The Cellulose Synthase Gene Superfamily and Biochemical Functions of Xylem-Specific Cellulose Synthase-Like Genes in *Populus trichocarpa*[^W][^OA]

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Wood from forest trees modified for more cellulose or hemicelluloses could be a major feedstock for fuel ethanol. Xylan and glucomannan are the two major hemicelluloses in wood of angiosperms. However, little is known about the genes and gene products involved in the synthesis of these wood polysaccharides. Using *Populus trichocarpa* as a model angiosperm tree, we report here a systematic analysis in various tissues of the absolute transcript copy numbers of cellulose synthase superfamily genes, the cellulose synthase (*CesA*) and the hemicellulose-related cellulose synthase-like (*Csl*) genes. Candidate *Csl* genes were characterized for biochemical functions in Drosophila Schneider 2 (S2) cells. Of the 48 identified members, 37 were found expressed in various tissues. Seven *CesA* genes are xylem specific, suggesting gene networks for the synthesis of wood cellulose. Four *CesA* genes are xylem specific, three of which belong to the *CslA* subfamily. The more xylem-specific *CslA* subfamily is represented by three types of members: *PtCslA1*, *PtCslA3*, and *PtCslA5*. They share high sequence homology, but their recombinant proteins produced by the S2 cells exhibited distinct substrate specificity. *PtCslA5* had no catalytic activity with the substrates for xylan or glucomannan. *PtCslA1* and *PtCslA3* encoded mannan synthases, but *PtCslA1* further encoded a glucomannan synthase for the synthesis of (1→4)-β-D-glucomannan. The expression of *PtCslA1* is most highly xylem specific, suggesting a key role for it in the synthesis of wood glucomannan. The results may help guide further studies to learn about the regulation of cellulose and hemicellulose synthesis in wood.

Most of the biomass produced in trees is the secondary xylem, or wood. Cellulose and hemicelluloses represent almost the entire polysaccharide components in walls of the secondary xylem cells (Sarkanen and Hergert, 1971; Higuchi, 1997). Lignin is the third major wall component of these cells. Wood in angiosperm trees generally contains 42% to 50% cellulose, 25% to 30% hemicelluloses, 20% to 25% lignin, and 5% to 8% extractives (Fengel and Wegener, 1984). Xylan and glucomannan comprise approximately 85% and 15% of the total hemicellulose, respectively (Timell, 1969; Fengel and Wegener, 1979, 1984). This lignocellulosic pool is a major carbon sink in forest ecosystems and accounts for roughly 20% of the terrestrial carbon storage (Schlesinger and Lichter, 2001), offering an enormous, renewable polysaccharide feedstock for materials and biofuels (Ragauskas et al., 2006). Trees are target energy crops in the United States (Wooley et al., 1999; McAlon et al., 2000).

Being abundant in wood, cellulose, xylan, and glucomannan can be readily purified for structure characterization. Cellulose is a linear polymer composed of (1→4)-linked β-D-Glc residues. Xylan is a polymer with a linear backbone composed entirely of β-D-Xyl residues connected through (1→4)-linkages and is partially acetylated and substituted with 4-O-methyl-GlcUA groups (Perila, 1961; Timell, 1964, 1969; Fengel and Wegener, 1984; Jacobs et al., 2002). Xylan in angiosperm wood is therefore also referred to as O-acetyl-4-O-methylglucuronoxylan. Glucomannan in angiosperm wood is essentially a pure linear polymer containing (1→4)-linked β-D-Glc and β-D-Man residues, with Glc:Man ratios of 1:1 to 1:3 (Timell, 1986; Jacobs et al., 2002).

Kimura et al., 1999; Dhugga, 2001; Doblin et al., 2002). Among the 10 CesA genes identified in the Arabidopsis genome (Richmond and Somerville, 2000), AtCesA8, AtCesA7, and AtCesA4, corresponding to irx1, irx3, and irx5 mutants, are believed to coordinate cellulose biosynthesis in the secondary walls (Taylor et al., 1999, 2000, 2003; Gardiner et al., 2003). Biochemical functions of CesA genes have not been determined, nor has plant cellulose synthase activity been demonstrated (Doblin et al., 2002; Peng et al., 2002).

Hemicelluloses are believed to be synthesized in the Golgi, mediated most likely by cellulose synthase-like (Csl) proteins (Carpita and McCann, 2000). However, functions of Csl proteins are largely uncharacterized. CesA and Csl proteins belong to a cellulose synthase superfamily within the glycosyltransferase (GT) family 2 (Dhugga, 2001; Keegstra and Raikhel, 2001; Coutinho et al., 2003; Somerville et al., 2004). In Arabidopsis, there are at least six Csl gene subfamilies (A–G), containing 29 members (Richmond and Somerville, 2000; Hazen et al., 2002). The biosynthesis of cell wall hemicelluloses may involve some of these Csls for backbone elongation and other GTs for side-chain addition (Cutler and Somerville, 1997; Richmond and Somerville, 2000; Perrin et al., 2001; Hazen et al., 2002; Coutinho et al., 2003; Dhugga et al., 2004; Girke et al., 2004; Liepman et al., 2005). Only three such GT genes have demonstrated biochemical functions, all associated with the biosynthesis of Arabidopsis xyloglucan in the primary cell walls (Edwards et al., 1999; Perrin et al., 2001; Hazen et al., 2002; Coutinho et al., 2003). Dhugga et al. (2004) first showed in guar (Cyamopsis tetragonoloba) seeds a gene encoding a β-mannan synthase (ManS) activity capable of synthesizing the (1→4)-β-D-mannan backbone of galactomannan. The expression of this ManS (CManS) gene is closely associated with guar endosperm development where the accumulation of galactomannan takes place (Dhugga et al., 2004). CManS gene is in the CslA subfamily. Liepman et al. (2005) were the first to confirm the biochemical functions of Arabidopsis CslA genes (AtCslA2, AtCslA7, and AtCslA9) in Drosophila Schneider 2 (S2) cells and postulated that all plant CslA genes encode enzymes with ManS activity and that AtCslA9 may also encode a β-glucosidase activity (GlcManS). The tissue-specific expression of these AtCslA genes, however, was not reported. A recent genetic study showed evidence for the participation of arabidopsis (Orzca sativa), or more monocot-specific, CslF genes in the biosynthesis of cell wall (1→3;1→4)-β-D-glucans that are normally absent from dicot species (Burton et al., 2006).

Eighteen CesA genes were identified in the Populus trichocarpa genome (Djerbi et al., 2005), but Csl genes in this genome are poorly annotated. Only a handful of CesA and Csl genes from tree species were studied for their expression patterns (Wu et al., 2000; Samuga and Joshi, 2002; Liang and Joshi, 2004; Nairn and Haselkorn, 2005; Geisler-Lee et al., 2006; Ranik and Myburg, 2006). None of the tree Csl genes have been characterized for the biochemical functions to determine those involved in the synthesis of wood hemicelluloses. This is due mainly to the unavailability of efficient heterologous expression systems prior to the one developed by Liepman et al. (2005) and a lack of knowledge for identifying xylem- or wood-specific Csl genes for functional analysis. We carried out a systematic, genome-wide analysis of all the possible cellulose synthase superfamily members in P. trichocarpa for the phylogenetic relationship and for the quantitative transcript abundance in various tissues. These analyses led to the identification of xylem-specific CslA gene members, whose recombinant proteins were produced in Drosophila S2 cells. One member having the most conspicuous xylem specificity encoded a GlcManS for the synthesis of (1→4)-β-D-glucosidase.

