This is to certify that the
dissertation entitled

Cell wall biosynthesis in Zea Mays

presented by

Henricus EG van Erp

has been accepted towards fulfillment
of the requirements for the

Ph. D. degree in Genetics

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11-28-2007
Date

CELL WALL BIOSYNTHESIS IN *ZEA MAYS*

By

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A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Genetics

2007

ABSTRACT

CELL WALL BIOSYNTHESIS IN *ZEA MAYS*

By

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For my thesis research I studied dark-grown maize seedlings as a model system for cell wall biosynthesis. When maize seedlings are grown in complete darkness an organ develops called the mesocotyl, which serves to position the seedling towards the light. After exposure to light, the rapid elongation of the mesocotyl is reduced and concomitant with this, certain enzymatic activities related to cell wall biosynthesis are down-regulated, including a Golgi-localized glucan synthase. I attempted to identify the glucan synthase with biochemical methods and characterized the product it synthesizes. I established that it synthesizes (1,4)-β-glucan. Because pure (1,4)-β-glucan is not known to be synthesized in the Golgi, the glucan synthase might be involved in the synthesis of other polysaccharides, such as xyloglucan or mixed-linkage glucan (MLG). The glucan synthase was successfully solubilized with digitonin and it still synthesized (1,4)-β-glucan. However, after chromatography glucose-6-phosphate and glucose-1,6-diphosphate were synthesized instead of (1,4)-β-glucan.

I studied the correlation of the light-regulation of cell wall biosynthetic enzymatic activities to transcript levels of the maize *Cellulose Synthase Like (CSL)* gene family. The *CSL* gene family encodes the enzymes involved in the synthesis of the (1,4)-β-linked glycan backbones of hemicelluloses such as xyloglucan, MLG and (1,4)-β-mannan. As a first step, I annotated the maize *CSL* gene family. The rice and maize *CSL* gene families are similar, except for a few differences. The *CSLC* gene family is expanded

from five genes in *Arabidopsis* and six in rice to potentially twelve in maize. Also, an atypical *CSL* gene was found called *CSLX*. The CSLX protein is closely related to the CSLG proteins from poplar, which suggests that the CSLX protein belongs to the CSLG subfamily. Assays for enzymatic activities related to cell wall biosynthesis such as callose synthase, xylan synthase, glucan synthase, mannan synthase, UDP-galactose incorporating activity and latent inosine diphosphatase (IDPase) were performed. Mannan synthase and UDP-galactose incorporation were strongly reduced by light-treatment. Some of the *CSLA* genes encode for mannan synthases. The reduction in mannan synthase activity correlated with the reduction in *CSLA* transcript levels after light-treatment. This suggests that mannan biosynthesis is regulated at the level of transcription.

ACKNOWLEDGEMENTS

I would like to thank Jonathan Walton for accepting me in his laboratory. He is an excellent adviser who always has time for scientific discussion. I would like to thank my committee members, Ken Keegstra, Kathy Osteryoung, Andreas Weber, Jonathan Walton, Beronda Montgomery-Kaguri and Marcus Pauly for giving me guidance and support during my Ph. D. I want to thank my past and previous lab members for the good times we had and the help with my experiments, especially Peter Kuhn and Melissa Borrusch. I want to thank the past and present members of the Keegstra lab for their help. I want to thank Prof. Jurgen Abel who spent a few weeks during two summers helping me with the identification of the (1,4)-β-glucan synthase. I want to thank our secretaries for arranging all my papers and making travel arrangements. I want to thank Jeannine Lee our genetics secretary for all her help and her nice parties. I want to thank Barb Sears, our current genetics program director, and the previous director Helmut Bertrand for leading the genetics program. I want to thank my friends for all the fun time we had and the helpful scientific discussions, especially Hiroshi Maeda. I want to thank my parents and my parents in law for their great support. I would like to thank my wife Young Nam Lee for supporting me.

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CHAPTER 1: LITERATURE REVIEW

Plant cell walls have diverse biological functions

The cell wall is a defining feature of plants and has a variety of functions including structural support and defense against pathogens. The cell wall gives plant cells the shapes they need to perform their function as shown by tracheary elements or petals (Carpita and McCann, 2000). Cell walls provide the force needed to cope with the turgor pressure exerted by the vacuole. When plant cells take up water in the vacuole pressure is exerted on the cell wall and subsequent loosening of the cell wall allows plant cells to expand. Cell walls have a role in defense against pathogens. After pathogen attack papillae are formed below the site of infection in order to provide a physical barrier. They are composed of callose, proteins and other components (Aist, 1976). The cell wall is a source of signaling/regulatory molecules. Xyloglucan fragments can inhibit auxin-induced elongation growth of pea epicotyls (York et al., 1984) and homogalacturonan oligosaccharides can induce the synthesis of phytoalexins in plants (Nothnagel et al., 1983). Polysaccharides can function in carbon storage in seeds. Examples of storage polysaccharides are (1,4)-β-mannan, xyloglucan and galactans (Buckeridge et al., 2000).

The importance of the cell wall in growth and development is shown by mutations in genes related to cell wall biosynthesis. Mutants in cellulose synthase genes involved in secondary cell wall biosynthesis show a collapsed xylem phenotype (Taylor et al., 1999, 2000, 2003). Mutations in genes involved in the synthesis of the pectic polysaccharide rhamnogalacturonan 2 (RG2), show severe growth defects and cell adhesion defects (O'Neill et al., 2001; Iwai et al., 2002).

Organization of the apoplast

The continuum of cell wall material and liquid between individual plant cells is called the apoplast. The cell walls of individual cells are connected with a cell wall layer composed of pectin and proteins called the middle lamella (Carpita and McCann, 2000). The next layer of cell wall material, which is deposited at the inside of the middle lamella, is called the primary cell wall. It is composed of cellulose, hemicelluloses and proteins and it provides strength but at the same time allows plant cells to expand. The secondary cell wall is deposited in certain cell types at the inside of the primary cell wall when plant cells stop elongating (Carpita and McCann, 2000). It is more rigid and generally contains more lignin. The development of xylem vessels composed of secondary cell walls was a major adaptation for land plants to assume upright growth and transport solutes over long distance (Bateman et al., 1998).

Different plants vary in their cell wall composition

A complicating factor in the understanding of cell wall biosynthesis is that different groups of plants have different types of primary cell walls, although the main component, cellulose, is identical in all plants. Two types of primary cell walls can be distinguished: type I and type II. Dicots and group B monocots have a type I cell wall. In type I cell walls xyloglucan is the major hemicellulose cross-linking the cellulose microfibrils, and structural proteins strengthen the primary cell wall. The monocots can be distinguished further based on the presence (group A) or absence (group B) of ester-linked ferulic acids in their primary cell walls. The group A monocots have a type II cell wall. In the type II cell wall, glucuronoarabinoxylan (GAX) is the major cross linking

hemicellulose, and xyloglucan and glucomannan are relatively minor component (Carpita et al., 2001, Smith and Harris, 1998). The type II cell wall of the group A monocots can be distinguished further based on the presence or absence of mixed-linkage glucan (MLG). Cell walls of the family of the Poales (grasses), which belongs to the group A monocots contains MLG (Carpita and Gibeaut, 1993, Smith and Harris, 1998).

Cell wall proteins and polysaccharides

Cellulose biosynthesis and the *CESA* gene family

Cellulose is the major load-bearing polymer in the primary cell wall and is synthesized at the plasma membrane in terminal rosette complexes (Kimura et al., 1999). The rosettes consist of six globules that each synthesize six β(1,4)-glucan chains. Together these 36 β(1,4)-glucan chains compose the cellulose microfibrils. The (1,4)-β-glucan chains are held together by hydrogen bonds and Vanderwaals interactions (Nishiyama et al., 2002, 2003) and their length varies between the primary and secondary cell wall. Generally the chains are shorter in the primary cell wall (2000-8000 glucose residues) than in the secondary cell wall (14,000-15,000 glucose residues) (Brown et al., 2004).

The first plant cellulose-synthase genes (*celA1* and *celA2*) were identified from a cotton cDNA library based on similarity to bacterial cellulose synthases from *Acetobacter xylinum* and *Agrobacterium tumefaciens* (Pear et al., 1996). Arioli et al. (1998) provided genetic evidence that *AtCESA1* is involved in cellulose biosynthesis. With immunogold labeling it was shown that the globules involved in cellulose

biosynthesis consist of cellulose synthase (CESA) proteins (Kimura et al., 1999). In vitro assays for cellulose biosynthesis were established by Lai-Kee-Him et al. (2002). They solubilized intact cellulose synthase complexes from *Rubus fruticosus* (blackberry) and synthesized cellulose in the presence of UDP-glucose. No Mg^{2+} was required for cellulose biosynthesis in vitro.

The CESA proteins contain two conserved domains. Domain A consists of 3 widely spaced Asp (D) residues and is thought to be involved in UDP-Glc binding. Domain B consists of a conserved domain, QXXRW, hypothesized to be part of the catalytic site (Richmond et al., 2000). Domain A is conserved in both processive- and non-processive glycosyltransferases. Only processive enzymes contain domain B (Richmond et al., 2000). Different CESA proteins are involved in cellulose biosynthesis in the primary and secondary cell walls. The CESA1, 3, and 6 proteins are involved in cellulose biosynthesis in the primary cell wall (Fagard et al., 2000; Arioli et al., 1998; Ellis et al., 2002; Burn et al., 2002). The CESA4, 7, and 8 proteins are involved in cellulose biosynthesis in secondary cell walls (Taylor et al., 1999, 2000, 2003; Zhong et al., 2003). The CESA proteins belong to the glycosyltransferase family (GT) 2 of carbohydrate active enzymes (www.cazy.org; Campell et al., 1997; Coutinho and Henrissat, 1999; Coutinho et al., 2003). For a review about cellulose biosynthesis see Somerville (2006).

Other proteins associated with the cellulose synthase complex are sucrose synthase and microtubules (Salnikov et al., 2001). Microtubules can guide the cellulose synthase complexes, but movement of the CESA complexes is not random in their absence, suggesting that these complexes can also be guided by polymerization of the

cellulose microfibrils (Paradez et al., 2006). Other proteins which might be involved in cellulose biosynthesis are the cellulase KORRIGAN (Nicol et al., 1998), the putative glycosylphosphatidylinositol (GPI) anchored protein COBRA (Schindelman et al., 2001), the kinesin-like protein FRAGILE FIBER 1 (FRA1) (Zhong et al., 2002), the novel plasma membrane protein KOBITO1 (Pagant et al., 2002) and the α-glucosidase I KNOPF (Gillmor et al., 2002).

Hemicelluloses and the *CSL* gene family

Cross-links between the cellulose microfibrils are formed by hemicelluloses such as xyloglucan, xylan, mixed-linkage glucan, and glucomannan, and pectin. The substrates for hemicellulose biosynthesis are nucleotide sugars, which are synthesized from UDP-glucose by nucleotide sugar-converting enzymes (Seifert, 2004). They are hypothesized to be in complexes with glycosyltransferases and nucleotide-sugar transporters at the Golgi membrane (Seifert, 2004). Once the hemicelluloses are synthesized in the Golgi, they are transported to the plasma membrane in vesicles, secreted, and incorporated into the cell wall (Moore et al., 1991).

Enzymes encoded by the *Cellulose Synthase Like* (*CSL*) (GT2) gene family are hypothesized to encode for the enzymes synthesizing the β(1,4)-linked hemicellulose backbones. The *CSL* gene family was identified based on homology to the *CESA* gene family and is composed of 30 members in Arabidopsis (Cutler and Somerville, 1997) and 37 members in rice (Hazen et al., 2002). In dicots such as Arabidopsis the *CSL* gene family consists of the *CSLA, B, C, D, E* and *G* subfamilies (Cutler and Somerville, 1997). In rice the *CSL* gene family consists of the *CSLA, C, D, E, F* and *H* subfamilies

(Hazen et al., 2002). The *CESA, CSLB, D, E, G, F* and *H* genes most likely originated from a cyanobacterial ancestor. The *CSLA* and *C* genes most likely originated from a different bacterial ancestor (Nobles and Brown, 2004).

For several *CSL* genes a function has been determined. AtCSLC4 is involved in the synthesis of the β(1,4)-linked glucan backbone of xyloglucan (Cocuron et al., 2007). OsCSLF2 is involved in the synthesis of mixed-linked glucan (Burton et al., 2006) and the CSLA proteins are involved in (gluco)mannan biosynthesis (Dhugga et al., 2004; Liepman et al., 2005; Suzuki et al., 2006; Liepman et al., 2007). For several *CSL* genes, a biological function has been found using a genetics approach. *AtCSLD3* is important for root hair growth in *Arabidopsis* (Wang et al., 2001; Favery et al., 2001) and pollen tube growth in *Nicotiana alata* (Doblin et al., 2001). *AtCSLA7* is involved in pollen-tube growth and embryogenesis in *Arabidopsis* (Goubet et al., 2003) and the *AtCSLA9* mutant *rat4* is resistant to transformation by *Agrobacterium tumefaciens* (Zhu et al., 2003).

Xyloglucan

Xyloglucan is present in the primary cell walls of all land plants. It is not present in charophycean green algae, indicating that xyloglucan might have been an important adaptation for plants to colonize land (Popper and Fry, 2003). The core structure of all xyloglucans is a β(1,4)-linked glucan backbone decorated with α-(1,6)-linked xylose residues. In the dicot *Arabidopsis* the xylose residues are spaced very regularly. Three consecutive glucose residues are decorated with α-(1,6)-linked xylosyl residues, followed by an unsubstituted glucose. The xylose residues in *Arabidopsis* can be decorated further

by β-(1,2)-linked galactose, which subsequently can be substituted with α-(1,2)-linked fucose.

The xyloglucan structure in monocots is different from dicots. In monocots only 30-40% of the glucose residues are substituted with xylose (Kato et al., 1982). As a result, a major difference between dicot and monocot xyloglucans is their solubility. Dicot xyloglucan is soluble in water, whereas monocot xyloglucan can only be dissolved in alkali solutions.

In vitro assays for dicot xyloglucan biosynthesis were developed by several researchers (Ray et al., 1980; Hayashi and Matsuda 1981; Gordon and Maclachlan, 1989). Hayashi and Matsuda (1981) determined that UDP-glucose and UDP-xylose are both necessary in order to synthesize xyloglucan in vitro. Incorporation of UDP-xylose into xyloglucan is stimulated by Mn^{2+}, but not by Mg^{2+} (Hayashi and Matsuda, 1981). It was determined that UDP-xylose stimulates the incorporation of UDP-glucose into xyloglucan and that UDP-glucose stimulates the incorporation of UDP-xylose into xyloglucan (Hayashi and Matsuda, 1981). This indicates that the synthesis of the β(1,4)-glucan backbone and the addition of xylosyl side chains need to occur simultaneously. The reason for this might be the insolubility of unsubstituted β(1,4)-glucan. A curious observation is that UDP-xylose only stimulates the incorporation of UDP-glucose into xyloglucan if the UDP-xylose concentration is lower than the UDP-glucose concentration (Hayashi and Matsuda, 1981; Gordon and Maclachlan, 1989). The reason for this is not clear. The β(1,4)-glucan backbone of xyloglucan is synthesized by members of the CSLC protein family (Cocuron et al., 2007). The xyloglucan xylosyltransferase (*XT*) (GT34) genes were identified by Faik et al. (2002) and Cavalier et al. (2006). An in vitro assay

for the galactosyltransferase was developed by Faik et al., (1997). Degalactosylated xyloglucan was used as a substrate for the galactosyltransferase. A galactosyltransferase (*MUR3*) (GT47) was identified by Madson et al. (2003). An in vitro assay for the fucosyltransferase was developed by Camarind and Maclachlan (1986). The fucosyltransferase (*FUT*) (GT37) was identified by Perrin et al. (1999) and further studied by Faik et al. (2000) and Vazin et al. (2002).

XTH proteins

It is thought that cross-linking of the cellulose microfibrils by xyloglucan is important in the regulation of cell elongation in dicots (Cosgrove, 2005). The hydrolysis and the subsequent reannealing of xyloglucan allow plant cells to expand while maintaining strength. The proteins involved in this process are the xyloglucan endotransglucosylase/hydrolases (XTH) (Rose et al., 2002). In Arabidopsis there are 33 XTH genes and their expression pattern is correlated with elongation growth (Yokoyama and Nishitani, 2001). In rice there are 29 members (Yokoyama et al., 2004), which suggests that xyloglucan also has an important role in cell elongation in grasses. XTH proteins are not active on substrates other than xyloglucan (Smith and Fry, 1991; Fry et al., 1992). However, Hrmova et al. (2007) showed that HvXTH5 can facilitate the formation of covalent bonds between xyloglucan and cellulose or MLG, although this was a minor activity of this protein.

Xylan

Xylans consist of a backbone of (1,4)-β-linked xylose residues, which can be substituted with arabinose, ferulic acid, glucuronic acid or methylglucuronic acid residues. Xylan can also be highly acetylated (Teleman et al., 2000). In both dicots and monocots the xylan backbone is decorated with arabinose and glucuronic acid residues. Group A monocots have arabinose at the O-3 position, glucuronic acid at the O-2 position of the xylose residues, and feruloyl groups at O-5 position of the arabinose residues. Group B monocots and all dicots have arabinose residues at the O-2 and O-3 position and glucuronic acid at the O-2 position (Carpita and McCann, 2000).

In dicots xylan is a minor component of the primary cell wall, but a major component of the secondary cell wall. In monocots xylan is a major component of both the primary and secondary cell walls (McNeil et al., 1984). In the primary cell wall of monocots it has a major role in cross-linking the cellulose microfibrils (Carpita et al., 2001). Unsubstituted xylans form a tight connection with cellulose microfibrils and highly substituted xylans form the cross links between the cellulose microfibrils (Carpita et al., 1983 and 2001).

In vitro xylan synthase assays were developed by Bailey and Hassid (1966). Bioinformatics approaches have identified candidate genes for the (1,4)-β-xylan synthase. Person et al. (2005) and Brown et al. (2005) analyzed micro-arrays for coexpression of genes with secondary cell wall *CESA* genes. Several of these genes showed an irregular xylem phenotype (irx) when mutated. Two examples are the *irx8* (GT8) and *irx9* (GT43) mutants, which have a decreased xylan and pectin content (Pena et al., 2007; Persson et al., 2007). Glucuronosyl transferase assays were developed by

Waldron and Brett (1983). The *IRREGULAR XYLEM 7 (IRX7)* or *FRAGILE FIBER 8 (FRA8) (GT47)* gene, might encode an enzyme that adds glucuronosyl residues to the xylan backbone. The *irx7/fra8* mutant has no glucuronic acid side chains, a reduction in xylan and cellulose, and an increase in pectin and xyloglucan (Zhong et al., 2005).

Mannan

Mannan polysaccharides are present in all plants. Algae and mosses, however, have more mannan in their cell walls than vascular plants (Popper and Fry, 2003). Mannan polysaccharides occur in several different forms, such as pure mannan, glucomannan and galactomannan. Pure mannan consists of (1,4)-β-linked mannose and glucomannan is composed of (1,4)-β-linked glucose and mannose. These polymers can have galactose attached by an α-(1,6)-glycosidic bond and acetyl groups at the C-2 or C-3 position of the mannose residues.

Glucomannan is a minor hemicellulose in the cell wall of angiosperms, but the major hemicellulose in secondary cell walls of gymnosperms where it constitutes 16-18% of the cell wall (Maeda et al., 2000). In *Arabidopsis* glucomannan is present in the secondary cell walls of xylem, xylem parenchyma and interfascicular fibers (Handford et al., 2003). In maize it is a minor cross-linking hemicellulose in the primary cell wall (Carpita et al., 2001). Mannan, glucomannan and galactomannan are also present as a storage polysaccharide in seeds (Buckeridge et al., 2000).

Galactomannan can be synthesized in vitro with UDP-galactose as a substrate and β(1,4)-mannan as an acceptor (Edwards et al., 1989). The first gene involved in galactomannan biosynthesis, the galactomannan galactosyltransferase (*GMGT*) (GT34),

was identified by Edwards et al. (1999). Glucomannan can be synthesized in vitro in the presence of GDP-glucose and GDP-mannose (Heller and Villemez, 1972; Liepman et al., 2005). The genes encoding for the enzymes involved in the synthesis of the mannan backbone are encoded by the *CSLA* gene family (Dhugga et al., 2004; Liepman et al., 2005; Suzuki et al., 2006; Liepman et al., 2007). Most likely all the members of the *CSLA* gene family are involved in mannan biosynthesis (Liepman et al., 2007).

Recently a mannan transglycosylase activity was discovered in flowers of kiwi fruit and in tomato fruit (Schröder et al., 2004). This is an indication that mannans might have a role in cell elongation comparable to xyloglucan.

Mixed-linkage glucan (MLG)

MLG is specific for the cell wall of the members of the Poales family (grasses) (Smith and Harris, 1998). It is composed of (1→3),(1→4)-β-D-linked glucose residues followed by blocks of (1→4)-β-D-glucan and (1→3)-β-D-glucan residues (Kato and Nevins, 1984). MLG is a developmentally regulated polysaccharide in certain tissues such as internodes of deep water rice (Sauter and Kende, 1992), and the maize coleoptile (Carpita et al., 1984). Because the amount of MLG in some tissues increases during rapid cell extension, it is thought that this polysaccharide contributes to increased extensibility of the cell wall (Carpita et al., 1984; Sauter and Kende, 1992). This is supported by the fact that MLG antibodies suppress auxin-induced elongation and also inhibit the degradation of MLG (Hoson and Nevins, 1989).

In vitro assays for MLG were established by Gibeaut and Carpita (1993) and Henry and Stone (1982). MLG synthase uses UDP-glucose as a substrate and requires

Mg^{2+} or Mn^{2+} for activity (Gibeaut and Carpita, 1993). Golgi-membranes were incubated with radiolabeled UDP-glucose, the products were collected, treated with lichenase and the hydrolysis products were analyzed with HPAEC-PAD. The radiolabeled hydrolysis products eluted at the same position as the MLG standards. This analysis showed that MLG was synthesized in vitro. The CSLF proteins are involved in the synthesis of MLG (Burton et al., 2006). *Arabidopsis* was transformed with the rice *CSLF* genes. With monoclonal antibodies against MLG and MLG specific enzymes it was shown that MLG was synthesized in *Arabidopsis* expressing *OsCSLF2*.

Callose

Callose is composed of (1,3)-β-D-linked glucose, and it is synthesized at the plasma membrane (Turner et al., 1998). Callose is involved in many processes, such as cell plate formation (Hong et al., 2001a), blocking of sieve plates in the phloem (Furch et al., 2007), pollen tube growth (Doblin et al., 2001) and papilla formation in response to wounding and pathogen infection (Nishimura et al., 2003; Jacobs et al., 2003).

Callose synthase activity was first detected in membrane preparations and requires Ca^{2+} (Feingold et al., 1958). Dhugga and Ray (1994) attempted to identify the callose synthase and found two proteins of 55 kDa and 70 kDa that correlated with callose synthase activity. Based on homology to the *FKS1* genes Hong et al. (2001a) annotated 12 putative callose synthase (*CalS*) genes in *Arabidopsis*. The *FKS1* genes encode for callose synthases in yeast (Douglas et al., 1994). The plant callose synthase genes were also annotated by Richmond (http://cellwall.stanford.edu/) and they named them *GLUCAN SYNTHASE-LIKE* (*GSL*) genes. *GSL* genes (GT48) are large and have 3

to 50 exons. The proteins they encode do not possess the conserved D, D, D, QXXRW domain found in the CESA protein family, which suggest a separate evolutionary origin (Hong et al., 2001a). Hong et al. (2001a) provided genetic evidence that the *GSL* genes encode for callose synthases. Over-expression of *GSL1* resulted in increased callose deposition at the cell plate. Additional genetic evidence came from the work of Nishimura et al. (2003) and Jacobs et al. (2003), who found that a mutation in *GSL5* causes *Arabidopsis* not to deposit callose in papillae after pathogen infection. Biochemical evidence that the GSL proteins are involved in callose biosynthesis comes from the work of Li et al. (2003a). A callose synthase was purified from barley and enriched more than 60-fold. The purified protein was shown to possess callose synthase activity by performing in gel callose synthase assays. The purified protein was sequenced and the peptide fragments corresponded to the amino-acid sequence predicted for the HvGSL1 protein (Li et al., 2003a).

Other proteins involved in callose biosynthesis are sucrose synthase (Amor et al., 1995), UDP-glucose transferase (UGT1) and Rop1, which is a GTPase. UGT1 might function to transfer UDP-glucose to the active site of callose synthase, because it does not posses a known UDP-Glc binding domain. UGT1 is regulated by Rop1. Rop1 only interacts with UGT1 in the GTP-bound form (Hong et al., 2001b) and can therefore function as a molecular switch for callose biosynthesis.

Pectin

Pectin is a minor component of the cell wall of Poaceae but a major component of the cell walls of other monocots and dicots. The major pectins are homogalacturonan

(HG), rhamnogalacturonan I (RG-I) and rhamnogalacturonan II (RG-II) (Ridley et al., 2000). Several genes involved in pectin biosynthesis have been identified using genetic and biochemical methods (Lerouxel et al., 2006).

Arabinogalactan proteins (AGPs)

AGPs are a diverse class of proteoglycans which have a role in plant development including xylem formation, somatic embryogenesis and plant pathogen interactions. AGPs consist of carbohydrates which are mainly composed of galactose and arabinose, and are attached to a protein backbone. No proteins involved in the biosynthesis of the glycan chains have been identified yet, but many genes encoding for the protein backbone have been isolated (Seifert et al., 2007).

Expansins

Expansins are proteins involved in the process of cell expansion as shown by their influence on extension growth in cucumber hypocotyls (Li et al., 2003b). Their exact mechanism of action is not known, but they are hypothesized to break the hydrogen bonds in the cellulose/xyloglucan network, thus allowing for cell expansion to take place. Initially two expansin subfamilies were discovered, called α- and β-expansins. Recently two new families have been found, which are called γ- and δ-expansins (Li et al., 2003b).

Summary

In summary, the plant cell wall is composed of a highly complex network of polysaccharides, and proteins. There are still many unknowns, such as the regulation of

cell wall biosynthesis, most of the enzymes involved in pectin and AGP biosynthesis, the enzymes synthesizing the xylan backbone, and the exact mode of action of expansins. However, advanced genomics and proteomics technologies are opening the door to new discoveries in this exciting but difficult area of plant science.

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CHAPTER 2: THE LIGHT-REGULATED GOLGI-LOCALIZED GLUCAN SYNTHASE FROM MAIZE: PROPERTIES, PRODUCT ANALYSIS AND SOLUBILIZATION

ABSTRACT

In maize, a Golgi-localized enzymatic activity uses UDP-glucose as a substrate and incorporates it into ethanol-insoluble products. This enzymatic activity is light and auxin regulated (Walton and Ray 1982a, 1982b). We hypothesized that this enzymatic activity might be involved in the biosynthesis of a hemicellulosic glucan. We determined that this UDP-glucose incorporating enzymatic activity is a (1,4)-β-glucan synthase and attempted to identify it using biochemical methods. We solubilized the (1,4)-β-glucan synthase activity using digitonin. After subsequent hydroxyapatite chromatography glucose was no longer incorporated into (1,4)-β-glucan but in glucose-6-phosphate (G6P) and glucose-1,6-diphosphate (G1,6dP) instead.

INTRODUCTION

Glucan synthases synthesize starch from ADP-glucose or (1,4)-β-glucans and (1,3)-β-glucans from UDP-glucose or GDP-glucose (Barber et al, 1964; Villemez et al., 1967; Ray et al., 1969; Tsai and Hassid, 1971; Ball et al., 2003). Biochemical identification of enzymes involved in cell wall biosynthesis is difficult due to their hydrophobic nature, instability and low levels (Meikle et al., 1991; Dhugga et al., 1994). However, this approach has been successfully used to identify galactomannan galactosyltransferase (Edwards et al., 1999), xyloglucan fucosyltransferase (Perrin et al.,

1999), callose synthase (Li et al., 2003) and galacturonan synthase involved in pectin biosynthesis (Sterling et al., 2006).

In this part of my research I attempted to identify a Golgi-localized glucan synthase using biochemical methods. As a first step I optimized the enzyme assay. As a second step, I determined the product it synthesizes. As a third step, I solubilized and partially purified the glucan synthase.

