MAP20, a Microtubule-Associated Protein in the Secondary Cell Walls of Hybrid Aspen, Is a Target of the Cellulose Synthesis Inhibitor 2,6-Dichlorobenzonitrile

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We have identified a gene, denoted PttMAP20, which is strongly up-regulated during secondary cell wall synthesis and tightly coregulated with the secondary wall-associated CESA genes in hybrid aspen (Populus tremula × tremuloides). Immunolocalization studies with affinity-purified antibodies specific for PttMAP20 revealed that the protein is found in all cell types in developing xylem and that it is most abundant in cells forming secondary cell walls. This PttMAP20 protein sequence contains a highly conserved TPX2 domain first identified in a microtubule-associated protein (MAP) in Xenopus laevis. Overexpression of PttMAP20 in Arabidopsis (Arabidopsis thaliana) leads to helical twisting of epidermal cells, frequently associated with MAPs. In addition, a PttMAP20-yellow fluorescent protein fusion protein expressed in tobacco (Nicotiana tabacum) leaves localizes to microtubules in leaf epidermal pavement cells. Recombinant PttMAP20 expressed in Escherichia coli also binds specifically to in vitro-assembled, taxol-stabilized bovine microtubules. Finally, the herbicide 2,6-dichlorobenzonitrile, which inhibits cellulose synthesis in plants, was found to bind specifically to PttMAP20. Together with the known function of cortical microtubules in orienting cellulose microfibrils, these observations suggest that PttMAP20 has a role in cellulose biosynthesis.

Wood formation is a complex developmental process, which marries the biosynthesis of cellulose and other cell wall polymers and leads to the formation of highly organized secondary cell walls in trees (Mellerowicz et al., 2001). Cellulose microfibrils deposited in ordered layers denoted S1, S2, and S3 constitute the main load-bearing component of secondary cell walls. Cellulose is synthesized by large protein complexes, rosettes, located at the plasmalemma (for review, see Somerville, 2006). Studies in different plant models indicate that several distinct catalytic subunits (CESA) are needed to form a functional cellulose synthesizing complex (Somerville, 2006). Furthermore, different sets of three CESA proteins are apparently employed for cellulose biosynthesis in primary and secondary cell walls in both angiosperms and monocots (Tanaka et al., 2003; Taylor et al., 2003; Burton et al., 2004; Djerbi et al., 2004; Persson et al., 2007).

The complexity and highly ordered structure of wood suggest that the deposition of secondary walls must be a tightly controlled and coordinated process. A large body of evidence indicates that cortical microtubules are somehow involved in the ordered deposition of cellulose microfibrils (Somerville, 2006). Microscopic evidence on different plant species indicates that the orientation of cellulose microfibrils in both primary and secondary walls is often parallel to the underlying cortical microtubule arrays (Hepler and Newcomb, 1964; Wooding and Northcote, 1964; Pickett-Heaps and Northcote, 1966; Hardham and Gunning, 1979; Abe et al., 1995; Chaffey et al., 1997; Funada et al., 1997; Chaffey et al., 1999; Funada et al., 2002; Haigler et al., 2005; Oda et al., 2005). It has also

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been demonstrated recently that the CESA complexes in the Arabidopsis (Arabidopsis thaliana) plasma membrane of the primary walled cells move at constant rates in linear tracts that coincide with cortical microtubules (Paredez et al., 2006).

In Arabidopsis, all three secondary cell wall-associated CESA proteins colocalize with bands of cortical microtubules in older xylem vessels (Gardiner et al., 2003), and it has been suggested that the microtubules guide the deposition of the cellulose synthase complexes in the plasma membrane. Unlike in primary walls, the presence of microtubules seems to be required for cellulose synthesis throughout the secondary cell wall synthesis (Gardiner et al., 2003; Wightman and Turner, 2008). Studies of developing wood cells in both conifers and angiosperms also reveal co-alignment in the orientation of microfibrils and microtubules during the formation of the successive cell layers S1 to S3 (Chaffey et al., 1997, 1999, 2002; Funada et al., 2002). The use of chemicals disrupting microtubules (Himmelspach et al., 2003) or inhibiting cellulose biosynthesis (Delmer, 1999) and dyes preventing the crystallization of cellulose microfibrils (Haigler et al., 2005) interrupts the intracellular communication between microfibrils and microtubules that governs the ordered deposition of cellulose microfibrils. The classical hypothesis on the connection of cellulose biosynthesis and cortical microtubules suggests that microtubules form barriers that constrain the paths of cellulose synthase complexes on the plasma membrane, thereby guiding the ordered deposition of the microfibrils (for review, see Giddings and Staehelin, 1991). However, there are also data to suggest that organized microtubules are required to establish rather than to maintain the pattern of cellulose microfibril deposition (Sugimoto et al., 2003; Roberts et al., 2004; Wasteneys, 2004), perhaps to correctly position CESA protein complexes in the plasma membrane (Gardiner et al., 2003; Wightman and Turner, 2008).

