Cortical microtubules (MTs) are evolutionarily conserved cytoskeletal components with specialized roles in plants, including regulation of cell wall biogenesis. MT functions and dynamics are dictated by the composition of their monomeric subunits, α- (TUAs) and β-tubulins (TUBs), which in animals and protists are subject to both transcriptional regulation and post-translational modifications (PTM). While spatiotemporal regulation of tubulin gene expression has been reported in plants, whether and to what extent tubulin PTMs occur in these species remain poorly understood. We chose the woody perennial *Populus* for investigation of tubulin PTMs in this study, with a particular focus on developing xylem where high tubulin transcript levels support MT-dependent secondary cell wall deposition. Mass spectrometry and immunodetection concurred that detyrosination, non-tyrosination and glutamylation were essentially absent in tubulins isolated from wood-forming tissues of *P. deltoides* and *P. tremula × alba*. Label-free quantification of tubulin isotypes and RNA-Seq estimation of tubulin transcript abundance were largely consistent with transcriptional regulation. However, two TUB isotypes were detected at noticeably lower levels than expected based on RNA-Seq transcript abundance in both *Populus* species. These findings led us to conclude that MT composition during wood formation depends exclusively on transcriptional and, to a lesser extent, translational regulation of tubulin isotypes.

**Keywords:** detyrosination, non-tyrosination, glutamylation, acetylation, tension wood, microtubules

**INTRODUCTION**

Microtubules (MTs) are filamentous cytoskeleton components made up of α- and β-tubulins. In plants, MTs play critical roles in regulating intracellular trafficking, morphogenesis, and cellulose microfibril deposition during cell wall formation (Wasteneys, 2002). In the woody perennial *Populus*, α- (TUAs) and β-tubulins (TUBs) are encoded by relatively large multi-gene families, with highly conserved amino acid sequences (88–98% identities), except for the hypervariable C-terminus. The C-terminal tails of tubulin are of interest because they are hotspots for post-translational modifications (PTMs) in animals and protists (MacRae, 1997; Westermann and Weber, 2003; Magiera and Janke, 2014). Although tubulin gene expression (Oakley et al., 2007) and transgenic manipulation (Swamy et al., 2015) have been reported in *Populus*, characterization at the protein level has been largely unexplored.
Detyrosination, polyglutamylation and polyglycylation comprise the most well-characterized C-terminal PTMs of animal tubulins, in addition to acetylation that occurs in the N-terminus (Magiera and Janke, 2014). Immunological evidence for most of these PTMs has been reported in plants: detyrosination in tobacco, maize, grapevine and soybean; non-tyrosination (A2) in tobacco; polyglutamylation in tobacco, maize and soybean; and acetylation in numerous angiosperms (Smertenko et al., 1997, 1998; Huang and Lloyd, 1999; Wang et al., 2004; Parrotta et al., 2009; Iovanović et al., 2010; Nakagawa et al., 2013; Gzyl et al., 2015; Hotta et al., 2016). However, immunological detection can lead to equivocal conclusions (Hotta et al., 2016), especially when using animal-derived tubulin PTM antibodies of unknown specificities against plant tubulins (Parrotta et al., 2009). By comparison, mass spectrometry (MS)-based proteomics analysis provides a higher resolution approach for identification and quantification of tubulin isotypes and PTM isoforms (Verdier-Pinard et al., 2009; Miller et al., 2010). For clarity throughout, ‘isotype’ refers to genetically encoded tubulins and ‘isoform’ refers to their PTM variants (Parrotta et al., 2014). MS-based analysis is imperative for substantiating findings from antibody-based results, which can be equivocal due to high levels of sequence homology among numerous tubulin isotypes and the variable nature of (some) PTMs. For instance, detyrosinated and polyglutamylated tubulin isoforms were detected in tobacco suspension cells by immunofluorescence microscopy and immunoblotting (Smertenko et al., 1997), but were deemed absent in a recent study, also with cultured tobacco cells, by immunoblot and MS analyses (Hotta et al., 2016). The absence of detyrosination and polyglutamylation signals was also reported for Arabidopsis cells in the latter study (Hotta et al., 2016), challenging the occurrence of tubulin C-terminal PTMs in plants.

