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Xylem-Specific and Tension Stress–Responsive Expression of Cellulose Synthase Genes from Aspen Trees

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Abstract

Genetic improvement of cellulose biosynthesis in woody trees is one of the major goals of tree biotechnology research. Yet, progress in this field has been slow owing to (1) unavailability of key genes from tree genomes, (2) the inability to isolate active and intact cellulose synthase complexes and, (3) the limited understanding of the mechanistic processes involved in the wood cellulose development. Here I report on the recent advances in molecular genetics of cellulose synthases (CesA) from aspen trees. Two different types of cellulose synthases appear to be involved in cellulose deposition in primary and secondary walls in aspen xylem. The three distinct secondary CesAs from aspen—PtrCesA1, PtrCesA2, and PtrCesA3—appear to be aspen homologs of *Arabidopsis* secondary CesAs AtCesA8, AtCesA7, and AtCesA4, respectively, based on their high identity/similarity (>80%). These aspen CesA proteins share the transmembrane domain (TMD) structure that is typical of all known "true" CesA proteins: two TMDs toward the *N*-terminal and six TMDs toward the *C*-terminal. The putative catalytic domain is present between TMDs 2 and 3. All signature motifs of processive glycosyltransferases are also present in this catalytic domain. In a phylogenetic tree based on various predicted CesA proteins from *Arabidopsis* and aspen, aspen CesAs fall into families similar to those seen with *Arabidopsis* CesAs, suggesting their functional similarity. The coordinate expression of three aspen secondary CesAs in xylem and phloem fibers, along with their simultaneous tension stress–responsive upregulation, suggests that these three CesAs may play a pivotal role in biosynthesis of better–quality cellulose in secondary cell walls of plants. These results are likely to have a direct impact on genetic manipulation of trees in the future.

Index Entries: Aspen; cellulose biosynthesis; cellulose synthase; trees; wood development.

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Introduction

Understanding the biosynthesis of cellulose, a major macromolecule in forest trees, is essential for developing important strategies needed to produce genetically improved trees. Such an approach is also necessary to meet our current and future demands for cellulose-based wood and paper products. Wood is a major agricultural commodity of which cellulose and lignin impart strength and rigidity to timber products, but lignin needs to be removed from paper pulp to produce high-quality paper products. Therefore, our long-term goal is to develop genetically improved trees with balanced proportions of cellulose and lignin that will benefit global forest products industries. To achieve this goal, we have been acquiring basic knowledge about the underlying genetic mechanisms controlling cellulose, and lignin biosynthesis, which will assist us in building better trees in the future. Here, I will review the current state of knowledge regarding the molecular mechanism of cellulose biosynthesis with special emphasis on trees.

Cellulose is by far the most abundant organic material on Earth *(1)*. One would therefore ask, is it necessary to improve cellulose production in trees? However, augmentation of cellulose content and improvement of cellulose quality in specific tree tissues such as in wood fibers definitely holds a greater promise. Cellulose is a homopolymer of glucose that forms unbranched β-1,4-D-glucan chains of which every alternate glucose molecule is flipped by 180° *(2)*. It has been suggested that subsequent to synthesis, many single cellulose chains instantly associate to form microfibrils, and many such microfibrils come together to form macrofibrils in the secondary walls *(3)*. The number of glucan chains in each such bundle is known to vary from 36 to more than 1200 in plant cell walls *(2)*. In addition, the number of glucose residues per cellulose chain, or degree of polymerization (DP), is another trait that shows significant variations from about 500 to 2000 in primary cell walls to about 14,000 in secondary cell walls *(4)*. The cellulose content also varies between primary and secondary cell walls. The primary walls contain only 1–10% cellulose, to perfectly fulfill its function in expanding and growing plant cells, whereas cellulose content in secondary walls exceeds 50%, to provide rigidity and strength to plant cell walls. In nature, cellulose is naturally aggregated in highly ordered crystalline form alternating with less-ordered amorphous form. The crystalline form of cellulose has better tensile strength and wood cellulose is about 60–70% crystalline *(5)*. Thus, plant cellulose is a highly heterogeneous material.

