scanning Confocal Microscopy

Images were collected with a spectral Leica SP2 AOBS confocal microscope (Leica Microsystems) equipped with an argon laser. GFP and FM4-64 were detected using a laser line 488 nm.

Results

KOR1 interacts with CESAs in the primary cell wall cellulose synthase complex

In order to test whether KORRIGAN has a role in cellulose biosynthesis in the primary cell wall, the interaction between KOR1 and three members of the primary cell wall cellulose-synthesizing rosette (CESA1, CESA3 and CESA6) were tested *in vivo* using the membrane based yeast two hybrid system. The results indicated that KOR1 is able to interact with all three of the CESA proteins as yeast colonies were able to grow on selective medium (Fig. 1). The lack of growth in the negative control indicated that the interaction with KOR1 was specific as an unrelated protein expressed as prey is not able to activate the system. Reverse

experiments, in which the different CESA proteins were the bait and the KOR1 the prey, confirmed these findings in that all combinations were able to induce the reporter genes, allowing the yeast to grow on selective medium (Figure 1). We conclude that KOR1 can interact with CESA1, CESA3, and CESA6 *in vivo*.

Interactions between KOR1 and CESA1, 3 or 6 were assessed *in planta*, using the bimolecular fluorescence complementation (BiFC) technique (Desprez et al., 2007). Expression of CESA1 together with KOR1 (both fused to YPF fragment) resulted in a strong fluorescent signal at the plasma membrane (Figure 2). The interactions between KOR1 and CESA3 (Figure 2D) or CESA6 (Figure 2E) also yielded fluorescence however the signal was detected less frequently in comparison the CESA1 (Figure 2). Positive and negative controls testing dimerization of PIP2-1 protein (Figure 2A) or interaction of CESA7-CESA7 (Timmers et al., 2009; Figure 2B) both yielded the expected result.

KOR1 interacts with CESAs in the secondary cell wall cellulose synthase complex

Reduction in cellulose content of the secondary cell wall in the *kor1* (*irx*) mutants also link KOR1 to cellulose synthesis in the secondary cell wall. To test a possible interaction between KORRIGAN and the secondary CESA proteins (CESA4, CESA7 and CESA8), the KOR1 was expressed as bait in combination with the secondary CESA as prey. The combination KOR1 with CESA4 or CESA8 activated the reporter genes, and therefore was able to grow on selective medium whereas no growth was detected when KOR1 and CESA7 were tested (Figure 1). The results were further confirmed by the interaction between the CESA proteins as bait and the KOR1 protein as prey.

The physical interaction of KOR1 with the secondary CESA protein found with the MbYTH system was also tested *in planta.* Both KOR1 and the different secondary CESA proteins were fused to fragments of the YFP and expressed in *Nicotiana benthamiana* leaves in different combinations. Restored fluorescence indicated interaction between the two fusion proteins. The combination CESA7 and KOR1 only showed a background fluorescent signal, whereas both CESA4 (Figure 2F) and CESA8 (Figure2G) show a distinct signal when expressed in combination with KOR1 (Figure 2). The interactions found *in planta* by the BiFC system confirmed the results found with the yeast two hybrid system.

In conclusion, KOR1 can interact with all the CESA proteins in the primary cellulose synthase complex *in planta* and two in the secondary cellulose synthase complex

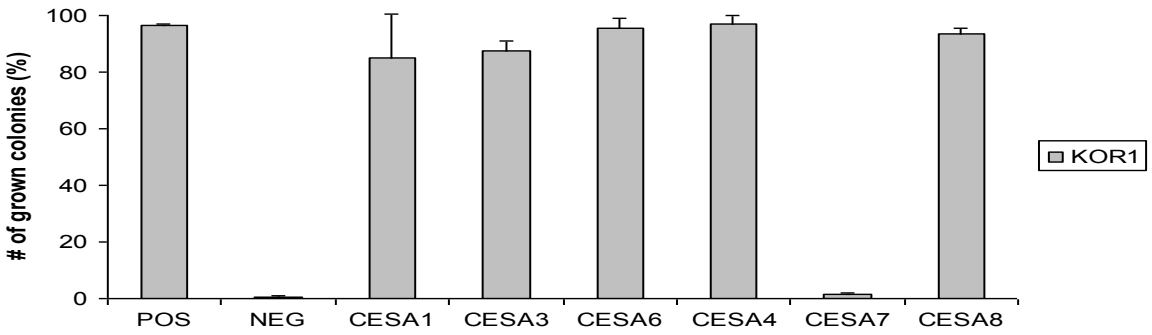


Figure 1: Interactions between KOR 1 and the different CESA proteins using the Membrane-based Yeast Two Hybrid**.** The bars represent the percentage of yeast colonies grown for 3 days on selective medium at 30 ºC. KOR1 was expressed in yeast as bait and the different CESA proteins as prey (as indicated in the legend).

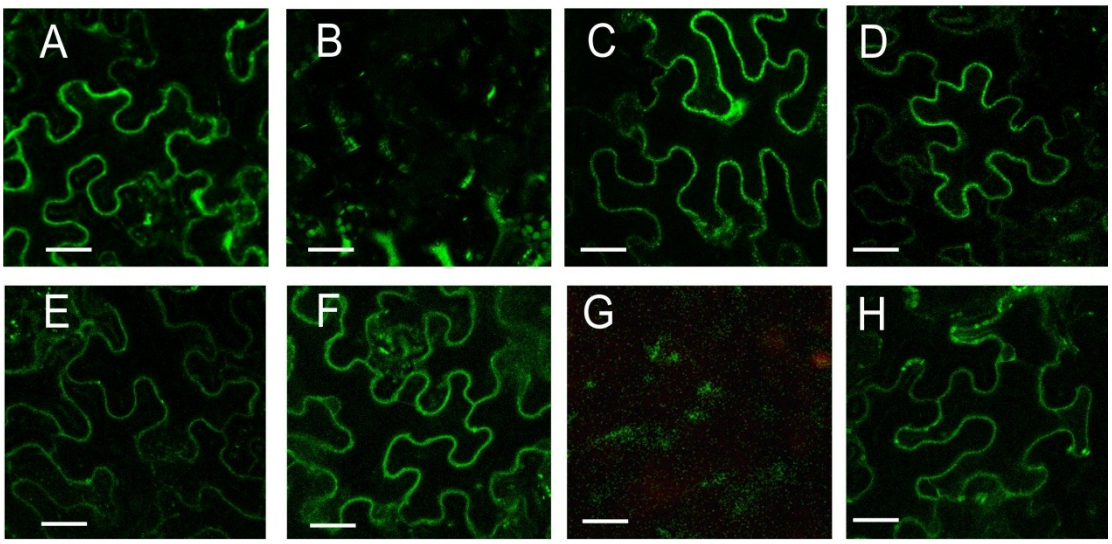


Figure 2: BiFC in *N. benthamiana* leaf epidermis shows *in vivo* dimerization between KORRIGAN and various CESA proteins. Confocal images are presented, showing YFP fluorescence indicating an interaction (positive control testing the dimerization of PIP2 (A), or lack of fluorescence indicating no interaction (B). The KOR1 expressed together with different CESA proteins; Test for interaction between KOR1 and CESA1 by fusing KOR1 to the N-terminus of YFP and CESA1 to the C-terminus of YFP (C). Test for interaction between KOR1 and CESA3 by fusing KOR1 to the N-terminus of YFP and CESA3 to the C-terminus of (D). Test for interaction between KOR1 and CESA6 by fusing KOR1 to the N-terminus of YFP and CESA6 to the C-terminus of YFP (E). Test for interaction between KOR1 and CESA4 by fusing KOR1 to the N-terminus of YFP and CESA1 to the C-terminus of YFP (F). Test for interaction between KOR1 and CESA7 by fusing KOR1 to the N-terminus of YFP and CESA7 to the C-terminus of (G). Test for interaction between KOR1 and CESA8 by fusing KOR1 to the N-terminus of YFP and CESA6 to the C-terminus of YFP (H). The scale bar is 100μm

The transmembrane domain is essential for interactions between KOR1 and CESA

The KOR1 protein is a membrane-anchored protein containing a short N-terminus located in the cytosol, a transmembrane domain (TMD), and an extracellular catalytic domain (Figure 3). In order to further characterize the interaction between KOR1 and the CESA proteins, several truncated KOR1 proteins were engineered. The N-terminal part of the protein together with the TMD was used to test whether the cytosolic portion of the protein (KOR1N; Figure 3B) is responsible for the interaction. The TMD with the C-terminal portion of the protein (KOR1C; Figure 3C) was tested for interactions between the catalytic domain and the CESA proteins. The TMD was also tested separately, by expressing only the TMD (KOR1TMD; Figure 3D). These truncated proteins were fused to the N-terminal portion of ubiquitin and used as prey, while the different CESA proteins were used as baits. All the truncated proteins of KOR1 interacted with all CESA proteins, except for the CESA7 *in vivo* (Figure4)

As all the tested KOR1 domains containing the TMD were able to interact as well as the full length KOR1, it was deduced that the TMD might be involved in these interactions. To determine whether this domain is essential for the interaction, another recombinant protein was made, in which the TMD was absent, resulting in a soluble protein (KOR1SOL AA 1 to 69 + 95 to 621, Figure 3E). No interactions were found between KOR1SOL and the CESA proteins (Figure 4).

To confirm these results *in planta*, truncated forms of KOR1 were tested for interaction with the primary (CESA1, CESA3, CESA6) and secondary (CESA4, CESA7, CESA8) CESAs using the BiFC assay. The results indicated that the fluorescent signal was comparable with the full length KOR1 protein (Figure 2). All the primary CESAs were able to interact with KOR1. CESA1 was able to interact with KOR1N, KOR1C and KOR1TMD (Figure 5) proteins. The same pattern was observed with CESA3 (Figure 5) and CESA6. The fluorescent signal was comparable with that obtained for the full length KOR1 protein (Figure 2). Both CESA4 (Figure 5) and CESA8 (Figure 5) of the secondary CESAs were able to also interact with the truncated forms of KOR1 except for CESA7 which did not show interaction with any of the KOR1 constructs. The alternate pairwise combinations all resulted in similar interaction patterns except for KOR1C-CESA1.

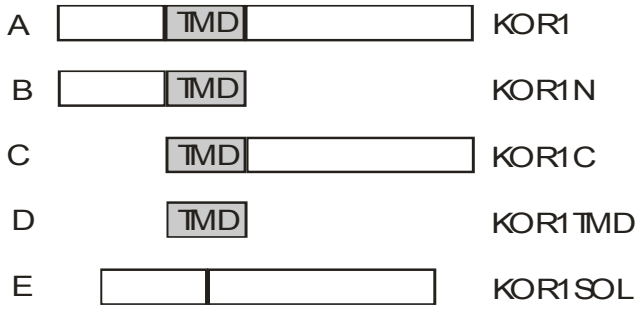


Figure 3: Representation of different truncated KORRIGAN proteins used in this study**.** A) KOR1 is the complete protein (AA 1 to 621), B) KOR1N: the N-terminal part with the TMD (AA 1 to 94), C) KOR1C: TMD plus the C-terminal part of KOR1 (AA 70 to 621), D)

KOR1TMD only the TMD of KOR1 (AA 70 to 94), E) KOR1SOL is the KOR1 protein without the TMD; in which the N-terminus is fused directly to the C-terminal part (AA 1 to 69 + 95 to 621).

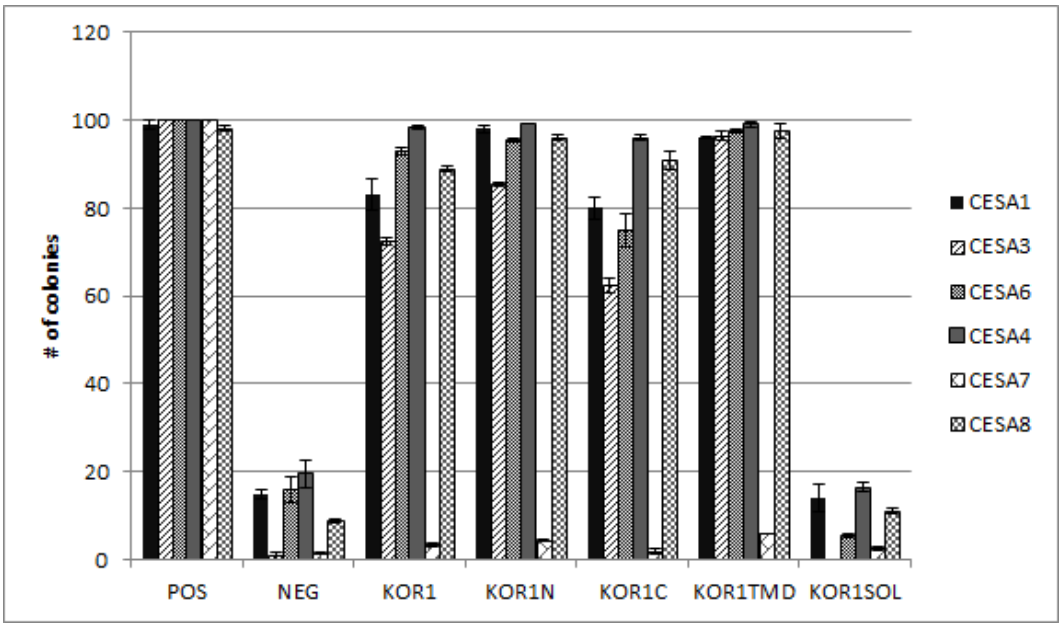


Figure 4: Interactions between the different KOR1 domains and the different CESA proteins using the Membrane-based Yeast Two Hybrid**.** The bars represent the percentage of yeast colonies grown for 3 days on selective medium at 30 ºC. The different CESA proteins were expressed in yeast as bait (as indicated in the legend) and the different KOR1 protein domains as prey.

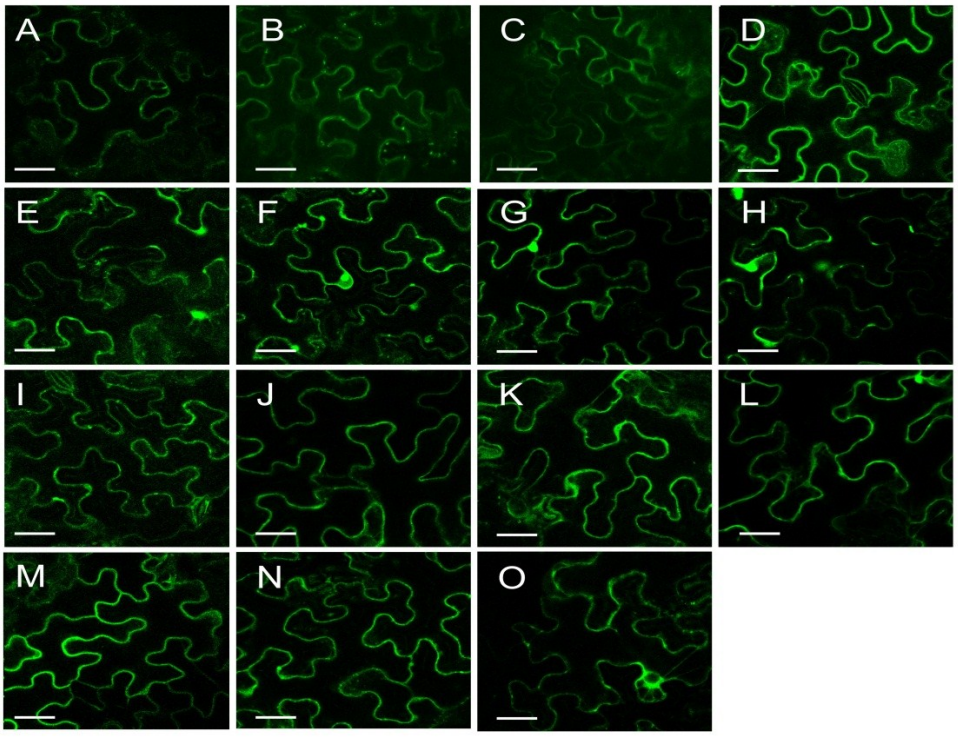


Figure 5: Bimolecular Fluorescence Complementation (BiFC) experiments in tobacco leaf epidermis. Confocal images are presented, showing YFP fluorescence indicating interaction.

Tests for interactions between the CESAs and truncated versions of KOR1 are shown. CESA1/KOR1N (Figure 5A), KOR1N/CESA1 (Fig 5B), CESA1/ KOR1C (Fig 5C), CESA3/KOR1N (Figure 5D), KOR1N/CESA3 (Fig 5E), KOR1C/ CESA3 (Fig 5F), CESA6/KOR1N (Figure 5G), KOR1N/CESA6 (Fig 5H), KOR1C/ CESA6 (Fig 5I), CESA4/KOR1N (Figure 5J), KOR1N/CESA4 (Fig 5K), KOR1C/ CESA4 (Fig 5L), CESA8/KOR1N (Figure 5M), KOR1N / CESA8 (Fig 5N), KOR1C/ CESA8 (Fig 5O). Scale bars = 100 µm.

KORRIGAN is able to form homodimers *in planta*

It is known that type II membrane proteins, like KOR1, often form dimers to perform their function. Using the MbYTH assay, KOR1 was expressed both as bait and as prey to test the ability of KOR1 to form homodimers. The results showed that KOR1 could form homodimers, as it resulted in the growth of yeast on selective medium (Figure 6). To test whether a protein domain can be found responsible for this interaction the truncated KOR1 proteins were tested for interaction with each other. Growth was found in all combinations of truncated proteins except for those which lack a TMD. The combination of the KOR1C and the KOR1N, showed a weak yet significantly higher than the background (Figure 6).

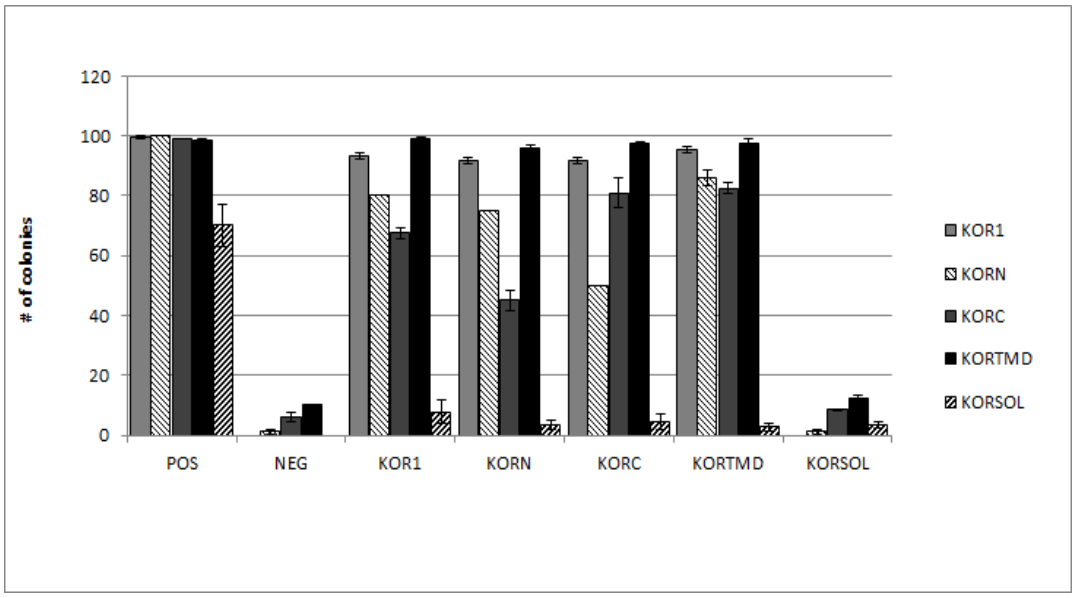


Figure 6: Interactions between the different KOR1 domains using the Membrane-based Yeast Two Hybrid**.** The bars represent the percentage of yeast colonies grown for 3 days on selective medium at 30 ºC. KOR1 domains represented in the legend were expressed in yeast as bait and the different KOR1 domains indicated at the x-axis as prey.

To confirm these findings the interactions were tested *in planta.* Our results showed that fluorescence was restored when two different fusion proteins (YFP/C-KOR1 and YFP/N-KOR1) were expressed in *Nicotiana benthamiana,* indicating the formation of homodimers of KOR1 proteins (Figure 7A). Different parts of the protein, KOR1N or KOR1C were also tested for interaction with the full-length or portions of KOR1 protein and fluorescence was found in all combinations tested. The partial proteins KOR1N and KOR1C could interact with the full-length KOR1 protein as well as with the respective truncated forms (Figure 7) and form homodimers (Figure 7), although the interaction between the KOR1N and KOR1C

(Figure 7D) was significantly weaker. As all (truncated) proteins in this test contained the TMD, these results indicate that this domain is also important in the dimerization of KOR1.

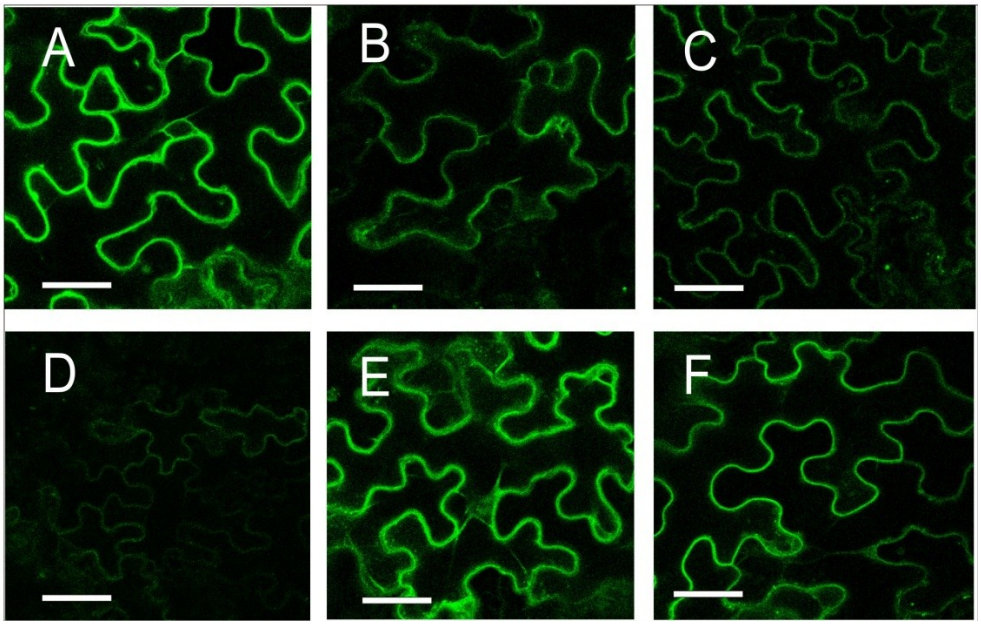


Figure 7: BiFC in *N. benthamiana* shows *in vivo* dimerization between KORRIGAN domains**.** Dimerization between the different truncated forms of KORRIGAN KOR1/KOR1 (A), KOR1/KOR1N (B), KOR1/KOR1C (C), KOR1N/KOR1C (D), KOR1N/KOR1N (E), KOR1C/KOR1C (F) The scale bar is 100μm.

KOR1 is present in the plasma membrane

To further characterize the role of KOR1 in cellulose synthesis, the localization of Kor1 was assessed by generating a green fluorescent protein fusion to the *KOR1* cDNA (GFP-KOR1) under the control of the endogenous *KOR1* promoter and expressing the chimeric protein in the *kor1-1* mutant background. Stable expression of the construct was found to nearly fully complement the growth defects exhibited by the *kor1-1* mutant (Supplemental Figure 1). Examination of GFP-KOR1 in the epidermis of 3-day-old etiolated hypocotyls revealed the presence of discrete particles found aligned at the surface of the cells, in the focal plane of the plasma membrane (Figure 8A). We confirmed the plasma membrane localization of these particles by testing their co-localization with the lipophilic tracer dye FM4-64 (Bolte et al., 2004) in the hypocotyl. GFP-KOR1 surface particles co-localized with FM4-64 at the plasma membrane (Figure 8B), while larger GFP-KOR1 particles were clearly intracellular (Figure 8B, arrows). In addition to these plasma membrane particles and intracellular compartments, GFP-KOR1 also weakly labeled the tonoplast (Figure 8A).

KOR1 plasma membrane particles have the same velocity as the cellulose synthase complex

Visualization of time series showed that GFP-KOR1 plasma membrane particles are motile (Figure 8). Their movement in the hypocotyl epidermis of 3-day-old etiolated seedlings using kymographs were analysed. The straight traces on the kymographs and cross-hatching pattern indicated that GFP-KOR1 plasma membrane particles migrate bi-directionally along linear

tracks with steady velocities (Figure 8D). These same properties characterize the movement of markers for primary cell wall cellulose synthase complexes (Paredez et al., 2006b; Desprez et al., 2007). The velocity of GFP-KOR1 plasma membrane particles were also quantified. Five hundred and forty-five measurements from 12 cells in 4 plants gave a mean velocity of 280 nm/min for GFP-KOR1 plasma membrane particles (range: 111 to 439 nm/min). Notably, the mean velocity and velocity range was similar to that observed for GFP-CESA3 and GFP-CESA6, two cellulose synthases involved in the deposition of the primary cell wall (277 nm/min and 272 nm/min, respectively) (Desprez et al., 2007).

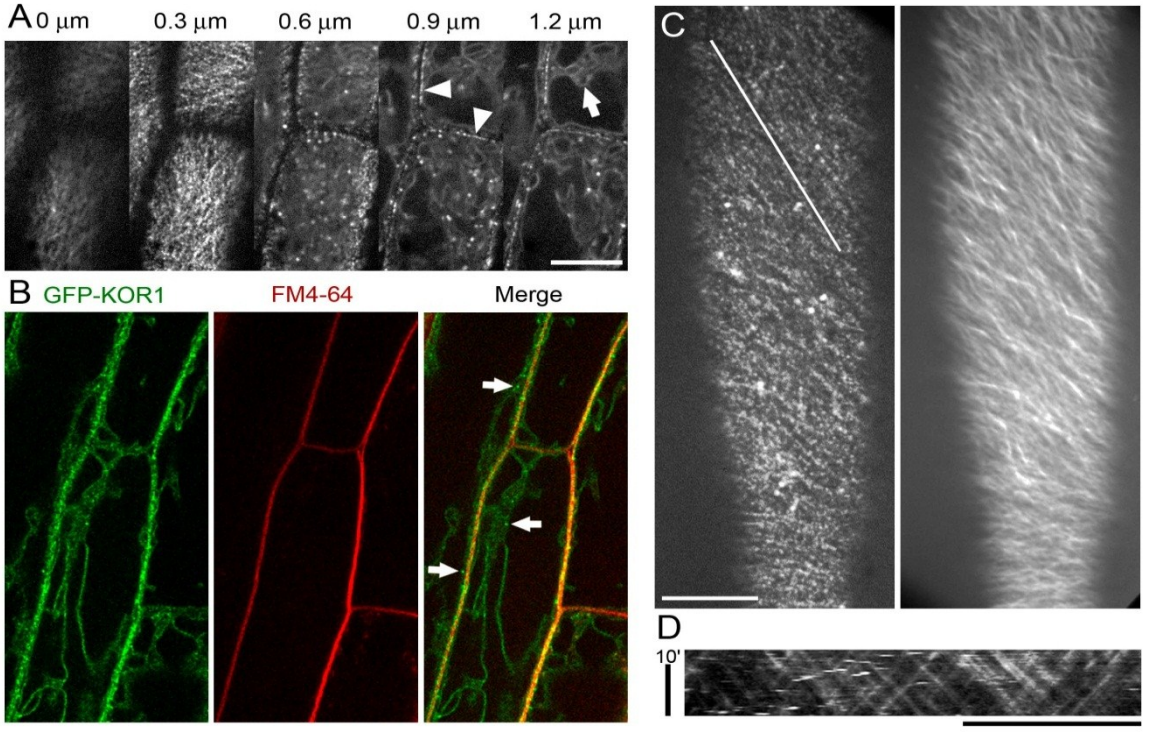


Figure 8: KOR1 is localized in the plasma membrane and in intracellular compartments. Images were acquired in the hypocotyl epidermis of 3-day-old etiolated Arabidopsis seedlings expressing GFP-KOR1 in the *kor1-1* mutant background. Scale bars = 10 microns except where noted. (A) Montage of successive optical sections (0.3 micron interval) of a z-stack through epidermal cells at the top of the hypocotyl. GFP-KOR1 particles are dense in the plasma membrane. In deeper optical sections, the plasma membrane labeling of GFP-KOR1 can also be visualized at the periphery of the cells (arrowheads). The arrow shows an example of GFP-KOR1 tonoplast labeling. (B) Cells at the top of the hypocotyl labeled with FM4-64. In the merged images, GFP-KOR1 clearly colocalizes with FM4-64 at the plasma membrane. (C) The left panel shows a single timepoint from a time series. GFP-KOR1 particles are aligned in organized rows in the plasma membrane. In the right panel, an average projection of the time series illustrates the movement of GFP-KOR1 particles in linear trajectories. (D) Kymograph along the trajectory indicated by the white line in (C). Position is represented along the z-axis, and time is represented along the y-axis. The cross-hatching pattern indicates that GFP-KOR1 particles move bi-directionally with steady velocities.

Discussion

KORRIGAN plays an important role in cellulose biosynthesis as shown by the reduced cellulose content in the *kor1* knock out mutants in Arabidopsis; however direct interaction

between KOR1 and CESA proteins had never been displayed before (Szyjanowicz et al., 2004). Despite previous studies where KOR1 did not co-immunoprecipatate with any of the primary CESAs (Desprez et al., 2007), more sensitive techniques such as split-ubiquitin assays and BiFC were used to assess whether interactions could be detected between KOR1 and primary and secondary CESAs. The results both *in vivo* and *in planta* demonstrated that KOR1 can indeed interact with the three primary (CESA1, 3 and 6) and two of the secondary CESAs (CESA4 and 8).

Mutational analysis has shown that KOR1 cycles between the plasma membrane, or phragmoplast, and in an intracellular compartment in dividing cells (Brummell et al., 1997; Nicol et al., 1998; Peng et al., 2002; Zuo et al., 2000; Molhoj et al., 2002). KOR1 was seen to be localized in the Golgi apparatus, early endosomes, and tonoplast of epidermal cells in the root meristem by using an N-terminal GFP-KOR fusion construct complementing the *kor1-1* mutant (Robert et al., 2005). Inhibition of cellulose synthesis by isoxaben promoted a redistribution of GFP-KOR1 away from early endosomes to compartments close to the plasma membrane in cells close to the plasma membrane facing the root surface at the later stages of cell elongation (Robert et al., 2005). A KOR1-GFP fusion construct expressed in tobacco BY2 cells showed GFP fluorescence present in intracellular organelles in interphase cells, whereas in dividing cells the fluorescence accumulated in the phragmoplast. Surprisingly, no fluorescence was observed in the plasma membrane. However, there are multiple lines of evidence suggesting that KOR1 exerts its activity at the plasma membrane. Biochemical fractionation studies indicate that KOR1 is present at the plasma membrane, as well as in other intracellular compartments (Brummell et al., 1997; Nicol et al., 1998). Substitution mutations in the polarized targeting motifs of KOR1 caused the fusion proteins to localize to the plasma membrane as well suggesting that KOR1 cycles between the plasma membrane and other compartments (Zuo et al., 2000; Molhoj, 2002). KOR1 is an integral membrane protein, whose substrate is likely cellulose (Molhoj et al., 2001; Master et al., 2004). Such an enzyme would be expected to be present at the plasma membrane, where it would act on cellulose already incorporated into the wall, or as it exits the cellulose synthase complex. In this study, we visualized GFP-KOR1 in discrete motile particles at the plasma membrane for the first time in living cells. These results showing KOR1 cycling through different intracellular compartments and especially through the plasma membrane suggest that KOR1 interacts or may be bound to the cellulose synthase complex.

The alterations in cellulose content in the *kor1-1* dwarf mutant together with the result that KOR1 interacted with CESA1, 3 and 6 indicated that KOR1 is directly involved in the synthesis of cellulose in the primary cell wall. The dwarf mutant *korrigan*, (*kor1-1*) phenotype results in a severe reduction in crystalline cellulose both in the primary and secondary cell wall (Nicol et al., 1998) underlining the importance of glucan trimming during cellulose biosynthesis. The movement of cellulose synthase complex was reported to be at least two-fold slower in *kor1-1* mutants than in controls (Paredez et al., 2008). It was also demonstrated that the *acw1* mutation effects cellulose accumulation in the cell wall and microfibril formation resulting in a 40% reduction in crystalline cellulose content compared to wild type plants.

