

Diverse roles of PtrDUF579 proteins in *Populus* and PtrDUF579-1 function in vascular cambium proliferation during secondary growth

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Abstract DUF579 (domain of unknown function 579) family proteins contain a DUF579 domain structure but vary greatly in their overall sequence similarity. Several DUF579 proteins have been found to play a role in cell wall biosynthesis in *Arabidopsis*, while DUF579 family genes have not yet been systematically investigated in *Populus*. In this study, the *Populus* DUF579 family proteins were found to be localized in different cell types and subcellular locations. The diverse expression patterns of the proteins indicate that they may perform different functions in *Populus*. Among the DUF579 family members, PtrDUF579-1 is found to be specifically expressed in vascular cambium zone cells where it is localized in the Golgi apparatus. Suppression of *PtrDUF579-1* expression reduced plant height and stem diameter size. Cambium cell division and xylem tissue growth was inhibited while secondary cell wall formation was unchanged in *PtrDUF579-1* suppressed plants. Cell walls analysis showed that the composition of the pectin fraction of the cambium cell wall was altered while other polysaccharides were not affected in *PtrDUF579-1* suppressed plants. This observation suggest cambium expressed PtrDUF579-1 may affect cell wall biosynthesis and be involved in cambium cell proliferation in *Populus*. Overall, DUF579 family proteins play a diverse set of roles in *Populus*.

Keywords DUF579 · Secondary growth · Cambium · *Populus*

Introduction

DUF579 proteins are found in all eukaryotic species, but their functions in yeast and mammals have been little characterized. In plants, DUF579 proteins have been reported to be involved in cell wall formation in *Arabidopsis* (Urbanowicz et al. 2012; Lee et al. 2012; Jensen et al. 2011; Brown et al. 2011). 10 DUF579 genes, divided into two subgroups, are present in the *Arabidopsis* genome (Brown et al. 2011). Protein sequence analysis shows that the homology of DUF579 proteins between the two subgroups is as low as 30 %. Recently, mutations in subgroup B DUF579 genes *IRX15* and *IRX15-L* were shown to affect glucuronoxylan (GX) biosynthesis and secondary cell wall structure (Jensen et al. 2011; Brown et al. 2011). Three DUF579 proteins from subgroup A have been biochemically characterized as glucuronoxylan methyltransferase (GXMT) in vitro. Mutations to these genes causes reduced methylation of the glucuronic acid residue in GX and changes to the lignin composition of secondary cell walls (Urbanowicz et al. 2012; Lee et al. 2012). Subcellular localization analysis shows that subgroup A GXMT proteins are localized in the Golgi while subgroup B *IRX15* proteins are localized in both the Golgi and in other uncharacterized vesicles (Urbanowicz et al. 2012; Jensen et al. 2011; Brown et al. 2011). DUF579 family proteins have converse effects on GX methylation depending on which subgroup they belong to. Knocking-out *GXMT/GXM* genes including *At1g33800*, *At4g09990* and *At1g09610* results in a reduction in GX methylation (Urbanowicz et al. 2012; Lee et al. 2012), while knocking-out *irx15* (*At3g50220*) and *irx15 l* (*At5g67210*) leads to an increase in GX methylation

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(Jensen et al. 2011; Brown et al. 2011). Furthermore, secondary cell wall defects are observed in *irx15* and *irx15l* double mutants in which the content and degree of polymerization (DP) of GX is reduced. In contrast, similar changes are not detected in *GXMT* knock-out plants (Urbanowicz et al. 2012; Jensen et al. 2011; Brown et al. 2011). Further studies are needed to determine whether *Arabidopsis* DUF579 proteins from different subgroups perform different biochemical functions in vivo.

DUF579 proteins have been identified in the secondary vascular tissue of *Populus* and proteomics indicate the proteins may function in secondary growth (Song et al. 2011; Kalluri et al. 2009). Recently, four *Populus* DUF579 proteins were found to exhibit glucuronoxylan methyltransferase activity, but with different substrate affinities and catalytic efficiencies (Yuan et al. 2014). In this study, the *PtrDUF579-1* protein was found to be specifically expressed in cambium zone cells. *PtrDUF579-1* was shown to play a role in regulating cambium cell division; a finding which sheds new light on the function of DUF579 during secondary growth in *Populus*.