Cellulose synthesis occurs on the plasma membrane of plant cells *(4)*. In freeze-fracture replicas from angiosperm tissues, discrete particle rosettes or terminal complexes (TC) coinciding with cellulose synthesis have been observed *(2)*. Kimura et al. *(6)* have recently used immunogold labeling of rosette terminal complexes to confirm that cellulose synthases (CesAs) are physically localized within these complexes. Since improve-

ments in secondary wall cellulose are economically more desirable and quantitative as well as qualitative variations in primary and secondary wall cellulose are postulated to be the result of different types of CesAs *(7)*, it is natural to search for such secondary CesAs. The first breakthrough in *CesA* gene cloning did not come from plants that make an abundant amount of cellulose, but from bacteria, such as *Acetobacter*, that produce extracellular cellulose. Active CesA complexes could be isolated from these bacteria, and the first *Acetobacter CesA* gene was cloned in the early 1990s *(8)*.

Through intensive search for conserved D, D, D . . . QXXRW signature that was proposed to be present in many processive glycosyltransferases such as bacterial *CesAs (9)*, Pear et al. *(10)* isolated the first cotton *CesA* cDNAs (GhCesAs) from a cDNA library prepared from cotton fibers that are highly active in secondary wall cellulose synthesis. Subsequently, with completion of *Arabidopsis* genome sequencing, at least 10 distinct *CesA* genes (*AtCesA-1* to *AtCesA-10*) with similarity to cotton *CesA* genes were identified *(11)*. Why so many CesAs are present in plant genomes is still an open area of question, but at least some members, such as *AtCesA-4*, *AtCesA-7*, and *AtCesA-8*, have been associated with secondary wall development *(12–14)*, indicating that different CesAs may indeed be involved in cell- or tissue-specific cellulose biosynthesis. Many reviews are available on these topics and interested readers may refer to them for further information *(1–3,11,15)*. Understanding the process of cellulose biosynthesis during wood development is one of the major goals of tree biotechnology research. Yet, the factors that control cellulose content and quality in wood cellulose are still unknown.

In response to tension stress, many angiosperm trees develop tension wood on the upper side of their leaning trunks and nonvertical branches *(5)*. A newly developed yet highly specialized gelatinous layer (G-layer) replaces one of the inner secondary walls (S2 or S3), filling the entire lumen of the tension wood fibers *(16)*. This thick G-layer, present in both xylem and phloem fibers, consists almost exclusively of cellulose (98.5%), has a small percentage of xylose (1.5%), and is devoid of lignin or pectin materials *(17,18)*. The G-layer cellulose is highly crystalline (>95%) and has a high degree of tensile strength associated with parallel orientation of microfibrils. This is the purest form of cellulose occurring in any woody tissues, and it rivals only with cotton fiber cellulose. Since cellulose-based forest product industries are vital to our economy and they mainly use wood as their raw materials (*see* Website: http://www.afandpa.org/news/COP_6_index.html), we are also interested in understanding the molecular mechanism of highly crystalline cellulose production during wood development of trees. Work has focused on quaking aspen (*Populus tremuloides*) trees for all the studies in which tension wood formation is well documented *(18)*. For the last 10 yr, researchers from our laboratory have successfully used quaking aspen as a model tree system for many molecular genetic explorations (*see* Website: http://forestry.mtu.edu/iwr/pbrc/),

and it is the most appropriate species for research in cellulose biosynthesis in trees.

As stated before, recent molecular genetic evidence from a variety of sources indicates that the CesA enzymes play an important role in cellulose biosynthesis in plants, and that at least two different types of CesAs are likely to be involved in cellulose biosynthesis of primary and secondary cell walls *(1,13,14)*. As compared with primary wall, secondary wall cellulose shows higher DP, higher crystallinity, and specialized orientation of microfibrils *(4,5)*, and these characteristics may be attributed to activities of secondary CesAs. To investigate the molecular basis of cellulose production during wood development as well as tension stress response in aspen trees, our group recently started experiments in cloning and characterization of *CesA* genes from aspen. We reported the first full-length cellulose synthase (*PtCesA*)(designated as *PtrCesA1* hereafter, as suggested by Delmer *[1]*) cDNA from developing xylem of aspen *(19)*. Northern blot and *in situ* hybridization analyses of *PtrCesA1* gene transcripts in various aspen tissues conclusively demonstrated that *PtrCesA1* expression is confined to developing xylem cells during normal stem development. Moreover, predicted PtrCesA1 protein showed a high degree of similarity (approx 90%) with other known secondary CesAs from developing cotton fibers (*GhCesA1*) and *Arabidopsis* xylem (AtCesA8 = irx1), indicating that PtrCesA1 may also play a role in secondary cell wall synthesis *(10,14)*. These observations were further confirmed by *PtrCesA1* gene promoter-β-glucuronidase (GUS) fusion analysis in transgenic tobacco plants that showed xylem-specific GUS expression in all plant tissues examined. During tension stress conditions, however, PtrCesA1 promoter-regulated GUS expression continued in xylem on the tension side and GUS expression was turned off in tissues undergoing compression on the opposite side of the bend. Our results suggested a unique role for *PtrCesA1* in cellulose biosynthesis in both tension-stressed and normal tissues in aspen *(19)*. Although transcriptional regulation of *PtrCesA1* gene in tension-stressed and normal xylem tissues was conclusively shown in our earlier work with transgenic tobacco, tobacco plants do not make tension wood in nature. We therefore transformed aspen tissues (which normally produce tension wood) with *PtrCesA1* promoter–GUS fusion. These transgenic aspen trees showed the same GUS expression profile, confirming that *PtrCesA1* gene is indeed xylem specific and tension stress–responsive in aspen background. Additional confirmatory data have been recently collected using an *in situ* mRNA hybridization technique in which gene-specific probe from *PtrCesA1* gene was shown to be confined to developing vascular tissues. Moreover, *PtrCesA1* gene expression was also observed to be upregulated on the tension-stressed side of aspen stem with simultaneous downregulation of *PtrCesA1* gene on the compressed side of the aspen stem (unpublished observations).

