Differential Expansion and Expression of α- and β-Tubulin Gene Families in *Populus*[1][W][OA]

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Microtubule organization is intimately associated with cellulose microfibril deposition, central to plant secondary cell wall development. We have determined that a relatively large suite of eight α-*TUBULIN* (*TUA*) and 20 β-*TUBULIN* (*TUB*) genes is expressed in the woody perennial *Populus*. A number of features, including gene number, α:β gene representation, amino acid changes at the C terminus, and transcript abundance in wood-forming tissue, distinguish the *Populus* tubulin suite from that of *Arabidopsis thaliana*. Five of the eight *Populus* TUAs are unusual in that they contain a C-terminal methionine, glutamic acid, or glutamine, instead of the more typical, and potentially regulatory, C-terminal tyrosine. Both C-terminal Y-type (*TUA1*) and M-type (*TUA5*) TUAs were highly expressed in wood-forming tissues and pollen, while the Y-type *TUA6* and *TUA8* were abundant only in pollen. Transcripts of the disproportionately expanded *TUB* family were present at comparatively low levels, with phylogenetically distinct classes predominating in xylem and pollen. When tension wood induction was used as a model system to examine changes in tubulin gene expression under conditions of augmented cellulose deposition, xylem-abundant *TUA* and *TUB* genes were up-regulated. Immunolocalization of TUA and TUB in xylem and phloem fibers of stems further supported the notion of heavy microtubule involvement during cellulose microfibril deposition in secondary walls. The high degree of sequence diversity, differential expansion, and differential regulation of *Populus TUA* and *TUB* families may confer flexibility in cell wall formation that is of adaptive significance to the woody perennial growth habit.

Polymers of the α-TUBULIN (TUA) and β-TUBULIN (TUB) proteins comprise dynamic arrays of cortical microtubules (MTs) that are continually reorganizing in response to developmental and environmental cues (e.g. Wasteneys, 2004), and are postulated to guide the deposition of cellulose microfibrils during cell wall formation in plants (Ledbetter and Porter, 1963). TUA and TUB proteins exhibit strong sequence conservation, with animal, plant, protist, and fungal isoforms typically sharing >88% amino acid sequence similarity (Fosket and Morejohn, 1992; Dutcher, 2001). Functional conservation of tubulins across kingdoms is supported by copolymerization of heterologous or chimeric TUA and TUB, either in vitro (Bondstar et al., 1986) or in vivo (Anthony and Hussey, 1999; Anthony et al., 1999). However, plant and animal tubulins differ in their sensitivities to various anti-MT drugs (Morejohn and Fosket, 1984), in accordance with plant- and vertebrate-specific TUA and TUB amino acid residues at consensus positions (Fosket and Morejohn, 1992). Functional heterogeneity of tubulin subunits within species, organs, or even cells is further manifest as spatiotemporally distinct gene products, many of which are subject to posttranslational modification (PTM; for review, see Luduena, 1998; McKean et al., 2001).

Spatiotemporal control of tubulin gene expression has been documented in all plant species examined. For instance, Arabidopsis (*Arabidopsis thaliana*) contains six *TUA*s encoding four distinct proteins (Kopczak et al., 1992). *ArathTUA1* is expressed specifically in pollen (Carpenter et al., 1992), while the other five are expressed in vegetative tissues (Kopczak et al., 1992; Abe et al., 2004). Of the nine Arabidopsis *TUB*s, *ArathTUB1* and *ArathTUB5* are preferentially expressed in roots and leaves, respectively, while *ArathTUB9* accumulates specifically in pollen (Snustad et al., 1992; Cheng et al., 2001). Rice (*Oryza sativa*) also contains a pollen-specific isoform (*OryzaTUB8*) and seven other *TUB*s that show variable expression during development (Yoshikawa et al., 2003). Strict temporal association of specific tubulin transcripts during cotton (*Gossypium hirsutum*) fiber development (Whittaker and Triplett, 1999; Li et al., 2002) or *Zinnia* tracheary element differentiation (Yoshimura et al., 1996) is consistent with the

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idea of a functional link between MTs and microfibril deposition during secondary wall synthesis. The five characterized *TUA*s of cotton are highly expressed in elongating fibers, but only *GoshiTUA2/3* and *GoshiTUA4* remain abundant following the onset of secondary cell wall synthesis (Whittaker and Triplett, 1999). In *Zinnia*, transcripts of *ZinelTUB1* and *ZinelTUB3* increase during trans-differentiation of mesophyll cells into tracheary elements, contrasting with the constitutive and weak expression of *ZinelTUB2* throughout the culture period (Yoshimura et al., 1996).

These data suggest a basis for examining the role of tubulin isoforms during secondary wall development in wood-forming tissues of trees, a process characterized by orderly deposition of extensive microfibril arrays. Changes in the alignment of cellulose microfibrils in developing cell walls of xylem and phloem have in fact been linked to changes in MT orientation in both hardwood and softwood tree species (Abe et al., 1995; Chaffey et al., 1997, 1999). A recent report on functional associations between cellulose synthase complexes and MTs by time-lapse visualization provides further evidence for a direct cytoskeletal guidance mechanism during cellulose deposition (Paredez et al., 2006). With the availability of the sequenced *Populus* genome, an in-depth analysis of the role of tubulin gene expression in wood-forming tissues can be initiated. We report here that the *TUA* and *TUB* gene families are differentially expanded in *Populus* compared to their herbaceous counterparts. Their encoded protein sequences are more diverse than in any other species reported so far, especially at the hypervariable C terminus. Both the TUA and TUB families contain spatiotemporally distinct isoforms, a small minority of which are highly expressed in wood-forming tissues undergoing secondary cell wall thickening. The abundance and multiplicity of xylem-expressed TUA and TUB isoforms certainly distinguish "wood" formation in *Populus* and Arabidopsis. Over the course of tree ontogeny, such flexibility of expression might reasonably be expected to contribute adaptively to perennial cellulose deposition and vascular development.

RESULTS

Cloning of Aspen *TUA* Genes

Eight *TUA* cDNAs, designated *TUA1* through *TUA8*, were isolated from quaking aspen (*Populus tremuloides* Michx.). *TUA1* was originally identified as a xylem-abundant (relative to phloem) cDNA fragment by mRNA differential display (Touchell et al., 2003) and its full-length cDNA subsequently isolated by screening an aspen xylem cDNA library. *TUA2* to *TUA8* were cloned from various aspen tissues using PCR-based approaches (see "Materials and Methods"). The assembly v1.1 of the recently released *Populus trichocarpa* genome (Tuskan et al., 2006) contains all eight *TUA* orthologs (annotated as *PoptrTUA1* through *PoptrTUA8*), with two additional truncated sequences, *PoptrTUA5tψ* and *PoptrTUA7tψ* (Table I), which show highest nucleotide identity to *PoptrTUA5* and *PoptrTUA7*, respectively, and are most likely pseudogenes. The open reading frames of the eight *TUA* genes range from 1,350 to 1,356 bp in size, and share 74% to 97% sequence identity. However, they contain distinct 3′-untranslated regions (UTRs), with sequence identity less than 62%. The eight predicted TUA proteins range in length from 449 to 551 amino acids, and show a very high degree of sequence homology to each other (88%–98% identity; Supplemental Fig. S1), except for their hypervariable C termini (Fig. 1). All plant TUA proteins identified so far contain a C-terminal Tyr residue (referred to as Y-type), which has been implicated in PTM control of tubulin stability in animal models. Interestingly, only three of the eight predicted aspen TUA proteins are Y-type. The other five terminate with either Met (i.e. M-type: class I TUA3, TUA5, and TUA7) or Glu/Gln (i.e. E/Q-type: class II TUA2 and TUA4; Fig. 1).