Results

DUF579 gene family in *Populus trichocarpa*

DUF579 genes from the *Populus trichocarpa* genome (<http://www.phytozome.net/poplar>) were catalogued. 12 DUF579 family members were identified and named *PtrDUF579-1* to *PtrDUF579-12* (Fig. 1). Ten of the 12 DUF579 proteins (except *PtrDUF579-11* and *PtrDUF579-12*) contain a predicted transmembrane signal peptide at their N-terminal and a DUF579 domain at their C terminal (Fig. S1). The 12 *PtrDUF579* genes were predicted to encode proteins with sizes that varied from 248 to 372 amino acids. Phylogenetic analysis showed that the 12 DUF579 proteins are organized into six pairs which can be classified into three groups (lettered A, B, C). Pairs within each group varied in the number of corresponding *Arabidopsis* homologs (Fig. 1). Group A includes 3 pairs: *PtrDUF579-1* and *PtrDUF579-2*, *PtrDUF579-3* and *PtrDUF579-4*, *PtrDUF579-5* and *PtrDUF579-6*. Group B contains 2 pairs: *PtrDUF579-7* and *PtrDUF579-8*, *PtrDUF579-9* and *PtrDUF579-10*. Group C consists of one pair: *PtrDUF579-11* and *PtrDUF579-12* (Fig. 1). The paired organization of DUF579 proteins in *Populus* is likely due to genome duplication (Tuskan et al. 2006). Despite sharing conserved domains, different pairs of DUF579s nonetheless display remarkable variation in overall sequence identity ranging from 17 to 69 % (Table S1). The high variation suggests that the family members may carry out different functions.

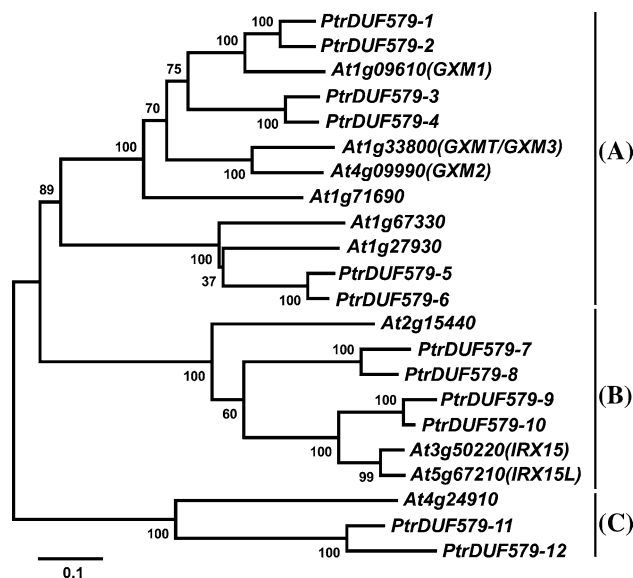


Fig. 1 Phylogenetic analysis of DUF579 proteins from *Populus* and *Arabidopsis*. The cladogram is constructed with neighbour-joining method after DUF579 protein sequences are aligned with ClustalW program. The DUF579 proteins are classified into three groups (a, b, c) according to branch length. Ptr, *Populus trichocarpa*; At, *Arabidopsis thaliana*

Expression profiling of *PtrDUF579* family genes

The expression profiles of the 6 *PtrDUF579* gene pairs were analyzed in various differentiating tissues, including shoot tip, young stem (the second internode), developing xylem, developing phloem and young leaf (Fig. 2). *PtrDUF579* family members were found to display distinct expression patterns in various tissues. High expression of *PtrDUF579-1* was detected in developing xylem and phloem tissues and shoot tip. Two pairs, *PtrDUF579-3* and *PtrDUF579-4*, *PtrDUF579-9* and *PtrDUF579-10*, were highly expressed in developing xylem. Expression of *PtrDUF579-11* and *PtrDUF579-12* was found to be xylem specific but at much lower levels compared to *PtrDUF579-3* and *PtrDUF579-9*. The other two gene pairs, *PtrDUF579-5* and *PtrDUF579-6*, *PtrDUF579-7* and *PtrDUF579-8*, were expressed in shoot tip, young stem, developing xylem, developing phloem and young leaf but at a lower level.