We recently cloned and characterized two more full-length CesA cDNAs from aspen xylem—*PtrCesA2* and *PtrCesA3*—that showed a high

Table 1
Percentage Identity/Similarity Among Various Secondary CesA Proteins from *Arabidopsis* and Aspen[a].

	AtCesA4	AtCesA7	AtCesA8	PtrCesA1	PtrCesA2	PtrCesA3
AtCesA4	—	69/77	69/76	69/76	69/78	**79/85**
AtCesA7		—	67/74	66/74	**87/92**	70/77
AtCesA8			—	**82/88**	67/75	68/76
PtrCesA1				—	64/74	68/75
PtrCesA2					—	69/77
PtrCesA3						—

[a]Homologous members from *Arabidopsis* and aspen are shown in bold.

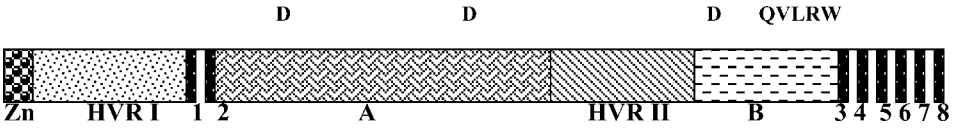

Fig. 1. Diagrammatic representation of PtrCesA proteins. Various domains are indicated as follows: Zn = zinc-binding domain; HVR I = *N*-terminal HVR; 1–8 = TMDs; subdomains A and B = highly conserved (70–90%) part of catalytic domains in relation to other CesA proteins; HVR II = central HVR. D, D, D, and QVLRW motifs are shown at the top.

degree of similarity to two other secondary CesAs from *Arabidopsis*—AtCesA7 (irx3) *(12)* and AtCesA4 *(13)*, respectively (unpublished observations) (GenBank accession nos. AY095297 and AF527387). *PtrCesA2* is 3280 bp long and contains an open reading frame of 3096 bp encoding a protein of 1032 amino acids. *PtrCesA3* is >3.4 kb long and encodes a protein of 1043 amino acids. A comparison among *Arabidopsis* secondary CesAs, AtCesA4, AtCesA7, and AtCesA8 with aspen PtrCesA1, PtrCesA2, and PtrCesA3 is provided in Table 1.

Based on these data it can be concluded that PtrCesA1, PtrCesA2, and PtrCesA3 are aspen homologs of *Arabidopsis* AtCesA8, AtCesA7, and AtCesA4, respectively. All three CesA proteins from aspen share the same transmembrane domain (TMD) structure that is typical of all known "true" CesA proteins *(15)*: two TMDs toward the *N*-terminal and six TMDs toward the *C*-terminal (Fig. 1). Moreover, the first hypervariable region, HVR I, resides in the *N*-terminal region immediately following the putative zinc-binding domain that is suggested to be involved in protein-protein interaction with other accessory proteins involved in cellulose biosynthesis *(20)*. The putative catalytic domain is present between TMD 2 and 3. All signature motifs of processive glycosyltransferases *(9)* are also present in this globular region, as shown in Fig. 1. The second hypervariable region, HVR II, which may have some specific function in regulating the quantity and quality of cellulose produced, further interrupts the catalytic domain into subdomains A and B.

