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Mannan synthase activity in the CSLD family

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Cellulose Synthase Like (CSL) proteins are a group of plant glycosyltransferases that are predicted to synthesize β-1,4-linked polysaccharide backbones. CSLC, CSLF and CSLH families have been confirmed to synthesize xyloglucan and mixed linkage β-glucan, while CSLA family proteins have been shown to synthesize mannans. The polysaccharide products of the five remaining CSL families have not been determined. Five *CSLD* genes have been identified in *Arabidopsis thaliana* and a role in cell wall biosynthesis has been demonstrated by reverse genetics. We have extended past research by producing a series of double and triple Arabidopsis mutants and gathered evidence that CSLD2, CSLD3 and CSLD5 are involved in mannan synthesis and that their products are necessary for the transition between early developmental stages in Arabidopsis. Moreover, our data revealed a complex interaction between the three glycosyltransferases and brought new evidence regarding the formation of non-cellulosic polysaccharides through multimeric complexes.

Key words: mannan, mannose, plant cell wall, glycosyltransferase, cellulose synthase like, CSL, biosynthesis, hemicellulose

Abbreviations: CSL, Cellulose Synthase Like; CESA, Cellulose Synthase; GDP, Guanosine diphosphate; UDP, Uridine diphosphates

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The plant cell wall is mainly composed of polysaccharides, which are often grouped into cellulose, hemicelluloses and pectin. Since the discovery of the first cellulose synthase (*CESA*) genes in cotton fibers,[1] the synthesis of cellulose has been extensively studied.[2] In contrast, the glycosyltransferases responsible for synthesizing hemicelluloses and pectin are still largely unidentified.[3,4,5] The *CESA* genes are members of a superfamily that includes genes with a high sequence similarity with *CESA* genes and are named *Cellulose Synthase Like* (*CSL*).[6] The *CSL* genes have themselves been grouped into nine families designated *CSLA*, *-B, -C, -D, -E, -F, -G, -H* and *–J* (**Figure 1A**).[5,6] Mannan and glucomannan synthase activity has been demonstrated in the CSLA family,[7,8,9] while members of the CSLC family have been implicated in synthesis of the xyloglucan backbone.[10] CSLF and CSLH, which are found only in grasses, are involved in synthesis of mixed linkage glucan.[11,12] The function of the remaining CSL families has not been determined. We have reported our research on the CSLD family in a recent publication.[13] Of all the *CSL* families, *CSLD* possesses the most ancient intron/exon structure and is the most similar to the *CESA* family.[6] *CSLD* genes are found in all sequenced genomes of terrestrial plants including *Physcomitrella* and *Selaginella* suggesting a highly conserved function throughout the plant kingdom (**Figure 1A**). Five genes (*CSLD1* to *CSLD5*) and one apparent pseudogene (*CSLD6*) have been identified in *Arabidopsis thaliana*.[14] Bernal et al.[14,15] studied knock-out mutants of the individual genes and presented evidence for a role in cell wall biosynthesis for each Arabidopsis CSLD. To elucidate the activity of the CSLD proteins and obtain further understanding of their biological role, we generated double mutants *csld2/csld3, csld2/csld5, csld3/csld5* and the triple mutant *csld2/csld3/csld5*. Immunochemical, biochemical and complementation assays brought evidence