RESULTS
Cellulose Synthase Gene Superfamily in the P. trichocarpa Genome

We identified in the P. trichocarpa genome database (http://www.jgi.doe.gov/poplar/) 48 gene models encoding transmembrane and D,D,D,QxxRW (Saxena et al., 1995; Campbell et al., 1997; Saxena and Brown, 1997) domains containing full protein sequences that are homologous to the Arabidopsis CesA and Csl proteins. Sequence analysis using the BLASTX program (Altschul et al., 1997) suggested that, of these 48 genes, 18 could be classified as CesA genes and were denoted as PtCesA (Supplemental Table S1). The number of PtCesA genes is consistent with that recently reported (Djerbi et al., 2005). The 18 PtCesA genes share 54% to 100% protein sequence homology with each other (Supplemental Table S2). Phylogenetically, they form nine groups, eight of which contain a pair of CesA genes with a nearly identical sequence (Fig. 1; Supplemental Table S2). In Arabidopsis, there are at least 10 CesA genes, which are widely recognized as AtCesA1 to 10 (http://cellwall.stanford.edu; Delmer, 1999; Richmond and Somerville, 2000; Aspeborg et al., 2005). The 18 PtCesA genes share 55% to 88% protein sequence homology with the 10 AtCesA genes (Supplemental Table S2). To be consistent with the currently accepted numbering system for the Arabidopsis CesA genes, the 18 PtCesA genes were named PtCesA1 to 18, with PtCesA1 to 10 being the most closely related homologs of AtCesA1 to 10, respectively (Supplemental Fig. S1). PtCesA11 and PtCesA1 have an identical sequence, and PtCesA12 to 18 are the other homologs of AtCesA1 to 10 (Fig. 1; Supplemental Fig. S1). For P. trichocarpa, the eight PtCesA pairs are: PtCesA11/PtCesA10, 2/5, 3/13, 6/9, 7/17, 8/18, 12/14, and 15/16 (Fig. 1). PtCesA4 is unique and not paired (Fig. 1; Djerbi et al., 2005) and shares approximately 65% protein sequence homology with all the other PtCesAs (Supplemental Table S2). It shares, however, 81%
protein sequence homology with the Arabidopsis
*AtCesA4*. *P. trichocarpa* and Arabidopsis *CesA* genes can
be classified into seven clades (Supplemental Fig. S1).
With the exception of clade VI, which does not contain
the *AtCesA* gene, the other six include *CesA* genes from
both species.

The remaining 30 of the 48 identified genes belong to
the *Csl* gene families and are denoted as *PtCsls* (Supplemental Table S1). They could be classified into
*PtCslA, B, C, D, E,* and *G* subfamilies (Fig. 1) according
to their protein sequence homology with the 29 known
*AtCsl* members that define these subfamilies (http://
cellwall.stanford.edu; Richmond and Somerville,
2000). *CslF, H,* and *J* subfamilies, which are believed
to be monocot-related *Csls* (Burton et al., 2006; Farrokhi
et al., 2006), are absent from the *P. trichocarpa* and
Arabidopsis genomes. Although these two genomes
share the same, more dicot-specific *Csl* subfamilies, the
numbers of the members in many subfamilies differ
between these two species. The *P. trichocarpa* genome
has fewer *CslA, CslB,* and *CslC* members but more
*CslD* members than does the Arabidopsis genome. We
also found 15 additional *P. trichocarpa* gene models that
have partial coding sequences with varying degrees of
homology with those of the Arabidopsis *CslC, D,* and
*G* subfamily genes (Supplemental Table S1). These
potential *Csl* genes are, however, not considered as the
*Csl* members in this study because of a lack of the full
coding sequences in the current gene models. The
identities of these 15 genes need to be verified.

Expression Profiling of *P. trichocarpa CesA* and *Csl* Genes

To identify xylem-related *PtCesA* and *PtCsl* genes, we
profiled the expression of all possible cellulose
synthase superfamily genes in various tissues of
*P. trichocarpa* by quantitative real-time PCR. To ensure
the transcript amplification is specific, each set of PCR
primers was designed so that their sequences would
match perfectly with the target sequence but differ in
at least three nucleotides from the sequences of all the
other superfamily members (Supplemental Table S3).
The amplification specificity was further validated
based on the generation of a single PCR product
from each set of primers and that distinct dissociation
curves were derived from the paired *PtCesAs*. We also
used pure plasmid DNAs from seven *PtCesA* and
*PtCsl* cDNA clones (see "Materials and Methods" for
details) as the transcript expression standards in tis-
sues examined. Together, these allowed us to deter-
mine the absolute transcript abundance quantified
based on the transcript copy numbers for each mem-
ber in a unit weight of the total RNA. Thus, our
approach enabled comparison of the expression of all
the superfamily members in different tissues. The tissues studied here were all from trees under normal growth in a greenhouse.

Of the 48 identified cellulose synthase superfamily genes, 37 were found expressed in the tested tissues (Table I), having transcript copy numbers ranging from approximately $10^3$ to $10^7$ copies total RNA. For CesA genes, PtCesA13 and PtCesA18 were found most highly expressed in the developing xylem, with numbers of transcript molecules being up to approximately $10^4$ times that in the other tested tissues. PtCesA18 is most homologous to a *Populus tremuloides* CesA characterized previously as a xylem-specific cellulose synthase gene (Wu et al., 2000). PtCesA4, 5, 7, 8, and 17 also are clearly xylem specific, even though their transcript abundances in xylem and in the other tested tissues were lower than PtCesA13 and PtCesA18 (Table I). Other than these seven xylem-specific CesA genes, the rest of the PtCesA genes (a total of eight) having detectable transcript molecules did not exhibit clear tissue specificity. Transcripts of *PtCesA3*, *PtCesA12*, and *PtCesA15* were either undetectable or essentially absent in the tested tissues. It is possible that these genes may be expressed only in a tissue- or cell-specific manner or under specific growth conditions. Among all the tested tissues, leaves in general had the lowest transcript level for all of the detectable PtCesA genes, consistent with the presence of only a small amount of the cellulose-enriched vascular systems in leaves.

The transcripts of 21 of the 30 PtCsl genes were detected in the tissues examined (Table I). Overall, the transcript abundance of Csl genes is lower than that of CesA genes, as observed previously for the Arabidopsis Csl genes (Hamann et al., 2004). PtCslA1, A2, A5, and D6 showed a strong preferential expression in the developing xylem, while PtCslC1 and C4 displayed a shoot-tip specificity. No tissue specificity was evident for the remaining 15 detected PtCsl genes (Table I) or

### Table I. Transcript copy numbers (× 10⁶/mg total RNA) of cellulose synthase superfamily gene members in various *P. trichocarpa* tissues

Values are means ± se of at least three independent PCR runs (see “Materials and Methods” for detailed RNA isolation and PCR primer design and reactions).