In our initial characterization of the Populus tubulin families, we described several Populus TUA genes that are predicted to harbor an unusual C-terminal Met, Gli or Gln instead of the evolutionarily conserved C-terminal Tyr (Oakley et al., 2007). This finding, along with discussion therein about the lack of an apparent homolog of tubulin Tyr ligase (TTL) in sequenced plant genomes (Oakley et al., 2007), raised the question whether the TUA detyrosination-tyrosination cycle is active in plants. Recently, we showed that detyrosination and non-tyrosination of TUA were negligible in P. tremula × alba based on immunoblotting and MS analysis (Swamy et al., 2015). In the present study, we expanded the investigation to survey tubulin isotypes and their PTMs in P. deltoides. We focused on developing xylem because it undergoes extensive MT-dependent secondary cell wall thickening (Funada, 2008) and exhibits very high tubulin transcript levels especially in tension wood (TW; Oakley et al., 2007). TW fibers formed in response to gravitational stimuli are characterized by a cellulose-enriched gelatinous layer with increased MT abundance compared to normal wood (NW) fibers (Plomion et al., 2001; Pilate et al., 2004). While tubulin transcript levels increased substantially during TW formation (Oakley et al., 2007), there are no reports on whether auxiliary mechanisms including PTM may also become engaged. We took advantage of the proteomics dataset from P. tremula × alba xylem (Swamy et al., 2015) for comparative analysis. In both cases, RNA-Seq-based de novo tubulin transcript assembly was undertaken in order to correct for sequence variations from the P. trichocarpa reference genome that could affect the accuracy of proteomics data analysis. The RNA-Seq data also permitted an assessment of xylem tubulin transcript abundance in both species. Our results indicated that C-terminal tubulin PTMs were undetectable in Populus xylem, in contrast to animal systems where their occurrence is commonplace. We interpret the results to suggest that genetically encoded diversity and other regulatory mechanisms supplant PTM modulation in Populus, even in MT-rich wood forming tissues.

**MATERIALS AND METHODS**

**Plant Materials**

Bulk samples of developing xylem were scraped into liquid nitrogen from the debarked trunks of 5-year-old, field-grown P. deltoides trees. TW xylem was obtained from the upper side of the trunk leaned at a 30–40° angle from the vertical axis for 4 weeks. Snap-frozen samples were stored at −80°C until use.

**Tubulin Purification**

Approximately 5 g of xylem tissue was ground into a fine powder in liquid nitrogen for tubulin purification using a modified DEAE-Sephadex chromatography method (Morejohn and Fosket, 1982; Smertenko et al., 1997). The tissue powder was suspended in 10 ml of PEM buffer (50 mM PIPES, pH 6.9; 0.5 mM MgCl₂; 1 mM EGTA and 1 mM DTT) with protease inhibitors (1 mM benzamidine HCl; 2 mM leupeptin; 15 mM pepstatin A; 1 mM phenylmethylsulfonyl fluoride; 1 mM sodium fluoride and 50 μM N-tosyl-L-phenylalanine chloromethyl ketone) and 2 mM GTP, and vortexed vigorously. The mixture was first clarified at 50,000 g for 10 min, and the supernatant ultracentrifuged at 100,000 g for 45 min, both at 2°C. The resulting supernatant was mixed with 0.5 volumes of PEM-equilibrated DEAE-Sephadex A50 containing 0.5 mM GTP and incubated at 4°C for 1 h with gentle agitation. The mixture was loaded into a polypropylene chromatography column (0.8 cm × 4 cm, BioRad), and washed with 3–5 volumes of 0.4 M KCl in PEM buffer containing 0.1 mM GTP. The bound tubulin proteins were then eluted with 0.8 M KCl in PEM buffer with 0.1 mM GTP. The protein-rich fractions were pooled and dialyzed against 1 L of 10 mM NH₄HCO₃ at 4°C overnight with one buffer change. The protein was concentrated using a Nanosep centrifugal column (MWCO 10K, Amicon) and the concentration was estimated using Bradford reagents (BioRad). Recombinant TUA1 in pET30a (Oakley et al., 2007) expressed in E. coli strain BL21 (DE3) was purified from inclusion body using BugBuster protein extraction reagent (Novagen), and quantified with BCA protein assay kit (Novagen).