MATERIALS AND METHODS

Growth of maize Seedlings

Maize seeds (variety DK355, Monsanto, or variety FR1061 X FR9661, Midwest Seed Genetics, St Carroll, IA) were imbibed at room temperature for 24 h in an Erlenmeyer flask shaking at 150 rpm under white light. The seeds were spread on top of trays of fine vermiculite soaked in water, covered with a thin layer of vermiculite and covered with plastic. The trays were kept in a completely dark room at 24°C and after two days the plastic cover was removed. On the evening of day three, one tray of seedlings was given a 15 min white light exposure (97 μMol s^{-1} m^{-2}), after which it was placed back in the dark room. Sixteen hours later the second cm of the mesocotyl below the first node was harvested under white light.

Isolation of total membranes

An equal weight of mesocotyl segments isolated from dark-grown and light-treated maize seedlings were ground in buffer (50 mM Tris buffer pH 8, 1 mM EDTA, 0.01 mM $MgSO_4$ and 250 mM sucrose). All steps of this procedure were performed at

4°C. The amount of buffer added was 3 ml/g of fresh weight. The mesocotyl segments were ground in a mortar and filtered through two layers of cheesecloth. The flow-through was centrifuged for 5 min at 10,000 rpm in a Sorval SS34 rotor. The supernatant was centrifuged for 20 min at 146,000x g in a Ti-50 rotor (Beckman Coulter). The pellet was resuspended by homogenization in grinding buffer at a final concentration of 1 ml/g of original fresh weight.

(1,4)-β-glucan synthase assay

One hundred µl of microsomal suspension (100-200 µg of protein) was added to a 15 ml Pyrex glass tube, $MgSO_4$ was added to a concentration of 20 mM and UDP-[^{3}H]glucose (34 Ci/mmol, Sigma) to 3.7-49 nM (below 49 nM the product synthesized was similar). The reaction mixture was adjusted with grinding buffer to a volume of 110-200 µl, vortexed, and incubated for 1 h at room temperature (21°C). Five ml of 70% ethanol at 4°C was added to the tube and vortexed. This mixture was filtered through a glass fiber filter (GF/A, 2.5 cm diameter, Cat. No. 1820025, Whatman) with a vacuum manifold (Millipore) to collect the radiolabeled ethanol-insoluble products. The tube was rinsed with 5 ml of ethanol and this was also filtered. Finally it was washed with 5 ml of ethanol and dried. The radioactivity on the dried filter was measured using a scintillation counter (LS5000 TDC, Beckman Coulter).

Callose synthase assay

Callose synthase assays were performed in the same manner as described for the (1,4)-β-glucan synthase except that the UDP-glucose concentration was 1 mM and no Mg^{2+} and Ca^{2+} were added.

Enzymatic hydrolysis of radiolabeled products

One ml of buffer was added to the dried filters containing the radiolabeled products. The specificity of the enzymes and buffers used are described in Table 1. Five µl of the respective enzyme solution was added to the filters soaked in buffer, after which they were incubated overnight at room temperature. Nine ml of 100% ethanol at 4°C was added, and this solution was filtered through GF/A filters. The filter remaining in the vial in which the enzyme treatment was performed was collected on the new filter. The vial in which the enzyme treatment was performed was rinsed once with 90% ethanol at 4°C. Finally the two filters were washed once with ethanol, dried and radioactivity was measured. The radioactivity remaining on the filter was compared to a control that was not treated with hydrolytic enzymes.

TABLE 1: Enzymes used, their catalog number, buffers used and substrates they hydrolyze

***obtained from Megazyme, **Roche, ***Sigma**

Enzyme name	Cat. No	Buffer used	Substrates hydrolyzed
alkaline phosphatase***	P7640	50 mM tris-glycine buffer pH 8.8	phosphate attached to many types of molecules
α-amylase (10 mg/ml)***	A6380	50 mM MOPS pH 7	starch
cellulase*	Lot 30201	50 mM acetate buffer pH 5	cellulose xyloglucan MLG ((1→3,1→4)-β-D-glucan) glucomannan xylan
Driselase from *Basidiomycetes sp.***	D8037	50 mM acetate buffer pH 5	callose xylan cellulose
endo-1,4-β-D-galactanase*	Lot 00901	50 mM acetate buffer pH 4	galactan polygalacturonic acid arabinan birch-wood xylan citrus pectin cellulose
β-1-(3,4,6)-galactosidase***	G1288	50 mM acetate buffer pH 5	releases β-1→3, β-1→4, and β-1→6-linked galactose from the non-reducing end of complex oligosaccharides

TABLE 1 Continued			
laminarinase*	Lot 90601	50 mM acetate buffer pH 4.5	(1→3)-β-D-glucan
lichenase*	Lot 30501	50 mM MES, pH 6	MLG
β-mannanase*	Lot 21101	50 mM tris-glycine buffer pH 8.8	Carob galactomannan
pectinase from *Aspergillus niger****	P2736	50 mM acetate buffer pH 4	pectin hemicelluloses cellulose
proteinase K** (20mg/ml)	12222300	50 mM Tris pH 8	proteins
xylanase M1*	Lot 70502	50 mM acetate buffer pH 5	xylan CM-cellulose 4M CM-cellulose 6M
xylanase M6*	Lot 51206	50 mM acetate buffer pH 5	xylan

Collection of hydrolysis products

The same procedure was followed as described for the enzymatic digestion of the radiolabeled products, except that after the addition of ethanol to a final concentration of 90%, the solution was collected in eppendorf tubes. The buffer/ethanol solution was dried under vacuum and the radiolabeled hydrolysis products were redissolved in 100 µl of water.

Total hydrolysis of the products

Two ml of 2 M trifluoroacetic acid (TFA) was added to a screw cab tube containing a filter with ethanol-insoluble product. The tube was heated for 2 h at 121°C

and subsequently cooled to room temperature. The TFA was evaporated and the filter was rinsed twice with 90% ethanol. The ethanol rinses were collected in an eppendorf tube and dried.

High-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD)

The HPAEC-PAD analysis (Dionex) was equipped with a 25 µl injection loop and an ED50 electrochemical pulsed amperometric detector (PAD). The radiolabeled digestion products were mixed with oligosaccharide standards (1-5 µg each) and analyzed using a CarboPac PA-1 column (4 x 250 mm) run at a flow rate of 1 ml a min. One or two min fractions were collected and neutralized with HCl before scintillation counting. The identity of the radiolabeled compounds was determined by co-elution with the standards. Table 2 describes the solutions and gradients used for each type of analysis.

TABLE 2: Gradients used for the HPAEC-PAD analysis of different products	
Type of products analyzed (program name)	**Gradient**
Monosaccharides	Isocratic at 18 mM NaOH for 20 min
Oligosaccharides (cellobiose, cellotriose, laminaribiose, xylobiose, xylotriose)	Isocratic at 90 mM NaOH for 30 min
Cellooligosaccharides 1 (glucose, cellobiose, cellotriose, cellotetraose, cellopentaose, cellohexaose)	Isocratic at 100 mM NaOH for 30 min, gradient of 100-500 mM NaOH in 10 min, isocratic at 500 mM NaOH + 500 mM NaOAc
Cellooligosaccharides 2 (glucose, cellobiose, cellotriose, cellotetraose, cellopentaose, cellohexaose)	A gradient from 0-500 mM NaOAc in 30 min and then constant 500 mM NaOH
Glucose-phosphates	Isocratic at 39% buffer B for 10 min, gradient from 39%-100% buffer B in 30 min. Buffer A: 59 mM NaOH, 196 mM NaOAc Buffer B: 50 mM NaOH, 75 mM NaOAc
Manno/xylooligosaccharides	Isocratic at 20 mM NaOH for 20 min, gradient from 20-90 mM NaOH in 20 min, isocratic at 500 mM NaOH
Mixed linkage glucan (MLG)	15 min gradient from 0.5 M NaOH to 0.5 NaOH and 0.25 M NaOAc

Solubilization of the (1,4)-β-glucan

Reactions were performed and the filters with the products were dried. Solubility of the (1,4)-β-glucan was tested by adding different solvents to filters containing ethanol-insoluble reaction products and incubating them overnight. Next day the solvents were removed and the radioactivity remaining on the filter was determined. Solutions used

were 0.2 N, 1 N, 2 N and 4 N NaOH, 0.5% ammonium oxalate, 15.8 M acetic / 1.5 M nitric acid, water, dimethyl sulfoxide (DMSO), DMSO plus 4.2 M 1-methylimidazole (MI) (Cat. No.: 336092, Sigma) and DMSO plus 0.5 M tetrabutylammonium fluoride (TBAF) (Cat. No.: 86843, Sigma). Ammonium oxalate (0.5% w/v) dissolves non-crystalline cellulose attached to a protein by heating cell wall material in this solution for 1 hour at 100°C (Peng et al., 2001). Acetic/nitric acid dissolves non-cellulosic (1,4)-β-glucan (Updegraff, 1969). MI and TBAF will dissolve crystalline cellulose (Heinze et al., 2001; Lu et al., 2003). DMSO plus TBAF dissolves cellulose up to a degree of polymerization of 650 within 15 minutes without any pretreatment at room temperature (Heinze et al., 2001). The acetic/nitric acid solution was prepared by mixing 150 ml 80% acetic acid and 15 ml concentrated nitric acid. The DMSO plus TBAF solution was prepared by mixing 6.6 g of TBAF and 33 ml of DMSO. The DMSO plus MI solution was prepared by adding 5 ml of MI to 10 ml of DMSO.

Selection of the optimal detergent for solubilization of the glucan synthase

(1,4)-β-glucan synthase activity was determined in the presence of different detergents. One hundred µl of microsomes were incubated with 0.01%, 0.1% or 1% detergent for 30 min on ice and assayed. Detergents used were Brij35 (Cat. No. 430A9-6, Sigma), digitonin (Cat. No. D141, Sigma), Triton X100 (Cat. No. 111037, Research Products International), Tween 20 (Cat. No. P7949, Sigma), CHAPS (Cat. No. C5070, Sigma), Zwittergent (Cat. No. 693030, Calbiochem) and n-octoglucoside (Cat. No. 494460, Calbiochem). Detergents that inhibited the glucan synthase activity the least were tested for their ability to solubilize this enzymatic activity. Microsomes were

incubated with different detergent concentrations and for different time periods. The detergent / microsome mixture was centrifuged at 146,000 rpm in a Ti-50 rotor for 20 min, and the supernatant and pellet were separated and both were assayed. Solubilization efficiency was determined by comparing the amount of ethanol-insoluble product synthesized by the supernatant to the ethanol-insoluble product synthesized by the pellet.

Optimized solubilization procedure for the glucan synthase

Microsomal membranes were resuspended in solubilization buffer (10 mM Tris pH 8, 1 mM $MgSO_4$, 4 mM DTT, 20% glycerol) by homogenization at a concentration of 1 ml/g of fresh weight. Digitonin was added to a final concentration of 0.5%, mixed with the microsomal solution and kept on ice for 20 min. This mixture was centrifuged for 20 min at 146,000 g and the supernatant was collected. The resulting pellet was resuspended in half the fresh weight volume of solubilization buffer and homogenized. Digitonin was added to a concentration of 0.5%; the mixture was incubated on ice for 20 min and centrifuged for 20 min at 146,000 g. The supernatant was collected, combined with the supernatant of the first solubilization and stored at -80°C for later use.

Hydroxyapatite chromatography

Hydroxyapatite chromatography (5 ml Econo-Pac CHT-II Cartridge, Cat. No. 732-0081, Biorad) was performed under the following conditions: a 30 min gradient was run from 1-100 mM potassium phosphate buffer, pH 8.0, at a flow rate of 0.5 ml/min, followed by a wash with 400 mM phosphate buffer pH 8.0. Two min (1 ml) fractions were collected and 100 µl was used for glucan synthase assays.

Measurement of protein concentrations

Protein concentrations were measured with the Bradford assay (Bradford, 1976). Five ml of Bradford solution was added to 10-100 µl of sample, vortexed and incubated at room temperature for 10 min. The Bradford stock solution contained 300 mg Coomassie Brilliant Blue G-250 (Cat. No. 27815, Fluka) dissolved in 150 ml 95% ethanol and 300 ml 85% phosphoric acid. The Bradford working solution was 15 ml of stock solution diluted to 100 ml with water. The OD_{595} was measured and the protein concentration was determined with bovine immunoglobulin as standard.

RESULTS

Optimization of the glucan synthase assay

The optimal $MgCl_2$ concentration for glucan synthase activity is 15 mM (Figure 1). Therefore all the glucan synthase assays were performed in the presence of 20 mM Mg^{2+}.

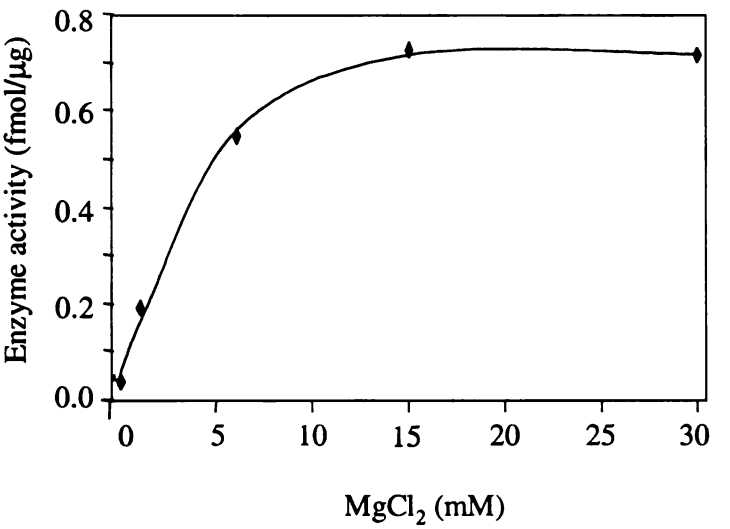


Figure 1. Effect of different $MgCl_2$ concentrations on glucan synthase activity (measured as fmol [^{3}H]glucose incorporated into ethanol-insoluble products per µg of protein) using microsomes isolated from dark-grown seedlings. This experiment was repeated several times and a representative result is shown in this figure.

The pH of the microsomal solution was adjusted by adding either KH_2PO_4 [pH 6.5, 7 and 7.5] or Tris [pH 7.5, 8 and 8.5]. The glucan synthase has a broad pH optimum from 7.5-8.0 (Figure 2). Therefore all the glucan synthase assays were performed at pH 8.

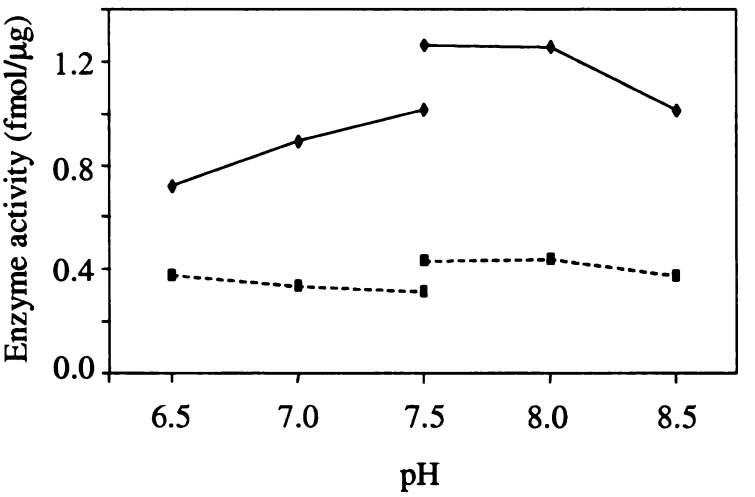


Figure 2. Effect of pH on glucan synthase activity; microsomes isolated from dark-grown (solid line), and light-treated (dashed line) seedlings. This experiment was repeated several times and a representative result is shown in this figure.

Glucan synthase activity is proportional to protein concentration

Glucan synthase assays were performed by adding increasing amounts of microsomes to the assay. Figure 3 shows that the specific activity is constant over a 5-fold range of protein concentration for microsomes isolated from both the dark-grown and light-treated seedlings.

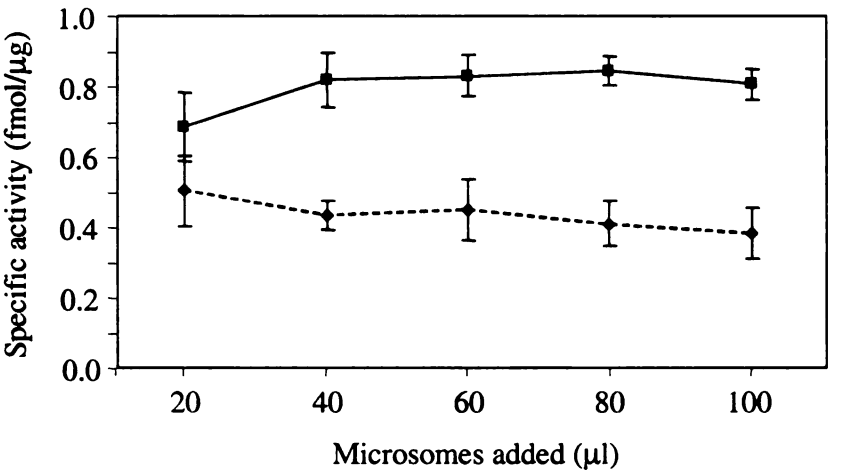


Figure 3. Specific glucan synthase activity over a 5-fold range of protein concentration. Data represent the mean (n = 3) ± SE for microsomes isolated from dark-grown (solid line) and (n = 2) ± SE for microsomes isolated from light-treated (dashed line) seedlings.

Researchers in the past have shown that glucose from UDP-glucose can be incorporated into several different products which can contain β(1,4), β(1,3) and both β(1,4) and β(1,3) linked glucose (Tsai et al., 1971; Gibeaut et al., 1993; Kudlicka et al., 1997). In order to determine in which products glucose is incorporated in the case of the glucan synthase, we performed assays at different UDP-glucose concentrations and treated the products with cellulase, laminarinase, or lichenase.

The composition of the product is dependent on the UDP-glucose concentration

A. Assays with microsomes isolated from dark-grown seedlings.

When assays were performed with 20 nM UDP-[^{3}H]glucose, the product was mainly solubilized by cellulase (Figure 4). Xylanase, proteinase K, or α-amylase did not solubilize the radiolabeled product by more than 25% (Figure 5 and data not shown). When the UDP-glucose concentration was 10 μM the products were partially solubilized by cellulase and partially by laminarinase. At 1 mM UDP-glucose the products were mainly solubilized by laminarinase (Figure 4).

B. Assays with microsomes isolated from light-treated seedlings.

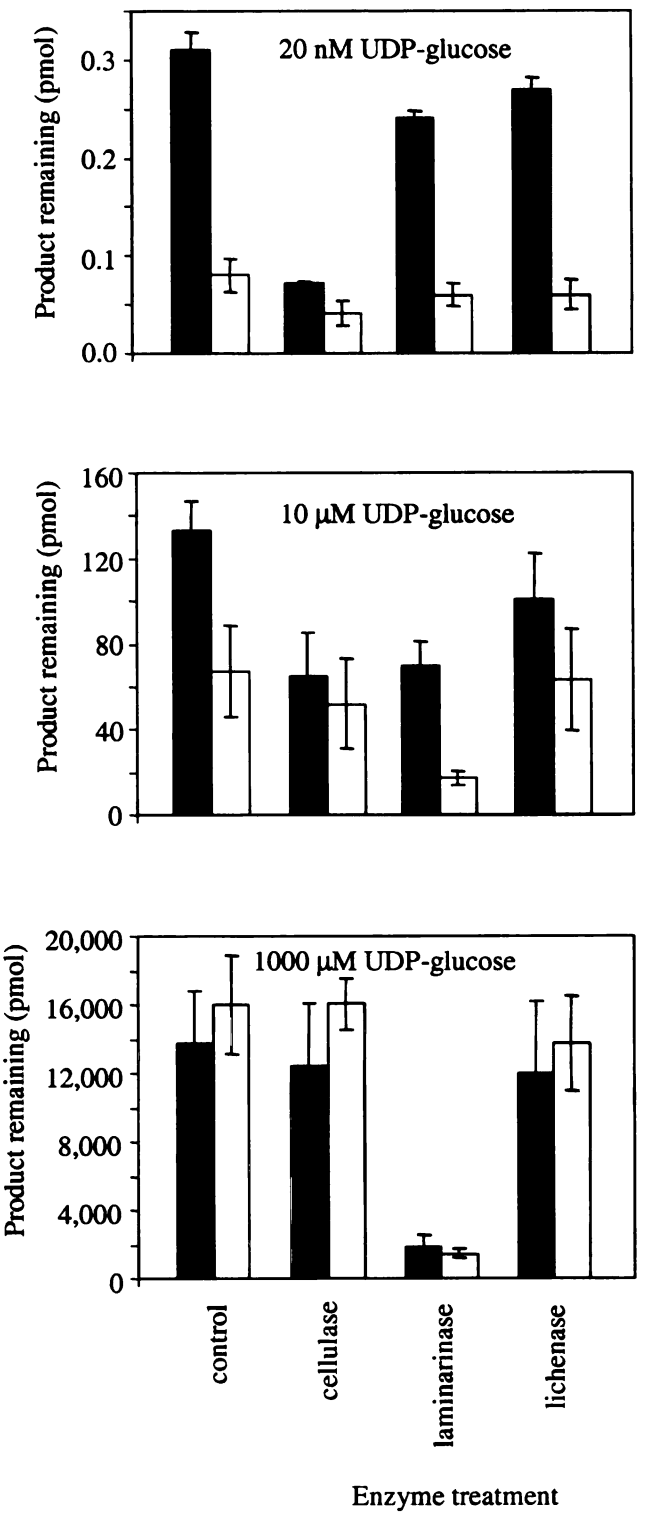
After light-treatment there was a ~70% reduction in activity at 20 nM UDP-Glc, but the product was still mainly cellulase-sensitive (Figure 4). After light-treatment there was a ~50% reduction in activity when the UDP-glucose concentration was 10 μM and the products were mainly solubilized by laminarinase. After light-treatment there was a ~15% increase in activity when the UDP-glucose concentration was 1 mM and the product was also mainly solubilized by laminarinase.

In summary, these results show that at low UDP-glucose concentration the product is mainly solubilized by cellulase and light has a large effect on activity. At high UDP-glucose concentration the product is mainly solubilized by laminarinase and light has no effect. These results can be explained in the following way. At low UDP-glucose concentrations a (1,4)-β-glucan synthase is active which has a low K_m and a low V_{max}. This enzyme is down-regulated by light. At high UDP-glucose concentrations a different

enzyme, callose synthase is active, which has a high K_m and a high V_{max}. Callose synthase is not affected by light.

Figure 4. Enzymatic sensitivity of the glucan synthase product made at different UDP-Glc concentrations. Solid bars: assays with microsomes from dark-grown seedlings; white bars: assays with microsomes from light-treated seedlings. Data represent the mean ($n = 3$) ± SE for assays performed at 20 nM or 10 μM UDP-glucose and ($n = 2$) ± SE for assays performed at 1000 μM UDP-glucose.

Figure 4


20 nM UDP-glucose
Product remaining (pmol)
0.3
0.2
0.1
0.0
10 μM UDP-glucose
Product remaining (pmol)
160
120
80
40
0
1000 μM UDP-glucose
Product remaining (pmol)
20,000
16,000
12,000
8,000
4,000
0
control
cellulase
laminarinase
lichenase
Enzyme treatment

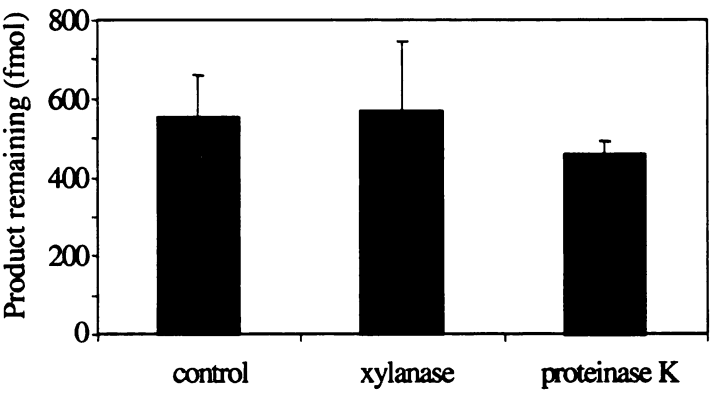


Figure 5. Products synthesized with microsomes isolated from dark-grown seedlings treated with xylanase M1 and proteinase K. Data represent the mean (n = 4) ± SE for the control and (n =3) ± SE for xylanase M1 and proteinase K.

The cellulase that was used for solubilizing the products was also active on other substrates (see Table 1). Therefore, to determine the chemical nature of the glucan synthesized by the light-regulated glucan synthase, further characterization of the product was necessary.

The product synthesized at 3.7 nM UDP-glucose is a (1,4)-β-glucan

The product solubilized by cellulase was analyzed by HPAEC-PAD using the oligosaccharide program (see Table 2). The major hydrolysis products have the same elution time as cellobiose and glucose (Figure 6), which indicates that the product in which [^{3}H]glucose is incorporated is a (1,4)-β-glucan both for microsomes isolated from dark-grown and light-treated seedlings.

The monosaccharide composition of the products was also analyzed. The products were hydrolyzed with TFA and analyzed by HPAEC-PAD using the monosaccharide program (see Table 2). This showed that the radiolabel was in glucose for assays performed with microsomes isolated both from dark-grown and light-treated seedlings (Figure 7).

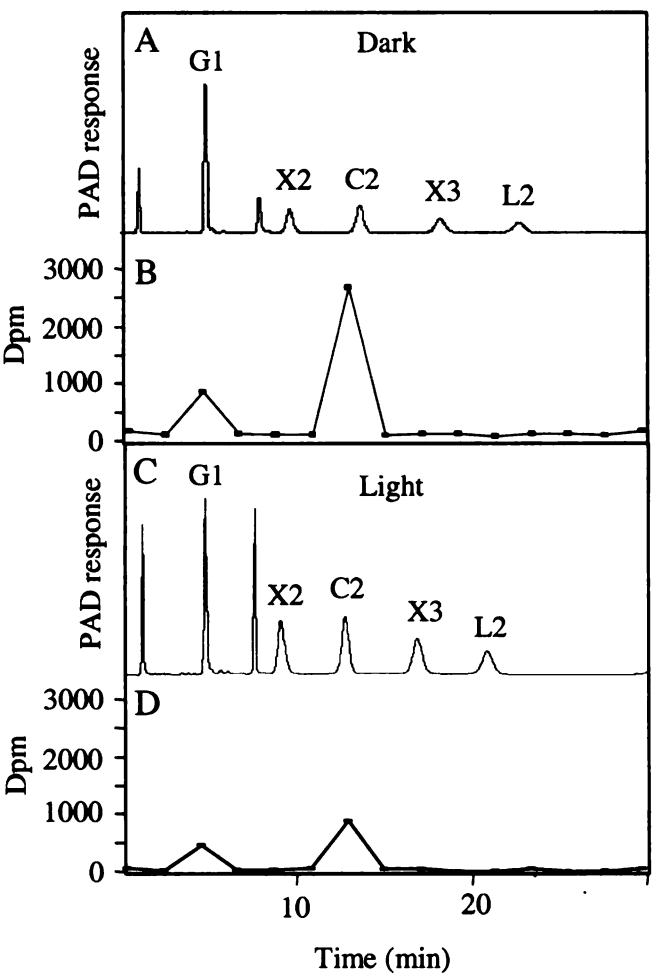


Figure 6. The glucan synthase products synthesized with microsomes isolated from dark-grown (B) and light-treated (D) seedlings were treated with cellulase and the hydrolysis products were analyzed by HPAEC-PAD. A and C, Oligosaccharide standards: G1 (glucose), X2 (xylobiose), C2 (cellobiose), X3 (xylotriose), L2 (laminaribiose). B and D, Radioactivity.