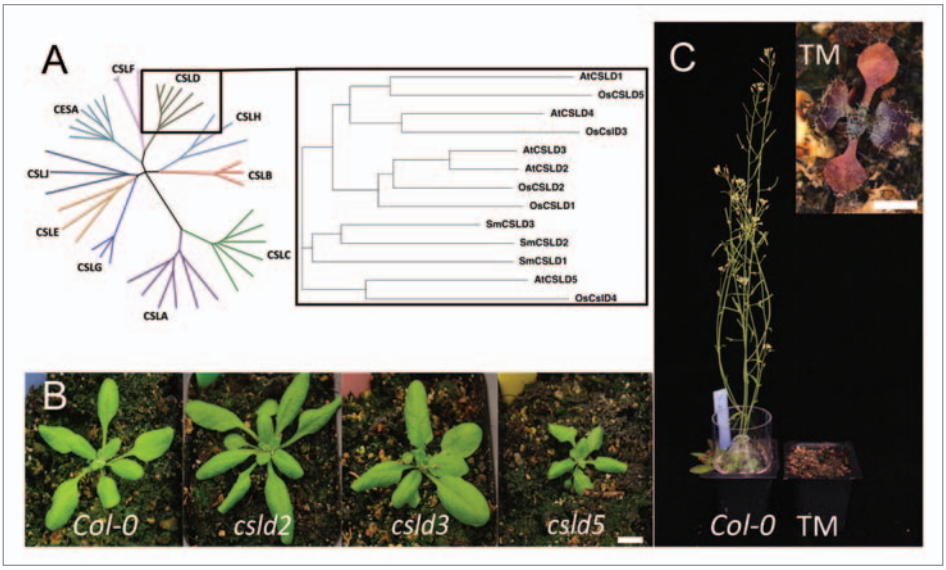

Figure 1. (A) Schematic representation of the CESA superfamily phylogeny. The inset on the right is a detailed phylogenetic tree of CSLDs from *Selaginella moellendorffii*, *Arabidopsis thaliana* and *Oryza sativa*. The figure is modified from Ulvskov and Scheller.[5] (B) Comparison of *csld2*, *csld3*, *csld5* with *Col-0* 20 days after germination. The inflorescences of *csld2* and *csld3* were similar to *Col-0* whereas *csld5* had a delayed growth. Scale bar: 1 cm. (C) *Col-0* and *csld2/csld3/csld5* (triple mutant, TM) 40 days after germination. After 40 days, the triple mutant was barely developed and, as shown in the magnified inset, displayed purple coloration indicating accumulation of anthocyanins, a typical stress response. Scale bar: 2 mm.

that CSLD5 or CSLD2 in concomitance with CSLD3 act as mannan synthases.

CSLD Proteins are Involved in Tip Growth and Early Development

Unlike the single mutations, which did not cause any drastic growth alteration (**Figure 1B**),[14,15] the double and triple mutants showed a reduced viability (as represented by *csld2/csld3/csld5* in **Figure 1C**). Anatomical studies revealed that the dwarf phenotype was correlated with an aberrant radial growth, suggesting perturbations in the development of cambial cells and indicating a crucial role for the three CSLD proteins at an early stage of development.[13] This was supported by root measurements that showed reductions in cell division and cell elongation in the triple mutant. Furthermore, aberrant root hair development indicated that CSLD2 and CSLD3 have key functions in specific tip-growing cell types.[13,15] A function in tip growing pollen tubes was likewise observed for CSLD1 and CSLD4.[15] Analysis of the sugar composition of the mutant cell walls did not show differences from wild type. However, immunodetection using the LM21 monoclonal antibody showed an absence of mannan in the secondary cell wall of xylem cells and the interfascicular fibers (**Figure 2A**). Although the correlation between the disruption of development and the absence of mannan in young and mature cells is unclear, the immunodetection provided a first clue about the possible polymer synthesized by the CSLD proteins.

Tobacco Expressing CSLD Proteins has High Mannan Synthase Activity

Tobacco microsomes from plants overexpressing Arabidopsis CSLD5 or CSLD2 and/or CSLD3 were isolated for in vitro enzymatic assay where mannose from GDP-mannose was transferred onto endogenous acceptors or mannose. The data indicated that CSLD5 and the joint activity of CSLD2 and CSLD3 lead to the synthesis of mannan (**Figure 2B**).