**Western blotting**

Seventy-five nanograms of purified tubulins were resolved on a 10% SDS-PAGE gel, and transferred onto an Immobilon-FL polyvinylidenedifluoride membrane (EMD Millipore). The membranes were incubated with the Blocking Buffer for...
Fluorescent Western Blotting (Rockland Immunochemicals) in phosphate-buffered saline, and then with primary and secondary antibodies in the same blocker containing 0.1% Tween 20. The following primary antibodies were used. Polyclonal antibodies (Open Biosystems) raised against recombinant TUA1 in rabbits (1:5000) and recombinant TUB15 in chickens (1:5000) recognize TUA and TUB proteins, respectively. Polyclonal rabbit antibodies raised against synthetic C-terminal peptides of TUA1 (anti-dY, ESPGDGEDGDEGDE at 1:1000; and anti-dEY, ESPGDGEDGDEG at 1:1000; Sigma Genosys) recognize detyrosinated and non-tyrosinated TUA isoforms as described previously (Swamy et al., 2015). Mouse-derived monoclonal antibodies for polyglutamylated (clone B3, 1:1500) and acetylated (clone 6-11B-1, 1:500) tubulins were obtained from Sigma. Hybridization signals were detected using IRDye 680RD-conjugated goat anti-rabbit IgG, 800CW-conjugated goat anti-mouse or 800CW-conjugated donkey anti-chicken secondary antibodies (1:15000, Li-cor) with an Odyssey infrared imaging system (Li-cor).

Mass Spectrometric Analysis

The SDS-PAGE bands containing tubulins were excised, destained and subjected to in-gel trypsin digestion as described (Shevchenko et al., 1996) at the proteomics core of Michigan State University. Peptides were fractionated by reverse phase HPLC using a Waters nanoAcquity UPLC. Eluted peptides were sprayed into a ThermoFisher LTQ Linear Ion trap mass spectrometer outfitted with a MICHRM Bioresources ADVANCE nanospray source. The top five ions in each survey scan are then subjected to data-dependent zoom scans followed by low energy collision induced dissociation (CID) and the resulting MS/MS spectra are converted to peak lists in BioWorks Browser v3.2 using the default LTQ instrument parameters. Peak lists were searched against all Populus tubulin sequences using the Mascot searching algorithm v2.1. The Mascot output was then analyzed using Scaffold, v1.7.0 to probabilistically validate protein identifications.

An independent set of purified tubulin samples was subjected to SDS-PAGE and in-gel CNBr digestion according to Xiao et al. (2010) at the Proteomics core of the Albert Einstein College of Medicine. Peptides were fractionated by reverse phase HPLC (Ultimate 3000, Dionex), followed by MALDI-TOF analysis (ABI 4800, Applied Biosystems) in both positive and negative ion mode as detailed in (Xiao et al., 2010). Populus tubulin sequences were used as the database. Data were processed with Data Explorer software (v4.9). Label-free quantitation of tubulin isoforms was performed according to Miller et al. (2012).