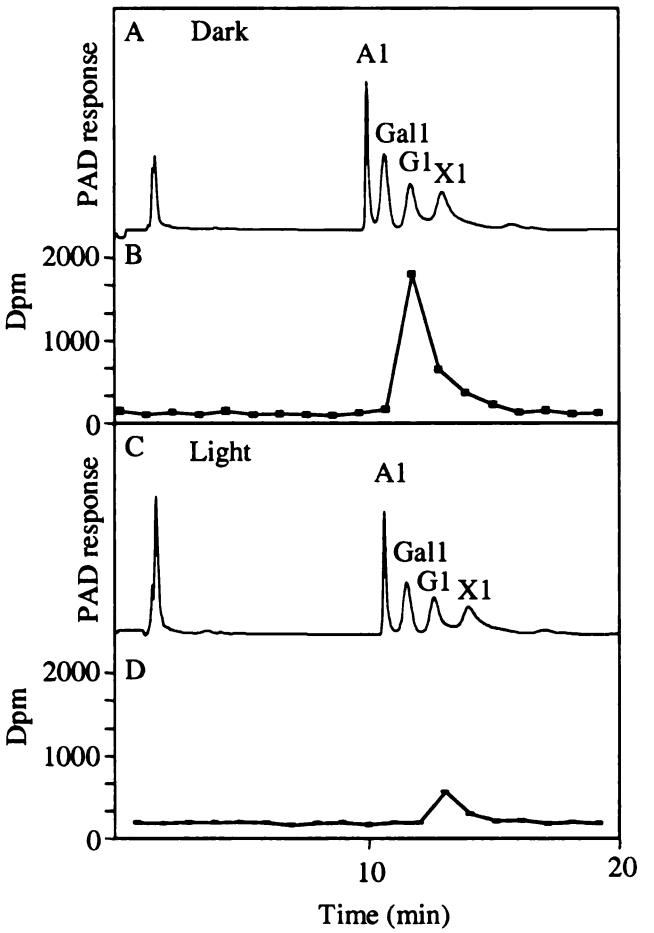


Figure 7. The glucan synthase products synthesized with microsomes isolated from dark-grown (B) and light-treated (D) seedlings were hydrolyzed with TFA and the products were analyzed by HPAEC-PAD. A and C, Monosaccharide standards: A1 (arabinose), Gal1 (galactose), G1 (glucose), X1 (xylose). B and D, Radioactivity.

At 1 mM UDP-glucose, the product synthesized is a (1,3)-β-glucan

The product synthesized at 1 mM UDP-glucose was analyzed in a similar way as described for the product synthesized at 3.7 nM UDP-[^{3}H]glucose. The hydrolysis

products elute with laminaribiose and glucose (Figure 8), which indicates that the product in which UDP-[^{3}H]glucose is incorporated is a (1,3)-β-glucan.

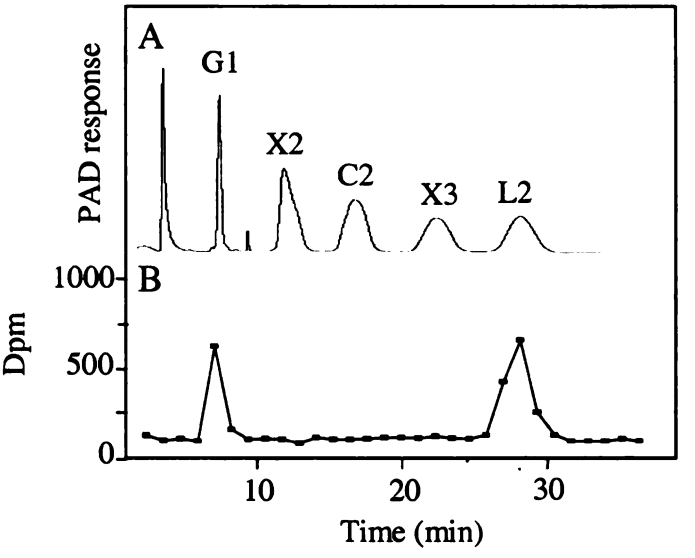


Figure 8. The glucan synthase product synthesized at 1 mM UDP-glucose was hydrolyzed with laminarinase and the products were analyzed by HPAEC-PAD. A, Oligosaccharide standards. B, Radioactivity.

The product in which [^{3}H]glucose is incorporated is non-crystalline (1→4)-β-glucan and is not attached to a protein.

Important properties of the (1,4)-β-glucan are its size and solubility. The (1,4)-β-glucan synthesized at low UDP-Glc concentration was solubilized by 4 N NaOH, but not by 0.2 N or 2 N NaOH. It became insoluble again after dilution of 4 N NaOH to 1 N (data not shown). This solubilization experiment indicates that the product has the properties of a hemicellulose. The product was not soluble in 0.5% ammonium oxalate (data not

shown) (Peng et al., 2001). The product was completely dissolved by Updegraff's reagent. The product was solubilized partially by DMSO, and DMSO plus MI (Figure 9). It was almost completely solubilized by DMSO plus TBAF (Figure 9). The product was reprecipitated again when the DMSO/TBAF solution was diluted tenfold with water (data not shown). These results are consistent with the product being non-crystalline (1,4)-β-glucan and not attached to a protein.

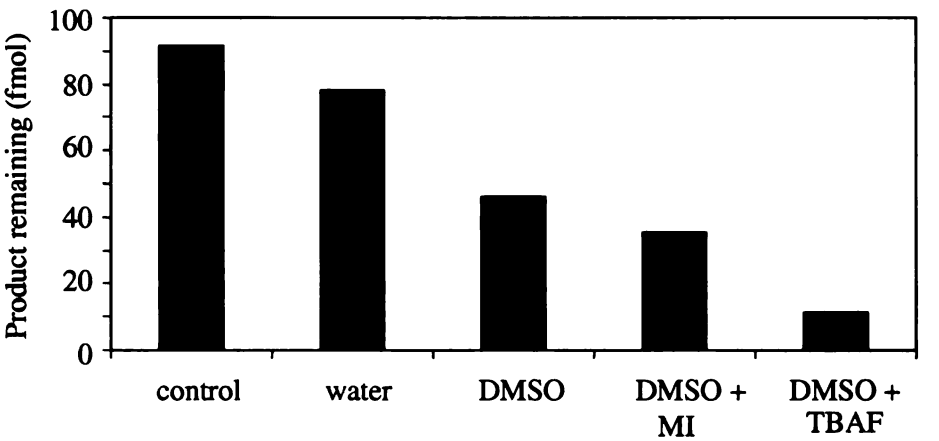


Figure 9. Solubility of the (1,4)-β-glucan in different solvents. Filters containing ethanol-insoluble products were incubated with the above mentioned solutions, filtered and the remaining radioactivity was determined.

Glucan synthase assays in the presence of UDP-xylose

(1,4)-β-glucan is found in xyloglucan and in cellulose (Haysashi, 1989). To test whether the glucan synthase is involved in xyloglucan biosynthesis assays were performed in the presence of both UDP-glucose and UDP-xylose. It has been shown that plant membranes can synthesize xyloglucan when UDP-glucose and UDP-xylose are

both added to the enzyme assay (Hayashi and Matsuda, 1981). If the maize (1,4)-β-glucan synthase is involved in xyloglucan biosynthesis, there is a possibility that addition of UDP-glucose and UDP-xylose to the enzyme assay together might result in xyloglucan biosynthesis in vitro. However, in vitro xyloglucan assays have been developed only for dicots and not for monocots.

Glucan synthase assays were performed in the presence of constant concentration of UDP-[^{3}H]glucose (49 nM) and varying concentrations of UDP-xylose. UDP-xylose inhibited incorporation of UDP-[^{3}H]glucose (half maximum inhibition at ~50 μM). The effect was similar for assays performed with microsomes isolated from dark-grown and light-treated seedlings (Figure 10). The inhibition of UDP-glucose incorporation by UDP-xylose has also been observed for xyloglucan biosynthesis in dicots. However, there is a stimulation of xyloglucan biosynthesis when the UDP-xylose concentration is lower than the UDP-glucose concentrations in the enzyme assays (Gordon and Maclachlan, 1989; Hayashi and Matsuda, 1981). I did not observe any stimulation in my enzyme assays by UDP-xylose, even at a concentration as low as 25 nM.

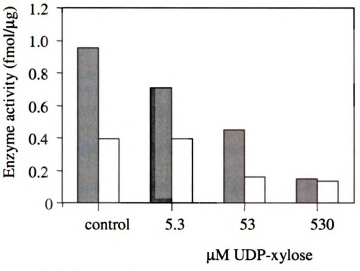


Figure 10. Glucan synthase assays in the presence of UDP-xylose. Assays were performed at constant UDP-Glc concentrations (49 nM) with microsomes isolated from dark-grown (gray bars), and light-treated (white bars) seedlings. No UDP-xylose was added to the control.

To test further the hypothesis that the maize (1,4)-β-glucan synthase is involved in xyloglucan biosynthesis, the products synthesized in the presence of UDP-[^{3}H]glucose and UDP-xylose (50 µM) were treated with Driselase. Driselase is known to release isoprimeverose from xyloglucan (Popper and Fry, 2003). The solubilized products were collected, mixed with oligosaccharide and isoprimeverose standards and analyzed by HPAEC-PAD with the oligosaccharide program (see Table 2). The radiolabeled hydrolysis products coeluted with cellobiose and glucose, but not with isoprimeverose (data not shown). No in vitro xyloglucan biosynthesis was observed.

The (1,4)-β-glucan synthase activity is not inhibited by cellulose synthase inhibitors

In order to determine if perhaps the (1,4)-β-glucan synthase is a cellulose synthase in transit to the plasma membrane, (1,4)-β-glucan synthase assays were performed in the presence of 2,6-dichlorobenzonitrile (DCB) and isoxaben. DCB and isoxaben are known to inhibit cellulose biosynthesis although it is not known if this inhibition is a direct or an indirect effect (Peng et al., 2001; Desprez et al., 2002, Scheible et al., 2001). DCB inhibits cellulose biosynthesis, but causes no accumulation of non-crystalline cellulose (Peng et al., 2001). DCB inhibits cellulose biosynthesis in barley cell cultures, but not hemicellulose biosynthesis (Sheletzky et al., 1992). Figure 11 shows that the (1,4)-β-glucan synthase activity is not inhibited by DCB or isoxaben.

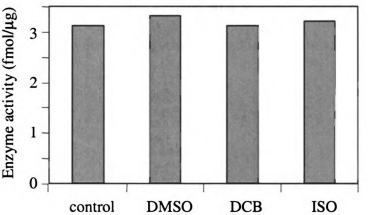


Figure 11. Glucan synthase assays performed in the presence of DCB and isoxaben (ISO). DCB and isoxaben were dissolved in DMSO and used at a concentration of 8 µM and 8 nM, respectively. The DMSO concentration was 0.8%.

From these results no definitive conclusion can be made if the (1,4)-β-glucan synthase is a cellulose synthase or not, because the mode of action of these herbicides is not known. If they inhibit cellulose biosynthesis directly then the (1,4)-β-glucan synthase is most likely not a cellulose synthase.

Solubilization of the (1,4)-β-glucan synthase

In order to fractionate proteins using chromatographic methods they have to be in solution. The (1,4)-β-glucan synthase is a membrane protein and therefore insoluble. In order to solubilize it I tested several different detergents for their effect on (1,4)-β-glucan synthase activity. The detergents which had the least inhibitory effect were tested for their ability to solubilize the (1,4)-β-glucan synthase. Most detergents when used at 0.01% concentration did not inhibit the activity. At a concentration of 1.0% most of the detergents inhibited the enzyme strongly, except Brij35, digitonin and Tween 20 (Table 3).

Table 3. The effect of different detergents on (1,4)-β-glucan synthase activity. The activity remaining after detergent treatment is given as % compared to the control (n = nonionic, z = zwitterionic; 100% activity = 10,000 dpm).

Detergent	Activity compared to control (%)		
	Detergent concentration		
	1%	0.1%	0.01%
Brij35 (n)	100	100	95
Digitonin (n)	63	44	143
Triton X100 (n)	2	8	28
Tween 20 (n)	35	50	91
CHAPS (z)	3	65	94
Zwittergent (z)	2	3	22
n-octoglucoside (n)	2	37	71

Brij35, digitonin, and Tween 20 were tested for their ability to solubilize the (1,4)-β-glucan synthase. Digitonin solubilized it most efficiently. Several different digitonin concentrations were tested and it was concluded that digitonin solubilized the activity most efficiently at a concentration of 0.25%-0.5% (0.0025-0.005 μg digitonin / μg protein). At this concentration 19% of (1,4)-β-glucan synthase activity which was present in the total microsomes was recovered (Figure 12).

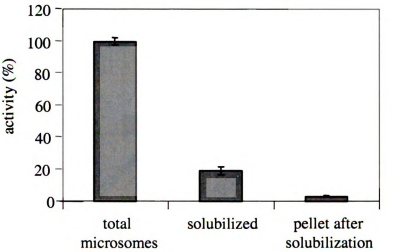


Figure 12. Comparison of (1,4)-β-glucan synthase activity after solubilization with 0.5% digitonin with the activity in the microsomes. Data represent the mean (n = 3) ± SE.

Enzyme assays were performed with the solubilized (1,4)-β-glucan synthase preparation and the products were treated with different hydrolytic enzymes. The products were solubilized more than 80% by cellulase. Laminarinase, xylanase, and lichenase appeared to solubilize the product but to a much lesser degree (Figure 13).

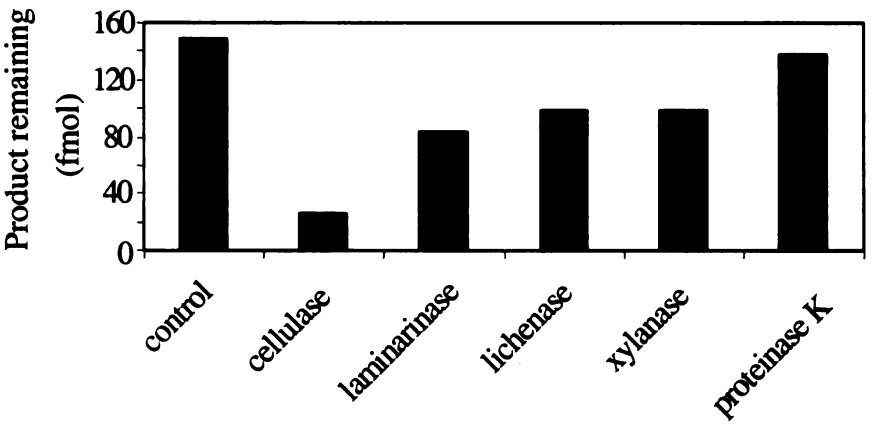


Figure 13. Enzyme treatment of the products synthesized by the solubilized (1,4)-β-glucan synthase preparation. This graph shows the remaining product after enzyme treatments.

The solubilized products were mixed with oligosaccharide standards and analyzed by HPAEC-PAD. Only cellulase gave any detectable product, which co-eluted with cellobiose and glucose (Figure 14). The other enzymes gave no detectable products (data not shown). Therefore, after solubilization the glucan synthase still synthesizes a (1,4)-β-glucan.

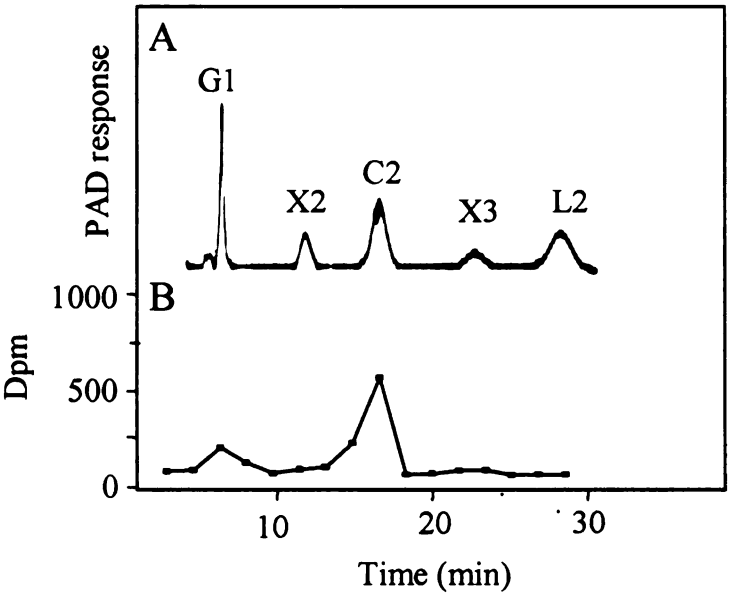


Figure 14. Analysis of the product synthesized by the solubilized (1,4)-β-glucan synthase. The product was treated with cellulase and the hydrolysis products were analyzed by HPAEC-PAD. A, Oligosaccharide standards. B, Radioactivity.

Fractionation of the solubilized (1,4)-β-glucan synthase

We attempted to fractionate the solubilized (1,4)-β-glucan synthase using anion exchange, hydrophobic interaction, size exclusion and hydroxyapatite chromatography. Apparent activity was recovered after hydroxyapatite chromatography (Figure 15). The total amount of ethanol-insoluble products synthesized after hydroxyapatite chromatography was comparable to the total amount of ethanol-insoluble products synthesized in the solubilized glucan synthase preparation before chromatography.

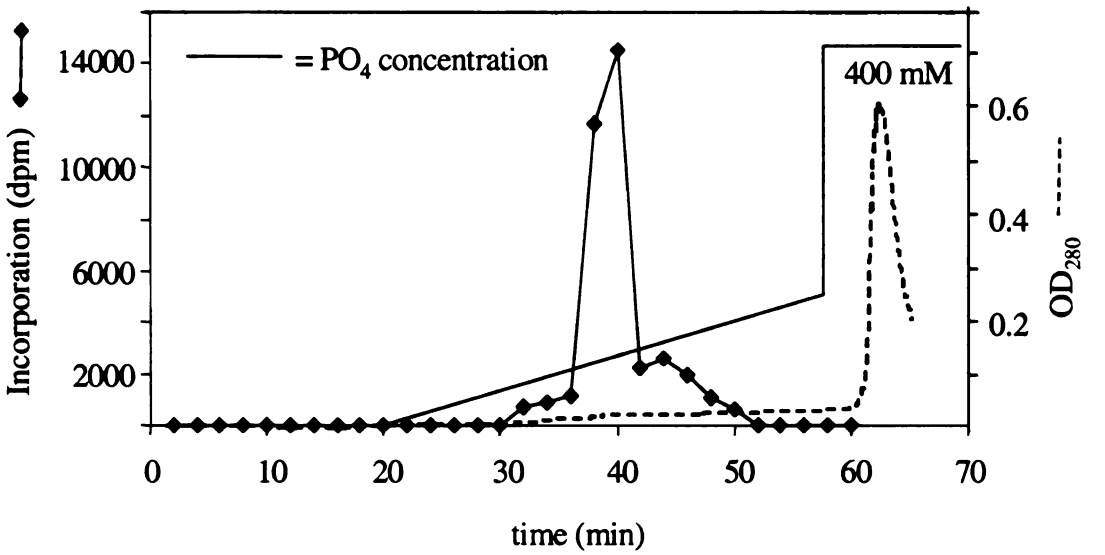


Figure 15. Hydroxyapatite fractionation of solubilized (1,4)-β-glucan synthase. A 30-minute gradient was run from 1 to 100 mM potassium phosphate, pH 8, followed by 400 mM potassium phosphate, pH 8 (solid line). The OD_{280} trace (dashed line) shows that the majority of protein is separated from the fractions containing the activity (diamonds).

The products synthesized after hydroxyapatite chromatography were precipitated with 70% ethanol, collected by centrifugation, and dried. Water was added to redissolve the products, cellooligosaccharide standards were added, and the products were analyzed by HPAEC-PAD using the cellooligosaccharide 1 program (see Table 2). Although the product before hydroxyapatite chromatography was not water-soluble (Figure 9), after hydroxyapatite chromatography the product was completely water-soluble. The radiolabeled product eluted near the cellopentaose and cellohexaose standards (Figure 16).

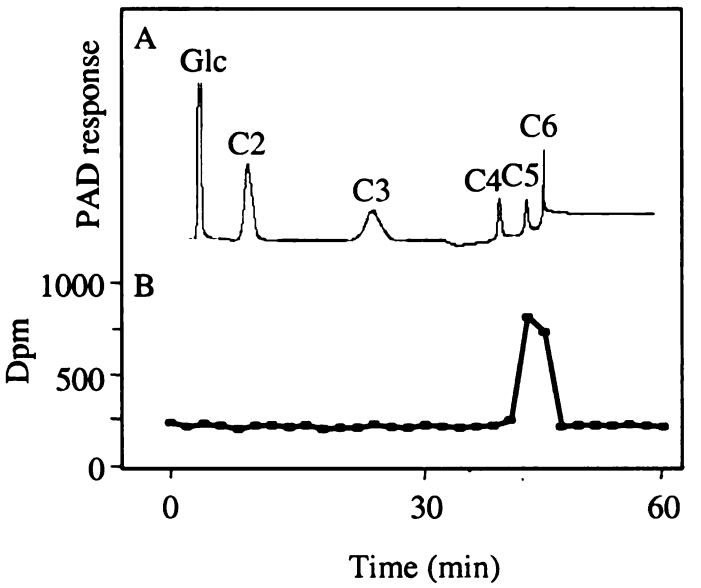


Figure 16. The ethanol-insoluble and water-soluble product synthesized after hydroxyapatite chromatography was analyzed by HPAEC-PAD. A, Oligosaccharide standards: Glc (glucose), C2 (cellobiose), C3 (cellotetriose), C4 (cellotetraose), C5 (cellopentaose), C6 (cellohexaose). B, Radioactivity.

To further characterize the product synthesized by the hydroxyapatite fractions, it was treated with different hydrolytic enzymes. The product was solubilized almost completely by xylanase M1. Cellulase, laminarinase, lichenase and proteinase K appeared to solubilize the product to some degree (Figure 17). This indicates that the product after hydroxyapatite chromatography is different from the product made before hydroxyapatite chromatography (Figs. 4 and 5).

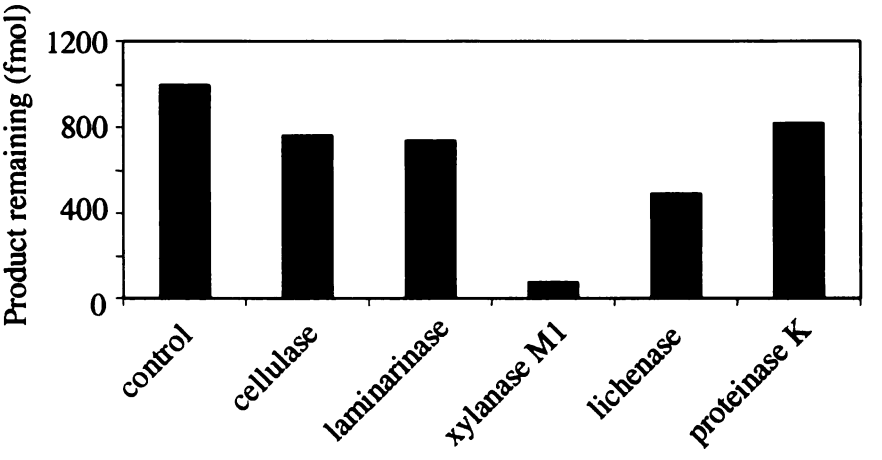


Figure 17. Enzyme sensitivity of the products synthesized after hydroxyapatite chromatography.

The solubilized products obtained after cellulase, laminarinase, or lichenase treatments were analyzed by HPAEC-PAD with the cellooligosaccharide 2 program. The products eluted mainly near the cellopentaose and cellohexaose standards. A minor change in elution time of the radiolabeled products was observed after cellulase treatment of the products (Figure 18). This change was not specific for cellulase, however, because the same observation was made after treating the product with endo-(1,3)-β-glucanase or lichenase (data not shown). From these data it was concluded that none of these enzymes actually hydrolyzed the products, and therefore after hydroxyapatite chromatography the product synthesized is not a (1,4)-β-glucan.

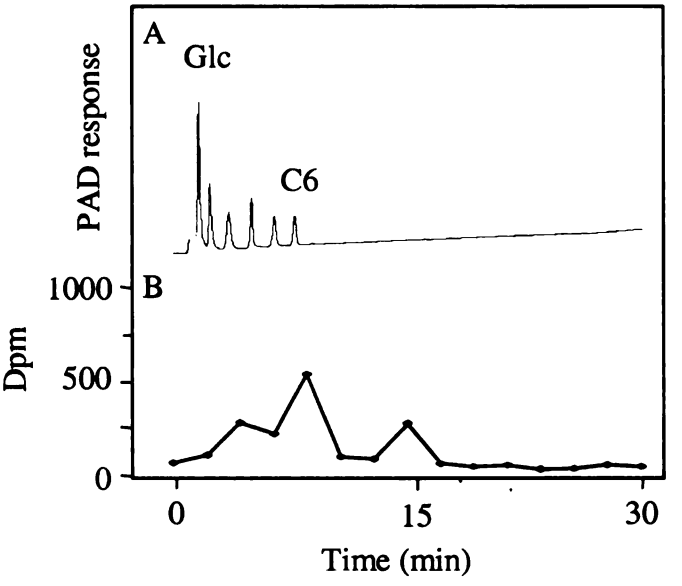


Figure 18. The product synthesized after hydroxyapatite chromatography was treated with cellulase and the hydrolysis products were analyzed by HPAEC-PAD. A, Oligosaccharide standards. B, Radioactivity.

After xylanase M1 treatment, glucose was the only product observed (data not shown). Because xylanase M6 did not hydrolyze the product, its not xylan. Alkaline phosphatase also hydrolyzed the product and glucose was released (Figure 19). These data suggested that after hydroxyapatite chromatography, the product might be a glucose-phosphate. Its hydrolysis by xylanase M1 could be due to contamination of this enzyme preparation by phosphatase.

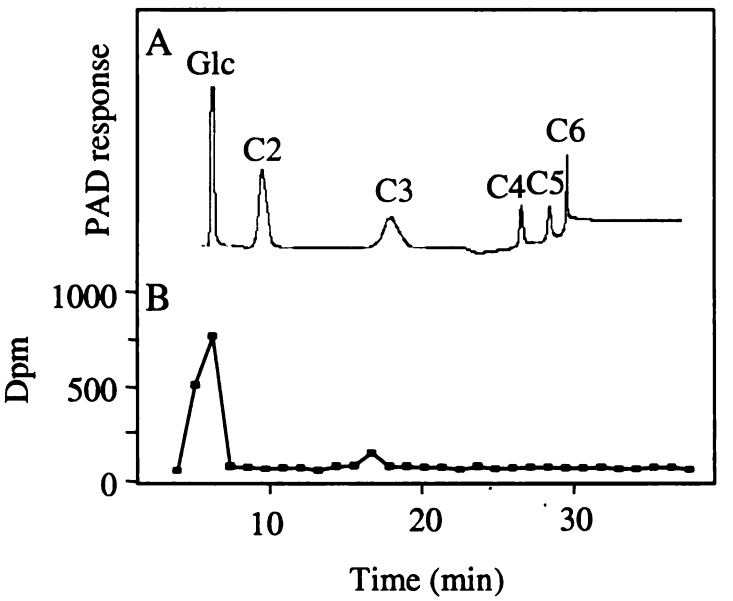


Figure 19. The product synthesized after hydroxyapatite chromatography was treated with alkaline phosphatase and the hydrolysis products were analyzed by HPAEC-PAD. A, Oligosaccharide standards. B, Radioactivity.

The product synthesized after hydroxyapatite chromatography was further characterized. Figure 20 shows that the products synthesized after hydroxyapatite chromatography eluted with glucose-6-phosphate (G6P) and glucose-1,6-diphosphate (G1,6dP). Therefore we conclude that after hydroxyapatite chromatography UDP-glucose is converted to G6P and G1,6dP. G6P and G1,6dP might form a complex with Mg^{2+} in the glucan synthase assay, which would make them insoluble in ethanol. Support for this comes from the fact that EDTA at a concentration greater than the Mg^{2+} concentration solubilizes the ethanol-insoluble products synthesized after hydroxyapatite chromatography (data not shown).