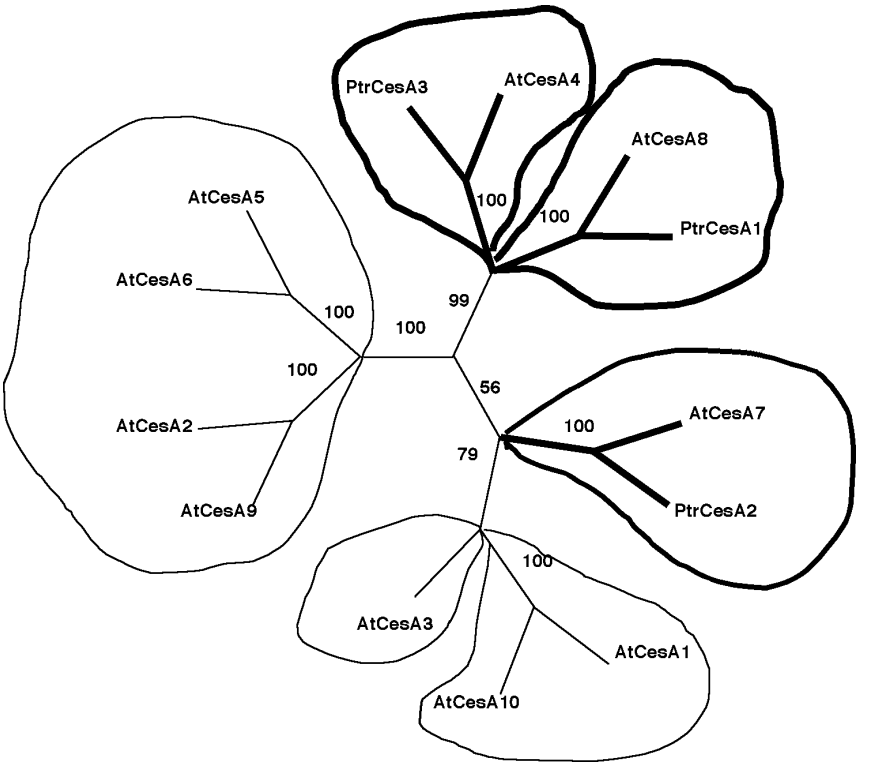

Fig. 2. Phylogenetic analysis of 13 full-length CesA proteins from *Arabidopsis* and aspen. DNA and protein sequence analysis was performed using various program routines from Genetics Computer Group (GCG) package version 10.2. Multiple sequence alignment of various CesA proteins was done with Pileup program from the GCG package. Cladograms were developed using PAUP 4.0b10 version (Phylogenetic Analysis Using Parsimony; Sinaur, Sunderland, MD) with parsimony analysis and heuristic search algorithm. Bootstrap analysis was done with 1000 replicates, and bootstrap values above 50 were considered for the development of unrooted tree. GenBank accession numbers (underlined) for *CesA* genes to deduce the protein sequence included in this figure are as follows: *AtCesA1*, AF027172; *AtCesA2*, AF027173; *AtCesA3*, AF027174; *AtCesA4*, AB006703; *AtCesA5*, AB016893; *AtCesA6*, AF062485; *AtCesA7*, AF088917; *AtCesA8*, AF267742; *AtCesA9*, AC007019; *AtCesA10*, AC006300; *PtrCesA1*, AF072131; *PtrCesA2*, unpublished; *PtrCesA3*, unpublished (the nomenclature used was proposed by Delmer *(1)* in which At = *Arabidopsis thaliana*; Ptr = *Populus tremuloides*). Bold lines indicate CesAs that are implicated in secondary cell-wall synthesis.

Since DNA sequence information regarding 10 full-length *CesA* genes from *Arabidopsis* and 3 three full-length *CesA* genes from aspen is currently available from which encoded proteins could be easily obtained by using *in silico* methods, we compiled a total of 13 deduced CesA proteins for the reconstruction of evolutionary events. The unrooted phylogenetic tree developed from these 13 sequences using 1000 bootstrap values is shown in Fig. 2. The most important observation is separation of the secondary cell wall–specific clade, as indicated by the thick delimiting lines. AtCesA7,

which is shown to be associated with secondary wall development in *Arabidopsis (12)*, now pairs with PtrCesA2, forming the first distinct branch of secondary CesAs. PtrCesA3 from aspen xylem groups with vascular tissue–specific *Arabidopsis* AtCesA4 *(13)* and forms the second branch of secondary CesAs. The third branch of the secondary clade consists of PtrCesA1 from aspen-developing xylem *(19)*, and AtCesA8 is associated with secondary wall development in *Arabidopsis (14)*. Thus, members of these groups seem to be associated with the secondary wall development in plants. The remaining three groups may be associated with primary cell wall development.