The assembly, bundling, and stability of microtubules depend on the activity of various microtubule-associated proteins (MAPs) and their regulatory kinases and phosphatases (Sedbrook, 2004; Wasteneys and Yang, 2004). Among plant MAPs, mainly from Arabidopsis, many have been shown to influence plant or organ morphology and/or cellulose microfibril orientation (Sedbrook, 2004). The findings so far largely concern the primary cell walls and include TOR1, which regulates the direction of organ growth (Buschmann et al., 2004); SKU6/SPRAL1, which is involved in directional cell expansion (Sedbrook, 2004); WAVE DAMPENED2 (WVD2), which modulates anisotropic cell expansion during organ growth (Yuen et al., 2003; Sedbrook, 2004; Perrin et al., 2007); and AtMAP70-5, which is seemingly essential for maintaining axial polarity and ensuring regular extension of plant organs (Korolev et al., 2007). There are few findings of MAPs affecting secondary walls in Arabidopsis (Mellerowicz and Sundberg, 2008). These include the proteins FRAGILE FIBER1 (FRA1) and FRA2, both affecting the patterning of cellulose microfibrils in the inner wall of interfascicular fibers (Zhong et al., 2002; Burk et al., 2007). However, owing to the extreme complexity of the microtubular networks, it is likely that many more plant MAPs remain to be discovered (Gardiner and Marc, 2003; Oda and Hasezawa, 2006).

To shed more light on the overall process of wood formation, we have employed functional genomics in hybrid aspen (Populus tremula × tremuloides). Bioinformatic analyses of EST sequences in various wood-specific EST libraries followed by expression profiling across different stages of wood formation led to the identification of a number of transcripts with high relative levels of expression in the woody tissues (Sterky et al., 1998, 2004; Hertzberg et al., 2001; Djerbi et al., 2004; Aspeborg et al., 2005; Geisler-Lee et al., 2006). In addition to enzymes and proteins with a predictable function, a large number of the genes discovered encoded proteins with unknown functions.

Here, we have characterized the product of one of these “unknown” genes and show that it is tightly coregulated with the secondary cell wall-associated CESA genes and that the corresponding protein binds specifically to microtubules. In addition, the cellulose synthesis inhibitor 2,6-dichlorobenzonitrile (DCB) binds specifically to this protein in vitro. The significance of these findings is discussed in light of current models of the communication between interphase cortical microtubules and cellulose microfibrils during cell wall biosynthesis.

RESULTS

Expression Analysis of PttMAP20

Data from microarray analysis of different poplar tissues and organs show that a small cytosolic protein, designated PttMAP20, is preferentially expressed in developing secondary xylem tissues (Fig. 1A). The expression pattern of PttMAP20 is remarkably similar to three previously identified xylem-associated PttCESA genes (Djerbi et al., 2004). To confirm this pattern of expression, high-resolution quantitative PCR (qPCR) expression analysis of PttMAP20 and PttCESA1, PttCESA3-2, and PttCESA9-2 was conducted across the wood-forming tissues in Populus tremula. Tissue samples were dissected into eight fractions from functional phloem toward maturing wood cells using tangential cryosectioning (Uggla et al., 2001), giving a spatial resolution of gene expression of about 90 μm in the radial direction (Fig. 1B, bottom). Indeed, PttMAP20 expression was very low in functional phloem tissues and primary walled cambial cells, while it increased in expanding cells and most dramatically at the onset of secondary wall biosynthesis (Fig. 1B, top). The peak values of PttMAP20 expression in fractions 4 to 6 corresponded to the peak rate of fiber wall thickening as measured from SEM images of the corresponding tissue (data not shown). Moreover, the qPCR analysis confirmed a very tight coexpression between PttMAP20.
and the secondary wall-specific CESA genes. The only deviation from this pattern was observed during late maturation of wood fibers, where the PtMAP20 expression dropped to a lower level somewhat earlier than that of the CESA genes. The qPCR analysis, signal intensities in the microarrays (data not shown), and the abundance of PtMAP20 transcripts in EST libraries (www.populus.db.umu.se) all indicate a high level of expression of MAP20 in woody tissues in poplar. Thus, it can be concluded that MAP20 is a highly expressed gene specifically associated to secondary wall formation and tightly coregulated with secondary wall CESA genes.