RNA-Seq Analysis

The total RNA was extracted with the Direct-zol RNA Kit (Zymo Research) using Plant RNA Reagent (Life Technologies) and quantified with the Qubit RNA HS Assay Kit on a Qubit fluorometer (Life Technologies). RNA-Seq libraries were prepared using the Illumina TruSeq Stranded RNA LT Kit and sequenced on an Illumina NextSeq 500 at the Georgia Genomics Facility of University of Georgia. The data are available at NCBI Sequence Read Archive under accession number SRP076604.
| Isotype | Residues | P. deltoides m/z | P. tremula × alba m/z | R. trichocarpa m/z |
|---------|----------|-----------------|----------------------|-------------------|
| TUA1    | 414–451  | EEGFESEARELDALEKDYEVEGAESPDGEDGDEGDEY | 4180.691 | EEGFESEARELDALEKDYEVEGAESPDGEDGDEGDEY | 4180.691 |
| TUA2    | 414–450  | EEGFESEARELDALEKDYEVEGAEGVDEDENEDYE | 4224.717 | EEGFESEARELDALEKDYEVEGAEGVDEDENEDYE | 4224.717 |
| TUA3<sup>a</sup> | 414–451 | EEGFESEARELDALEKDYEVEGAETAEQGDDEGEEYM<sup>*</sup> | 4222.738 | EEGFESEARELDALEKDYEVEGAETAEQGDDEGEEYM<sup>*</sup> | 4194.707 |
| TUA4    | 414–450  | EEGFESEARELDALEKDYEVEGAEGVDDEEDNEDYE | 4166.712 | EEGFESEARELDALEKDYEVEGAEGVDDEEDNEDYE | 4166.712 |
| TUA5    | 414–451  | EEGFESEARELDALEKDYEVEGAESQODQDEGDEY<sup>*</sup> | 4186.657 | EEGFESEARELDALEKDYEVEGAESQODQDEGDEY<sup>*</sup> | 4166.712 |
| TUA6    | 414–449  | EEGFESEARELDALEKDYEVEGAEGGDGEDEEGEDY | 4204.637 | EEGFESEARELDALEKDYEVEGAEGGDGEDEEGEDY | 4204.637 |
| TUA7<sup>a</sup> | 414–451 | EEGFESEARELDALEKDYEVEGAETAEQGDDEGEEYM<sup>*</sup> | 4194.707 | EEGFESEARELDALEKDYEVEGAETAEQGDDEGEEYM<sup>*</sup> | 4208.722 |
| TUA8    | 414–449  | EEGFESEARELDALEKDYEVEGAEGGDGEDEEDDDY | 4024.637 | EEGFESEARELDALEKDYEVEGAEGGDGEDEEDDDY | 4024.637 |
| TUB1    | 416–449  | NDVSEYQQYQDATDEEYEDEEEGYQDYQ | 4038.559 | NDVSEYQQYQDATDEEYEDEEEGYQDYQ | 4038.559 |
| TUB2<sup>a</sup> | 416–450 | NDVSEYQQYQDATDEEYEDEEEGYQDYQ | 4252.655 | NDVSEYQQYQDATDEEYEDEEEGYQDYQ | 4238.630 |
| TUB3    | 416–447  | NDVSEYQQYQDATDEEYEDEEEGYQDYQ | 3688.400 | NDVSEYQQYQDATDEEYEDEEEGYQDYQ | 3688.400 |
| TUB4    | 416–446  | NDVSEYQQYQDATDEEYEDEEEGYQDYQ | 3960.400 | NDVSEYQQYQDATDEEYEDEEEGYQDYQ | 3690.400 |
| TUB5    | 416–444  | NDVSEYQQYQDATDEEYEDEELHDM<sup>*</sup> | 3475.373 | NDVSEYQQYQDATDEEYEDEELHDM<sup>*</sup> | 3475.373 |
| TUB6<sup>b</sup> | 416–443 | NDVSEYQQYQDATDEEYEDEELHDM<sup>*</sup> | 3364.320 | NDVSEYQQYQDATDEEYEDEELHDM<sup>*</sup> | 3364.320 |
| TUB7<sup>b</sup> | 416–445 | NDVSEYQQYQDATDEEYEDEELHDM<sup>*</sup> | 3524.414 | NDVSEYQQYQDATDEEYEDEELHDM<sup>*</sup> | 3524.414 |
| TUB8    | 416–445  | NDVSEYQQYQDATDEEYEDEELHDM<sup>*</sup> | 3692.500 | NDVSEYQQYQDATDEEYEDEELHDM<sup>*</sup> | 3692.500 |
| TUB9<sup>a</sup> | 416–442 | NDVSEYQQYQDATDEEYEDEELHDM<sup>*</sup> | 3132.271 | NDVSEYQQYQDATDEEYEDEELHDM<sup>*</sup> | 3104.240 |
| TUB10<sup>a</sup> | 416–444 | NDVSEYQQYQDATDEEYEDEELHDM<sup>*</sup> | 3424.378 | NDVSEYQQYQDATDEEYEDEELHDM<sup>*</sup> | 3122.214 |
| TUB11<sup>a</sup> | 416–444 | NDVSEYQQYQDATDEEYEDEELHDM<sup>*</sup> | 3408.403 | NDVSEYQQYQDATDEEYEDEELHDM<sup>*</sup> | 3408.403 |
| TUB12<sup>a</sup> | 416–444 | NDVSEYQQYQDATDEEYEDEELHDM<sup>*</sup> | 3437.373 | NDVSEYQQYQDATDEEYEDEELHDM<sup>*</sup> | 3437.373 |
| TUB13   | 411–442  | NDVSEYQQYQDATDEEYEDEELHDM<sup>*</sup> | 3692.500 | NDVSEYQQYQDATDEEYEDEELHDM<sup>*</sup> | 3692.500 |
| TUB14<sup>a</sup> | 416–446 | NDVSEYQQYQDATDEEYEDEELHDM<sup>*</sup> | 3524.414 | NDVSEYQQYQDATDEEYEDEELHDM<sup>*</sup> | 3524.414 |
| TUB15   | 416–450  | NDVSEYQQYQDATDEEYEDEELHDM<sup>*</sup> | 3838.506 | NDVSEYQQYQDATDEEYEDEELHDM<sup>*</sup> | 3838.506 |
| TUB16<sup>a</sup> | 416–450 | NDVSEYQQYQDATDEEYEDEELHDM<sup>*</sup> | 4101.591 | NDVSEYQQYQDATDEEYEDEELHDM<sup>*</sup> | 4101.591 |
| TUB17   | 416–449  | NDVSEYQQYQDATDEEYEDEELHDM<sup>*</sup> | 3843.506 | NDVSEYQQYQDATDEEYEDEELHDM<sup>*</sup> | 3843.506 |
| TUB18   | 416–446  | NDVSEYQQYQDATDEEYEDEELHDM<sup>*</sup> | 4038.559 | NDVSEYQQYQDATDEEYEDEELHDM<sup>*</sup> | 4038.559 |
| TUB19   | 416–446  | NDVSEYQQYQDATDEEYEDEELHDM<sup>*</sup> | 4252.655 | NDVSEYQQYQDATDEEYEDEELHDM<sup>*</sup> | 4252.655 |
| TUB20<sup>a</sup> | 416–450 | NDVSEYQQYQDATDEEYEDEELHDM<sup>*</sup> | 4238.630 | NDVSEYQQYQDATDEEYEDEELHDM<sup>*</sup> | 4238.630 |