The mannan synthase activity of CSLD members was unexpected since β-1,4-glucan synthase activity had been observed in the CSLC, CSLF and CSLH families, which are all more distant to the CESA family than the CSLD members (**Figure 1A**). Thus, based on the phylogenetic data and the protein structure it was assumed that CSLDs would exhibit a similar glucan synthase activity.[13] Moreover, *csla* knock-out mutants displayed an absence of glucomannan in their mature stems, suggesting an exclusive role in mannan synthesis by the CSLA family in this organ.[7] Nevertheless, our anatomical and complementation studies have highlighted that, firstly, the CSLD proteins are active in specific cell structure such as the tip of root hairs and, secondly, they function at an earlier stage of development than CSLAs. Thus, it is likely that CSLA and CSLD functions do not overlap in Arabidopsis tissues. Moreover, CSLD and CSLA families may synthesize different classes of mannan polysaccharides. Indeed, the mannan superclass encompasses a range of polymers containing β-1,4-mannose residues in their backbone with a degree of substitution that varies from species to cell type. Depending of the core and the substitution, these polysaccharides are named mannan, galactomannan, glucomannan or galactoglucomannan.[5] A careful comparison of the evidence suggests that CSLAs and CSLDs are in fact responsible for synthesis of different types of polymers. The biochemical activity studies of CSLA proteins from different species have shown that these proteins readily use GDP-glucose as well as GDP-mannose and hence efficiently synthesize glucomannans.[7,8] Our studies indicated a much lower activity

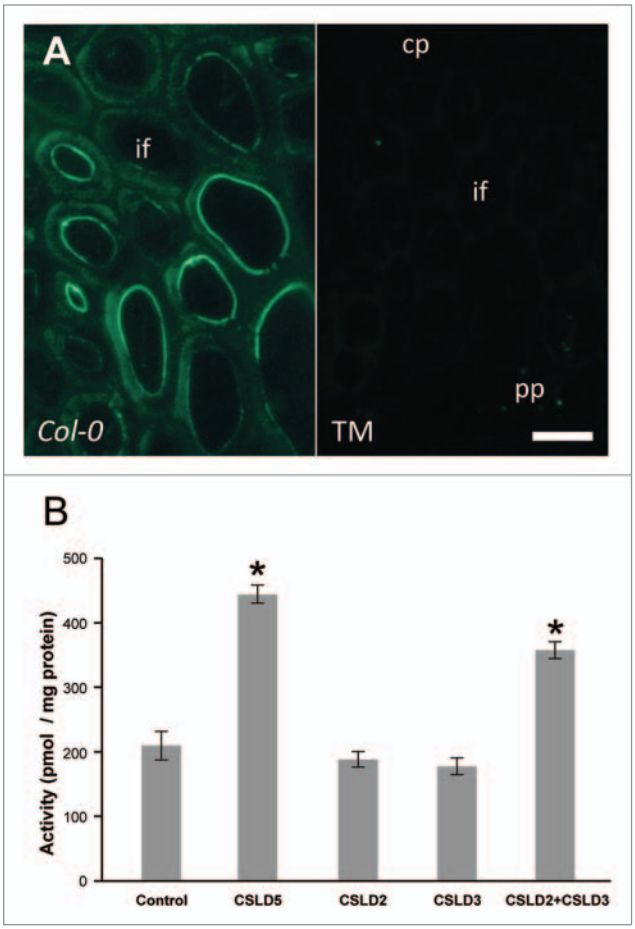

Figure 2. (A) Immunodetection of mannan epitope in the interfascicular fibers of *Col-0* and *csld2/csld3/csld5* (TM). In wild type transverse stem sections, the mannan epitope was strongly detected in the interfascicular fibers whereas in the triple mutant no antibody binding was observed in the cell wall of equivalent cells. cp, cortical parenchyma; if, interfascicular fibers; pp, pith parenchyma. Scale bar: 10 µm. (B) Radiochemical mannosyltransferase activity-assay of tobacco microsomes isolated from plants transiently expressing Arabidopsis CSLD5 or CSLD2 and/or CSLD3 as YFP fusion proteins. GDP-^{14}C-mannose was used as substrate to be transferred onto endogenous acceptors. Compared to the control plants, the microsomes with Arabidopsis CSLD5 or both CSLD2 and CSLD3 showed a significantly higher mannosyltransferase activity. Data are mean ± SE [n = 4, *indicates significant difference from control ($p < 0.001$, *t*-test)].