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Leaf</th>
<th>Shoot Tip</th>
<th>Phloem</th>
<th>Xylem</th>
</tr>
</thead>
<tbody>
<tr>
<td>PtCesA1/PtCesA11</td>
<td>67.1 ± 0.49</td>
<td>307.1 ± 9.29</td>
<td>155.0 ± 7.01</td>
<td>61.9 ± 0.27</td>
</tr>
<tr>
<td>PtCesA2</td>
<td>7.8 ± 1.41</td>
<td>42.9 ± 2.92</td>
<td>57.6 ± 0.93</td>
<td>36.5 ± 1.65</td>
</tr>
<tr>
<td>PtCesA4</td>
<td>1.1 ± 0.12</td>
<td>9.52 ± 1.17</td>
<td>7.5 ± 1.20</td>
<td>33.5 ± 1.38</td>
</tr>
<tr>
<td>PtCesA5</td>
<td>19.7 ± 2.01</td>
<td>82.3 ± 4.46</td>
<td>56.6 ± 0.89</td>
<td>148.1 ± 8.08</td>
</tr>
<tr>
<td>PtCesA6</td>
<td>6.7 ± 0.74</td>
<td>43.9 ± 2.16</td>
<td>16.4 ± 2.20</td>
<td>25.0 ± 1.59</td>
</tr>
<tr>
<td>PtCesA7</td>
<td>3.9 ± 0.24</td>
<td>14.6 ± 1.21</td>
<td>16.4 ± 0.17</td>
<td>249.0 ± 33.7</td>
</tr>
<tr>
<td>PtCesA8</td>
<td>2.8 ± 0.94</td>
<td>22.7 ± 3.83</td>
<td>18.3 ± 0.69</td>
<td>378.0 ± 20.86</td>
</tr>
<tr>
<td>PtCesA9</td>
<td>87.4 ± 1.10</td>
<td>237.0 ± 19.21</td>
<td>169.1 ± 26.30</td>
<td>231.1 ± 19.91</td>
</tr>
<tr>
<td>PtCesA10</td>
<td>82.4 ± 10.81</td>
<td>351.1 ± 20.31</td>
<td>123.1 ± 5.45</td>
<td>577.1 ± 40.11</td>
</tr>
<tr>
<td>PtCesA12</td>
<td>1.3 ± 0.11</td>
<td>3.3 ± 0.32</td>
<td>3.3 ± 0.85</td>
<td>11.1 ± 0.92</td>
</tr>
<tr>
<td>PtCesA13</td>
<td>41.8 ± 3.20</td>
<td>133.0 ± 2.43</td>
<td>73.6 ± 2.85</td>
<td>1080.1 ± 323.1</td>
</tr>
<tr>
<td>PtCesA14</td>
<td>53.6 ± 14.51</td>
<td>47.4 ± 1.96</td>
<td>27.9 ± 0.55</td>
<td>60.0 ± 19.9</td>
</tr>
<tr>
<td>PtCesA16</td>
<td>31.1 ± 2.05</td>
<td>80.3 ± 3.21</td>
<td>53.4 ± 0.57</td>
<td>27.9 ± 5.27</td>
</tr>
<tr>
<td>PtCesA17</td>
<td>1.7 ± 0.44</td>
<td>5.17 ± 0.55</td>
<td>5.9 ± 0.38</td>
<td>115.0 ± 8.06</td>
</tr>
<tr>
<td>PtCesA18</td>
<td>7.8 ± 0.84</td>
<td>56.2 ± 10.51</td>
<td>38.9 ± 1.04</td>
<td>1090.0 ± 347.0</td>
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<tr>
<td>PtGslA1</td>
<td>17.7 ± 5.35</td>
<td>12.1 ± 0.31</td>
<td>8.32 ± 1.87</td>
<td>196.1 ± 4.02</td>
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<tr>
<td>PtGslA2</td>
<td>4.06 ± 0.59</td>
<td>18.0 ± 2.12</td>
<td>6.0 ± 1.32</td>
<td>190.1 ± 37.81</td>
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<tr>
<td>PtGslA3</td>
<td>10.0 ± 0.85</td>
<td>28.2 ± 3.88</td>
<td>13.0 ± 0.80</td>
<td>28.5 ± 14.01</td>
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<tr>
<td>PtGslA5</td>
<td>60.9 ± 11.61</td>
<td>179.0 ± 16.21</td>
<td>94.2 ± 8.99</td>
<td>331.1 ± 37.40</td>
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<tr>
<td>PtGslC1</td>
<td>16.2 ± 2.97</td>
<td>102.1 ± 10.01</td>
<td>21.0 ± 1.28</td>
<td>20.3 ± 4.19</td>
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<tr>
<td>PtGslC2</td>
<td>13.9 ± 0.35</td>
<td>24.2 ± 1.48</td>
<td>8.9 ± 0.09</td>
<td>21.7 ± 7.54</td>
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<tr>
<td>PtGslC3</td>
<td>10.8 ± 0.42</td>
<td>18.5 ± 1.19</td>
<td>7.49 ± 1.65</td>
<td>3.18 ± 0.68</td>
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<tr>
<td>PtGslC4</td>
<td>9.8 ± 0.94</td>
<td>42.4 ± 5.86</td>
<td>9.67 ± 1.36</td>
<td>13.4 ± 3.02</td>
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<tr>
<td>PtGslC5</td>
<td>8.8 ± 0.63</td>
<td>16.9 ± 4.19</td>
<td>8.2 ± 1.23</td>
<td>14.0 ± 3.07</td>
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<tr>
<td>PtGslD1</td>
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<td>2.7 ± 0.58</td>
<td>3.1 ± 0.96</td>
<td>1.41 ± 0.29</td>
</tr>
<tr>
<td>PtGslD2</td>
<td>4.5 ± 0.50</td>
<td>5.9 ± 0.41</td>
<td>7.2 ± 0.16</td>
<td>15.7 ± 1.20</td>
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<tr>
<td>PtGslD4</td>
<td>2.1 ± 0.11</td>
<td>4.6 ± 0.70</td>
<td>2.7 ± 0.17</td>
<td>1.5 ± 0.47</td>
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<tr>
<td>PtGslD6</td>
<td>14.6 ± 1.54</td>
<td>60.9 ± 0.82</td>
<td>41.7 ± 0.82</td>
<td>177.1 ± 4.36</td>
</tr>
<tr>
<td>PtGslD8</td>
<td>10.1 ± 2.15</td>
<td>4.63 ± 1.43</td>
<td>2.25 ± 0.31</td>
<td>0.69 ± 0.39</td>
</tr>
<tr>
<td>PtGslD9</td>
<td>2.9 ± 0.91</td>
<td>2.8 ± 0.28</td>
<td>1.3 ± 0.13</td>
<td>0.2 ± 0.09</td>
</tr>
<tr>
<td>PtGslD10</td>
<td>7.4 ± 0.86</td>
<td>7.9 ± 0.66</td>
<td>1.7 ± 0.19</td>
<td>2.9 ± 0.39</td>
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<td>PtGslE1</td>
<td>4.6 ± 0.03</td>
<td>2.9 ± 0.50</td>
<td>0.7 ± 0.03</td>
<td>1.9 ± 0.84</td>
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<td>PtGslE2</td>
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<td>0.4 ± 0.03</td>
<td>3.9 ± 0.35</td>
<td>1.2 ± 0.12</td>
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<td>PtGslE3</td>
<td>9.1 ± 0.19</td>
<td>7.2 ± 1.38</td>
<td>0.3 ± 0.08</td>
<td>1.8 ± 0.93</td>
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<td>PtGslG4</td>
<td>9.3 ± 3.30</td>
<td>5.9 ± 0.33</td>
<td>3.8 ± 0.74</td>
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<td>PtGslG5</td>
<td>4.8 ± 0.42</td>
<td>9.6 ± 0.31</td>
<td>8.2 ± 0.24</td>
<td>0.9 ± 0.41</td>
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</table>
for the potential PtCsl genes represented by the 15 short gene models (Supplemental Table S4).