<sup>a</sup>Sequences deviating from the *P. trichocarpa* reference are shown in red. <sup>b</sup>Sequences in gray were not recovered from de novo transcript assembly; instead, the *P. trichocarpa* reference sequences were used. <sup>*</sup>Modification of Met to homoserine lactone during CNBr reaction.
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FIGURE 2 | Tubulin isotype and transcript abundance in xylem. (A) Label-free MS quantification of TUA (left panel) and TUB (right panel) reporter peptides in normal wood (NW) and tension wood (TW) of *P. deltoides* (*n* = 2). (B) TUA and TUB transcript abundance based on RNA-Seq analysis of *P. deltoides* NW and TW samples (*n* = 2). (C) Label-free MS quantification of TUA and TUB based on a previously published *P. tremula × alba* NW xylem dataset (Swamy et al., 2015). (D) TUA and TUB transcript abundance based on RNA-Seq analysis of *P. tremula × alba* NW samples (*n* = 3).

Fosket, 1982). Based on SDS-PAGE and Coomassie Brilliant Blue staining, the purified proteins were enriched in a doublet of ∼50 kDa, close to the expected size for tubulins (Figure 1A). Western blotting confirmed that the lower band was TUA, and the upper one was TUB (Figure 1B). The yield of tubulins was 40–60 µg/g fresh-weight of developing xylem. The excised tubulin bands were subjected to in-gel cyanogen bromide (CNBr) digestion for proteomics analysis by MS.

A label-free method (Miller et al., 2012) was applied to determine the relative abundance of tubulin isotypes in NW and TW samples of *P. deltoides* using isotype-specific C-terminal reporter peptides (Table 1). RNA-Seq data from the same series of samples were used for de novo assembly of (expressed) *P. deltoides* tubulin transcripts in order to correct for sequence polymorphisms that deviate from the *P. trichocarpa* reference gene models (see “Materials and Methods” and Table 1). TUA1 and TUA4/5 were the most abundant isotypes in both NW and TW (Figure 2A). The predicted *m/z* for the TUA4 and TUA5 reporter peptides is indistinguishable by MS (Table 1). However, as TUA4 transcripts were barely detected in xylem based on RNA-Seq analysis (Figure 2B), the TUA4/5 reporter peptide signal probably represents (and hence was assigned to) TUA5 (Figure 2A). The TUB signals were much lower than the TUA signals, consistent with the immunoblot results shown in Figure 1. TUB15 and TUB 16 were the predominant TUB isotypes in *P. deltoides* xylem. The MS abundances of TUA and TUB isotypes were largely similar between NW and TW samples of *P. deltoides* (Figure 2A). We also analyzed the proteomics dataset of wild-type (WT) *P. tremula × alba* xylem (NW) described in Swamy et al. (2015), with the same RNA-Seq-based sequence curation as above (Figure 2C). The TUA and TUB isotype profiles were generally consistent between species, with the exception of TUB9 which was among the top three most abundant C-terminal TUB peptides in *P. tremula × alba* xylem (Figure 2C), but was undetected in *P. deltoides* (Figure 2A).