It must also be mentioned that the *kor1-1* mutant stems that showed distinct primary cell wall defects also showed signs of severely collapsed xylem similar to the *irx2* mutants in the secondary cell wall (Szyjanowicz et al., 2004) showing that KOR1 is also involved in the secondary cell wall. The interaction with CESA4 and CESA8 indicates that KOR1 is also part of the rosette structure of the secondary cell wall. The fact that CESA7 didn't interact with KOR1 is supported by previous co-immunoprecipitation experiments, using an epitope-tagged form of KOR. The study on the *irx2* mutant of KOR showed that KOR did not co-purify with (AtCesA7) *irx3* (Szyjanowicz et al., 2004). By checking the interactions of the other CESAs in the secondary cell wall (CESA4 and CESA8) with KOR1 both *in vivo* and *in planta* we demonstrate that there is indeed a physical interaction between KOR1 and the CESAs in the secondary cell wall except for CESA7. Other reports have also indicated that KOR1 plays a role in secondary cell wall development. Co-expression of *Populus KOR* orthologue with the three secondary cell wall *CESA* genes has also been reported (Bhandari et al., 2006). It was also shown that over-expression of the putative *AtKOR* orthologue in hybrid aspen (PttCel9A1) causes a decrease in cellulose crystallinity (Master et al., 2004). Furthermore, two *irx2-1* and *irx2-2* KOR1 mutants showed reduced cellulose synthesis (30% of the WT) in the secondary cell wall (Szyjanowicz et al., 2004) similar to the *irx* mutants.

Interestingly, not all the CESAs in the primary and secondary cell wall have a similar interaction pattern with KOR1. The different reactions between the primary and the secondary cell wall proteins is difficult to explain as the specific functions of the different CESA proteins are not known. As previously mentioned CESA1 was the most efficient integrator in the primary cell wall whereas CESA7 from the secondary cell wall did not interact with KOR1 suggesting that specific CESA isoforms may have unique roles in recruiting KOR1. In other words the binding of KOR1 to the different CESA proteins is specific, as KORRIGAN does not bind to all of them. Not only does this imply that the methods used are sensitive enough to specifically determine interactions between these highly homologous proteins, it also suggests that the KOR1 protein has a specific position within the rosette.

Despite the extensive research on KOR1, its exact role in cellulose biosynthesis is still unknown. It has been suggested that KOR1 may determine the length of individual cellulose chains, either during cellulose synthesis, or subsequent to microfibril assembly or termination of the complex by releasing the cellulose microfibril from the cellulose synthase complex before the complex is internalized from the plasma membrane (Sommerville, 2006; Molhoj et al., 2001). Further detailed analysis on the development of the secondary cell wall cellulose with X-ray diffraction showed that down regulation of hybrid poplar *KOR* gene (*Pa×gKOR)* leads to less cellulose production similar to *irx* mutants, but surprisingly results in an increase in the crystallinity index of the secondary cell wall. Alternatively, overexpression of an exogenous *AtKOR* gene in poplar decreases cellulose crystallinity. These results are suggested to provide evidence for a role for KOR in the splitting of the macrofibril into individual microfibrils, and any lack of function could prevent the macrofibril from being dispersed into the microfibrils (Malony and Mansfield, 2010).

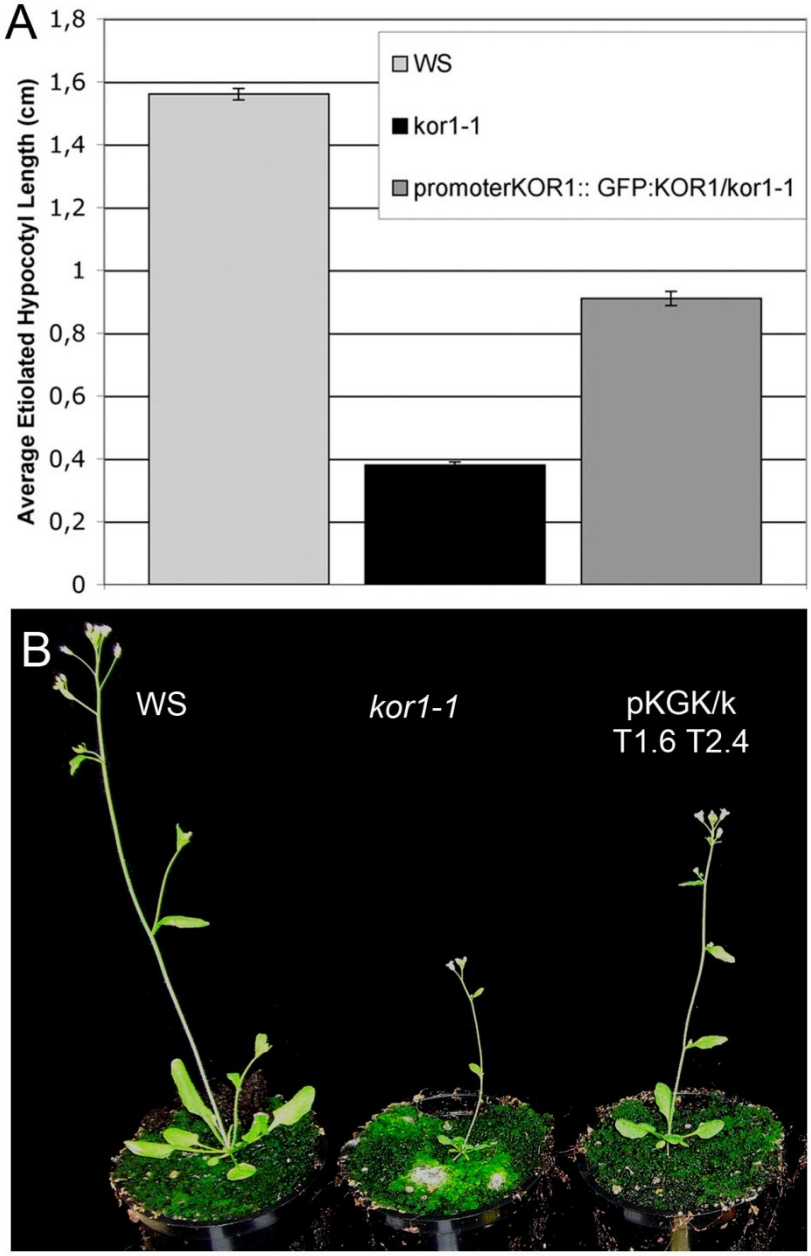
Evidence of direct interaction between KOR1 and the CESAs in both the primary and secondary cell wall challenges the suggested roles for KOR downstream of cellulose synthesis

(Maloney and Mansfield, 2010a; Szyjanowicz et al., 2004; Takahashi et al., 2009). Some studies have suggested that the *acw1* mutation in KOR1 gene which shows defects in primary cell wall formation, reduced cellulose content and a delay in the formation of cellulose microfibrils at the restrictive temperature of 31°C, may affect cellulose synthesis indirectly due to changes occurring in the other cell wall carbohydrates as it also results in increased pectin synthesis (Sato et al., 2001). As defects in the cellulose–hemicellulose network and the modification of pectin during cell wall synthesis would all affect cell wall assembly (McCann and Roberts 1994; Hayashi 1991; Rose and Bennett 1999; Ito and Nishitani 1999; Fujino et al. 2000 ; Carpita and McCann 2000), they suggest that if KOR1 activity contributes to cell wall rearrangement and causes changes in the plant cell wall carbohydrates then a reduction in this activity might explain the changes in cell wall assembly observed in the *acw1* mutant (Sato et al., 2001). However, the delay in cellulose microfibril formation by *acw1* protoplasts at the non-permissive temperature (31 ºC) is consistent with the hypothesis that the effect of gene function on cellulose synthesis is direct. Identification of the substrate and product of KOR1 activity should provide further insight into the role of this enzyme in cell wall synthesis (Sato et al., 2001).

A more detailed view on the interaction between KOR1 and the CESA proteins using truncated versions of KOR1 revealed that all portions which contain the TMD were able to interact and this led to the conclusion that KOR1 transmembrane domain is required for the interaction with CESAs. These data suggest that KOR1 and CESA associate in the CSC in the plasma membrane, between the TMD of KORRIGAN and the CESA proteins. Considering that some proteins required for cellulose synthesis are found in sterol-rich lipid rafts (Morel et al., 2006), it is possible that KOR1 and CESAs traffic to the plasma membrane separately, but are then concentrated in specific lipid domains, favouring interactions. This domain is also important in the homodimerisation of the KOR1 protein as all partial proteins containing this domain are able to interact, however other domains of the KOR1 protein might also play a role in the dimerization as the combination KOR1C and KOR1N only showed a weak interaction in both assays. The function of this dimerization is thus far unknown, however one could speculate that the dimer enables a more efficient hydrolysis of the glucan chains or binding to the cellulose, or the interlinking of KORRIGAN might result in a more stable rosette complex.

In conclusion, we have determined that the KOR1 protein is expressed in the same cells as GFP-CESA3 and interacts with both primary and secondary CESA proteins both *in vitro* and *in planta* at the plasma membrane proving the hypothesis that KOR1 makes up a part of the rosette structure. The physical interaction also indicates that KOR1 is directly involved in cellulose biosynthesis, and probably does so in the form of a homodimer. Furthermore, our study showed that the TMD of KOR1 is essential in the interaction with the different CESA proteins.

Supplemental Figures



Supplemental Figure 1: *promoterKOR1-GFP-KOR1* partially complements the *kor1-1* mutant phenotype. (A) Comparison of etiolated hypocotyl length after 5 days growth between wild-type (WS), the *kor1-1* mutant, and homozygous *promoterKOR1-GFP-KOR1* expressed in the *kor1-1* mutant. Means and standard error are shown for N ≥ 46. (B) Comparison of phenotypes after 3 weeks growth in greenhouse conditions between wild-type (WS), the *kor1-1* mutant, and homozygous *promoterKOR1-GFP-KOR1* expressed in the *kor1-1* mutant (pKGK/k). Expression of *promoterKOR1-GFP-KOR1* partially complements the small stature and small organ size of the *kor1-1* mutant.

Supplementary Table 1: Primers used for the MbYTH system

Gene	Primer for bait amplification
CESA1	5'AAGACTGCAGAATGGAGGCCAGTGCCGGC3' 5'AACAGGCGCCCTAAAAGACACCTCCTTTGCC3'
CESA3	5'AGAACCATGGAATGGAATCCGAGGAGAAACC3' 5'AAGAACTAGTTCAACAGTTGATTCCACTTCC3'
CESA6	5'AGAACCATGGAATGAACACCGGTGGTCGG3' 5'AAGAACTAGTTCACAAGCAGTCTAAACCA3'
KOR1	5'AAGACGTCATGTACGGAAGAGATCCATGGGG3' 5'TTTACTAGTCAAGGTTTCCATGGTGCTGGTGG3'
KOR1C	5'AAAGACGTCAAGATCTTCGTCTGGACTGTTGGT3' 5'TTTACTAGTCAAGGTTTCCATGGTGCTGGTGG3'
KOR1N	5'AAGACGTCATGTACGGAAGAGATCCATGGGG3' 5'TTTACTAGTTTAACGATCAAGGTAATGAA3'
KOR1 TMD	5'AAAGACGTCAAGATCTTCGTCTGGACTGTTGGT3' 5'TTTACTAGTTTAACGATCAAGGTAATGAA3'
KOR1 SOL	5'AAGACGTCATGTACGGAAGAGATCCATGGGG3' 5'GCGGCTAACGATAATACAACC3'/5'ACTGTGCCGCGTCATCATCC3' 5'ACTGTGCCGCGTCATCATCC3' 5'TTTACTAGTCAAGGTTTCCATGGTGCTGGTGG3'
Gene	Primer for prey amplification
CESA1	5'AAGAGGCCATTACGGCCATGGAGGCCAGTGCCGGC3' 5'AAGAGGCCGAGGCGGCCAAGTAAAAGACACCTCCTTTGCCAT3'
CESA3	5'AGAACGGCCATTACGGCCATGGAATCCGAAGGAGAAACC3' 5'GAGGCCGAGGCGGCCGTCAACAGTTGATTCCACATTCCAGAAT3'
CESA6	5'AGAACGGCCATTACGGCCATGAACACCGGTGGTCGGTTAATCGC3' 5'GAGGCCGAGGCGGCCGTCACAAGCAGTCTAAACCACAGATCTCGAGAAT3'
KOR1	5'AACAGGCCATTACGGCCATGTACGGAAGAGATCCATGGGG3' 5'AAGAGGCCGAGGCGGCCATCAAGGTTTCCATGGTGCTGGTGG3'
KOR1C	5'AACAGGCCATTACGGCCAAGATCTTCGTCTGGACTGTTGGT3' 5'AAGAGGCCGAGGCGGCCATCAAGGTTTCCATGGTGCTGGTGG3'
KOR1N	5'AACAGGCCATTACGGCCATGTACGGAAGAGATCCATGGGG3' 5'AAGAGGCCGAGGCGGCCTTTAACGATCAAGGTAATGAA3'
KOR1 TMD	5'AACAGGCCATTACGGCCAA ' 5'AAGAGGCCGAGGCGGCC
KOR1 SOL	5'AACAGGCCATTACGGCCATGTACGGAAGAGATCCATGGGG3' 5'GCGGCTAACGATAATACAACC3' 5'ACTGTGCCGCGTCATCATCC3' 5'AAGAGGCCGAGGCGGCCATCAAGGTTTCCATGGTGCTGGTGG3'

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Chapter 4

Specific Isoforms of Sucrose Synthase are Integral Components of the Primary and Secondary Cellulose Synthase Complexes

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Abstract

The cellulose synthases proteins are the catalytic subunits of the cellulose synthase rosette complex. However, these proteins do not work alone. In addition to CESA proteins, cellulose biosynthesis requires the action of other proteins one of which is sucrose synthase (SUS). In plants sucrose synthase plays a major role in energy metabolism by breaking down sucrose to UDP-glucose and fructose. The UDP-glucose supplied by sucrose synthase is channelled to the cellulose synthase complex in the plasma membrane for cell wall biosynthesis. In the model plant *Arabidopsis thaliana* the *SUS* gene family is thought to contain six putative members *AtSUS1* to *6*. However it has not been demonstrated before which isoforms are responsible for providing the UDP-glucose for the cellulose synthase complex. Using both *in vitro* and *in planta* protein interaction assays, we studied the interactions between each of the CESA proteins in the primary and secondary cell walls with the sucrose synthase isoforms in Arabidopsis. We demonstrate that not all the sucrose synthase isoforms are able to interact with the catalytic subunits of the rosette complex. In order to further verify the direct interaction between the CESAs and SUS isoforms, we show that GFP-SUS6 expressed under its own promoter is colocalized with Td-tomato-CESA6. In order to monitor possible changes in the cell wall composition caused by the loss of sucrose synthase isoforms, FT-IR microspectroscopy carried out on various *sus* knockout mutants showed a global increase in polysaccharide content for some of the mutant knockouts. Altogether, our data supports a role for sucrose synthase in providing UDP-glucose for the Cellulose Synthase Complex at the plasma membrane.

Introduction

Cellulose is a linear homopolymer of β-1,4-linked glucose residues synthesized by the cellulose synthase complex (Pauly and Keegstra, 2008) with the CESAs being the catalytic subunit of the complex in vascular plants (Taylor et al., 2000; Richmond and Somerville, 2000, 2001). Genetic complementation and biochemical studies on CESAs have shown that cellulose biosynthesis involves the function of up to three distinct non redundant CESAs (Taylor et al. 2000; Scheible et al. 2001; Burn et al. 2002) with CESA1, CESA3, and CESA6-like proteins present in the primary cell wall complexes (Doblin et al., 2002; Desprez et al., 2007; Persson et al., 2007) and CESA4, CESA7, and CESA8 required for the secondary cell wall complex (Scheible et al., 2001; Taylor et al., 2000, 2003) in Arabidopsis.

One of the requirements for robust cellulose synthesis is the supply of its substrate UDP-glucose (Carpita and Delmer, 1981) also used for the biosynthesis of xyloglucan and callose (Amor et al., 1995; Haigler et al., 2001). In plants, UDP-glucose (UDP-Glc) can potentially be synthesized by two different pathways. One route involves the enzyme UDP-glucose pyrophosphorylase which catalyses the reversible reaction of glucose to UDP-glucose (Flores-Diaz et al., 1997). Levels of this enzyme are usually very high in plant cells, but it probably functions primarily in the direction of UDP-glucose degradation, mostly in non-photosynthetic tissues (Kleczkowski, 1994). Another route for the direct production of UDP-glucose is by the sucrose synthase enzyme (SUS). Sucrose synthase can catalyse the conversion of sucrose to fructose and UDP-glucose required for cellulose biosynthesis (Chourey and Nelson, 1979; Geigenberger and Stitt, 1993). The optimal pH for cleavage of sucrose is 6-6.5 and for synthesis is pH 8-9 (Chourey and Nelson, 1979; Tsai, 1974). The reaction catalysed by SUS is freely reversible, but the high levels of this enzyme and steady-state measurements of levels of its substrates and products in non-photosynthetic tissues suggest that it functions primarily in the direction of sucrose degradation and UDP-glucose synthesis (Xu et al., 1989; Geigenberger et al., 1993; Amor et al., 1995).

The sucrose synthase enzyme (SUS) is believed to have different roles in plants. One is linked to sucrose hydrolysis in sink tissues, and another concerns its role as the primary provider of UDP-glucose for cell wall biosynthesis (Amor et al., 1995; Zrenner et al., 1995; Nakai et al., 1998; Koch, 2004; Coleman et al., 2009; Fujii et al., 2010). SUS is mainly restricted to tissues that metabolize sucrose (Konishi et al., 2004) with its localisation possibly determining its function. Several lines of evidence indicate that SUS occurs in both soluble and membrane-associated forms. While a soluble form of SUS is generally involved in the cytoplasmic metabolism of sucrose, a membrane associated form of SUS may have various functions. A tonoplast-associated SUS may be involved in sucrose transport from vacuole to the cytosol in sucrose-storing organs (Etxeberria and Gonzalez, 2003). SUS bound to the Golgi membrane is thought to provide UDP-glucose for xyloglucan synthesis (Buckeridge et al., 1999; Konishi et al., 2004). SUS associated with the plasma membrane is thought to provide UDP-glucose directly for cellulose and callose synthesis and has been studied in a number of species (Winter and Huber, 2000; Haigler et al 2001; Komina et al., 2002). Plasma membrane associated SUS was first described in cotton fibres and maize kernels (Amor et al., 1995; Carlson and Chourey 1996). However this association of sucrose synthase with the membrane

is not well understood with phosphorylation/dephosphorylation of the SUS enzyme suggested to play a role in its association as well as its location and function (Winter et al., 1997; Angenout et al., 2006).

One hypothesis about how the cellulose biosynthesis is controlled is through a model proposing a direct association between SUS and the plasma membrane cellulose synthase rosettes complex (Supplementary Figure 1). In this model, a membrane bound SUS is linked physically to the complex and plays an important role in channelling UDP-glucose to the cellulose synthase complex (Amor et al., 1995; Fennoy et al., 1998; Koch, 2004). The biochemical features of this model would allow UDP-glucose provided by SUS to feed directly into cellulose formation and would allow the rapid recycling of UDP (Purcell et al., 1998; Koch, 2004; Supplementary Figure 1). In this model, SUS could provide not only a direct pathway of carbon transfer from newly imported sucrose to UDP-glucose, but also by using less ATP it would bemore energy efficient (Nolte et al., 1995). Therefore, by forming a putative complex with cellulose synthase at the plasma membrane, SUS has the potential capacity to channel carbon directly from sucrose to cellulose (Amor et al., 1995).

SUS has shown to affect cellulose biosynthesis in various plants. Transgenic cotton plants with decreased SUS activity affected crystalline cellulose deposition by reducing it to about 63% of wild-type (Tang and Sturm, 1999; Ruan, 2007). The overexpression of *SUS* genes in poplar (Coleman et al. 2009) and tobacco (Coleman et al. 2006) has revealed an effect on biomass production suggesting a link to cell wall formation and cellulose biosynthesis. The positive effect of SUS on cellulose production appears to be dependent on the SUS isoform used in various plants with a study in pea indicating that different SUS isoforms are associated with different metabolic fates of sucrose (Barratt et al., 2001). Altogether these observations support a role for SUS in carbon partitioning. The SUS isoforms are encoded by a small multigene family and have been identified in many plants. In *Arabidopsis thaliana*, six isoforms of SUS have been identified and extensive studies have been made of the tissue specific expression patterns of all six isoforms (Baud et al., 2004; Bieniawska et al., 2007). Surprisingly, Arabidopsis *sus* knockout lines have shown little or no clearly obvious phenotypes as silencing of several *SUS* genes together in Arabidopsis, including a quadruple knockout, had no obvious effects on plant growth (Baud et al., 2004; Bieniawska et al., 2007; Barratt et al., 2009) which could possibly be due to an overlap of function between the isoforms.

Decades of research have suggested that SUS plays a role in plant metabolism and may be involved in cellulose biosynthesis. However, clear evidence for a direct interaction between sucrose synthase and the cellulose-synthesizing complex was lacking and remained an important question relative to enzyme function. In this study using the membrane-based yeast two-hybrid system (MbYTH; Fetchko and Stagljar, 2004), which has already been proven to be a powerful tool to detect interactions between proteins involved in cell wall biosynthesis (Timmers et al., 2009), and the bimolecular fluorescence complementation method (BiFC) we show that sucrose synthase is directly associated with the CESAs. Our results show that not all the SUS isoforms are able to physically interact with the rosette complex. SUS1, SUS3, SUS4 and SUS6 show interaction with the CESAs however SUS5 shows no interaction

neither *in vivo* nor *in planta,* suggesting specificity in interaction and function between the SUS isoforms in Arabidopsis. Considering the lack of an obvious phenotype in the *sus* mutant knockouts of Arabidopsis, interestingly, FT-IR microspectroscopy carried out on the single, double and quadruple *sus* mutants showed an increase in carbohydrate content in some mutants however further analysis is required to determine which polysaccharides have increased. To further support the role of sucrose synthase in cellulose biosynthesis, we studied the localization of SUS6 fused to green fluorescent protein (GFP) under the endogenous SUS6 promoter into pCESA6::tdTomato::CESA6 seedlings by means of vacuum infiltration (Marion et al., 2008). The transient assay demonstrated the colocalization of GFP-SUS6 with tdTomato-CESA6 in epidermal cells from Arabidopsis cotyledons. Our data demonstrates that the interaction between SUS isoforms and the CESA proteins is specific, and based on their function may interact at the plasma membrane. The new information provided here will help shed light on the role of SUS in cellulose synthesis.

Materials and Methods

Constructs for the MbYTH system

The constructs for the MbYTH system concerning the primary and secondary cellulose synthases were described previously (Timmers et al., 2009; Chapter2). The full-length cDNAs for the Arabidopsis *SUS* genes (*SUS1, SUS3, SUS4, SUS5* and *SUS6*) were obtained from Prof. Dr. Alison Smith at John Innes Centre., UK. *SUS2* was not available at the time, thus was not used. The cDNA's of the mentioned genes were amplified by PCR using the Phusion DNA Polymerase (Finnzymes, Helsinki, Finland) with suitable primers (Supplementary Table 1). The resulting PCR-products were digested and ligated into the pTFB1 vector (Bait) and the pADSL-Nx vector (Prey) (Dualsystems Biotech AG). Bait and prey expression is regulated by the TEF1 and ADH1 promoter, respectively. The sequences of the inserts were confirmed by Sanger sequence analysis. Both the bait and prey protein were fused N-terminally to the Cub-TF reporter cassette of the vector p TFB1 and NubG cassette of the vector p ADSL-Nx respectively.

Membrane based yeast two hybrid screen

The interactions between the CESAs and SUS isoforms were assayed using the split-ubiquitin membrane-based yeast two-hybrid (Johnsson and Varshavsky, 1994; Reinders et al., 2002) using yeast NYM51 strain in the Split Ubiquitin System kit (Dualsystems Biotech AG). The assays were performed according to supplier instructions (DUAL membrane Kit 1). The bait and prey constructs were co-transformed into the yeast strain NMY51 (Dualsystems Biotech AG) according to the provided transformation procedure (DUAL membrane Kit 1). The yeast, containing both plasmids, was plated onto synthetic medium lacking leucine and tryptophan (SD med.-L-T), and grown at 30ºC for three days. To quantify the interactions between different preys, 100 colonies of each combination were spotted onto selection medium containing the appropriate amount of 3-ammonium-triazole (3-AT) and grown at 30ºC for three days. The number of spots grown was then counted. Detection of β-galactosidase activity was performed with the filter-lift assay (Breeden and Nasmyth, 1985).

Constructs for Split-YFP

The full-length cDNA of the *CESA* and the *SUS* genes (*SUS1, SUS3, SUS4, SUS5* and *SUS6*) were generated through Phusion DNA Polymerase (Finnzymes, Helsinki, Finland) with suitable primers (Desprez 2007; Timmers et al., 2009; Supplemetary Table 1). Coding sequences of the genes were cloned into the Gateway-compatible destination vectors pBIFc-2 and pBIFc-3 plasmids regulated by the constitutive *35S* promoter (Hu et al., 2002). The N-terminal and the C-Terminal fragments of YFP were both fused to the N terminus of the coding sequences of the CESAs and the SUS. As a positive control, the aquaporin PIP2-1 (Boursiac et al., 2005; Desprez et al., 2007) was used, as Aquaporins are known to form homotetramers in the plasma membrane (Murata et al., 2000). As a negative control PIP2-1 chimera was co-expressed with the corresponding CESA constructs.

Bimolecular Fluorescence Complementation screen

Leaves of 3-week-old plants (*Nicotiana benthamiana*) were transformed by infiltration as described (Voinnet et al., 2003). Upon interaction between the two proteins, the fragments restore fluorescence. YFP fluorescence was detected 3 days after infiltration by using the 514-nm laser line of a SP2 AOBS confocal laser scanning microscope (Leica, Solms, Germany) equipped with an argon laser. To check the YFP reconstitution, spectral analysis was performed with the 496-nm laser line. The fluorescence with all constructs was detected at the same photo-multiplier tube (PMT) settings (760), except for the negative interactions for which the PMT was increased up to 880.

Promoter:GFP Constructs

35S promoter constructs

Standard molecular cloning techniques were performed essentially as described (Sambrook and Russell, 2001). Constructs were made by using Gateway cloning technology (Invitrogen). The *SUS1, SUS3, SUS4, SUS5, SUS6* genes were first cloned into to pDONR221 (Life Technologies, Carlsbad, CA, USA) through Phusion DNA Polymerase (Finnzymes, Helsinki, Finland) with suitable primers (Supplementary Table1), and then to a Gateway-compatible pgwb6 vector under the regulation of 35S constitutive promoter. After sequence verification the final expression vector was electroporated in *Agrobacterium tumefaciens* strain C58 mp90.

SUS Endogenous promoter constructs

The *SUS6* promoter sequence was amplified from genomic DNA of Arabidopsis wild-type Col-0 by PCR using Phusion DNA Polymerase (Finnzymes, Helsinki, Finland) with suitable primers (Supplementary Table 1) and cloned into the *HindIII- XbaI* site of the GFP pGWB6 vector after the removal of the 35S promoter. An LR reaction was performed to obtain the *SUS* prom-GFP-*SUS* construct (GFP-SUS6). After sequence verification the final expression vector was electroporated in *Agrobacterium tumefaciens* strain C58 mp90.

Vacuum infiltration

For transient expression in Arabidopsis seedlings, the Agrobacterium strains containing the construct used were grown as described (Marion et al., 2008), prior to infiltration, diluted in 5% sucrose, 200 Mm acetosyringone to an OD600 of 2.0, and mixed 1:1. The seedlings of 3–4 d old prom*CESA6*::tdTomato::*CESA6* of Arabidopsis (Gutierrez and Lindeboom, unpublished, Laboratory of plant cell biology Wageningen University) were transformed via vacuum infiltration (Marion et al., 2008) and the seedlings were examined for fluorescence 3 days post-infiltration.

Plant material and *in vitro* growth conditions

The transgenic Arabidopsis lines used for fluorescence microscopy were all of the Colombia ecotype. prom*CESA6*::tdTomato::*CESA6* (Gutierrez and Lindeboom, unpublished, Laboratory of plant cell biology Wageningen University) was a complemented mutant of the prom*CESA6*::tdTomato::*CESA6* in a CESA6 *prc1-1* background. The single (*sus1, sus3, sus4 ,sus5, sus6*), double (*sus5/6*) and quadruple mutant knockouts (*sus1/2/3/4*) were provided by Prof. Dr. Alison Smith from John Innes, UK as previously described (Baud et al., 2004; Bieniawska et al., 2007). Plants were grown in dark as described (Refrégier et al., 2004). For imaging, seedlings were cultured in chambers as described (Chan et al., 2007).

Spinning disk analysis

Spinning disk analysis and image analysis were performed as described (Crowell et al., 2009).

Laser Scanning Confocal Microscopy

Images were collected with a spectral Leica SP2 AOBS confocal microscope (Leica Microsystems) equipped with an argon laser. GFP were detected using a laser line 488 nm

FT-IR Microspectrometry

Four-day-old seedlings were squashed between two barium fluoride windows and rinsed abundantly with distilled water for 2 min before drying at 37°C for 20 min. For each mutant, 20 spectra were collected from individual hypocotyls of seedlings from four independent cultures (five seedlings from each culture; Mouille et al. 2003). Normalization of the data and statistical analyses were performed as described (Mouille et al., 2003). Normalization of the data set and statistical analyses were performed using the statistical language R version 2.6. (R Development Core Team, 2006). To normalize the spectra, the baseline, estimated using a linear regression involving 10 points at each end of the spectrum, was subtracted from each absorbance value, and the area was set to 1 by dividing each absorbance value by the sum of all absorbance values. To determine the significant difference of the composition and the structure between mutants and the wild type, Student t-test was performed.

Results

The primary and secondary cell wall CESAs interact with the sucrose synthase isoforms

In order to test whether SUS has a role in cellulose biosynthesis, the interaction between the non-redundant members of the primary cell wall cellulose synthases (CESA1, CESA3 and CESA6) and the secondary cell wall CESAs (CESA4, CESA7 and CESA8) with the SUS isoforms (SUS1, SUS3, SUS4, SUS5, and SUS6) were tested *in vivo* using the membrane based yeast two hybrid system. Interactions were tested with CESA1, 3,4,6,7 and 8 proteins fused to C-terminal part of the ubiquitin (Cub) and the transcription factor (bait), whereas the SUS isoforms were fused to the N-terminal part of the ubiquitin (Nub; preys). Upon interaction between the bait and the prey the transcription factor (TF) is released into the nucleus where it activates reporter genes allowing the yeast to grow on selective medium. The results indicated that all three of the primary and the secondary CESA proteins were able to interact with the SUS isoforms as most yeast colonies were able to grow on selective medium except for AtSUS5 (Figure 1). The lack of growth in the negative control indicated that the interaction with the SUS isoforms were specific as an unrelated protein expressed as prey is not able to activate the system.

To verify that CESA1, 3, 4, 6, 7 and 8 can interact with the SUS isoforms *in planta*, we employed the Bimolecular Fluorescence Complementation (BiFC) technique (Desprez et al., 2007). Using this system, the interaction between the CESAs and SUS1, SUS3, SUS4, SUS5 and SUS6 were tested in *Nicotiana benthamiana*. Two YFP fragments, either YFP/N or YPF/C, each linked to the N-terminus of the proteins, were transiently expressed in the plant. Expression of CESA1, 3,4,6,7 and 8 fused to the C-terminus of YFP together with the SUS1, SUS4 and SUS6 (fused to the N-terminus of YPF fragment) resulted in strong fluorescent signals at the plasma membrane (Figure 2). The interactions between CESA1, 3, 4, 6, 7 and 8 fused to the to the C-terminus of YFP with the SUS3 (fused to the N-terminus of YPF fragment) resulted in somewhat weaker fluorescent signals (Figure 2) while the interactions between the CESAs fused to the to the C-terminus of YFP with SUS5 (fused to the N-terminus of YPF fragment) resulted in no fluorescent at all which was comparable to the negative control. The alternate pairwise combinations all resulted in similar interaction patterns (data not shown).

In conclusion, the CESA protein in the primary and secondary cellulose synthase complex can interact with specific SUS isoforms (SUS1, SUS3, SUS4 and SUS6) *in vivo* and *in planta*. However, SUS5 shows no interaction with the cellulose synthase catalytic subunits (CESAs) in the primary and secondary cell wall in the membrane based yeast two hybrid and only showed a background fluorescent signal *in planta*.