**Immunolocalization of PtMAP20**

Indirect immunofluorescence experiments using affinity purified antibodies raised against recombinant PtMAP20 (see Supplemental Figs. S1 and S2 and Supplemental Materials and Methods S1 for details) was used to localize the protein in wood-forming tissues. However, signals corresponding to the anti-PtMAP20 antibodies were only observed if 6 M urea was used to pretreat the samples. This treatment, which has been used previously for retrieving difficult epitopes of other proteins (Shi et al., 1993, 1994; Ito et al., 1998), led to poor quality of tissue preservation, making it hard to draw conclusions about the subcellular localization of PtMAP20. Strong signals corresponding to the anti-PtMAP20 antibodies were detected in all cell types, the strongest being observed in developing xylem cells (fibers, vessel elements, and ray parenchyma cells) at the stage of secondary cell wall thickening (Fig. 2A).

The microtubules were also visualized in the same tissues using a monoclonal antibody, DM1A, specific for mammalian α-tubulins (Blose et al., 1984). Signals specific for α-tubulin were detected in all stem cells except for the sieve tube cells (Fig. 2B, arrow). In developing wood cells, the α-tubulin signals were moderate in the cambium, lower in the radial expansion zone, and strongest during secondary wall deposition (Fig. 2B). Although the signals specific for both α-tubulin and PtMAP20 coincided in the same cell types in developing wood, the α-tubulin signals were clearly more distinct than the PtMAP20 signals in the cambium, where microtubules have a major role in cell division and primary wall deposition.

**Sequence Analysis and Bioinformatics**

The full-length sequence of PtMAP20 was obtained by sequencing two overlapping cDNA clones: A089p68u, corresponding to the microarray ID PU02741, and A068p39u. The cDNA sequence was used to search the Populus genome database (http://genome.jgi-psf.org/Poptr1/Poptr1.home.html) and was shown to match the gene model eugene3.00440209 (denoted estExt_fgnes34_pg.C_440200 in the Populus genome release 1.1). The translated protein sequence of PtMAP20 revealed a protein of 176 amino acids with an estimated molecular mass of 20.8 kD and a pI of 9.65. A BLASTP search with PtMAP20 as the query sequence revealed similar proteins with unknown
function in Arabidopsis, *Medicago truncatula*, and rice (*Oryza sativa*), while a TBLASTN search against the National Center for Biotechnology Information (NCBI) database dbEST produced a list of homologous ESTs in diverse plant species, including conifers (data not shown). Further sequence analysis revealed that the residues 62 to 118 of the *PttMAP20* gene sequence correspond to the Pfam TPX2 model PF06886. The TPX2 domain was first identified in the microtubule-targeting protein for the kinesin-like protein 2 of *Xenopus laevis* (TPX2; Wittmann et al., 2000). A screen of the *Populus trichocarpa* genome identified altogether 19 gene models containing a TPX2 domain. In addition to *PttMAP20*, another two of these poplar genes, here designated MAP20-like genes 1 and 2 (*PttMAP20-H1* and *PttMAP20-H2*; Fig. 3B), were also relatively highly expressed during secondary cell wall biosynthesis (data not shown).

The closest relatives of *PttMAP20* are the hypothetical protein ABE90152.1 from *M. truncatula* and the Arabidopsis-expressed protein At5g37478 (Fig. 3A). According to the current annotation, the At5g37478 protein comprises only 96 amino acid residues and lacks a part of the N-terminal region of the conserved TPX2 domain (Fig. 3B). However, reannotation of this gene led to an alternative gene model, hereafter referred to as At5g37478_alt, which corresponds to a protein of 145 amino acids, including the complete TPX2 region (Fig. 3A). This annotation is also supported by two homologous ESTs (CN734834 and CD830242 in the dEST) from oilseed rape (*Brassica napus*). A comparison of *PttMAP20* with At5g37478_alt shows that the C termini of the two proteins are highly conserved, while the N-terminal sequences preceding the TPX2 domain are more divergent.

A phylogenetic tree was constructed using the conserved TPX2 domains of all identified plant TPX2-like proteins and representative examples of animal TPX2-containing proteins (Fig. 3B). The plant sequences were found to fall in a clade separate from the animal TPX2-containing proteins, suggesting that the TPX2 domain diversified after the evolution of animals. Among the previously characterized plant MAPs, only the 23-kD WVD2 (Perrin et al., 2007) contains a TPX2 domain. WVD2 has been previously classified in a family of proteins containing a KLEEK domain (Yuen et al., 2003), and the phylogenetic analysis carried out here suggests that the KLEEK family is a large subfamily within the family of TPX2-containing proteins (Fig. 3B; Supplemental Fig. S3).