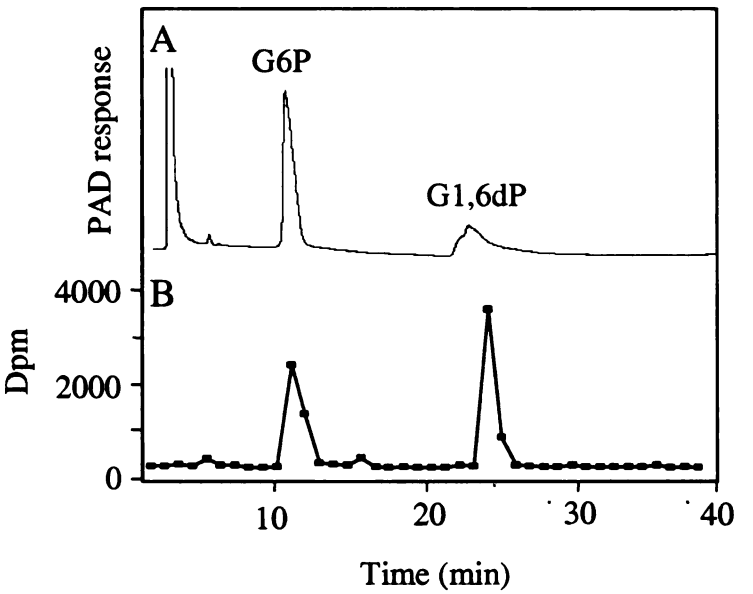


Figure 20. The water soluble products synthesized after hydroxyapatite chromatography were analyzed by HPAEC-PAD. A, glucose-6-phosphate (G6P) and glucose-1,6-disphosphate (G1,6dP) standards. B, Radioactivity.

DISCUSSION

In this study the biochemical properties of a Golgi-localized (1,4)-β-glucan synthase in the maize mesocotyl were studied, the product it synthesizes was characterized, and an attempt was made to purify the responsible protein.

It was determined that the pH optimum of the (1,4)-β-glucan synthase is 7.5-8 and that it requires a high (>15 mM) Mg^{2+} concentration for maximal activity. Glucan synthases have been studied in the past and there are several possible products in which glucose could be incorporated, including cellulose (Kudlicka et al., 1997), mixed-linkage-glucan (Gibeaut et al., 1993), xyloglucan (Bauer et al., 1973) or callose (Kudlicka et al., 1997).

I determined that microsomes isolated from both dark-grown and light-treated seedlings synthesize a (1,4)-β-glucan at a low UDP-Glc concentration and a (1,3)-β-glucan at a UDP-Glc concentration of 1 mM. The (1,4)-β-glucan synthase activity is affected by exposure of the seedlings to light 16 hours before extraction, in contrast to the (1,3)-β-glucan synthase activity, which is only affected somewhat by light.

Further analysis of the β(1,4)-glucan showed that the product is non-crystalline cellulose because the product was soluble in 4 N NaOH. Most likely the β(1,4)-glucan synthesized in our reactions is not attached to a protein because it could not be solubilized in 0.5% ammonium-oxalate. However, from these experiments we can not definitively conclude that the β(1,4)-glucan synthesized in our reactions is not attached to a protein. The reason being that the method we used for dissolving β(1,4)-glucan in ammonium-oxalate was different than the method Peng et al., (2001) used. Peng et al., (2001) boiled cell wall material for 1 hour in 0.5% ammonium oxalate while in our case we overnight incubated the β(1,4)-glucan in 0.5% ammonium oxalate at room temperature. Its possible that in our case the β(1,4)-glucan is attached to a protein, but that our method did not dissolve the β(1,4)-glucan because of the lower temperature at which we performed the incubation. Another factor which could influence the results we obtained in comparison to the results other researchers obtained is that in our case the β(1,4)-glucan was synthesized in vitro and was subsequently ethanol precipitated on a glass fiber filter. Other researchers used cell wall material synthesized in vivo for their experiments.

Attempts were made to determine if the (1,4)-β-glucan synthase is involved in xyloglucan or cellulose biosynthesis, but the results were inconclusive. A possible

explanation for these inconclusive results is that the assay conditions used were optimized for in vitro xyloglucan biosynthesis in dicots, because no assay for in vitro xyloglucan biosynthesis in monocots has been established.

In order to determine if the (1,4)-β-glucan synthase is a cellulose synthase on its way to the plasma-membrane, enzyme assays were performed in the presence of the cellulose synthase inhibitors DCB and isoxaben. Neither of these compounds inhibited (1,4)-β-glucan synthase activity in vitro, which might indicate that this enzyme is not a cellulose synthase. It is not known, however, what the exact mechanism of actions of these herbicides is, so no definitive conclusions can be made. Most likely these herbicides inhibit cellulose biosynthesis indirectly. This is supported by the observation that resistance to isoxaben is mediated by mutations which are not in the active site of the CESA3 and CESA6 proteins (Scheible et al., 2001; Desperez et al., 2002) and by the observation that loss of function mutations in genes related to cellulose biosynthesis such CESA2, COBRA, KORRIGAN, and CESA6 resulted in increased sensitivity to DCB and isoxaben (Somerville, 2006).

The (1,4)-β-glucan synthase was successfully solubilized with digitonin and the product synthesized was still (1,4)-β-glucan. The (1,4)-β-glucan synthase activity recovered after solubilization by digitonin was low, however. A possible explanation is that digitonin might inhibit (1,4)-β-glucan synthase activity by changing the conformation of the enzyme or by removing other factors which might stimulate activity.

After hydroxyapatite chromatography G6P and G1,6dP were synthesized, but no (1,4)-β-glucan. G1P can be synthesized from UDP-glucose by UDP-glucose pyrophosphorylase (UGPase). G6P can be synthesized from G1P by phosphoglucomutase

(Sowokinos et al., 1993). UGPase is a PPi and Mg^{2+}-dependent enzyme activity which catalyzes the following reversible reaction: UDP-glucose + PPi ⇔ glucose-1-P + UTP (Sowokinos et al., 1993). One plausible explanation is that UDP-glucose is first converted into G1P by UGPase. G1P is subsequently converted into G6P by phosphoglucomutase. The PPi could have leaked from the hydroxyapatite column, which consists of calcium phosphate. In vitro G1,6dP is an activator of phosphoglucomutase, but G1,6dP is not found in plants, however (Galloway et al., 1985). In animals and bacteria G1,6dP can be synthesized by phosphoglucomutase and is an intermediate in the conversion of G1P into G6P (Naught et al., 2005). In plants G1,6dP might also be synthesized by phosphoglucomutase and serve as an intermediate in the conversion of G1P into G6P. The UDP-glucose pyrophosphorylase and phosphoglucomutase proteins were present in the hydroxyapatite fractions with activity. This supports the hypothesis that UDP-glucose is converted in G1P and subsequently in G6P by these enzymes. Under standard glucan synthase assay conditions no UDP-glucose pyrophosphorylase and phosphoglucomutase activity was detected in the solubilized (1,4)-β-glucan synthase preparation. However, after hydroxyapatite chromatography this is the only activity present under standard glucan synthase assay conditions. One possibility is that there is no PPi present in the solubilized glucan synthase fraction, which is necessary for UDP-glucose pyrophosphorylase activity. After chromatography UDP-glucose pyrophosphorylase might be activated by leaking of PPi from the hydroxyapatite column. Under standard glucan synthase assay conditions the pmol of [^{3}H]-glucose precipitated by ethanol after hydroxyapatite chromatography is similar to the pmol [^{3}H]-glucose precipitated by ethanol in the solubilized (1,4)-β-glucan synthase preparation. In case

G6P and G1,6dP are synthesized by a different enzyme than the (1,4)-β-glucan synthase I would expect the pmol of [^{3}H]-glucose precipitated to be different. An alternative possibility is that the glucan synthase becomes damaged or is separated from an acceptor or necessary auxiliary protein following chromatography and that it converts UDP-glucose into G1P instead of synthesizing (1,4)-β-glucan.

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CHAPTER 3: ANNOTATION OF THE ZEA MAYS *CSL* GENE FAMILY

ABSTRACT

The maize (Zm) *CSL* gene family was annotated based on orthology to the rice (Os) *CSL* gene family using NCBI (public) and MaizeSeq (proprietary) EST collections and available genome sequence (public). Maize has all of the *CSL* subfamilies which are present in rice. There are, however, several differences between the two. The *CSLC* subfamily, for instance is expanded from six members in rice and five in *Arabidopsis* to potentially twelve members in maize. The *CSLH* subfamily is reduced from three members in rice to one in maize. Maize has one atypical CSL, called ZmCSLX, which is related to *Populus trichocarpa* (poplar) CSLG1 and G3. Several ESTs related to the *ZmCSLX* ORF were found in other monocot species such as *Sorghum bicolor* (sorghum), *Saccharum sp* (sugar cane) and *Hordeum vulgare subsp vulgare* (barley).

INTRODUCTION

The first cellulose synthase genes were discovered in *Acetobacter xylinum* (Wong et al., 1990). Based on sequence similarity to these bacterial cellulose genes the plant cellulose synthase (*CESA*) genes were discovered (Pear et al., 1996; Arioli et al., 1998). Based on sequence similarity to the *CESA* family, a related gene family called the *CSL* family was described in *Arabidopsis* (Richmond and Somerville, 2000). *Arabidopsis* has 10 *CESA* genes and 29 *CSL* genes, which together compose the *CESA* superfamily. The *Arabidopsis* (At) *CSL* gene family is divided into 6 subfamilies called *CSLA*, *B*, *C*, *D*, *E* and *G*. The rice *CSL* gene family was annotated by Hazen et al (2002) and is composed

of the *CSLA*, *C*, *D*, *E*, *F* and *H* subfamilies and it has 34 members. The rice *CSL* gene family is missing the *CSLB* and *G* subfamilies but has two cereal specific subfamilies called *CSLF* and *H*. All the members of the *CESA* superfamily have two conserved domains, several widely spaced aspartate residues (D) and the QXXRW motif. These two domains are hypothesized to be the sugar-nucleotide binding site and the active site, respectively (Richmond and Somerville, 2000). The CSL proteins are involved in the synthesis of the sugar backbones of at least some of the hemicelluloses. The CSLA proteins, for instance, are (1,4)-β-(gluco)mannan synthases (Dhugga et al., 2004; Liepman et al., 2005; Suzuki et al., 2006; Liepman et al., 2007), AtCSLC4 and TmCSLC synthesize the (1,4)-β-linked glucose backbone of xyloglucan (Cocuron et al., 2007), the CSLD proteins might be involved in cellulose biosynthesis in tip growing cells (Doblin et al., 2001; Wang et al., 2001; Roberts and Bushoven, 2007) and OsCSLF2 is involved in mixed-linkage-glucan (MLG) biosynthesis (Burton et al., 2006). It is not known however, if all the members of a CSL subfamily catalyze the synthesis of the same polymer. The function of the other CSL proteins is not known.

MATERIALS AND METHODS

Annotation of the maize CSL protein family

The *ZmCSL* gene family was annotated based on homology to the OsCSL protein family (Hazen et al., 2002; http://waltonlab.prl.msu.edu//CSL_updates.html). The Iowa State Maize Assembled Genome Island 4 (MAGI4) database (http://magi.plantgenomics.iastate.edu/; Fu et al., 2005) was searched (tblastn) using OsCSLA1, C1, D1, E1, F1 and H1 proteins as queries. Proteins encoded by the MAGIs

were determined using the FGENESH monocot *ab initio* gene prediction program (Yao et al., 2005; www.softberry.com). The predicted proteins were aligned with the OsCSL proteins using ClustalW, MegAlign (DNASTAR). MAGIs belonging to the same *CSL* subfamily were aligned (criteria: match size 12, mismatch percentage 80, minimum sequence length 100) using SeqMan (DNASTAR). Sequences which aligned were assembled into contigs. These genomic contigs were used to search for additional genomic sequence in the MAGI4 database, at the public database at TIGR (http://www.tigr.org/), and at the Plant Genome Database (http://www.plantgdb.org/). The genomic contigs were also used to search the NCBI EST database (http://www.ncbi.nlm.nih.gov/, blastn, database est_others, *Zea mays*) or the ESTs in the MaizeSeq database (http://www.maizeseq.org/, blastn). The ESTs were assembled in contigs and aligned with the genomic contigs using Spidey (http://www.ncbi.nlm.nih.gov/spidey/) in order to determine the intron-exon structure of the genes. The annotation was refined by comparing the ZmCSL proteins with their closest rice orthologs using ClustalW. Images in this dissertation are presented in color.

Phylogenetic analysis

Proteins were aligned with the ClustalX using default settings (Multiple Alignment Mode) (Thompson et al., 1997). Phylograms were constructed with the heuristic search method in PAUP and a phylogram corresponding to 1000 bootstrap replicates was constructed (Version 4.0b10, Sinauer Associates, Sunderland, MA). The phylogenetic trees were modified with the Treeview program (Page, 1996).

RESULTS

The genomic, cDNA and amino acid sequences for each *ZmCSL* gene can be found at http://waltonlab.prl.msu.edu//. The list of genomic sequences used for the annotation can be found in Appendix I, Table 8. A detailed description of the annotation of each *CSL* gene can be found in Appendix I, Table 9. The number of genes in the maize and rice *CSL* gene families is comparable for the *CSLA, D, E,* and *F* subfamilies. The *ZmCSLC* gene family is expanded from six in rice to twelve in maize including one gene, *ZmCSLC9*, which is most likely a pseudogene. *ZmCSLA11*, *E2* and *E4* are probably also pseudogenes, because they have stop codons in their coding regions and there are no ESTs in the NCBI and MaizeSeq EST databases. Rice has three *CSLH* genes, maize only one. One atypical *CSL*, *ZmCSLX,* was found which does not have a homolog in rice (Table 4).

Table 4: The number of *CSL* genes for each subfamily in rice and maize			
	Rice	**Maize** (Full length: full length genomic DNA and/or cDNA is available for this gene. Partial: partial genomic DNA and/or cDNA).	
***CSL* subfamily**		**Full length**	**Partial**
A	9	6 (*ZmCSLA1, 3, 6, 7, 8, 9, 10*)	5 (*ZmCSLA2, 4, 5, 11* (pseudogene))
C	6	7 (*ZmCSLC1, 3, 4, 5, 6, 7, 11*)	5 (*ZmCSLC2, 8, 9* (pseudogene), *10, 12*)
D	5	4 (*ZmCSLD1, 2, 4, 5*)	1 (*ZmCSLD3*)
E	3	2 (*ZmCSLE1, 3*)	2 (*ZmCSLE2* (pseudogene), *E4* (pseudogene))
F	8	5 (*ZmCSLF1, 2, 4, 6, 7*)	2 (*ZmCSLF3, 5*)
H	3	1 (*ZmCSLH1*)	0
X	0	1 (*ZmCSLX*)	0

Phylogenetic analysis of the CSLA and CSLC protein families of different plant species

Figure 21 shows a phylogram constructed with the full length CSLA proteins of maize, rice (www.prl.msu.edu/walton/CSL_updates.htm), poplar *(Populus trichocarpa)*

(Suzuki et al., 2006), moss *(P. patens)* (Roberts and Bushoven, 2007), *Arabidopsis,* guar *(Cyamopsis tetragonoloba)* (Dhugga et al., 2004) and loblolly pine *(Pinus taeda)* (Liepman et al., 2007) and the full length CSLC proteins of *Arabidopsis*, nastursium (*Tropaeolum majus*, Tm), maize, moss, rice and poplar. The ZmCESA1, 2, 3 and 4 proteins were used as an outgroup. In Appendix II, Table 11 the Genbank locus numbers of the *Arabidopsis* CSL proteins are mentioned, in Table 12 the JGI protein IDs of the poplar CSL proteins, in Table 13 the JGI protein IDs for the *Physcomitrella* CSL proteins and in Table 14 the GenBank locus number for the ZmCESA proteins.

For almost every rice CSLA or C protein, there are one or two corresponding maize orthologs. Conversely, there are one or two rice orthologs for each maize protein. Therefore it can be concluded that there is a strong conservation between these two protein families in rice and maize. Nine CSLA and CSLC genes in maize do not have full length genomic or cDNA sequence available (Table 4). Only after these genes are completely annotated can a definitive conclusion about the similarity between these two gene families be made. The same can be observed for the *Arabidopsis* and poplar CSLA and CSLC protein families. For almost every *Arabidopsis* CSLA or C protein, there are one or two corresponding poplar orthologs. Conversely, there are one or two poplar orthologs for each *Arabidopsis* protein. Therefore it can be concluded that there is a strong conservation between these two protein families in *Arabidopsis* and poplar. The strong conservation between maize and rice on the one hand and the *Arabidopsis* and poplar CSLA and CSLC protein families on the other hand reflects the evolutionary divergence between cereals and dicots. This evolutionary divergence of the CSLA and

CSLC protein families might reflect the structural differences between cereal and dicot cell walls.

Some of the CSLA proteins from rice and maize (OsCSLA2, 3, 4, 5, 7, 11 and ZmCSLA1, 6, 7, 10), on the one hand, and *Arabidopsis* (AtCSLA1, 3, 7, 10, 11, 14, 15), on the other, form distinct clades. These cereal and *Arabidopsis* specific CSLA clades are weakly supported, however (bootstrap value of <50). AtCSLA2; OsCSLA1, 9; PtCSLA4, 5 and ZmCSLA3, 9 cluster in a mixed clade, which is weakly supported (bootstrap value of <50). The remainder of the CSLA proteins from rice, maize, poplar, pine tree, and *Arabidopsis* (AtCSLA9; OsCSLA6; PpCSLA1, 2; PtCSLA1, 2, 3; PtaCSLA1, 2; CtMan and ZmCSLA8) do not cluster in any specific clade. In conclusion, dicots and cereals might have some CSLA proteins in common but might also have unique CSLA proteins. This might reflect biochemical specialization due to the different structures of their cell walls.

The AtCSLC4 and TmCSLC proteins are involved in synthesizing the β(1,4)-glucan backbone of xyloglucan (Cocuron et al., 2007). Figure 21 shows that some of the CSLC proteins from rice and maize (OsCSLC1, 7; ZmCSLC3, 4, 6) on the one hand, and moss (PpCSLC1, 2 3) on the other, form distinct clades. The rice and maize-specific clade and the moss-specific clades are weakly supported (bootstrap value of <50). The clustering in these distinct clades might reflect biochemical specialization due to the different structures of moss, dicot and cereal cell walls. Xyloglucan in cereals is less xylosylated than xyloglucan in dicots (Kato et al., 1980b, 1981b, 1982; Zablackis et al., 1995; Gibeaut et al., 2005). The cereal specific group of CSLC proteins might be involved in the synthesis of the less xylosylated type of xyloglucan found in cereals. Two

other major clades consists of both dicot and cereal CSLC proteins. Clade 1 consists of the AtCSLC5, 8; OsCSLC2, 3; PtCSLC1, 2 and ZmCSLC1, 11 proteins. Clade 2 consists of the AtCSLC12; OsCSLC9, 10; PtCSLC3, 4; ZmCSLC5, 7 proteins. These clades are weakly supported, however (bootstrap value <50). The rice and maize CSLC proteins that belong to these mixed clades might be involved in the synthesis of the β(1,4)-glucan backbone of galactose-containing xyloglucan such as found in rice endosperm and barley cell walls (Shibuya and Misaki, 1978; Kato et al., 1981). In maize the presence of galactosylated xyloglucan has not been shown, however. There is one minor clade consisting of AtCSLC6 and PtCSLC5, which is weakly supported (bootstrap value 56).

Figure 21. Parsimony phylogram of *Arabidopsis* (At), guar (Ct), maize (Zm), moss (Pp), pine tree (Pta), poplar (Pt) and rice (Os) CSLA proteins and the CSLC proteins of *Arabidopsis,* maize, moss, nasturtium (Tm), poplar and rice. The colors in this figure correspond to different species. The ZmCESA1, 2, 3 and 4 proteins were used as an outgroup. Four ZmCSLA proteins and five ZmCSLC proteins were excluded because full length sequences are not available.

Figure 21

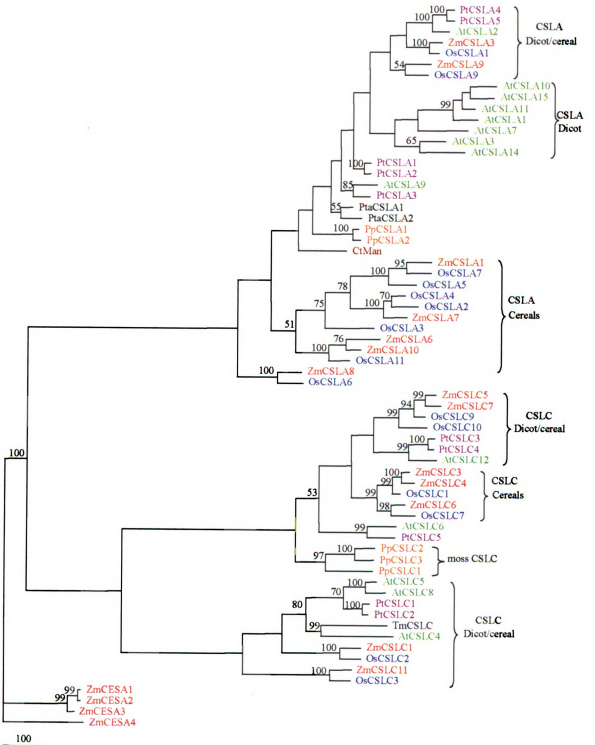

PtCSLA4
PtCSLA5
AtCSLA2
ZmCSLA3
OsCSLA1
ZmCSLA9
OsCSLA9
CSLA
Dicot/cereal
AtCSLA10
AtCSLA15
AtCSLA11
AtCSLA1
AtCSLA7
AtCSLA3
AtCSLA14
CSLA
Dicot
PtCSLA1
PtCSLA2
AtCSLA9
PtCSLA3
PtaCSLA1
PtaCSLA2
PpCSLA1
PpCSLA2
CtMan
ZmCSLA1
OsCSLA7
OsCSLA5
OsCSLA4
OsCSLA2
ZmCSLA7
OsCSLA3
ZmCSLA6
ZmCSLA10
OsCSLA11
CSLA
Cereals
ZmCSLA8
OsCSLA6
ZmCSLC5
ZmCSLC7
OsCSLC9
OsCSLC10
PtCSLC3
PtCSLC4
AtCSLC12
CSLC
Dicot/cereal
ZmCSLC3
ZmCSLC4
OsCSLC1
ZmCSLC6
OsCSLC7
CSLC
Cereals
AtCSLC6
PtCSLC5
PpCSLC2
PpCSLC3
PpCSLC1
moss CSLC
AtCSLC5
AtCSLC8
PtCSLC1
PtCSLC2
TmCSLC
AtCSLC4
CSLC
Dicot/cereal
ZmCSLC1
OsCSLC2
ZmCSLC11
OsCSLC3
ZmCESA1
ZmCESA2
ZmCESA3
ZmCESA4
100

Figure 22 shows which CSLA proteins encode for mannan synthases. None of the CSLA proteins belonging to the cereal-specific clade of CSLA proteins has been shown to encode mannan synthase, while many members of the other clades of CSLA proteins are proven mannan synthases (Dhugga et al., 2004; Liepman et al., 2005 and 2007; Suzuki et al., 2007). None of the CSLA proteins in the cereal-specific clade have been tested for mannan synthase activity. This raises the question if the CSLA proteins cereal-specific clade are mannan synthases or if they have other biochemical activities.

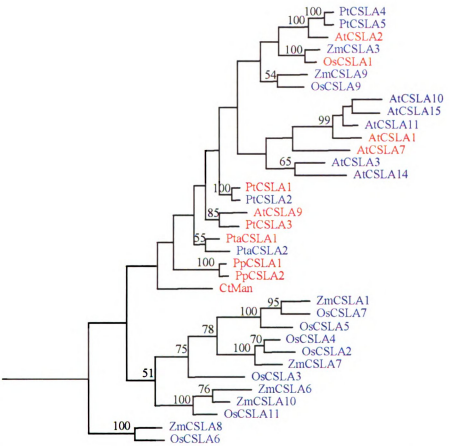


Figure 22. Parsimony phylogram of *Arabidopsis* (At), guar (Ct), rice (Os), maize (Zm), moss (Pp) and poplar (Pt) CSLA proteins. The tree is identical to figure 21, but colored by function rather than taxonomy. Those that have been determined to encode for (1,4)-β-mannan synthases are shown in red and blue means that they have not been tested.

Phylogenetic analysis of the CSLB, D, E, F, H and G protein families of different plant species

Figure 23 shows a parsimony phylogram of the *Arabidopsis*, maize, moss, *Nicotiana tabacum* (Nt), poplar and rice CSLB, D, E, F, G, H and X proteins. For almost every rice CSLD, E, F and H protein there are one or two maize orthologs. Conversely,

there are one or two rice orthologs for each maize gene. Therefore it can be concluded that there is a strong conservation between these four protein families in rice and maize. For almost every *Arabidopsis* CSLB, D, E and G protein, there are one or two corresponding poplar orthologs. Conversely, there are one or two poplar orthologs for each *Arabidopsis* protein. Therefore it can be concluded that there is a strong conservation between these two protein families in *Arabidopsis* and poplar.

The CSLD protein family clusters in four major clades. Three of these clades consist of cereal and dicot CSLD proteins and one clade is specific for moss. Clade 1 consists of the AtCSLD2, 3; OsCSLD1, 2; ZmCSLD2, 5 and PtCSLD5, 6 proteins and is weakly supported (bootstrap value <50). Clade 2 consists of the AtCSLD1, 4; OsCSLD3, 5; ZmCSLD4 and PtCSLD7, 8, 9, 10 proteins and is weakly supported (bootstrap value 56). Clade 3 consists of the AtCSLD5; OsCSLD4; ZmCSLD1 and PtCSLD1, 2 proteins and is strongly supported (bootstrap value 89). The moss specific clade consists of PpCSLD1, 2, 3, 4, 5, 6, 7 and 8 and is strongly supported (bootstrap value of 89). The biological significance of these clades is not known, however. Besides these four major clades PtCSLD3 and 4 cluster together in a specific clade which is weakly supported (bootstrap value of 60) and AtCSLD6 clusters by itself (bootstrap value of 100).

The analysis of the maize CSL protein family supports the conclusion that the CSLF proteins are found only in cereals (Fig. 23). The maize and rice CSLF proteins cluster in a separate clade which is weakly supported (bootstrap value of 53).

The CSLB family is not present in rice or maize, but only in dicots and is strongly supported (bootstrap value of 98). The CSLB family is divided into two different clades.

One consists of *Arabidopsis* CSLB proteins and the other consists of poplar CSLB proteins. These clades are weakly supported (bootstrap value of 58). Rice and maize have a CSLB-like family, which was named CSLH by Hazen et al. (2002) and is strongly supported (bootstrap value of 98).

The CSLE protein family consists of a cereal specific clade (OsCSLE1, 2, 6; ZmCSLE1, 3), a poplar specific (PtCSLE2, 3) clade and a mixed *Arabidopsis* and poplar clade (AtCSLE1, PtCSLE1). The cereal specific clade and the poplar specific clade are weakly supported (bootstrap value of 58). The *Arabidopsis* and poplar specific clade is strongly supported (bootstrap value of 100).

The CSLG family is also not present in rice (Hazen et al., 2002). Maize however, has a protein, which we call ZmCSLX, which clusters with all of the other members of the CSLG family. This clade is strongly supported (bootstrap value of 86).

Figure 23. Parsimony phylogram of the *Arabidopsis* (At), maize (Zm), moss (Pp), tobacco (*Nicotiana tabacum* (Nt)), poplar (Pt) and rice (Os) CSLB, D, E, F, G, H and X proteins. The ZmCSLA1, 3 and ZmCSLC1, 3 proteins were used as an outgroup. One ZmCSLD, two ZmCSLE and two CSLF proteins were excluded because full length sequences are not available. The colors in this figure indicate the different species.