Localization of *PtrDUF579* protein

One member (*PtrDUF579-1*, *PtrDUF579-3* and *PtrDUF579-9*) from three pairs of *PtrDUF579* genes was selected to produce specific antibodies. Specific peptides (Fig. S1) were synthesized and used to raise antibodies. The specificity of the antibodies was examined and verified by Western blot against the total proteins extracted from developing

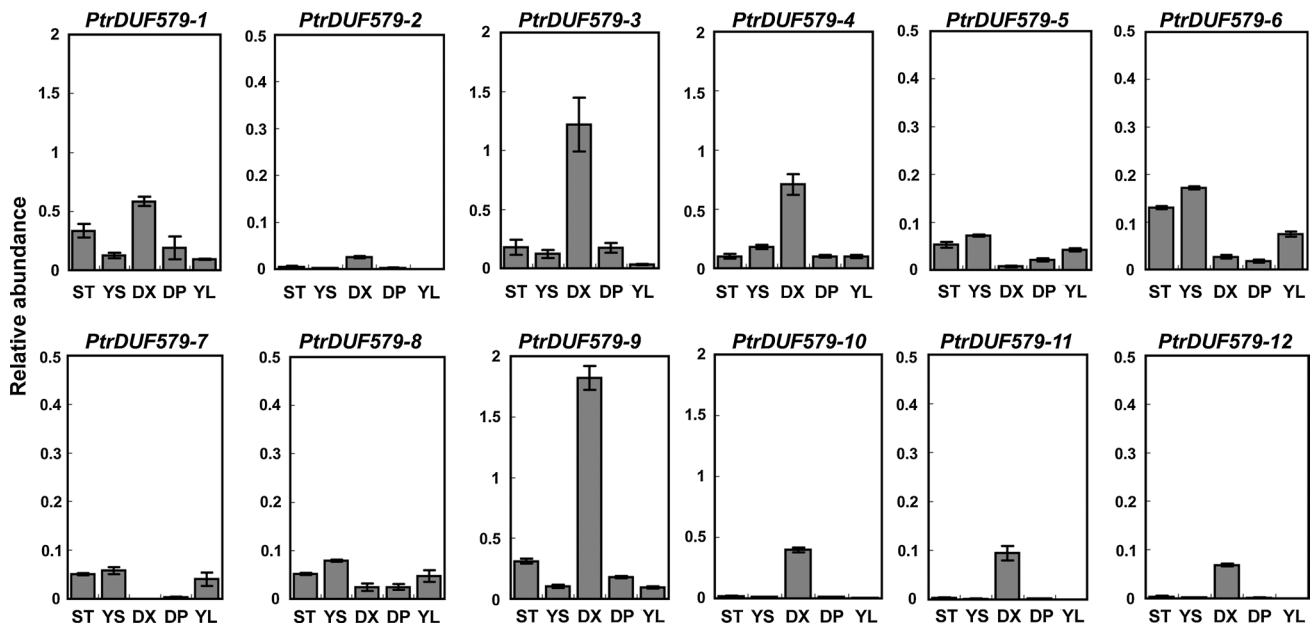


Fig. 2 Expression of *PtrDUF579* genes in various tissues of *P. trichocarpa*. ST, shoot tip; YS, young stem (the second internode); DX, developing xylem; DP, developing phloem; YL, young leaf

xylem and phloem tissues (Fig. S2). Anti-PtrDUF579-1 antibodies recognized two protein bands, one displayed a molecular size similar to the predicted molecular weight of PtrDUF579-1 (32.5 kDa) while the other indicated a larger size in both tissues. Anti-PtrDUF579-3 and anti-PtrDUF579-9 antibodies detected the protein only in the developing xylem and not in the developing phloem. The detected proteins showed a molecular size about 39 and 40 kDa (Fig. S2), which were slightly larger than their predicted molecular weights (PtrDUF579-3: 34.1 kDa and PtrDUF579-9: 35.5 kDa), suggesting a possible modification to the PtrDUF579 proteins in secondary vascular tissues.

Microsomal membrane and soluble proteins were extracted from developing xylem tissues. PtrDUF579-1 was detected in both protein fractions via Western blot analysis. PtrDUF579-9 was also detected in both protein fractions while PtrDUF579-3 was detected only in the membrane fraction (Fig. 3a). However, the size of the PtrDUF579-9 band in the membrane fraction was slightly larger than that in the soluble fraction, likely reflecting differences in modifications to PtrDUF579-9 in membrane versus in cytosol. Immunolocalization showed that PtrDUF579-1 was detected specifically in cambium cells but not in other types of xylem cells (Fig. 3b, e). PtrDUF579-1 was also detected in procambium cells (Fig. S3). PtrDUF579-3 was detected in xylem fiber cells, vessel cells and ray cells but barely detected in cambium, phloem or cortex cells (Fig. 3c, f). PtrDUF579-9 was detected in xylem fiber cells and ray cells (Fig. 3d, g). Observed differences in

membrane association and cell type localization suggest PtrDUF579-1, PtrDUF579-3 and PtrDUF579-9 play different roles in plants despite being all expressed in xylem tissue. Specially, PtrDUF579-1 may function in cambium zone cells.