Another interesting conclusion can be drawn from this sequence analysis. Assuming that this phylogenetic tree reflects structural (and possibly functional) differences among various *CesA* gene family members, one can trace the possible minimum number of functional classes of *CesA* genes in plants. The 10 *Arabidopsis* CesAs actually form only six phylogenetic groups (group 1: AtCesA1 and AtCesA10; group 2: AtCesA3; group 3: AtCesA2, AtCesA5, AtCesA6, and AtCesA9; group 4: AtCesA8; group 5: AtCesA4; and group 6: AtCesA7). It must be acknowledged that other alternative interpretations of this phylogenetic tree are also possible. The three known aspen CesA genes cluster around the same secondary CesA groups (groups 4–6) among a total of six groups. Considering the hypothesis that presence of more than one CesA is involved cellulose synthesis in plants *(14,21)* and six subunits together make up a rosette complex where each subunit further contains six CesA proteins *(2)*, it is tempting to speculate that three primary CesA groups (groups 1–3) form functional rosettes in primary walls and three secondary CesA groups (groups 4–6) form rosettes in the secondary walls, as shown in Fig. 3. This would also explain why even a slight alteration in just one *CesA* gene could seriously impact the process of cellulose biosynthesis in *Arabidopsis* (*12,14,22)*.

In the near future, we would like to examine whether expression patterns of PtrCesA2 and PtrCesA3 genes are also xylem specific and tension stress–responsive in aspen similar to PtrCesA1. Taylor et al. *(14)* have recently shown that *AtCesA7* (irx3) and *AtCesA8* (irx1) coordinately express in xylem tissues of *Arabidopsis* and that they both may cooperatively control cellulose synthesis in secondary cell walls of xylem tissues. Both these genes have also been genetically proven to be involved in secondary cell-wall formation in *Arabidopsis (12,14)*. We would therefore like to explore if PtrCesA1 and PtrCesA2 proteins that show a high degree of identity/similarity to AtCesA7 and AtCesA8 proteins, respectively, are also coordinately expressed in xylem tissues in a tension stress–responsive manner. Furthermore, expression of the third secondary *CesA* gene from *Arabidopsis*, *AtCesA4* has recently been shown to be associated with vascular tissue development *(13)* and has a high degree of similarity to aspen PtrCesA3, as shown in Table 1. Although cooperation of this third group of CesA with the other two secondary CesA groups has not yet been previously suggested, we would like to explore if this third group of secondary CesA from

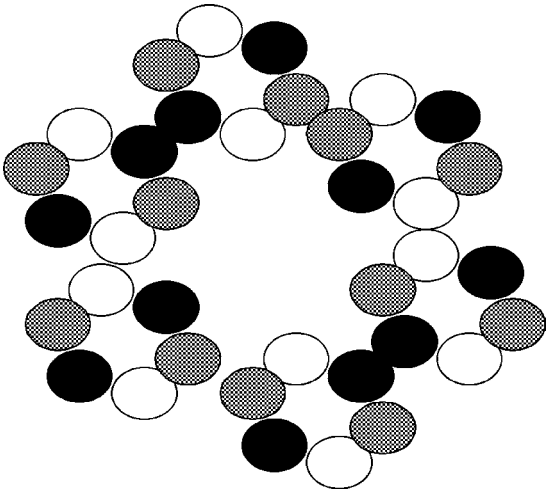

Fig. 3. Cross-sectional schematic diagram of rosette structures consisting of six subunits each made up of three types of CesAs shown as open circles, closed circles, and checkered circles. Both primary and secondary walls may have different groups of three CesA proteins.

aspen, PtrCesA3, is also xylem specific and tension stress–responsive. Preliminary data with *in situ* mRNA hybridization of gene-specific probes from PtrCesA2 and PtrCesA3 to young aspen stem tissues have suggested that both of these genes are also expressed in the xylem tissues similar to PtrCesA1 (unpublished observations).

More experiments with tension stress–responsive behavior of aspen stems are currently in progress. These investigations may help us to obtain a more accurate picture of molecular events occurring in normal xylem development as well as in response to tension stress in angiosperm trees. We hypothesize that secondary CesAs from aspen, PtrCesA1, PtrCesA2, and PtrCesA3 coordinately express at the transcriptional and translational levels in developing xylem tissues of aspen and work cooperatively to produce cellulose with high DP in secondary walls of normal xylem. In addition, we believe that they may also regulate the high crystallinity of cellulose during tension stress conditions through compensatory metabolic shifts between cellulose and lignin biosynthesis.

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