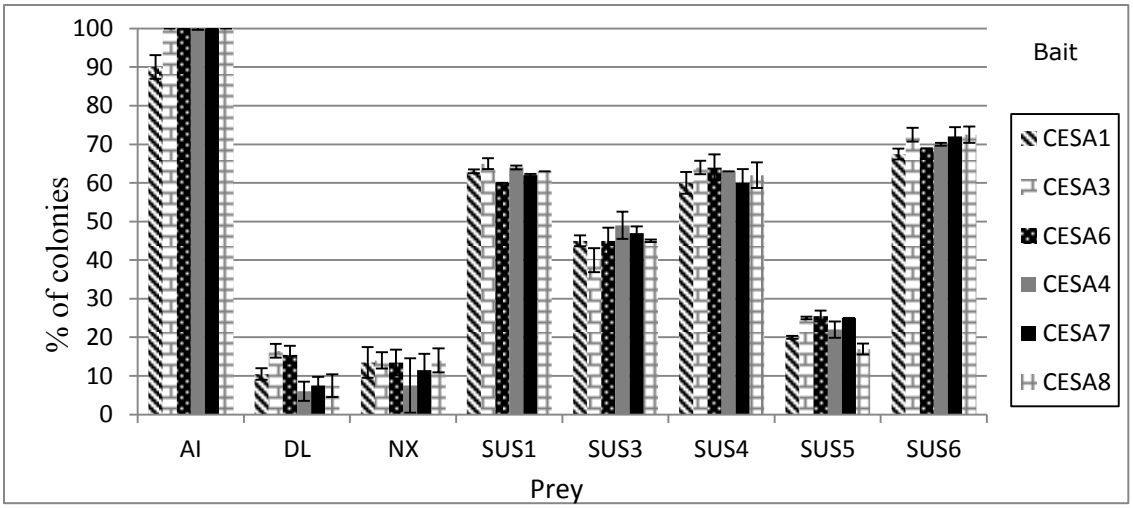


Figure 1: Interactions between the SUS isoforms and the different CESA proteins using the Membrane-based Yeast Two Hybrid. The bars represent the percentage of yeast colonies grown for 3 days on selective medium at 30 ºC. The different CESA proteins were expressed in yeast as bait and the SUS isoforms as prey.

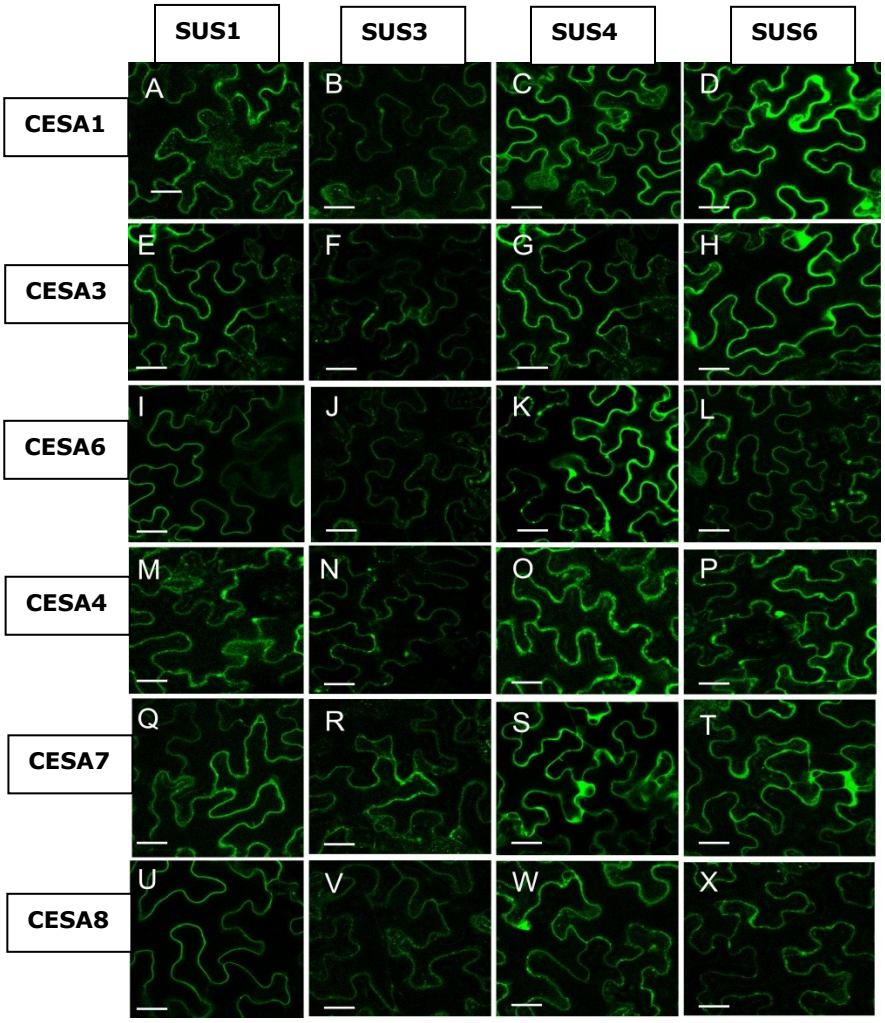


Figure 2: BiFC in *N. benthamiana* leaf epidermis shows *in vivo* dimerization between the SUS isoforms and the CESA proteins. Confocal images are presented, showing YFP

fluorescence indicating an interaction (positive control testing the dimerization of PIP2 (1), or lack of fluorescence indicating no interaction (2). The SUS isoforms expressed together with different CESA proteins; Test for interaction between SUS isoforms (SUS1, SUS3, SUS4, SUS5 and SUS6) and CESA isoforms (CESA1, CESA3, CESA6, CESA4, CESA7 and CESA8) by fusing SUS to the N-terminus of YFP and CESA to the C-terminus of YFP. The scale bar is 100µm.

SUS6 isoform shows colocalization with CESA6

To determine the subcellular localization of the SUS isoforms in Arabidopsis and to investigate whether they indeed have a role in cellulose biosynthesis, GFP fused *SUS1*, *SUS3*, *SUS4*, *SUS5* and *SUS6* genes were cloned under the control of the 35S CaMV in the pgwb6 vector. Surprisingly, most of the expressing GFP yielded no fluorescence in the Arabidopsis seedlings as overexpression probably led to feedback mechanism that down regulated the *SUS* gene however a very weak signal was detected in *SUS6*.

Since the former GFP-SUS constructs under the control of a 35S promoter showed a weak signal, to examine the localization of SUS in Arabidopsis, GFP-SUS6 was cloned under the control of its own endogenous promoter. The GFP fluorescence signal was determined by infiltration into *Nicotiana benthamiana* and was clearly detectable in the transient assay *Nicotiana benthamiana* plants, 3 days after infiltration with Leica SP2 AOBS confocal microscope and yielded a strong fluorescence (Figure 4A).

Subsequently, the GFP-SUS6 construct cloned under its endogenous promoter was vacuum infiltrated into the stable line of prom*CESA6*:tdtomato:*CESA6* to check for colocalization with the CESAs with the spinning disk microscope which resulted in a signal consistent with the interaction results between SUS6 isoforms and CESA6 showing GFP-SUS6 fusion protein colocalizing with CESA6 (Figure 3 B, C and D).

In conclusion, SUS6 shows colocalization with CESA6, suggesting that the SUS6 isoform would provide UDP-glucose for the CESAs. It must be mentioned however that the migration of CESA6 and SUS6 was not detected.

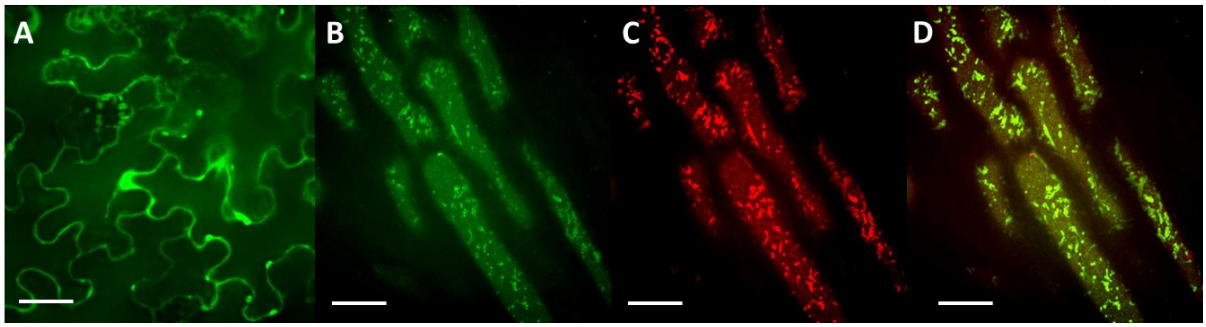


Figure 3: (A) GFP signal from SUS6 gene cloned under its endogenous promoter infiltrated into *Nicotiana benthamiana* scale bar is 100µm. (*B*), Localization of GFP-*SUS6*, (*C*), TdTomato-CESA6 and (*D*) merge (Bar = 10 mm).

FTIR analysis

Fourier transform infrared (FT-IR) microspectroscopy has previously been described as a screening method to identify cell wall deficiencies in mutants (Chen et al, 1998; Mouille et al., 2003). To determine possible structural changes in the cell walls of the *sus* knockout mutants, FT-IR microspectroscopy (Mouille et al., 2003) was performed on single *sus* mutant (*sus1, sus3, sus4, sus5 and sus6*) as well as double (*sus5/6*) and quadruple (*sus1/2/3/4*) knockout mutants. The spectra obtained are averaged from analyses on 20 samples (Figure 4). In order to obtain information on the spectral differences between the wild type and *sus* mutants, the average spectrum from the mutants was compared with that of the wild type Col-0 line. A student t-test was used to determine the significance of the difference between the average values for every individual wave number of the spectrum (Figure 5). The t-value was plotted against the wave numbers of the spectrum. A series of peaks were seen at specific wave numbers that mostly show non-significant differences in absorbance values between the mutants and the wild type line. FT-IR microspectroscopy carried out on hypocotyls of single (*sus1, sus3, sus4, sus5* and *sus6*) double *(sus5/6*) and quadruple (*sus1/2/3/4*) *sus* knockout mutants according to Student t-test, revealed differences in the "carbohydrate fingerprint region" of the spectrum (800-1200 cm^{-1}) in *sus1*, *sus4* and *sus1/2/3/4* mutants (Figure5 A). Between the single mutants, *sus1* mutant knockout showed a different pattern compared to the other single knockouts in some wavelengths with differences seen in the wavelengths related to glycosilic linkage (1160±30 cm^{-1}) and protein content (1550 to 1650 cm^{-1}), the latter was also detected in the double sus56 mutant. By comparing the double (*sus5/6*) and quadruple (*sus1/2/3/4*) *sus* mutants, the quadruple mutant (*sus1/2/3/4*) also displayed an increase in glycosidic linkage (1160cm^{-1}).

In conclusion, FT-IR analysis shows that there is change in cell wall content in *sus1, sus4* and *sus1234* compared to the wild type suggesting an increase in polysaccharide content in *sus1* and *sus4*. This approach did not allow us to conclude about the nature of this polysaccharide fraction.

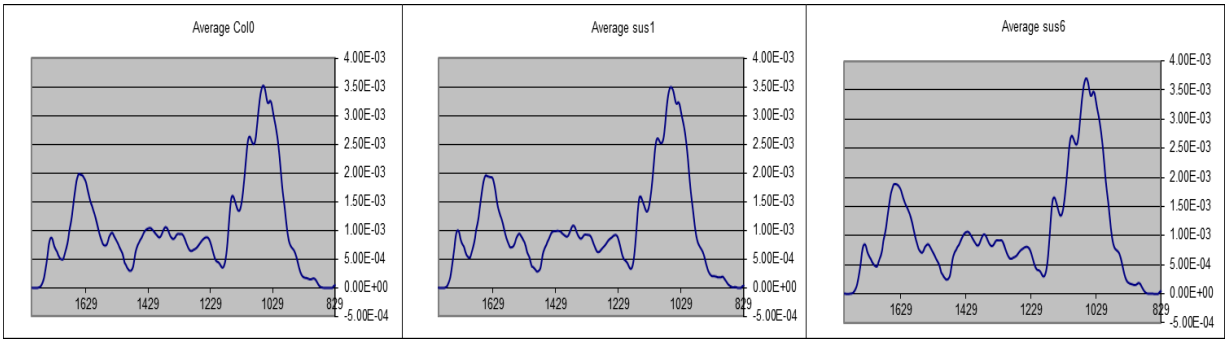


Figure 4: FT-IR microscopy on hypocotyl sections of wild type, sus1 and sus6 mutant. Data were averaged and referenced against spectra from an empty area of the disc. The X-axis is the frequency in wavenumbers (cm^{-1}) and y axis relative absorbance.

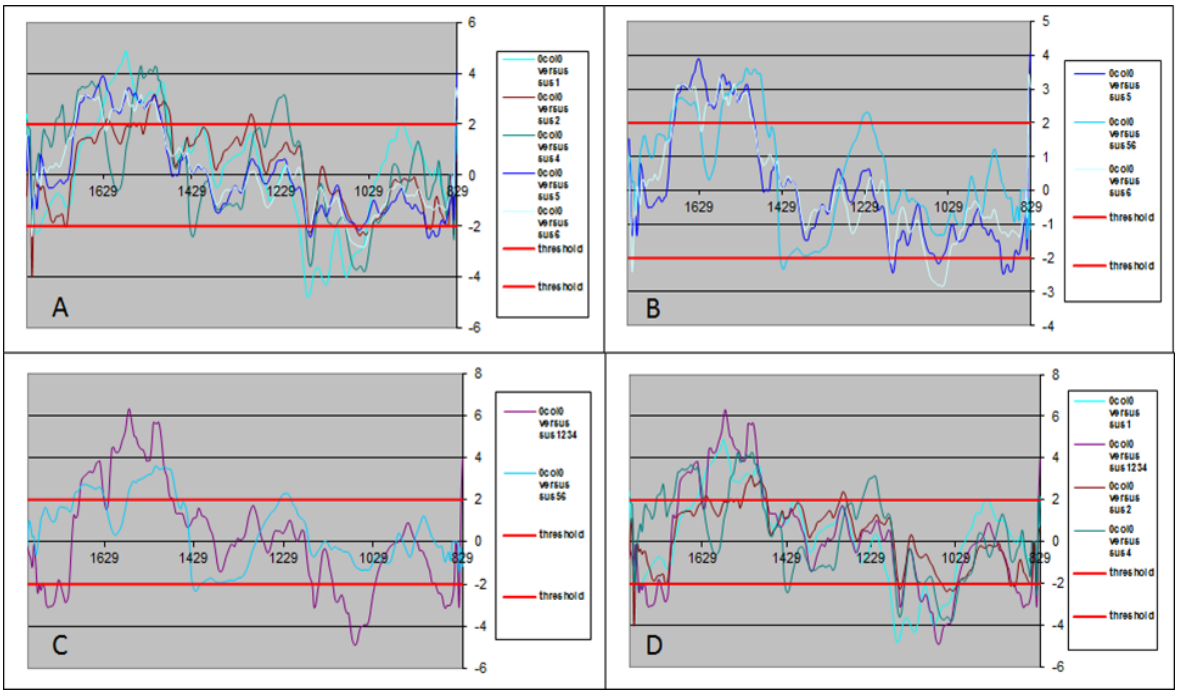


Figure 5: Student's t-test for changes in cell wall composition of *sus* knockout mutants.t-value for the comparison between the sucrose synthase single (*sus1, sus3, sus4, sus5* and *sus6*), double (*sus56*) and quadruple knockout mutants (*sus1234*) with Col-0 (y-axis) is plotted against the wave numbers (x-axis). Horizontal lines refer to the P=0.95 significance threshold. (A) Student's t-test: t-value for the comparison between the sucrose synthase single (*sus1, sus3, sus4, sus5* and *sus6*) and Col-0. (B) Student's t-test: t-value for the comparison between the sucrose synthase single (*sus5, sus6*) double (*sus56*) with Col-0 (y-axis) is plotted against the wave numbers (x-axis). (C) Student's t-test: t-value for the comparison between the sucrose synthase double (*sus5/6*) and quadruple knockout mutants (*sus1/2/3/4*) with Col-0 (y-axis) is plotted against the wave numbers (x-axis). (D) Student's t-test: t-value for the comparison between the sucrose synthase single (*sus1, sus2, sus4*) and quadruple knockout mutants (*sus1/2/3/4*) with Col0 (y-axis) is plotted against the wave numbers (x-axis).

Discussion

Specific expression and redundancy of SUS

Research carried out in various plants has shown that the level of *SUS* expression is strongly associated with cellulose production (Geisler-Lee et al., 2006) and this association may be isoform specific. An increase in cellulose production has been detected in cells expressing a modified mung bean isoform of sucrose synthase in the *Acetobacter xylinum* by preventing the accumulation of UDP, which inhibits cellulose production in the bacteria (Nakai et al., 1999; Coleman et al., 2009). A decrease in sucrose synthase activity has also resulted in cellulose deficiency in plants. Two SUS isoforms (Susy*Dc1 and Susy*Dc2) were identified in carrot showing different expression patterns, while *Susy*Dc2* was exclusively expressed in flowers, *Susy*Dc1*was expressed in stems, roots, flower buds, flowers, and maturing seeds (Sturm et al., 1999) with the highest level of expression found to be in strongly used sinks for sucrose, such as growing stems and tap root tips (Sturm et al., 1999). Down- regulation of *Susy*Dc1* expression by antisense mRNA resulted in decreased cellulose content and much

smaller transgenic carrot plants (Tang and Sturm 1999). Similarly in maize which is encoded by three distinct *SUS* (*SH1, SUS1, and SUS3*) genes (Koch et al., 1996; Carlson et al., 2002). Out of the two biochemically similar SUS isoforms (SH1 and SUS1), the SUS1 isoform plays the dominant role in providing the substrate for cellulose biosynthesis, whereas the SUS3 isoform is needed mainly for generating precursors for starch biosynthesis (Chourey et al., 1998). Evidence indicating that SUS is highly enriched near the plasma membrane in tracheary elements undergoing vigorous secondary cell wall thickenings has also been shown (Salnikov et al. 2001, Salnikov et al. 2003) again further supporting a role for sucrose synthase in cell wall biosynthesis. Studies investigating gene expression patterns in poplar have identified SUS as being associated with cellulose biosynthesis and affecting the formation of tension wood where increased cellulose deposition occurs (Hertzberg et al., 2001; Andersson-Gunneras et al., 2006; Coleman et al., 2009). Overexpression of the *Gossypium hirsutum* sucrose synthase (*SUS*) gene in hybrid poplar (Populus alba × grandidentata) showed increased deposition of cellulose in the secondary cell wall (thicker xylem) and cell wall crystallinity (Coleman et al., 2009). The association of sucrose synthase with cellulose formation has also been demonstrated in cotton as suppression of the expression of a SUS isoform (SUS A) in transgenic cotton ovules decreased fibre elongation (Ruan et al., 2003) and affected cellulose deposition (Ruan, 2007). Recently, another sucrose synthase isoform (SUS C) has been detected and found in abundance in cotton fibre during secondary cell wall biosynthesis indicating an essential role for sucrose synthase in providing UDP-glucose for cellulose biosynthesis (Brill et al., 2011).

Surprisingly, silencing of several *SUS* genes, including a quadruple knockout in Arabidopsis showed no obvious effect on plant growth (Bieniawska et al., 2007; Barratt et al., 2000) except for a double knockout of *sus1/sus4* which only showed a phenotype of growth retardation and accumulation of sugars when roots are subjected to hypoxia showed (Baud et al., 2004) with the final conclusion being that Sucrose synthase activity measured at pH 9.5 in the sucrose synthetic direction in Arabidopsis leaves is too low to account for cellulose biosynthesis thus sucrose synthase is not essential for normal growth and probably does not play a role in cellulose deposition in Arabidopsis (Barratt et al., 2009). However, a recent study has refuted these claims by pointing out that the measurements of SUS activity was carried out in conditions far from optimal and in the sucrose synthetic direction (PH=9) rather than sucrose cleavage (PH=7). After measuring SUS activity in the sucrose breakdown (UDPG synthesis) direction (PH=7) in the leaves of WT, the double knockout mutants (*sus5/6*) and quadruple mutant plants (*sus1/2/3/4*), it was shown that SUS activity measured in the sucrose cleavage pathway in wild type leaves was nearly10-fold higher than what was previously reported (Baroja-Fernández et al., 2011). At the transcriptomic level *SUS5* and *SUS6* expression levels in quadruple mutant leaves, and *SUS1-4* expression levels in *sus5/6* double mutant leaves were comparable to those of the WT (Baroja-Fernández et al., 2011). Most importantly, SUS activity in the leaves and stems of both *sus1/2/3/4* and *sus5/6* mutants was found to be nearly 85% of that occurring in WT leaves showing that SUS activity in the quadruple and *sus5/6* double mutants is sufficient to support normal cellulose biosynthesis and exceeds the minimum required to support normal cellulose biosynthesis in stems. Furthermore, as neither the quadruple mutant (*sus1/2/3/4*) nor the double mutant (*sus5/6*)

showed differences in cellulose content or cell wall structure in stems when compared with wild-type Arabidopsis thaliana plants (Barratt et al. 2009) except for *sus1/4* double knockout phenotype under hypoxia, the idea that *SUS* genes may be able to partially take over the role of other sucrose synthase isoforms could be possible. Since, there are so many sucrose synthase isoforms in Arabidopsis, redundancy between the SUS isoforms and an overlap in function between the *SUS* genes with some being able to take over in the absence of the others could be envisaged.

By using FT-IR microspectroscopy - a non-invasive, non-destructive method that provides information about structure and bonding of the organic molecules analysed (McCann et al. 1997). We showed that there was no significant difference between most of the single and double mutants with the wild-type plants in the vasculature and surrounding regions nor were changes in other polysaccharide compositions seen. However, the changes that were detected in the single mutants of *sus1*, *sus4* and the quadruple mutant(*sus1/2/3/4*) by showing increase in polysaccharide content confirms the results reported previously of the double knockout mutant of *sus1/4* showing increased sugar content and impaired growth under hypoxia. The cellulose content in the mutants was measured and was shown not to have increased (Barrat et al., 2009). Our FT-IR analysis was carried out on 4 day old seedlings which would detect changes occurring in the polysaccharide content of the knockout mutants. Nonetheless, further analysis would be required to know which polysaccharide has been increased. The fact that an obvious phenotype was not detected in the single knockouts of *sus1* and *sus4* could be due to the fact that the changes in carbohydrate content was not drastically different or easily detectable and it required a double knockout of the mentioned mutant for a phenotype to be detected. It has been shown that SUS1 and SUS4 are together necessary for tolerance of hypoxic conditions and that increases in transcript levels of *SUS1* and *SUS4* were detected in both single and double mutants with the single k.o. mutants showing less increase than the double probably due to redundancy between the isoforms (Baud et al., 2004; Bieniawska et al., 2007; Barratt et al. 2009).

Specific interactions between CESA and SUS

SUS proteins have been suggested to play a direct role in cellulose biosynthesis. Through mass spectrometry and immunoblotting the catalytic unit of CESA was found to be enriched with Sucrose synthase, thus was suggested that SUS is an integral component of the cellulose synthase rosette in bean hypocotyls (Fujii et al., 2010). Despite efforts to show this association, direct interaction between the SUS isoforms and CESAs had never been demonstrated before. Using the split-ubiquitin assay and BiFC, we show that specific SUS isoforms can interact with CESAs in the Arabidopsis primary and secondary cell walls, both *in vivo* and *in planta*.

Both the *in vivo* and *in planta* interaction data presented here show that most sucrose synthases are physically associated with the rosette complex. The fact that SUS1, SUS3, SUS4 and SUS6 all show interaction with the CESAs indicate possible redundancy between the SUS isoforms and overlap in function. It would be interesting to see whether a pentuple knockout mutant of *sus1/2/3/4/6* would result in any obvious changes in cellulose deposition

or cell wall structure. Despite the redundancy seen, the fact that no interaction is detected with SUS5 probably depicts specificity in relation to function or localization between the Arabidopsis sucrose synthase isoforms which has been suggested in various crop plants.

Visualization of GFP-SUS6 in presence of Td-tomato-CESA6

The biosynthesis of cellulose in cell wall construction is a complex process. The plasma membrane bound rosette complex is assembled in the Golgi apparatus (Haigler and Brown Jr, 1986). These rosette complexes composed of CESAs are subsequently transported through the cytoplasm from the Golgi to the plasma membrane where they are activated for cellulose synthesis by using UDP-glucose as a substrate so the SUS isoforms would have to provide the substrate for cellulose biosynthesis in these subcellular locations and most probably at the plasma membrane.

Our subcellular localization studies further confirmed that SUS are found in the same locations as cellulose synthases, as the localization is most probably in the plasma membrane it is the plasma membrane associated form of SUS that provides the UDP-glucose and has a role in cellulose biosynthesis. As the subcellular localization experiments were carried out through transient expression of the genes, the signal was weak thus the production and analysis of stable transformants will provide a better tool to further explore the localisation of sucrose synthases and their relationship with the CESAs and more importantly cellulose in the cell wall.

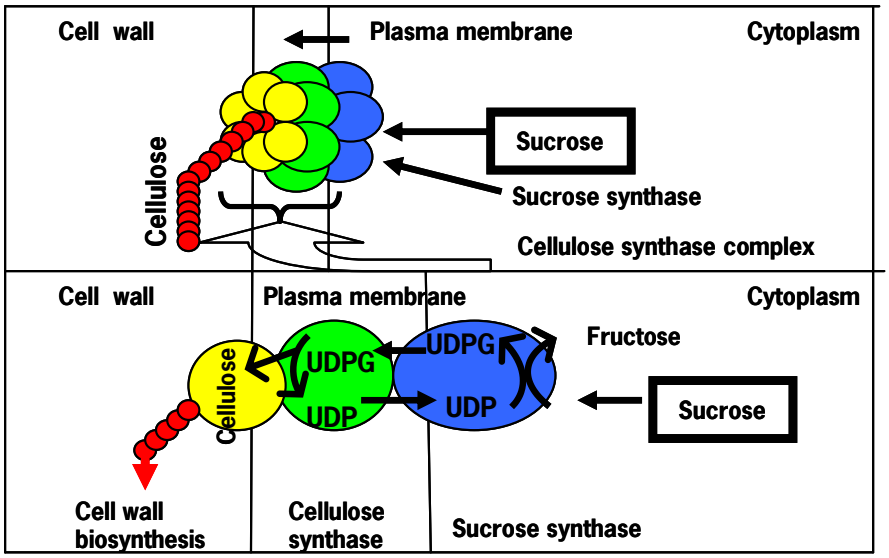
Several lines of evidence indicate that sucrose synthase has both soluble and membrane-associated forms (Amor et al, 1995; Carlson and Chourey, 1996; Winter et al., 1997). Contradicting evidence has proposed that phosphorylated or dephosphorilation causes the association or release of SUS from the membrane resulting in a soluble form of sucrose synthase however the exact role of sucrose synthase phosphorylation/ dephosphorilation in this association remains elusive. If phosphorylation were to be a determining factor for its association with the membrane, by analysing the phosphorylation sites present in the Arabidopsis sucrose synthase isoforms it is apparent that SUS5 does not follow the common pattern of phosphorylation as it does not contain the same phosphorylation sites as the other isoforms which could be an indication for its lack of interaction with the CESAs (see General Discussion for more details).

We have shown based on our interaction studies and colocalization experiments with the CESAs that sucrose synthase does associate with cellulose synthase complex most probably at the plasma membrane and the Golgi. However the question remains whether sucrose synthase is the sole provider of the UDP-glucose for cellulose biosynthesis or is any other enzymes responsible for this provision as well. It would be interesting to see how the plant would react in the complete absence of sucrose synthase isoforms, would the complete knockout of all the sucrose synthase isoforms result in a collapsed cell wall, would the it lead to cell death and prove lethal for the plant or would other enzymes be able to compensate for the loss of the sucrose synthases? The entry of sucrose into distinct biochemical pathways is regulated through its hydrolysis by the invertase enzymes as well as sucrose synthase. Invertase, which cleaves sucrose into glucose and fructose, can indirectly lead to the synthesis of UDP-glucose

as it would require an extra ATP to produce UDP-Gl for cellulose or callose biosynthesis. Thus sucrose synthase (SUS) is regarded as the more energy conservative of the two enzyme reactions. Nevertheless, it has been shown that loss of two closely related, cytosolic invertase isoforms in Arabidopsis show dramatically retarded growth and extreme reduction in root growth also suggesting that the roots resemble cell-wall biosynthesis defective mutants, however a stronger phenotype is detected at higher sugar concentrations in cell wall mutants, whereas growth of *cinv1/cinv2* on glucose restored root extension in double mutants to approximately half of WT showing the opposite effect (Barratt et al., 2009). This phenotype could be due to a lack of substrate for cell-wall synthesis or it might be a consequence of a strong inhibition of root growth alone. Thus, at present, the role of invertase, if any, in modulating cellulose production remains unclear and more in depth analysis would be required to examine its role.

In conclusion we have demonstrated a direct association between sucrose synthase isoforms and cellulose synthases in Arabidopsis suggesting a role in cellulose biosynthesis. Undoubtedly further in depth analysis based on the production of stable transformant lines of GFP-SUS6 as well as the characterization of a complete knockout of the *SUS* genes would be noteworthy and necessary to know more about the involvement of Sucrose synthase in the sucrose–cellulose conversion processes in Arabidopsis.

Supplementary data



Supplementary Figure 1: Regulation of sucrose synthase through sub-cellular localization. A rapidly growing body of evidence supports an association between sucrose synthase and rosettes of the plasma membrane cellulose synthase complex, much as originally proposed by Delmer and co-workers (Amor et al., 1995). Adopted from Koch, 2004.

Supplementary Table1:

Gene	Primer for sucrose synthase prey amplification
Susy1NX	5'GTCGCCATTACGGCCATGGCAAACGCTGAACGTATGATAACGC3' 5'GAGAGGCCGAGGCGGCCGCAATCATCTTGTGCAAGAGGAACAGC3'
Susy3NX	5'GTGGCCATTACGGCCATGGCAAACCCTAAGCTCACTAGG3' 5'GAGAGGCCGAGGCGGCCGTCAGTCATCGGCGGTTGAAG3'
Susy4NX	5'AACAGGCCATTACGGCCATGTACGGAAGAGATCCATGGGG3' 5'AAGAGGCCGAGGCGGCCTTTAACGATCAAGGTAATGAA3'
Susy5NX	5'GTGGCCATTACGGCC ATGGAAATGACATCTGGATCGTTAGGCAATGGGATCCCAGAAGCGATGGGG3' 5'GAGGCCGAGGCGGCC TTAAGCACCAAACAACCTGAAACTCAATCGCGGCTGTGTTC3'
Susy6NX	5'GTGGCCATTACGGCC ATGTCATCTTCATCTCAAGCTATGCTTCAAAAGTCGGATTCCATCGCTGA3' 5'GAGGCCGAGGCGGCC TTAATACTCTTGAGCCGAGTTAGCACCAAAGAACCTCTG3'
Gene	Primer for sucrose synthase gateway compatible constructs
Susy1-pdonr207	5' GGGGACAAGTTTGTACAAAAAAGCAGGCTCCATGGCAAACGCTGAACGTATGATAACGCG 3' 5' GGGGACCACTTTGTACAAGAAAGCTGGGTCTCAATCATCTTGTGCAAGAGGAACAGCCTG 3'
Susy3-pdonr207	5' GGGGACAAGTTTGTACAAAAAAGCAGGCTCCATGGCAAACCCTAAGCTCACTAGGGTTCTAAG 3' 5' GGGGACCACTTTGTACAAGAAAGCTGGGTCTCAGTCATCGGCGGTTGAAGGAACAGTTTTCAC3'
Susy4-pdonr207	5' GGGGACAAGTTTGTACAAAAAAGCAGGCTCCATGGCAAACGCAGAACGTGTAATAACGCGAG 3' 5' GGGGACCACTTTGTACAAGAAAGCTGGGTCTCACTCTTCATGAGCAAGAGGAACAGCTTGAG3'
Susy5-pdonr207	5' GGGGACAAGTTTGTACAAAAAAGCAGGCTCCATGGAAATGACATCTGGATCGTTAGGCAATGG T3' 5' GGGGACCACTTTGTACAAGAAAGCTGGGTCTTAAGCACCAAACAACCTGAAACTCAATCGC3'
Susy6-pdonr207	5'GGGGACAAGTTTGTACAAAAAAGCAGGCTCC ATGTCATCTTCATCTCAAGCTATGCTTCAAAAGTCG3' 5'GGGGACCACTTTGTACAAGAAAGCTGGGTCTTAATACTCTTGAGCCGAGTTAGCACCAAAGAAC3'

Supplementary Figure 2:. Expression of the sucrose synthase isoforms in various tissues.