with GDP-glucose suggesting that mannan rather than glucomannan would be the product.[13] This biochemical difference is consistent with the mutant phenotypes. The *csla* mutants studied by Goubet et al.[7] had little mannose in the stems and enzymatic fingerprinting indicated that glucomannan was essentially absent. However, the fingerprinting analysis also showed that pure mannooligosaccharides were more or less at wild-type levels in the mutants. Likewise, a polyclonal anti-mannan antibody showed the virtual absence of mannan.[7] However, this antibody is known to preferentially bind glucomannans rather than pure mannans.[16] In contrast, the LM21 antibody used in our study preferentially detects unsubstituted pure mannans and has a lower affinity for glucomannan.[17] Thus, these observations lead us to propose that the bulk glucomannan polysaccharides in Arabidopsis are synthesized by members of the CSLA family while the pure mannans are synthesized by CSLDs. According to the enzymatic fingerprinting, the pure mannans are much less abundant, consistent with our inability to detect significant changes in sugar composition of the cell walls from *csld* mutants. Nevertheless, the CSLD proteins clearly have a vital function as evidenced by the strong phenotype of the triple mutant, and this is in contrast with the *csla* stem glucomannan mutants, which had no morphological or developmental phenotype. However, it should be noted that the Arabidopsis *csla7* mutant has a strong embryolethal phenotype indicating an essential role of glucomannan in the embryo.[18]

Interestingly, the catalytic site of CSLA9 has been shown to face the Golgi lumen whereas CSLC4 transfers UDP-glucose to xyloglucan chain acceptors in the cytoplasm before the transport of its resulting product into the Golgi.[19] In silico data suggest that CSLDs would have a similar topology to CSLC4 and CESAs rather than CSLA9. Thus, the CSLD proteins are predicted to use cytoplasmic GDP-mannose whereas the CSLA proteins would depend on one or more transporters of GDP-mannose and/or GDP glucose.

CSLD Protein Complexes

CSLD2 and CSLD3 only showed mannan synthase activity when co-expressed. This confirmed previous assumptions regarding the interaction between the two proteins. Indeed, microarray data has already shown that CSLD2 and CSLD3 are strongly co-expressed in *Arabidopsis thaliana*.[15] Our complementation studies and also the severe phenotypes of the *csld2/csld5* and *csld3/csld5* double mutants proved the non-redundancy of the two genes.[13]

Several other studies have mentioned the existence of non-cellulosic glycosyltransferase complexes but so far direct evidence has only been reported for glucuronoarabinoxylan synthase. Zeng et al. have isolated three glycosyltransferases in wheat, proved their cooperative transferase activities in glucuronoarabinoxylan synthesis and demonstrated that the three proteins, TaGT43-4, TaGT47-13 and TaGT75-4, form a complex.[20] The dimerization between ARAD1 and ARAD2 involved in arabinan biosynthesis has also been confirmed but this data has not yet been published (unpublished data). Indirect evidence suggested that GAUT1/GAUT7 probably form a homogalacturonan synthase complex[3] and the slight reduction of (gluco)

mannan antibody binding in *csla2/csla3* stems also suggests a cooperative activity between the two CSLA proteins.[7] Here, our study showed that the CSLD2 and CSLD3 mannosyltransferases were only active when co-expressed, strongly suggesting that they function in a complex. Bimolecular fluorescence complementation studies suggested homo- or heterodimerization between CSLD2, CSLD3 and CSLD5. Unfortunately, the limits of the technique—i.e. a propensity of false positives—require additional evidence to validate the direct interaction between these proteins and our attempts to co-immunoprecipitate a complex remain inconclusive.

Numerous enzymes have been tested for glycosyltransferase activity without success. Indeed, many efforts over the last ten years to discover the glycosyltransferases involved in cell wall biosynthesis mostly focused on the enzymes as single active units rather than components in complexes. Future research will need to focus on new strategies that will take into account the possible modulation of activity through protein-protein interactions.