**Binding of PttMAP20 to in Vitro-Assembled Microtubules**

The presence of a TPX2 domain suggested that *PttMAP20* might be a MAP. To test this experimentally,
the PttMAP20 cDNA was expressed in Escherichia coli, and the corresponding protein was purified (Supplemental Fig. S1) and used for binding studies with in vitro polymerized bovine microtubules (BMTs). After incubation with BMTs and centrifugation, PttMAP20 was recovered in the pellet fraction together with BMTs (Fig. 4A, lane 4P), while it remained in the supernatant fraction in the absence of BMTs (Fig. 4, lane 5S). BMTs in the absence of other proteins were found in the pellet (Fig. 4, lane 3P), while bovine serum albumin (BSA; used as a nonbinding control protein) remained in the supernatant, whether incubated with or without BMTs (Fig. 4, lanes 1S and 2S, respectively). An image of the entire gel is shown in Supplemental Figure S4.

Overexpression of PttMAP20 in Plants

To study the effects of PttMAP20 in vivo, we generated stable Arabidopsis plants expressing PttMAP20 under the cauliflower mosaic virus 35S promoter and confirmed the overexpression by reverse transcription (RT)-PCR (Fig. 5A). Seedlings of these plants displayed left-handed twisting of cotyledon petioles (Fig. 5B) and shorter roots (data not shown), and etiolated hypocotyls of two independent lines displayed a right-handed helical twisting of the epidermal cell layers (Fig. 5C).

To corroborate the binding of PttMAP20 to microtubules in vivo, we examined the subcellular localization of PttMAP20 by in vivo imaging of PttMAP20-yellow fluorescent protein (YFP) protein transiently expressed in tobacco (Nicotiana tabacum) leaves (Fig. 6). Confocal microscopy observations of the YFP signal clearly show that the fusion protein localizes to interphase microtubules in leaf epidermal pavement cells and that the net-like structure is labeled uniformly throughout its length (Fig. 6).

Interactions of DCB and/or [3H]DCPA with PttMAP20 and Microtubules

Because the cellulose synthesis inhibitor DCB has been shown to bind to other plant proteins similar in size to PttMAP20 (Delmer et al., 1987), we investigated whether recombinant PttMAP20 could be labeled with a photoactive and radioactive DCB analog, 2,6-dichlorophenylazide ([3H]DCPA; Delmer et al., 1987). Incubation of increasing concentrations of PttMAP20 with 0.75 μM [3H]DCPA led to a gradually increasing signal when the mixtures were irradiated by UV light (Fig. 7A, lanes 1–5). Control experiments in which [3H]DCPA, UV irradiation, or both were omitted gave no signal (Fig. 7A, lanes 6–8). The specificity of the labeling was confirmed by competition experiments showing that the labeling of PttMAP20 by [3H]DCPA decreased with increasing concentrations of nonradioactive DCPA (Fig. 7B) or DCB (Fig. 7C). The amount of [3H]DCPA bound to PttMAP20 dropped by nearly 50% when 2 mM competing DCPA was added in the photoaffinity labeling reaction mixture together with 0.75 μM [3H]DCPA (Fig. 7B). A slightly lower competing effect was obtained with DCB in the same conditions (approximately 25% decrease of bound [3H]DCPA in the presence of 2 mM DCB; Fig. 7C), probably because DCPA, but not DCB, is structurally identical to [3H]DCPA. The amount of bound [3H]DCPA dropped by as much as 90% in the presence of a 25-fold excess of either of the competing reagents (Fig. 7, B and C), thereby demonstrating the specificity of the labeling. Interestingly, the presence of DCB up to a final concentration of 200 μM did not influence the binding of PttMAP20 onto microtubules (Fig. 7D). Furthermore, no effect on microtubule polymerization was detected in the presence of up to 7,500 μM DCB (Fig. 7E).
DISCUSSION

The small cytosolic poplar protein, PttMAP20, was first identified by the strong up-regulation of the expression of the corresponding gene during the formation of secondary xylem in poplar. Standard sequence comparisons revealed that PttMAP20 shares a small conserved domain with a large multidomain MAP, TPX2, from *Xenopus* (Wittmann et al., 1998, 2000). The original TPX2 protein sequence contains two short domains predicted to adopt a coiled coil structure often involved in protein-protein interactions, one at the N terminus (residues 171–208) and one at the C terminus (650–684). The C-terminal coiled coil domain is highly conserved in all TPX2-like proteins, and it is thus called the TPX2 domain. Proteins containing a TPX2 domain have so far been identified in animals, fungi, and plants, but functional data concerning these proteins is scarce. The full-length animal TPX2 protein binds to and nucleates microtubules, and it interacts with proteins using microtubules as a landing place to perform their function. (Wittmann et al., 1998, 2000; Kufer et al., 2002; Brunet et al., 2004). However, it is not clear whether it is the TPX2 domain or other parts of the full-length protein that are responsible for its binding to microtubules (Brunet et al., 2004). The present bioinformatic analysis reveals many other TPX2 containing proteins in plants.

Figure 3. (Continued.)
However, beyond their TPX2 domains, these proteins show little similarity with each other.