Enzymatic Activities of Plant Protein Extracts for the Synthesis of Hemicelluloses

The more xylem-specific expression of several PtCesA and PtCsl member genes may suggest their involvement in the biosynthesis of wood cellulose and hemicelluloses. Because the lack of a reliable method for determining the biochemical activities of cellulose synthase gene products (Doblin et al., 2002; Peng et al., 2002), we focused on the biochemical functions of PtCsl genes. We first investigated the enzymatic activities for the biosynthesis of the hemicellulose backbones in tissues where the expression of PtCsls has been quantified (Table I). GDP-[U-14C]Man, GDP-[U-14C]Glc, and UDP-[U-14C]Xyl, which supply the possible backbone monomers of major wood hemicelluloses, were used as the substrates. Total crude protein was extracted from the young shoot, leaf, developing xylem, and phloem tissues (see “Materials and Methods” for details). Each crude extract preparation was separated into soluble and microsomal fractions, which were analyzed separately for the catalytic activities with these three substrates. The incorporation of radioactivity into the 70% ethanol-insoluble polymeric product was quantified as the enzyme activity. As expected, enzyme activities could not be detected in soluble protein preparations. In contrast, microsomal fractions from the developing xylem displayed strong activities with these three substrates, with UDP-[U-14C]Xyl and GDP-[U-14C]Man being the better substrates than GDP-[U-14C]Glc (Fig. 2). These activities in the microsomes from the other tested tissues were low or negligible (Fig. 2). These results verified the presence in the developing xylem of enzymes converting these monosaccharides into polymers, possibly xylan and glucomannan. These wood hemicellulose activities are likely encoded by the xylem-specific PtCsl genes. We then focused on the PtCslA family, which as a whole has the most conspicuous xylem specificity (Table I). CslA family members were shown to encode ManS or GlcManS activities (Dhugga et al., 2004; Liepman et al., 2005).

Cloning of PtCslA Genes and Expression in Drosophila S2 Cells

There are five members in the PtCslA subfamily, PtCslA1 to 5. We conducted a phylogenetic analysis of CslA members from P. trichocarpa, Arabidopsis, and rice, and of a CslA, CtManS, from guar (Dhugga et al., 2004). Except for PtCslA3 and AtCslA9, which are grouped together, the other CslA members are grouped in a more species-specific manner (Fig. 3). This suggests that plant CslA members may exhibit species-specific biochemical functions. The five PtCslA members are divided into three subgroups, with PtCslA1 and PtCslA2 (94% sequence homology) being in one subgroup and PtCslA4 and PtCslA5 (93% homology) in another (Fig. 1). We selected PtCslA1, PtCslA3, and PtCslA5, one member from each of the three subgroups in the PtCslA subfamily, for cloning and functional analysis. Based on the predicted transcript sequences, we designed PCR primers to amplify a P. trichocarpa xylem cDNA library and identified several full and partial PtCslA cDNA coding sequences. Further cloning resulted in the isolation of three full coding sequences, which matched with the predicted transcripts of PtCslA1, PtCslA3, and PtCslA5 genes encoding predicted protein sequences of 522, 533, and 538 amino acids. These protein sequences all contain the conserved motifs characteristic of the processive GTs (Saxena et al., 1995, 2001). These proteins share 71% to 79% sequence homology with each other, 66% to 71% with CtManS (Dhugga et al., 2004), and 71% to 83% with the Arabidopsis AtCslA9, a ManS (Liepman et al., 2005). Using gene-specific sequences in the 5′-untranslated region (UTR) as probes, we carried out northern-blot analysis of the transcript levels of these three PtCslA genes in various P. trichocarpa tissues and confirmed a keen xylem specificity for PtCslA1 (Fig. 4). The expression of PtCslA3 and PtCslA5 in xylem could not be detected on a northern blot but was conspicuous for PtCslA5 in the other vascular system-containing tissues examined. Overall, these northern results are consistent with the transcript copy numbers determined by the real-time PCR (Table I).

To characterize the biochemical function of PtCslA1, PtCslA3, and PtCslA5 genes, we constructed the V5-tagged open reading frame (ORF) transgene for each of these genes and a LacZ as control and expressed them in Drosophila S2 cells using a pCoBlast vector system (Liepman et al., 2005). After these cells
were transfected with the expression construct, the stably transformed cell lines were selected and induced by copper sulfate for recombinant protein production. Contents from the whole cell lysates, supernatant after ultracentrifugation, and microsomal fraction were analyzed by western blotting using anti-V5-HRP antibodies as the probe. As shown in Figure 5, the recombinant proteins from all three PtCslA genes were expressed in the microsomal fraction. The V5-tagged PtCslA fusion proteins migrated on the protein gel more rapidly than expected, which also was observed for Arabidopsis Csl fusion proteins produced in the S2 cells (Liepman et al., 2005).

**Catalytic Activities of the Recombinant Proteins**

Microsomal preparations from the S2 cells that produce the three PtCslA and the LacZ recombinant proteins were tested for the catalytic activity with GDP-[U-14C]Man, GDP-[U-14C]Glc, UDP-[U-14C]Xyl, or the mixture of GDP-Man and GDP-Glc with one being U-14C-labeled at the monosaccharide. The concentration and radioactivity for each individual nucleotide sugar used were the same in either the single or mixed substrate cases (Fig. 6). None of these PtCslA recombinant proteins could mediate the conversion of UDP-[U-14C]Xyl. PtCslA1 exhibited a strong activity converting GDP-[U-14C]Man into 70% ethanol-insoluble, radioactive polymeric products, suggesting a possible ManS function for PtCslA1. PtCslA3 recombinant protein also had such activity. Having only a background activity with GDP-[U-14C]Man as the control LacZ protein, PtCslA5 may not be a ManS.

PtCslA1 also utilized GDP-[U-14C]Glc but preferred GDP-[U-14C]Man. PtCslA3 and PtCslA5 had essentially no such activity. Furthermore, PtCslA1 was the only one of these three CslAs exhibiting conspicuous activity capable of converting GDP-[U-14C]Man and GDP-Glc into an ethanol-insoluble polymeric product. This product is likely a [U-14C]Man-labeled gluco-mannan whose radioactivity was diluted, as compared to the radioactivity in the product from GDP-[U-14C]Man alone, due the incorporation of the nonradioactive Glc residues. Similarly, a [U-14C]Glc-labeled gluco-mannan was the probable product of PtCslA1-mediated conversion of a mixture of GDP-Man and GDP-[U-14C]Glc. These results are consistent with those of the reactions mediated by the Arabidopsis
Gene expression in various *P. trichocarpa* tissues. Total RNA samples were isolated from six tissues as shown. RNA loading levels are indicated by the 18S rRNA transcript signals stained with ethidium bromide. The gene-specific fragments were identified and used as the hybridization probes. PtCslA1 is expressed specifically in the developing xylem.

AtCslA9 recombinant protein produced by the S2 cells (Liepman et al., 2005). Thus, PtCslA1 also is likely a GlcManS.