Figure 23

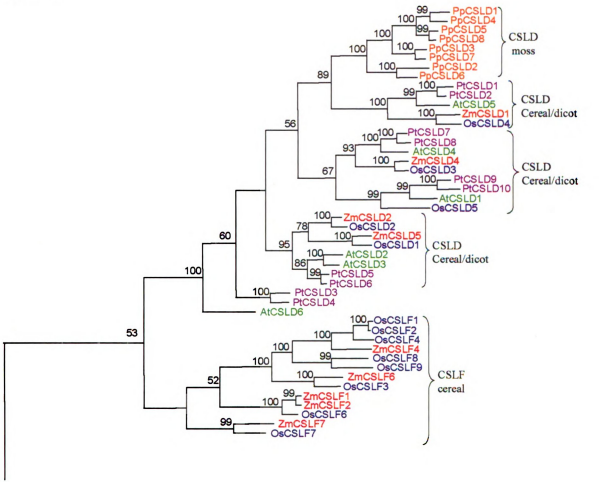
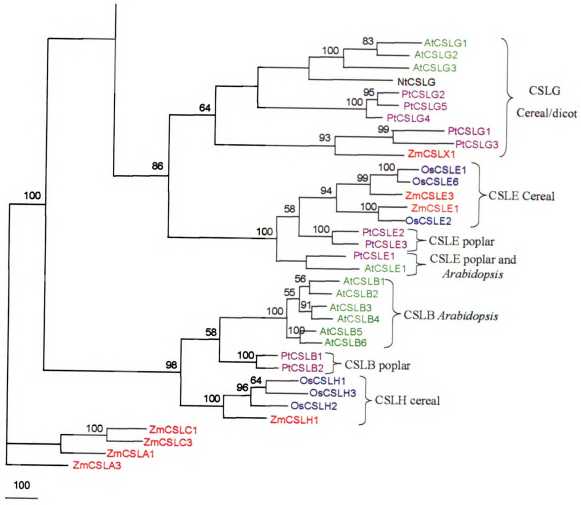

PpCSLD1
PpCSLD4
PpCSLD5
PpCSLD8
PpCSLD3
PpCSLD7
PpCSLD2
PpCSLD6
CSLD
moss
PtCSLD1
PtCSLD2
AtCSLD5
ZmCSLD1
OsCSLD4
CSLD
Cereal/dicot
PtCSLD7
PtCSLD8
AtCSLD4
ZmCSLD4
OsCSLD3
PtCSLD9
PtCSLD10
AtCSLD1
OsCSLD5
CSLD
Cereal/dicot
ZmCSLD2
OsCSLD2
ZmCSLD5
OsCSLD1
AtCSLD2
AtCSLD3
PtCSLD5
PtCSLD6
CSLD
Cereal/dicot
PtCSLD3
PtCSLD4
AtCSLD6
OsCSLF1
OsCSLF2
OsCSLF4
ZmCSLF4
OsCSLF8
OsCSLF9
ZmCSLF6
OsCSLF3
ZmCSLF1
ZmCSLF2
OsCSLF6
ZmCSLF7
OsCSLF7
CSLF
cereal

Figure 23 continued



ZmCSLX

The properties of the ZmCSLX protein suggest that it is a member of the CSL family. It has the D, D, D and the QXXRW motifs present in all of the members of the CESA superfamily. It is predicted to have four transmembrane domains by the TMHMM Server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM-2.0/). When a blast search (GenBank, tblastn, nr) was performed using CSLX as a query, a CSLG protein from *Nicotiana tabacum* (tobacco) had the highest score ($2e^{-122}$) (as of November, 2006). Figure 23 shows that ZmCSLX clusters with the PtCSLG1 and 3 proteins. The reason why the highest blast scores were not for poplar CSLG1 or G3, but for a tobacco CSLG, is that the poplar proteins are not available in Genbank but only at the Joint Genome Institute (JGI) website. To determine if other cereals have orthologs of ZmCSLX the SAMI (Sorghum Assembled genoMic Islands) database was searched (http://magi.plantgenomics.iastate.edu/, tblastn) with the *ZmCSLX* ORF as query (Table 5). Several related genomic sequences were found. Two ESTs related to this gene were found in the Sorghum EST database (GenBank, est_others, *Sorghum bicolor*, tblastn). For rice and wheat, no ESTs were found. Sugarcane has one EST and barley three (Table 5). The high percentage of identity (Table 5) suggests that these are true orthologs. The proteins encoded by these ESTs cluster most closely with ZmCSLX when aligned with the maize CSLA, C, D, E, F and H proteins, and with the *Arabidopsis*, poplar and tobacco CSLG proteins (data not shown). It cannot yet be concluded, however, that these monocots have CSLX proteins, because no full length sequences are available yet. In conclusion, the CSLG protein family, which was previously thought to be specific for dicots, might also be present in some monocots.

Table 5: Genomic DNA and EST sequences related to the *ZmCSLX* ORF in different monocot species (family Poaceae)

***% maximal identity at nucleotide level**

****% coverage at nucleotide level**

Species	Genomic DNA	ESTs
Sorghum	CW233534, CW233535, CW457534, CW457535, CW494817, CW494818, fsbb001f286e18.R, SAMIv2_25399	CF430961 (*92%, **19%) CF431079 (*97%, **13%)
Barley		CA000498 (*85%, **9%) CA002678 (*97%, **5%) CA011599 (*95%, **13%)
Sugar cane		CA096252 (*95%, **16%)

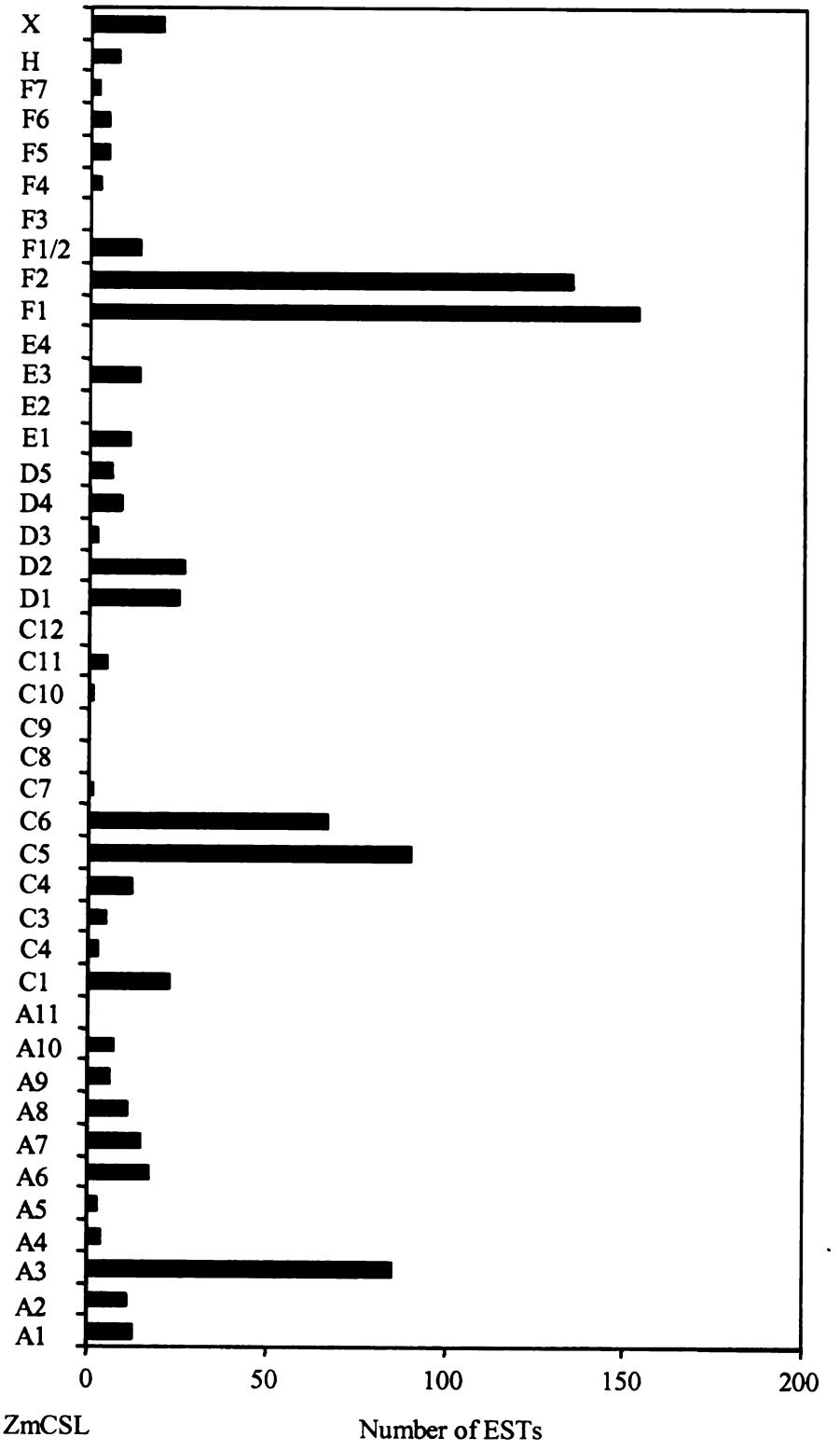
ZmCSL ESTs

The ESTs for each *ZmCSL* are described in Table 6. The number of ESTs in GenBank (http://www.ncbi.nlm.nih.gov/, blastn, est_others, *Zea mays*) and MaizeSeq (http://www.maizeseq.org/, blastn) databases was determined by searching with *ZmCSL* cDNAs as queries. An interesting observation is that the numbers of ESTs for each *CSL* gene varies significantly for each *CSL* subfamily and within each subfamily (Figure 24). A total of 828 ESTs belonging to maize *CSL* genes were found in GenBank and MaizeSeq as of November, 2006. Six hundred and six of these ESTs (66%) belong to five

members of the *ZmCSL* gene family (*ZmCSLA3*, *C5*, *C6*, *F1* and *F2*). *ZmCSLF1* and *F2* account for 36% of all the maize ESTs in GenBank and MaizeSeq. The accession numbers of the ESTs for each *ZmCSL* are in Appendix I, Table 10.

Figure 24. Distribution of ESTs belonging to maize *CSL* genes in GenBank (blue bar) and MaizeSeq (purple bar) as of November 2006. F1/F2 indicates that these ESTs aligned with identical regions in the *CSLF1* and *F2* genes, which makes it impossible to determine from which gene they originate.

Figure 24


X
H
F7
F6
F5
F4
F3
F1/2
F2
F1
E4
E3
E2
E1
D5
D4
D3
D2
D1
C12
C11
C10
C9
C8
C7
C6
C5
C4
C3
C4
C1
A11
A10
A9
A8
A7
A6
A5
A4
A3
A2
A1
0
50
100
150
200
ZmCSL
Number of ESTs

DISCUSSION

The annotation of the *ZmCSL* gene family showed that it is very similar to the *OsCSL* gene family. For almost every maize CSL protein there are one or two rice homologs, and there are one or two maize homologs for every rice CSL protein. When the rice and maize CSL proteins were aligned with CSL proteins from other plant species, clustering in monocot specific clades was observed for the CSLA, C and E proteins. The CSLD proteins did not show this type of clustering, instead the monocot and dicot CSLD proteins clustered in several mixed clades. My phylogenetic analysis confirms that CSLB proteins are only present in dicots and that the CSLH and F proteins are only present in monocots (Hazen et al., 2002).

Many of the CSLA proteins are mannan synthases (Dhugga et al., 2004; Liepman et al., 2005, 2007; Suzuki et al., 2006) but none of the CSLA proteins in the cereal-specific clade have been shown to encode for mannan synthases. This raises the question if the CSLA proteins in this cereal-specific clade are mannan synthases. Cereal and dicot cell walls have a different structure. Could it be that the cereal specific CSLA proteins are involved in synthesis of a cereal-specific polymer? This could be tested by expressing them and determining their biochemical activity. Handford et al. (2003) showed that mannan epitopes are abundantly present in *Arabidopsis*. In maize mannan is only a minor component of the cell wall however (Carpita et al., 2001). Investigation of mutants indicates that mannan has a role in embryo development in *Arabidopsis* (Goubet et al., 2003) and is important for *Agrobacterium* infection of *Arabidopsis* (Zhu et al., 2003).

Several members of the CSLC protein family (AtCSLC4 and TmCSLC) are involved in the synthesis of the β(1,4)-linked glucose backbone of xyloglucan (Cocuron

et al., 2007). The ZmCSLC protein family has twelve members in maize in comparison to six in rice and five in *Arabidopsis* and poplar. Xyloglucan is a minor hemicellulose in maize and other cereals (Carpita et al., 2001; Gibeaut et al., 2005) in comparison to dicots (Zablackis et al., 1995), so it is surprising that maize has so many CSLC proteins.

The CSLC proteins cluster in several different clades. Five of the rice and maize CSLC proteins cluster in a cereal-specific clade. The members of this clade might be involved in the synthesis of the β(1,4)-glucan backbone of the cereal type of xyloglucan. The majority of xyloglucan in cereals is less xylosylated than dicot xyloglucan and contains no galactose and fucose residues on the xylosyl side chains of the β(1,4)-glucan backbone (Bauer et al., 1973; Kato et al., 1980b, 1981b, 1982). The remainder of the maize and rice CSLC proteins clusters with dicot CSLC proteins. These maize and rice CSLC proteins might be involved in the synthesis of the galactosylated xyloglucan found in, for instance, rice endosperm and barley cell walls (Shibuya and Misaki, 1978; Kato et al., 1981). Galactosylated xyloglucan has not yet been reported in maize, however. The three moss CSLC proteins cluster in a separate clade. The clustering of these moss CSLC proteins in a separate clade might reflect the evolutionary distance between mosses and other land plants. Moss cell walls also contain xyloglucan (Popper and Fry, 2002), so these CSLC proteins might be involved in the synthesis of the β(1,4)-linked glucose backbone of xyloglucan in mosses.

The CSLD proteins cluster in four major clades. Three of these clades consist of dicot and monocot CSLD proteins. One clade consists only of moss CSLD proteins. It is not known what the biological significance of the separate clades with dicot and monocot CSLD proteins is. It could be that the CSLD proteins belonging to the three monocot and

dicot specific clades are expressed in specific tissues or that they have a different biochemical function. A knock-out mutant in Kojak/AtCSLD3 showed reduced elongation of root hairs and the root hair tips leaked cytoplasm. This indicates tensile strength of the root hair cell walls was reduced (Favery et al., 2001; Wang et al., 2001). Based on these observations the authors suggested that the KOJAK/AtCSLD3 protein might be involved in the synthesis of β(1,4)-glucan in tip-growing cells such as roothairs (Favery et al., 2001; Wang et al., 2001). Pollen tubes in *Nicotiana alata* consist of callose and cellulose. Two of the major glycosyltransferases expressed in these pollen tubes were a callose synthase and NaCSLD1. This suggests that NaCSLD1 is involved in cellulose biosynthesis in pollen tubes in *N. alata* (Doblin et al., 2001). *CSLD* genes are also highly expressed in auxin-treated cultures of *P. patents* (Roberts et al., 2007). Tip growth is important in *P. patents,* so this supports the hypothesis that the CSLD proteins are involved in cell wall biosynthesis in tip growing cells.

The biochemical functions of the CSLE proteins are not known. The CSLE protein family consists of a cereal specific clade, a poplar specific clade and a mixed *Arabidopsis* and poplar clade. The biological significance of these different clades is not known.

The CSLF proteins, which are specific for cereals (Hazen et al., 2002) are involved in mixed-linkage glucan biosynthesis (Burton et al., 2006). Arabidopsis was transformed with the *OsCSLF2* gene and MLG was detected in the cell walls with monoclonal antibodies and enzymatic analysis.

One atypical ZmCSL was found which was provisionally called ZmCSLX. Rice apparently does not have a CSLX, but sorghum, sugarcane and barley do. It cannot yet be

concluded, however, that *ZmCSLX* genes are present or absent in other monocot species besides maize because the complete coding region is currently available only for *ZmCSLX*. The ZmCSLX protein clustered with the CSLG proteins of *Arabidopsis*, poplar and tobacco, so ZmCSLX probably belongs to the CSLG protein family. Previously it was thought that the CSLG family was specific for dicots because no CSLG ESTs and genomic sequences were found for rice or maize (Richmond and Somerville, 2001; Hazen et al., 2002). My results indicate that CSLG proteins might also be present in monocots. The biochemical function of the CSLG family is not known. Expression analysis with GUS fusions of AtCSLG1 and G2 showed their expression is consistent with a role in xylem cell wall formation (Richmond and Somerville, 2001).

The ZmCSLH protein family has one member in maize in comparison to three in rice. We do not know the function of the CSLH proteins. The *CSLH* subfamily is not found in dicots (Hazen et al., 2002), which is confirmed by my phylogenetic analysis. CSLH proteins might be involved in the synthesis of a monocot-specific polysaccharide such as MLG.

The number of ESTs in GenBank and MaizeSeq are unequally distributed among the *ZmCSL* genes of every *CSL* subfamily. *ZmCSLA3*, *C5*, *C6*, *F1* and *F2*, for instance, are represented by a large number of ESTs, especially in GenBank. One possible reason for this is that the ESTs originate from libraries that are constructed from pooled multiple tissues, and it is not known what percentage of the RNA came from what tissue. ESTs from *CSL* genes that are highly expressed in certain tissues might dominate the libraries. The abundance of the ESTs seems to be more equal in MaizeSeq than in GenBank. One

reason for this could be that the ESTs in the MaizeSeq database mainly consist of full length cDNAs, which might be assembled from individual ESTs.

For predicting the structure of the genes encoded by the genomic sequences, the FGENESH gene prediction program trained for monocots was used (Yao et al., 2005). FGENESH predicted the gene structure of 17 (41%) of the 41 *CSL* genes correctly. Yao et al. (2005) found that FGENESH predicted 50% of the gene structures of maize genes correctly. Several reasons why the gene predictions by FGENESH are not always correct are that it is not suitable for the prediction of genes with very small exons (<100 bp), large introns, or non-canonical splice sites. The major source of problems in the FGENESH prediction for the *ZmCSL* gene family was related to incomplete genomic sequence, large intron size, small exon size, and incorrect prediction of the start or end of an exon. Alternative splice sites were not a major cause of the incorrect predictions of FGENESH in the case of the *ZmCSL* gene family. There is only one intron in the 41 genes of the *ZmCSL* family which has a non-canonical splice site. The second intron of *ZmCSLF4* has a non-canonical splice site, GC/AG instead of GT/AG, which was predicted incorrectly by FGENESH. One rice gene, *OsCSLA4*, also has a non-canonical splice site.

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CHAPTER 4: LIGHT-REGULATION OF THE MAIZE *CSL* GENE FAMILY AND ENZYMATIC ACTIVITIES RELATED TO CELL WALL BIOSYNTHESIS

ABSTRACT

To gain a better understanding of the developmental regulation of cell wall biosynthesis, light-regulation of cell-wall-related enzymatic activities were analyzed in the maize mesocotyl. (1,4)-β-glucan synthase activity was reduced by ~50%, (1,4)-β-mannan synthase by ~85%, and galactose incorporating activity by ~80% by exposure of mesocotyls to a 15 min light pulse (97 μEinstein s^{-1} m^{-2}) 16 hour before harvest. The decline of the (1,4)-β-mannan synthase and the galactose incorporating activities was gradual (half life of ~4 hours), suggesting that these enzyme activities are regulated by transcription of the corresponding genes. An attempt was made to correlate these activities with the light-regulation of the mRNA levels of the *ZmCSL* genes in the mesocotyl. Many of the *ZmCSLA* and *C* genes, but only a few of the *ZmCSLD*, *E* and *F* genes are expressed in the mesocotyl. Many but not all of them are light-regulated. The transcript levels of only two of the nine *CSLA* genes expressed in the mesocotyl are reduced by light-treatment, whereas the mannan synthase activity is reduced by >85% by light-treatment. Quantitative RT-PCR showed that the major *CSLA* expressed in the maize mesocotyl is *CSLA3*. The transcript levels of *CSLA3* are reduced by seven-fold after light-treatment, which correlates with the strong reduction in mannan synthase activity after light-treatment.

INTRODUCTION

An excellent system for studying the relation of plant growth by an environmental factor such as light is the maize seedling. When seedlings are grown in complete darkness, a structure called the mesocotyl develops, which serves to position the meristem at the soil surface. Once the dark-grown seedlings receive a short light-treatment, the rapid elongation of the mesocotyl is reduced. One enzymatic activity in the mesocotyl, which is light- and auxin-regulated, is the Golgi-localized glucan synthase of maize (Walton and Ray 1982a and b). In Chapter 2 it was shown that this enzyme synthesizes (1,4)-β-glucan.

In this chapter, the regulation of glycan synthases related to cell wall biosynthesis, including the Golgi-localized (1,4)-β-glucan synthase, (1,4)-β-mannan synthase, a galactose-incorporating activity, (1,4)-β-xylan synthase, and callose synthase were studied. An attempt was made to correlate this with the light-regulation of transcription of the *ZmCSL* gene family.

MATERIALS AND METHODS

Genomic DNA isolation

One gram of tissue that had been frozen in liquid nitrogen and stored at -80°C was ground, resuspended in 10 ml hexadecyltrimethylammonium bromide (CTAB) buffer and incubated at 65°C for 30 min. CTAB buffer (2%) was prepared by dissolving 2 g CTAB, 8.2 g NaCl, and 1 ml β-mercaptoethanol in 100 ml 100 mM Tris, pH 8.0. The CTAB buffer was heated to 65°C in order to dissolve it after the addition of NaCl. The genomic

DNA was extracted twice with an equal volume of chloroform: isoamyl alcohol (24 : 1) for 5 min, followed by centrifugation at 3000 g for 10 min. The aqueous phase was collected, isopropanol was added to 0.8 volumes, and the genomic DNA was precipitated by centrifugation at 3000 g for 10 min. The pellets were washed with 70% ethanol, and the genomic DNA was air dried and resuspended in 10 ml TE buffer (10 mM Tris, pH 7.5, 1 mM EDTA).

RNA isolation

Tissue frozen in liquid nitrogen and stored at -80°C was ground, transferred to eppendorf tubes (100 mg per tube) and 1 ml of TRIzole reagent (Cat. No 15596, Invitrogen) was added. All following incubation steps were performed at room temperature and the centrifugations at 4°C, unless otherwise mentioned. The mixture was incubated for 30 min, centrifuged for 10 min at 12,000 g and 0.2 ml of chloroform was added. The tubes were shaken for 15 s, incubated for 2 min, and centrifuged at 12,000 g for 15 min. The aqueous phase was transferred to a new tube and the RNA was precipitated with isopropyl alcohol by centrifugation at 12,000 g for 10 min. The RNA was washed with 75% ethanol, precipitated by centrifugation at 7,500 g for 5 min, air dried, and redissolved in 243 µl diethylpyrocarbonate (DEPC)-treated water. The RNA was DNAse treated by adding 27 µl of 10X DNAse buffer (100 mM Tris pH 7.5, 25 mM $MgCl_2$, 5 mM $CaCl_2$) and 1 µl of RNase free DNAse (Cat. No. 85897570-36, Roche), incubated for 30 min, and heated for 10 min at 85°C in order to inactivate the DNAse. The RNA concentration was calculated by measuring OD_{260}.

Reverse transcription

RNA (1-3.4 μg) isolated from the second cm of the mesocotyl below the first node of dark-grown and light-treated seedlings was reverse-transcribed into cDNA with the Superscript III kit (Cat. No. 18080-051, Invitrogen). The volume of the RNA solution was adjusted to 8 μl using DEPC-treated water. One μl of 50 μM oligodT primer and 1 μl of 10 mM dNTP mixture were added followed by incubation at 65°C for 5 min. Two μl of 10X reverse transcriptase buffer, 4 μl 25 mM $MgCl_2$, 2 μl 0.1 mM DTT, 1 μl RNAse OUT and 1 μl of Superscript III reverse transcriptase were added followed by incubation at 50°C for 50 min. The reaction was terminated by heating at 85°C for 5 min.

Primer design

Primers for *ZmCSL* genes were designed based on the open reading frames using the PrimerSelect program (DNAstar, Madison, WI) (parameters for primer design were the following: primer length of 17-24 bp; product length of 400-600 bp; melting temperature of 39.1-70.2 °C). The primers were tested on other *ZmCSL* genes using the PrimerSelect program in order to determine their specificity. Only primers that did not give a predicted PCR product with the other *ZmCSL* genes were synthesized (Integrated DNA Technologies, Coralville, IA). The sizes of the amplified DNA fragments were determined by agarose gel electrophoresis. Primers amplifying the correct size fragments from genomic DNA were selected for PCR on cDNA.

Genomic PCR

The PCR reaction mixture was prepared by mixing 10 µl 10X PCR buffer minus $MgCl_2$, 2 µl 10 mM dNTP mixture (Cat. No. 12048400, Roche), 3 µl 50 mM $MgCl_2$, 2.5 µl of the forward and reverse primer (50 µM), 9 µl of genomic DNA solution, 0.33 µl Taq polymerase (5 U / µl) (Cat. No. 18038-018, Invitrogen) and 71 µl ddH_2O. PCR was performed under the following conditions on a Gradient 40 Robocycler (Stratagene): 3 min at 94°C (1 cycle), 1 min 94°C, 1 min 55°C, 1 min 72°C (33 cycles) and 10 min at 72°C (1 cycle).

Semi-quantitative RT-PCR

The PCR reaction mixture was prepared by mixing 10 µl 10X PCR buffer minus $MgCl_2$, 2 µl 10 mM dNTP mixture, 3 µl 50 mM $MgCl_2$, 2.5 µl of forward and reverse primer (50 µM), 1 µl of cDNA solution (50 ng / µl RNA equivalent), 0.33 µl Taq polymerase (5 U / µl) and 79 µl ddH_2O. PCR was performed under the following conditions on a Gradient 40 Robocycler: 3 min at 94°C (1 cycle), 1 min 94°C, 1 min 55°C, 1 min 72°C (30 cycles) and 10 min 72°C (1 cycle). Ten µl samples were taken after 22, 26, and 30 cycles and analyzed by agarose gel electrophoresis in order to determine if the PCR reaction was in the linear range.

Agarose gel electrophoresis

Agarose gel electrophoresis was performed with 150 ml 1.3% Tris-Acetate-EDTA (TAE) agarose gels stained with ethidium bromide (15 µl of 5 mg/ml stock solution). A stock of 10X TAE buffer, pH 8.2, was prepared by mixing 48.4 g Tris, 10.9 g glacial

acetic acid, 2.9 g EDTA, and deionized water to a final volume of 1 l. The agarose gels were run in TAE buffer for 30 min at 94 V and imaged with a Gel Doc EQ System (BioRad).

Real-time quantitative RT-PCR

Primers were designed as described under primer design in this materials and methods section. Sequences of the primers used for quantitative PCR can be found in supplementary Table 18. Primers with an efficiency between 90-110% were selected and the primer concentration in the PCR reactions was 300 nM. An equivalent of 5 ng of reverse transcribed RNA and 7.5 µl of 2X SYBR® Green I dye (Cat. No. 4309155, Applied Biosystems) was added to each reaction. RNA was isolated from the second centimeter of the maize mesocotyl of dark and light-treated maize seedlings and reverse transcription into cDNA was performed as described under "Reverse transcription" in this Materials and Methods section. The final reaction volume was 15 µl. PCR reactions were performed on the ABI PRISM® 7700 Sequence Detection System (PE Biosystems) with the following cycling parameters: 2 min at 50 °C, 1 cycle, 10 min 95 °C, 1 cycle and 15 sec 95 °C, 1 min 60 °C for 40 cycles. The relative expression compared to *EF1α* was determined with the $2^{-\Delta\Delta C}{}_t$ method (Livak and Schmittgen, 2001).

Enzyme assays

Enzyme assays with 100 µl of microsomal solution (prepared as described in the materials and methods of chapter 1) were performed in 15 ml Corex conical glass tubes. Glucan synthase assays were performed with 20 mM Mg^{2+} and 49 nM UDP-[^{3}H]glucose

(34 Ci / mmol, Sigma). Callose synthase assays were performed with 49 nM UDP-[^{3}H]glucose and 1 mM UDP-glucose. Xylan synthase assays were performed with 2.0 mM $MgSO_4$, 4.1 mM $MnCl_2$ and 1.2 μM UDP-[^{14}C]xylose (238.4 mCi / mmol, Perkin-Elmer). Mannan synthase assays were performed with 2.0 mM $MgSO_4$, 4.1 mM $MnCl_2$ and 1.4 nM GDP-[^{14}C]mannose (260 mCi / mmol, Amersham). Assays with 1.37 nM UDP-[^{14}C]galactose (367 mCi / mmol, Perkin-Elmer) were performed with 1.2 mM $MgSO_4$. Inosine diphosphatase (IDPase) assays were performed by mixing 0.4 ml assay mixture and 50 μl microsomal solution. The assay mixture was prepared by mixing 13.2 ml stock solution and 1.65 ml 3% digitonin. The stock solution was prepared by mixing 30 ml 80 mM Tris, pH 7.5, 200 μl 1 M $MgCl_2$, 66 mg IDP and 6.2 ml water. This mixture was incubated for 45 min; the reactions were stopped by adding 1 ml 14% trichloroacetic acid (TCA) followed by centrifugation for 20 min at 1000 g. One ml of supernatant was removed and 1 ml of freshly prepared Taussky-Shorr reagent was added. Taussky-Shorr reagent was prepared by mixing 5% (w/v) $FeSO_4$, 1% (w/v) NH_4MO_4 and 1 N H_2SO_4 (Taussky and Shorr, 1953). This mixture was vortexed and OD_{710} was measured after 10 min.