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Chapter 5

Identifying New Interactors with the Cellulose Synthase Protein Complex Involved in Cellulose Biosynthesis in the Primary Plant Cell Wall

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Abstract

Cellulose is one of the major components of the plant cell wall and is synthesized at the plasma membrane by a rosette complex. In Arabidopsis at least three cellulose synthases (CESAs) are required to form a functional rosette complex which comprise of CESA1, 3 and 6 in the primary cell wall and CESA4, 7 and 8 in the secondary cell wall. Although the CESAs are quite well characterized, it has been difficult to identify other proteins that are involved in cellulose biosynthesis and are part of the rosette complex. In this study, a protein interaction approach was used to identify other protein members of the rosette complex. A library screen was performed with the membrane-based yeast two-hybrid system using each of the three different CESA proteins as bait (CESA 1, 3 and 6). This resulted in a list of candidates, some of which showed interaction with two or the three CESA proteins. The list consists of proteins known to be involved in cell wall metabolism, such as the endo-chitinase-like gene *CTL1*, whereas some other proteins had no previous link to cellulose biosynthesis and are promising targets for future research as being part of the rosette complex.

Introduction

The thin primary cell wall facilitates the growth, adaptability and development of the plant cell wall and is composed of cellulose microfibrils that are cross-linked to hemicellulosic polysaccharides (mainly xyloglucans) as well as pectin polysaccharides which is a source of cell signalling for defence and controls porosity. In addition, primary cell walls contain structural (cross linking and wall loosening) and enzymatic proteins (Carpita and Gibeaut, 1993) all involved in the maintenance of the mechanical properties of the wall.

Crucial phases during cell wall development are the expansion, division and extension of the cell. The cellulose–hemicellulose network plays a leading role in determining the extensibility of cell walls, and enzymes such as xyloglucan endotransglucosylase/hydrolases (Fry et al., 1992; Nishitani et al., 1992) and expansins (McQueen Mason et al., 1992) act on this network and control the process of cell growth. During cell division, the biosynthesis of cellulose occurs through the assembly of cellulose microfibrils with microfibrils cross linking the hemicelluloses synthesized in the Golgi apparatus at practically the same time (McCann and Roberts, 1991; Carpita and Gibeaut, 1993; Lima et al., 2001). After division, plant cells continue to grow by extension and this process also involves cellulose microfibrils since important changes in the mechanical properties of the wall have to be performed in order to allow elongation in a specific direction (Cosgrove, 2000; Whitney et al., 1995; Lima et al., 2001). In order to stop growth, cross links between polymers can be made and this is thought to be performed by phenolic compounds and structural proteins such as extensins (Fry et al., 1988). The formation and growth of the cell wall is not an independent synthesis of each of the preserved carbohydrates, but instead a symphony between different enzymes with molecular interactions of cellulose with pectins crosslinking with hemicelluloses (Jarvis, 1992; Sarkar et al., 2009). The structural assembly and preservation of the complex cell wall architecture involves the collaboration of many plant cell wall related genes. In Arabidopsis, several dozen of these gene families have been identified in the genome database, each family consisting of 30 to one-hundred members (Carpita and MacCann, 2000).

Cellulose is synthesised in the primary cell wall by a rosette complex composed of at least three different CESA proteins (CESA1, CESA3 and CESA6). Hexameric rosette complexes containing CESA proteins and their binding partners are found in the plasma membrane and in intracellular organelles giving us insight into the localisation of the CESAs and their potential interactors .The CESAs are retained in the endoplasmic reticulum (ER) until they are assembled into complexes (Gardiner, 2003). When the complex is assembled, the cellulose synthase complex traffic from the ER presumably to the Golgi secretory pathway and are subsequently transported from the Golgi via cytoplasmic vesicles to the plasma membrane, where they are activated for cellulose synthesis (Haigler and Brown, 1986; Gardiner, 2003). The Golgi bodies pause on cortical microtubules during the delivery of cellulose synthase complex to the plasma membrane which regulated by major cytoskeletal components in higher plants, actin filaments and cortical microtubules (Crowell et al., 2009; Guitierrez et al., 2009). Cortical microtubules undeniably play the leading role in regulating the dynamics of

cellulose synthase complexes, by targeting their secretion through interactions with the Golgi apparatus (Crowell, 2009) and guiding their movement through the plasma membrane (Paradez, 2006).

Although these three CESA's are essential, alone they are not able to produce cellulose *in vitro*. Cellulose synthesis, maintenance, modification and degradation require various other proteins as well as the CESAs (Carpita et al., 2001) some of which belong to multigene families (Henrissat et al., 2001). Despite belonging to large families consisting of many genes, the individual members detected somewhere in the cellulose synthesis pathway often have distinct patterns of expression among plant cells and tissues (Taylor et al., 1999, 2000; Fagard et al., 2000; Peng et al., 2000; Sarria et al., 2001; Burton et al., 2006; Persson et al., 2007).

Except for cellulose which is synthesized at the plasma membrane, other cell wall polysaccharide biosynthesis enzymes are located in the cytoplasm, the Golgi and other parts of the cell and are synthesized in cytosolic compartments and subsequently transferred to the cell wall. Nevertheless, it is obvious that these enzymes have a direct impact on the events taking place in the cell wall and by cross linking with the cellulose microfibrils they affect cellulose biosynthesis.

Many genes that are co-expressed with the primary and secondary wall *CESA* genes are thought to affect cellulose production (Brown et al., 2005; Persson et al., 2005). Reverse genetics have uncovered some proteins that contribute to the production of cellulose through interactions with cellulose synthase complex. Proteins like sucrose synthase (SUS), β-(1,4)-glucanase KORRIGAN (KOR1), endo-chitinase-like protein (CTL1/POM1), COBRA or COBRA-like proteins (COB), kobito (KOB1), cytoskeleton-related proteins like tubulins (Gutierrez et al., 2009), Cellulose Synthase-Interactive protein 1 CSL1 (Nicol et al., 1998*;* Pagant et al., 2002*;* Roudier et al., 2005*;* Song et al., 2010*;* Gu et al., 2010) have all been suggested to play a role in cellulose biosynthesis. Some of these proteins are linked to the cellulose machinery due to a reduced cellulose content in the relative mutants (Lane et al., 2001*;* Schindelman et al., 2001; Sato et al., 2010*;* Zhong et al., 2002; Gu, 2010; Pagant et al., 2002). Although these mutants have an effect on the cellulose synthesis, it is unknown whether the corresponding proteins are an integral part of the cellulose synthesizing machinery or periodically interact with the CESAs. Based on the current understanding of cellulose synthesis their role in influencing cellulose biosynthesis could either be direct or indirect. Thus far the specific function of most of these proteins during cellulose deposition remains elusive.

To characterize the protein complexes involved in cellulose biosynthesis, a yeast two-hybrid library screen was performed to identify proteins that physically interact with each of the primary CESAs (#1, #3, and #6). Interaction with constitutive components of the rosette structure, the CESA proteins, might be an indication that a particular protein is involved in, or its activity is coordinated with cellulose biosynthesis. The membrane-based yeast two-hybrid system (MbYTH) was used to determine the interaction between the cell wall CESA proteins (Timmers et al., 2009) and has proved to be a valuable tool to analyse interactions between

CESAs. In this study, we extend this approach by using CESAs as baits to identify other constituents of the rosette complex.

Materials and Methods

Constructs for the Membrane based Yeast two Hybrid library screen

The full-length *A. thaliana* cDNAs were obtained from the Riken Bioresource Center (Seki et al., 1998; Seki et al., 2002) AtCESA1 (RAFL09-89-G08), AtCESA3 (RAFL05-19-M03), and AtCESA6 (RAFL05-02-P19). The cDNA's of the CESA genes were amplified by PCR using the Phusion DNA Polymerase (Finnzymes, Helsinki, Finland) with suitable primers (Timmers et al., 2009; Supplementary Table 3). The bait protein was fused N-terminally to the Cub-TF reporter cassette of the (Bait) vector pTFB (Dualsystems Biotech AG). The bait expression is regulated by the TEF1 promoter. The sequences of the inserts were obtained by Sanger sequence analysis. The library, a NubG Arabidopsis cDNA-library (Dualsystems P02210), was constructed from six-day-old seedlings with a mixture of dark grown (etiolated) seedlings and seedlings exposed to blue and far red light. The library consists of 1.7*107 independent clones with an average insert size of 1.7 kb (ranges from 1.2-2.5kb).

Membrane based Yeast two Hybrid screen

The yeast strain NMY51 (Dualsystems Biotech AG) was transformed according to the protocol (DUAL membrane Kit 1). Yeast containing the bait plasmids were transformed with the *Arabidopsis thaliana* library plasmids on SD medium (lacking leucine, tryptophan, and histidine) containing the appropriate concentration of 3-ammonium-triazole for CESA1, CESA3, and CESA6 baits. After five days of growth at 30ºC, the grown colonies were scored. Detection of β-galactosidase activity was performed with the filter-lift assay (Breeden and Nasmyth, 1985). The interactors were identified by PCR, Sanger sequence analysis and consecutively a blast search on the NCBI website (Altschul et al., 1997). With all yeast two hybrid library screens, there is a possibility of detecting a subset of common false positives representing nonspecific DNA-binding proteins that are repeatedly found in different screens interacting with unrelated bait proteins and should be disconsidered. The list of known auto-activators can be found on the Dual membrane website (*www.Dualsystems.com*).

Results

Identifying candidate proteins interacting with the cellulose synthesizing complex

In order to find other members of the primary CESA complex, each of the primary CESA proteins was used as bait to screen a plasmid library, which expressed full length cDNAs of Arabidopsis as preys. Only colonies able to grow in the absence of the four auxotrophic markers, and able to activate the *LacZ* gene, were considered as potential interactors with the CESA complex. The preys expressed in these colonies were identified using colony-PCR and subsequent sequence analysis. The corresponding genes were identified by highest similarity to genes of the *A. thaliana* genome available at the NCBI database (Altschul *et al*., 1997).

This resulted in a large list of proteins with 100, 264 and 678 colonies detected interacting with CESA1, CESA3 and CESA6 respectively. Out of the many colonies picked a total number of 20, 80 and 200 of those proteins were found interacting more than once with CESA1, 3 and 6 respectively. After eliminating the repeats, the list of genes resulted in 80 proteins interacting with CESA1, 114 with CESA3 and 250 with CESA6 while some showed interaction with two or all three of the CESAs (Figure 1). The list of interactors contained proteins with diverse functions, which were divided into 10 groups based on sub cellular localization (Figure 1A) and in 11 groups based on the predicted function or metabolic pathway involved (Figure 1B).

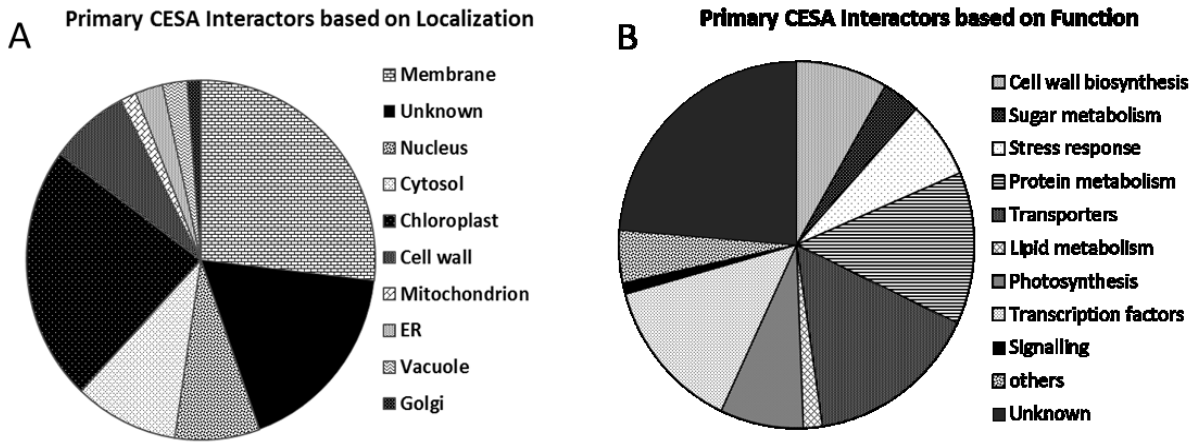

Figure1: Grouping of all the interactors with CESA1, CESA3 and CESA6 based on localization in the cell (A) or protein function (B).

The encoded interactors were distributed into various classes most importantly cell wall metabolism, protein metabolism, sugar metabolism, stress response, signalling, transcription factors, transporter proteins and proteins of unknown function with transporter proteins, those involved in protein metabolism and proteins of unknown function constituting the most abundant classes. The size limitation of the cDNA library, which is restricted to 2.5kb, most probably explains the absence of the CESA proteins, as they are 3kb in size. The absence of various other proteins known to interact with the CESAs such as KOR1 in turn suggests that the screen is not saturated or that KOR1 is not present in the library.

Biological relevance of the protein interactions

In spite of the power of MbYTH to characterize protein-protein interactions, as in all heterologous analyses systems, the biological relevance of the identified interactors requires further evaluation. One of the drawbacks of this system is the false positives generated by auto-activation. These false positives have been identified (see M&M) and consequently discarded resulting in a smaller list of candidate genes (Supplementary Table 1). Thirty proteins were related to auto-activation of the system and were subsequently discarded from the list.

Taking the CESA rosette complex pathway into consideration for possible interactors involved in cellulose biosynthesis, proteins which do not reside in the Golgi, ER, cytosol, plasma membrane or cell wall, are therefore considered as unlikely candidates for the primary CESA complex. Using this criteria and discarding the candidates with unknown localisation, the number of candidate genes decreased from 395 (Figure 1) to 183 (Figure 2A; Supplementary Table 2), as the original list of proteins contained several chloroplast, mitochondria, vacuolar proteins as well as proteins which were solely found in the nucleus. The biologically relevant interactors were also distributed into classes of cell wall biosynthesis, protein metabolism, sugar metabolism, stress response, signalling, transporter proteins, proteins of unknown function as well as another group of proteins classified as others consisting of structural proteins. Similarly, Transporter proteins, those involved in cell wall biosynthesis and proteins of unknown function constituted the most abundant classes.(Figure 2B).

The involvement in cell wall biosynthesis has been reported for several of the interacting proteins. Some of them can be directly linked to cellulose biosynthesis based on their enzymatic function (Table 1). Interestingly, not all CESA isoforms had a similar number of interactors. Many proteins were found more than once (Supplementary Table 1 and 2), some were found interacting with two different CESA isoforms. A total of 5 proteins consisting of xyloglucan endotransglucosylase/hydrolases (XTH15 and XTH16) and defence response proteins (a peroxidase protein and PBP1) as well as a sterol methyltransferase protein showed interaction with all three CESA proteins. Only 2 interacted with both CESA1and CESA3 while a total of 8 proteins interacted with both CESA1 and CESA6 and 16 proteins were found to only interact with both CESA3 and CESA6 (Figure 3; Table 1).

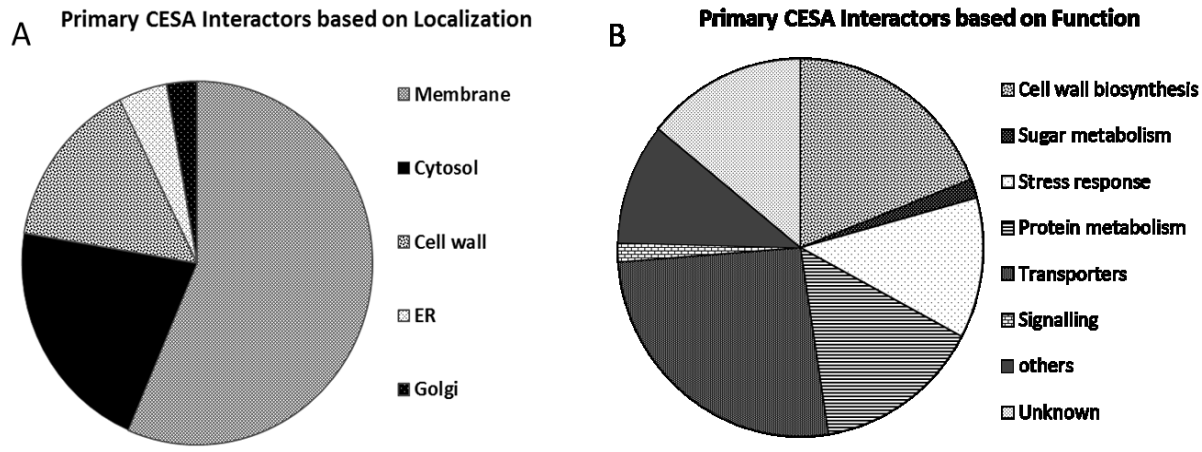

Figure 2: Grouping of the biologically relevant interactors with CESA1, CESA3 and CESA6 based on localization (A) and protein function (B) in the cell.

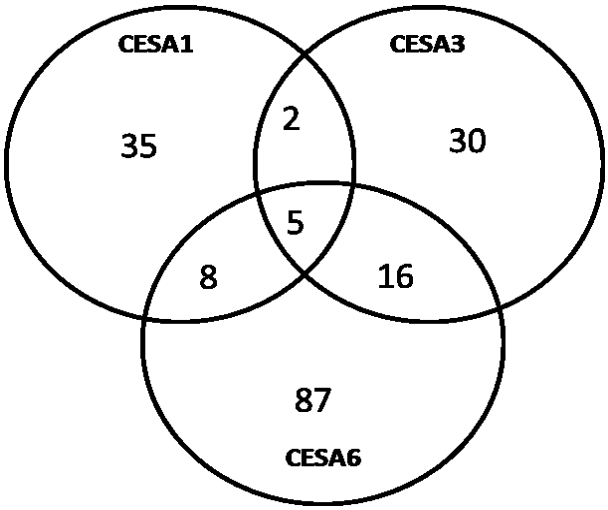

Figure3: Number of biologically relevant candidates found interacting with the different CESA proteins as bait**.**

Table 1: List of relevant interactors thought to have a role in cell wall biosynthesis and candidates that show interaction with two or more primary CESA proteins.

Group	Locus	Name	Localisation	Function	Family/Function/Activity	cesainteractors(# interactions)
cell wall biosynthesis	*AT1G01800*		Cell wall	NAD(P)-binding Rossmann-fold superfamily protein	Protein binding/ oxidoreductase activity	6(5)
	AT1G47960	AtC/VIF1	Cell wall	cell wall / vacuolar inhibitor of fructosidase 1		6
	AT2G06850	AtXTH4	Cell wall	Xyloglucanendotransglucosylase/hydrolase 4	GH family 16	1,6(10)
	AT3G45970	AtEXLA1	Cell wall	Expansin-like A1	Plant cell wall loosening	6
	AT3G54590	AtHRGP1	Cell wall	Hydroxyproline-rich glycoprotein	constituent of cell wall	6(4)
	AT4G14130	AtXTH15	Cell wall	Xyloglucanendotransglucosylase/hydrolase 15	GH family 16	1,3(6),6(12)
	AT1G05850	AtPOM1/CTL1	Endomembrane	Chitinase family protei/lignin biosynthesis	GH family 19	3(3),6
	AT4G25260		Endomembrane	Plant invertase/pectin methylesterase inhibitor	Pectin metabolism	6(2)
	AT5G13870	AtXTH5	Cell wall	Xyloglucanendotransglucosylase/hydrolase 5	GlyTransferase family 16	6(2)
	AT5G48100		Cell wall	Methylesterase/invertase inhibitors	Pectin metabolism	3
	AT1G32170	AtXTH30	Cell wall	Xyloglucanendotransglucosylase/hydrolase 30	GH family 16	3(2),6(6)
	AT2G28950	*AtEXP6*	Cell wall,	Expansin A6	Plant cell wall loosening	1
	AT3G23730	AtXTH16	*Cell wall,*	Xyloglucanendotransglucosylase/hydrolase16	GH family 16	1,3(2),6(6)
	AT1G21310	AtEXT3	Cell wall/endomembrane	Extensin 3	Structural constituent of cell wall	6

	AT2G42840	AtPDF1	Cell-wall	Protodermal factor 1	cell-wall protein	1
	AT4G24220	AtAWI31	Cytosol	NAD(P)-binding Rossmann-fold superfamily protein,VEIN PATTERNING 1	Xylem and phloem pattern formation	6(4)
	AT2G27370	AtUPF0497	Plasma membrane	Uncharacterised protein family	*Cell wall modification/CASP3*	3
	AT4G30270	AtXTH24	Plasma membrane	Xyloglucanendotransglucosylase/hydrolase 24	GH family 16	1,6(6)
	AT1G11820		Endomembrane	Hydrolase activity, hydrolyzing O-glycosyl compounds	O-Glycosyl hydrolases family 17 protein	6
	AT3G19820	AtDWARF1	Plasma membrane / vacuole	cell elongation protein	Secondary cell wall biogenesis/lignin metabolic process	6(2)
	AT1G29050	AtTBL38	Endomembrane	Trichome birefringence-like 38	Other members have shown to be involved in the synthesis and deposition of secondary wall cellulose	3,6
	AT1G76930	AtEXT4	Endomembrane	Extensin 4	Structural constituent of cell wall	6
	AT1G18580	AtGAUT11	Golgi	Galacturonosyltransferase 11	GT family 8	6(9)
	AT3G61130	AtGAUT1	Golgi	Galacturonosyltransferase 1/pectin synthase	GT family 9	1,6(15)
	AT5G03760	*AtCSLA9*	Golgi	Cellulose synthase like A9	Mannan synthase activity	1
	AT1G12780	AtUGE1	Cytosol/plasma membrane	UDP-D-glucose/UDP-D-galactose 4-epimerase 1		3(2),6(2)
	AT3G46440	AtUXS5	*Cytosol*	UDP-XYL synthase 5	Substrate for many cell wall carbohydrates including hemicellulose and pectin	3
	AT5G59290	ATUXS3	*Cytosol*	UDP-glucuronic acid decarboxylase 3	Methylesterase inhibitor (PMEI)	3(5)
	AT5G48900		Endomembrane	putative pectatelyase 20	GH family 28	1
	AT4G23820		Endomembrane	Pectin lyase-like superfamily protein	Unknown	6(2)
	AT5G15780		Endomembrane	Pollen Ole e 1 allergen and extensin family protein		6
	AT5G26000	AtBGLU38	Cell wall	thioglucosideglucohydrolase 1	Extensin family	6
	AT3G09260	AtBGLU23,	ER , membrane, ribosome	beta-glucosidase 23	Actin cytoskeleton organization	3(3),6
	AT1G71790		Cytoplasm	(actin filament)capping protein Capz superfamily	Protein binding	6(2)
	AT1G77760	AtNR1	Cytosol	Nitrate reductase	Lignin toolbox	1
Protein metabolism	*AT4G26570*	AtCBL3	Membrane	Calcineurin B-like 3	Protein binding	1,6
	AT5G58060	AtGP1	cytosol	SNARE-like superfamily protein	Intracellular protein transport/nucleotide binding	3(2),6(2)
	AT1G01620	AtPIP1C	Plasma membrane	Plasma membrane intrinsic protein 1C	Vesicle mediated transport	3(9),6(24)
	AT1G04750	AtVAMP7B	Plasma membrane	Vesicle-associated membrane protein 721	Vesicle mediated transport	6
	AT3G53420	AtPIP2A	Plasma membrane	Plasma membrane intrinsic protein 2A	Water channel activity	3,6
	AT3G61430	AtPIP1A	Plasma membrane	Plasma membrane intrinsic protein 1A	Intracellular protein transport	3(4),6(2)

Transporters	*AT4G17170*	AtRAB2	Plasma membrane	RAB GTPase homolog B1C	Water channel activity	1,6(2)
	AT4G23400	AtPIP1D	Plasma membrane	Plasma membrane intrinsic protein 1;5	Water channel activity	3,6(2)
	AT4G35100	AtPIP3	Plasma membrane	Plasma membrane intrinsic protein 3	*Protein transport*	3,6(7)
	AT5G60660	AtPIP2F	Plasma membrane	Plasma membrane intrinsic protein 2;4	Protein trafficking	3,6
	AT2G33120	ATVAMP722	Membrane	Synaptobrevin-related protein 1	*Tansmembrane transport*	3
	AT2G39010	AtPIP2;6	Membrane	Plasma membrane intrinsic protein 2E	Tansmembrane transporter activity	3,6(8)
	AT2G40380	AtPRA1.B2	ER	Prenylated RAB acceptor 1.B2	Intracellular protein transport/nucleotide binding	1,6
Stress response	*AT1G30230*		Plasma membrane	Glutathione S-transferase	*Defence response*	1,6
	AT2G37130		cytosol	Peroxidase superfamily protein	*Defence response*	1,3,6(4)
	AT3G16420	AtPBP1	cytosol/nucleus	PYK10-binding protein 1	1-aminocyclopropane-1-carboxylate oxidase activity	1,3(10),6(11)
Unknown	*AT5G42860*		Plasma membrane			3,6(2)
	AT1G35660		Plasma membrane		*carbohydrate binding*	3,6
	AT2G36410	AtDUF662	Plasma membrane	Family of unknown function	Structural molecule activity	1,6
	AT1G76090	AtSMT3	ER	Sterol methyltransferase 3		1,3(2),6(2)
Others	*AT1G04820*	AtTUA4	Cell wall	Tubulin alpha-4 chain	Structural constituent of cytoskeleton	1,3
	AT5G26667	(CMP/UMP KINASE)	cytosol	Uridylate kinase activity	Phospholipase activator activity	1,3

The list of biologically relevant interactors resulted in a large number of candidates interacting with the primary CESAs (Supplementary Table2). In order to know more about the candidates , the GeneCAT co-expression tool (http://genecat.mpg.de; Mutwil et al., 2008) was used to compare expression patterns with the CESAs which resulted in some candidates such as POM1/CTL1 and Actin 2 showing similar expression patterns to the CESAs (Figure 4 A, B and C). The expression pattern of KORRIGAN which has already been shown to interact with the CESAs was also added for better clarity. Further analysis was carried out to show the degree of co-expression between the CESAs and the interacting proteins using the GeneCAT co-expression tool (http://genecat.mpg.de; Mutwil et al., 2008) based on Pearson Correlation Coefficient (r-value) between each of the genes which was turned into distance and resulted in a large list of genes in the expression tree (data not shown). The genes most closely co-expressed with the CESAs are displayed as interesting candidates for further study (Table 2; Figure 4D). KOR1 and the sucrose synthases which have already been shown to be interacting directly with the CESAs and are suggested to have a role in cellulose production were added to the list as a comparison in co-expression distance. Korrigan1 was clustered very close to the primary CESAs (Figure 4D) while sucrose synthases were clustered quite far away (data

not shown). Thus these closely related genes do not rule out the possibility of other candidates located further away in the co-expression dendogram interacting with the CESAs and having a role in cellulose biosynthesis.

Table 2: Biologically relevant candidates found through the membrane based yeast two hybrid library screen, closely co-expressed with the primary CESAs.

Group	Locus	Name	Localisation	Function	Family/ Function/ Activity
Cellwall biosynthesis	*AT2G06850*	AtXTH4	Cell wall	Xyloglucanendotransglucosylase/hydrolase 4	GH family 16
	AT3G19820	AtDWARF1	Plasma membrane / vacuole	cell elongation protein	Secondary cell wall biogenesis/lignin metabolic process
	AT2G28950	AtEXP6	Cell wall,	Expansin A6	Plant cell wall loosening
	AT5G48900		Endomembrane	putative pectatelyase 20	GH family 28
	AT1G29050	AtTBL38	Endomembrane	Trichome birefringence-like 38	Other members have shown to be involved in the synthesis and deposition of secondary wall cellulose
	AT4G23820		Endomembrane	Pectin lyase-like superfamily protein	Unknown
	AT1G05850	AtPOM1/CTL1	Endomembrane	Chitinase family protei/lignin biosynthesis	GH family 19
	AT4G30270	AtXTH24	Plasma membrane	Xyloglucanendotransglucosylase/hydrolase 24	GH family 16
Protein metabolism	*AT3G49670*	AtBAM2	Plasma membrane	Leucine-rich receptor-like protein kinase family protein	Protein phosphorilation
	AT2G41430	AtERD15	Cytoplasm	Dehydration-induced protein	
	AT5G55730	AtFLA1	Plasma membrane	Fasciclin-like arabinogalactan 1	
Signalling	*AT2G14890*	AtAGP9	membrane	Arabinogalactan protein 9	Lipid metabolism/oxidation-reduction process
Stress response	*AT2G31570*	AtGPX2	cytosol	Glutathione peroxidase 2	Glutathione peroxidase activity
	AT1G62380	AtACO2	cell wall/plasma membrane/ytosol	ACC oxidase 2	Oxidative strees response
Sugar metabolism	*AT3G08030*	AtDUF642	cell wall	Hypothetical protein	Response to brassinosteroid stimulus
Transporter	*AT1G70940*	ATPIN3	Membrane	Auxin efflux carrier family protein	Vesicle-mediated transport
	AT1G01620	AtPIP1C	Plasma membrane	Plasma membrane intrinsic protein 1C	Vesicle mediated transport
	AT4G35100	AtPIP3	Plasma membrane	Plasma membrane intrinsic protein 3	Protein transport
	AT4G23400	AtPIP1D	Plasma membrane	Plasma membrane intrinsic protein 1;5	Water channel activity
	AT2G37170	AtPIP2B	plasma mmbrane	Plasma membrane intrinsic protein 2	Transmembranetrasnport

	AT2G45960	AtPIP1;2	Plasma membrane	Plasma membrane intrinsic protein 1B	Water channel activity
	AT3G53420	AtPIP2A	Plasma membrane	Plasma membrane intrinsic protein 2A	Water channel activity
	AT3G61430	AtPIP1A	Plasma membrane	Plasma membrane intrinsic protein 1A	Intracellular protein transport
	AT4G35750	SEC14	cytosol	SEC14 cytosolic factor family protein / Phosphoglyceride transfer family protein	
	AT3G45600	TET3	Endomembrane	Tetraspanin3	Sugar transmembrane transporter activity
Unknown	*AT3G27390*		Plasma membrane	Hypothetical protein	
Others	*AT4G21450*		Plasma membrane	vesicle-associated membrane family protein	Glycine hydroxymethyltransferase activity
	AT3G18780	actin 2	Membrane	Structural constituent of cytoskeleton	cell wall biogenesis
	AT5G23860	AtTUB8	Membrane	Tubulin beta 8	Structural constituent of cytoskeleton
	AT5G12250	AtTUB6	Membrane	Beta-6 tubulin	Structural constituent of cytoskeleton

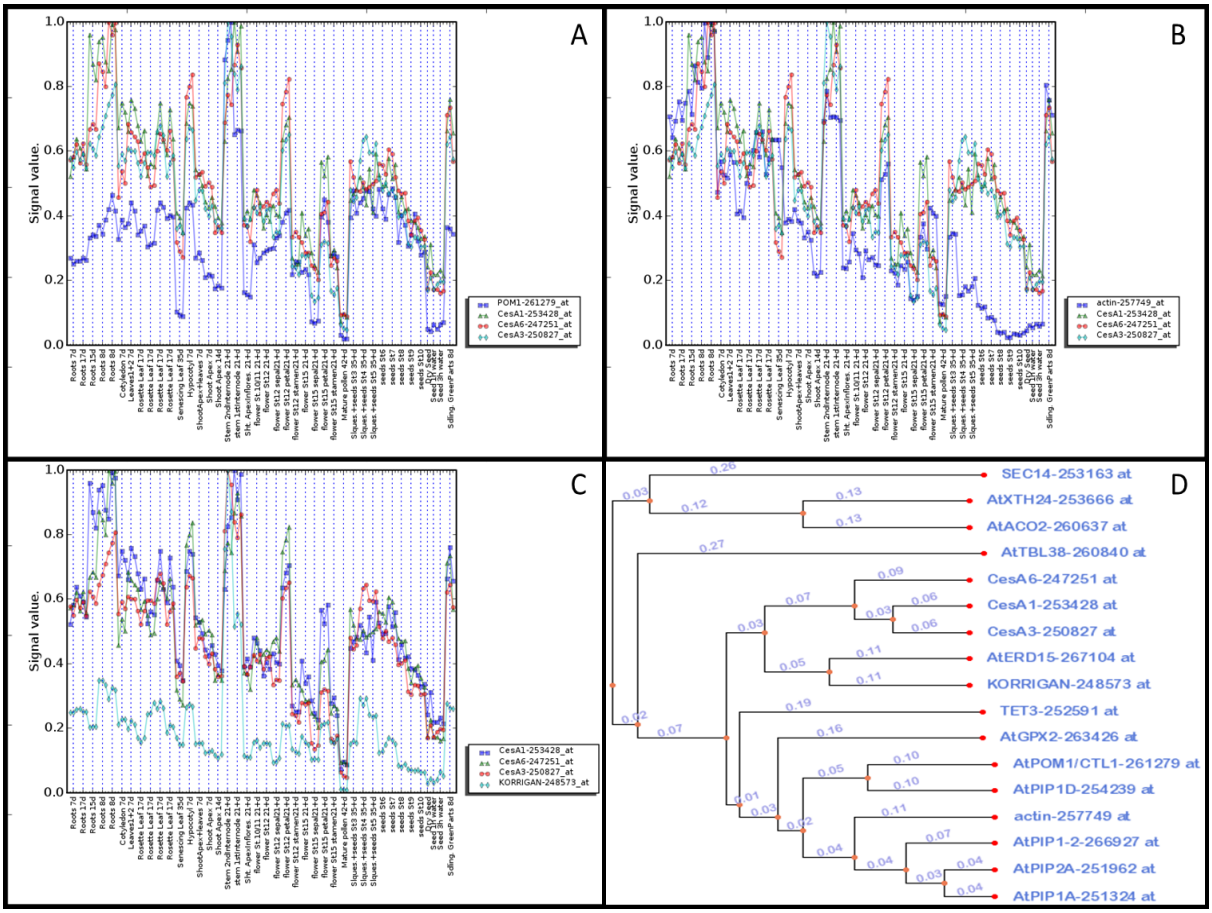

Figure 4: (A) The expression profile of POM1/CTL1 with the primary CESAs, (B) The expression profile of Actin2 with the primary CESAs, (C) The expression profile of KORRIGAN with the primary CESAs, (D) The expression tree of the most closely expressed candidates found in our library screen as well as KORRIGAN with the primary CESAs

Discussion

Several proteins are suggested to be physically linked to the rosette structure. However, so far other than CESAs, the cellulase KOR1 and certain sucrose synthases isoforms have been demonstrated to have direct interaction (Chapter 3 and Chapter 4).