Analysis of the PtCslA1-Mediated in Vitro Reaction Products

To further verify the recombinant protein functions, we isolated the in vitro enzyme reaction products and analyzed their composition and linkage types. We focused on the PtCslA1-mediated products from GDP-Man and from mixtures of GDP-Man and GDP-Glc. In vitro polymers produced from the PtCslA1 reaction with GDP-[U-14C]Man were hydrolyzed by trifluoroacetic acid, and the products were added with a mixture of six wood monosaccharides as the elution carrier and separated by HPAEC-PAD HPLC (Wright and Wallis, 1995) and the elution fractions were counted for radioactivity. Only the Man eluents were radioactive (data not shown). This indicates that PtCslA1 is likely a ManS converting Man from GDP-Man into a pure mannan and that the protein preparations from the S2 cells do not epimerize GDP-[U-14C]Man into GDP-[U-14C]Glc. Similarly, analysis of the in vitro polymers from the mixed substrates confirmed the isolation of only radioactive Man from polymers derived from the mixed GDP-[U-14C]Man and GDP-Glc and of exclusively radioactive Glc from the mixed GDP-Man and GDP-[U-14C]Glc substrate pool (data not shown). Thus, PtCslA1 may also possess a GlcManS activity for polymerizing GDP-Man and GDP-Glc into, most likely, a glucomannan.

The in vitro polymers were then hydrolyzed by linkage-specific glycanases: endo-β-D-(1→4)-mannanase, endo-β-D-(1→4)-glucanase (cellulase), and endo-β-D-(1→3)-glucanase (Fig. 7). In the absence of these hydrolytic enzymes (buffer in Fig. 7), essentially no radioactivity could be released into the 70% ethanol solution from the radioactive in vitro polymers. The radioactive polymer from GDP-[U-14C]Man could be effectively digested by endo-β-D-(1→4)-mannanase, releasing over 80% of its radioactivity into solution as mostly monomeric Man units (Wright and Wallis, 1995). The remaining radioactivity was quantitatively retained in the undigested portion of the polymer. However, this GDP-[U-14C]Man-derived polymer could not be hydrolyzed by either endo-β-D-(1→4)- or (1→3)-glucanases, as essentially no radioactivity could be detected in the solution (Fig. 7). The composition and linkage analyses, therefore, point to an in vitro ManS function for PtCslA1 mediating the conversion of GDP-Man into a pure (1→4)-β-D-mannan. The polymer from the mixed GDP-[U-14C]Man and GDP-Glc substrates could also be hydrolyzed by endo-β-D-(1→4)-mannanase with a high efficiency as revealed by the level of the radioactivity in solution (Fig. 7). When this polymer was digested with endo-β-D-(1→4)-glucanase, however, the solution radioactivity decreased drastically, indicative of the polymeric substrate being a [14C]Man-containing glucomannan having strictly β-D-(1→4)-linked Glc residues. Indeed, this polymer could not be digested by endo-β-D-(1→3)-glucanase. The polymer from the substrate pool of GDP-Man and GDP-[U-14C]Glc also responded positively to endo-β-D-(1→4)-mannanase digestion, resulting in a significant release of the radioactivity. Most likely, the radioactivity was derived from the [14C]Glc units that were originally surrounded by Man residues and released after mannanase-mediated hydrolysis of these Man residues. These results also suggest that the oligomeric Glc (or glucan) residues in this polymer are probably infrequent. This is supported by

![Image](84x571 to 251x711)

**Figure 4.** Northern-blotting analysis of PtCslA1, PtCslA3, and PtCslA5 gene expression in various *P. trichocarpa* tissues. Total RNA samples were isolated from six tissues as shown. RNA loading levels are indicated by the 18S rRNA transcript signals stained with ethidium bromide. The gene-specific fragments were identified and used as the hybridization probes. PtCslA1 is expressed specifically in the developing xylem.

![Image](323x166 to 538x316)

**Figure 5.** Western-blotting analysis of PtCslA1, PtCslA3, and PtCslA5 recombinant proteins expressed in Drosophila S2 cells. Protein samples (50 μg) from the whole lysates (1), supernatant after ultracentrifugation (2), and microsomal fraction (3) from the S2 cells were separated on SDS-PAGE. LacZ was used as a control. The sizes of the protein markers (M) are indicated. The signals were detected with the antibodies recognizing the V5-tag fused with the recombinant proteins. The expressed PtCslA1, PtCslA3, and PtCslA5 recombinant proteins (marked with arrowheads) were localized in the microsomal fraction (lane 3 in each protein group).
activities with the substrates tested. PtCslA5 had essentially no catalytic can, and glucomannan synthase activities, while PtCslA3 exhibited three independent experiments. None of the recombinant proteins

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Figure 6. Enzymatic activities of the recombinant proteins from Droso phila S2 cells for the synthesis of hemicelluloses. The microsomal fraction (200–300 μg proteins) of the S2 cells expressing PtCslA1, PtCslA3, PtCslA5, or LacZ protein was incubated with 2 mM GDP-[U-14C]Glc (7.7145 Bq/nmol), 2 mM UDP-[U-14C]Xyl (9.25 Bq/nmol), 2 mM GDP-[U-14C]Man (7.722 Bq/nmol) + 2 mM GDP-Glc, or 2 mM GDP-MAN + 2 mM GDP- [U-14C]Glc. The 70% ethanol-insoluble polymeric product was isolated and counted for the radioactivity for calculating the enzymatic activity as indicated. Values are means ± SDs of at least three independent experiments. None of the recombinant proteins could utilize UDP-Xyl. However, PtCslA1 had putative mannan, glu can, and glucomannan synthase activities, while PtCslA3 exhibited mainly mannan synthase activity. PtCslA5 had essentially no catalytic activities with the substrates tested.

DISCUSSION

Transcript Abundance Profiling of PtCesA and PtCsl Genes Identified Xylem-Specific Members in P. trichocarpa

We report here a systematic analysis of all the possible cellulose synthase superfamily member genes in a tree species. The identified PtCesA genes form several phylogenetic pairs (Fig. 1), an observation being suggested as a result of gene duplication during Populus evolution (Djerbi et al., 2005). However, most of the paired PtCesA genes, which presumably have a similar function, have distinguishable tissue expression patterns (Table I). These pairs include PtCesA1(11)/PtCesA10, 2/5, 6/9, 12/14, and 15/16. For example, for the PtCesA1(11)/10 pair, PtCesA1(11) is more shoot-tip specific, whereas PtCesA10 has a higher xylem specificity. While PtCesA2 lacks tissue specificity, its paired homolog, PtCesA5, is apparently more xylem specific. These results may imply a more tissue- or cell type-specific division of function for the CesA genes following their duplication in the P. trichocarpa genome.

In contrast to the paired PtCesA as having diverged expression patterns, the PtCesAs of the 7/17 pair have well-matched expression patterns, as do those of the 8/18 pair (Table I). They are all clearly xylem specific. Notably, PtCesA4 (another xylem-specific CesA), the PtCesA7/17 pair, and the PtCesA8/18 pair are the homologs of Arabidopsis AtCesA4, 7, and 8, respectively. The gene products of these three AtCesA genes are known as the set of the three cellulose synthases required for the biosynthesis of cellulose in the secondary cell walls (Taylor et al., 1999, 2000, 2003). The redundant xylem-specific expression of PtCesA7 and PtCesA17 and of PtCesA8 and PtCesA18 is consistent with the massive production of cellulose in xylem secondary cell walls for wood formation. However, there is another xylem-specific CesA, the PtCesA13. It is one of the two PtCesA genes having the highest transcript level in the developing xylem (Table I). Perhaps more than three CesAs or a set of CesAs that is different from Arabidopsis are required for the biosynthesis of cellulose in xylem cell walls of P. trichocarpa, or of trees in general. Interestingly, while PtCesA13 has a high transcript abundance in xylem, the transcripts of its paired homolog, PtCesA3, were undetectable in all tested tissues. Because all these transcripts were

the endo-β-D-(1→4)-glucanase digestion of the polymer that resulted in the release of a low level of radioactivity (Fig. 7). The Glc linkages in this polymer are not the β-D-(1→3) type, as the polymer remained intact after reaction with endo-β-D-(1→3)-glucanase. These polymer linkage analyses further support the composition characterization that the PtCslA1 recombinant protein has a GlcManS activity capable of polymerizing Man from GDP-Man into GDP-Glc into a (1→4)-β-D-glucomannan.