Our present data show that PttMAP20 binds to microtubules, both in vitro and in vivo, and that its overexpression in Arabidopsis leads to helical growth phenotypes. Similar phenotypes are frequently observed in plants with altered microtubule dynamics, for example, plants overexpressing tagged tubulin or MAPs, and dominant-negative tubulin mutants (Sedbrook and Kaloriti, 2008). Overexpression of the Arabidopsis microtubule-destabilizing factor MAP18 produces left-handed helical twisting of epidermal cell layers of hypocotyl (Wang et al., 2007), whereas overexpression of the microtubule-bundling protein WVD2 results in right-handed root twisting and left-handed leaf petiole twisting (Perrin et al., 2007). In addition, map65-3/ple mutants, with defects in the microtubule-bundling protein MAP65-3, have short expanded roots (Müller et al., 2004), probably caused by defective cytokinesis.

DCB is a herbicide that is known to inhibit cellulose synthesis, but the mechanism of action of the drug is not known. Early observations of the effects of DCB in higher plants revealed dwarfed plant phenotypes (Sabba and Vaughn, 1999, and refs. therein), inhibition of cell wall formation and cytokinesis (Meyer and Herth, 1978), and disruption of the ability of cell plates to flatten out and become rigid (Vaughan et al., 1996). Habituation of plants for growth in the presence of DCB reveals accumulation of noncrystalline β-1,4-glucan (Encina et al., 2002; Garcia-Angulo et al., 2006). The effect of DCB is fully reversible, and the removal of DCB from the growth medium induces patches in the plasma membrane that accumulate fibrillar material. Interestingly, the accumulation of this material seems to coincide with regions of close contacts with the plasma membrane and cortical microtubules (Meyer and Herth, 1978). In algae, DCB seems to interfere with the assembly of the linear terminal complexes implicated in cellulose biosynthesis (Mizuta and Brown, 1992). DCB treatment has also been shown to cause changes in the number of intact rosettes at the plasma membranes of moss (Funaria hygrometrica) and wheat.
and accumulation of cellulose synthase subunits within localized regions at the plasma membrane of Arabidopsis hypocotyls (DeBolt et al., 2007). Recently, DCB treatment has been shown to cause cessation of the CESA mobility at the plasma membrane of Arabidopsis (DeBolt et al., 2007). A common theme between these different observations seems to be that DCB inhibits cellulose synthesis by disrupting the ordered deposition of crystalline cellulose rather than inhibiting the polymerization of the β-1,4-glucan chains per se.

Before this work, the only hint toward the molecular function of DCB was that it was shown to bind to small proteins of 12 to 18 kD, the amount of which seemed to increase significantly at the onset of secondary cell wall synthesis in different plants (Delmer et al., 1987). It was suggested that the receptor protein for DCB might act as a regulator of cellulose synthesis, but its identity and the mode of action of DCB have remained unknown. DCB induces changes in the cortical microtubule networks in the fucoid alga Pelvetia compressa (Bisgrove and Kropf, 2001) and Arabidopsis roots (Himmelspach et al., 2003). This observation led Bisgrove and Kropf (2001) to suggest that the DCB binding protein might be associated with microtubules. Here, we have shown that a small poplar protein, PttMAP20, is a target for DCB in poplar and that it indeed binds to microtubules. This suggests for the first time, to our knowledge, a direct link between a specific MAP and cellulose biosynthesis, and provides a possible explanation for the action of DCB.

Even though DCB binds specifically to PttMAP20, this does not prevent the binding of the protein to

![Figure 6](image-url)

**Figure 6.** Confocal microscopy images of tobacco leaf epidermal cells transiently expressing PttMAP20 fused to YFP. The images were recorded 3 to 5 d after the agroinfiltration. Scale bar = 20 μm. Shown is the decoration of the tobacco microtubules by the PttMAP20-YFP fusion proteins in vivo.

![Figure 7](image-url)