Detection of Tubulin PTMs

We searched the *P. deltoides* MS spectra of C-terminal polypeptides for evidence of tubulin detyrosination, non-tyrosination or glutamylation. The tyrosinated (unmodified) C-terminal peptide (residues 414–451) of TUA1 was detected with an *m/z* of 4180.5, very near the theoretical monoisotopic mass (MH+) of 4180.69 (Figures 3A,B). Its authenticity was previously confirmed by MALDI-TOF/TOF analysis.
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FIGURE 3 | MS analysis of TUA detyrosination and non-tyrosination. Mass spectra of the C-terminal CNBr peptides of TUA1 (A–E), its detyrosinated (TUA1dY, F–J) or non-tyrosinated (TUA1dEY, K–N) isoforms. The primary axis is scaled to the most abundant peptide in each panel, with intensity on the secondary axis. Insets show mass spectra of the respective C-terminal peptide. The monoisotopic mass is denoted by a triangle. Insets in (A–E,I,N) show consistent isotopic profiles. The peptide identities in (C,I,N) were previously confirmed by MALDI-TOF/TOF (Swamy et al., 2015). Peptide signals in all other insets (F–H,J–M) lacked clear isotopic distribution and are deemed background noise.

using enriched tubulins purified from NW xylem of WT P. tremula × alba (Swamy et al., 2015) (Figure 3C). In that same study, we also identified and MALDI-TOF/TOF-confirmed C-terminal peptides of detyrosinated (dT, m/z 4017.4) and non-tyrosinatable (dEY, m/z 3888.6) isoforms from transgenic P. tremula × alba ectopically expressing the dY and dEY PTM mimics of TUA1, respectively (Swamy et al., 2015) (Figures 3I,N). However, the MS signal for either PTM peptide was near background in P. deltoides NW and TW samples or in WT P. tremula × alba (Figures 3F–H,K–M) (Swamy et al., 2015). This was corroborated by Western blot analysis of purified xylem tubulins from both Populus species using PTM-specific antibodies raised against the dY (anti-dY) or dEY (anti-dEY) C-terminal peptide of TUA1. As reported previously (Swamy et al., 2015), the anti-dY and anti-dEY immunosignals were clearly detected with xylem tubulins purified from dY- and dEY-expressing transgenics, respectively (Figures 4A,B). The faint signals observed in the other samples likely represent background hybridization as seen for the recombinant TUA1 protein purified from Escherichia coli that was included as a negative control (Figures 4A,B). The results suggest that detyrosination and non-tyrosination of TUA were either absent or negligible in Populus xylem.

Polyglutamylation of Populus tubulins was examined by immunoblotting using a commercial monoclonal antibody raised against polyglutamylated TUs of sea urchin (Lytechinus pictus) (Gagnon et al., 1996). A weak signal that overlapped with the anti-TUA signal was detected for all samples, including recombinant TUA1, indicative of background hybridization likely due to the TUA origin of the monoclonal antibody (Figures 4C,D). We then searched the MALDI mass spectra for peptide masses that were 129 Da (the mass of a Glu residue) larger than the C-terminal
peptide masses of TUAs and TUBs in both *Populus* species. No such signals were found above background levels, suggesting that tubulin glutamylation is unlikely to occur in wood-forming tissues of *Populus*.

Acetylation of TUAs was studied using a commercial monoclonal antibody raised against acetylated TUA of sea urchin. As with the other immunoblot analyses described above, only background hybridization signals were detected regardless of tissue (NW or TW) or genotype (Figures 5A,B). A shot-gun LC-MS/MS analysis of trypsin-digested tubulin identified an N-terminal peptide shared by TUA1 and TUA5 that appeared to be acetylated at Lys-40 due to a mass shift of 42 Da (Figure 5C). Although this peptide was identified with a 94% probability, the acetylated Lys-40 signals (b5 and b6 ions) were very low. Unmodified Lys-40 is susceptible to trypsin cleavage, and accordingly, we identified a shorter N-terminal TUA1/TUA5 peptide with an exposed Lys-40 (Figure 5D). As TUA1 and TUA5 are abundant in *Populus* xylem, we interpret the immunoblot and MS data to suggest that both isotypes were subjected to very low levels of acetylation at the conserved Lys-40 residue.