Phylogenetic Analysis of the TUA Family

Phylogenetic analysis of representative algal and plant full-length TUA proteins is shown in Figure 2. Plant TUAs form two distinct classes, with the eight *Populus*, six Arabidopsis, and four rice isoforms evenly distributed between the two classes. Although the bootstrap values for most nodes were low, likely due to high degree of sequence similarity, a similar topology was observed using the neighbor-joining or minimum-evolution methods with interior-branch test (only the minimum-evolution tree is shown). In general, monocot and dicot TUAs cluster separately within a class (Fig. 2). In class I, the xylem-originated PoptrTUA1 clusters with fiber-specific GoshiTUA2, GoshiTUA3, and GoshiTUA4 from cotton, consistent with involvement in secondary cell wall development. However, the three Arabidopsis class I members cluster closely with potato (*Solanum tuberosum*; SoltuTUA1, ABB02631) and two monocot TUAs from maize (*Zea mays*; ZeamaTUA3, CAA44861) and green foxtail (*Setaria viridis*; SetviTUA1, CAE52514) in a separate branch. Similarly, the class II family also includes a third and distinct branch containing both dicot and monocot TUA members, one of which (ArathTUA1) was shown to exhibit pollen-specific expression (Carpenter et al., 1992).

The four *Populus* class II *TUA*s probably descend from one ancient gene that gave rise to the progenitors of *PoptrTUA2/4* and *PoptrTUA6/8* during the eurosid genome-wide duplication, and eventually to the modern complement of *PoptrTUA2*, *PoptrTUA4*, *PoptrTUA6*, and *PoptrTUA8* following the salicoid duplication (Tuskan et al., 2006). This duplication was dated between 6 and 10 million years ago (mya), based on synonymous substitution rates of 1.5×10^{-8} and 9.1×10^{-9} mutations per site per year for dicot genes by Koch et al. (2000) and Lynch and Conery (2000), respectively. Two of the class II Arabidopsis members, *ArathTUA3* and *ArathTUA5*, are tandem repeats encoding identical

Table I. *List of TUA and TUB genes identified from the Populus genome*

Gene	JGI Gene Model	Locus
PoptrTUA1	gw1.II.3483.1	LG_II:8299090–8300967
PoptrTUA2	estExt_fgenesh4_pm.C_LG_III0736	LG_III:18595875–18598862
PoptrTUA3	estExt_Genewise1_v1.C_LG_I4174	LG_I:27533386–27535822
PoptrTUA4	estExt_fgenesh4_pg.C_LG_I0041	LG_I:292815–295515
PoptrTUA5	eugene3.00090803	LG_IX:4925341–4927829
PoptrTUA6	eugene3.00130559	LG_XIII:4169981–4173491
PoptrTUA7	estExt_Genewise1_v1.C_LG_XVII1003	LG_XVII:4138330–4140210
PoptrTUA8	estExt_fgenesh4_pg.C_LG_XIX0417	LG_XIX:5204194–5207599
PoptrTUA5tψ	gw1.III.2686.1	LG_III:12748887–12749356
PoptrTUA7tψ	gw1.VII.1672.1	LG_VII:3417187–3417348
PoptrTUB1	eugene3.00011551	LG_I:17121298–17123342
PoptrTUB2	estExt_fgenesh4_pm.C_LG_IX0574	LG_IX:8108706–8110629
PoptrTUB3	eugene3.00010923	LG_I:7896652–7899456
PoptrTUB4	eugene3.00030977	LG_III:11846773–11849592
PoptrTUB5	fgenesh4_pg.C_LG_VI000763	LG_VI:6461827–6463345
PoptrTUB6	eugene3.00161038	LG_XVI:10159508–10161158
PoptrTUB7	estExt_Genewise1_v1.C_LG_I6025	LG_I:34930733–34933465
PoptrTUB8	eugene3.01400079	scaffold_140:531237–534474
PoptrTUB9	eugene3.00010909	LG_I:7810326–7812139
PoptrTUB10	grail3.0018029802	LG_III:11963516–11965115
PoptrTUB11	eugene3.00120398	LG_XII:3902075–3904191
PoptrTUB12	estExt_fgenesh4_pm.C_LG_XV0128	LG_XV:3923544–3925244
PoptrTUB13	eugene3.00011808	LG_I:19840697–19843574
PoptrTUB14	estExt_fgenesh4_pm.C_LG_IX0458	LG_IX:6223860–6226800
PoptrTUB15	estExt_Genewise1_v1.C_LG_I1970	LG_I:19849822–19852686
PoptrTUB16	estExt_fgenesh4_pm.C_LG_IX0457	LG_IX:6218943–6221998
PoptrTUB17	eugene3.00060314	LG_VI:2140954–2144202
PoptrTUB18	eugene3.00160267	LG_XVI:1699400–1702625
PoptrTUB19	estExt_Genewise1_v1.C_LG_II0459	LG_II:1312352–1316028
PoptrTUB20	grail3.0002068402	LG_V:16706215–16709216
PoptrTUB8tψ	eugene3.00121178	LG_XII:13261307–13261577
PoptrTUB18tψ	grail3.0047019501	LG_III:15713547–15713716

proteins. This duplication was estimated to have occurred only 3 to 5 mya. None of the other *Populus* and Arabidopsis *TUA* genes is associated with genome-wide or tandem duplications, suggesting that they may have originated from other lineage-specific segmental gene duplication events. For instance, *PoptrTUA5* and *PoptrTUA7* seem to have duplicated independently around the same time as the recent *Populus* whole-genome duplication event, followed by truncations of *PoptrTUA5tψ* and *PoptrTUA7tψ*. *ArathTUA2* and *ArathTUA4* also encode identical proteins, and appear to have duplicated as an independent event 5 to 8.5 mya.

Gene Structure of the *TUA* Family

TUA genomic sequences were retrieved from the *Populus* genome portal v1.1 for gene structure analysis. The exon-intron splice junctions of all eight full-length *TUA* genes follow the GT-AG rule, but class I and class II *PoptrTUA*s differ both in number and position of introns (Fig. 3A). Three introns are located within the coding region of the class I members, whereas four are present in class II genes. While all intron positions are conserved within each subfamily, only the second intron position is common to all *PoptrTUA*s (Fig. 3A). Analysis of available genomic sequences from public databases also showed a conserved exon-intron structure among the class II Arabidopsis, maize, and rice *TUA* genes, but this is not the case for the class I family. Of the three Arabidopsis class I members, only *ArathTUA2* exhibits conserved position of all three introns. The positions of introns 1 and 3 are conserved in *ArathTUA4*, whereas only the position of the first intron is conserved in *ArathTUA6* (Kopczak et al., 1992). Other class I *TUA* genes from maize (Montoliu et al., 1990), rice (The Institute for Genomic Research Rice Database), green foxtail (Delye et al., 2004), and pea (*Pisum sativum*; Brierley et al., 1995) all contain three introns. Taken together, the distinct phylogenetic relationship and gene structures among class I and class II *TUA* members suggest their divergence before the separation of monocot and dicot plants.