**Figure 7.** A, Labeling of recombinant PttMAP20 by [3H]DCPA. Lanes 1 to 5, 1 ng, 10 ng, 100 ng, 1 μg, and 10 μg (respectively) of purified PttMAP20 were labeled in the presence of 0.75 μM [3H]DCPA as described in “Materials and Methods”; lanes 6 to 8, control experiments performed using 1 μg of PttMAP20 but omitting UV irradiation (lane 6), [3H]DCPA (lane 7), or both (lane 8). B, Inhibition of the binding of [3H]DCPA to PttMAP20 by competition with DCPA. The experiments were performed using 0.75 μM [3H]DCPA, 1 μg of PttMAP20, and increasing concentrations of DCPA as indicated in the plot. The amount of bound [3H]DCPA was determined by measuring the radioactivity in the PttMAP20 bands excised from SDS-PAGE gels (liquid scintillation; see “Materials and Methods” for experimental details). C, As in B, but using DCB as a competing molecule. A maximum of 5% variation was obtained for each point in B and C after replicate experiments. D, Effect of DCB on the binding of PttMAP20 onto BMT and on the polymerization of BMT. Increasing concentrations of DCB were incubated in the presence of 1 μg of recombinant PttMAP20 for 20 min. The mixture were subsequently added to preassembled BMT and subjected to the spin-down binding assay (see “Materials and Methods”). The presence of tubulin and PttMAP20 in the pellet and supernatant was assessed by SDS-PAGE analysis. 1, No DCB; 2 to 4, 2 μM, 20 μM, and 200 μM DCB, respectively. E, The effect of DCB on the polymerization of microtubules as evidenced by SDS-PAGE, stained by Coomassie Blue. Increasing concentrations of DCB (0.75 μM, 7.5 μM, 75 μM, 750 μM, and 7,500 μM, lanes 3–7, respectively) were mixed with monomeric α- and β-tubulins before initiating the polymerization reaction as described in “Materials and Methods.” Control reactions with buffer or methanol replacing DCB are shown in lanes 1 and 2, respectively. Both supernatant and pellet fractions are shown for all samples.
microtubules. This suggests that DCB interferes with some other important interaction of PttMAP20 during cellulose synthesis. Several lines of evidence indicate that there may be a direct interaction between the microtubules and at least some of the CESA proteins. Perhaps the strongest evidence is provided by live-cell imaging experiments showing that the cellulose synthase complexes move precisely in register with microtubules (Paredez et al., 2006). Two different mutations of the CESA genes, the RSW1 affecting CESA1 and a null mutation of CESA2, have been shown to exhibit altered microtubule organization (Sugimoto et al., 2003; Chu et al., 2007), in addition to the disassembly of the rosette complexes and reduction of cellulose crystallinity. Finally, mutations affecting two other proteins, KORRIGAN and PROCUSTE1, both known to be involved in cellulose synthesis, have been shown to influence microtubule orientation (Paredez et al., 2008).

The putative ortholog of PttMAP20 is the only apparent target of DCB in e.g. cotton (Gossypium hirsutum; Delmer et al., 1987). It is thus conceivable that the inhibition of cellulose synthesis by DCB is a result of the inactivation of PttMAP20 and its orthologs in other plants. Because DCB does not interfere with the binding of PttMAP20 to microtubules, we speculate that PttMAP20 may be directly or indirectly associated with the cellulose synthase complex. The disruption of the proposed interaction by DCB would thus act to decouple cellulose biosynthesis and the cortical microtubule arrays. It is clear that further experimental work is required to corroborate this hypothesis, but it is consistent with data indicating that mutations in the CESA proteins can mimic the action of DCB (see above), although there is no evidence of DCB binding directly to the CESA proteins.

CONCLUSION

We have discovered a novel MAP, PttMAP20, which is strongly up-regulated in developing xylem tissues in poplars. The tight coregulation of the PttMAP20 gene with the secondary cell wall-associated CESA genes and the finding that the cellulose synthesis inhibitor DCB binds specifically to PttMAP20 suggest functional association with cellulose biosynthesis. Because microtubules are known to be somehow involved in coordinating the ordered deposition of cellulose microfibrils, we propose that PttMAP20 has a role in coupling the machinery of cellulose synthesis with cortical microtubules in developing secondary walls in poplar.

MATERIALS AND METHODS

Expression Analyses

Expression Profiling on Microarrays

Expression data from microarray experiments were obtained from UPSC-BASE (www.upscbase.db.umu.se/), experiment number 30 (courtesy of Göran Sandberg), using PU numbers PttCESA1: PU07566; PttCESA3: PU07525, PU07543, PU30161 (mean values); PttCESA9: PU06755; PttMAP20: PU00458, PU02741. The data was normalized against a sample containing an equal amount of RNA from all tissues and organs analyzed, and average level of expression was calculated in cases when a gene was represented by several spots on the microarray.

qPCR

Stem samples from Populus tremula were collected from a natural population of approximately 5-m-high trees growing outside Umeå, immediately frozen in liquid nitrogen, and stored at −70°C. Tangential sections (30 μm) were collected across the wood-forming tissues using a cryomicrotome (Ugglea et al., 2001) and pooled into eight samples. Total RNA extraction, cDNA synthesis, and SYBR green qPCR were performed using Bio-Rad Aurum Plant Mini kit, Bio-Rad iScript cDNA synthesis kit, and Bio-Rad SYBR Green qPCR mix, respectively, following the manufacturer’s instructions. All qPCR data were expressed as −ΔCt values (where −ΔCt is CF(GENE) − CF(CTRL GENE)). The data were normalized by using two housekeeping genes, ubiquitin (Pt794626, fgenesh4_pg.C_scaffold_13026000001) and elongation factor 1α (Pt815174, estExt_fgenesh4_pg.C_LG_I1178). Mean Ct values for these two genes were used when calculating −ΔCt values. Each sample was analyzed in three technical replicates. Primers used for gene expression analysis were designed from Populus trichocarpa gene model sequences (see Supplemental Table S1 for a list of all primers). The PCR program included initial denaturation at 95°C for 3 min; 40 cycles of 10 s at 95°C, 30 s at 55°C, and 30 s at 72°C; and a final melt curve analysis (incremental heating from 54°C to 95°C).