Figure 7. Analysis of the PtCesA1 recombinant protein-mediated in vitro reaction products with linkage-specific glycans. The 70% ethanol-insoluble polymeric product from PtCesA1-mediated reaction with GDP-[U-14C]Man, GDP-[U-14C]Man + GDP-Glc, or GDP-Man + GDP-[U-14C]Glc was isolated. Each type of reaction product was hydrolyzed by endo-β-D-(1→4)-mannanase (shown as mannanase), endo-β-D-(1→4)-glucanase (cellulase), and endo-β-D-(1→3)-gluc anase (1,3-glucanase), respectively. Buffer without any hydrolitic enzyme was used in the control reaction. Values of the 14C radioactivity released from the hydrolysis of the in vitro product as percentage of the original radioactivity incorporated in the in vitro polymer are shown. Values are means ± SDs of at least three independent experiments. The analyses confirmed that the PtCslA1 recombinant protein had a mannan synthase activity capable of polymerizing Man from GDP-Man into a (1→4)-β-D-mannan and a glucomannan synthase activity for polymerizing Man from GDP-Man and Glc from GDP-Glc into a (1→4)-β-D-glucomannan.
analyzed for tissues under normal growth, it is unknown whether the expression of those PtCesA genes with undetectable or low transcripts is only inducible under specific growth conditions (Wu et al., 2000). Our transcript analyses provide evidence for growth- or development-regulated transcriptional control of cellulose synthesis in *P. trichocarpa*. The quantitative transcript abundances may help guide further studies to learn about this control.

The transcript levels of a majority of the PtCsl genes in the tissues examined were too low to be conclusive about the expression patterns (Table I). The transcripts of only five Csl genes, PtCslA1, PtCslA2, PtCslA5, PtCslD1, and PtCslD6, could be detected at significant levels. In this study, we focused on the PtCslA family; the function of PtCslD6, the only xylem-specific PtCsl gene outside the PtCslA family, remains to be characterized.

**Putative XylS Activities in Stem-Developing Xylem of *P. trichocarpa***

We detected in the microsomes from the developing xylem strong activities converting UDP-Xyl or GDP-Man into 70% ethanol-insoluble polymers (Fig. 2). However, the similar plant protein activities observed for these two substrates are inconsistent with the fact that the quantity of xylan is normally severalfold higher than that of glucomannan in the angiosperm wood (Timell, 1964, 1969; Fengel and Wegener, 1979, 1984). It is possible that the plant protein reaction products from either substrate are a similar mixture of radioactive xylan and mannan. This assumes the presence of sugar nucleotide exchange reactions that would effectively interconvert UDP-[U-14C]Xyl and GDP-[U-14C]Man. Although this might explain the similar enzyme activities observed, such an epimerization activity has never been reported (Leloir, 1951; Adams, 1976; Dalessandro and Northcote, 1977). Indeed, a radioactive xylan polymer with no incorporation of Man residues was synthesized from UDP-[14C]Xyl by microsomal proteins from corn cobs (Bailey and Hassid, 1966), further negating the activities for an interconversion of UDP-Xyl and GDP-Man. The similar UDP-Xyl- and GDP-Man-utilizing activities observed may have other implications. It may suggest that the in vivo xylan synthase activity involves protein partners (Zhong et al., 2005) or other factors (Dhugga, 2005; Liepman et al., 2005) forming an effective machinery for the biosynthesis of xylan. The microsomal protein preparation may have disrupted such machinery, making the in vitro xylan synthase activity far less efficient than its native state. This proposition needs to be validated.

**In Vitro Enzymatic Activities of PtCslA Members Are Distinct from Each Other***

Based on the sequence-defined subfamily grouping (http://cellwall.stanford.edu; Richmond and Somerville, 2000), there are only three types of members in the PtCslA subfamily, represented by PtCslA1, PtCslA3, and PtCslA5, respectively. They share high (71%–79%) protein sequence homology, but their recombinant proteins produced by the S2 cells show distinct catalytic activities. PtCslA1 and PtCslA3 encode a ManS activity, whereas PtCslA5 has essentially no such activity (Fig. 6). The in vitro polymer products of the recombinant PtCslA1 with GDP-Man are a pure mannan, suggesting the absence in the system of the epimerization of the three sugar nucleotides that were used as the substrates. These three types of sugars are the possible backbone constituents of the only two significant hemicelluloses known in the angiosperm wood. The fact that none of these sugars can be utilized by PtCslA5, even though a good level of this protein was produced by the S2 cells (Fig. 6), provides evidence that PtCslA5 may not be associated with the biosynthesis of the two wood hemicelluloses. But remarkably, PtCslA5 has, among all the detectable Csl gene members in *P. trichocarpa*, the highest transcript copy numbers in all tissues tested (Table I). In particular, its expression is more associated with the vascular systems in *P. trichocarpa* (Table I; Fig. 4). Whether PtCslA5 is associated with the biosynthesis of wood hemicelluloses remains to be elucidated. Perhaps roles for PtCslA5 gene and gene product can be better revealed using the plant systems.

The ManS activity of PtCslA3 recombinant protein is specific for mannan synthesis (Fig. 6). Pure mannan, however, has never been isolated from angiosperm tree species, due probably to its low quantity. This is consistent with the low levels of PtCslA3 transcripts in all tissues tested (Table I). It is possible that the low level of mannan may act as signaling molecules (Liepman et al., 2005) instead of a structure polysaccharide. Indeed, complex polysaccharides have numerous biological, including signaling and growth-regulating, properties (Creelman and Mullet, 1997).

Recombinant protein activity assays and in vitro product characterizations validated that, of all the three PtCslA gene types, PtCslA1 represents the most conspicuous one that encodes both ManS and GlcManS activities capable of mediating the synthesis of (1→4)-β-D-mannan and (1→4)-β-D-glucosamin (Figs. 6 and 7). These in vitro functions are similar to those of the Arabidopsis AtCslA9 (Liepman et al., 2005), which shares 80% sequence homology with PtCslA1.

Surprisingly, AtCslA9 and PtCslA3 share higher sequence homology at 83%, but PtCslA3 does not have in vitro GlcManS activities (Fig. 6). PtCslA3 acts instead more like the Arabidopsis AtCslA7 (Liepman et al., 2005) and both are more of a ManS. These two proteins having a seemingly similar function share, however, only 60% sequence homology. More intriguingly, Arabidopsis AtCslA2 and PtCslA5 share 84% sequence homology, but AtCslA2 is a ManS and also is likely a GlcManS (Liepman et al., 2005), whereas PtCslA5 is neither a ManS nor a GlcManS (Fig. 6). Evidently, sequence homology of proteins between...
species may not be a general indicator for functional similarity. This may be a consequence of gene function divergence following speciation. In addition, the numbers of the members in certain Csl subfamilies, including the CslA subfamily, differ between \textit{P. trichocarpa} and Arabidopsis (see “Results”). These variations in gene function and diversification may reflect the requirement of different types and quantities of the hemicelluloses by herbaceous and by woody dicots. In \textit{P. trichocarpa}, all the three possible types of CslA genes are more vascular tissue specific, but likely only one is required for the synthesis of wood glucomannan.