Evidence of involvement in cellulose biosynthesis was already present for some of the candidates detected in our library screen such as genes involved in polysaccharide synthases (cellulose synthase-like, xyloglucan endo-transglycosylase hydrolase, CTL1) as well as structural and enzymatic proteins (expansins) alongside structural constituents of the cytoskeleton (actin). Some candidates showed to be interacting with more than one CESA namely XTH 15 and XTH16, PER21, PBP1 and a sterol methyltransferase protein which interacted with all three CESA proteins making them interesting for further investigation. Candidates closely co-expressed with the primary *CESA*s also consisted of interesting proteins with some already known to be involved in cellulose biosynthesis and some which would require further in depth study. Since the protein interactions were tested *in vitro* in a heterologous expression system, additional experiments are needed to establish the relevance of the interactions *in planta*. Knowledge on *in vivo* localization of protein can be used to identify false positives and confirm the potential biological relevance of a candidate. Additional knowledge of an interactor can also help confirm its involvement in cellulose biosynthesis.

In plants, cellulose plays a key role in structural support. The cellulose synthase machinery is associated with the cytoskeleton. The arrangement of the deposition of wall microfibrils is influenced by microtubules. Recently, it has been found that, while cortical microtubules position CESA complex delivery to the plasma membrane (Gutierrez et al. 2009), the acto-myosin system traffics CESA-containing Golgi bodies (Crowell et al. 2009, Gutierrez et al. 2009, Akkerman et al., 2011). Cortical F-actin has frequently been suggested to have a role in cellulose biosynthesis (Heath and Seagull, 1982). When actin antagonists are applied to differentiating cotton fibers both microtubules and newly deposited secondary wall thickenings occur in abnormal orientations relative to the cell axis (Kobayashi et al., 1988;Seagull, 1990; Salnikov, 2001). This finding implicates a role for the actin cytoskeleton in the secretion during cell wall formation and suggests that the actin cytoskeleton is also important for cellulose synthesis (Wightman and Turner, 2008; Crowell et al., 2009; Gutierrez et al., 2009; Akkerman et al., 2011). Actin filaments and endomembrane dynamics are critical for the distribution of cellulose synthase, showing that enzymes are transported through Golgi bodies and/or vesicles moving along actin filaments (Wightman and Turner, 2010; Cai et al., 2011). Direct interaction of the CESAs with actin (actin 2, AT3G18780) detected in our library screen is supported by previous experiments that show disruption of the actin cytoskeleton resulted in aggregation and reduced motility of the primary wall CESA-containing Golgi bodies, and in an uneven distribution of CESA complexes at the plasma membrane. Thus, the actin cytoskeleton is central for the distribution of CESA complexes in the cell (Crowell et al., 2009; Gutierrez et al., 2009).

Proteins like CTL1 seem to be implemented in cellulose production. The direct interaction between the CESAs and CTL1 (AT1G05850) found in our library screen is supported by data showing that *CTL1* is co-expressed with primary *CESA* genes (Gu et al., 2010). CTL1 had initially been suggested to play a role in chitin synthesis, however not only have no chitinase activity of heterologously expressed CTL1 proteins been detected to date (Zhong et al., 2002; Hermans et al., 2010), but also, *ctl1* mutants show incomplete cell walls and a strong reduction in cellulose content (Zhong et al., 2002; Mouille et al., 2003) and has been suggested to play a role in establishing interactions between cellulose microfibrils and hemicelluloses by affecting the assembly of the glucan chains (Sanchez et al., 2012).

Another important component thought to be required for cellulose biosynthesis is the gene that controls a trait referred to as Trichome Birefringence (TBR; Potikha and Delmer, 1995). The highly ordered cellulose found in the cell walls of Arabidopsis trichomes displays strong birefringence under polarized light, whereas the Arabidopsis *tbr* mutant displays no such birefringence with reduced cellulose content in *tbr* mutant trichomes (Potikha and Delmer, 1995). Several genes responsible for the TBR trait have been identified and characterized by mutational analysis showing severely reduced crystalline cellulose in trichomes. TBR belongs to a plant-specific, poorly described gene family (*TBR-like*) with 46 members in Arabidopsis. The *TBR* and *TBR-like3* (*TBL3*) are transcriptionally coordinated with the primary and secondary cellulose synthase genes. It has been demonstrated that *TBR* and *TBL3* influence secondary wall cellulose deposition with *TBL3* also involved in pectin modification. TBR also displays extraordinary co-expression with primary *CESA* genes, such as *CESA3 or CESA6*. Furthermore, etiolated *tbr* hypocotyls show phenotypes characteristic for primary *cesa* mutants (Bischoff et al., 2010). Due to the fact that many other genes from this family have not yet been characterized it may be interesting to further analyse the *Trichome Birefringence-Like*38 gene (AT1G29050) from the *TBL* family which has been detected interacting in our library screen and shows to be closely expressed with the primary CESAs.

The integral membrane DWARF1 protein (DIMINUTO1 or CBB1) found in our library screen (Klahre et al., 1998) is a brassinosteroid known to be involved in cell elongation (Takahashi et al. 1995; Kauschmann et al. 1996). Its recessive mutant has very short hypocotyls, leaves, stems, and roots due to the severe reduction in cell length (Takahashi et al. 1995) with 23% reduction in cellulose content and smaller, disintegrated xylem vessels in the inflorescence stem (Hossain zakir 2011). Brassinosteroids are known hormones which play a crucial role in plant growth and development (Hardtke 2007) with mutants showing a dwarf phenotype due to reduced cell size (Kauschmann et al., 1996) as well as changes in the transcript levels of several genes related to cell division, elongation and cell wall alterations i.e. KORRIGAN, the xyloglucan endotransglycosylases and expansins (Sanchez-Rodrıguez et al. 2010). Brassinosteroids have been shown to induce the expression of cellulose synthases (Salas Fernandez et al. 2009). In cotton, fibre initiation as well as elongation of cultured cotton ovules requires brassinosteroids (Luo et al., 2007) and in Arabidopsis, relative mutants display dramatically dwarfed phenotypes (Szekeres et al., 1996). By checking the expression levels of the CESAs in respected mutants and overexpression lines, brassinosteroids were

shown to promote the expression of most *CESA* genes with chromatin immunoprecipitation (ChIP) analysis demonstrating that the BES1 transcription factor activated by brassinosteroids can bind to the promoter regions of nine *CESA* genes *in vivo*, again supporting a potential role for these hormones in cellulose biosynthesis (Xie et al., 2011).

The cell walls of higher plants are mainly comprised of polysaccharides however they also contain variable amounts of proteins which can function either structurally or enzymatically. Proteins such as expansins, xyloglucan endotransglucosylase/hydrolase (XTHs), glycine-rich proteins (GRPs), hydroxyproline-rich proteins (HRGPs) consisting of proline-rich proteins (PRPs), and extensins which represent a sub-family of HRGPs (Chen et al., 1985). It is thought that they strengthen the cell walls in normal plant development (Fry, 1988; Carpita and Gibeaut, 1993). Interestingly some of these proteins were detected in our library screen indicating a direct physical interaction with the CESAs and possibly a direct role in cellulose biosynthesis.

Expansins are cell-wall-loosening proteins that have been proposed to be involved in the control of cell enlargement (Cosgrove, 2003) and are classified into four families (EXPA, EXPB, EXLA and EXLB) based on their phylogenetic relationship (Sampedro and Cosgrove, 2005). Expansins synergistically enhance the hydrolysis of crystalline cellulose by cellulases. Because glucan accessibility is the rate-limiting step in cellulase action, this result could indicate that expansin promotes the release of glucans on the surface of the cellulose microfibril, making them available for enzymatic attack (Cosgrove, 2005). Not only have expansins been shown to directly interact with the CESAs in our library screen (AT3G45970 and AT2G28950), but they were also found to associate with hemicellulose-coated cellulose microfibrils *in vitro* and are thought to induce cell wall loosening by disrupting the non-covalent bonds between cellulose and matrix polysaccharides (McQueen-Mason and Cosgrove, 1995).

In addition to expansins, the xyloglucan endotransglycosylase/hydrolase (XETs) -an abundant hemicellulose in primary cell walls- play important roles in cell wall loosening by coating and tethering the load-bearing paracrystalline cellulose microfibrils in the cellulose–xyloglucan network (Fry et al ., 1992; Pauly et al., 1999; Cosgrove, 2005; Mellerowicz et al., 2008). This would allow the cellulose microfibrils to move relative to each other for cell expansion (Fry et al. 1992; Van Sandt, 2007). The XETs are a member of a large family of GH (Glycosyl hydrolase) plant enzymes called xyloglucan endotransglucosylase/hydrolase (XTHs). Some of the proteins of this vast family seem to have a endotransglycosylation activity while some have a hydrolase activity (Bourquin et al. 2002; Eklöf and Brumer 2010). The GH9 members (endo-glucanases or cellulases) from Arabidopsis are only involved in polysaccharide hydrolysis (Sinnott, 1990; Urbanowicz et al., 2007) with KOR1 being an example of a membrane-bound endoglucanases GH family 9 involved in cellulose formation (Chapter 3). The proteins encoded by XTH genes comprise a subfamily of GH16 (Cantarel et al., 2009). GH16 enzymes cleave β-1,3 or β-1,4 bonds in various glucans and galactans. In Arabidopsis**,** the XTH gene family contains 33 genes with different expression patterns but their function is unclear (Yokoyama and Nishitani, 2001). Xyloglucan endotransglucosylase/hydrolases are a

class of enzymes that mediate the construction and restructure of the cellulose/xyloglucan framework by splitting and reconnecting xyloglucan molecule crosslinking among cellulose microfbrils. Remodeling of cellulose microfbrils within cell-wall matrices is realized to be one of the most critical steps in the regulation of cells expansion in plants. The XTHs (*XTH4, XTH5, XTH15, XTH16, XTH24 and XTH30*) found interacting with the CESAs in our library screen are members of the xyloglucan endotransglycosylase hydrolase family 16. The candidate AT3G23730 (*XTH15*) was seen to interact with all three primary cell wall CESAs and considering the high sequence homology between *XTH15* and *XTH16* (Rose et al., 2002; Sasidharan et al., 2010), a similar interaction pattern of *XTH16* (AT4G14130) with all three primary cell wall CESAs is not surprising and would suggest a similar function albeit still unknown. Members of this family were found to internally cleave xyloglucan strands that are not tightly stuck to the surface of cellulose to allow for cell loosening and subsequent strengthening of the cell wall (Uozu et al., 2000). Taking these examples into consideration it may well be possible that other unknown members of the GHs also have a role in cellulose biosynthesis and that the idea of hemicellulose hydrolysis and cellulose synthesis occurring simultaneously is possible (Lima et al., 2001).

Another one of the proteins found interacting with all the three CESA proteins was a protein belonging to the peroxidase super family, *AtPrx21* (AT2G37130). Peroxidases belong to a large multigene family of 73 members in Aabidopsis (Passardi et al., 2004) and like XTHs are important cell wall enzymes thought to be related to wall loosening and wall tightening through peroxidative cross-linking of wall proteins and polysaccharides (Brady and Fry, 1997; Boudet, 2000; Passardi et al., 2005). They could either release reactive oxygen species able to cut polysaccharides or restrict growth by cross-linking structural proteins or polysaccharides. These processes also contribute to localized wall strengthening in response to pathogens (Lamb and Dixon, 1997). Treatment of wild type plants with a cellulose biosynthesis inhibitor (2, 6-dichlorobenzonitrile, DCB) showed significant increase in peroxidase activity by 2- and 4- fold and resulted in alteration of the cell wall structure (Colville and Smirnoff, 2008) which may be due to a direct effect of peroxidases on cellulose biosynthesis supporting the direct interaction detected between the CESAs and Prx21 in our library screen. Furthermore, as peroxidases are defense proteins, it has been suggested that defense responses are induced by disruption of cell wall synthesis or structure (Colville and Smirnoff, 2008). However, the exact role of peroxidases in cellulose biosynthesis remains elusive and requires further study.

SMT3, a sterol methyltransferase- is another one of the mentioned candidates interacting with the three primary cell wall CESAs. Sterols are membrane components and have a role in regulating membrane fluidity and permeability (Hartmann, 1998). They also serve as a precursor for brassinosteroids as growth promoting plant steroid hormones (Bishop and Koncz, 2002). The most abundant plant sterols differ mainly by the number of carbon additions at the C-24 position, which are catalyzed by sterol methyltransferases (SMTs) (Benveniste, 1986). Arabidopsis has three *SMT* genes (*SMT1*, *SMT2*, and *SMT3*; Husselstein et al., 1996; Bouvier-Nave et al., 1997; Diener et al., 2000; Carland et al., 2002). The protein

encoded by SMT1, catalyses the first methylation step and serves as a branch point between cholesterol and the more abundant sterols and brassinosteroids (Diener et al., 2000). SMT2 and SMT3 work together in the second methyl-addition step which distinguishes the sterols from brassinosteroid precursors (Bouvier-Nave et al., 1997). As a result of SMT activities, cholesterol, stigmasterol, campesterol, and sitosterol accumulate. To date, several mutants defective in the early steps of the biosynthetic pathway, such as *cotyledon vascular patterning1* (*cvp1)* and *sterol methyltransferase1 (smt1)*, have been described (Carland et al., 2002). Transmission electron microscopy (TEM) revealed common defects in cell wall formation in the *smt1/cph* (*sterol methyltransferase 1/cephalopod)* mutant. Consistent with a defect in cellulose synthesis as the cause of cell wall defects, the sterol biosynthesis mutant exhibit reduced levels of cellulose coupled with ectopic deposits of both callose and lignin. The *cvp1* mutant that shows defects in cotyledon vascular patterning was found to be caused by a mutation in *SMT2* (Carland et al., 2002). The deficiency of *SMT2* in the *cvp1* mutant resulted in a vein pattern defect by interfering with the pattern of cellulose synthesis in the walls of provascular cells. SMT2 and SMT3 are highly homologous, act on the same substrate, and exhibit similar but distinguishable expression patterns and as the *cvp1* mutant plants are viable, then probably these proteins are functionally redundant and SMT3 activity can substitute for the loss of SMT2 (Carland et al., 2002). A null *smt3* mutant appeared similar to the wild type, but a *cvp1/smt3* double mutant showed enhanced defects relative to *cvp1* mutants, such as discontinuous cotyledon vein pattern, and defective root growth, loss of apical dominance and sterility. Thus sterol production is suggested to be critical for both cell elongation and cell wall expansion during the building of the primary cell wall and raises the possibility that there is direct link between plasma membrane sterols and cellulose synthesis (Carland et al., 2002). Furthermore, the major plant sterol sitosterol was suggested as a primer for cellulose synthesis, which is initiated with the conjugation of Glc to sitosterol, forming sitosterol-β-glucoside (Peng et al., 2002). Because sitosterol is reduced greatly in *cvp1*, both sitosterol-β-glucoside and cellulose microfibrils might be reduced as a consequence, causing aberrations in the pattern of cell wall extensibility that normally guide the elongation and morphology of provascular and vascular cells (Peng et al., 2002; Carland et al., 2002). Alternatively, sterols in addition to sitosterol may have roles in cellulose synthesis.

This study revealed a long list of candidate genes considered promising for future research with some possibly being an integral component of the cellulose synthase complex and others with a putative function which cannot be directly linked to cellulose biosynthesis to date. Future research would have to reveal whether their interaction with the primary CESAs is biologically relevant and what their exact position and role is in the cellulose synthase complex. Numerous genes encoding proteins with unknown function were also identified some of which share structural domains and some were the so-called proteins with domains of unknown function (DUF). Since a few of these unknown proteins can only be found in plants and are found interacting with cell wall polysaccharides, they would be considered a target of choice for future studies, however it cannot be ruled out that more proteins are involved in cellulose biosynthesis in the primary cell wall as a few known interactors like KOR1 were not identified in this screen.

Supplementary Material

Supplemetary Table1:

Group	Locus	Name	Localisation	Function	Family/Function/Activity	cesainteractors (# interactions)
cell wall biosynthesis	*AT1G01800*		Cell wall	NAD(P)-binding Rossmann-fold superfamily protein	Protein binding/ oxidoreductase activity	6(5)
	AT1G47960	AtC/VIF1	Cell wall	cell wall / vacuolar inhibitor of fructosidase 1		6
	AT2G06850	AtXTH4	Cell wall	Xyloglucanendotransglucosylase/hydrolase 4	GH family 16	1,6(10)
	AT3G45970	AtEXLA1	Cell wall	Expansin-like A1	Plant cell wall loosening	6
	AT3G54590	AtHRGP1	Cell wall	Hydroxyproline-rich glycoprotein	constituent of cell wall	6(4)
	AT4G14130	AtXTH15	Cell wall	Xyloglucanendotransglucosylase/hydrolase 15	GH family 16	1,3(6),6(12)
	AT1G05850	AtPOM1/CTL 1	Endomembrane	Chitinase family protei/lignin biosynthesis	GH family 19	3(3),6
	AT4G25260		Endomembrane	Plant invertase/pectin methylesterase inhibitor	Pectin metabolism	6(2)
	AT5G13870	AtXTH5	Cell wall	Xyloglucanendotransglucosylase/hydrolase 5	GlyTransferase family 16	6(2)
	AT5G48100		Cell wall	Methylesterase/invertase inhibitors	Pectin metabolism	3
	AT1G32170	AtXTH30	Cell wall	Xyloglucanendotransglucosylase/hydrolase 30	GH family 16	3(2),6(6)
	AT2G28950	*AtEXP6*	Cell wall,	Expansin A6	Plant cell wall loosening	1
	AT3G23730	AtXTH16	*Cell wall,*	Xyloglucanendotransglucosylase/hydrolase16	GH family 16	1,3(2),6(6)
	AT1G21310	AtEXT3	Cell wall/endomembrane	Extensin 3	Structural constituent of cell wall	6
	AT2G42840	AtPDF1	Cell-wall	Protodermal factor 1	cell-wall protein	1
	AT4G24220	AtAWI31	Cytosol	NAD(P)-binding Rossmann-fold superfamily protein,VEIN PATTERNING 1	Xylem and phloem pattern formation	6(4)
	AT2G27370	AtUPF0497	Plasma membrane	Uncharacterised protein family	*Cell wall modification/CASP3*	3
	AT4G30270	AtXTH24	Plasma membrane	Xyloglucanendotransglucosylase/hydrolase 24	GH family 16	1,6(6)
	AT1G11820		Endomembrane	Hydrolase activity, hydrolyzing O-glycosyl compounds	O-Glycosyl hydrolases family 17 protein	6
	AT3G19820	AtDWARF1	Plasma membrane / vacuole	cell elongation protein	Secondary cell wall biogenesis/lignin metabolic process	6(2)
	AT1G29050	AtTBL38	Endomembrane	Trichome birefringence-like 38	Other members have shown to be involved in the synthesis and deposition of secondary wall cellulose	3,6
	AT1G76930	AtEXT4	Endomembrane	Extensin 4	Structural constituent of cell wall	6
	AT1G18580	AtGAUT11	Golgi	Galacturonosyltransferase 11	GT family 8	6(9)
	AT3G61130	AtGAUT1	Golgi	Galacturonosyltransferase 1/pectin synthase	GT family 9	1,6(15)
	AT5G03760	*AtCSLA9*	Golgi	Cellulose synthase like A9	Mannan synthase activity	1
	AT1G12780	AtUGE1	Cytosol/plasma membrane	UDP-D-glucose/UDP-D-galactose 4-epimerase 1		3(2),6(2)
	AT3G46440	AtUXS5	*Cytosol*	UDP-XYL synthase 5	Substrate for many cell wall carbohydrates including hemicellulose and pectin	3

	AT5G59290	ATUXS3	*Cytosol*	UDP-glucuronic acid decarboxylase 3	Methylesterase inhibitor (PMEI)	3(5)
	AT5G48900		Endomembrane	putative pectatelyase 20	GH family 28	1
	AT4G23820		Endomembrane	Pectin lyase-like superfamily protein	Unknown	6(2)
	AT5G15780	Extensin family	Endomembrane	Pollen Ole e 1 allergen and extensin family protein		6
	AT5G26000	AtBGLU38	Cell wall	thioglucosideglucohydrolase 1		6
	AT3G09260	AtBGLU23,	ER , membrane, ribosome	beta-glucosidase 23	Actin cytoskeleton organization	3(3),6
	AT1G71790		Cytoplasm	(actin filament)capping protein Capz superfamily	Protein binding	6(2)
	AT1G77760	AtNR1	Cytosol	Nitrate reductase	Lignin toolbox	1
protein metabolism	*AT1G76790*		Cytosol	O-methyltransferase family protein	Ptructural constituent of ribosome	6
	AT1G43170	AtRP1		Ribosomal protein 1	Structural constituent of ribosome	3
	AT1G14320	AtRPL10	Cytosol	Ribosomal protein L16p/L10e family protein	*Structural constituent of ribosome*	6(2)
	AT1G72370		Cytosol	40s ribosomal protein SA	Sructural constituent of ribosome	1
	AT2G41840		Cytosol	Ribosomal protein S5 family protein	*Structural constituent of ribosome*	3(2)
	AT5G02740		Cytosol	Ribosomal protein S24e family protein	Structural constituent of ribosome	1
	AT3G62870		Cytosol/membrane	60S ribosomal protein L7a-2"	*Structural constituent of ribosome*	1
	AT5G07090	AtRPS4A	Plama membrane/ cytosol	Ribosomal protein S4 family protein	Structural constituent of ribosome	6
	AT3G53870		Membrane	Ribosomal protein S3 family protein	*Structural constituent of ribosome*	6
	AT4G36130		Membrane / cytosol	Ribosomal protein L2 family	Nucleotide binding	6
	AT4G27680		Endomembrane	26S proteasome regulatory particle chain RPT6-like protein	Translation elongation factor	6(4)
	AT4G20360	AtRABE1b		RAB GTPase homolog E1B	Protein folding/heat shockproten	6
	AT1G75310	AtAUL1		auxin-like 1 protein	Ubiquitin-protein ligase activity /response to chitin /protein ubiquitination	6
	AT2G35000		Endomembrane	E3 ubiquitin-protein ligase ATL9		3
	AT3G19910			E3 ubiquitin ligase BIG BROTHER-like protein	Protein binding	6
	AT2G41430	AtERD15)	Cytoplasm	Dehydration-induced protein		6
	AT1G10620		Unknown	Protein kinase superfamily protein/protein phosphorylation		3
	AT2G36570	Leucine-rich repeat protein kinase family protein	Plasma membrane	Protein serine/threonine kinase activity protein phosphorilation	Leucin-rich repeat protein kinase	6(3)
	AT4G03390	AtSRF3	Plasma membrane	STRUBBELIG-receptor family 3	Protein kinase/protein phosphorylation	6(2)
	AT5G10930	AtCIPK5	Unknown	CBL-interacting protein kinase 5	Protein kinase	6
	AT1G30270	AtCIPK23	Plasma membrane	CBL-interacting protein kinase 23		6(2)
	AT3G49670	AtBAM2	Plasma membrane	Leucine-rich receptor-like protein kinase family protein	Protein phosphorilation	6
	AT2G30040	AtMAPKKK14		Mitogen-activated protein kinase kinasekinase 14	Protein phosphorylation /protein kinase	6
	AT4G01595				Protein binding	3

	AT3G53570	AtFC1		FUS3-complementing gene 1/serine/threonine-protein kinase AFC1	*Protein phosphorylation/protein kinase*	6
	AT5G35580			Protein kinase superfamily protein	kinase activity /protein autophosphorylation	6
	AT2G39660		Cytoplasm/nucleus/plasma membrane	serine/threonine-protein kinase BIK1-BOTRYTIS--INDUCED KINASE1	Homologous to serpin (serine protease	1
	AT1G03230		Cell wall	Eukaryotic aspartyl protease family protein	Protein binding	6
	AT5G16840	AtBPA1		Binding partner of acd11 1	*Protein dephosphorylation*	6
	AT1G64040	AtTOPP3	Cytoplasm/ nucleus	Serine/threonine protein phosphatase 3	Protein binding	6(2)
	AT4G26570	AtCBL3	Membrane	Calcineurin B-like 3	Protein phosphorylated amino acid binding	1,6
	AT1G35160		Plasma membrane/cytosol	14-3-3 PROTEIN G-BOX FACTOR14 PHI	Glycine hydroxymethyltransferase activity	1
	AT4G13930	AtSHM4	Membrane/ cytosol	serine hydroxymethyltransferase 4	S-adenosylmethioninesynthetase 1	3
	AT1G02500		Membrane/cell wall,	S-adenosylmethionine biosynthetic process		1
	AT4G13940			*adenosylhomocysteinase 1-ATSAHH1; DL3010W; EMB1395; EMBRYO DEFECTIVE*	Methionine adenosyltransferase activity	1
	AT4G01850	AtSAM-2	cell wall / nucleus/ cytosol	S-adenosylmethioninesynthetase 2		3
	AT2G36880		Plasma membrane	Methionine Adenosyltransferase 3	S-adenosylmethioninesynthetase	1
	AT1G64660	AtMGL	cytosol	Methionine gamma-lyase		6
	AT3G17390		cell wall	Methionine Adenosyltransferase 4	Amino acid metabolic process	1
	AT2G37170	AtPIP2B	plasma mmbrane	Plasma membrane intrinsic protein 2	Transmembranetrasnport	6(3)
	AT1G79990	AtF19K16.s	cytosol	coatomer subunit beta'-1	Role in transport	3(6)
	AT5G58060	AtGP1	*cytosol*	SNARE-like superfamily protein	Intracellular protein transport/nucleotide binding	3(2),6(2)
	AT3G01340		*cytosol*	Transducin/WD40 repeat-like superfamily protein	Member of the SNARE superfamily proteins	3
	AT3G11820	AtSYR1	Plasma membrane	Syntaxin of plants 121	Member of the SNARE superfamily proteins	6(2)
	AT5G08080	AtSYP132	Plasma membrane	Syntaxin of plants 132	Water channel activity	6
	AT1G01620	AtPIP1C	Plasma membrane	Plasma membrane intrinsic protein 1C	Vesicle mediated transport	3(9),6(24)
	AT1G04750	AtVAMP7B	Plasma membrane	Vesicle-associated membrane protein 721	Vesicle mediated transport	6
	AT1G08560	AtSYP111	Plasma membrane	Syntaxin of plants 111	Water channel activity	3
	AT2G45960	AtPIP1;2	Plasma membrane	Plasma membrane intrinsic protein 1B	Water channel activity	3
	AT2G45960	AtPIP1B	Plasma membrane	Plasma membrane intrinsic protein 1B	Protein targeting to membrane	6(4)
	AT3G52400	AtSYP122	Plasma membrane	Syntaxin of plants 122, SNARE family protein	Water channel activity	3
	AT3G53420	AtPIP2A	Plasma membrane	Plasma membrane intrinsic protein 2A	Water channel activity	3,6
	AT3G61430	AtPIP1A	Plasma membrane	Plasma membrane intrinsic protein 1A	Intracellular protein transport	3(4),6(2)
	AT4G02080	AtASAR1	Plasma membrane	Secretion-associated RAS super family 2	*ER to Golgi vesicle-mediated transport*	6
	AT4G17170	AtRAB2	Plasma membrane	RAB GTPase homolog B1C	Water channel activity	1,6(2)
	AT4G23400	AtPIP1D	Plasma membrane	Plasma membrane intrinsic protein 1;5	Water channel activity	3,6(2)
	AT4G35100	AtPIP3	Plasma membrane	Plasma membrane intrinsic protein 3	*Protein transport*	3,6(7)

Transporters	*AT5G59150*	AtRABA2D	Plasma membrane	RAB GTPase homolog A2D	Water channel	6
	AT5G60660	AtPIP2F	Plasma membrane	Plasma membrane intrinsic protein 2;4	Protein trafficking	3,6
	AT1G26670	AtVTI1B	plasma membrane and trans-Golgi network	Vesicle transport v-SNARE family protein	Water channel	6
	AT2G37170	AtPIP2B	plasma mmbrane	Plasma membrane intrinsic protein 2	Transmembranetrasnport	6(3)
	AT1G16010	AtMGT2	Membrane	Magnesium transporter 2	Transmembrane transporter activity	6
	AT1G47603	AtPUP19	Membrane	Purine permease 19	Water channel	6
	AT1G52180		Membrane	Aquaporin-like superfamily protein	Transmembrane transport	6(7)
	AT1G55730		Membrane	Vacuolarcation/proton exchanger 5	Transmembrane transporter activity	1
	AT1G60960		Membrane	Fe(2+) transport protein 3	Transporter activity	1
	AT1G70940	ATPIN3	Membrane	Auxin efflux carrier family protein	Vesicle-mediated transport	3(2)
	AT2G33120	ATVAMP722	Membrane	Synaptobrevin-related protein 1	*Tansmembrane transport*	3
	AT2G34250	SecY	Membrane	SecY protein transport family protein	Water channel activity	6
	AT2G39010	AtPIP2;6	Membrane	Plasma membrane intrinsic protein 2E	Tansmembrane transporter activity	3,6(8)
	AT5G05820	*Nucleotide-sugar transporter family protein*	Membrane	*Nucleotide-sugar transporter family protein*	Water channel activity	1
	AT5G47450	AtDELTA-TIP3	Membrane	Tonoplast intrinsic protein 2;3	Transporter activity	6(7)
	AT5G15100	ATPIN8	Membrane	Auxin efflux carrier family protein	L-prolinetransmembrane transporter activity	6
	AT2G39890	ATPROT1	membrane, plasma membrane	Proline transporter 1	Vesicle-mediated transport	3
	AT2G40380	AtPRA1.B2	ER	Prenylated RAB acceptor 1.B2	Intracellular protein transport/nucleotide binding	1,6
	AT4G30600		ER	Signal recognition particle receptor alpha subunit family protein	Phospholipase activator activity	6
	AT2G47170	AtARF1A1C	Golgi	Ras-related small GTP-binding family protein	Protein transporter activity	6(2)
	AT4G32760	ENTH	Golgi/plasma membrane	GAT family protein	Nucleotide binding	6
	AT2G26060		heteromeric G-protein complex	WD40 repeat-EMBRYO DEFECTIVE 1345	Nucleotide binding	1
	AT1G55680		*heterotrimeric G-protein complex*	*Transducin/WD-40 repeat-containing protein*	Might be involved in protein sorting to the vacuole	1
	AT1G08190	AtATVAM2	Intracellular	Vacuolar protein sorting 41		6(2)
	AT1G69250	AtNTF2	Intracellular	Nuclear transport factor 2 (NTF2) family protein with RNA binding	Putative transport protein	1
	AT1G03060	AtSPI	Unknown	Beige/BEACH domain ;WD domain, G-beta repeat protein	Response to karrikin /ABC transporter I family member 20	3
	AT5G02270	ATNAP9	Unknown	Non-intrinsic ABC protein 9	Water channel	6
	AT2G36830	AtGAMMA-TIP	Vacuole	Gamma tonoplast intrinsic protein	Vesicle-mediated transport	6(30
	AT1G79590	AtSYP52	Vacuole	Syntaxin of plants 52	*Protein transport,*	6
	AT5G45130	ATRAB5A	Vacuole membrane	RAB homolog 1	Water channel	6(3)
	AT3G26520	AtTIP2	Vacuole/chloroplast	Tonoplast intrinsic protein 2	Water channel	3(16),6(30)