In Silico Identification of *Populus TUB* Genes

Twenty *TUB* genes, designated *PoptrTUB1* through *PoptrTUB20*, were identified from the *P. trichocarpa* genome sequence and confirmed to be transcriptionally active in aspen (see below). The manually curated gene models are listed in Table I. The predicted open reading frames range in size from 1,335 to 1,356 bp, and share 76% to 96% nucleotide identity. Their 3′-UTRs are

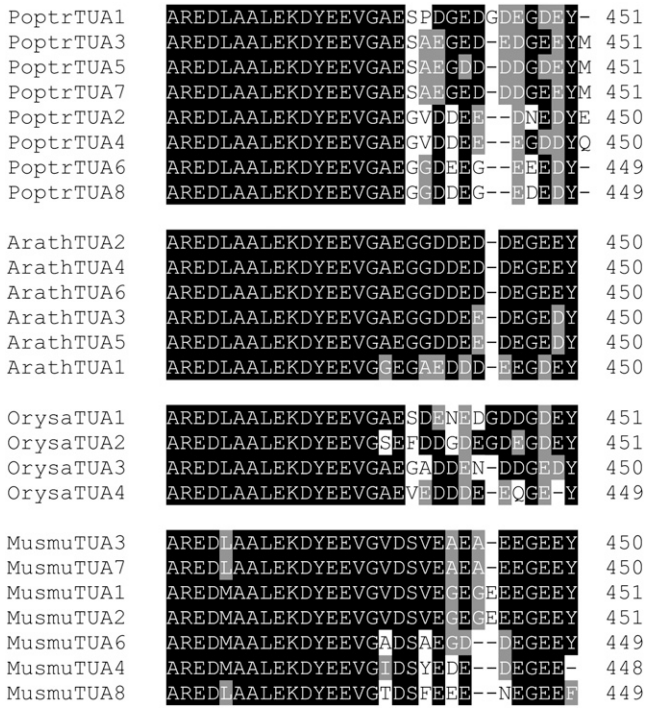

Figure 1. C-terminal sequence alignments of TUA families from *Populus*, Arabidopsis, rice, and mouse. GenBank accession numbers of the Arabidopsis, rice, and mouse proteins are provided in Supplemental Table S2.

more variable, exhibiting less than 68% sequence identity (not shown). The hypothetical PoptrTUB proteins vary from 444 to 451 amino acids in length, and, like PoptrTUAs, are highly conserved (89%–98% sequence identity overall; Supplemental Fig. S2), except for the hypervariable C termini (Fig. 4). Compared to the *PoptrTUA* family, the *PoptrTUB* family has undergone significant expansion, involving both eurosid and salicoid genome-wide duplication events, along with tandem gene duplication, giving rise to 10 pairs of highly homologous *PoptrTUB* genes. The tandem gene duplication event involving *PoptrTUB13* and *PoptrTUB15* is estimated to have occurred 4 to 7 mya, and the second event involving *PoptrTUB14* and *PoptrTUB16* 6 to 10.5 mya.

Phylogenetic analysis reveals at least four distinct classes of plant TUBs (Fig. 5). Class I and class I-like comprise by far the largest cluster, containing half of the *Populus* (10 out of 20) and known maize (four out of eight) TUB families, along with a single Arabidopsis member (ArathTUB6). Four TUBs (PoptrTUB9–PoptrTUB12) are designated as class I-like, as they lack a 5′-UTR intron that is characteristic of class I members (Fig. 3B) and do not form a tight cluster with class I TUBs (Fig. 5). Class II consists primarily of dicot TUBs, including six *Populus* and four Arabidopsis isoforms, and one each from rice, maize, and green foxtail. Class III and Class IV each contain two *Populus* and two Arabidopsis isoforms arising from genome-wide duplications. Class III members are unique in that many contain an insertion of one to three amino acids at position 39, and others have amino acid hypervariability at or near this position. Several class III and class IV members, including *ArathTUB9* (Cheng et al., 2001), *OrysaTUB8* (Yoshikawa et al., 2003), *ZeamaTUB3*, and *ZeamaTUB4* (Rogers et al., 1993), were found to be preferentially expressed in pollen. The *Populus* genome also contains two truncated *TUB* pseudogenes, designated *PoptrTUB8tψ* and *PoptrTUB18tψ* based on their closest homologs, *PoptrTUB8* and *PoptrTUB18*, respectively. The *PoptrTUB18-PoptrTUB18tψ* duplication probably occurred around the salicoid genome duplication, while the *PoptrTUB8-PoptrTUB8tψ* duplication was more recent, dated 3 to 5 mya based on molecular clock analysis.

Gene Structure of the *TUB* Family

All *PoptrTUB* genes have two introns located at conserved positions (Fig. 3B), analogous to the gene structure of the Arabidopsis TUB family previously reported (Snustad et al., 1992). This intron structure is also observed in 12 other plant *TUB* genes for which genomic sequences are available (data not shown). The only known exceptions are class II maize *ZeamaTUB1* and rice *OrysaTUB2*, both of which contain only the first intron, while the closely related *SetviTUB2* from green foxtail has two. This suggests that the second intron was lost independently in two different lineages. The first event occurred after the ancestral divergence of rice and maize/green foxtail in the lineage leading to rice. The second event occurred after the ancestral divergence of maize and green foxtail in the lineage leading to maize. Class I *TUB*s, such as *PoptrTUB13* to *PoptrTUB18* (Fig. 3B), *ArathTUB6*, *SetviTUB1*, *ZeamaTUB2*, *ZeamaTUB5*, *OrysaTUB4*, and *OrysaTUB6*, contain an additional intron within the 5′-UTR. In the case of *OrysaTUB4*, this leader intron has been shown to positively regulate transcription both in vivo and in vitro (Morello et al., 2002). Taken together, the presence of four distinct classes with members from both dicot and monocot species suggests that the angiosperm TUB family members may have originated from a single ancestral gene.

Expression Patterns of *TUA* and *TUB* Transcripts

Expression of the eight *TUA* and 20 *TUB* genes in various aspen tissues was analyzed by quantitative real-time reverse transcription (RT)-PCR using gene-specific primers (Supplemental Table S1). The class I *TUA* members, with the exception of *TUA7*, were most abundantly expressed in developing xylem of field-grown trees. The Y-type *TUA1* was by far the most abundant transcript in developing xylem, consistent with its origin as a xylem-specific cDNA, followed by the M-type *TUA5* (Fig. 6A). *TUA5* was also highly expressed in root tips. Transcripts of the other two M-type class I isoforms were detected at a lower level,

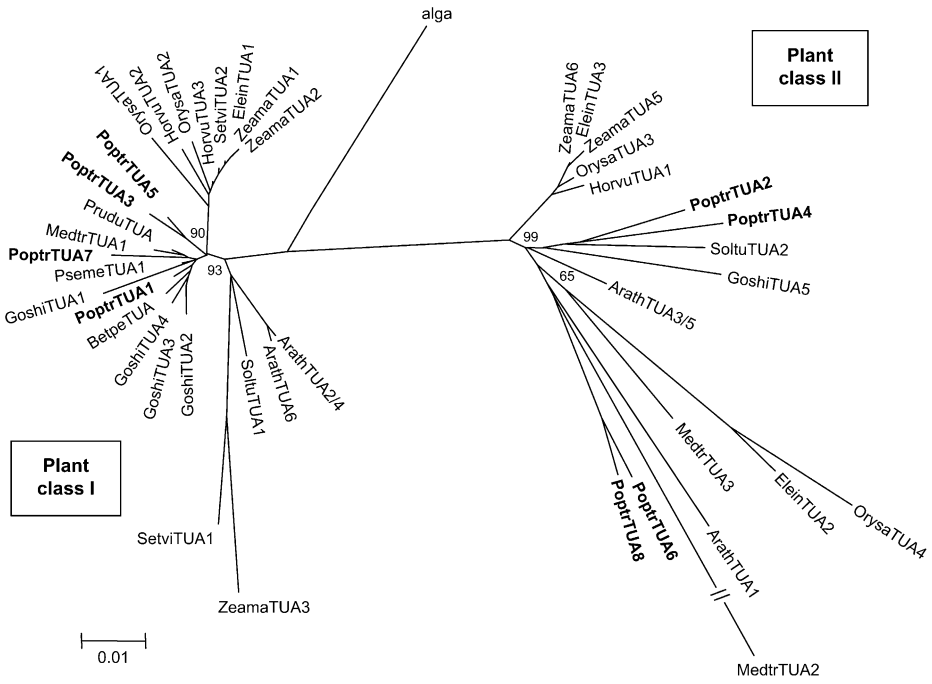

Figure 2. Minimum-evolution tree of representative full-length plant TUA proteins. The green algal (*Chlamydomonas reinhardtii*) TUA (AAN87017) was used as the outgroup. Numbers on major branches indicate percentage support of interior-branch test using 1,000 replicates. GenBank accession numbers of the sequences included are provided in Supplemental Table S2.

with *TUA3* primarily in xylem and shoot apices, and *TUA7* in apices and young leaves. It appears that both Y- and M-type class I members were abundant in xylem, whereas M-type class I TUAs predominated in all other vegetative tissues. Expression of class II *TUA*s was very weak, with no apparent preference among vegetative tissues. Aspen pollen grains contained a high level of tubulin transcripts, including both Y- (*TUA1*) and M-type (*TUA5* and *TUA7*) class I TUA members, and, most notably, the Y-type class II *TUA6/8* that were negligibly expressed elsewhere (Fig. 6A). Not surprisingly, the predicted TUA6 and TUA8 proteins cluster closely with the pollen-specific ArathTUA1 in phylogenetic analysis (Fig. 2), suggesting functional conservation. Because of the high degree of sequence identity, RT-PCR primers were initially designed to amplify both *TUA6* and *TUA8*, and detection of both transcripts was then confirmed by sequencing of randomly selected RT-PCR clones (data not shown). Additional gene-specific primers were designed to distinguish *TUA6* and *TUA8* expression in pollen. Real-time RT-PCR analysis showed that both genes were well expressed in pollen, but *TUA8* transcripts were 3-fold more abundant than *TUA6* (Fig. 6A, inset).