\textit{PtCslA1} Likely Encodes a Key GlcManS for the Synthesis of Wood Glucomannan

Although \textit{PtCslA1} encodes an in vitro ManS activity, the presence of pure mannan in wood of angiosperm trees has not been demonstrated, to our knowledge. But glucomannan, or (1→4)-\beta-d-glucan, can be readily purified from angiosperm wood. (1→4)-\beta-d-Glucomannan does not contain long stretches of mannan or glucan residues. Instead, it has alternated Man and Glc residues with Glc:Man ratios of 1:1 to 1:3 (Timell, 1986; Jacobs et al., 2002). This alternated polymerization of Man and Glc in glucomannan synthesis was also projected by the radioactivity products after enzymatic hydrolysis of the in vitro glucomannan (Fig. 7). Thus, the observed in vitro “mannan” and “glucan” synthesis functions for \textit{PtCslA1} recombinant protein may not be important for the biosynthesis of glucomannan in vivo. The in vivo function of \textit{PtCslA1} may simply be that of a GlcManS for the synthesis of one of the two important structure hemicelluloses in the angiosperm wood. This proposition is in line with the devoted expression of \textit{PtCslA1} in the secondary xylem (Fig. 4; Table I), the main tissue type where glucomannan deposits. Furthermore, based on the incorporation (Fig. 6) and release (Fig. 7) of the radioactive Glc and Man units, the Glc:Man ratio in the in vitro (1→4)-\beta-d-glucan was estimated to be approximately 1.2 to 1.3, consistent with the structure of angiosperm wood’s glucomannan.

Glucomannan is a minor but significant component in angiosperm wood. In contrast, in conifer wood, glucomannan, which is slightly branched with Gal residues, is the predominant hemicellulose (Timell, 1986; Jacobs et al., 2002). The exact physiological function of glucomannan in trees is not clear. However, glucomannan is a highly undesirable trait in conifer wood for pulp and paper production. Nearly 60% of all pulp produced in the United States is manufactured from conifers, and lower limits on chemical/energy intensities for pulp production from these species have been reached (Nilsson et al., 1995). During pulping of conifer wood, glucomannan (approximately 20\% of the wood weight) is almost completely degraded at the onset of the process, consuming approximately 25\% of the chemicals intended to remove lignin to produce woodpulp (Rydholm, 1965; Gellerstedt, 2001). Thus, engineered conifers with less glucomannan are expected to drastically lower the chemical intensity limits for pulp production. As energy feedstock, on the other hand, wood modified for an increased quantity of glucomannan, the six-carbon sugar pool, would be highly desirable for improved ethanol yield. The identification of genes encoding ManS and GlcManS by the previous and current studies may help facilitate these biotechnological applications to improving the economics of the wood and future wood-based biofuel industries.

\textbf{MATERIALS AND METHODS}

\textbf{Reagents and Enzymes}

\textit{GDP-[14C]Man (9.6 GBq/mmol) and UDP-[14C]Xyl (9.8 GBq/mmol) were obtained from Perkin-Elmer, GDP-[14C]Glc (11.1 GBq/mmol) from American Radiolabeled Chemicals, and nonradioactive UDP-Xyl from Carboxsource Services. Enol-\beta-d-(1→4)-mannanase purified from \textit{Bacillus sp.}, endo-\beta-d-(1→4)-glucanase (cellulase) from \textit{Aspergillus niger}, and endo-\beta-d-(1→5)-glucanase from \textit{Trichoderma sp.} were obtained from Megazyme. Oligonucleotide primers were synthesized by MWG Biotech. Vectors, \textit{Escherichia coli} cells, Drosophila S2 cells, culture media, and anti-V5-HRP antibody were purchased from Invitrogen, SuperSignal West Pico chemiluminescent from Pierce, Bradford protein assay concentrate from Bio-Rad, Immobilon-P membrane from Millipore, \textit{Aspergillus niger} and \textit{Trichoderma sp.} were identified by searching through the current database with the BLASTX program (Altschul et al., 1997). The predicted protein sequences of the BLASTX-identified gene models were further screened for completeness by analyzing their transmembrane domain profiles with TMHMM (http://www.cbs.dtu.dk/services/TMHMM/) and the presence of the conserved motifs that are common to Cesa and Csl genes (Saxena et al., 1995; Saxena and Brown, 1997). Cellulose synthase superfamily members of Arabidopsis and rice (\textit{Oryza sativa}) were obtained from the GenBank based on the annotation of The Arabidopsis Information Resource (TAIR; http://www.arabidopsis.org) and the The Institute for Genomic Research Rice Genome Annotation project (http://www.tigr.org/tdb/ezk1/osa1/). Phylogenetic trees and their associated bootstrap values were analyzed using the ClustalW multiple sequence alignment program (Thompson et al., 1994). The neighboring-join trees were created based on the distance matrices derived from the results of multiple sequence alignments using the default settings, followed by 1,000 bootstrap trials to evaluate the qualities of the trees.

\textbf{RNA Isolation and Gel-Blot Analysis}

Leaf (from four to six internodes), stem-developing phloem, stem-developing xylem, young shoot tip (one to three internodes), and fine root tissues were isolated from these tissues, and the RNA quality was examined by UV spectrogram scan and gel electrophoresis, as we did previously (Lu et al., 2005). The total RNA was used for northern blotting and real-time PCR analysis. RNA gel blotting and hybridization were performed under high stringency conditions (65\%C) as described previously (Hu et al., 1999). A 5’-end fragment including the 5’-UTR was selected from each of the three
Gene-Specific PCR Primer Design and Real-Time PCR Analysis of Gene Transcript Copy Numbers

Based on the identified PiCtCsl and PiCt genes, PCR primers were designed so that the sequences of each set of primers would match perfectly with the target PiCtCsl or PiCt sequence but differ in at least three nucleotides from the sequences of all the other superfamily members (Supplemental Table S3). Each designed primer set was expected to amplify a PCR product from a specific exon with a size of approximately 100 bp in length.

Total RNA was treated with RNase-free DNase I and purified by RNeasy plant RNA isolation kit. Total RNA (200 ng) was reverse transcribed using the manufacturer’s manual. Real-time PCR was conducted using an Applied Biosystems 7300HT sequence detection system (Shi and Chiang, 2005). For each reaction, the 25-μL mixture contained the first-strand cDNA (equivalent to 100 pg of total RNA), 5 pmol each of the forward and reverse primers, and 2X SYBR Green PCR master mix and reverse transcription-PCR kit according to the manufacturer’s instructions.

The PCR amplification efficiencies for all seven of these standard genes were highly reproducible, validating the appropriateness of the standard genes also for other applications. The Ct values for each gene were calculated by the amplification efficiencies of the standard genes and used to quantify the copy numbers of the target gene transcripts. Thus, copy numbers in 10^3, 10^4, 10^5, 10^6, 10^7, 10^8, and 10^9 copies were derived with the use of the standard genes.