	AT4G17340	AtDELTA-TIP2	Vacuole/chloroplast	Tonoplast intrinsic protein 2;2	Water channel	3,6(42)
	AT3G16240	AtDELTA-TIP1	Vacuole	Delta tonoplast integral protein		6(17)
	AT4G35750	SEC14	cytosol	SEC14 cytosolic factor family protein / Phosphoglyceride transfer family protein		3,6
	AT3G45600	(TET3)	Endomembrane	Tetraspanin3	Sugar transmembrane transporter activity	6
	AT3G48740	AtSWEET11	Plasma membrane	Nodulin MtN3 family protein	Sugar transmembrane transporter activity	6
	AT5G23660	AtMTN3	Plasma membrane	MTN3-like protein	actin filament-based movement	6
	AT3G26680	AtSNM1		DNA repair metallo-beta-lactamase family protein	L-ascorbate peroxidase activity	3
	AT5G54280	ATM2	Plasma membrane	myosin 2		6
Stress response	*AT1G07890*	AtAPX1		Ascorbate peroxidase 1	Glutathione peroxidase activity	6(3)
	AT2G31570	AtGPX2	cytosol	Glutathione peroxidase 2	Glutathione peroxidase activity	6(3)
	AT4G11600	AtGPX6	ER	Glutathione peroxidase 6	*Peroxidase activity*	6(4)
	AT3G63080	ATGPX5	plasma membrane /ER	Glutathione peroxidase 5	Metallo-hydrolase/oxidoreductase superfamily protein	6
	AT3G10850	AtGLX2	cytosol/cytoplasm	Hydroxyacylglutathione hydrolase activity	Glutathione transferase activity	6
	AT1G78380		Membrane	*Glutathione S-transferase TAU 19*		1
	AT2G29450	ATGSTU1	Plasma membrane/ cytosol	Glutathione S-transferase tau 5	Translation elongation factor EF1B/ribosomal protein S6	6(2)
	AT1G30230		Plasma membrane	Glutathione S-transferase	*Defence response*	1,6
	AT2G37130		cytosol	Peroxidase superfamily protein	*Defence response*	1,3,6(4)
	AT3G16420	AtPBP1	cytosol/nucleus	PYK10-binding protein 1	1-aminocyclopropane-1-carboxylate oxidase activity	1,3(10),6(11)
	AT1G62380	AtACO2	cell wall/plasma membrane/ytosol	ACC oxidase 2	Oxidative strees response	6(3)
	AT3G32980	ATprx12	cell wall,	*Peroxidase 32*	Oxidative strees response	1
	AT3G28200		cell wall/ ytosol	*Peroxidase 31*	Defence response	1
	AT5G54500	AtFQR1	Membrane	Flavodoxin-like quinonereductase 1	Peroxidase activity	6
	AT1G49570		Endomembrane	Peroxidase superfamily protein	Defense response by callose deposition in cell wall	3
	AT4G26850	AtVTC2	cell wall	Mannose-1-phosphate guanylyltransferase (GDP)s		3(2)
	AT4G39260	AtCCR1	cell wall/plasma membrane	Glycine rich protein 8	callose synthase	6(2)
	AT2G31960	ATGSL3	Plasma membrane	*Glucan synthase-like 3*	Glycolysis	6
	AT1G13440	*GAPC2*	Membrane	Glyceraldehyde 3-phosphate dehydrogenase		1
	AT1G31970	AtDEA(D/H)	cytosol	RNA helicase family protein	Defense response	6(2)
	AT1G45616	(AtRLP6)	Membrane	Receptor like protein 6	Extracellular polysaccharide biosynthetic	6
	AT4G00560	F6N23.	Endomembrane	Rossmann-fold NAD(P)-binding domain-containing		1
	AT1G03870	AtFLA9	Plasma membrane	Fasciclin-like arabinogalactan protein 9 - FLA9		1
Signalling	*AT5G44130*	AtFLA13	Plasma membrane	Fasciclin-like arabinogalactan protein 13 precursor		6
	AT5G55730	AtFLA1	Plasma membrane	Fasciclin-like arabinogalactan 1		6
	AT2G14890	AtAGP9	membrane	Arabinogalactan protein 9	Lipid metabolism/oxidation-reduction process	6(2)
	AT4G18770	AtMYB98			Nucleic acid binding	1
	AT5G53950	AtANAC098		Arabidopsis NAC (No Apical Meristem) domain containing protein 98	Secondary cell wall biogenesis	6

Transcription factors	*AT1G51600*	GATA28		Transcription factor 28	Response to chitin	3(6)
	AT1G51700	AtDOF1		DOF zinc finger protein 1	*Phloem and xylem development*	6(2)
	AT1G80780			Polynucleotidyltransferase, ribonuclease H-like superfamily protein	GATA transcription factor 20	1,6(4)
	AT1G32870	AtNAC13	Nucleus/cytoplasm	NAC domain protein 13	Translation elongation factor activity	6
	AT3G11400	At3G1-EIF3G1	Plasma membrane	Translation initiation factor 3G1	Translation initiation factor activity	1
	AT1G01200	AtRABA3	Nucleus/cell plate	RAB GTPase homolog A3	Translation initiation factor activity	6
	AT5G18110	AtNCBP	Cytosol	Novel cap-binding protein	Translation initiation factor activity	6(2)
	AT2G39990	AtEIF2	Cytoplasm/nucleus	Eukaryotic translation initiation factor 2	Membrane trafficking	6
Sugar metabolism	*AT1G47770*	T2E6		Beta-galactosidase related protein		6
	AT2G01290	(RPI2) ribose-5-phosphate isomerase 2	cytoplasm	Ribose-5-phosphate isomerase activity/cell death	carboxylesterase activity	6(2)
	AT3G48690	ATCXE12	cytosol	Alpha/beta-Hydrolases superfamily protein		6
	AT3G08030	AtDUF642	*cell wall*	Hypothetical protein	*Response to brassinosteroid stimulus*	1
Unknown	*AT4G08950*	AtEXO1	cell wall	Phosphate-responsive 1 family protein		6(2)
	AT2G28370	AtUPF0497	Plasma membrane			6
	AT3G27390		Plasma membrane	*Hypothetical protein*		1
	AT3G51610		Plasma membrane			6
	AT4G35240	AtDUF630 and AtDUF632	*Plasma membrane*			3
	AT5G11680		*Plasma membrane*	*Hypothetical protein*		1
	AT5G42860		Plasma membrane			3,6(2)
	AT4G22750		Endomembrane	DHHC-type zinc finger family protein		6(2)
	AT4G34881		Endomembrane			3
	AT4G36230		Endomembrane			3
	AT1G08630	AtTHA1	Unknown	Threonine aldolase 1		3
	AT1G11650	AtRBP45B	Unknown	RNA-binding (RRM/RBD/RNP motifs) family protein		6
	AT1G22750	AtDUF1475	Unknown			3
	AT1G23710	AtDUF1645	Unknown			6
	AT1G24050		Unknown	RNA-processing, Lsm domain/Anticodon-binding domain-containing protein		6
	AT1G27290		Unknown			3
	AT1G27921		Unknown	Potential natural antisense gene, locus overlaps with AT1G27920		3(2)
	AT1G58235		Unknown			3(2),6(2)
	AT1G72600		Unknown	Hydroxyproline-rich glycoprotein family protein		6
	AT2G18115		Unknown	Pseudogene, glycine-rich protein		3
	AT2G24550		Unknown			3
	AT2G26110	AtDUF761	Unknown			3
	AT2G35170		Unknown	Histone H3 K4-specific methyltransferase SET7/9 family protein		1

AT2G38310	AtPYL4	Unknown	PYR1-like 4		3,6
AT2G42760		Unknown			6(2)
AT2G42900		Unknown	Plant basic secretory protein (BSP) family protein	Prolyl-4-hydroxylases, ATP4H1	6
AT2G43080	AtP4H1	Unknown	PROLYL 4-HYDROXYLASE/P4H isoform 1		6
AT2G47780		Unknown	Rubber elongation factor protein		3
AT3G61090		Unknown	Putative endonuclease or glycosyl hydrolase		3(3)
AT5G20700	DUF581	Unknown		Protein binding	3
AT5G24890		Unknown	Protein binding		3
AT5G25280		Unknown	Serine-rich protein-related		3
AT5G27860		Unknown	*Hypothetical protein*		1
AT5G45350		Unknown	Proline-rich family protein		6
AT5G58575		Unknown	Hypothetical protein	Response to chitin	6(2)
AT5G66070	NEP1	Unknown	NEP1-interacting protein-like 1/		6(4)
AT1G35660		Plasma membrane		*carbohydrate binding*	3,6
AT4G32130	AtDUF2012	ER		Acid phosphatase activity	6
AT1G04040		*cell wall,*	*HAD superfamily, subfamily IIIB acid phosphatase*		1
AT2G36410	AtDUF662	Plasma membrane	Family of unknown function	Structural molecule activity	1,6
AT4G21450		Plasma membrane	vesicle-associated membrane family protein	Glycine hydroxymethyltransferase activity	3
AT5G46700	AtTRN2	Plasma membrane	Tetraspanin family protein		1
AT1G54215		Endomembrane	Proline-rich family protein	Involved in sterol biosynthesis/xylem and phloem pattern formation/	3
AT1G20330	AtCVP1	ER	COTYLEDON VASCULAR PATTERN 1, sterol methyltransferase 2	Transferase activity	3
AT5G04530	KCS19	Membrane	*3-ketoacyl-CoA synthase 21*	Phosphatase activity	1
AT5G44020	MRH10	Membrane	HAD superfamily, subfamily IIIB acid	Multicellular organismal development	1
AT3G23810	SAHH2	Plasma membrane		S-adenosylmethionine-dependent methyltransferase activity	1
AT1G76090	AtSMT3)	ER	Sterol methyltransferase 3		1,3(2),6(2)
AT2G31740			S-adenosyl-L-methionine-dependent methyltransferases superfamily protein	Microtubule cytoskeleton organization	3
AT1G20090	AtARAC4	Plasma membrane	Rac-like GTP-binding protein		6
AT1G25520	Uncharacterized protein family	Membrane	*Response to karrikin/putative transmembrane protein*		6
AT5G29020	transposable element gene	Unknown	Unknown		3,6(2)
AT1G71340	glycerophosphodiesterphosphodiesterase domain-containing protein				1
AT5G10450	*14-3-3-like protein GF14 lambda*			*cell tip growth,*	1
AT3G07880	SCN1 |	cytoplasm	SUPERCENTIPEDE1		6
AT3G44310	Nitrilase 1	Plasma membrane	Indole-3-acetonitrile nitrile hydratase activity		1
AT3G18780	*actin 2*	Membrane	Structural constituent of cytoskeleton	cell wall biogenesis	1
AT1G78580	AtTPS1	cytoplasm	Alpha,alpha-trehalose-phosphate synthase [UDP-forming] 1		6(2)

	AT5G53870	AtENODL1	Plasma membrane	Early nodulin-like protein 1		6(2)
	AT1G65930	cytosolic NADP+-dependent isocitrate dehydrogenase	cytosol	isocitrate dehydrogenase (NADP+) activity		6
	AT1G78590	(NADK3) NAD(H) kinase 3	cytosol	NAD+ kinase activity		6
	AT5G19990	(ATSUG1)regulatory particle triple-A ATPase 6A		Ubiquitin-dependent protein catabolic process		3
	AT1G26110	AtDCP5 | protein decapping 5	cytosol	De-adenylation-independent decapping of nuclear-transcribed mRNA	Hydrolase activity, acting on ester bonds	6
	AT4G16690	(ATMES16)	cytosol	Methyl esterase 16	catalytic activity	6
	AT5G12210	(AtRGTB1)	cytosol	RAB geranylgeranyltransferase beta subunit 1	Uridylate kinase activity	6
	AT5G26667	(CMP/UMP KINASE)	*cytosol*	Uridylate kinase activity	Phospholipase activator activity	1,3
	AT5G14670	(ARFA1B)	cytosol	ADP-ribosylation factor A1B	*Phenylpropanoid metabolic process*	6(2)
	AT2G22990	SNG1	ER	Sinapoylglucose 1		3
	AT4G22753	(SMO1-3)	ER	Fatty acid biosynthetic process/sterol 4-alpha methyl oxidase 1-3		6
	AT1G09660	RNA-binding KH domain-containing protein	Unknown	RNA-binding KH domain-containing protein		6
	AT1G09920	TRAF-type zincfinger-related	Unknown	Ubiquitin fusion degradation UFD1 family protein		6
	AT1G17620	Late embryogenesis abundant (LEA) hydroxyproline-rich glycoprotein family	Unknown	Unknown		6
	AT1G65960	(GAD2) glutamate decarboxylase 2	Unknown	Glutamate metabolic process		6
	AT1G65970	(TPX2) thioredoxin-dependent peroxidase 2	Unknown	cell redox homeostatis	Structural constituent of cytoskeleton	6
	AT1G04820	AtTUA4	Cell wall	Tubulin alpha-4 chain	Structural constituent of cytoskeleton	1,3
	AT5G19770	AtTUA3	Cell wall/plasma membrane	Tubulin alpha-3	Structural constituent of cytoskeleton	6(2)
	AT5G12250	AtTUB6	Membrane	Beta-6 tubulin	Structural constituent of cytoskeleton	3(3)
	AT5G23860	AtTUB8	Membrane	Tubulin beta 8	Structural constituent of cytoskeleton	3
	AT5G62690	AtTUB2	Membrane	Tubulin beta chain 2	Structural constituent of cytoskeleton	3
	AT5G62700	AtTUB3	Membrane	Tubulin beta chain 3	Microtubule-based process	3
	AT1G75780	AtTUB1	Membrane	Tubulin beta-1 chain		3

Supplementary Table 2: Biologically relevant interactors

Group	Locus	Name	Localisation	Function	Family/ Function/ Activity	cesainteractors(# interactions)
cell wall biosynthesis	*AT1G01800*		Cell wall	NAD(P)-binding Rossmann-fold superfamily protein	Protein binding/ oxidoreductase activity	6(5)
	AT1G47960	AtC/VIF1	Cell wall	cell wall / vacuolar inhibitor of fructosidase 1		6
	AT2G06850	AtXTH4	Cell wall	Xyloglucanendotransglucosylase/hydrolase 4	GH family 16	1,6(10)
	AT3G45970	AtEXLA1	Cell wall	Expansin-like A1	Plant cell wall loosening	6
	AT3G54590	AtHRGP1	Cell wall	Hydroxyproline-rich glycoprotein	constituent of cell wall	6(4)
	AT4G14130	AtXTH15	Cell wall	Xyloglucanendotransglucosylase/hydrolase 15	GH family 16	1,3(6),6(12)

	AT1G05850	AtPOM1/CTL1	Endomembrane	Chitinase family protei/lignin biosynthesis	GH family 19	3(3),6
	AT4G25260		Endomembrane	Plant invertase/pectin methylesterase inhibitor	Pectin metabolism	6(2)
	AT5G13870	AtXTH5	Cell wall	Xyloglucanendotransglucosylase/hydrolase 5	GlyTransferase family 16	6(2)
	AT5G48100		Cell wall	Methylesterase/invertase inhibitors	Pectin metabolism	3
	AT1G32170	AtXTH30	Cell wall	Xyloglucanendotransglucosylase/hydrolase 30	GH family 16	3(2),6(6)
	AT2G28950	AtEXP6	Cell wall,	Expansin A6	Plant cell wall loosening	1
	AT3G23730	AtXTH16	Cell wall,	Xyloglucanendotransglucosylase/hydrolase16	GH family 16	1,3(2),6(6)
	AT1G21310	AtEXT3	Cell wall/endomembrane	Extensin 3	Structural constituent of cell wall	6
	AT2G42840	AtPDF1	Cell-wall	Protodermal factor 1	cell-wall protein	1
	AT4G24220	AtAWI31	Cytosol	NAD(P)-binding Rossmann-fold superfamily protein,VEIN PATTERNING 1	Xylem and phloem pattern formation	6(4)
	AT2G27370	AtUPF0497	Plasma membrane	Uncharacterised protein family	Cell wall modification/CASP3	3
	AT4G30270	AtXTH24	Plasma membrane	Xyloglucanendotransglucosylase/hydrolase 24	GH family 16	1,6(6)
	AT1G11820		Endomembrane	Hydrolase activity, hydrolyzing O-glycosyl compounds	O-Glycosyl hydrolases family 17 protein	6
	AT3G19820	AtDWARF1	Plasma membrane / vacuole	cell elongation protein	Secondary cell wall biogenesis/lignin metabolic process	6(2)
	AT1G29050	AtTBL38	Endomembrane	Trichome birefringence-like 38	Other members have shown to be involved in the synthesis and deposition of secondary wall cellulose	3,6
	AT1G76930	AtEXT4	Endomembrane	Extensin 4	Structural constituent of cell wall	6
	AT1G18580	AtGAUT11	Golgi	Galacturonosyltransferase 11	GT family 8	6(9)
	AT3G61130	AtGAUT1	Golgi	Galacturonosyltransferase 1/pectin synthase	GT family 9	1,6(15)
	AT5G03760	AtCSLA9	Golgi	Cellulose synthase like A9	Mannan synthase activity	1
	AT1G12780	AtUGE1	Cytosol/plasma membrane	UDP-D-glucose/UDP-D-galactose 4-epimerase 1		3(2),6(2)
	AT3G46440	AtUXS5	Cytosol	UDP-XYL synthase 5	Substrate for many cell wall carbohydrates including hemicellulose and pectin	3
	AT5G59290	ATUXS3	Cytosol	UDP-glucuronic acid decarboxylase 3	Methylesterase inhibitor (PMEI)	3(5)
	AT5G48900		Endomembrane	putative pectatelyase 20	GH family 28	1
	AT4G23820		Endomembrane	Pectin lyase-like superfamily protein	Unknown	6(2)
	AT5G15780	Extensin family	Endomembrane	Pollen Ole e 1 allergen and extensin family protein		6
	AT5G26000	AtBGLU38	Cell wall	thioglucosideglucohydrolase 1		6
	AT3G09260	AtBGLU23,	ER , membrane, ribosome	beta-glucosidase 23	Actin cytoskeleton organization	3(3),6
	AT1G71790		Cytoplasm	(actin filament)capping protein Capz superfamily	Protein binding	6(2)
	AT1G77760	AtNR1	Cytosol	Nitrate reductase	Lignin toolbox	1
protein metabolism	*AT1G76790*		Cytosol	O-methyltransferase family protein	Ptructural constituent of ribosome	6
	AT1G14320	AtRPL10	Cytosol	Ribosomal protein L16p/L10e family protein	Structural constituent of ribosome	6(2)
	AT1G72370		Cytosol	40s ribosomal protein SA	Sructural constituent of ribosome	1
	AT2G41840		Cytosol	Ribosomal protein S5 family protein	Structural constituent of ribosome	3(2)
	AT5G02740		Cytosol	Ribosomal protein S24e family protein	Structural constituent of ribosome	1
	AT3G62870		Cytosol/membrane	60S ribosomal protein L7a-2"	Structural constituent of ribosome	1
	AT5G07090	AtRPS4A	Plama membrane/ cytosol	Ribosomal protein S4 family protein	Structural constituent of ribosome	6
	AT3G53870		Membrane	Ribosomal protein S3 family protein	Structural constituent of ribosome	6
	AT4G36130		Membrane / cytosol	Ribosomal protein L2 family	Nucleotide binding	6

	AT4G27680		Endomembrane	26S proteasome regulatory particle chain RPT6-like protein	Translation elongation factor	6(4)
	AT2G35000		Endomembrane	E3 ubiquitin-protein ligase ATL9		3
	AT2G41430	AtERD15)	Cytoplasm	Dehydration-induced protein		6
	AT2G36570	Leucine-rich repeat protein kinase family protein	Plasma membrane	Protein serine/threonine kinase activity protein phosphorilation	Leucin-rich repeat protein kinase	6(3)
	AT4G03390	AtSRF3	Plasma membrane	STRUBBELIG-receptor family 3	Protein kinase/protein phosphorylation	6(2)
	AT1G30270	AtCIPK23	Plasma membrane	CBL-interacting protein kinase 23		6(2)
	AT3G49670	AtBAM2	Plasma membrane	Leucine-rich receptor-like protein kinase family protein	Protein phosphorilation	6
	AT1G03230		Cell wall	Eukaryotic aspartyl protease family protein	Protein binding	6
	AT1G64040	AtTOPP3	Cytoplasm/ nucleus	Serine/threonine protein phosphatase 3	Protein binding	6(2)
	AT4G26570	AtCBL3	Membrane	Calcineurin B-like 3	Protein phosphorylated amino acid binding	1,6
	AT1G35160		Plasma membrane/cytosol	14-3-3 PROTEIN G-BOX FACTOR14 PHI	Glycine hydroxymethyltransferase activity	1
	AT4G13930	AtSHM4	Membrane/ cytosol	serine hydroxymethyltransferase 4	S-adenosylmethioninesynthetase 1	3
	AT1G02500		Membrane/cell wall,	S-adenosylmethionine biosynthetic process		1
	AT4G01850	AtSAM-2	cell wall / nucleus/ cytosol	S-adenosylmethioninesynthetase 2		3
	AT2G36880		Plasma membrane	Methionine Adenosyltransferase 3	S-adenosylmethioninesynthetase	1
	AT1G64660	AtMGL	cytosol	Methionine gamma-lyase		6
	AT3G17390		cell wall	Methionine Adenosyltransferase 4	Amino acid metabolic process	1
Transporters	*AT2G37170*	AtPIP2B	plasma mmbrane	Plasma membrane intrinsic protein 2	Transmembranetrasnport	6(3)
	AT1G79990	AtF19K16.s	cytosol	coatomer subunit beta'-1	Role in transport	3(6)
	AT5G58060	AtGP1	cytosol	SNARE-like superfamily protein	Intracellular protein transport/nucleotide binding	3(2),6(2)
	AT3G01340		cytosol	Transducin/WD40 repeat-like superfamily protein	Member of the SNARE superfamily proteins	3
	AT3G11820	AtSYR1	Plasma membrane	Syntaxin of plants 121	Member of the SNARE superfamily proteins	6(2)
	AT5G08080	AtSYP132	Plasma membrane	Syntaxin of plants 132	Water channel activity	6
	AT1G01620	AtPIP1C	Plasma membrane	Plasma membrane intrinsic protein 1C	Vesicle mediated transport	3(9),6(24)
	AT1G04750	AtVAMP7B	Plasma membrane	Vesicle-associated membrane protein 721	Vesicle mediated transport	6
	AT1G08560	AtSYP111	Plasma membrane	Syntaxin of plants 111	Water channel activity	3
	AT2G45960	AtPIP1;2	Plasma membrane	Plasma membrane intrinsic protein 1B	Water channel activity	3
	AT2G45960	AtPIP1B	Plasma membrane	Plasma membrane intrinsic protein 1B	Protein targeting to membrane	6(4)
	AT3G52400	AtSYP122	Plasma membrane	Syntaxin of plants 122, SNARE family protein	Water channel activity	3
	AT3G53420	AtPIP2A	Plasma membrane	Plasma membrane intrinsic protein 2A	Water channel activity	3,6
	AT3G61430	AtPIP1A	Plasma membrane	Plasma membrane intrinsic protein 1A	Intracellular protein transport	3(4),6(2)
	AT4G02080	AtASAR1	Plasma membrane	Secretion-associated RAS super family 2	ER to Golgi vesicle-mediated transport	6
	AT4G17170	AtRAB2	Plasma membrane	RAB GTPase homolog B1C	Water channel activity	1,6(2)
	AT4G23400	AtPIP1D	Plasma membrane	Plasma membrane intrinsic protein 1;5	Water channel activity	3,6(2)
	AT4G35100	AtPIP3	Plasma membrane	Plasma membrane intrinsic protein 3	Protein transport	3,6(7)
	AT5G59150	AtRABA2D	Plasma membrane	RAB GTPase homolog A2D	Water channel	6
	AT5G60660	AtPIP2F	Plasma membrane	Plasma membrane intrinsic protein 2;4	Protein trafficking	3,6
	AT1G26670	AtVTI1B	plasma membrane and trans-Golgi network	Vesicle transport v-SNARE family protein	Water channel	6
	AT2G37170	AtPIP2B	plasma mmbrane	Plasma membrane intrinsic protein 2	Transmembranetrasnport	6(3)

	AT1G16010	AtMGT2	Membrane	Magnesium transporter 2	Transmembrane transporter activity	6
	AT1G47603	AtPUP19	Membrane	Purine permease 19	Water channel	6
	AT1G52180		Membrane	Aquaporin-like superfamily protein	Transmembrane transport	6(7)
	AT1G55730		Membrane	Vacuolarcation/proton exchanger 5	Transmembrane transporter activity	1
	AT1G60960		Membrane	Fe(2+) transport protein 3	Transporter activity	1
	AT1G70940	ATPIN3	Membrane	Auxin efflux carrier family protein	Vesicle-mediated transport	3(2)
	AT2G33120	ATVAMP722	Membrane	Synaptobrevin-related protein 1	Tansmembrane transport	3
	AT2G34250	SecY	Membrane	SecY protein transport family protein	Water channel activity	6
	AT2G39010	AtPIP2;6	Membrane	Plasma membrane intrinsic protein 2E	Tansmembrane transporter activity	3,6(8)
	AT5G05820	Nucleotide-sugar transporter family protein	Membrane	Nucleotide-sugar transporter family protein	Water channel activity	1
	AT5G47450	AtDELTA-TIP3	Membrane	Tonoplast intrinsic protein 2;3	Transporter activity	6(7)
	AT5G15100	ATPIN8	Membrane	Auxin efflux carrier family protein	L-prolinetransmembrane transporter activity	6
	AT2G39890	ATPROT1	membrane, plasma membrane	Proline transporter 1	Vesicle-mediated transport	3
	AT2G40380	AtPRA1.B2	ER	Prenylated RAB acceptor 1.B2	Intracellular protein transport/nucleotide binding	1,6
	AT4G30600		ER	Signal recognition particle receptor alpha subunit family protein	Phospholipase activator activity	6
	AT2G47170	AtARF1A1C	Golgi	Ras-related small GTP-binding family protein	Protein transporter activity	6(2)
	AT4G32760	ENTH	Golgi/plasma membrane	GAT family protein	Nucleotide binding	6
	AT2G26060		heteromeric G-protein complex	WD40 repeat-EMBRYO DEFECTIVE 1345	Nucleotide binding	1
	AT1G55680		heterotrimeric G-protein complex	Transducin/WD-40 repeat-containing protein	Might be involved in protein sorting to the vacuole	1
	AT1G08190	AtATVAM2	Intracellular	Vacuolar protein sorting 41		6(2)
	AT1G69250	AtNTF2	Intracellular	Nuclear transport factor 2 (NTF2) family protein with RNA binding	Putative transport protein	1
	AT4G35750	SEC14	cytosol	SEC14 cytosolic factor family protein / Phosphoglyceride transfer family protein		3,6
	AT3G45600	(TET3)	Endomembrane	Tetraspanin3	Sugar transmembrane transporter activity	6
	AT3G48740	AtSWEET11	Plasma membrane	Nodulin MtN3 family protein	Sugar transmembrane transporter activity	6
	AT5G23660	AtMTN3	Plasma membrane	MTN3-like protein	actin filament-based movement	6
	AT5G54280	ATM2	Plasma membrane	myosin 2		6
Stress response	*AT2G31570*	AtGPX2	cytosol	Glutathione peroxidase 2	Glutathione peroxidase activity	6(3)
	AT4G11600	AtGPX6	ER	Glutathione peroxidase 6	Peroxidase activity	6(4)
	AT3G63080	ATGPX5	plasma membrane /ER	Glutathione peroxidase 5	Metallo-hydrolase/oxidoreductase superfamily protein	6
	AT3G10850	AtGLX2	cytosol/cytoplasm	Hydroxyacylglutathione hydrolase activity	Glutathione transferase activity	6
	AT1G78380		Membrane	Glutathione S-transferase TAU 19		1
	AT2G29450	ATGSTU1	Plasma membrane/ cytosol	Glutathione S-transferase tau 5	Translation elongation factor EF1B/ribosomal protein S6	6(2)
	AT1G30230		Plasma membrane	Glutathione S-transferase	Defence response	1,6
	AT2G37130		cytosol	Peroxidase superfamily protein	Defence response	1,3,6(4)
	AT3G16420	AtPBP1	cytosol/nucleus	PYK10-binding protein 1	1-aminocyclopropane-1-carboxylate oxidase activity	1,3(10),6(11)
	AT1G62380	AtACO2	cell wall/plasma membrane/ytosol	ACC oxidase 2	Oxidative strees response	6(3)
	AT3G32980	ATprx12	cell wall,	Peroxidase 32	Oxidative strees response	1
	AT3G28200		cell wall/ ytosol	Peroxidase 31	Defence response	1
	AT5G54500	AtFQR1	Membrane	Flavodoxin-like quinonereductase 1	Peroxidase activity	6
	AT1G49570		Endomembrane	Peroxidase superfamily protein	Defense response by callose deposition in cell wall	3

	AT4G26850	AtVTC2	cell wall	Mannose-1-phosphate guanylyltransferase (GDP)s		3(2)
	AT4G39260	AtCCR1	cell wall/plasma membrane	Glycine rich protein 8	callose synthase	6(2)
	AT2G31960	ATGSL3	Plasma membrane	Glucan synthase-like 3	Glycolysis	6
	AT1G13440	GAPC2	Membrane	Glyceraldehyde 3-phosphate dehydrogenase		1
	AT1G31970	AtDEA(D/H)	cytosol	RNA helicase family protein	Defense response	6(2)
	AT1G45616	(AtRLP6)	Membrane	Receptor like protein 6	Extracellular polysaccharide biosynthetic	6
	AT4G00560	F6N23.	Endomembrane	Rossmann-fold NAD(P)-binding domain-containing		1
	AT1G03870	AtFLA9	Plasma membrane	Fasciclin-like arabinogalactan protein 9 - FLA9		1
Signalling	*AT5G44130*	AtFLA13	Plasma membrane	Fasciclin-like arabinogalactan protein 13 precursor		6
	AT5G55730	AtFLA1	Plasma membrane	Fasciclin-like arabinogalactan 1		6
	AT2G14890	AtAGP9	membrane	Arabinogalactan protein 9	Lipid metabolism/oxidation-reduction process	6(2)
Sugar metabolism	*AT2G01290*	(RPI2) ribose-5-phosphate isomerase 2	cytoplasm	Ribose-5-phosphate isomerase activity/cell death	carboxylesterase activity	6(2)
	AT3G48690	ATCXE12	cytosol	Alpha/beta-Hydrolases superfamily protein		6
	AT3G08030	AtDUF642	cell wall	Hypothetical protein	Response to brassinosteroid stimulus	1
Unknown	*AT4G08950*	AtEXO1	cell wall	Phosphate-responsive 1 family protein		6(2)
	AT2G28370	AtUPF0497	Plasma membrane			6
	AT3G27390		Plasma membrane	Hypothetical protein		1
	AT3G51610		Plasma membrane			6
	AT4G35240	AtDUF630 and AtDUF632	Plasma membrane			3
	AT5G11680		Plasma membrane	Hypothetical protein		1
	AT5G42860		Plasma membrane			3,6(2)
	AT4G22750		Endomembrane	DHHC-type zinc finger family protein		6(2)
	AT4G34881		Endomembrane			3
	AT4G36230		Endomembrane			3
	AT1G35660		Plasma membrane		carbohydrate binding	3,6
	AT4G32130	AtDUF2012	ER		Acid phosphatase activity	6
	AT1G04040		cell wall,	HAD superfamily, subfamily IIIB acid phosphatase		1
	AT2G36410	AtDUF662	Plasma membrane	Family of unknown function	Structural molecule activity	1,6
	AT4G21450		Plasma membrane	vesicle-associated membrane family protein	Glycine hydroxymethyltransferase activity	3
	AT5G46700	AtTRN2	Plasma membrane	Tetraspanin family protein		1
	AT1G54215		Endomembrane	Proline-rich family protein	Involved in sterol biosynthesis/xylem and phloem pattern formation/	3
	AT1G20330	AtCVP1	ER	COTYLEDON VASCULAR PATTERN 1, sterol methyltransferase 2	Transferase activity	3
	AT5G04530	KCS19	Membrane	3-ketoacyl-CoA synthase 21	Phosphatase activity	1
	AT5G44020	MRH10	Membrane	HAD superfamily, subfamily IIIB acid	Multicellular organismal development	1
	AT3G23810	SAHH2	Plasma membrane		S-adenosylmethionine-dependent methyltransferase activity	1
	AT1G76090	AtSMT3)	ER	Sterol methyltransferase 3		1,3(2),6(2)
	AT1G20090	AtARAC4	Plasma membrane	Rac-like GTP-binding protein		6
	AT1G25520	Uncharacterized protein family	Membrane	Response to karrikin/putative transmembrane protein		6
	AT3G07880	SCN1 |	cytoplasm	SUPERCENTIPEDE1		6

	AT3G44310	Nitrilase 1	Plasma membrane	Indole-3-acetonitrile nitrile hydratase activity		1
	AT3G18780	actin 2	Membrane	Structural constituent of cytoskeleton	cell wall biogenesis	1
	AT1G78580	AtTPS1	cytoplasm	Alpha,alpha-trehalose-phosphate synthase [UDP-forming] 1		6(2)
	AT5G53870	AtENODL1	Plasma membrane	Early nodulin-like protein 1		6(2)
	AT1G65930	cytosolic NADP+-dependent isocitrate dehydrogenase	cytosol	isocitrate dehydrogenase (NADP+) activity		6
	AT1G78590	(NADK3) NAD(H) kinase 3	cytosol	NAD+ kinase activity		6
	AT1G26110	AtDCP5 | protein decapping 5	cytosol	De-adenylation-independent decapping of nuclear-transcribed mRNA	Hydrolase activity, acting on ester bonds	6
	AT4G16690	(ATMES16)	cytosol	Methyl esterase 16	catalytic activity	6
	AT5G12210	(AtRGTB1)	cytosol	RAB geranylgeranyltransferase beta subunit 1	Uridylate kinase activity	6
	AT5G26667	(CMP/UMP KINASE)	cytosol	Uridylate kinase activity	Phospholipase activator activity	1,3
	AT5G14670	(ARFA1B)	cytosol	ADP-ribosylation factor A1B	Phenylpropanoid metabolic process	6(2)
	AT2G22990	SNG1	ER	Sinapoylglucose 1		3
	AT4G22753	SMO1-3	ER	Fatty acid biosynthetic process/sterol 4-alpha methyl oxidase 1-3		6
	AT1G04820	AtTUA4	Cell wall	Tubulin alpha-4 chain	Structural constituent of cytoskeleton	1,3
	AT5G19770	AtTUA3	Cell wall/plasma membrane	Tubulin alpha-3	Structural constituent of cytoskeleton	6(2)
Others	*AT5G12250*	AtTUB6	Membrane	Beta-6 tubulin	Structural constituent of cytoskeleton	3(3)
	AT5G23860	AtTUB8	Membrane	Tubulin beta 8	Structural constituent of cytoskeleton	3
	AT5G62690	AtTUB2	Membrane	Tubulin beta chain 2	Structural constituent of cytoskeleton	3
	AT5G62700	AtTUB3	Membrane	Tubulin beta chain 3	Microtubule-based process	3
	AT1G75780	AtTUB1	Membrane	Tubulin beta-1 chain		3

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Chapter 6

General Discussion

Understanding the biosynthesis of wall polysaccharides components has attracted considerable interest in light of the fundamental importance of these molecules as the earth's most abundant source of terrestrial biomass. Cell wall polysaccharides an important renewable resource of biopolymers with diverse industrial applications. Cellulose synthesized by the cellulose synthase complex, is one of the main polysaccharides found in plants and is responsible for a structural role. The initial discovery of the multi subunit cellulose synthase complex at the plasma membrane in green algae showed that it was associated with the ends of cellulose microfibrils (Brown and Montezinos, 1976). Subsequently, the rosette terminal complexes comprised of six particles were visualised in higher plants like *Zea mays* (Mueller and Brown, 1980). Since those findings, extensive research via numerous analytical techniques has been carried out in the cellulose biosynthesis field. However there still remain many unanswered questions in the cellulose biosynthetic pathway. It is currently unclear why Arabidopsis plants contain so many *CESA* genes. The CESA proteins known to be involved in primary wall formation are grouped separately from those involved in secondary wall formation based on phylogeny, indicating that CESA may not be functionally analogous (Doblin et al., 2002). It is not known whether all the CESAs have catalytic activity and a synthesis role or if some have other roles in the complex like structural roles. It is not known if there is any overlap in function between the CESAs or whether there are differences and specificities in function between the CESAs. It may be that one CESA can catalyse chain initiation with the sitosterol glucoside primer whereas another one catalyses the elongation of the chain (Read and Bacic, 2002). This hypothesis also relates to the assembly of the complex, it is unknown how many different CESA isoforms are required for cellulose production within a cell and what the relationship is between the CESAs in the rosette. Are the CESA triads composing the rosette complex proposed to function together within heteromeric terminal complexes in a fixed structure? If so, then how are the CESAs positioned in the rosette, are they strategically placed or is it just random? The objective of the research presented in this thesis was to generate more in-depth knowledge in cellulose biosynthesis and to this aim better characterize and understand the cellulose synthase complex and its components by notably identifying the various interacting proteins forming the complex in the plant cell wall.