Determination of the amount of MLG in maize cell walls

Ten second centimeter mesocotyl segments of dark-grown and light-treated maize seedlings were collected in eppendorf tubes and frozen in liquid nitrogen. The samples were boiled for 20 minutes in 1 ml of ddH_20, ground and centrifuged for 6 min at 14,000 rpm at 4°C. The supernatant was removed and the pellet was washed three times with water, once with ethanol and once with acetone. The pellet was dried and weighed. One

ml of 25 μM Bis-Tris (pH 6.5), 4 μl of 2% sodium azide and 10 μl of lichenase were added to the pellet and this mixture was incubated overnight at 30°C. Ethanol was added to a final concentration of 70%, centrifuged for 10 min at 14,000 g and the supernatant was collected. The pellet was washed twice with 70% ethanol and the supernatants were combined. The supernatant was dried under vacuum and the pellet was dissolved in one ml of water. Twenty μl of sample was analyzed by HPAEC-PAD with the MLG program (see Table 2, Chapter 2). When MLG is hydrolyzed by a MLG-specific enzyme such as lichenase characteristic hydrolysis products including G4G3G, G4G4G3G and G4G4G4G3G are released. The PAD response peak of each of these oligosaccharides was integrated and taken as a measure for the amount of MLG present in the maize mesocotyl.

RESULTS

Light-regulation of the maize *CSL* gene family

Amplification of *ZmCSL* genes from genomic DNA

Most (83%) of the *ZmCSL* genes could be amplified from genomic DNA using gene-specific primers (Table 6; Appendix III, Tables 15, 16 and 17). The following *ZmCSL* genes could not be amplified from genomic DNA although for each of these genes multiple primer pairs were tested: *C2*, *C10, C12, D4, F1* and *F7*. In the case of *CSLC2*, *C10* and *C12* this could be due to the incomplete annotation of these genes. The *CSLD4, F1* and *F7* genes were completely annotated however, so the reason why these genes could not be amplified from genomic DNA is not clear.

Semi-quantitative RT-PCR

Nine *ZmCSLA* genes, nine *ZmCSLC* genes, four *ZmCSLD* genes, two *ZmCSLE* genes, two *ZmCSLF* genes, and none of the *ZmCSLH* and *ZmCSLX* genes could be amplified from cDNA prepared from RNA from dark-grown mesocotyls (Table 6).

Table 6: PCR amplification of *ZmCSL* genes from genomic DNA and cDNA. n.d. means not determined.		
ZmCSL	**Amplified from genomic DNA**	**Amplified from cDNA**
A1	Yes	Yes
A2	Yes	No
A3	Yes	Yes
A4	Yes	Yes
A5	Yes	Yes
A6	Yes	Yes
A7	Yes	Yes
A8	Yes	Yes
A9	Yes	Yes
A10	Yes	Yes
C1	Yes	Yes
C2	No	No
C3	Yes	Yes
C4	Yes	Yes
C5	Yes	No
C6	Yes	Yes
C7	Yes	Yes
C8	Yes	No
C9	No	No
C10	Yes	No
C11	Yes	No
C12	No	No
D1	Yes	No
D2	Yes	Yes
D3	Yes	No
D4	No	No
D5	Yes	No
E1	Yes	Yes
E2	n.d.	n.d.
E3	Yes	No
E4	n.d.	n.d.
F1	No	No
F2	Yes	No
F3	Yes	No
F4	Yes	No
F5	Yes	Yes
F6	Yes	Yes
F7	No	No
H1	Yes	No
X1	Yes	No

The transcript levels of *ZmCSLA1, 4, 5, 6, 7, 8* and *10* were not or weakly affected by light, and the transcript levels of *ZmCSLA3* and *9* were strongly increased by light (Figure 25). The transcript levels of *ZmCSLC1* were not affected by light, *ZmCSLC3* and *6* weakly, and *ZmCSLC4* and *7* strongly. The transcript levels of *ZmCSLE1* and

ZmCSLF5 were not affected by light whereas the transcript levels of *ZmCSLD2* and *ZmCSLF6* were only weakly affected by light. The transcript levels of *Elongation Factor 2α* (*EF2α*), a control gene, were not affected by light.

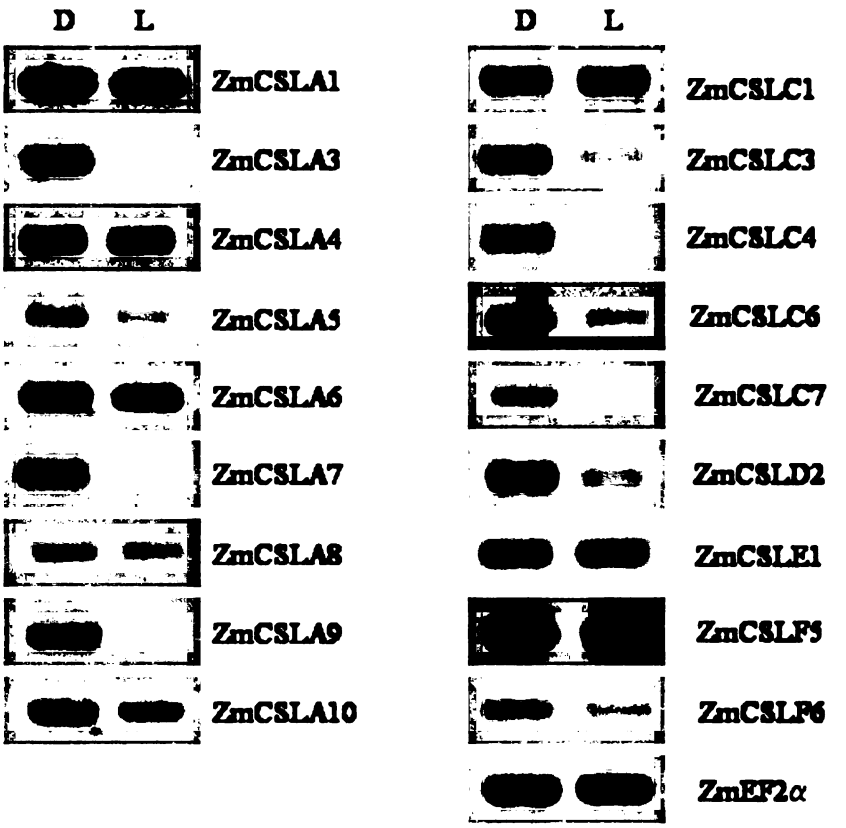

Figure 25. Ethidium-stained agarose gel showing RT-PCR products of the *ZmCSL* gene family. PCR was performed for 30 cycles as described in materials and methods. This number of cycles was not saturating for any of the genes. The source of mRNA was the second centimeter of the mesocotyl of dark-grown (D) or light-treated (L) seedlings.

Real-time quantitative RT-PCR

Quantitative RT-PCR was performed with cDNA reverse transcribed from RNA isolated from dark and light-treated maize seedlings using gene specific primers (See Appendix IV Table 18). The relative expression compared to *EF1α* was determined with the $2^{-\Delta\Delta C}{}_t$ method (Livak and Schmittgen, 2001). Figure 26 shows that the major *CSLA* expressed in the maize mesocotyl is *CSLA3* (57% of total *CSLA* transcript levels) and its transcript levels were reduced approximately seven-fold by light (Table 7). The transcript levels of *CSLA7* were reduced approximately 13-fold by light. The total reduction in *CSLA* transcript levels after light-treatment was approximately 60%. The transcript levels of *CSLA1*, *A6* and *A9* were not affected by light (Fig. 26). The transcript levels of *CSLA4*, *5*, *8* and *10* were weakly affected by light (Fig. 26). The transcript levels of *CSLA4* were increased approximately 2-3 fold and the transcript levels of *CSLA8* and *A10* were reduced by approximately 2-3 fold by light.

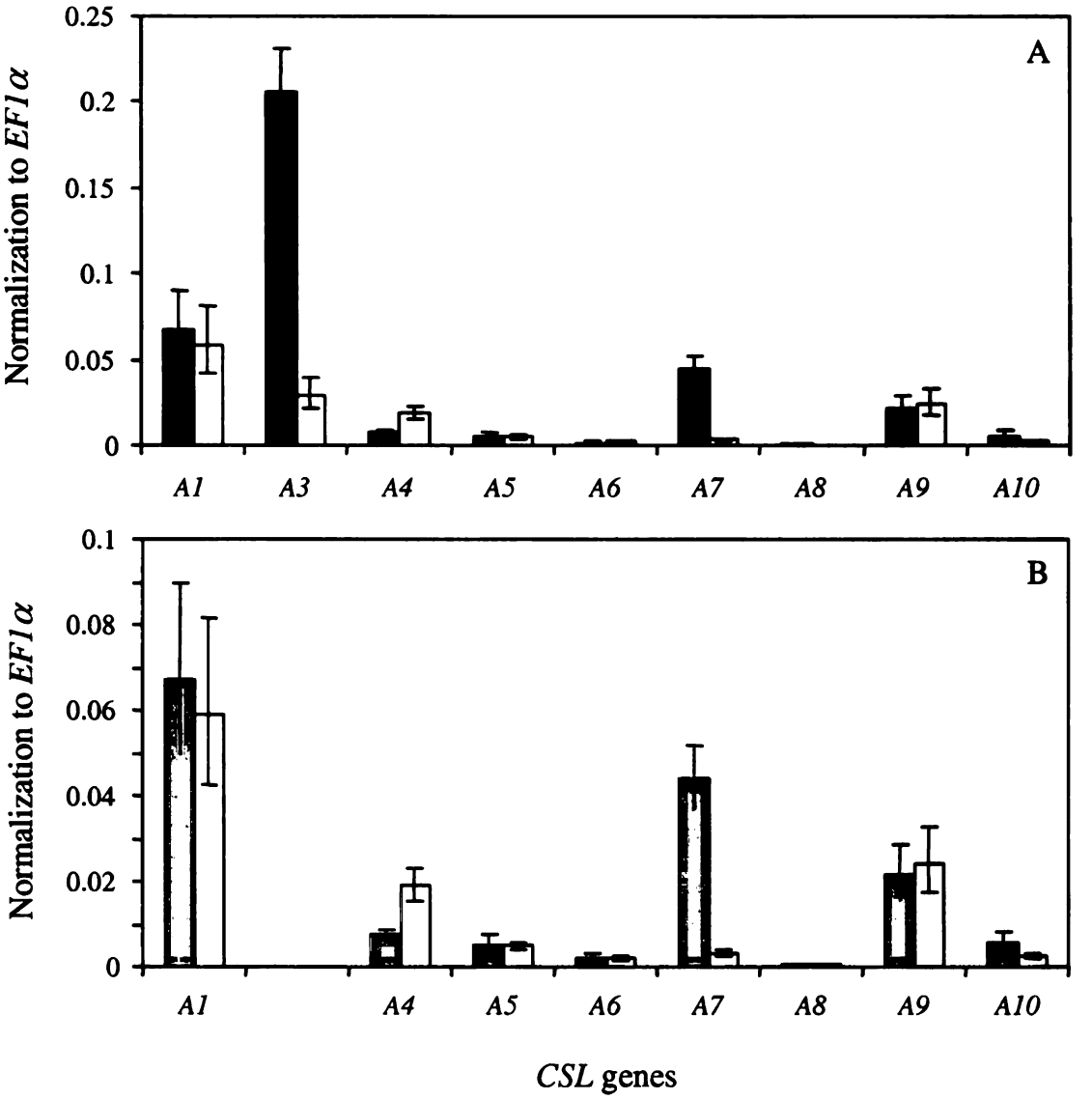

Figure 26. Real-time quantitative RT-PCR results for the maize *CSLA* gene family. The relative expression level was normalized to *EF1α*. Grey bar: dark-treated maize seedlings. White bar: light-treated seedlings. A: *CSLA3* included; B: the same figure but excluding *CSLA3* (expanded y-scale). Data represent the mean (n = 2-3) ± SE.

Figure 27 shows the quantitative RT-PCR results for the *CSLC, D, E* and *F* genes. Table 7 shows the fold change in gene expression compared to *EF1α*. The transcript levels of *CSLC1, E1* and *F5* were increased by approximately 4, 6 and 9-fold respectively. The transcript levels of *CSLC3, C7* and *F6* were reduced by 2-3, 2 and 2-3-fold respectively. The transcript levels of *CSLC4* were strongly reduced after light-treatment. No conclusion could be made about the light-dependence of *CSLC6* transcript levels because the efficiency of the primer pair used was not in the acceptable range.

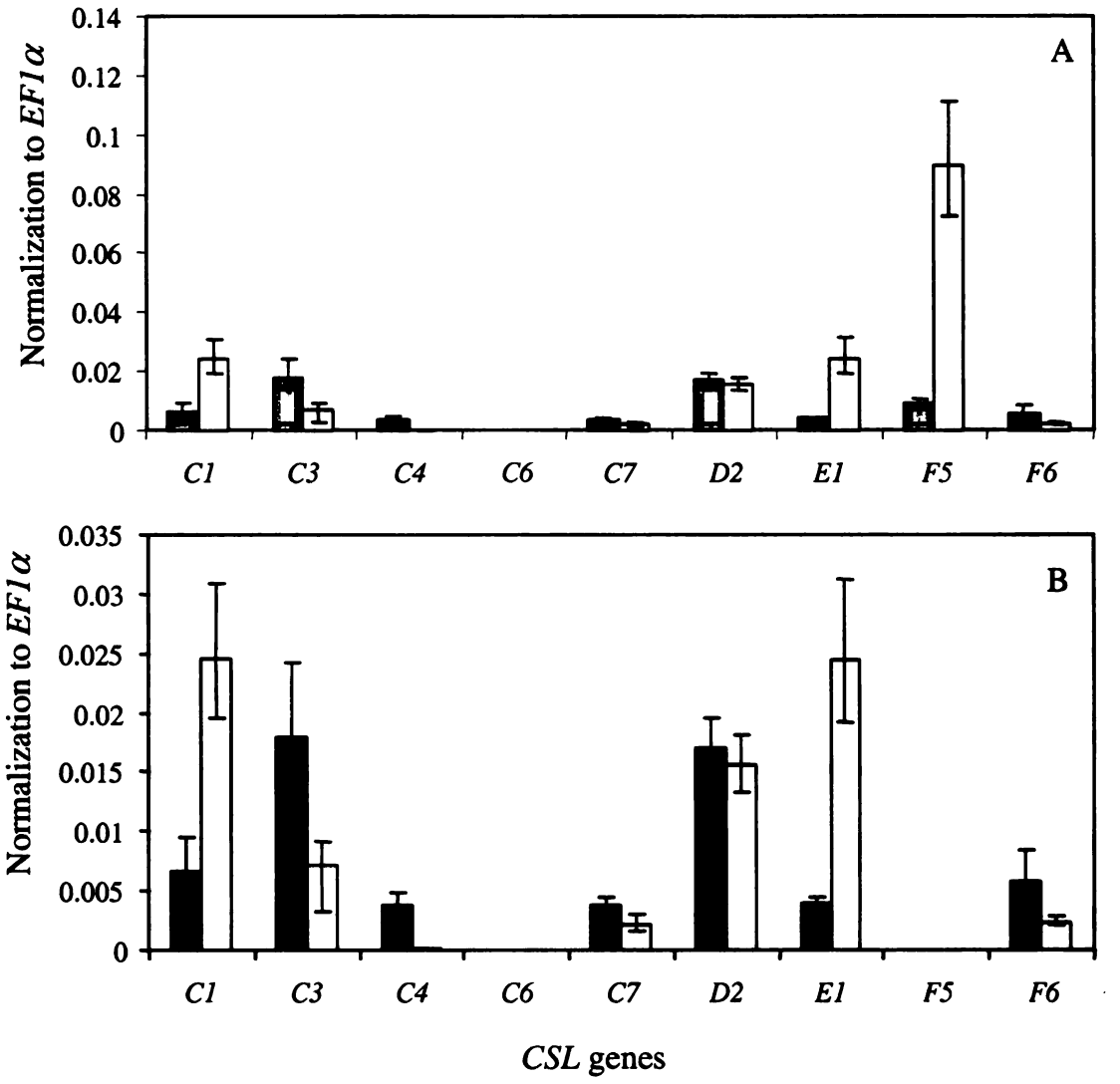

Figure 27. Real-time quantitative RT-PCR results for the maize *CSLC, D, E* and *F* gene family. The relative expression was compared to *EF1α*. Grey bar: dark-treated maize seedlings. White bar: light-treated seedlings. A: *CSLF5* included; B: the same figure but excluding *CSLF5* (expanded y-scale). Data represent the mean (n = 2-3) ± SE.

Table 7: Change in transcript levels compared to *EF1α* determined with real-time quantitative RT-PCR	
ZmCSL	Fold change
A1	-1.1
A3	-7.0
A4	+2.5
A6	+1.2
A7	-13.3
A8	-2.5
A9	+1.1
A10	-2.4
C1	+3.7
C3	-2.5
C4	-19.7
C6	n.d.
C7	-1.7
D2	-1.1
E1	+6.1
F5	+9.4
F6	-2.4

Light-regulation of enzymatic activities related to cell wall biosynthesis.

Time course experiments were performed to determine if there was a correlation between the light-regulation of *ZmCSL* transcripts and enzymatic activities related to cell wall biosynthesis. Enzymes assayed were (1,4)-β-glucan synthase, (1,4)-β-mannan synthase, UDP-galactose incorporating activity, (1,4)-β-xylan synthase and (1,3)-β-glucan synthase. As a control, latent IDPase, a Golgi-localized enzymatic activity which is not affected by light, was used (Ray et al., 1969; Walton and Ray 1982a; Mitsui et al., 1994). The enzyme assays were stopped after 0, 15, 30, 45 and 60 min and radioactive incorporation into ethanol-insoluble products was determined. Walton and Ray (1982a) found that the IDPase activity was reduced by around 15% after light-treatment and callose synthase by 25%. In the experiments described here an increase in callose synthase activity of 15-55% was observed. Mannan synthase, xylan synthase, glucan

synthase and the galactose-incorporating activity were reduced by 85-90%, 15%-25%, 48%-53% and 75%-83% respectively (Figure 28).

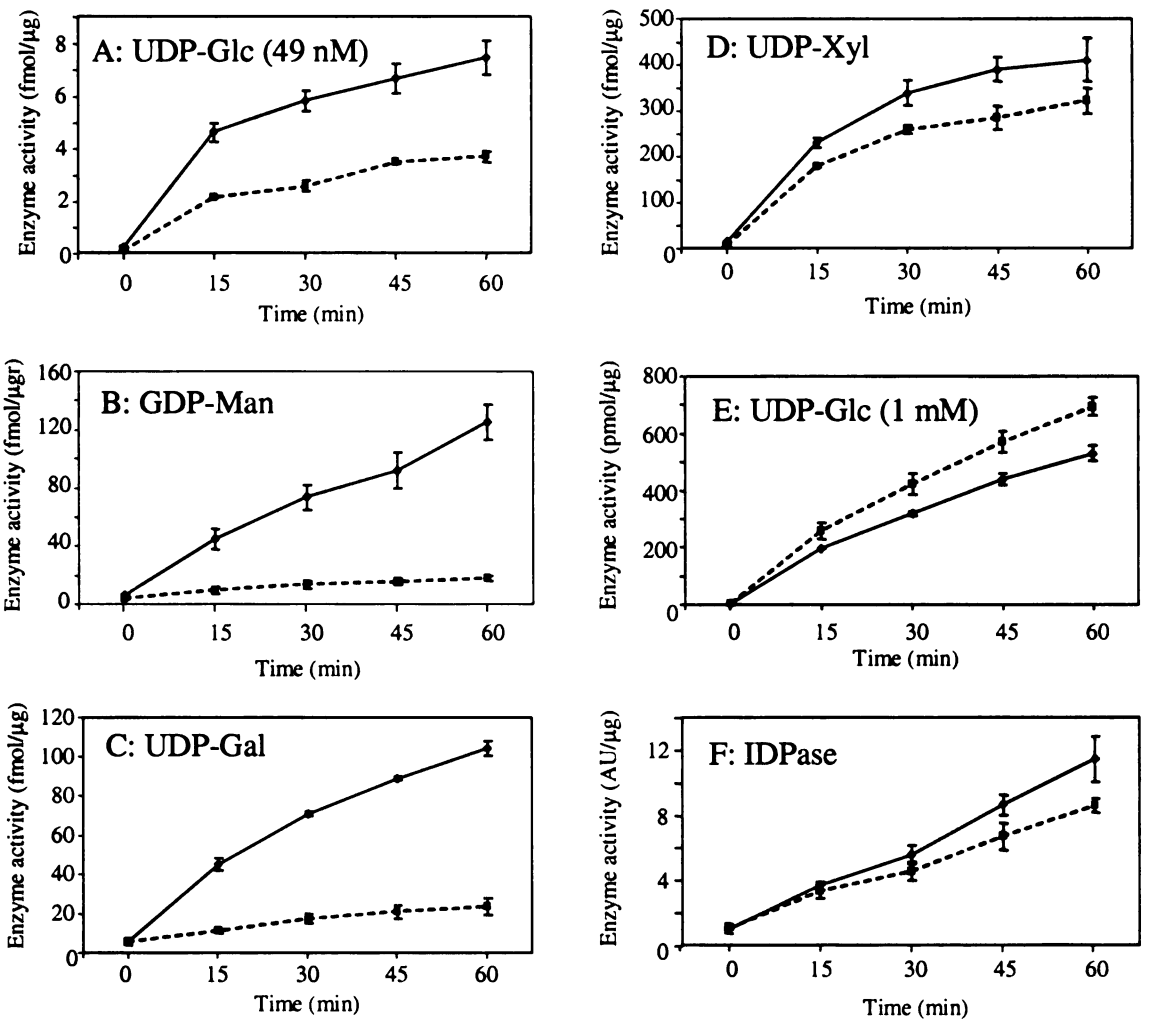

Figure 28. (1,4)-β-glucan synthase (A), (1,4)-β-mannan synthase (B), UDP-Gal incorporating activity (C), (1,4)-β-xylan synthase (D), (1,3)-β-glucan synthase (E), and latent IDPase (F) activities in microsomes from dark-grown (solid line) or light-treated mesocotyls (dashed line). Reactions were stopped after 0, 15, 30, 45 or 60 min. Data represent the mean (n = 2) ± SE.

Analysis of the radiolabeled products

The products in which glucose was incorporated at 4 to 49 nM was a (1,4)-β-glucan (Figure 6 and 7, Chapter 2) and at 1 mM a (1,3)-β-glucan (Figure 8, Chapter 2). The products synthesized in the presence of GDP-[^{14}C]mannose were fully solubilized by commercial mannanase (see Table 1, Chapter 2). The hydrolysis products were analyzed by HPAEC-PAD using the manno/xylooligosaccharide program (see Table 2, Chapter 2). Figure 29 shows that the radiolabeled hydrolysis products coeluted with mannose, mannobiose and mannotriose, which indicates that the product is (1,4)-β-mannan.

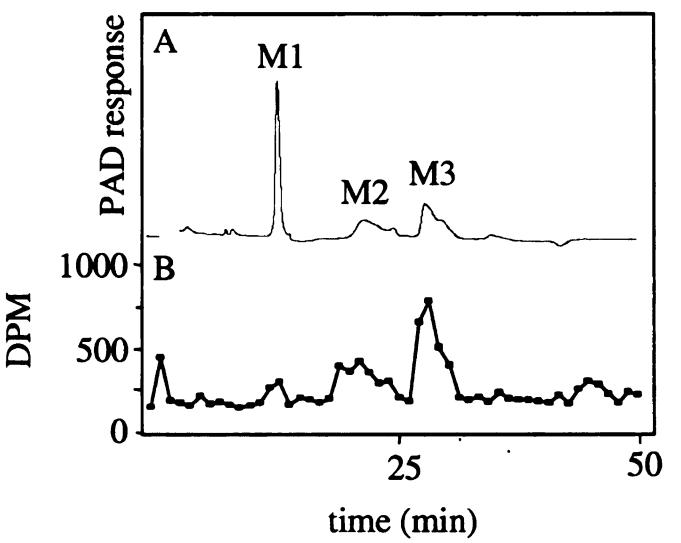

Figure 29. The product synthesized from UDP-[^{14}C]mannose was treated with endo-(1,4)-β-mannanase and the hydrolysis products were analyzed by HPAEC-PAD. A, Oligosaccharide standards: M1 (mannose), M2 (mannobiose), M3 (mannotriose). B, Radioactivity.

The products synthesized in the presence of UDP-[^{14}C]xylose were treated with endo-(1,4)-β-xylanase and the hydrolysis products were analyzed by HPAEC-PAD with the manno/xylooligosaccharide program (see Table 2, Chapter 2) (Figure 30). Figure 30 shows that the radiolabeled hydrolysis products coeluted with xylose and xylobiose, which indicates that UDP-[^{14}C]xylose is incorporated into a (1,4)-β-xylan.

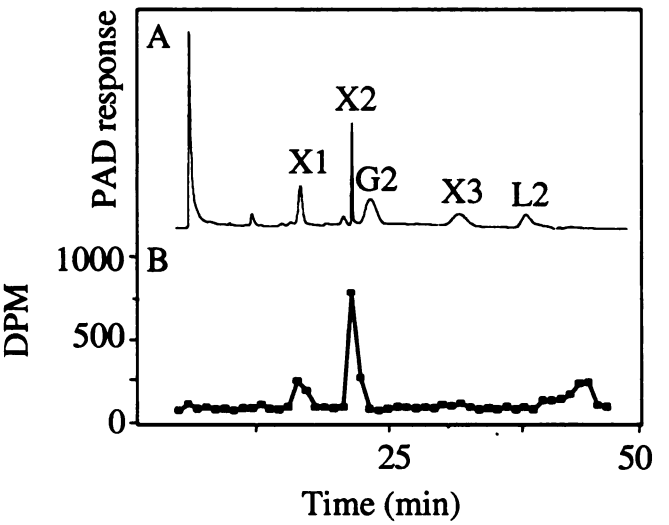

Figure 30. The product synthesized from UDP-[^{14}C]xylose was treated with xylanase M6 and the hydrolysis products were analyzed by HPAEC-PAD. A, Oligosaccharide standards: X1 (xylose), X2 (xylobiose), G2 (cellobiose), X3 (xylotriose), L2 (laminaribiose). B, Radioactivity.

The product in which [^{14}C]galactose was incorporated was treated with several different hydrolytic enzymes such as endo-1,4-β-D-galactanase and β-1-(3,4,6)-galactosidase (see Chapter 2, Table 1), none of which hydrolyzed the product.

The only enzyme mixture that hydrolyzed this product was pectinase from *Aspergillus niger* (See Table 1, Chapter 2) (data not shown). Monosaccharide analysis of the hydrolyzed product showed that the radiolabel was still in galactose (data not shown). Attempts to further characterize the galactose-containing product were unsuccessful.

Correlation between the levels of the *ZmCSLA* transcripts and (1,4)-β-mannan synthase activity

Whereas (1,4)-β-mannan synthase activity was reduced by 85-90% by light, the transcript levels of only two of nine *ZmCSLA* genes were strongly reduced by light. This apparent discrepancy can be explained by the difference in expression levels of the different CSLA proteins. *CSLA3* accounts for 57% of the *CSLA* transcript levels and its transcript levels are reduced seven-fold after light-treatment. This reduction in *CSLA3* transcript levels could result in a comparable reduction in the amount of *CSLA3* mRNA translated into CSLA3 protein. This could explain the strong reduction in mannan synthase activity after light-treatment (Fig. 28).