Crude Plant Protein Extraction and Microsomal Protein Preparation from P. trichocarpa

The tissue sample (1.5 g) was ground in liquid nitrogen and homogenized at 4°C in 30 mL of extraction buffer: 50 mM HEPES-KOH, pH 7.5, containing 0.4 M Suc, 5 mM MgCl₂, 2 mM dihædrothiol (DTT), 1 mM phenylmethylsulfonyl fluoride, 1 mg/mL pepstatin A, 1 mg/mL leupeptin, and 2% (v/v) Polyclar-AT. The homogenate was centrifuged at 1,000 g for 10 min at 4°C and filtered with Miracloth. The filtrate was centrifuged at 100,000 g for 1 h to collect the microsomal fractions (Osakabe et al., 1999). The microsomal debris was then resuspended in 0.5 mL of the extraction buffer using a glass homogenizer. Protein concentrations were determined by the Bio-Rad protein assay system.

PtCtCsl cDNA Cloning and Heterologous Expression in Drosophila S2 Cells

PtCtCslA1, PtCtCslA3, and PtCtCslA5 cDNAs were PCR cloned. Based on the predicted cDNA sequences, gene-specific primer sets (PtCtCslA1: 5’-end primer, ACCATGGGTGTTCCTCggccgtgccgatg, and 3’-end primer, AGATGTCGGGGAAGATGGTGC; PtCtCslA3: 5’-end primer, ACCATGGGAGGGCTA and 3’-end primer, AATATGGTACCAAGATGTCAT) were designed to amplify the coding sequences, which were subsequently cloned into pMT/V5-His-TOPO vector. P. trichocarpa xylem lambda phage cDNA library was used as the template and ExTag polymerase was employed to ensure the sequence authenticity of the PCR products. The cDNA clones were purified and verified by full sequencing from both directions. Following this approach, PtCtCsa1, PtCtCsa1B, PtCtCslC1, and PtCtCslC2 cDNAs were also cloned and sequenced.

Drosophila S2 cells were cotransfected with the pCtCslA vector and the pMT/V5-His-TOPO vector containing the target PtCtCsl vector into Drosophila S2 cells were selected in Schneider’s Drosophila medium containing 25 mg/L of blasticidin. Protein expression was induced with the addition of copper sulfate (0.75 mM) for 24 h. The induced cells were harvested by centrifugation at 500 g for 5 min followed by microsomal protein preparation.

Microsomal Protein Preparation from Drosophila S2 Cells

The S2 cells from 32-mL cultures were harvested and then disrupted by sonication in 7 mL of an extraction buffer (50 mM HEPES-KOH, pH 7.5, containing 0.4 M Suc, 5 mM MgCl₂, 2 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 1 mg/mL pepstatin A, and 1 mg/mL leupeptin) for 2 min at 4°C. The microsomal protein preparation was performed as described above for the plant cells.

Immunoblot Analysis of Recombinant Proteins

Protein preparation (50 μg) was incubated in standard SDS-PAGE buffer for 15 min at 4°C. After separation on SDS-PAGE, proteins were transferred to an Immobilon-P membrane by electrophoretic transfer (Li et al., 2001). The membrane was blocked with bovine serum albumin for 1 h at room temperature and treated with anti-V5-HRP antibody overnight at 4°C. The signal was detected by using SuperSignal West Pico chemiluminescent substrate. After detection, the membrane was stained with Coomasie Brilliant Blue R-250 to verify uniform loading and transfer.

Activity Assays for Microsomal Protein Preparations from Plant and S2 Cells

Enzyme assays were performed essentially according to Liepen et al. (2005). Briefly, the assay was carried out in 60 μL of reaction mixture containing assay buffer (50 mM HEPES-KOH, pH 7.5, containing 2.5 mM DTT, 2.5 mM MgCl₂, 5 mM MrCl₃, and 6% glycerol), microsomal proteins (200–300 μg), and substrate(s). The following substrate concentrations were used. For individual substrate reactions: 2 mM GDP-[U-14C]Man (7.72 Bq/μmol), 2 mM GDP-[U-14C]Glc (7.71 Bq/μmol), or 2 mM UDP-Xyl (9.25 Bq/μmol). Mixed substrate reactions contained 2 mM GDP-[U-14C]Man (7.72 Bq/μmol) and 2 mM GDP-Glc; 2 mM GDP-Man and 2 mM GDP-[U-14C]Glc (7.71 Bq/μmol). Reaction mixtures were incubated at 25°C for 30 min, and the reaction was terminated by adding 1 mL of 70% ethanol containing 2 mM EDTA. Carob galactomannan (200 μg) was added as a carrier. Products were precipitated at −20°C and pelleted at 16,000 g for 10 min. Pellets were washed with four times with 70% ethanol containing 2 mM EDTA to remove excess radiactivity. Washed pellet was then resuspended in water and counted using a Beckman Coulter LS 6500 liquid scintillation counter.

Characterization of Polysaccharide Products

The identity of the radioisotope-labeled products was examined by both acid hydrolysis and linkage-specific hydrolyase digestion. Product pellet was harvested from large scale of reactions (10-fold scaled up from the normal reaction described above) and washed with 70% ethanol containing 2 mM EDTA as described above and dried. The pellet was hydrolyzed in 0.1 mL of 2 M trifluoroacetic acid at 120°C for 1 h. After addition of a mixture of Ara, Rha, Gal, Glc, Xyl, and Man standards, the hydrolysates were filtered and subjected (20 μL) to an anion-exchange chromatography ( Dionex) consisting of an AS50 anion exchanger, a GP40 gradient pump, a CarboPac PAI column, and an ED40 column.
electrochemical detector using water as the eluent at a flow rate of 1.2 mL min\(^{-1}\). Before each sample injection, the column was washed and equilibrated with 200 \(\mu\)M NaOH for 20 min, with 400 \(\mu\)M NaOH as the post column mobile phase at a flow rate of 0.15 mL min\(^{-1}\). The system afforded a well separation and detection of the six sugars. The eluents corresponding to each sugar were collected and counted (Beckman Coulter LS 6500).

For hydrolyase digestions, manufacturer’s protocols were followed. The in vitro product pellet was resuspended in 150 \(\mu\)L of buffer [0.1 \(\mu\)M Gly-NaOH, pH 8.8, for endo\(-\beta\)-1\((\rightarrow4)\)-mannanase, 0.1 \(\mu\)M AcONa-AcOH, pH 4.5, for endo-cel lulase, and 0.1 \(\mu\)M AcONa-AcOH, pH 4.0, for endo\(-\beta\)-1\((\rightarrow3)\)-glucanase] and hydrolyzed with 10 \(\mu\)L of the hydrolytic enzyme for 1 h at 40°C. After hydrolysis, 1.3 mL of 70% ethanol containing 2 \(\mu\)L EDTA and 200 \(\mu\)g carob galactomannan were added to the reaction mixture, which was precipitated at \(-20^\circ\)C for 1 h and pelleted at 16,000g for 10 min. The radioactivities of the supernatant and pellet were counted (Beckman Coulter LS 6500) separately.

### Supplemental Data

The following materials are available in the online version of this article.

**Supplemental Figure S1.** Cladogram of the CesA genes from Arabidopsis and poplar genomes.

**Supplemental Table S1.** The gene loci of PtCesA and PtCsl members.

**Supplemental Table S2.** CesA sequence homology between Arabidopsis and *P. trichocarpa*.

**Supplemental Table S3.** List of real-time PCR primers.

**Supplemental Table S4.** Transcript copy numbers of the partial putative *PtCsl* genes.

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