TUB9 and *TUB15* represent the predominant *TUB* species in developing xylem, followed by *TUB16* and *TUB13* (Fig. 6). Transcripts of *TUB15* were also detected in roots and pollen grains. The other *TUB* genes were expressed at low levels in vegetative tissues, but many of the class II, III, and IV *TUB* members were well expressed in pollen (Fig. 6C). Pollen expression of class III *TUB19* and *TUB20* and class IV *TUB7* and *TUB8* is consistent with their phylogenetic clustering with other pollen-specific TUB isoforms from Arabidopsis, rice, and maize (Fig. 5). We noticed that expression of *TUB* paralogs, such as class I *TUB9* versus *TUB10*, *TUB13* versus *TUB15*, and *TUB14* versus *TUB16*, differed, consistent with the possibility of functional divergence. This has also been noted in Arabidopsis for its class III members, with *ArathTUB1* exhibiting root-preferential expression and *ArathTUB5* being leaf abundant (Snustad et al., 1992).

Expression of *TUA* and *TUB* Transcripts in Tension Wood

Tension wood is an angiosperm-specific response to gravitational stress, characterized by altered microfibril structure and properties due to the presence of a gelatinous layer that is composed primarily of cellulose (Norberg and Meier, 1966). An increased abundance of MTs, in parallel with microfibrils, has been noted in tension wood fibers (Fujita et al., 1974; Prodhan et al., 1995). We therefore measured *TUA* and *TUB* expression in tension wood tissue. As shown in Figure 7, xylem-abundant *TUA1* and *TUA5* were up-regulated 2- to 4-fold in tension wood. Transcripts of weakly expressed class II *TUA4* and *TUA8* were also induced. Similarly, expression of xylem-preferential *TUB9*, *TUB15*, and *TUB16* was up-regulated by tension stress, as was the less abundant *TUB3*, *TUB4*, *TUB7*, and *TUB19*. Similar up-regulation of three secondary cell wall-associated cellulose synthase genes and one Korrigan endoglucanase in experimentally induced tension wood has also been reported in aspen (Bhandari et al., 2006). Thus, xylem-abundant *TUA* and *TUB* genes are coordinately regulated with cellulose biosynthesis machinery.

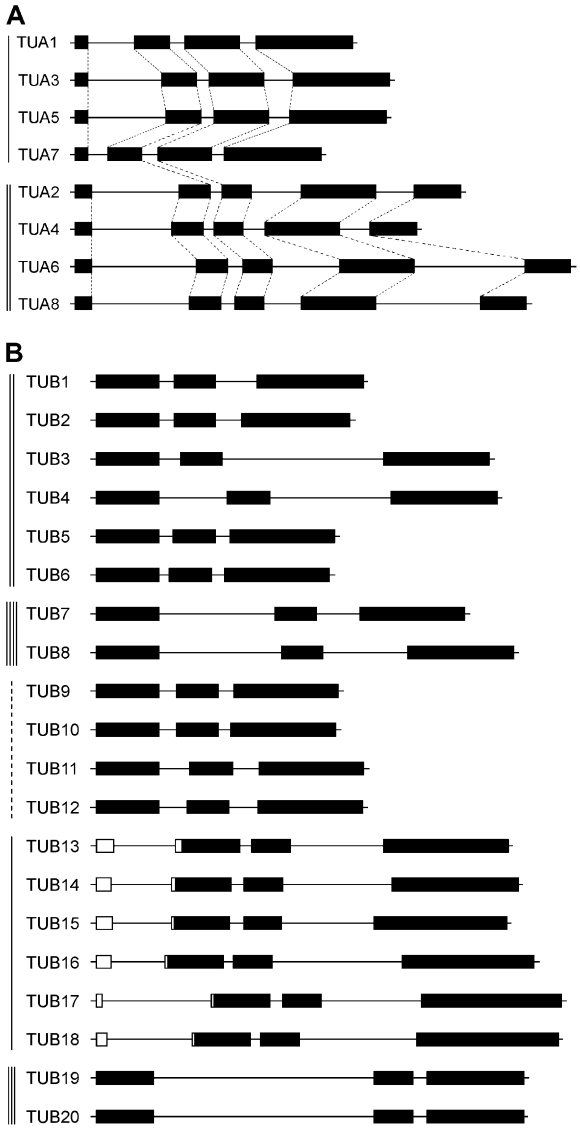

Figure 3. *Populus TUA* and *TUB* gene structure. A, Class I *TUA*s contain three introns, while class II *TUA*s have four. Intron positions are conserved within each class, but only the second intron is conserved in all *TUA* genes (dashed lines denote position conservation). B, All *TUB* genes have two introns within their coding sequences at conserved positions. Class I members contain an additional intron in the 5′-UTR. Class designation is marked by vertical lines on the left, with a dashed line representing the class I-like TUB group.

Fiber-Specific Localization of Tubulins in Developing Stems

Cellular distribution of tubulin proteins in aspen stem sections was investigated by immunolocalization (Fig. 8). Using a polyclonal anti-TUA antibody raised against recombinant aspen TUA1, no specific signal was detected at the third internode (data not shown), but conspicuous staining in developing phloem fibers and newly formed xylem fibers was observed at the sixth internode (Fig. 8A). The signal intensified in stem sections undergoing secondary cell wall thickening (Fig. 8, B, C, E and F). In xylem, the strongest signal was found in developing fibers, with weak staining observed in some ray parenchyma and newly developed vessel cells adjacent to the cambial zone. Strong signals were also found in phloem fibers with extensive secondary cell wall development. Weak signals were observed in ray initials of the cambial zone and in some newly developed phloem cells. Similar immunolocalization patterns were observed using a commercially available polyclonal anti-TUB peptide-antibody (abcam ab15568; Fig. 8, E and F). The weaker TUB signals from thin-walled ray parenchyma, phloem,