**RNA-Seq Analysis of TUA and TUB**

The RNA-Seq data were also processed to estimate tubulin transcript abundance in xylem of both *P. deltoides* and *P. tremula × alba*. Because TUA and TUB genes share high degrees of sequence identity, we extracted up to 200 bp sequences spanning the hypervariable C-termini and 3′-UTRs from the de novo transcript assemblies as references for read mapping (see “Materials and Methods” and Supplementary Data 1). This was intended to minimize ambiguous read alignment in the highly conserved coding region. The overall TUA and TUB transcript profiles were similar between *P. deltoides* and *P. tremula × alba*, suggesting a conserved transcriptional regulation in this genus (Figures 2B,D). The transcript abundance estimates by RNA-Seq were largely congruent with the isotype profiles observed in both species (Figure 2), suggesting transcriptional regulation. Exceptions to this general trend were two TUBs with discordant patterns. Low MS signals were detected for TUB18 despite its very high transcript levels in both species, and TUB17 exhibited a similar though less pronounced pattern. The data suggested involvement of post-transcriptional or translational regulation. As mentioned above, the TUB9 reporter peptide was detected in hybrid aspen *P. tremula × alba*, but not *P. deltoides* (Figures 2A,C). Accordingly, TUB9 transcripts were present at relatively higher levels in *P. tremula × alba* (75th percentile of all TUBs) than in *P. deltoides* (50–55th percentile) xylem (Figures 2B,D). The results hint at a taxon-specific fine tuning of TUB regulation in *Populus*.

The proteomics data also help curate gene model annotation of the reference genome. We previously cloned all eight TUA cDNAs from *P. tremuloides* (Oakley et al., 2007). The experimentally identified TUA5 mRNA (GenBank
accretion (Figures 2–5). This discrepancy might be attributed to a shorter TW induction period (4 weeks) in the present experiment than that (~3 months) reported in Oakley et al. (2007). It could also reflect varying degrees of strain due to large (5-year-old, present study) versus small trees (2-year-old, previous work) in response to bending, although genetic or ontogenetic influences could not be excluded. Future work is needed to investigate the underlying differences in TW response. Despite the absence of treatment effects, tubulin proteins and transcripts remained highly enriched in both NW and TW xylem of *P. deltoides* (see Figure 1 and discussion above). The TW sample thus served as an independent replicate for tubulin PTM assessment.

Lys-40 acetylation of TUA1/TUA5 was the only tubulin PTM confirmed by MS/MS in *Populus* xylem, although the immunosignals were negligible using a commercial monoclonal antibody that has been used to detect acetylated TUAs in a wide range of plant species (Huang and Lloyd, 1999; Tran et al., 2012; Nakagawa et al., 2013; Gzyl et al., 2015). Tubulin acetylation was detected at high levels in tobacco cell cultures but at much lower levels in *Arabidopsis* cell cultures (Hotta et al., 2016). Variable levels of tubulin acetylation were also reported in a broad survey of 15 angiosperm species (Nakagawa et al., 2013) or between different tissues (Wang et al., 2004). Although plants lack apparent homologs of the animal acetyltransferase MEC-17 that catalyzes TUA Lys-40 acetylation (Akella et al., 2010), *Arabidopsis* histone acetyltransferase EL3P and histone deacetylase HDA14 have been shown to be highly enriched on MTs and may take part in TUA acetylation/deacetylation (Tran et al., 2012). Data mining of a multi-tissue (leaf, xylem, bark and root) RNA-Seq dataset (Xue et al., 2016) suggested that the *Populus* EL3P and HDA14 orthologs were mainly expressed in leaves. Their transcripts were barely detected (FPKM ≤ 5) in developing xylem of greenhouse-grown *P. tremula* × *alba* (Swamy et al., 2015; Xue et al., 2016). In field-grown *P. deltoides*, EL3P orthologs were also poorly expressed, but deacetylase HDA14 orthologs were detected at higher levels (FPKM ~ 15). Poor expression of acetyltransferases and deacetylases, or higher deacetylase than acetyltransferase transcript levels could both explain the low levels of TUA acetylation that we observed in NW and TW tissues.