In order to gain further knowledge of the cellulose synthase complex and its components, isolating the CESA rosettes protein complex from a living cell, followed by characterizing their constituents through biochemical analysis *in vitro* would be a suitable method. This procedure has proven challenging as the enzyme complex is highly unstable and has limited the possibility of purifying the intact complex. Furthermore, considering that the interaction is not tested *in vivo*, deviated conditions might cause association and dissociation of proteins, bringing about false results. The abundant *in vitro* synthesis of cellulose in its native crystalline form has proven difficult to date probably due to it being under complex cellular control with multiple-proteins involved in its synthesis (Blanton and Haigler, 1996). Cellulose synthase activity was unambiguously identified in a solubilized membrane preparation from blackberry (Lai Kee Him et al., 2002); however, these preparations did not constitute a purified cellulose synthase complex. Affinity purification has been used to identify multimeric protein complexes, but lacks information on the exact physical association within

them. Using a blue native polyacrylamide gel electrophoresis (BN-PAGE) of solubilized Arabidopsis cell extracts, a complex of 840 kDa in size was detected containing the primary CESAs (Wang et al., 2008) however, the purification of the complex which was larger than the predicted size of the cellulose synthase complex assembled purely from six CesAs was not carried out and its composition was not determined. Purification of a secondary cell wall CESA-containing complex under non denaturing conditions through affinity purification of an intact epitope-tagged complex was also attempted, however it only resulted in the detection of CesA-CesA interactions and more information on the complex and other proteins associating with the CESAs could not be determined (Atanassov et al., 2009).

One approach for a more detailed view on the proteins involved in the cellulose complexes was to embark on protein-protein interaction methods. The membrane-based yeast two-hybrid system (MbYTH) was chosen to study the *in vitro* interactions of proteins involved in the synthesis of cellulose and the bimolecular fluorescence complementation assay (BiFC) was used to confirm these interactions *in planta*. Although other protein interaction methods such as co-immunoprecipitation and pull down assays could have been used, the high throughput MbYTH system was chosen, as it not only enables the verification of interactions between proteins already known to interact but also enables the discovery of new interactors. It must be mentioned that the *in vivo* membrane based yeast two hybrid would require other methods to validate the interaction actually occurring *in planta,* which explains the use of the non-invasive fluorescent-based BiFC techniques as it benefits from the absence of background signal, shows high specificity, high stability and allows detection of protein-protein interaction in living cells (Hu et al., 2002). Moreover, accurate assessment of protein–protein interactions show values of up to 90% when cross-referencing two or more methodologies (Mering et al., 2002).

The presence of mixed CESA complexes *in planta*

To get more insight on the CESAs in the primary and secondary cellulose synthase complex and whether there were any similarities in function or positioning of the CESAs in the primary and secondary cellulose synthase complex, the parallels between the primary and secondary cellulose synthase complex were investigated. To this aim, a series of yeast-two-hybrid and bimolecular fluorescence experiments between the primary and secondary CESAs were carried out to assess how the different CESAs could interact. In order to further understand the effects of incorporating primary CESA isoforms into the secondary cellulose synthase complex and the secondary CESA isoforms into the primary cellulose synthase complex, expression of N-terminal GFP fusion constructs of secondary *CESA* coding sequences with the promoter region of the primary *CESA* sequences and vice versa were assessed. Our data demonstrates that the CESA proteins known to assemble into complexes in either primary or secondary cell wall biosynthesis are able to interact with CESA proteins that compose the other type of complex but, this interchangeability between the two complexes is limited. The physical interaction detected between the CESAs in the primary and secondary complex in pairwise combinations both in yeast and in the *Nicotiana benthamiana* showed that the CESAs could be present in the same complex if they were to be co-expressed. Additional selectivity was shown to exist between the CESAs within the plant cell wall

because of the specific complementation of certain primary *cesa* mutants with certain secondary constructs and vice versa. Out of the primary CESAs, only CESA7 was able to be incorporated into the cellulose synthase complex at the plasma membrane in the *cesa3* mutant (*je5*) by partially rescuing the phenotype and out of the secondary CESAs, only CESA1 was able to compensate partially for the lack of growth in the *cesa8* knockout mutant line.

This specificity indicates both parallels and differences between the primary and secondary CESAs in function or positioning in the complex as only specific CESAs can, albeit partially, take over the role of other CESAs. One interpretation of these results is that each isoform within the CESA-complex would have specific positions which could either be consistent and distinct spatial locations in the structure of the complex, or they could be assigned due to strong interaction affinities between CESA classes during assembly of the complex. From these results, it appears the CESA3 and CESA7 can gain access to the same position in the complex. The same can probably be said for CESA1 and CESA8; however analysis on GFP-fused constructs of the relative genes should be assessed to determine whether this is also the case.

The presence of three distinct CESAs have been shown to be essential for the function of the cellulose synthase complex both in the primary and secondary cellulose synthase complex (Taylor et al., 2000; Scheible et al., 2001; Taylor et al., 2003; Desprez et al., 2007; Persson et al., 2007). The stoichiometry for the three main primary CESA proteins (CESA1, CESA3 and CESA6) of the primary cellulose synthase complex was determined at 1:1:1 (Dr. E Crowell, INRA, France, personal communication) and based on gene expression the stoichiometry of the CESAs for the secondary cellulose synthase complex indicated a 1:1:1 ratio (Persson et al., 2005). Taking these factors into consideration, each of the CESA isoforms are considered equally essential for the assembly of the complex, therefore an individual function in the synthesis of cellulose for each one of the CESAs comprising the complex would be envisaged. However, determining their function based on our promoter swap results and the current data available would be difficult. Interestingly, the fact that there was no complete rescue apparent in the mutants and just a partial compensation was seen between the specific CESAs demonstrates a uniqueness in function in the three primary and three secondary CESAs, as no primary CESA was able to fully substitute a secondary cell wall CESA and *vice versa*. Reinforcing the idea that the composition of the CESAs in the cellulose synthase complex is specific and the biosynthesis of cellulose is more complex than previously thought. Despite the results obtained, the function of the individual CESAs still remains unknown, it is unknown whether all of the CESA proteins are catalytically active or if some of them instead serve a strictly structural or regulatory role. Differences between the catalytic activity of the CESAs were made more apparent with the velocity of GFP-CESA7 containing complexes in the *je5* background appearing to be slightly faster (30%) than the velocity of complexes containing GFP-CESA3 in the same background, suggesting greater catalytic activity of CesA7. It cannot be excluded that this difference in velocity could also be due to GFP-CESA7 trying to out compete the native CESA3 in the weak *cesa3* k.o. (*je5*) mutant background for the inclusion in the cellulose synthase complex.

Despite the observation of the GFP-fused CESAs moving from the Golgi to the plasma membrane for the incorporation in the cellulose synthase complex and subsequent cellulose biosynthesis. The process of final assembly of the complex is still considered a mystery. It is not known how the subunits would assemble in an orderly manner to form the large final cellulose synthase complex. However, the possible location of the final assembly of the complex seemed to be made clearer by detecting the location of GFP-CESA7 in the wild type plants at the Golgi bodies and small CESA compartments (SMaCCs). It was demonstrated that GFP-CESA7 was not able to enter the plasma membrane and was restricted to the Golgi bodies and SMaCCs due to wild type CesA3 being able to outcompete GFP-CESA7 for incorporation into the cellulose synthase complex. Thus the final assembly of the cellulose synthase complex could occur in the SMaCCs or just after insertion into the membrane.

Compared to previously gained knowledge, our data shines new light in the composition of the cellulose synthase complex by demonstrating that additional selectivity exists within the CESAs in the cellulose synthase complex, either through directed assembly or competition for interacting partners It also showcases specialization in the function or regulation of CESA families and helps in understanding the composition of CESA complexes during the transition from primary to secondary cell wall deposition, and the requirements for assembly and intracellular trafficking of CESA complexes by suggesting that the synthesis of cellulose during the transition between the primary and secondary cell wall may involve the action of mixed primary-secondary complexes

Identification of proteins interacting with the cellulose synthase complex

The function of the proteins other than the CESAs shown to be important for cellulose biosynthesis also remain unknown with many of those proteins physically interacting with the catalytic subunits of the cellulose synthase complex (CESAs) and some even showing co-localization with the CESAs. An attempt to isolate the cellulose synthase complex resulted in a prediction that the complex might contain at least 15 polypeptides, suggesting that proteins other than CESA are required for cellulose biosynthesis and deposition (Kudlicka and Brown, 1997; Saxena and Brown, 2005) with KOR1 and sucrose synthases amongst those proteins.

The interaction of KORRIGAN1 with the complex

Cellulase activity is known to be essential for both eukaryotic and prokaryotic cellulose synthesis (Mathysse et al., 1995; Peng et al., 2002; Delmer, 1999) although the precise role is still not known. In many prokaryotes, the cellulose synthase and endoglucanase genes required for cellulose synthesis form an operon (Romling, 2002; Standal et al., 1994; Matthysse et al., 1995). The presence of both the cellulose synthase and the soluble endoglucanase proteins detected in and around the operon are shown to be required for normal *in vivo* cellulose production in bacteria with the endoglucanase being suggested to function as a chain terminator during cellulose biosynthesis (Delmer, 1999; Saxena and Brown, 2005). Membrane anchored endoglucanases were also identified in plants, based on sequence homology to the bacterial cellulases and were referred to as KORRIGAN (Brummell et al., 1997; Nicol et al., 1998; Zuo et al., 2000; Lane et al., 2001; Sato et al.,

2001). The mutant phenotype for the knockout of this gene in Arabidopsis resembles the CESA mutants (Nicol et al., 1998; Lane et al., 2001; Sato et al., 2001; Zuo et al., 2000; Szyjanowicz et al., 2004). A dwarfed phenotype with reduced elongation of the root and radially expanded cells as well as reduced cellulose content (Lane et al., 2001, Szyjanowicz et al., 2004, Zuo et al., 2000) suggests that KOR1 has a central role in cellulose biosynthesis; however its exact function is still under discussion. KOR1 has been suggested to act as an editor for the correct association of glucan chains in the cellulose microfibrils by removing incorrectly positioned defective chains (Delmer, 1999; Mølhøj et al., 2002; Szyjanowicz et al., 2004; Somerville, 2006; Taylor, 2008). In this scenario, if KOR1 works in the removal of defective molecules, overexpression of *KOR1* would generate cellulose with increased crystallinity. It has also been postulated that KOR1 may be involved in the formation of smaller microfibrils from large macrofibrils which have been suggested to be synthesized first and subsequently divided into smaller microfibrils in one cellulose synthesis scenario (Ding and Himmel, 2006).

Since the mentioned endoglucanase is detected in both bacteria and plants and considered to have a role in cellulose production. It was thought that the KOR1 protein might be an additional component of the rosette complex. To confirm this hypothesis, the interactions between the CESA proteins and KOR1 were tested using sensitive techniques such as MbYTH and the split-YFP. It was demonstrated both *in vitro* and *in planta* that KOR1 was able to bind to all the primary cell wall CESA proteins. Interestingly, the strongest interactor between the primary CESAs was CESA1, suggesting that this CESA isoform may have a unique role in recruiting KOR1. By checking the interactions of the other CESAs in the secondary cell wall (CESA4 and CESA8) with KOR1 both *in vivo* and *in planta* we demonstrated a physical interaction between KOR1 and the CESAs in the secondary cell wall except for CESA7. The fact that CESA7 didn't interact with KOR1 is supported by previous co-immunoprecipitation experiments, using an epitope-tagged form of KOR or IRX3 (AtCesA7) which showed that KOR did not co-purify with (AtCesA7) *irx3* (Szyjanowicz et al., 2004) in the *irx2* mutant. Using Bioluminescence Resonance Energy Transfer **(**BRET) technology, an advanced cell based technology used for protein-protein interaction, a lack of interaction between CESA7 and KOR1 in Arabidopsis was also demonstrated further confirming our results. However it was inaccurately concluded that due to the absence of interaction between CESA7 and KOR1 there is no direct interaction between KOR1 and the secondary cellulose synthase complex (Maloney, 2010) which we clearly disprove. The specificity in interactions of the CESAs with KOR1 suggest that the different CESA proteins might have distinct functions, thus based on their different function the requirement for the cellulose KORRIGAN would differ, which is also implied by their specificity in interaction and positioning in the complex. A tight association of CESA and KOR1 in the plasma membrane would support a function in cellulose chain termination to release the cellulose microfibril from the synthase complex (Delmer, 1999; Szyjanowicz et al., 2004; Taylor, 2008).

In this study, the localization and dynamics of GFP-KOR1 expressed under the control of its endogenous promoter in the *kor1-1* mutant background in living cells was also determined as

GFP-KOR1 was detected in discrete motile particles at the plasma membrane. Surprisingly, it has previously been shown that overexpression of GFP-KOR1, driven by the Cauliflower mosaic virus *35S* promoter in a wild-type background, yielded a slightly different localization pattern from that observed here, with no detectable label at the plasma membrane (Robert et al., 2005). However, introgression of the *kor1-1* mutant into the overexpression lines restores the presence of GFP-KOR1 at the plasma membrane (Dr. Vernhettes, INRA, France, personal communication). This result suggests that the localization of GFP-KOR1 is sensitive to the expression level of the protein. As KORRIGAN has been shown to be present in various parts of the cell such as the Golgi apparatus and the plasma membrane, it could also be postulated for KOR1 to be present mainly in the Golgi apparatus and subsequently secreted to the plasma membrane with a short lifetime. Overexpression of KOR1 orthologs provokes reduced cell elongation and accumulation of non-crystalline cellulose (Takahashi et al., 2009). An abnormally high accumulation of KOR1 proteins may trigger a signal for growth arrest. GFP-KOR1 was detected in particles in the same cells and at the same time as GFP-CESAs in the epidermis of etiolated hypocotyls (Crowell et al., 2009) and were shown to migrate along linear trajectories with comparable velocities as those observed for GFP-CESA3 or GFP-CESA6 (Paredez et al., 2006b; Desprez et al., 2007) and further supports a role for KORRIGAN in cellulose biosynthesis. Likewise, the trajectories of GFP-KOR1 plasma membrane particles were highly similar to those of GFP-CESA3 in the same cell types (Dr. Vernhettes, INRA, France, personal communication).

All together the data presented here support a new model in which both KOR1 and CESAs are part of the cellulose synthase complex by showing that KOR1 is present in the plasma membrane, where it interacts with primary and secondary cell wall CESA proteins. This new information will help shed light on the role of KOR1 in cellulose synthesis, and the new GFP-KOR1 marker line generated, will provide useful tools to further explore the relationship between KOR1 and the primary cell wall cellulose synthase complex.

The interaction of sucrose synthase isoforms with the complex

Another protein suggested to be interacting with the cellulose synthase complex is the sucrose synthase. SUS which is involved in sucrose metabolism is also considered to have multiple functions within a cell. It has long been suggested to have a role in cellulose biosynthesis by providing UDP-Glc as a substrate for the production of cellulose. The expression of plant-specific sucrose synthase in the bacterium *Acetobacter xylinum* has shown to enhance cellulose production (Nakai et al., 1999) and overexpression of sucrose synthase genes in cotton increased fibre length and strength (Jiang et al., 2011) while an increase in biomass production was seen in poplar (Coleman et al., 2009b) and tobacco (Coleman et al., 2006, 2009) all linking sucrose synthase to cell wall formation and cellulose biosynthesis (Guerriero et al., 2010). However, clear evidence for a direct interaction between sucrose synthase and the cellulose-synthesizing complex was lacking and remained an important question, thus by using both *in vitro* and *in planta* protein interaction assays, it was demonstrated that most of the sucrose synthase isoforms (SUS1, SUS3 , SUS4, SUS6) were able to physically interact with the cellulose synthases in Arabidopsis except for SUS5 which showed no interaction,

suggesting specificity in interaction and possibly function between the SUS isoforms in Arabidopsis.

Sucrose synthase is mainly present in the cytoplasm, but has also been detected in other organelles in the plant (Etxebarria and Gonzalez, 2003; Subbaiah et al., 2006). It has also been located in sites, either close to the plasma membrane or in cell walls in various plants, where cellulose synthesis is high (Salnikov et al., 2001, 2003; Albrecht and Mustroph, 2003; Persia et al., 2008; Guerriero et al., 2010), thus, channelling the UDP-Glc substrate directly to the cellulose synthases located at the plasma membrane for the production of cellulose. By generating a GFP-fused *SUS6* gene under its own endogenous promoter, its co-localisation with fluorescently tagged CESA6 was demonstrated (Chapter 4) and would be expected at the plasma membrane based on the common model for cellulose biosynthesis (Chapter 4).

The plasma membrane location for an interaction between these two proteins is also based on their association with microtubules and actin. Both sucrose synthase and the CESAs are shown to be associated with the microtubules and actin. The SUS proteins are linked to microtubules and actin suggesting a cytoskeleton association (Winter et al., 1998; Salnikov et al., 2001) and the CESAs are known to be distributed in the cell by microtubules and actin filaments (Cai et al., 2011). It has been suggested that the actin filaments transport CESA-containing Golgi bodies and deliver them to sites of cell wall synthesis (Wightman and Turner, 2008). This interaction with actin/microtubules could possibly be a general requirement for the interaction of SUS with the CESAs (Cai et al., 2011) as they may mediate the distribution of the SUS isoforms while determining specific localizations for the SUS proteins. A similar role for microtubules has been suggested for CSL1 which is thought to bridge CESA complexes and cortical microtubules and is already known to directly interact with the CESAs (Li et al., 2012). However, it could also be postulated that the interaction between SUS and the CESAs could also occur in areas other than the plasma membrane such as the cytoplasm and intracellular organelles, where the CESAs are also present and its co-localisation with CESA6 has been determined (Chapter 4). If the delivery of the cellulose synthase complex to the plasma membrane were to occur in the SMaCCs, then the incorporation of a cytosolic SUS with the SMaCCs while transporting the CESAs and depositing them at the plasma membrane can be envisaged. In such a model a direct interaction between the SUS and the CESAs would occur at the SMaCCs thus providing the UDP-Glc substrate for cellulose biosynthesis. However further analysis on stable transformants would be required to determine the exact co-localisation and movement pattern of the SUS isoforms with the CESAs.

It has been proposed that each individual SUS isoform can be targeted to these diverse subcellular compartments. Although no overarching mechanism for controlling the sub-cellular localization and association has been resolved, phosphorylation has been suggested as a possible regulatory mechanism. Sucrose synthase is known to have both soluble and membrane-associated forms (Amor et al,. 1995; Winter et al., 1997). However, whether these two forms are encoded by the same gene or by different genes is not clear (Haigler et al., 2001). Some authors have suggested that this association is regulated by protein phosphorylation/dephosphorylation (Nakai et al., 1998, Tanase et al., 2002) and some argue

that even phosphorylation could be dependent on the isoform (Chourey et al., 1998, Barratt et al., 2001, Komatsu et al., 2002). The fact that sucrose synthase genes are highly conserved in both DNA sequence and gene structure (Sturm et al., 1999; Komatsu et al., 2002) and their proteins are also highly conserved in amino acid sequences (Sturm et al., 1999; Barratt et al., 2001) suggests that instead of distinct genes encoding soluble and membrane-associated sucrose synthases, post-translational modification, such as phosphorylation, could operate on both forms of the enzyme. *In vitro* studies in maize, has shown that phosphorylation of SUS at specific serine residues in maize, causes the release from the membrane while dephosphorylation of SUS promotes membrane association (Shaw et al., 1994; Huber et al., 1996; Winter et al., 1997). On the other hand, contradicting evidence has shown in maize that both membrane- and soluble- associated sucrose synthase were phosphorylated at a specific serine phosphorylation site (Hardin et al., 2003, 2004). Other examples show a lack of direct relationship between membrane type location, and degree of phosphorylation (Pozueta-Romeroa et al., 2004). Thus, the role of sucrose synthase phosphorylation/ dephosphorilation remains elusive and requires further investigation; however the relevance of phosphorylation to enzymatic activity cannot be overruled. To date, the underlined assumption is that SUS activity and cellular location are modulated in parallel through phosphorylation/ dephosphorylation of the enzyme.

In the SUS1 isoform in maize, the serine 15 (S15) residue is considered the major phosphorylation site (Huber et al., 1996; Winter et al., 1997) while serine 170 (S170) is suggested to have a relatively minor role (Huber et al., 1996; Hardin et al., 2003). Sucrose synthase cDNA sequences cloned from monocotyledonous and dicotyledonous species show that the consensus sequence of S15 (L/I XRXXS*XR/K) and that of S170 (LNRHLS*) phosphorylation site at the N-terminal end of the protein is conserved in most plants such as soybean, tomato and maize (Asano et al., 2002), thus it would seem likely that they are regulated in the same way (Huber et al., 1996; Zhang and Chollet, 1997; Zhang et al., 1999; Komina et al., 2002; Duncan et al., 2006; Anguenot et al., 2006). The exact physiological significance of the phosphorylation of the S15 at the N-terminal end of the protein is still not fully understood. The more minor S170 phosphorylation site is suggested to control the regulation of sucrose synthase enzyme level in the cell (Hardin et al., 2003)

It is interesting to note that the same consensus sequence as the S15 in maize SUS1 isoform was detected in SUS1, SUS3 and SUS4, but not in SUS2 and SUS5 while in SUS6 it is modified into T/KXXS* (Baud et al., 2004). The serine phosphorylation residue in SUS1and SUS4, SUS3 as well as SUS6 was positioned at S13, S11 and S14, respectively. By aligning the amino acid sequences of the SUS isoforms. The S170 consensus sequence was detected in SUS2, SUS3 and SUS4 and the same consensus sequence was found at the S167 position in SUS1; however it was not detected in SUS5 or SUS6 suggesting that they may be phosphorylated at some other site. The absence of the major phosphorylation site detected in the SUS5 Arabidopsis isoforms is interesting to note as it could be connected to its lack of interaction and association to the cellulose synthases (Figure 1). If we were to assume that phosphorylation/dephosphorylation would be responsible for the localization of the sucrose synthase isoforms to the plasma membrane and if the membrane associated isoforms were to

be in favour of the production of UDP-Glc for cellulose biosynthesis then the lack of this specific residue in SUS5 could explain the absence of its interaction with the CESAs. It must be added that this hypothesis is highly speculative and would require further experimental evidence to prove by using antibodies specific for the phosphorylated form to detect phosphorylation in the interacting proteins. By mutating the serine residues thought to be phosphorylated it would be possible to check for changes in interaction between the proteins and also assess its localization.

Figure 1: The alignment of the amino acid sequences of the SUS isoforms highlighting the Serine residue thought to be responsible for the phosphorylation/dephosphorylation of the protein.

Thus far the new information provided here confirms a previously suggested role for sucrose synthase in carbon partitioning and its association with the cellulose synthase complex. Furthermore, our data demonstrates colocalisation of SUS with the CESAs and the involvement of specific SUS isoforms in cellulose biosynthesis.

Identification of motives critical for protein-protein interactions

The interaction of the proteins in the cellulose synthase complex and those associating with it is vital for the orderly formation and proper function of the complex. Thus knowing more about where this interaction occurs in the proteins would help to understand the mechanisms involved in the formation and assembly of the complex.

Various regions in the cellulose synthase protein such as the RING finger at the N-terminal site, the cytosolic loop between the second and third transmembrane domain and the C-Terminal region of the protein have been suggested to be required for the CESA-CESA interactions. The cytosolic amino terminus containing RING-finger has been implicated in mediating protein-protein interaction (Saurin et al., 1996). The N-terminal region of all the CESA proteins contain a conserved double zinc-finger motif highly homologous to the RING-finger domain protein , and was shown to homodimerize and interact with the RING-finger of another CESA protein in cotton suggesting that the RING finger domain might be responsible for the interaction between these proteins (Kurek et al., 2002). However, mutations in the essential cysteines in the RING-finger motif of Arabidopsis CESAs did not result in a complete disruption of the interactions (Timmers et al., 2009). Thus, it was concluded that while the RING-finger may have a role in the CESA interaction, it was not essential for the

interaction between the different CESA isoforms and other more specific interaction regions should be assessed.

To further analyse what domains are more important for the assembly of the complex and whether the interaction of these domains would help incorporate the CESAs into the complex, a series of domain swapping experiments were carried out with wild-type and mutations of AtCESA1 and AtCESA3 proteins in their respective mutants. This resulted in the catalytic and C-terminal domains being more important than other regions for the specific incorporation of the CESAs into the cellulose synthase complex (Wang et al., 2006). Thus by comparing the C-terminal cytosolic region of the Arabidopsis CESAs which was conserved in CESA3, 4, 6, and 7, but not in CESA1 and 8, it became more apparent that while CESA3 and 7 showed more similarity to each other than with the other CESAs, the same region was less conserved in CESA1 and CESA8 compared to the other CESAs (Chapter 2) which could be a reason for the specify in the partial rescue of the *cesa3* mutant by CESA7 and, the *cesa8* knockout by CESA1. It could be speculated that the interaction between the C-terminus of one CESA with another might help mediate complex assembly and similarities and differences in the C-terminus of the CESAs would determine their interaction or substitution for one another.

Despite asserting certain domains for the interaction of the CESAs with each other, the domains responsible for the interaction between the CESAs and other proteins were not identified. The localization of the RING-motif in the N-terminal site of the CESAs at the cytoplasmic face of the plasma membrane could suggest it having a role in recruiting proteins other than the CESAs such as sucrose synthases. The presence of a leucine zipper coiled coil domain thought to have a role in protein-protein interaction was detected in the maize SUS1 and SUS3 isoforms *in silico* and alongside further experimental analysis was suggested to be the domain responsible for interacting with other proteins (Carlson et al., 2002; Duncan and Huber, 2007; Jayashree et al., 2008) thus would be interesting for further study to check for its interaction with the CESAs.

Interestingly no specific protein-protein interaction domain was detected in the KOR1 protein based on sequence analysis, thus truncated forms of the protein were assessed to detect the protein domain responsible for its interaction with the CESAs. It was shown that the KOR1 transmembrane domain was required for the interaction with CESA1 as all partial proteins containing this domain were able to interact supporting an interaction at the plasma membrane. The lack of interaction in the absence of TMD led to the conclusion that this domain is essential for the interaction with the CESA proteins (Chapter 3).

Other proteins found interacting with the complex

To date, CESA proteins are the only known components of cellulose synthase. Through the MbYTH system, a number of non CESA proteins were found interacting together with the primary CESAs in the cellulose synthase complex. The screen resulted in a list of proteins with very diverse catalytic functions. Proteins thought to affect cellulose biosynthesis and proteins speculated to associate with this complex such as CTL1, those related to the cell wall

structure and the cytoskeleton such as actin and tubulin as well as proteins located in the pathway of cellulose synthesis were listed. Interestingly, some of these proteins have previously been detected *in silico* and suggested to have a role in cellulose biosynthesis based on expression analysis (Persson et al., 2007) while others have also been shown to interact with the CESAs using pull down assays (Song et al., 2010). The MbYTH has shown to be a powerful tool to detect candidate genes involved in the cellulose synthesis pathway. Surprisingly, the list did not include some of the proteins already found interacting with the CESA proteins in previous studies, like KOR1 possibly due to incomplete screening of the library or absence of the genes in the library. The size limitations of the cDNA library which is restricted to 2.5kb could be another factor for the absence of some genes. Considering that the candidates are picked up in a heterologous system in yeast, simultaneous expression of two proteins in one cell might enable them to interact thus finding the real interactors that are actually present and interact with the CESAs in the same place at the same time *in vivo* would require further analysis.

As well as the CESAs in the cellulose synthase complex, cellulose biosynthesis requires the incorporation of a diverse set of proteins and regulatory mechanisms. The commonly viewed model of cellulose biosynthesis in plants constitutes the plasma membrane associated form of sucrose synthase (SUS) positioned interacting at the plasma membrane with the cytoplasmic portion of the cellulose synthase thus directly channelling UDP-Glc substrate to the hexagonal cellulose synthase rosette complex for glucan chain formation (Chapter 5). The KOR1 cellulase is also shown to be present at the plasma membrane interacting with the CESAs probably in the form of a dimer (Chapter 4) by editing the elongating glucan chain conversion to cellulose microfibrils and cleaving the defective glucan chains (Delmer and Haigler, 2002; Molhoj et al., 2002) on the cell wall side. However, other suggested protein components of the cellulose synthase complex or those associating with the complex at certain developmental stages and their stoichiometry are not completely known. Despite the detection of direct interaction between the CESAs and various candidates in our library screen, their roles in the formation of the cellulose synthase complex are still not clear (Doblin et al., 2002).