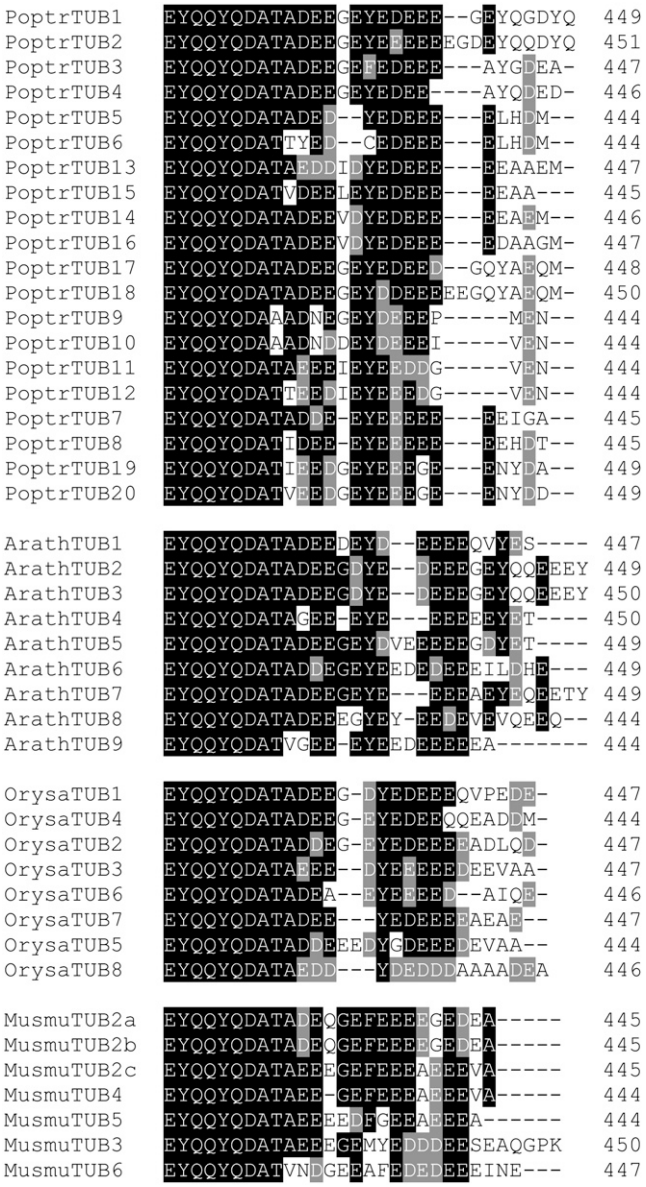

Figure 4. C-terminal sequence alignments of TUB families from *Populus*, Arabidopsis, rice, and mouse. GenBank accession numbers of the Arabidopsis, rice, and mouse proteins are provided in Supplemental Table S2.

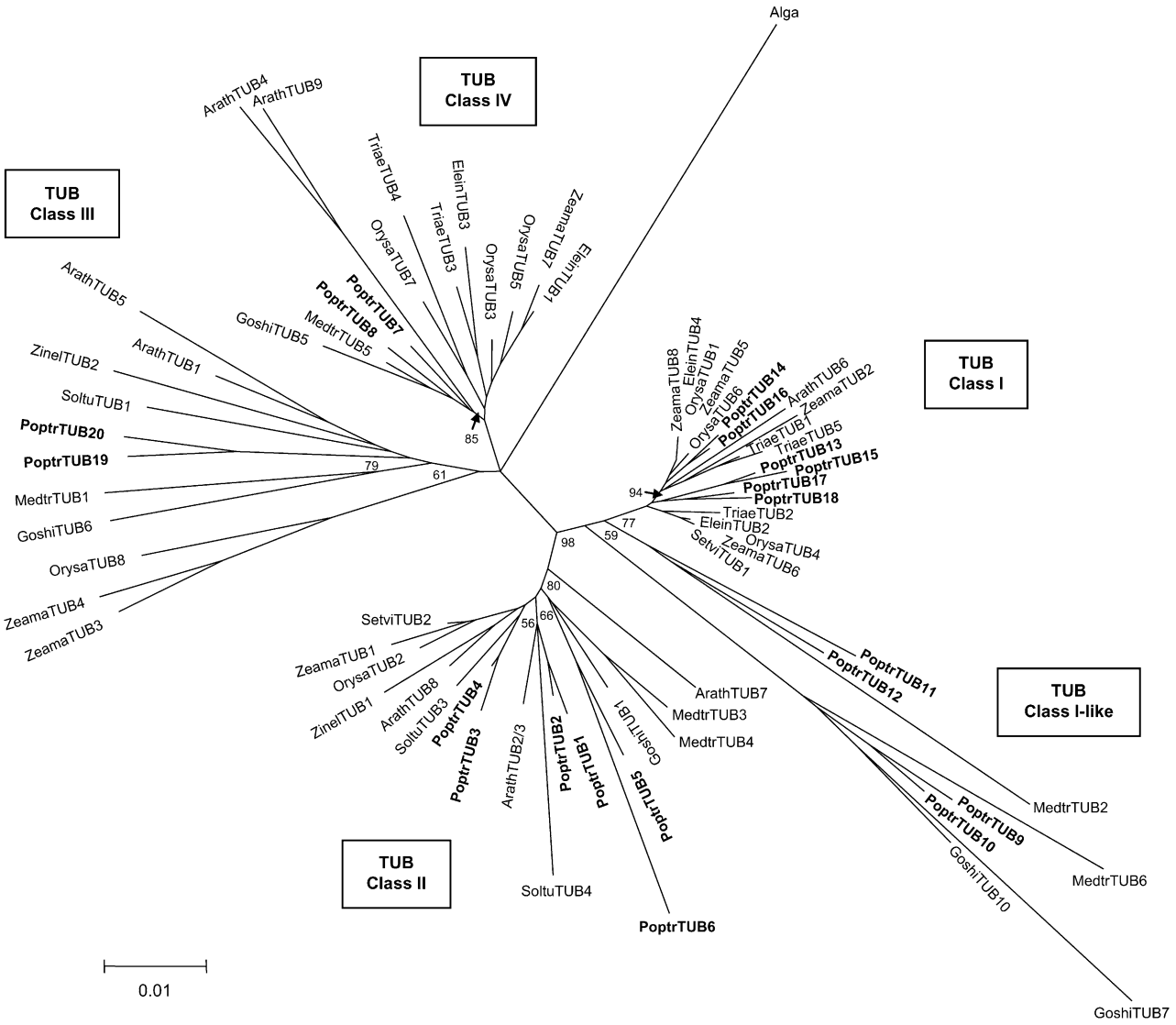

Figure 5. Minimum-evolution tree of representative full-length plant TUB proteins. The green algal (*C. reinhardtii*) TUB (P04690) was used as the outgroup. Numbers on major branches indicate percentage support of interior-branch test using 1,000 replicates. GenBank accession numbers of the sequences included are provided in Supplemental Table S2.

and pith cells (Fig. 8, E and F versus B and C) may reflect differential sensitivity of single-epitope (TUB) versus purified recombinant fusion protein (TUA) antigens. Although we do not ascribe the anti-TUA and anti-TUB signals to specific tubulin isoforms, the results show nevertheless that the most abundant tubulin species in wood-forming tissues are localized to fiber cells.

DISCUSSION

In mammalian systems (e.g. human and mouse), the TUA and TUB families are identical in size, with seven genes each (Sullivan, 1988; Stanchi et al., 2000). The family sizes, however, vary in higher plants, with six *TUA* and nine *TUB* genes in Arabidopsis, expanding to eight TUAs and 20 *TUB*s in the recently sequenced woody perennial *Populus*. We now know that *Populus* experienced two genome-wide gene duplication (eurosid and salicoid) events, followed by a series of chromosomal reorganizations, involving reciprocal tandem/terminal fusions and translocations (Tuskan et al., 2006). Interestingly, all 20 *PoptrTUB* genes are associated with genome-wide or tandem gene duplication events, whereas only four of the eight *PoptrTUA* genes are so derived. What led to the distinct patterns of gene retention (with regard to TUB) and/or loss (with regard to TUA) for two families of proteins that form 1:1 heterodimers is not known. Quantitative RT-PCR detection and sequence confirmation of all