Time-course of the light-inhibition of enzymatic activities related to cell wall biosynthesis

In order to get a better insight into the mechanism of regulation of the glycan synthase activities by light, time-course experiments for inhibition of enzyme activity after light-treatment were performed for the (1,4)-β-mannan synthase, (1,4)-β-xylan synthase and the UDP-galactose incorporating activities (Figure 31). A similar time-course for the (1,4)-β-glucan synthase was performed by Walton and Ray, (1982a). Five

trays of dark-grown maize seedlings were light-treated for 15 minutes and second centimeter mesocotyl segments were collected immediately from one tray of seedlings. The other four trays of light-treated seedlings were placed back in complete darkness and the mesocotyl segments were collected after 1, 3, 6 and 12 hours. Figure 31 shows that the (1,4)-β-mannan synthase and the UDP-galactose incorporating activities, gradually declined after light-treatment. The (1,4)-β-xylan synthase activity was not affected by light-treatment. These data suggest that the (1,4)-β-mannan synthase and the UDP-galactose incorporating activity are probably regulated at the level of transcription rather than by a rapid post-translational process such as phosphorylation. This conclusion is consistent with the quantitative RT-PCR results showing light-induced reduction in *CSL* mRNA levels (Figures 26 and 27).

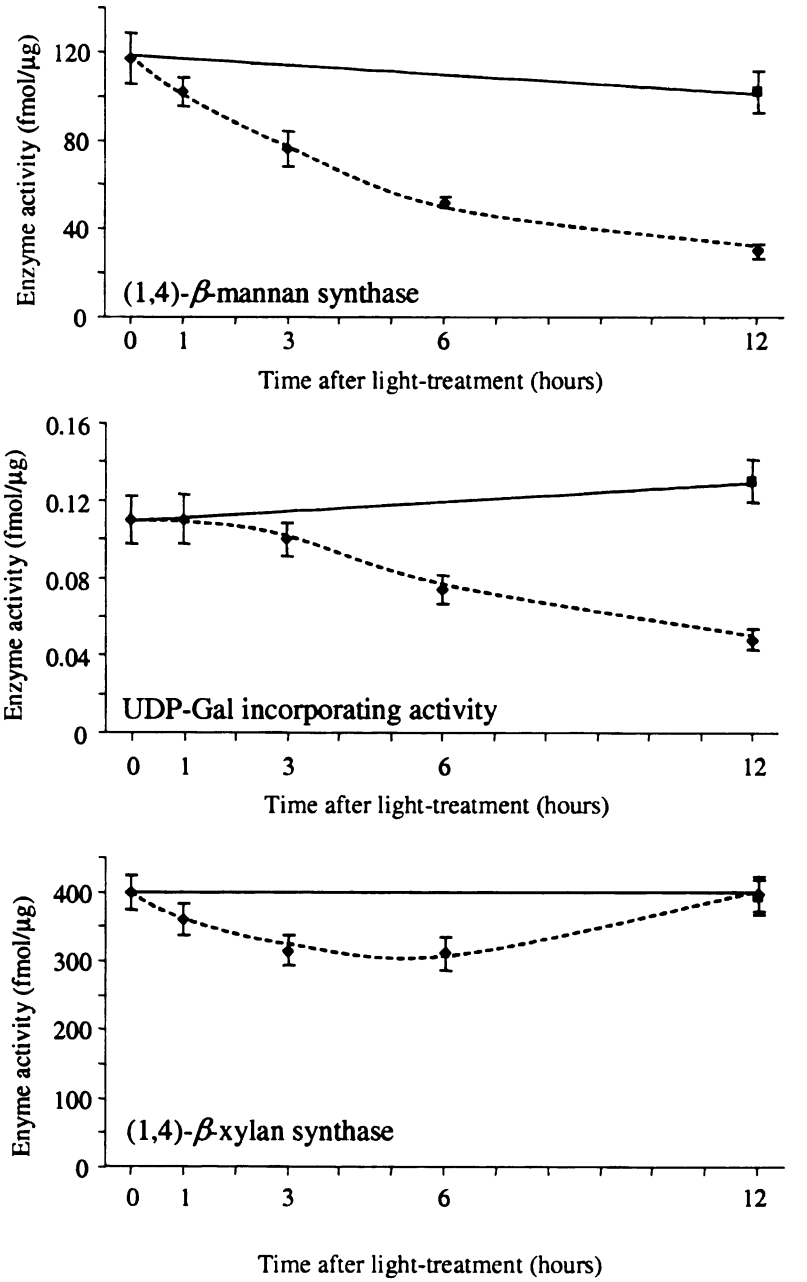

Figure 31. Time-course of inhibition of enzymatic activities after light-treatment for (1,4)-β-mannan synthase, the UDP-galactose incorporating activity and (1,4)-β-xylan synthase. Solid line: enzyme assays performed with microsomes isolated from dark-grown seedlings. Dashed line: enzyme assays performed with microsomes isolated from light-treated seedlings. Data represent the mean (n = 2) ± SE.

Analysis of MLG in cell walls of dark-grown and light-treated seedlings

The *CSLF* gene family is involved in MLG synthesis (Burton et al., 2006). In order to determine if there is a correlation between the *CSLF* transcript levels and the amount of MLG, MLG levels were determined. Figure 32 shows the result of the MLG analysis of the cell walls of second centimeter mesocotyl segments of dark-grown and light-treated maize seedlings. There is no difference in MLG levels between the dark-grown and light-treated maize seedlings. The transcript levels of *CSLF6* correlate with the MLG levels (Fig. 27). The transcript levels of *CSLF5*, however, do not correlate with the MLG levels. The transcript levels of *CSLF5* are strongly increased after light-treatment in contrast to the MLG levels. This discrepancy can be explained in several ways. It could be the *CSLF5* is not involved in MLG biosynthesis or that MLG biosynthesis might not be regulated at the level of transcription. Another possibility is that the turnover of MLG might be slow, so it is hard to see newly synthesized MLG against the background of MLG which is synthesized previously.

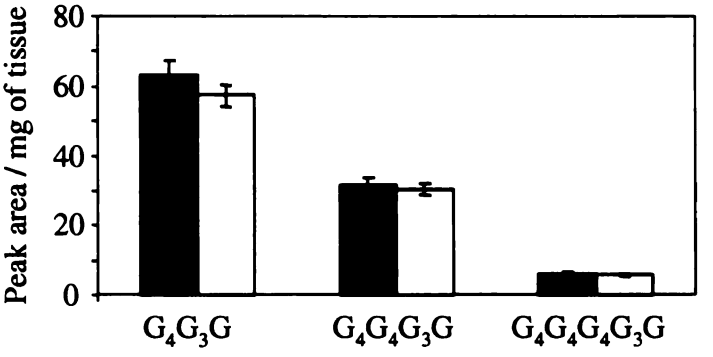

Figure 32. Amount of MLG in cell walls in dark-grown and light-treated seedlings. The integrated peak area of the PAD response per mg of dried cell wall material for each of three different oligosaccharides (G4G3G, G4G4G3G, G4G4G4G3G) released after lichenase treatment is shown. Data represent the mean (n = 6) ± SE.

DISCUSSION

In this part of my research I investigated the light-regulation of expression of the *ZmCSL* genes and enzymatic activities related to cell wall biosynthesis. Expression of 18 of the 34 *ZmCSL* genes was detected in the maize mesocotyl. Nine of them are *ZmCSLA*, 5 are *CSLC*, 1 is *CSLD*, 1 is *CSLE* and 2 are *CSLF* genes.

It is curious that maize has 11 *ZmCSLA* genes, although (1,4)-β-mannan is a minor polysaccharide in maize (Carpita et al., 2001). After light-treatment of the dark-grown seedlings there was a more than 85-90% reduction in (1,4)-β-mannan synthase activity. This is the first time to my knowledge that mannan synthase activity has been shown to be regulated by light. In contrast, transcript levels of most of the *CSLA* genes were not affected by light-treatment. There are several possible explanations for the

observed discrepancy between *CSLA* transcript levels and mannan synthase activity. The most likely explanation is that *CSLA3* is expressed more highly than the other *CSLA* genes and is strongly light-regulated. In this case it is assumed that there is a strong correlation between *CSLA3* transcript levels, CSLA3 protein levels and CSLA3 mannan synthase activity.

It has not yet been shown that *CSLA3* encodes a mannan synthase. However, CSLA3 is most likely involved in mannan biosynthesis because it clusters with CSLA proteins from other plant species that have been demonstrated to be mannan synthases. The closest ortholog of ZmCSLA3 is OsCSLA1, which is a mannan synthase (Liepman et al., 2007). The gradual decrease of the (1,4)-β-mannan synthase activity after light-treatment of dark-grown seedlings supports the hypothesis the mannan biosynthesis is regulated at the level of transcription. In case mannan biosynthesis would be regulated at the post-transcriptional level, for example by reversible phosphorylation, I would not expect this gradual decrease in mannan synthase activity after light-treatment, but a more abrupt change in activity.

Possible candidates genes for encoding the light-regulated (1,4)-β-glucan synthase are the members the *CSLC* gene family, because AtCSLC4 and TmCSLC are involved in the synthesis of the (1,4)-β-glucan backbone of xyloglucan (Cocuron et al., 2007). *ZmCSLC1* transcript levels are not affected by light, *ZmCSLC3* and *6* transcript levels are weakly and *ZmCSLC4* transcript levels are strongly reduced after light-treatment. The reduction in transcript levels of the *CSLC* genes correlates with the reduction in (1,4)-β-glucan synthase activity after light-treatment.

The CSLD proteins might be involved in (1,4)-β-glucan biosynthesis in tip-growing cells (Doblin et al., 2001; Favery et al., 2001; Wang et al., 2001; Roberts et al., 2007). Doblin et al. (2001) showed that the main glycosyltransferases expressed in tobacco pollen tubes were *NtCSLD1* and *NtGSL1*. Pollen tubes cell walls mainly consist of cellulose and callose. From these data they concluded that CSLD proteins might be involved in β(1,4)-glucan biosynthesis in tip-growing cells. Support for this hypothesis comes from the work of Favery et al. (2001) and Wang et al. (2001). They showed that a mutation in *AtCSLD3* caused the root hair tips to leak cytoplasm and to rupture, which indicates the tensile strength is changed. Root hairs elongate by tip growth. One *ZmCSLD*, *ZmCSLD2*, was expressed in the second centimeter of the maize mesocotyl. It is possible that *ZmCSLD2* encodes for the light-regulated Golgi-localized (1,4)-β-glucan synthase. However, the transcript levels of *ZmCSLD2* were not affected by light treatment, which does not correlate with the down-regulation of the (1,4)-β-glucan synthase activity after light-treatment.

Of the *ZmCSLF* genes only *F5* and *F6* are expressed in the maize mesocotyl. The CSLF proteins are involved in MLG biosynthesis (Burton et al., 2006). We analyzed the MLG content of the second centimeter of the maize mesocotyl before and after light-treatment of dark-grown maize seedlings. No difference in MLG content was found (Figure 34). The transcript levels of *ZmCSLF5* were strongly increased after light-treatment. This is not consistent with the observation that MLG levels do not change after light-treatment. If *ZmCSLF5* encodes for a MLG synthase I would not expect that the transcript levels of this gene would be strongly increased after light-treatment. One possibility is that CSLF5 is involved in the synthesis of a different polysaccharide.

Another possibility is that changes in MLG levels caused by the increase in *CSLF5* transcript and protein levels are not visible against the background of already synthesized MLG.

Of the *CSLE* genes, only *CSLE1* is expressed in the mesocotyl, and its transcript levels are strongly increased after light-treatment. The biochemical function of the CSLE proteins is not known. *ZmCSLX* and *H* are not expressed in the maize mesocotyl. The *CSLH* subfamily is specific for cereals (Hazen et al., 2000) and is hypothesized to be involved in the synthesis of a cereal-specific polysaccharide such as MLG. Because *ZmCSLH* is not expressed in the maize mesocotyl it cannot be involved in the synthesis of MLG in the mesocotyl. Whether ZmCSLH is involved in the synthesis of MLG in other tissues in maize remains to be determined. The biochemical function of ZmCSLX is also not known.

The semi-quantitative PCR results do not correspond very well to the results obtained with quantitative RT-PCR. A major discrepancy for instance was *CSLA9*. The semi-quantitative PCR results showed that the transcript levels of *CSLA9* are strongly light-regulated. However, the quantitative RT-PCR experiments showed no difference in transcript levels. The reason for this discrepancy is not known. Semi-quantitative PCR also does not give accurate information about the real transcript levels. A good example of this is *CSLF5*. From the semi-quantitative PCR data it is hard to conclude that there is an increase in transcript levels after light-treatment in contrast to quantitative RT-PCR, which showed a nine fold increase.

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CHAPTER 5: FUTURE DIRECTIONS

The future for research in cell wall biosynthesis is bright. Biofuels research is attracting a large amount of money for cell wall research. Progress is also being made in the sequencing of the genomes of grasses. Rice is sequenced and other grasses in the process of being sequenced are maize and *Brachypodium distachyon* (*Brachypodium*). This will create good conditions for studying cell wall biosynthesis in grasses.

An important question to be answered in the field of cell wall biosynthesis is which genes encode for the enzymes synthesizing the cell wall polysaccharides and how their synthesis is regulated. Sequencing of the maize genome will allow us to finish the annotation of the maize *CSL* gene family and determine the number of *CSL* genes. Pseudogenes can be determined by investigating if there are stop codons in their open reading frames. Alternative splice variants can be found by comparing EST sequences with the genomic DNA sequences. We might be able to establish the biochemical functions of some of the maize *CSL* genes by expressing them in heterologous expression systems.

It would be interesting to determine the biochemical functions of the CSLA proteins of maize by heterologous expression, especially the members of the cereal specific clade of CSLA proteins. None of the CSLA proteins in this cereal specific clade has been tested for mannan synthase activity yet and all the CSL proteins which are mannan synthases cluster separately from these cereal CSLA proteins. Knock-outs in the *CSLA3* and *A7* genes could be investigated. The transcript levels of *CSLA3* and *7* are strongly reduced after light-treatment, which correlates with the reduction in mannan synthase activity after light-treatment. There might be a reduction in mannan synthase

activity in a *CSLA3* knock-out and in case mannan is important for elongation growth, there might be a growth related phenotype. The levels of mannan in the maize mesocotyl are low however, so any phenotype related to elongation growth might be subtle.

An interesting question is why there are so many *CSLC* genes in maize in comparison to *Arabidopsis* and rice. AtCSLC4 and TmCSLC are involved the synthesis of the glucan backbone of xyloglucan. Some of the *CSLC* genes could be pseudogenes or not all of them encode for xyloglucan glucan synthases. The transcript levels of *CSLC1* were increased after light-treatment and the transcript levels of *CSLC4* were strongly reduced after light-treatment. Knock-outs in these genes could be investigated for phenotypes related to growth. The *CSLC* genes could be heterologously expressed in order to determine if they encode for xyloglucan glucan synthases.

Other candidates for investigating knockouts are the *CSLD2* and *E1* genes. Only one *CSLD* and *E* gene are expressed in the maize mesocotyl and the transcript levels of *CSLE1* are strongly upregulated after light-treatment.

OsCSLF2 has been shown to be involved in MLG biosynthesis. *CSLF5* and *F6* might be involved in MLG biosynthesis in the maize mesocotyl, but the strong increase in transcript levels of *ZmCSLF6* after light-treatment contradicts this hypothesis. The MLG levels are not changed after light-treatment of the maize mesocotyl, so the question is why the transcript levels of *CSLF5* would be increased if this gene encodes for a MLG synthase? It would be interesting to investigate knockouts in *CSLF5* and *F6*. Knockouts of these genes could give interesting phenotypes related to growth or mesocotyl elongation. By heterologously expressing these proteins we might be able to determine their function.

Once the maize genome is sequenced, promoters of the *CSL* genes can be analyzed for the presence of transcription factor binding sites which could be involved in auxin- or light-regulation. Some of the promoter elements which are expected to be found are auxin response elements, and promoter elements involved in light-regulation such as GATA boxes, GT1 and G-boxes. Especially investigation of the promoters of the *CSL* genes whose transcript levels are strongly affected by light could be interesting.

APPENDIX I: ANNOTATION OF THE MAIZE *CSL* GENE FAMILY

Table 8: Genomic sequences used for the annotation of the *ZmCSLA* subfamily	
Maize gene	**Genomic sequences** MAGI_... : genomic sequence comes from MAGI3 database MAGI4_... : genomic sequence comes from MAGI4 database
ZmCSLA1	MAGI4_62945, MAGI4_119251, MAGI4_119252, MAGI4_119253
ZmCSLA2	MAGI4_126476, MAGI4_138561, ZmGSStuc11-12-04.14465.2
ZmCSLA3	MAGI4_122017
ZmCSLA4	MAGI4_90697, MAGI4_90698, MAGI4_90700
ZmCSLA5	MAGI4_96931, MAGI4_96932, AZM5_99705
ZmCSLA6	MAGI4_143329, MAGI4_143330
ZmCSLA7	MAGI_21246, MAGI_79371, ZmGSStuc11-12-04.116.1
ZmCSLA8	MAGI4_125022
ZmCSLA9	MAGI4_106644
ZmCSLA10	MAGI4_146469, MAGI4_157680, MAGI4_157681, AZM5_15751, AZM5_18549
ZmCSLA11	MAGI4_103458

Table 8 Continued	
ZmCSLC1	MAGI4_123263, MAGI4_123264
ZmCSLC2	MAGI4_70285, ZMMBBc0414A15.
ZmCSLC3	MAGI_106507, MAGI_106508, MAGI_106509, MAGI4_72417, MAGI4_72418, MAGI4_72419, AZM5_86073, BH227815, CW003073, CW007807, ZmGSStuc11-12-04.7424.1
ZmCSLC4	MAGI4_121535
ZmCSLC5	MAGI4_77380, MAGI4_77381, MAGI4_77382, MAGI4_103479, CZ386891
ZmCSLC6	MAGI4_78784, MAGI4_163203
ZmCSLC7	MAGI4_72790, MAGI4_72791, MAGI4_72792, ZmGSStuc11-12-04.6288.2
ZmCSLC8	MAGI4_119258
ZmCSLC9	MAGI4_118095
ZmCSLC10	MAGI4_19169, APZT30938.R, ZMMBLb0010H15.R
ZmCSLC11	MAGI4_34342, MAGI4_94745, MAGI4_113710 , APTA61386.R, CZ330204, CZ400294, ZmGSStuc11-12-04.21281.1
ZmCSLC12	MAGI4_91218
ZmCSLD1	MAGI4_83198, MAGI4_83199, ZmGSStuc11-12-04.6504.1
ZmCSLD2	MAGI4_67442, MAGI4_110509, MAGI4_123679, MAGI4_151659, PUHOB07.R, ZmGSStuc11-12-04.51425.1, ZmGSStuc11-12-04.91725
ZmCSLD3	MAGI4_96519, MAGI4_96520
ZmCSLD4	MAGI_100286, MAGI4_40890, MAGI4_40891, MAGI4_40892, AZM5_30134, ZmGSStuc11-12-04.10994.1
ZmCSLD5	MAGI4_154948, MAGI4_154949

Table 8 Continued	
ZmCSLE1	MAGI_62646, MAGI_144905, MAGI4_126139, MAGI4_144906, AZM_5329, AZM5_95772, CC784651
ZmCSLE2	MAGI4_14602, AZM4_23029, AZM_5330, CC428870, PUHUD52.F
ZmCSLE3	MAGI4_116066, MAGI4_153810, CZ323910
ZmCSLE4	MAGI4_48170, ZmGSStuc11-12-04.30141.1, CC984189, CG338953, CZ359872
ZmCSLF1	MAGI_7632, MAGI_7634, MAGI_7635, MAGI4_93926, MAGI4_93927, MAGI4_93920, AZM5_9281
ZmCSLF2	MAGI4_93921, MAGI4_93922, MAGI4_93923, MAGI4_93924
ZmCSLF3	MAGI4_10379
ZmCSLF4	MAGI4_105268
ZmCSLF5	MAGI4_105266, MAGI4_105270
ZmCSLF6	MAGI4_159174
ZmCSLF7	MAGI_38566, MAGI4_91023, MAGI4_91024, CC386831
ZmCSLH	MAGI4_89273
ZmCSLX	MAGI_82244, MAGI4_4386, MAGI4_4387, MAGI4_4388, ZmGSStuc11-12-04.9135.1

Table 9: Detailed description of the annotation for each *ZmCSL* gene.	
Maize gene	**Description of annotation** In some cases the genomic DNA or cDNA for a *CSL* was divided into several pieces. In these cases the genomic or cDNA was numbered according to the orientation in regards to the 5' end of the gene with number 1 the part of the genomic DNA or cDNA closest to the 5' end of the gene. The following fragments of genomic or cDNA are labeled 2 or 3. Proteins encoded by this genomic DNA were predicted using the FGENESH gene monocot gene prediction program unless otherwise mentioned.
ZmCSLA1	FGENESH does not predict exon 5 which is from bp 3922 to 4032 in the genomic DNA. The genomic DNA of exon 8, intron 8, and exon 9 is missing. The cDNA sequence is full length.
ZmCSLA2	The 3' part of genomic DNA and cDNA is missing. The DNA for exon 1, intron 1 and part of exon 2 is complete. The FGENESH prediction is correct.
ZmCSLA3	FGENESH incorrectly predicts 3 genes, (bp 2578-3587, 4420-5003 and 7335-8897) in the genomic DNA. These 3 genes are in fact 1 gene. The start of exon 3 is at bp 4417 instead of bp 4420. FGENESH incorrectly predicts an exon from bp 4996-5003 in the genomic DNA. The FGENESH prediction for the start site of exon 4 is incorrect. The start site is at bp 7325 in genomic DNA instead of bp 7335. The genomic and cDNA sequences are full length.
ZmCSLA4	Part of the genomic DNA of intron 3 is missing. The FGENESH prediction for the start site of exon 4 start is incorrect. The start site is not at bp 391 in genomic DNA *ZmCSLA4.2*, but at bp 429. Exon 6 starts at bp 922 in *ZmCSLA4.2*. Part of the genomic DNA and cDNA of exon 6 and intron 6 are missing. The FGENESH prediction for the start site of exon 7 is incorrect. The start site is not at bp 258 in genomic DNA *ZmCSLA4.3*, but at bp 183.

Table 9 Continued	
ZmCSLA5	The FGENESH prediction for the start site of exon 1 is incorrect. The correct start site is at bp 1559 in genomic DNA *ZmCSLA5.1*, not at bp 1933. FGENESH incorrectly predicts 3 extra exons (bp 2575-2661, 2991-3146, 3226-3354) in genomic DNA *ZmCSLA5.1*. The genomic DNA for intron 2 and intron 3 is missing and the genomic DNA and cDNA for exon 3 is missing. The FGENESH prediction for the 3' end of exon 8 is incorrect. The 3' end of exon 8 is at bp 1922 in genomic DNA *ZmCSLA5.2* instead of bp 1910.
ZmCSLA6	FGENESH dicot incorrectly predicts the start site of exon 1 at bp. 224. The correct start site is at bp 344 in the genomic DNA. FGENESH dicot predicts 5 extra exons (bp 1337-1768, 1918-2103, 2321-2568, 2765-3080, 3157-3235 in the genomic DNA) of intron 2. The FGENESH dicot prediction for the start site of exon 3 is incorrect. The start site is at bp 3445 instead of bp 3372 in the genomic DNA. FGENESH dicot does not predict exon 4, which is from bp 3793-3913 in the genomic DNA. The FGENESH dicot prediction for the end of exon 8 is at bp 548 instead of bp 5051 in the genomic DNA. FGENESH dicot does not predict exon 9, which is from bp 5410 to 5572 in the genomic DNA. The genomic and cDNA sequences are full length.
ZmCSLA7	FGENESH predicts an extra exon (bp 1809 to1895 in the genomic DNA) in intron 1. FGENESH does not predict exon 6, which is from bp 3642-3744 in the genomic DNA. The genomic and cDNA sequences are full length.
ZmCSLA8	FGENESH incorrectly predicts an exon from bp 11843-11849. The start site of exon 1 is unclear. The genomic and cDNA sequences are not full length.
ZmCSLA9	FGENESH incorrectly predicts an exon from bp 2574-2596 in the genomic DNA. It does not predict exons 5 (bp 2972-3109) and exon 6 (bp 3230-3345). The genomic and cDNA sequences are full length.

Table 9 Continued	
ZmCSLA11	FGENESH does not predict the exon which is from bp 1088-1197 in the genomic DNA. The exon which is from bp 1542-1656 in the genomic DNA has a stopcodon at bp 1585. The genomic and cDNA sequences are not full length. This might be a pseudogene.
ZmCSLC1	FGENESH is correct. The genomic DNA and cDNA sequences are full length.
ZmCSLC2	FGENESH is correct. Part of the 5' end of the genomic DNA and cDNA is missing. The genomic and cDNA sequences are not full length.
ZmCSLC3	FGENESH is correct. The genomic and cDNA sequences are full length.
ZmCSLC4	FGENESH combines 2 genes into 1 gene. The correct stopcodon is at bp 6951 in the genomic DNA. The genomic and cDNA sequences are full length.
ZmCSLC5	FGENESH misses exon 3 (bp 3644-3935 in genomic DNA). The genomic DNA for exon 5 is missing, but the cDNA sequence is full length.
ZmCSLC6	The FGENESH prediction for exon 3 is incorrect. The start site of this exon is at bp 3566 in the genomic DNA instead of bp 3671. The genomic and cDNA sequences are full length.
ZmCSLC7	The FGENESH prediction for exon 3 is incorrect. The start site of this exon is at bp 2880 in the genomic DNA instead of bp 2958. The genomic and cDNA sequences are full length.
ZmCSLC8	The FGENESH prediction is incorrect for the exon which is from bp 1632 to 1940 in the genomic DNA. The end of this exon is at bp 1940 instead of bp 2030. The genomic and cDNA sequences are not full length.

Table 9 Continued	
ZmCSLC9	The FGENESH prediction for exon 2 is incorrect. The end of this exon is at bp 965 in the genomic DNA instead of bp 937. This exon contains a stopcodon in exon 2 at bp 940 in the genomic DNA. FGENESH makes two exons out of exon 3 which is from bp 1056 to 1362 in the genomic DNA. This exon contains two stopcodons. One at bp 1180 in the genomic DNA and one at bp 1233 in the genomic DNA. FGENESH incorrectly predicts an exon from bp 1482-1739 in the genomic DNA. The genomic and cDNA sequences are not full length. The 3' end of this gene is missing. This gene might be a pseudogene because of the stopcodons in the genomic DNA. Another possibility is that the errors in the genomic DNA are due to low coverage (1X) of this region in the maize genome.
ZmCSLC10	The FGENESH prediction for the exon which is from bp 283 to 645 in the genomic DNA is incorrect. The end of this exon is at bp 645 instead of bp 666. The genomic and cDNA sequences are not full length.
ZmCSLC11	The FGENESH prediction is incorrect for exon 1 and 2. The start of exon 1 is at bp 3209 in the genomic DNA instead of bp 3275. The end of exon 2 is at bp 4475 in the genomic DNA instead of bp 4600. A part of the genomic DNA of intron 2 is missing. The predicted start site of exon 3 is incorrect. The start site is at bp 543 instead of bp 495. FGENESH predicts two extra exons at the 3' end of the gene. This gene ends at bp 1857 in the genomic DNA. The genomic DNA is not full length, but the cDNA sequence is.
ZmCSLC12	FGENESH incorrectly predicts the end of the exon encoded by this genomic DNA at bp 890 instead of bp 896. The genomic and cDNA sequences are not full length.
ZmCSLD1	The FGENESH prediction for the start site of exon 2 is incorrect. The start site of exon 2 is at bp 2004 in instead of bp 1980 in the genomic DNA. The genomic DNA and cDNA sequences are full length.

Table 9 Continued	
ZmCSLD2	The FGENESH prediction is incorrect for the end of exon 1. The end of exon 1 is at bp 1520 in the genomic DNA instead of bp 1523. Part of the genomic DNA of exon 2 and intron 1 is missing. The FGENESH prediction is incorrect for the end of exon 2. This exon ends at bp 460 instead of bp 232 in genomic DNA *ZmCSLD2.2*. Part of the genomic DNA of exon 3 and intron 3 is missing. The cDNA for this gene is full length.
ZmCSLD3	The FGENESH prediction is correct. Part of the 5' end of the genomic and cDNA sequences are missing.
ZmCSLD4	The FGENESH prediction is correct. The genomic and cDNA sequences are full length.
ZmCSLD5	The FGENESH prediction is correct. The genomic and cDNA sequences are full length.
ZmCSLE1	The FGENESH prediction is correct. The cDNA sequence is full length, but part of genomic DNA of the middle part of the gene is missing. Genomic DNA *ZmCSLE1.1* has a deletion of a thymidine base at bp 2748 which causes a frameshift in exon 3. This deletion is not present in the ESTs. Genomic DNA *ZmCSLE1.2* contains a stopcodon at bp 300 due to a basepair change from A to G. This stopcodon is not present in the ESTs.
ZmCSLE2	FGENESH incorrectly predicts the end of the exon which is from bp 647 to 685 in the genomic DNA at bp 647 instead of 749. The genomic and cDNA sequences are not full length. There is a stopcodon in the genomic DNA at bp 724. This might be a pseudogene.
ZmCSLE3	FGENESH is correct. The genomic and cDNA sequences are full length.