Immunolocalization Experiments

Free-hand transverse and longitudinal radial sections were prepared from poplar stems, between the 10th and 15th nodes from the top. The sections were quickly transferred to the fixative solution (50 mM PIPES, 5 mM MgSO4, 5 mM EGTA, and 4% paraformaldehyde; pH 7.0) and fixed for 30 min at room temperature. The fixative was replaced by 6% urea, and the sections were incubated for 30 min at room temperature as described before (Shi et al., 1993, 1994; Ito et al., 1998). All steps thereafter were done on a slow shaking table. The sections were washed three times for 5 min with PBS (pH 7.4) containing 0.1% Tween 20 and blocked with 5% nonfat milk in PBS for 30 min at 25°C. The sections were then quickly quenched in 0.5% Toluidine blue for 15 s to block the background signals from lignin, washed four times in PBS- Tween for 10 min, and mounted on to the slide with Vectashield (Vector Laboratories) for confocal microscopy (LSM510, Zeiss). Fluorescence signals were observed using an excitation wavelength of 488 nm and a 505- to 530-nm emission filter. The image transmitted with the visible light channel was superimposed onto the fluorescence image to reveal anatomical details of the tissues examined.

Sequencing and Bioinformatics

The full-length protein coding sequence of PttMAP20 was obtained by sequencing the cDNA clones A006p39u and A089p68u on both strands using a primer walking with unlabeled universal and gene-specific primers. The GenBank accession no. of the PttMAP20 sequence is DQ661742. Similarity searches were performed using the BLAST (http://www.ncbi.nlm.nih.gov/blast/) and T BLASTN (http://www.ncbi.nlm.nih.gov/public documents/vibe/details/NcbiTblastn.html; Alschul et al., 1990, 1997), and the EST sequences were retrieved from the NCBI database dbEST (http://www.ncbi.nlm.nih.gov/dbEST/). Gene models for rice (Oryza sativa) were downloaded from The Institute for Genomic Research release 4 (http://rice.tigr.org/tdb/e2k1/osaita/) and Arabidopsis (Arabidopsis thaliana) models came from...
The Arabidopsis Information Resource (genome release 6; www.arabidopsis.org). Poplar sequences included ESTs and contigs from PopulusDB (Sterky et al., 2004) and trichocarpa gene models from JGI (http://genome.jgi-psf.org/Poptri1/Poptri1.home.html). PTX2-containing proteins from Xenopus laevis, Gallus gallus, mouse (Mus musculus), and human (Homo sapiens) were retrieved from UniProt (Wu et al., 2006). Proteins containing the PTX2 domain were identified using HMMSearch in the HMmer package (http://hmmer.wustl.edu) and the PTX2 model from Populus (Finn et al., 2006). Protein hits with an E-value of $10^{-10}$ were considered significant. The hit on At5g28646 had an E-value of 0.00036 and was studied more closely, which revealed that this was an incorrect prediction of Q84ZT9. WAVE-DAMPENED. All Populus hits had significant hits to transcripts from PopulusDB. Alternative transcripts were removed, three in rice and four in Arabidopsis, in favor of longer gene models.

The multiple alignment of PttMAP20 and the Arabidopsis and Medicago truncatula homologs was performed by Kalig (Lassmann and Sonnhammer, 2005), available at http://msa.cgb.ki.se/cgi-bin/msa.cgi. FGENESH (Salamov and Solovyev, 2000) was used to predict genes and gene products in the region 14899001 to 14490001 on the Arabidopsis chromosome 5. Phylogenetic analysis was performed using MrBayes (Ronquist and Huelsenbeck, 2003). Four parallel MCMC chains were run for 10 million iterations, thinning was 100, and the first 10% iterations were discarded as "burnin" before a consensus tree was computed.