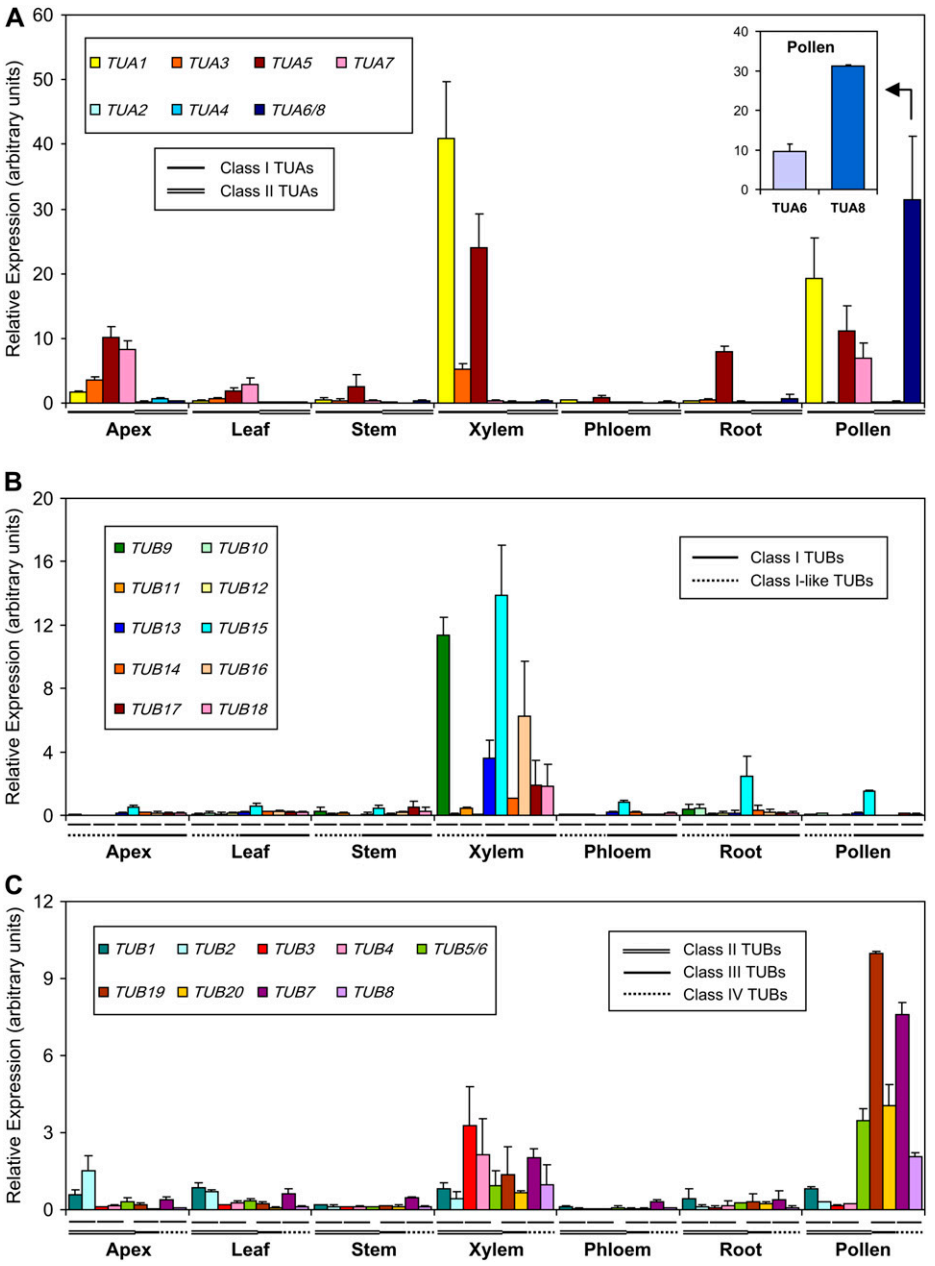

Figure 6. Relative *TUA* and *TUB* transcript levels in various aspen tissues. Expression levels were normalized to the geometric mean of three housekeeping genes. Error bars represent the measurement range of two biological replicates. A, Relative expression of *TUA*s. Both *TUA6* and *TUA8* transcripts were detected by the *TUA6/8* primers. Inset, Gene-specific detection of *TUA6* and *TUA8* transcript levels in pollen. B and C, Relative expression of *TUB*s. Both *TUB5* and *TUB6* transcripts were detected by the *TUB5/6* primers. Different *TUA* and *TUB* classes are denoted below the *x* axis by solid, double, or dashed lines as shown in the legend of each section. Hairlines immediately below the *x* axis in B and C specify paralogous *TUB* gene pairs. Primer sequences are provided in Supplemental Table S1.

20 *TUB* amplicons in various aspen tissues excludes the possibility that any *TUB* gene is not expressed.

When viewed in the context of coordinated regulation of TUA and TUB in other species, the selective expansion of the *TUB* gene family in *Populus* is interesting. In yeast (*Saccharomyces cerevisiae*), overexpression of *TUA* or *TUB* can lead to arrest of cell division, but a lethal phenotype was observed only in *TUB*-overexpressing strains, suggesting that transcript levels of *TUB* in excess of *TUA* are uniquely toxic (Weinstein and Solomon, 1990). Cotransformation of *TUA* and *TUB* was an absolute requirement for recovery of transgenic maize and tobacco (*Nicotiana tabacum*), as overexpression of either *TUA* or *TUB* alone was lethal (Anthony and Hussey, 1998, 1999). In the case of aspen, attempts to transgenically manipulate *TUA1* expression also failed to produce viable transformants via *Agrobacterium* transformation and organogenesis (R.V. Oakley and C.-J. Tsai, unpublished data), suggesting that tight regulation of the *TUA*:*TUB* transcript ratio is also important in *Populus*. Indeed, real-time RT-PCR analysis suggests that compensatory expression may be important to maintain a viable functional balance of *TUA*:*TUB* in *Populus*. In xylem, for instance, fewer *TUA* (e.g. *TUA1* and *TUA5*) than *TUB* (e.g. *TUB9*, *TUB13*, *TUB15*, and *TUB16*) genes

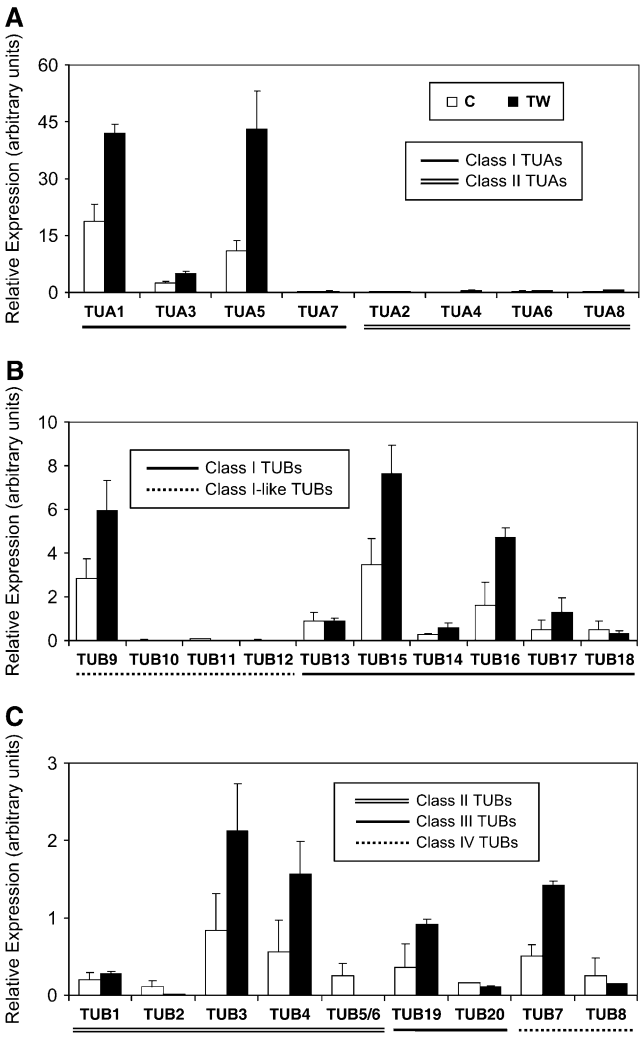

Figure 7. Relative transcript levels of *TUA* and *TUB* genes in developing xylem of normal (C) and tension wood (TW) tissues from aspen. Expression levels were normalized to the geometric mean of three housekeeping genes. Error bars represent the measurement range of two biological replicates.

were expressed, but the relative transcript levels of the two xylem-predominant *TUA*s were much higher than those of the *TUB*s. The same principle appeared to operate in pollen. The finding that xylem-abundant TUA, but not TUB, isoforms are also highly expressed in pollen is interesting, and may be viewed as a possible outcome of the differential evolution of these two gene families in *Populus*. The smaller TUA family appears to exhibit broader functionality, in this case, regulating wood formation as well as pollen development, whereas the large TUB family exhibits genewise redundancy and classwise functional specialization. Taken together, the variations in *TUA* and *TUB* family size and the quantitative differences in expression of individual gene family members lead us to hypothesize that transcriptional regulation is an important aspect of MT function in *Populus*.