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References

1. Pear JR, Kawagoe Y, Schreckengost WE, Delmer DP, Stalker DM. Higher plants contain homologs of the bacterial celA genes encoding the catalytic subunit of cellulose synthase. Proc Natl Acad Sci USA 1996; 93:12637-42.
2. Carpita NC. Update on mechanisms of plant cell wall biosynthesis: how plants make cellulose and other (1->4)-β-D-glycans. Plant Physiol 2011; 155:171-84.
3. Mohnen D. Pectin structure and biosynthesis. Curr Opin Plant Biol. 2008; 11:266-77.
4. Harholt J, Suttangkakul A, Scheller HV. Biosynthesis of pectin. Plant Physiol 2010; 153:384-95.
5. Scheller HV, Ulvskov P. Hemicelluloses. Annu Rev Plant Biol 2010; 61:263-89.
6. Richmond TA, Somerville CR. The cellulose synthase superfamily. Plant Physiol 2000; 124:495-98.
7. Goubet F, Barton CJ, Mortimer JC, Yu X, Zhang Z, Miles GP, et al. Cell wall glucomannan in Arabidopsis is synthesised by CSLA glycosyltransferases, and influences the progression of embryogenesis. Plant J 2009; 60:527-38.
8. Liepman AH, Wilkerson CG, Keegstra K. Expression of cellulose synthase-like (Csl) genes in insect cells reveals that CslA family members encode mannan synthases. Proc Natl Acad Sci USA 2005; 102:2221-26.
9. Dhugga KS, Barreiro R, Whitten B, Stecca K, Hazebroek J, Randhawa GS, et al. Guar seed beta-mannan synthase is a member of the cellulose synthase super gene family. Science 2004; 303:363-66.
10. Cocuron JC, Lerouxel O, Drakakaki G, Alonso AP, Liepman AH, Keegstra K, et al. A gene from the cellulose synthase-like C family encodes a beta-1,4 glucan synthase. Proc Natl Acad Sci USA 2007; 104:8550-55.
11. Burton RA, Wilson SM, Hrmova M, Harvey AJ, Shirley NJ, Medhurst A, et al. Cellulose synthase-like CslF genes mediate the synthesis of cell wall (1,3;1,4)-beta-D-glucans. Science 2006; 311:1940-42.
12. Doblin MS, Pettolino FA, Wilson SM, Campbell R, Burton RA, Fincher GB, et al. A barley cellulose synthase-like CSLH gene mediates (1,3;1,4)-beta-D-glucan synthesis in transgenic Arabidopsis. Proc Natl Acad Sci USA 2009; 106:5996-01.
13. Yin L, Verhertbruggen Y, Oikawa A, Manisseri C, Knierim B, Prak L, et al. The cooperative activities of CSLD2, CSLD3, and CSLD5 are required for normal Arabidopsis development. Mol Plant 2011; In press.
14. Bernal AJ, Jensen JK, Harholt J, Sorensen S, Moller I, Blaukopf C, et al. Disruption of ATCSLD5 results in reduced growth, reduced xylan and homogalacturonan synthase activity and altered xylan occurrence in Arabidopsis. Plant J 2007; 52:791-802.
15. Bernal AJ, Yoo CM, Mutwil M, Jensen JK, Hou G, Blaukopf C, et al. Functional analysis of the cellulose synthase-like genes CSLD1, CSLD2, and CSLD4 in tip-growing Arabidopsis cells. Plant Physiol 2008; 148:1238-53.
16. Handford MG, Baldwin TC, Goubet F, Prime TA, Miles J, Yu X, et al. Localisation and characterisation of cell wall mannan polysaccharides in *Arabidopsis thaliana*. Planta 2003; 218:27-36.
17. Marcus, S.E, Blake AW, Benians TAS, Lee KJD, Poyser C, Donaldson L, et al. Restricted access of proteins to mannan polysaccharides in intact plant cell walls. Plant J 2010; 64:191-203.
18. Goubet F, Misrahi A, Park SK, Zhang ZN, Twell D, Dupree P. AtCSLA7, a cellulose synthase-like putative glycosyltransferase, is important for pollen tube growth and embryogenesis in Arabidopsis. Plant Physiol 2003; 131:547-57.
19. Davis J, Brandizzi F, Liepman AH, Keegstra K. Arabidopsis mannan synthase CSLA9 and glucan synthase CSLC4 have opposite orientations in the Golgi membrane. Plant J 2010; 64:1028-37.
20. Zeng W, Jiang N, Nadella R, Killen TL, Nadella V, Faik A. A glucurono(arabino)xylan synthase complex from wheat contains members of the GT43, GT47, and GT75 families and functions cooperatively. Plant Physiol. 2010; 154:78-97.