**Arabidopsis Transformation by Means of Floral Dipping**

Arabidopsis was transformed according to the floral dip method (Clough and Bent, 1998) with Agrobacterium tumefaciens strain C58C1 (pCH32) harboring a binary vector with PttMAP20 fused to a myc tag at the C terminus. The Arabidopsis and Medicago homologs were cloned from genomic DNA prepared from seedlings according to our previously described protocol (Rajangam et al., 2006). Agrobacterium tumefaciens strain C58C1 was grown at 28°C in Luria-Bertani medium supplemented with 50 μg mL$^{-1}$ kanamycin and 5 μg mL$^{-1}$ tetracycline to stationary phase. Bacteria were sediments by centrifugation at 5,000g for 15 min at room temperature and resuspended in infiltration solution (10 mM MES and 10 mM MgCl$_2$ supplemented with 150 μg mL$^{-1}$ acetosyringone). Cells were left in this medium for 2 to 3 h and then infiltrated into the suberial part of fully expanded N. benthamiana leaves, using a 2-mL needleless syringe. After agroinfiltration, tobacco plants were put back in the growth chambers. All the Agrobacterium strains harbored the pCH32 helper plasmid (Hamilton and Baulcombe, 1999). Bacterial suspensions were adjusted to a final OD$._{600}$ of 1.0 for the suppressor and 0.5 to 0.7 for the other construct.

**Confocal Microscopy**

Confocal microscopy of the tobacco leaves was performed 3 to 5 d after agroinfiltration on a Zeiss confocal microscope, LSM 510 META. Sections of leaves were mounted on slides in a water/glycerol solution. Images were acquired with an argon/krypton laser and the following settings for the microscope: for YFP, excitation of laser line 514 nm, emission filter BP 530 to 600 nm using a Plan-Apochromat 63×/1.4 oil objective lens.

**Binding of PttMAP20 to Microtubules**

Protein binding to taxol (Paclitaxel)-stabilized BMTs was assayed using the Microtubule Binding Protein Spin Down Assay kit of Cytoskeleton. Then 20 μL of the microtubule suspension was mixed with 1 μg of pure recombinant PttMAP20, followed by centrifugation through a cushion of 60% glycerol at 100,000g. The pellet and supernatant fractions were carefully separated after centrifugation, and the proteins in both fractions were analyzed by 10% SDS-PAGE (NuPAGE, Invitrogen). The influence of DCB on the binding of PttMAP20 onto BMTs was tested by incubating 1 μg of pure recombinant PttMAP20 with 0 to 200 μM of DCB at room temperature for 20 min, after which the solutions were added to preassembled BMTs and subjected to the spin-down binding assay.

**Labeling of PttMAP20 by \[^3H\]DCPA**

\[^3H\]DCPA was custom synthesized by PerkinElmer, as previously described (Cooper et al., 1987). The labeling experiments were performed at room temperature in a final volume of 20 μL. Standard reaction mixtures consisted of 50 mM HEPES, pH 7.0, 300 mM NaCl, 1 μg of pure PttMAP20, and 0.75 μM \[^3H\]DCPA dissolved in 10% ethanol (final concentrations). In control experiments, \[^3H\]DCPA was replaced by ethanol. Control experiments were carried out by supplementing the standard reaction with a final concentration of 2 μM, 20 μM, 200 μM, 2 μg, or 20 μg DCB (in methanol) or DCPA (in ethanol). Controls were performed by replacing DCPA and DCB by ethanol or methanol, respectively. The mixtures were incubated on ice for 5 min, subjected to UV illumination (250 nm) for 10 min, and analyzed by SDS-PAGE. After Coomassie Blue staining, the gels were incubated for 30 min in the Amplify reagent (Amersham Biosciences), excised from the gels, and deposited in bottles containing 4 mL scintillation liquid. The amount of \[^3H\]DCPA bound to PttMAP20 was determined by measuring the corresponding radioactivity using a liquid scintillation counter.

**Assembly of BMTs in the Presence of DCB**

One hundred-microgram batches of monomeric α- and β-tubulins in 20 μL of the General Tubulin buffer (Cytoskeleton) were mixed with increasing concentrations of DCB (0.75-7,500 μM) dissolved in dimethyl sulfoxide, and incubated on ice for 10 min. A final concentration of 10 μM GTP was then added to initiate polymerization of BMTs, followed by analysis of the extent of polymerization using the Microtubule Binding Protein Spin Down Assay kit (Cytoskeleton).

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession number DQ661742.
Supplemental Data

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

Supplemental Figure S1. SDS-PAGE analysis of recombinant PttMAP20 expressed in E. coli during different stages of purification. Sequence alignment of the TPX2 domains.

Supplemental Figure S2. Western blotting of proteins in plant tissue extracts and E. coli protein extracts using the PttMAP20 antibodies.

Supplemental Figure S3. Alignment of the TPX2 domains.

Supplemental Figure S4. PttMAP20 in vitro binding to BMTs.

Supplemental Table S1. Primers used for the qPCR experiment of PttMAP20 and PttCESA genes.

Supplemental Materials and Methods S1. Production of recombinant PttMAP20, specific antibodies, and associated protein chemistry.

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