The identification of five novel *Populus* TUAs with a unique C-terminal Met, Glu, or Gln residue is of particular interest with regard to tyrosination/detyrosination of TUA, a PTM that has been extensively characterized in mammalian systems (MacRae, 1997). It involves the cyclic removal and re-attachment of the C-terminal Tyr (or Phe in MmTUA8; Fig. 1) by tubulin-specific carboxypeptidase and tubulin Tyr ligase (TTL), respectively (Idriss, 2000). Detyrosinated TUA can give rise to Δ2-TUA (also called nontyrosinatable TUA), which is excluded from the tyrosination/detyrosination cycle due to loss of the C-terminal TTL recognition motif GEE(Y) (Rudiger et al., 1994). Interestingly, the TTL motif is poorly conserved in Arabidopsis, rice, and *Populus* isoforms (Fig. 1). This suggests that detyrosination of plant TUAs, if it occurs, may not be reversible. Consistent with this is the lack of a clear TTL homolog in plants. The single-copy Arabidopsis (At1g77550) and *Populus* (Joint Genome Institute [JGI] gene model eugene3.00280021) genes annotated as "TTL family protein" encode polypeptides of 855 to 869 amino acids that are much larger than the well-characterized mammalian TTL of 370 amino acids (Ersfeld et al., 1993). Although detyrosinated and nontyrosinatable TUAs have been reported in tobacco suspension cells using mammalian PTM-specific antibodies (Smertenko et al., 1997), they have not been detected in planta (Duckett and Lloyd, 1994; Gilmer et al., 1999). PTMs afford a means to modulate tubulin diversity and MT dynamics in animals (for review, see MacRae, 1997; Westermann and Weber, 2003). Given the greater sequence diversity at the C terminus of *Populus* TUA and TUB isoforms, it would be of interest to investigate the role and extent of tubulin PTMs in this woody perennial. Such investigation will likely depend on proteomics approaches and development of plant tubulin PTM-specific antibodies.

Our results showed that both TUA and TUB families in *Populus* contain distinct isoforms, some with particularly strong expression in tissues undergoing secondary cell wall thickening and others with strong expression in pollen. Pollen-specific tubulin isoforms are phylogenetically conserved (Figs. 2 and 5), although the role of MTs in pollen development remains elusive. Recent characterization of pollen-specific kinesin-related motor proteins (Cai et al., 2000; Romagnoli et al., 2003) and MT-associated protein (Huang et al., 2007) support MT's participation with actin filaments in directing vesicle and organelle transport in elongating pollen tubes. MTs likely play a role in deposition of pollen cell wall components, e.g. pectins, callose, and cellulose, and in maintenance of the cylindrical shape of the pollen tube (Cai et al., 2005).

Several lines of evidence suggest that the xylem-predominant TUA and TUB isoforms are specifically associated with cellulose synthesis during secondary cell wall formation. First, TUA and TUB protein immunolocalization signals are strongest in xylem and phloem fiber cells of aspen stems undergoing secondary wall thickening (Fig. 8). Second, several of the

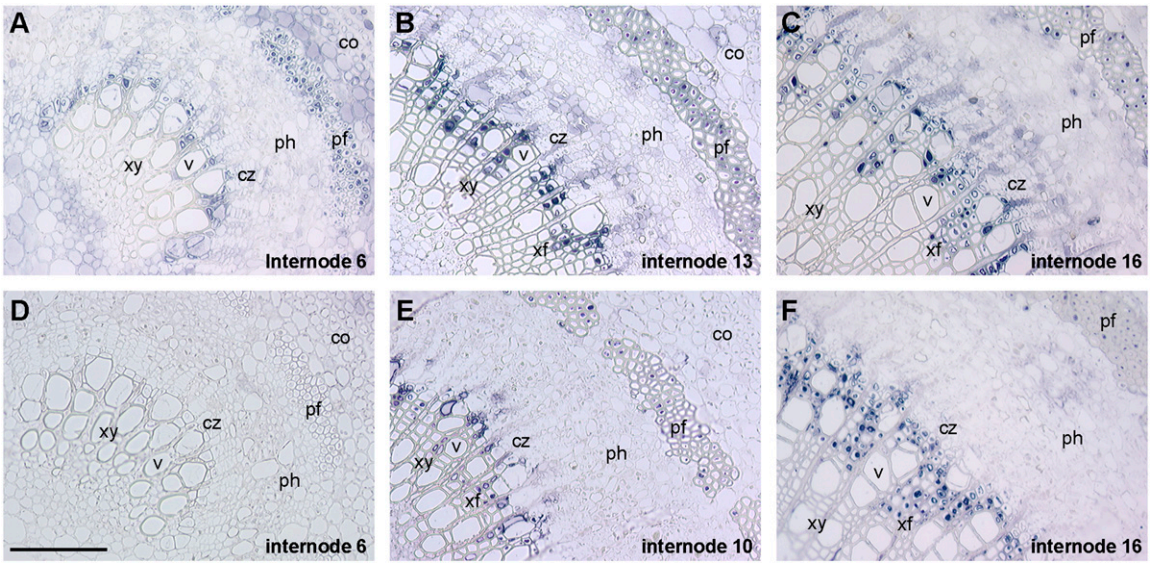

Figure 8. TUA and TUB protein localization in aspen stem sections. The most abundant TUA and TUB immunosignals are detected in xylem and phloem fibers of developing stem sections, using polyclonal antibodies raised against recombinant aspen TUA1 (A–C) or TUB peptide (E and F). A preimmune serum was used as control (D). Scale bar = 100 μm. co, Cortex; cz, cambial zone; pf, phloem fiber; ph, phloem; v, vessel; xf, xylem fiber; xy, xylem.

xylem-abundant and tension wood-induced *TUA* and *TUB* genes were among the most abundant transcripts detected by microarray or EST analysis during normal and tension wood formation in *Populus* (Hertzberg et al., 2001; Pilate et al., 2004; Andersson-Gunneras et al., 2006). Third, allelic variation in a loblolly pine (*Pinus taeda*) *TUA* gene was found to be significantly associated with earlywood microfibril angle in an association genetics study of wood quality traits (Gonzalez-Martinez et al., 2007). The deduced protein sequence of this pine *TUA* (EST contig 8045 available at http://biodata.ccgb.umn.edu/nsfpine/contig_dir20/) is identical to PsemeTUA1 from Douglas fir (*Pseudotsuga menziesii*) and clusters closely with class I *Populus* TUAs (Fig. 2). Finally, silencing the *Eucalyptus* homolog of *PoptrTUB9* has recently been shown to affect cellulose microfibril angle using induced somatic sector analysis (Spokevicius et al., 2007). Available molecular evidence thus supports in situ and, recently, in vivo visualization studies for a role of cortical MTs in the deposition and orientation of cellulose microfibrils (Ledbetter and Porter, 1963; Giddings and Staehelin, 1991; Paredez et al., 2006). In wood fibers undergoing active secondary cell wall synthesis, the process appears to involve specialized TUA and TUB subunits.