Table 9 Continued	
ZmCSLE4	The FGENESH prediction is incorrect for the exon which is from bp 3325 to 3464 in the genomic DNA. The start site of this exon is at bp 3325 instead of bp 3327. At bp 3303 there is a stopcodon in the genomic DNA. This might be a pseudogene. The genomic and cDNA sequences are not full length.
ZmCSLF1	FGENESH is correct. The genomic and cDNA sequences are full length.
ZmCSLF2	FGENESH is correct. The genomic and cDNA sequences are full length.
ZmCSLF1 and ZmCSLF2	These ESTs align with both CSLF1 and CSLF2.
ZmCSLF3	The predicted start site of the exon which is from bp 834-1502 in the genomic DNA is incorrect. The correct start site it at bp 759. The end of the exon which is from bp 1580-2317 in the genomic DNA is incorrect. The genomic and cDNA sequences for the end of this exon are missing. The genomic and cDNA sequences are not full length.
ZmCSLF4	The FGENESH prediction is incorrect for exon 2 which is predicted to end at bp 1882, instead the correct end is at bp 1956 in the genomic DNA. FGENESH incorrectly predicts an exon from bp 2819-2877. The genomic and cDNA sequences are full length.
ZmCSLF5	FGENESH is correct. Part of the 5' genomic and cDNA sequence is missing.
ZmCSLF6	FGENESH is correct. The genomic and cDNA sequences are full length.
ZmCSLF7	FGENESH is correct. The genomic and cDNA sequences are full length.
ZmCSLH	FGENESH is correct. The genomic DNA is of a piece of exon 9 is missing, the cDNA sequence is full length.
ZmCSLX	FGENESH is correct. The genomic and cDNA sequences are full length.

Table 10: ESTs found for each *CSL* in GenBank and MaizeSeq as of November 2006	
Maize gene	**ESTs**
ZmCSLA1 13 ESTs	AY111707, BE509763, BE639162, CB179595, CF637647, CX054069, DN219895, EB819007EC877128, EC901950 , MRT4577_113780, 61012.1, 112651.1
ZmCSLA2 11 ESTs	AI712277, BG267241, CO459150, CO529865, EB822275, MRT4577_160794, MRT4577_178541, 40199, 92880, 395289
ZmCSLA3 85 ESTs	BE345572, BI543108, BI542892, BI502667, BM337360, CB885661, CB605095, CD986535, CD986776, CF623308, CF647714, CF646849, CK827009, CO534845, CO521720, DN203737, DN210538, DN220659, DR785175, DR793529, DR796360, DR802009, DR810267, DR819129, DR824882, DR827942, DR830824, DR959515, DR960996, DR962628, DR972154, DT645077, DT652063, DT944501, DT947934, DV029937, DV032430, DV508950, DV521309, DV522106, DV528686, DV530970, DV533083, DY232094, DY236276, DY618629, DY686234, DY689456, DY689950, EB403285, EB403767, EB404299, EB405782, EB676269, EB701629, EB705890, EB816589, EC875141, EC882568, EC885731, EC886357, EC897088, EE017356, EE036839, EE040917, EE041738, EE164658, EE165758, EE167563, EE185452, EE187985, EE190120, EE287464, EE291877, MRT4577_29888C.1, 46987.2, 12762.2, 3447.3, 220844, 306862, 318020, 44615.1, 46986.1, 73611.4, 129440.1
ZmCSLA4 4 ESTs	CF650426, MRT4577_170140C.1, MRT4577_122156C.1, 112873.1
ZmCSLA5 3 ESTs	DR808454, EE156170, MRT4577_95044C.1
ZmCSLA6 17 ESTs	AI966916, AI629471, AW146710, BM331755, BM337273, BM339419, CO439336, DN206568, DR804900, DR959523, DR960322, DV028649, DV491468, DV512882, MRT4577_44468C.1, 6799.1, 148484.1

Table 10 Continued	
ZmCSLA7 15 ESTs	BM080245, CF040341, CO448087, CO451898, DN225331, DR970075, DV534833, EC885334, EC885995, EC891529, EE160654, MRT4577_40487C.1, MRT4577_40488C.1, 939.1, 136903.1
ZmCSLA8 11 ESTs	DR972654, DT645477, DT646829, DY537262, DY623006, EB642842, EE019504, EE188078, EE287562, MRT4577_105165C.1, 69248.1
ZmCSLA9 6 ESTs	CO441048, CO454463, MRT4577_167180C.1, 55086.1, 55087.2, 125237.1
ZmCSLA10 7 ESTs	CK368643, CK827890, CO526201, MRT4577_145084C.1, MRT4577_152087C.1, 324543, 35933.1
ZmCSLA11 0 ESTs	
ZmCSLC1 23 ESTs	AI491631, AI782918, AI967143, AI999860, BM349218, CB351253, CF004102, DR806804, DV020708, DV520752, DV523387, DV942945, DW961997, EE177147, EE287163, EE289125, MRT4577_280C.1, MRT4577_74050C.1, 15495.1, 503149, 51996.2, 51997.1, 116485.1
ZmCSLC2 3 ESTs	CF637490, CK371550, EE042682
ZmCSLC3 6 ESTs	CA828196, BQ279719, DN204842, EE025542, MRT4577_77404C.1
ZmCSLC4 11 ESTs	BU092694, BU092832, BQ487075, CA831369, EC892727, EE020946, EE175501, DY402635, MRT4577_177513C.1, 36147.1, 116763.1

Table 10 Continued	
ZmCSLC5 90 ESTs	AW134434, BF727705, BM498825, BQ578082, BQ048622, BT018527, CD440675, CD443934, CF044437, CF650782, CK144562, CO441868, CO457702, CO523572, CO527804, CO528077, CO531797, DR791573, DR791826, DR798460, DR805276, DR822059, DR825906, DR961560, DR970669, DT649058, DT941621, DV023358, DV024073, DV031784, DV165615, DV168144, DV170219, DV170453, DV170486, DV171001, DV508366, DV511482, DV529562, DV530255, DV536340, DV549730, DY535126, DY538898, DY539559, DY690607, EB163654, EB400980, EB406179, EB674364, EB676456, EB702758, EC874460, EC881320, EC881465, EC883184, EC893104, EC899426, EC901681, EC903967, EC904132, EE013636, EE018349, EE019352, EE020315, EE021227, EE042191, EE044776, EE046495, EE153400, EE156704, EE156726, EE159137, EE164327, EE172128, EE174453, EE176046, EE285929, EE291585, EE294171,MRT4577_41982C.1, MRT4577_53439C.1, MRT4577_53441C.1, MRT4577_99427C.1, 31567.2, 45744.1, 64532.2, 76758.3, 145181.1, 152721.1
ZmCSLC6 67 ESTs	CD986188, CF650598, CF919899, CF649783, CO526243, CO532188, CO461994, DR787544, DR797174, DR791495, DR802201, DR809519, DR812129, DR813649, DR829637, DR960832, DT942330, DT653933, DT654270, DV170447, DV503853, DV506512, DV514162, DV514533, DV514967, DV534763, EB158905, EB164184, EB402572, EB673906, EB813228, EB821678, EC874262, EC874577, EC875492, EC876358, EC877605, EC878679, EC889570, EC889068, EC894672, EE010659, EE012971, EE018864, EE024111, EE024645, EE026179, EE033570, EE033766, EE033832, EE033894, EE036521, EE040400, EE043759, EE156566, EE162034, EE165420, EE168545, EE170986, EE171280, EE178640, EE179176, EE288312, EE291735, MRT4577_112624, 61530.1, 139048.1

Table 10 Continued	
ZmCSLC7 1 EST	DV033286
ZmCSLC8 0 ESTs	
ZmCSLC9 0 ESTs	
ZmCSLC10	DR969222
ZmCSLC11 5 ESTs	CF635266, EE022110, EE186486, EE186309, MRT4577_144971C.1
ZmCSLC12 0 ESTs	
ZmCSLD1	CO533241, DR960265, DR828710, DR789710, DR786107, DT649020 , DT945255, DV164427, DV165563, DV025799, DV527723, DW467865, EB70160, EB818933, EC899381, EE024752, EE038233, EC887069, MRT4577_16017C.1, MRT4577_40058C.1, MRT4577_168633C.1, 22837.1, 31393.1, 39225.1, 117161.1
ZmCSLD2 27 ESTs	AI657474, CA404833, CD439402, CD445140, CD940648, CD957767, CD997962, CF024310, CK348127, DN225988, DN229925, DV495290, DV520872, DY622790, EB158461, EB167441, EE021092, EE030414, MRT4577_37569C.1, MRT4577_62600C.1, MRT4577_83752C.1, 20788.1, 28076.1, 35884.1, 49466.1, 139902.1, 148928.1
ZmCSLD3 2 ESTs	MRT4577_23057C.1, 38389.1
ZmCSLD4 9 ESTs	DR970587, DV534771, DY621894, EB637344, EE179918, MRT4577_24124C.1, MRT4577_78194C.1, 38192.1, 118619.1

Table 10 Continued	
ZmCSLD5 6 ESTs	AI857200, CK828171, DV491157, EE679307, 87531.1, 92873.1
ZmCSLE1 11 ESTs	BE761748, BM073901, DN209030, CO441675, CF051386, CD964407, CD964287, DY539742, EC881460, MRT4577_63661C.1, 38622.1
ZmCSLE2 0 ESTs	
ZmCSLE3 14 ESTs	CF627793, DR819529, DR964066, EC893493, EE011424, EE016689, EE017015, EE163100, EE170793, EE286791, MRT4577_144142C.1, 38833.1, 66548.2, 153926.1
ZmCSLE4 0 ESTs	

Table 10 Continued	
ZmCSLF1 153 ESTs	AI783230, AI795546, AI973329, AI999933, AW017656, AW042388, AW146811, BG265599, BM268865, BM333387, BM335019, BM335870, BM338286, BM347364, BM347399, BM347728, BM348968, BM349583, CD439674, CF647371, CO447585, CO520666, CO520904, CO529267, DN215438, DR821260, DR822182, DR826199, DR791112, DR791522, DR797652, DR796453, DR799904, DR799649, DR805715, DR814323, DR816712, DR817841, DR818247, DR820048, DR821261, DR822183, DR826200, DR954675, DR954860, DR956075, DR957779, DR969573, DT644307, DT649889, DT650208, DT652176, DT653968, DT938200, DT939993, DT941682, DT941786, DT944692, DV028925, DV029265, DV032475, DV167670, DV174682, DV504205, DV504499, DV504537, DV506225, DV506644, DV511477, DV512176, DV513987, DV519602, DV526195, DV527458, DV529942, DY530223, DV532277, DV534084, DV539057, DY235230, DY237207, DY237682, DY532292, DY536211, DY538305, DY619806, DY622103, DY624109, DY685856, DY690078, EB161045, EB163591, EB164004, EB399654, EB406715, EB637507, EB640049, EB641560, EB675223, EB702598, EB816752, EB820888, EB822621, EC874350, EC877358, EC885025, EC888233, EC892638, EC893592, EC893771, EC898151, EC899578, EC899898, EE021776, EE022726, EE023779, EE023856, EE024627, EE026187, EE027359, EE038746, EE040298, EE041443, EE043103, EE044201, EE045406, EE047322, EE153371, EE153947, EE169422, EE169470, EE173970, EE174283, EE174942, EE179257, EE185535, EE188221, EE189572, EE189911, EE287777, EE287836, EE287876, EE681777, 1522.2, 1751.6, 209037, 48889.1, 63576.3, 63577.4, 67452.5, 69791.1, 76585.8, 125340.1

Table 10 Continued	
ZmCSLF2 135 ESTs	AI673968, AW065348, BI135345, BM173697, BM417109, BM417198, BU049237, BU049339, CF058879, CF245147, CF244964, CD439166, CD976130, CF245147, CF623166, CF638045, CF648617, CK368125, CN071362, CO522999, CO533529, CO530432, CO519007, DR789101, DR792651, DR796147, DR805904, DR810237, DR821815, DR822597, DR829412, DR958628, DR969118, DT651821, DT652126, DT939385, DT947848, DV020239, DV020568, DV025107, DV027989, DV029421, DV033990, DV163120, DV164891, DV168557, DV172188, DV502129, DV518408, DV523120, DV528761, DV530271, DY235522, DY238535, DY530979, DY532999, DY533783, DY538486, DY539155, DY540904, DY542917, DY621079, DY685480, EB166098, EB167134, EB167397, EB401995, EB408246, EB637641, EB639474, EB701422, EB702456, EB705868, EB708252, EB819329, EC874832, EC877563, EC879225, EC879243, EC879391, EC879735, EC882382, EC889264, EC890410, EC894591, EC900348, EC900569, EC902928, EC903762, EE019913, EE020366, EE021716, EE027053, EE027323, EE032940, EE033079, EE033159, EE033838, EE033977, EE040866, EE041414, EE041512, EE042092, EE043783, EE046599, EE153305, EE153672, EE153921, EE156356, EE158385, EE160408, EE162564, EE164474, EE165945, EE167050, EE168402, EE170861, EE176042, EE179789, EE183456, EE183688, EE185850, EE186177, EE186178, EE188636, EE189897, EE190081, EE285694, EE290853, EE293194, EE295301, 67453.6, MRT4577_132210C.1
ESTs which are similar between ZmCSL*F1* and *ZmCSLF2*	CF058879, EC898208, EE012676, EE017551, EE018725, EE021716, EE038868, EE047285, EE163229, EE189814, EE285619, EE286893, EE289400, EE293869,
ZmCSLF3 0 ESTs	

Table 10 Continued	
ZmCSLF4 3 ESTs	EE172734, EE031146, MRT4577_91428C.1
ZmCSLF5 5 ESTs	EE292309, EC885590, EC886228, MRT4577_153693C.1, MRT4577_160801C.1
ZmCSLF6 5 ESTs	EC893050, EE042523, MRT4577_108931C.1, MRT4577_185512C.1, 24052.1
ZmCSLF7 2 ESTs	EE014434, MRT4577_11227C.1
ZmCSLH1 8 ESTs	DV518628, EC891386, EE012543, EE018922, EE186369, MRT4577_12320C.1 MRT4577_16629C.1, MRT4577_119932C.1
ZmCSLX 20 ESTs	BM382035, BT017533, CA401527, CD443089, CD953565, CF648387, DT644979, DV519225, EB701720 , EB676365 , EC901471 , EC878414, EC877425, EE014664, EE048062, EE171665, EE289753, MRT4577_56702C.1, 92886.1, 92889.1

APPENDIX II: GENBANK LOCUS NUMBERS AND JGI PROTEIN IDS

Table 11: GenBank locus number for *Arabidopsis* CSL proteins.

***Arabidopsis* protein**	**Locus**
AtCSLA2	AT5G22740
AtCSLA3	AT1G23480
AtCSLA7	AT2G35650
AtCSLA9	AT5G03760
AtCSLA10	AT1G24070
AtCSLA11	AT5G16190
AtCSLA14	AT3G56000
AtCSLA15	AT4G13410
AtCSLC4	AT3G28180
AtCSLC5	AT4G31590
AtCSLC6	AT3G07330
AtCSLC8	AT2G24630
AtCSLC12	AT4G07960
AtCSLB1	AT2G32610
AtCSLB2	AT2G32620
AtCSLB3	AT2G32530
AtCSLB4	AT2G32540
AtCSLB5	AT4G15290
AtCSLB6	AT4G15320
AtCSLC4	AT3G28180
AtCSLC5	AT4G31590
AtCSLC6	AT3G07330
AtCSLC8	AT2G24630
AtCSLC12	AT4G07960

Table 11 continued	
AtCSLD1	AT2G33100
AtCSLD2	AT5G16910
AtCSLD3	AT3G03050
AtCSLD4	AT4G38190
AtCSLD5	AT1G02730
AtCSLD6	AT1G32180
AtCSLE1	AT1G55850
AtCSLG1	AT4G24010
AtCSLG2	AT4G24000
AtCSLG3	AT4G23990

Table 12: JGI protein IDs for the poplar CSL proteins	
Poplar protein	**Protein ID**
PtCSLA1	686549
PtCSLA2	687416
PtCSLA3	589559
PtCSLA4	594843
PtCSLA5	556940
PtCSLB1	572982
PtCSLB2	684214
PtCSLC1	353078
PtCSLC2	578365
PtCSLC3	692569
PtCSLC4	694461
PtCSLC5	692052
PtCSLD1	552489

Table 12 continued	
PtCSLD2	700418
PtCSLD3	554065
PtCSLD4	590064
PtCSLD5	573858
PtCSLD6	703843
PtCSLD7	595034
PtCSLD8	78520
PtCSLD9	350683
PtCSLD10	48556
PtCSLE1	550222
PtCSLE2	343986
PtCSLE3	560094
PtCSLG1	698018
PtCSLG2	554513
PtCSLG3	80778
PtCSLG4	350373
PtCSLG5	350372

Table 13: JGI protein IDs for the *Physcomitrella* CSL proteins	
***Physcomitrella* protein**	**Protein ID**
PpCSLA1	DQ417756
PpCSLA2	DQ417757
PpCSLC1	DQ898147
PpCSLC2	DQ898148
PpCSLC3	DQ898149
PpCSLC4	DQ898150

Table 13 continued	
PpCSLC5	DQ898151
PpCSLC6	DQ898152
PpCSLD1	DQ898147
PpCSLD2	DQ898148
PpCSLD3	DQ898149
PpCSLD4	DQ898150
PpCSLD5	DQ898151
PpCSLD6	DQ898152
PpCSLD7	DQ898153
PpCSLD8	DQ898154

Table 14: GenBank locus number for the ZmCESA proteins	
ZmCESA	**Locus**
ZmCESA1	AAF89961
ZmCESA2	AAF89962
ZmCESA3	AAF89963
ZmCESA4	AAF89964

APPENDIX III: GENE SPECIFIC PRIMERS FOR SEMI-QUANTITATIVE RT-PCR

Table 15: Gene specific primers for the *ZmCSLA* genes

Gene name	Forward primer (5' → 3')	Reverse primer (5' → 3')
ZmCSLA1	ATCCCGGAGCTTTACCTACCAGT	AAGAACAATTGCCAGTGAAGT
ZmCSLA2	CGTATATCATCGTAGAAGAATCCA	CGCAGCCAGAGCCCCGTGACC
ZmCSLA3	CCGTCTGCGCCTTCTTTGGATTC	GGTGATGGCCGAGGGGATGTAGAC
ZmCSLA4	TGAACCAGTGAAGCCAACAGAATG	AACCGCCCCCTACCCACAC
ZmCSLA5	TCCCAAAGAATTCCTGATGACAA	ATGGGCGACAACCTTCCTAATG
	CATATCGCCGCCAACAACATC	TCTACCAAGCTTCTCCGTGACAAC
	GAGGTGTCGCTGTGGAGGAA	AGAAGAGGAAGATGGCAACTAAA A
ZmCSLA6	GAGAGTGTCGCTGTGGAGTAAAAT	ACCCAAAACCAACAACAAGGAACG
ZmCSLA7	ATTGGGGCGACTGTATGGAAGAA	AGCGAAGGCCTGGAGGAAGATGTA
	GGCCAGCAAGAAAATCAACATAAA	TGCCCGCACAGCCAAGTC
	TCGGTGAAGCAGGAGGATG	ATTGGCACCAGATGGATAGACC
ZmCSLA8	TTGCCCGGAGAATCGTAGG	TGGGGAAGAACAAAGAGGTAAAC A
	GACCTGCCCTCCTGTTCAAGA	ATAAGTAGTCAAAGCACGCAGAGG
	GTGAATGCCAACGACTGCT	AAGGTGCCTACGATTCTCC
	AGCTGGCTCTTTCTTATGC	GCGGTACTGCTAGGACTGGT
	CTGCTGGAGTATGGAGAACG	GCGGTACTGCTAGGACTGGT
ZmCSLA9	CGCCGTGACGTTCGTGTTTTACTG	ATAGCCAATGCCGACGATGAAGAA
ZmCSLA10	GGAAGGTCGCTGCCCACACG	GCCGACATACCCAAAGCCAACAAC
	GCTGCAGGGCTGGAAGTTTGTTT	ACTCGTCCACCAGCATCCAGAAGA
	CTAGCACGTTCAAGGCATAC	ACGAGGGGTTCTGTTAGC

Table 16: Gene specific primers for the *ZmCSLC* genes.		
Gene name	**Forward primer**	**Reverse primer**
ZmCSLC1	GACCTTTTCCGGCTGTGCT	CATGCGAGGAATCAACTTATCTGT
ZmCSLC2	No primers	
ZmCSLC3	TGCTTTGTGGATATTATCAAATCGAA	CCCCGATGAAACTATATAACCCTTC
ZmCSLC4	GCTTTGTGGATATTATCAAGTCGAAG	CGTGTTTGCGTTGAATCATAT
ZmCSLC5	AGGCATTGGAGGACTCAGGTGGAT	TACGGGATGATGAAGGGGAACGAC
ZmCSLC6	AATTGTTTAGGCTCTGCTTTGTGG	AACCCATTATTCAACTGCTATCAATCC
ZmCSLC7	GTGCCTTGGCTGCTTCTACATCC	CCGGGTCAGCAGGTTCTCATC
ZmCSLC8	GGCCATCCAGAAGCTGTCCAG	CTTGAGGTTGCCGGCCTTGTA
ZmCSLC9	CAACCTTGACTGCCCGAAATC	GAATCATGGCAGAGCAACAAACTT
	ATCCTATGCCTTGGCTACTTCTAC	ATGGGGTGTTATCTTGGCTTCC
	CGCCTACGTTGTGCTCTTCCT	ATGGGGTGTTATCTTGGCTTCC
ZmCSLC10	No primers	
ZmCSLC11	GCGGGCGGCTTCTGGGGTGTC	ACGCGGGGATACGAGGCTTGATGC
	CGACAACGGTGCAGGAGAAC	CCGCAGCAGGAAGAAGAGCAT
	CGACAACGGTGCAGGAGAAC	ATGAGCACGGGGATGTAGCA
	CGACAACGGTGCAGGAGAAC	GCAGAAGAGCGTGAAGGAGTAGA
ZmCSLC12	No primers	

Table 17: Gene specific primers for the *ZmCSLD, E, F, H, X* and *ZmEF2α* genes.

n.d. means not determined

Gene name	Forward primer	Reverse primer
ZmCSLD1	GGCTGCCGATGCTGGTGTA	TTGTTCTCCCTGTCCGTCTTCTTT
ZmCSLD2	CGATACGCCGACGCTCTGA	TGAAGGGGCCATTTGACAT
	GGAGGAGGGTCAAGAGGGAGTATG	GCGGAAAGCTTGCGAGTTGTAG
ZmCSLD3	GCACGGCGCCCATCAACCTCA	CGTACACGGCGCGGGACACA
ZmCSLD4	No primers	
ZmCSLD5	GCGCCTGTCGCTGGTCA	ATCCGACTTGCCTGTTGGATTGT
ZmCSLE1	GGATGGATGGGGTGGAATGTGTTA	CGGGCTGTACTTTGAGAGGGAGAT
	GGTGGAGCGAGAATGCAAGTTTTA	TTCCTCGCCACCCTAGTCCAATCT
ZmCSLE2	No primers	
ZmCSLE3	TCACGGGGCTGGCGATACACTGC	TCTGCCCGTTCCACCACCCTCTCA
ZmCSLE4	n.d.	
ZmCSLF1	No primers	
ZmCSLF2	CGTTCACGGCGATCTTCCTCAT	CCGCCGGCCACCTTTAGC
	GGCTCGTGAACCCCGTCCCGTAAT	GGAGAGGCCGTCGTCGCTGAGGTC
	CGCGCATCAACGGGCTGGAGAA	CGCCTGCGAGTTGTTGATGTAGTG
ZmCSLF3	GGCACGGGCAGCATGAGA	CAGCCCAGCAGCAGCACC
ZmCSLF4	GCGGCGCCGACGACGAGAG	GCCGGGCAGCTTGTTGTTGGACT
	CGCATGCGCAGGGAATACGAAGAG	GCGGAAAGCCTGCGAGTTGTTGAC
	TCCGGCGGCGAGTCCAACAA	CCACGTCGCCTTCATCACCATACC
ZmCSLF6	GCTGCGGGCCTCTGCTCTCCT	TTGGTGGCGGTGTGATTGTCCTGT
	TGTGCGCTGGTCTGGTGGGTCTTT	GTCATTGTCGTCCGCGGCTGTTTG
	TGGCCCGGCACATGGATTGAT	AGCTTCGGCCGTGATGTTTTCTGC
ZmCSLF7	No primers	
ZmCSLF8	GGAACATGGTTTGACCCTGCTG	CAAACTTGCTGGGGTTGTCC

TABLE 17 CONTINUED		
ZmCSLH	CGAGTCGGCGAGGAGCATCATCA	ACAGCGCCAGCGGGACGACGAACC
ZmCSLX	CGGCGACGGCGTTGAGGAGATT	GCCGGGGCTGCGTAAGGATGC
	CGCGCCCTACGTGCTGGTCCTC	GCGGTGCCGGCCTCGTATGC
ZmEF2α	TGGCCAGACCCGTGAGCAT	ACGGACAGCAAATCGACCAAGAG

APPENDIX IV: GENE SPECIFIC PRIMERS FOR REAL-TIME QUANTITATIVE RT-PCR

Table 18: Gene specific primers for the ZmCSLA, C, D, E, F and EF1α genes

Gene name	Forward primer (5' → 3')	Reverse primer (5' → 3')
ZmCSLA1	ATCCCGGAGCTTTACCTACCAGT	AAGAACAATTGCCAGTGAAGT
ZmCSLA3	CTCGGCCATCACCCTCCTCAACT C	CCCAAGACACAATGCGAAAACCAG
ZmCSLA4	TGAACCAGTGAAGCCAACAGAAT G	AACCGCCCCCTACCCACAC
ZmCSLA5	TCCCAAAGAATTCCTGATGACAA	ATGGGCGACAACCTTCCTAATG
ZmCSLA6	GAGAGTGTCGCTGTGGAGTAAAA T	ACCCAAAACCAACAACAAGGAACG
ZmCSLA7	TCGGTGAAGCAGGAGGATG	ATTGGCACCAGATGGATAGACC
ZmCSLA8	TTGCCCGGAGAATCGTAGG	TGGGGAAGAACAAAGAGGTAAACA
ZmCSLA9	CGCCGTGACGTTCGTGTTTTACTG	ATAGCCAATGCCGACGATGAAGAA
ZmCSLA10	GCTGCAGGGCTGGAAGTTTGTTT	ACTCGTCCACCAGCATCCAGAAGA
ZmCSLC1	TATCGGCATCGGGTGTTGAGGAC T	AGAATGCCACCGGTGTTGCTGTTT
ZmCSLC3	CCCCGATGAAACTATATAACCCT TC	CCCCGATGAAACTATATAACCCTTC
ZmCSLC4	CGTGTTTGCGTTGAATCATAT	CGTGTTTGCGTTGAATCATAT

Table 18 continued		
ZmCSLC6	AACCCATTATTCAACTGCTATCAATCC	AACCCATTATTCAACTGCTATCAATCC
ZmCSLC7	GTGCCTTGGCTGCTTCTACATCC	CCGGGTCAGCAGGTTCTCATC
ZmCSLD2	CGATACGCCGACGCTCTGA	TGAAGGGGCCATTTGACAT
ZmCSLE1	GGATGGATGGGGTGGAATGTGTTA	CGGGCTGTACTTTGAGAGGGAGAT
ZmCSLF6	GCTGCGGGCCTCTGCTCTCCT	TTGGTGGCGGTGTGATTGTCCTGT
ZmCSLF8	CAAACTTGCTGGGGTTGTCC	CAAACTTGCTGGGGTTGTCC
ZmEF1α	TGCGGAGCTCATTACCAAGATT	GCTCACCAGATGTTCGGATAAGTC