None of the C-terminal PTMs frequently reported for animal tubulins, including dehydroxylation, non-tyrosination and glutamylation, was detected by MS or immunoblotting above background noise in *Populus* xylem. Low levels of tubulin C-terminal PTM immunosignals were previously reported for tobacco suspension cells (Smertenko et al., 1997), maize leaves, roots, pollen and anthers (Wang et al., 2004) and soybean seedlings (Gzyl et al., 2015). However, a recent study employing an improved (tubulin binding protein-based) affinity purification of tubulins from tobacco and *Arabidopsis* cell cultures showed no evidence of C-terminal PTMs by immunoblot or MS analyses (Hotta et al., 2016), similar to our observations in *Populus* xylem. It thus appears that tubulin C-terminal PTMs are absent or rare in plants unlike in animals or protists. What underlies this evolutionary distinction between plants and other eukaryotes remains unclear, but several considerations can be offered. First,
reversible PTMs are thought to increase tubulin pool diversity, and hence the flexibility of MT function (Westermann and Weber, 2003). This diversity may be fulfilled by genetically encoded tubulins in \textit{Populus} and other plant species that are more numerous and heterogeneous than their animal counterparts. As we reported previously, five of the eight \textit{Populus} TUAs encode a unique C-terminal Met (TUA3, TUA5 and TUA7), Glu (TUA2) or Gln (TUA4), instead of the typical Tyr (TUA1, TUA6 and TUA8) necessary for participation in the detyrosination-tyrosination cycle (Oakley et al., 2007). MS detection of the C-terminal peptide of TUA1 (Y-type), TUA5 (M-type) and TUA2 (E-type) provided a convincing argument for a genetically diverse pool of TUA in poplar xylem, regardless of PTM activities.

Second, tubulin C-terminal tails exposed on the outer surface of MTs interact with MT-associated proteins (MAPs), and as such, their PTMs are thought to modulate MT–MAP interactions (Magiera and Janke, 2014). Analysis of sequenced genomes has shown that MAP families are generally more diverse in animals than in plants, both in number of families and number of genes within each family (Gardiner, 2013). The extent to which tubulin C-terminal PTMs parallel the complexity of MAP–MT interactions during evolution is an interesting possibility to consider. One exception to the MAP family diversity noted above is that the kinesin family has significantly expanded in plants (Lee and Liu, 2004). Kinesins and other motor proteins play important roles in self-organization of plant MTs in the absence of canonical centrosomes (Wasteneys, 2002). Plant kinesins have expanded functions from cell cycle to morphogenesis to signal transduction (Li et al., 2012). Thus, MT multiplexity in plants can also be fulfilled by mechanisms other than tubulin C-terminal PTMs.

Vertebrate tubulin PTMs are under strict spatiotemporal regulation (Westermann and Weber, 2003; Janke and Bulinski, 2011). Thus, another explanation for the lack of PTM signals in poplar xylem could be their conditional occurrence in certain tissues and cells, or during specific developmental stages that were not captured in the present investigation. In closing, we found no evidence of tubulin C-terminal PTMs in developing xylem of \textit{Populus} undergoing extensive MT-dependent cell wall biogenesis. Our data suggest that transcriptional, post-transcriptional and/or translational regulation of a genetically more diverse tubulin repertoire features more prominently than tubulin PTMs during wood formation in \textit{Populus}.

**AUTHOR CONTRIBUTIONS**

C-JT and SH designed the research, PS performed tubulin purification, HH performed Western blotting and analyzed proteomics data, XG and L-JX performed RNA-Seq analysis, HH, SH, and C-JT wrote the manuscript. All authors read and approved the manuscript.

**FUNDING**

This research was supported by the Office of Biological and Environmental Research within the Department of Energy (grant no. DE-SC0008470).

**ACKNOWLEDGMENTS**

The authors thank Nicholas Rohr and Bob Schmitz for Illumina RNA library construction, the Georgia Genomics Facility for RNA-Seq, Roden Oakley for the initial tubulin purification, Doug Whitten at the Michigan State University for shotgun proteomics analysis, and Hui Xiao at the proteomics core of the Albert Einstein College of Medicine for MALDI-TOF PTM analysis.

**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: http://journals.frontiersin.org/article/10.3389/fpls.2016.01493

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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