Exactly how MTs exert an influence over microfibril deposition remains unclear, but it likely involves multiple factors, including an assortment of MT-associated proteins (Burk and Ye, 2002; Zhong et al., 2002) and biophysical feedback signals from cellulose microfibrils (Fisher and Cyr, 1998; Himmelspach et al., 2003). Perennial secondary growth is the most prominent of the vegetative growth features distinguishing woody species from herbaceous annuals. Although many aspects of wood formation appear to be conserved between *Populus* and Arabidopsis, e.g. lignin and cellulose biosynthetic gene networks (Ehlting et al., 2005; Geisler-Lee et al., 2006), the underlying cytoskeletal processes orchestrating cellulose MF deposition and orientation may depend on tubulins in ways that are less conserved. *TUA* and *TUB* gene expression has not been associated with secondary growth in Arabidopsis (Ko et al., 2004; Ehlting et al., 2005). The discrepancy in tubulin regulation appears to coincide with differential evolution of the secondary cell wall-associated TUA and TUB isoforms. The Arabidopsis class I TUA members are unusual in that two of them lack the conserved class I gene structure, and that they cluster separately from the main dicot class I branch, including the fiber-specific poplar and cotton TUAs. In the case of TUB, the class I and class I-like group is underrepresented in Arabidopsis, with a lone member. By contrast, *Populus* possesses a disproportionately large group of class I and I-like TUBs (10 of 20), encompassing all of the xylem- and tension wood-predominant isoforms. Taken together, our data suggest that increased tubulin isoform diversity along with elevated transcript abundance characterize wood and fiber formation in *Populus*. This may be in accordance with expanded transcriptional and posttranslational regulation of tubulin dynamics to sustain long-term, perennial growth of large woody plants. Future investigations into tubulin expression and possible PTMs in response to the life-long continua of developmental and biotic and abiotic cues trees face may be necessary to shed additional light on the functional significance of the expanded tubulin gene families in *Populus*.

MATERIALS AND METHODS

Plant Materials

Shoot apices, young leaves, stems (internodes 10–14 from the apex), and root tips (terminal 5 mm) were obtained from greenhouse-grown aspen (*Populus tremuloides*). Developing secondary xylem and phloem were collected from wild aspen trees on the campus of Michigan Technological University during the peak growing season (July to early August) of 1999 through 2004. Developing tension wood xylem was harvested from the upper side of aspen trees bent at a 30 to 40 degree angle from the vertical axis for 3 months. Reproductive branches were taken from individual wild aspen trees in the vicinity of Houghton in April 2006, and maintained in a greenhouse until flowering. Pollen shed from mature male catkins was collected. Tissues were snap-frozen in liquid nitrogen and stored at −80°C until use.

DNA Cloning and Sequencing

Total RNA was extracted according to Chang et al. (1993) and treated with TURBO DNase (Ambion) to remove contaminating DNA. A partial *TUA1* fragment was first identified by mRNA differential display following the protocols of Touchell et al. (2003) and the full-length cDNA obtained by screening of an aspen xylem cDNA library (Ge and Chiang, 1996), according to standard recombinant DNA techniques (Sambrook and Russell, 2001). PCR-based approaches were used to clone other tubulin fragments, using cDNA synthesized with SuperScript II reverse transcriptase and an oligo(dT) primer according to the manufacturer's instructions (Invitrogen). Partial cDNA fragments of the other *TUA* genes were obtained by RT-PCR of phloem (*TUA2*, *TUA5*), apex (*TUA3*), or xylem (*TUA4*, *TUA6*, *TUA7*, *TUA8*) cDNA, using degenerate or gene-specific primers (Supplemental Table S1). Full-length cDNA clones were obtained by RT-PCR using gene-specific primers (Supplemental Table S1) or by 5′-RACE using the SMART RACE cDNA amplification kit (CLONTECH). PCR products were purified using the UltraClean PCR clean-up kit (MoBio) and cloned into the pCRII vector (Invitrogen). Positive clones were sequenced fully from both directions using the CEQ dye terminator cycle sequencing quick start kit and the CEQ8000 genetic analysis system (Beckman Coulter).

Sequence Analysis

Full-length amino acid sequences were aligned by ClustalW 1.82 (Chenna et al., 2003) and displayed with BOXSHADE 3.21 (http://www.ch.embnet.org/software/BOX_form.html). Phylogenetic analysis was performed with MEGA 3.1 (Kumar et al., 2004), using the neighbor-joining or minimum-evolution methods. Rooted trees were generated from the interior-branch test with 1,000 iterations, using the equal-input model (heterogeneous patterns) and the complete-deletion option for handling alignment gaps. Similar topologies were obtained when the C-terminal hypervariable regions were excluded from the sequence alignment and phylogenetic analyses (not shown). The accession numbers of sequences included in the phylogenetic analyses are listed in Supplemental Table S2. Genomic sequences corresponding to the eight *TUA* and 20 *TUB* genes were retrieved from the *Populus* genome portal (v1.1) hosted at the JGI and the exon-intron structure displayed using the Gene Structure visualization tool (http://warta.bio.psu.edu/cgi-bin/Tools/StrDraw.pl).

Estimation of Gene Duplication Dates

Protein coding regions of genes were aligned and nucleotide substitution rates were estimated using the distance measures of Nei and Gojobori, and the Jukes-Cantor correction implemented in MEGA 3.1. Synonymous substitution rates were used for calculating divergence and duplication times (T) of genes using $k = K_s/2T$, where k represents absolute rate of synonymous substitution per site per year for dicots, and K_s is the estimated number of synonymous substitutions per site between homologous sequences using the Kimura two-parameter method as described by Gaut et al. (1996) and Ramakrishna et al. (2002). This estimation was similarly applied to dating of gene duplications in *Populus* and Arabidopsis (*Arabidopsis thaliana*).

Real-Time RT-PCR Analysis

Relative transcript abundance of all tubulin isoforms in various aspen tissues was analyzed by real-time RT-PCR using the ABsolute QPCR SYBR Green Mix (Abgene) and the Mx3000P real-time PCR system (Stratagene). Gene-specific primers (Supplemental Table S1) flanking 94- to 294-bp amplicons near the 3′-UTRs were designed based on both cloned and JGI-predicted cDNA sequences and, whenever possible, GenBank *Populus* EST sequences. Each reaction was performed in duplicate with two biological replicates, using cDNA synthesized from 2.5 ng of total RNA. The specificity of amplification was assessed by dissociation curve analysis at the end of each run using the MxPro software (Stratagene), and confirmed by cloning and sequencing of the PCR products, including the *PoptrTUA6/8* and *PoptrTUB5/6* pairs that could not be distinguished by RT-PCR primers. Relative target transcript levels normalized to the geometric mean of three housekeeping genes (*ACTIN*, *ELONGATION FACTOR1β*, and *UBIQUITIN*) were determined using the ΔCT method (Tsai et al., 2006).

Immunolocalization

Immunolocalization was conducted according to Li et al. (2001) using butyl methyl methacrylate-embedded thin (3-μm) stem sections. Polyclonal anti-TUA antibodies were raised in rabbits using affinity-purified aspen TUA1 recombinant protein (Alpha Diagnostic). The anti-TUB antibodies (ab15568) were obtained from Abcam, and were raised against a synthetic human TUB peptide (416–430) that is highly conserved among PoptrTUBs and corresponds to the C-terminal helix H12 on the outside surface of the MT according to the electron crystallography (Nogales et al., 1998). Anti-TUA antibodies were used at 1/10,000 dilution and anti-TUB serum at 1/2,000 dilution in immunohybridization. Preimmune serum was used as the control. Hybridization signal was colorimetrically detected using nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate. Sections were then mounted in glycerol and images were recorded using a Nikon E-400 microscope equipped with a digital imaging system.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers AY229881 and AY229882, EF583813 to EF583816, and EF584828 to EF582849.

Supplemental Data

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

Supplemental Figure S1. Sequence alignment of full-length *Populus* TUA proteins.

Supplemental Figure S2. Sequence alignment of full-length *Populus* TUB proteins.

Supplemental Table S1. List of primers used for cloning and real-time RT-PCR analysis.

Supplemental Table S2. List of proteins and their GenBank accession numbers used in sequence alignment and phylogenetic analyses.

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