The cytoskeleton related proteins detected in the library screen is consistent with previous reports indicating a role for cortical microtubules in the deposition of cellulose microfibrils (Baskin, 2001; Somerville, 2006) on the cytoplasmic side of the cell. The cellulose synthase complex is shown to associate with cortical microtubules. It has been postulated that the CESAs move along linear tracks that coincide with the orientation of cortical microtubules (Zhong et al., 2002; Paredez et al., 2006). Other microtubule associated proteins like CSL1 are suggested to bridge CESA complexes and cortical microtubules (Gu et al., 2010) which highlights the role of organized microtubules in the regulation and deposition of cellulose. As well as microtubules, actin is also shown to be important for cellulose biosynthesis. The motility and movement of the CESAs in the Golgi bodies were reduced and uneven distribution of CesA complexes at the plasma membrane was caused by disrupting the actin cytoskeleton (Wightman and Turner, 2008; Crowell et al., 2009; Gutierrez et al., 2009).

Interestingly interaction between the primary cell wall CESAs and candidates responsible for the production of other cell wall biopolymers such as pectin and hemicellulose (e.g., xyloglucan, arabinoxylan) have also been detected in our library screen. Layers of cellulose microfibrils entwined with hemicellulose and pectin polysaccharides form a network (Carpita & Gibeaut, 1993). Despite knowledge on the individual cell wall polymers and their architecture (McNeil et al., 1984; McCann and Roberts, 1991; McCann et al., 1992; Carpita and Gibeaut, 1993; Vincken et al., 1997; Bacic et al., 1998; Schindler, 1998), little is known about their conformations in the wall and the interplay between the polymers in the formation of the cell wall. It is not known how they are associated with each other in a mature wall to make a functional network with high tensile strength, resilience and ability to expand in surface area.

The cell wall polysaccharides comprising the network are thought to be tightly regulated and act cooperatively. Great variability in the composition and organization of these cell wall polysaccharides and proteins is seen depending on cell type, and developmental stages (Somerville et al., 2004). This difference and change in the polysaccharides and protein conformation is to help with selective modification of cell wall structure during synthesis, growth, elongation and subsequent re-organization of the cell. Interference in one polysaccharide has shown to affect the abundance and conformation of other cell wall polymers. Reduced cellulose production in *prc1* mutant (*CesA6*) also results in increased hemicellulose (Fagard et al., 2000). In potato the silencing of *CESA4* resulted in an increase in pectins (Oomen et al., 2004). The *acw1* mutant of KORRIGAN which showed reduced cellulose content and defects in the primary cell wall also showed increased pectin synthesis (Sato et al., 2001). Thus some form of tight-knit regulation and feedback mechanism between the proteins involved in the synthesis of the cell wall polysaccharides and proteins could be envisaged which could be triggered by a change in a protein or polysaccharide.

Some of the proteins seen interacting with the primary cell wall CESAs are known to have roles in the synthesis of other cell wall polymers. This interaction could be an indication of mediation between the polysaccharides to be able to adapt to constant change and remodeling in the cell wall. It seems reasonable to think that the construction, maintenance and restructuring of the cell wall architecture could be achieved with the collaborative action of many enzymes, such as xyloglucan endotransglycosylases (XTH) and expansins, which loosen cell walls (Cosgrove, 2005) as well as those responsible for the production of cell wall polysachharides like cellulose synthase (CESA). Other proteins involved in this mediation of cell wall feedback signalling would consist of CTL1 (CHITINASE-LIKE) proteins which plays a key role in establishing interactions between cellulose microfibrils and hemicelluloses (Sánchez-Rodríguez et al., 2012).

One of the main interactions between the cell wall polymers is between cellulose and hemicellulose. The hemicellulose xyloglucan is thought to bind to the cellulose microfibril surface or cross link adjacent microfibrils together to form the strong load bearing network (Hayashi, 1989; McCann et al., 1990). Thus, xyloglucan synthesizing proteins are key in the upkeep of cell wall strength and extensibility (Fry, 1989; Rose et al., 2002). Xyloglucan endo-transglycosylases (XTH) are a group of enzymes from glycoside hydrolase family 16

(Coutinho and Henrissat, 2006). They catalyze wall-loosening by rearranging xyloglucans through cleavage or by religating the existing xyloglucans in the wall. Many XTH proteins were detected interacting with the primary CESAs in our library screen (Chapter 5) suggesting an association between cellulose biosynthesis and cell wall loosening. In situations when cellulose synthesis is inhibited repression of XTH genes could be a strategy to prevent cell wall loosening (Bischoff et al., 2009).

Pectin is also known to participate in the cross-linking of cell wall polymers. The binding reactions between pectins and other wall components might be involved in wall assembly during deposition. Pectin consists of a backbone with various side chains attached. It has been suggested that its interaction with the cell wall polymers is through the side chains consisting of either arabinogalactan polymers or linear chains of arabinans and/or galactans. Pectin side chains have been shown to bind to cellulose which may be of considerable significance in the modelling of primary cell walls of plants as well as in the process of cell wall assembly (Iwai et al., 2001; Oechslin et al., 2003; Vignon et al., 2004; Zykwinska et al., 2005; Zykwinska et al., 2007). The *Arabidopsis* galacturonosyltransferase (GAUT1) which is known to synthesize homogalacturonan (HG) was detected in our library screen. Proteomic analysis has shown that the cellulase KOR1 involved in cellulose biosynthesis co-immunoprecipates with GAUT1 (Atmojo et al., 2011). The Korrigan *acw1* mutant has shown defects in both cellulose and pectin composition (Fagard et al., 2000) further supporting an association between them. The interaction of both proteins (GAUT1 and the CESAs) which are known to be involved in different pathways demonstrates the tight association of cell wall components.

Despite the demonstrated interaction between the CESAs with XTHs and GAUT1 the location of where this interaction could take place is also interesting to note. Xyloglucan hemicelluloses and pectins are known to be synthesized in the Golgi apparatus and then transported in vesicles to the cell surface. It has even been suggested that pectin-xyloglucan complexes are formed in the Golgi apparatus (Popper and Fry, 2008). Since the XTHs, GAUT1 and the CESAs have all been detected in the Golgi apparatus and all are membrane proteins, their interaction could occur at the Golgi membrane.

The cell walls of higher plants are mainly comprised of polysaccharides; however they also contain variable amounts of proteins which can function either structurally or enzymatically. One of the proteins known to be involved in the signalling feedback between the cell wall and the cytoplasm which has also been detected in our library screen is the Fasciclin like arabinogalactan proteins (FLAs). These Arabinogalactans proteins are part of the Hydroxyproline-rich glycoproteins (HRGPs) in plant cell walls and are mostly anchored to the plasma membrane (Lee et al., 2005), making an interaction with the CESAs at the plasma membrane more likely. The Hydroxyproline-rich glycoproteins are thought to interact with cellulose as it is deposited, and subsequently send signals back to the cytoplasm to regulate its synthesis (Seifert and Roberts, 2007; MacMillan et al., 2010). Based on the expression profile of FLAs during cotton fibre development and Arabidopsis stem development, a function in either primary cell wall development or secondary cell wall deposition has been suggested (Liu et al., 2008; Minic et al., 2009).

Other proteins such as expansins and extensins are thought to accommodate the loosening and subsequent strengthening of the cell walls in normal plant development (Fry, 1988; Carpita and Gibeaut, 1993). Interestingly some of these proteins were detected in our library screen indicating a direct physical interaction with the CESAs and possibly a direct role in cellulose biosynthesis. The expansion of constrained cell walls during cell wall growth requires the bonds between cellulose microfibrils and xyloglucan chains as well as those with the pectin side chains to break and allow the wall to stretch. Proteins like XTHs and expansins are thought to accommodate the loosening to allow for the expansion of the cell wall (Chapter 5). After cell expansion, the proteins present in the wall are cross linked with the cell wall polysaccharides resulting in rigidification of the wall (Passardi et al., 2004). Hydroxyproline-rich proteins (HRGPs), such as extensins are known to be involved in this process (Hall and Cannon, 2002). Extensins, like other cell-wall proteins, are incorporated into the insoluble polysaccharide network and are believed to have a structural role, forming the scaffolding upon which the cell-wall architecture is formed. These proteins are woven between the polysaccharide networks (Carpita and Gibeaut, 1993) and strengthen the wall (Brady et al., 1996). The knockout of AtEXT3(AT1G21310) detected in our library screen, has resulted in a lethal *root-*, *shoot-*, *hypocotyl-defective* (*rsh*) mutant which shows a defective cell wall assembly that could be ascribed to the absence of AtEXT3 seedling (Hall and Cannon, 2002; Cannon et al., 2008). Further analysis would be required to understand its exact role and where the association with the CESAs which comprise the cellulose synthase complex would occur *in planta*.

Many more proteins have been picked up in our library screen which could potentially have a role in cellulose biosynthesis; some have even been suggested to be responsible for the synthesis of other polysaccharides. However, it must be mentioned that further in depth analysis of these interactions would be required to fully conclude a direct association between them.

The future of cellulose synthase complex and cellulose biosynthesis

The data from this thesis has contributed in furthering our understanding of the cellulose synthase complex and its catalytic subunits, the CESAs as well as proteins interacting with the complex in the context of its direct synthesis. Data demonstrated in this thesis provides experimental proof for previously suggested models for cellulose biosynthesis and the direct association of proteins like KORRIGAN and SUS with the CESAs. Despite prior information and the data produced in this thesis, many questions surrounding the composition of the cellulose synthase complex and the accurate placement of proteins associated with the complex as well as their cellular localisation remain unanswered. Isolating an intact, pure, and fully functional complex would be the definitive method of answering some of these questions.

To further confirm the protein-protein interaction results determined in this thesis which studied the interaction in isolation, using techniques such as FLIM-FRET to analyse the protein-protein interaction of the candidates *in vivo* so that the spatial organization and quantification of the interactions can be characterized in a living cell would be beneficial

(Lleres et al., 2007). To further analyse the formation, localisation and function of the proteins comprising the complex or interacting with it, many paths could be taken for further exploration. Knowing more about the structure of the proteins, their domains, the folding and oligomerization of the protein would help in understanding its association with the complex. Analysing the post-translational modifications (i.e. phosphorylation/dephosphorylation) of the proteins would help to assess whether it would influence the formation, localization and function of the protein. Gaining more information on the transport of proteins inside the cell between organelles, their delivery to the plasma membrane, incorporation into the complex and secretion to the cell wall as well as focusing on the transcriptional regulation of cellulose synthase forming genes and studying the signalling and feedback between the extracellular environment and the intracellular organelles would help to understand how cellulose is synthesized and how this synthesis adapts to bigger changes occurring in the cell wall (Mizrachi et al., 2011).

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Summary

One of the characteristics of the plant kingdom is the presence of a structural cell wall. The cell walls are generally categorized into two types, primary cell walls and secondary cell walls. Both primary and secondary cell walls of higher plants are comprised of various polysaccharides, enzymes and proteins. The primary cell wall polysaccharides are comprised mainly of cellulose, hemicellulose and pectins, while the secondary cell wall consists of cellulose, hemicellulose and lignin. Cellulose is the major structural component of both the primary and secondary cell walls. In higher plants cellulose is synthesized by so called rosette protein complexes with cellulose synthases (CESAs) as catalytic subunits of the complex. The CESAs are divided into two distinct families, three of which are thought to be specialized for the primary cell wall and three for the secondary cell wall.

The objective of the research presented in this thesis was to generate more in-depth knowledge in cellulose biosynthesis and to this aim better characterize and understand the cellulose synthase complex and its components by notably investigating the similarities and differences between the CESAs in the primary and secondary cellulose complex and identifying the various interacting proteins forming the complex in the plant cell wall.

In Chapter 2, the parallels between the cellulose synthase complex of the primary and secondary cell wall are analysed and the potential ability of primary and secondary CESAs to form a functional rosette complex was investigated. The membrane-based yeast two-hybrid (MbYTH) and biomolecular fluorescence (BiFC) systems were used to assess the interactions between 3 primary (CESA1, CESA3, CESA6) and 3 secondary (CESA4, CESA7, CESA8) *Arabidopsis thaliana* CESAs. The results showed that all primary CESAs can physically interact both *in vitro* and *in planta* with all secondary CESAs. Although CESAs are broadly capable of interacting in pairwise combinations they are not all able to form functional complexes *in planta*. Analysis of transgenic lines showed that CESA7 can partially rescue CESA3 in the primary cell wall. GFP-CESA protein fusions revealed that when CESA3 was replaced by CESA7 in the primary rosette the velocity of the mixed complexes was slightly faster than the native primary complexes. Isoxaben treatment gave insights in the final assembly of rosette complexes in the small microtubule associated compartments (SMaCCs). CESA1 in turn can partly rescue CESA8, resulting in an increase of cellulose content relative to the *cesA8ko*. These results demonstrate that sufficient parallels exist between the primary and secondary complexes for cross-functionality and open the possibility that mixed complexes of primary and secondary CESAs may occur at particular times. These results demonstrate that additional selectivity exists within the plant cell, either through directed assembly or competition for interacting partners

In addition to the CESA proteins, cellulose biosynthesis almost certainly requires the action of other proteins, although few have been identified and little is known about the biochemical role of those that have been identified. One of these proteins is KORRIGAN (KOR1), a membrane-bound endo-1,4-β-D-glucanase with a single trans membrane domain and two putative polarized targeting signals in the cytosolic tail. In Chapter 3, the direct association of KORRIGAN with the CESA proteins in the primary and secondary cell wall were assessed to confirm its presence in the rosette structure. Using various methods, both *in vitro* and *in planta*, we have shown that KOR1 specifically interacts with the primary cell wall CESA proteins and with two secondary cell wall CESA proteins. This interaction was studied in more detail to identify the domain responsible for the interaction. Moreover, the localization and dynamics of KOR1 fused to green fluorescent protein (GFP), expressed under its endogenous KOR1 promoter in the *kor1-1* mutant background was analysed. This showed that GFP-

KOR1 is expressed in the same cells as GFP-CESA3 in the epidermis of etiolated hypocotyls further supporting a model in which KOR1 participates in the Cellulose Synthase Complex at the plasma membrane.

In addition to CESA proteins, cellulose biosynthesis requires the action of other proteins one of which is sucrose synthase (SUS) which is suggested to provide the UDP-Glucose substrate for cellulose biosynthesis. In Chapter 4 the interactions between each of the CESA proteins in the primary and secondary cell walls with the sucrose synthase isoforms in Arabidopsis were tested to confirm its role as the UDP-Glucose substrate provider for cellulose biosynthesis. Using both *in vitro* and *in planta* protein interaction assays it is shown that not all the sucrose synthase isoforms are able to interact with the rosette complex. In order to further verify the direct association of the CESAs with the Sucrose synthase isoforms the localization of SUS6 fused to green fluorescent protein (GFP), expressed under its endogenous SUS6 promoter in a fluorescently tagged CESA6 mutant background was analysed. Colocalization of SUS6 and CESA6 was shown at the plasma membrane and the Golgi apparatus supporting a role for sucrose synthase in providing UDP-Glucose for the Cellulose Synthase Complex at the plasma membrane.

Although the CESA's are quite well characterized, it has been difficult to identify other proteins that are involved in cellulose biosynthesis and part of the rosette complex. In Chapter 5, in order to identify new candidates involved in cellulose biosynthesis, a library screen was performed with the membrane-based yeast two-hybrid system using each of the three primary CESA proteins as bait (CESA 1, 3 and 6). Several criteria were used to discriminate between the proteins found which resulted in a large list of 183 proteins appearing more relevant in having a role in cellulose biosynthesis with 30 of them highlighted based on close expression patterns to the CESAs. Some of the candidates showed interaction with at least two out of three CESA proteins with the list consisting of some proteins known to be involved in cell wall metabolism, such as the endo-chitinase-like gene CTL1 alongside other proteins not known to have had previous links to cellulose biosynthesis thus making them promising targets for future research.

Overall, Chapter 6 discusses the data from this thesis and how it has contributed in furthering our understanding of the cellulose synthase complex and its catalytic subunits, the CESAs as well as proteins interacting with the complex in the context of its direct synthesis.

Samenvatting

Eén van de kenmerken waarin planten zich onderscheiden van andere organismen is dat ze een celwand bezitten. Er zijn twee soorten celwanden, primaire en secundaire. Alhoewel ze beiden bestaan uit suikers, enzymen en structurele eiwitten is de samenstelling verschillend. Primaire celwanden bestaan hoofdzakelijk uit cellulose, hemicellulose en pectine terwijl secundaire celwanden bestaan uit cellulose, hemicellulose en lignine. Cellulose dat in zowel primaire als secundaire celwanden de belangrijkste structurele component is wordt gesynthetiseerd in rozet eiwit complexen waarbij cellulose synthasen (CESAs) de enzymatische activiteit verzorgen. De CESAs zijn onderverdeeld in twee specifieke families met elk drie leden voor zowel de primaire als secundaire cel wanden.

Het doel van het in dit proefschrift beschreven onderzoek was om meer inzicht te verkrijgen in de cellulose biosynthese via een gedetailleerd onderzoek van de cellulose synthase complexen en de verschillende componenten (eiwitten en enzymen) die deel uitmaken van deze complexen. Met name de identificatie van inter-acterende eiwitten in de verschillende complexen en de verschillen en/of overeenkomsten tussen de CESas in de primaire en secundaire celwand werden onderzocht.

In Hoofdstuk 2 zijn de resultaten beschreven van experimenten die moesten uitwijzen of leden van de CESAs uit de primaire celwand complexen in staat zijn om ook functionele rozet complexen voor de secundaire celwand te vormen en *vice versa*. Twee verschillende analyse systemen werden gebruikt om de interactie van de 3 primaire (CESA1, CESA3 en CESA6) en de drie secundaire (CESA4, CESA7 en CESA8) cellulose synthasen te onderzoeken: het 'membrane based yeast two hybrid' systeem (MbYTH) en biomoleculaire fluorescentie microscopie (BiFC). De resultaten laten zien dat de CESAs van de primaire celwand zowel *in vitro* als *in planta* fysiek interacties aan kunnen gaan met al de CESAs van de secundaire celwand. Alhoewel CESAs in principe in staat zijn om in paarwijze combinaties met elkaar te interacteren geldt niet dat alle CESAs in de plant functionele complexen met elkaar kunnen vormen. Analyse van transgene lijnen liet zien dat CESA7 de activiteit van CESA3 in de primaire celwand gedeeltelijk kan complementeren. GFP-CESA eiwit fusies toonden aan dat wanneer CESA3 vervangen werd door CESA7 in het primaire celwand rozet complex de snelheid van cellulose synthese van de gemengde complexen iets hoger lag dan die van de oorspronkelijke primaire rozet complexen. Behandeling met Isoxaben werd gebruikt om te zien hoe de rozet complexen werden geassembleerd in zogenaamde "small microtubule associated compartments" (SMaCCs). CESA1 op zijn beurt is in staat om gedeeltelijk de activiteit van CESA8 te vervangen hetgeen resulteert in een toename van de hoeveelheid cellulose relatief ten opzichte van het cellulose gehalte in de mutant *cesA8ko*. Deze resultaten laten duidelijk zien dat er veel parallellen bestaan tussen de primaire en secundaire complexen voor wat betreft kruisfunctionaliteit. Dit maakt het zeer waarschijnlijk dat ook *in planta* op gezette momenten in de ontwikkeling van de plant er gemengde complexen van primaire en secundaire CESAs in de celwanden voor komen. Deze resultaten laten ook zien dat er additionele selectiviteit is in de plantencel voor wat betreft de directe samenstelling van de complexen met verschillende CESAs of vanwege het feit dat bepaalde CESAs bij gelijktijdige aanwezigheid met elkaar in concurrentie zijn voor een plaats in de cellulose synthasen complexen. Naast de CESA eiwitten zijn ook andere eiwitten noodzakelijk in het cellulose synthetiserende complex, zowel structurele eiwitten als eiwitten met een enzymatische activiteit, om tot een goede cellulose biosynthese te komen. Tot nu toe zijn er echter zeer weinig eiwitten geïdentificeerd en is over hun biochemische rol in het proces nog minder bekend. Een van de eiwitten is KORRIGAN (KOR1), een membraan-gebonden endo-1,4-β-D-glucanase met een enkele trans membraan domein en twee mogelijke signaal sequenties in het cytosolische deel.

In Hoofdstuk 3 is de directe associatie tussen KORRIGAN en de CESA eiwitten in de primaire en secundaire celwand in meer detail bestudeerd. Door verschillende analyse methoden te gebruiken kon worden aangetoond, zowel *in vitro* als *in planta*, dat KOR1 interacties aangaat met alle primaire celwand CESA eiwitten maar met maar twee van de drie CESA eiwitten van de secundaire celwand. Het domein in KOR1 dat verantwoordelijk is voor de directe interactie kon worden geïdentificeerd. Daarnaast werd met behulp van het GFP (green fluorescent protein) indicator gen, onder controle van zijn eigen (KOR1) promoter, tot expressie gebracht in de *kor1-1* mutante achtergrond aangetoond dat dit GFP-KOR1 in dezelfde cellen tot expressie komt als GFP-CESA3. Dit ondersteund dus het model waarin KOR1 deel uitmaakt van het Cellulose Synthase Complex in de plasma membraan.

Naast de CESA eiwitten zijn er voor een goede cellulose biosynthese andere eiwitten (enzymen) noodzakelijk. Een daarvan is sucrose synthase (SUS) dat verantwoordelijk wordt geacht voor de aanlevering van het substraat UDP-glucose noodzakelijk voor cellulose biosynthese. In Hoofdstuk 4 zijn de resultaten beschreven van de studies naar de interacties tussen elk van de CESA eiwitten uit de primaire en secundaire celwand met verschillende sucrose synthase isovormen in Arabidopsis. Met zowel *in vitro* als *in planta* eiwit interactie toetsen kon worden aangetoond dat slechts enkele sucrose synthase isovormen in staat zijn om interactie met CESAs te vertonen in het rozet complex. Om een directe associatie van CESAs met sucrose synthase isovormen aan te tonen werd één van de sucrose synthasen (SUS6; gefuseerd met GFP) tot expressie gebracht onder zijn eigen promoter en getransformeerd naar een CESA6 fluorescent gemerkte mutante achtergrond teneinde localisatie van dit complex zichtbaar te kunnen maken. Co-localizatie van SUS6 en CESA6 was duidelijk aan te tonen zowel aan/in de plasma membraan als in het Golgi complex hetgeen de rol voor sucrose synthase als leverancier voor het substraat UDP-glucose voor het Cellulose Synthase Complex bevestigd.

Alhoewel de CESA's redelijk goed gekarakteriseerd zijn is het tot nu toe zeer moeilijk gebleken andere eiwitten te identificeren die deel uitmaken van het rozet complex en betrokken zijn bij de cellulose biosynthese. In Hoofdstuk 5, worden experimenten beschreven waarbij met behulp van een zogenaamde 'library screen' met het MbYTH systeem en de drie primaire CESA eiwitten als beet (CESA 1, 3 and 6) verschillende potentiële eiwit kandidaten geïsoleerd en geïdentificeerd werden. Verschillende criteria werden gebruikt om in de lange lijst van eiwit kandidaten de meest waarschijnlijk kandidaten te kunnen aanwijzen. Dit resulteerde uiteindelijk in een lijst van 183 kandidaat eiwitten waarvan er 30 op grond van hun op CESAs gelijkende gen expressiepatronen de meest voor de hand liggende kandidaat-genen lijken te zijn om verder onderzoek aan en mee te verrichten. Sommige van de kandidaat-genen vertoonden interactie met twee van de drie CESA eiwitten en bleken voor een deel te bestaan uit eiwitten waarvan, uit andere studies, bekend was dat ze op de een of andere wijze bij de celwand biosynthese betrokken moesten zijn zoals bijvoorbeeld het endo-chitinase-like gen CTL1. Van een groot deel van de andere eiwitten was een link met celwanden of cellulose biosynthese niet bekend.

Concluderend kan gesteld worden dat de resultaten beschreven in dit proefschrift ons begrip van het Cellulose Synthase Complex en de rol van de verschillende CESAs in de primaire en secundaire celwand complexen aanzienlijk heeft verbeterd. Daarnaast is er een eerste stap gezet om de exacte samenstelling van de eiwit complexen te ontrafelen doordat een groot aantal kandidaat eiwitten zijn geïdentificeerd en geïsoleerd waarmee gerichter toekomstige studies kunnen worden uitgevoerd.

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I would also like to thank Marian and Pierre for being my paranymphs! I am looking forward to sharing the special day with you.

Although I was already accustomed to the European lifestyle, being away from my family and friends was still tough. Luckily I soon found groups of friends from a wide variety of cultures who made the distance more bearable. The surprising mix of various cultures at Wageningen was a wonderful opportunity to get to know different people with different backgrounds and cultures. Some are still here and some have already left. I am very happy that my friendship with those who have already left has extended beyond our shared time in Wageningen and hope that it will be the

same for those still here. I would like to thank you all for your company and for being sources of laughter and support during these years. Arwa, thank you for the good times we had all through this journey, especially near the end. Mirjana, Anitha, Anna, Maria Del mar, Giulia, Yusuf, Gert, thanks for the fun and laughter you brought with you. Joana, thank you for the great time in Lisbon and a big thank you to your mom for her kindness and hospitality. Paula and Cesar, Natalia and Bjorn, Sharmistha and Animesh, Hulya and Serdal, Rafael and Marcela, Madhurri and Manoj, Natalia Carreno, Johan Bucher, Peter, Ram, Bjorn Kloosterman, Brigitte, Luis, Nico, Ningwen, Antoine, Gerard Bisterbosch, Mathieu, Sabaz, Zheng, Marten, Efstathios, Thijs, Long, Eugene, Paweena, Dongli, Suxian, Koen and many many others, thanks for the great company and the incredible memories.

I cherished the moments with my Iranian friends both in Wageningen and around the world, with whom I celebrated dinners, birthday parties, graduation ceremonies and many more. Thank you for making life in the Netherlands feel more like home. I would also like to thank all my friends back home for all the joy and laughter they provided from afar.

My deepest gratitude goes to my family without whom I have nothing. I am grateful for your unconditional love throughout my life and for moulding me into the person I am today. Despite the thousands of miles distance between us, you were with me every step of the way. I am not exaggerating when I say this dissertation would have simply been impossible without your constant encouragement and support. I am beyond happy to have you here today! I am indebted to my father for his love, wisdom and constant support. Daddy Kuimarce, thank you for being my DAD! I draw strength from you being next to me. It is an honour to be your daughter and carry your name. Words cannot describe my gratitude to my mother, Maman Nozhat you are the biggest source of inspiration in my life. Thank you for your love, understanding, endless patience and encouragement. I would not have contemplated this road were it not for you leading by example and instilling within me a love of creative pursuits. I hope I have made you just as proud of me as I am of you. My dearest Sahar, You are the love and fun of my life! I am blessed to have such a strong and caring twin sister. Thank you for being my support system from afar! My mere expression of thanks does not suffice. I love you all beyond words.

There are many many more people I would like to thank for playing a part in my time at Wageningen. I may have missed your names here, but you are always remembered and appreciated.

Thank you

About the author

Nasim Mansoori Zangir was born on 21st of September 1978 in Tehran, Iran. After finishing high school she started her academic studies for her BSc degree in the field of Agronomy and Plant Breeding at Tehran University. She then obtained her Master's degree in Plant Breeding at Tarbiat Modares University in Tehran. She started her PhD in the Plant Breeding Department, Bio-based economy group at Wageningen University in 2008. This thesis presents the outcome of her four years PhD research on "Characterising the Cellulose Synthase Complexes of Cell Walls".

Education Statement of the Graduate School

Experimental Plant Sciences

The Graduate School EXPERIMENTAL PLANT SCIENCES

Issued to: **Nasim Mansoori Zangir**
Date: **25 June 2012**
Group: **Plant Breeding, Wageningen University & Research Centre**

1) Start-up phase	*date*
► **First presentation of your project**	
Protein complexes in plant cell walls	Sep 08, 2008
► **Writing or rewriting a project proposal**	
Charchterization of membrane bound protein complexes involved in the cell wall	Jul 2008
► **Writing a review or book chapter**	
► MSc courses	
► **Laboratory use of isotopes**	
Subtotal Start-up Phase	*7,5 credits**

2) Scientific Exposure	*date*
► **EPS PhD student days**	
EPS PhD student day, Leiden University	Mar 26, 2009
EPS PhD student day, Utrecht University	Jun 01, 2010
International PhD Retreat, Cologne (Germany)	Apr 15-17, 2010
EPS PhD student days, Wageningen University	May 20, 2011
► **EPS theme symposia**	
EPS theme symposia 3 "Metabolism and Adaptation", University of Amsterdam	Feb 18, 2009
EPS theme symposia 3 "Metabolism and Adaptation", Leiden University	Feb 19, 2010
EPS theme symposia 3 "Metabolism and Adaptation", Wageningen University	Apr 26, 2011
► **NWO Lunteren days and other National Platforms**	
ALW meeting Lunteren Experiment Plant Sciences	Apr 07-08,2008
ALW meeting Lunteren Experiment Plant Sciences	Apr 06-07, 2009
ALW meeting Lunteren Experiment Plant Sciences	Apr 19-20, 2010
ALW meeting Lunteren Experiment Plant Sciences	Apr 04-05, 2011
ALW meeting Lunteren Experiment Plant Sciences	Apr 02-03, 2012
► **Seminars (series), workshops and symposia**	
European flying seminars: Richard Vierstra, Simon Gilroy, and Zhenbiao Yang	Apr - June, 2008
Raising the BAR for Arabidopsis Research: Using Large-scale Data Sets for Hypothesis Generation'	Jun 13, 2008
Mechanism and function of active DNA demethylation in Arabidopsis	Nov 03, 2008
Seminar Series Plant Sciences -1 and -2	Sep - Oct 2009
seminars of Tal Dagan, Wim Soppe and Valerie Williamson	Oct 2009
Agrigenomics seminar	Mar 03, 2010
Plant Research Day (Plant Breeding)	Jun 17, 2008
Plant Research Day (Plant Breeding)	2010
Plant Research Day (Plant Breeding)	Mar 08, 2011
Plant Research Day (Plant Breeding)	Feb 28, 2012
► **Seminar plus**	
► **International symposia and congresses**	
International cell wall meeting ,Porto, Portugal	Jul 25-30, 2010
International workshop Systems Biology and Design - Wageningen	Oct 06-08, 2010
9th International Botanical Microscopy Meeting	Apr 16-21, 2011

Fourth Conference on Biosynthesis of Plant Cell wall	Oct 02-06, 2011
► **Presentations**	
Specific isoforms of susy are integral components of the cellulose synthase complex, yeast two hybrid approach, PhD retreat Cologne (poster)	Apr 15-17, 2010
Specific isoforms of susy are integral components of the cellulose synthase complex- Cell wall meeting - Porto (poster)	Jul 25-30, 2010
Korrigan interacts specifically with the components of the cellulose synthase complex in the primary cell wall-phd plant retreat (poster)	Apr 15-17, 2010
Sucrose synthase in the cellulose synthase complex- INRA-versailles-France (oral)	Dec 03, 2010
Cellulose biosynthesis complexes in Arabidopsis -Joint meeting with cell biology department WUR (oral)	Jan 14, 2011
Characterisation of the memebrane bound protein complexes in cellulose biosynthesis-ALW meeting Lunteren (oral)	Apr 05 2011
KORRIGAN interacts specifically with the components of the cellulose synthase complex in the primary and secondary cell wall - Japan (oral)	Oct 05 2011
Characterizing the cellulose synthase complex in Arabidopsis'-ALW meeting Lunteren Experiment Plant Sciences (oral)	Apr 03 2012
► **IAB interview**	Feb 2011
► **Excursions**	
Visit KEYGENE breeding company	Jan 26, 2012
Subtotal Scientific Exposure	*22,1 credits**

3) In-Depth Studies	*date*
► **EPS courses or other PhD courses (higly recommended)**	
Gateway to Gateway Technology	Nov 17-21, 2008
A Dip into EBI's Data Resources course - Cambridge - UK	Jul 08-11, 2009
Bioinformatics-a user's approach	Aug 30-Sep 03, 2010
Systems biology summer school -Venice -Italy	Sep 12-16, 2011
► **Journal club**	
Weekly literature discussion at plant breeding	2008-20012
► **Individual research training**	
protein-protein interaction assay - Split-YFP - INRA - Versailles - France	Jul 2010
Subcellular localization of Sucrose Synthase Isoforms - INRA - Versailles -France	Nov/Dec 2010
FTIR Analysis- INRA - Versailles - FRANCE	Oct/Nov 2011
Subtotal In-Depth Studies	*10,8 credits**

4) Personal development	*date*
► **Skill training courses**	
Mobilising your scientific network	Jun 03 & 10, 2010
Presentation skills	May 12 & 26, 2010
PhD competence assessment	2008
► **Organisation of PhD students day, course or conference**	
Lab trip Plant Breeding	Sep 17 2010
► **Membership of Board, Committee or PhD council**	
Subtotal Personal Development	*3,8 credits**

TOTAL NUMBER OF CREDIT POINTS*	**44.2**

Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS which comprises of a minimum total of 30 ECTS credits

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

Cover picture: An outsider’s grasp on cellulose biosynthesis in plant cell walls.

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