Subcellular localization of PtrDUF579-1

To further examine the subcellular localization of PtrDUF579-1, the Golgi apparatus marker GmMan1 was co-expressed with PtrDUF579-1:mCherry in tobacco leaf. Colocalization of PtrDUF579-1:mCherry and the Golgi apparatus marker (Fig. 4a–d) indicates that PtrDUF579-1 is located in the Golgi apparatus during cambium cell proliferation.

Suppression of *PtrDUF579-1* affects secondary growth

PtrDUF579-1 suppressed plants were generated by introducing *Antisense-PtrDUF579-1* construct into *Populus*. A total of thirty-five independent transgenic lines were generated and confirmed. Expression of *PtrDUF579-1* was found to be down-regulated in twelve of the transgenic lines, among which Line 2 and Line 6 were selected for additional detailed analysis (Fig. 5a). The transgenic plants (Line 2 and Line 6) displayed shorter stems and internodes (Fig. 5a, b, Fig. S4). In addition, the growth of the stem diameter was reduced ~28 % compared to wild type (Fig. 5c, d). *PtrDUF579-1* expression was decreased by about 85 % (Fig. 5e). Meanwhile, expression of

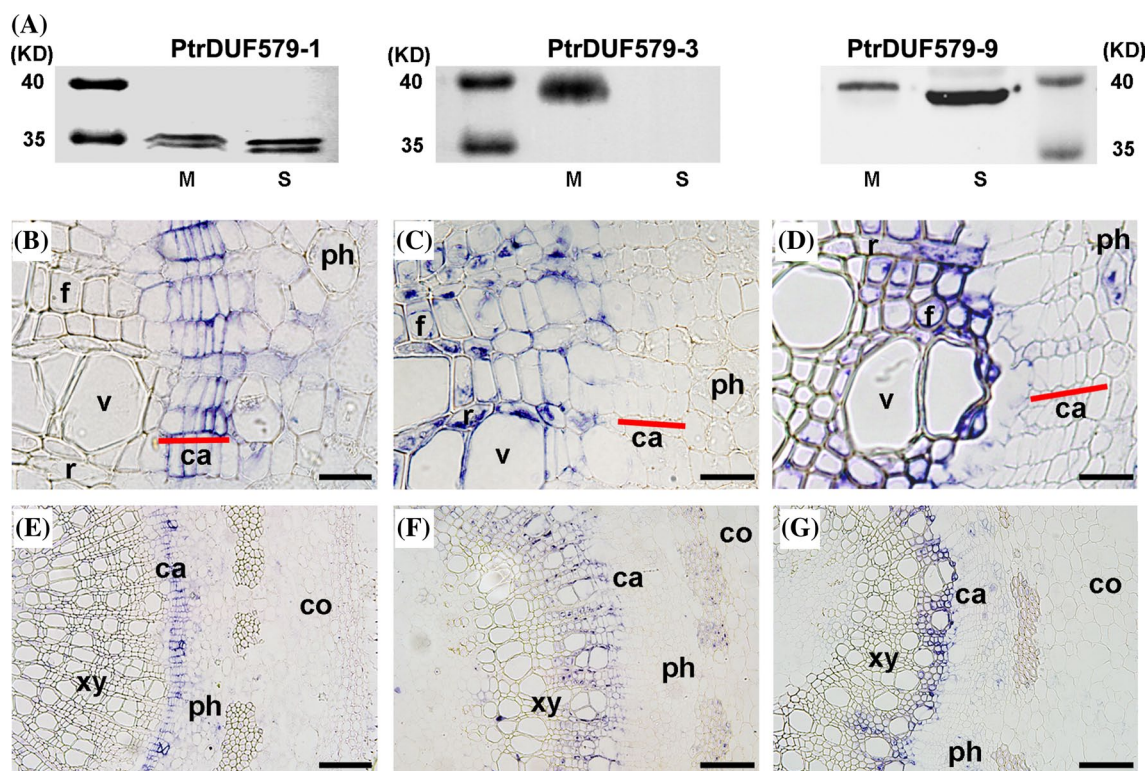


Fig. 3 PtrDUF579 protein localization in secondary vascular cells. **a** Detection of DUF579s in microsomal (*M*) and soluble (*S*) proteins from developing xylem. **b–g** Cross-sections of *Populus* stem from the 12th internode were hybridized with specific DUF579 antibodies, **b, e** PtrDUF579-1 is specifically localized in cambium cells.

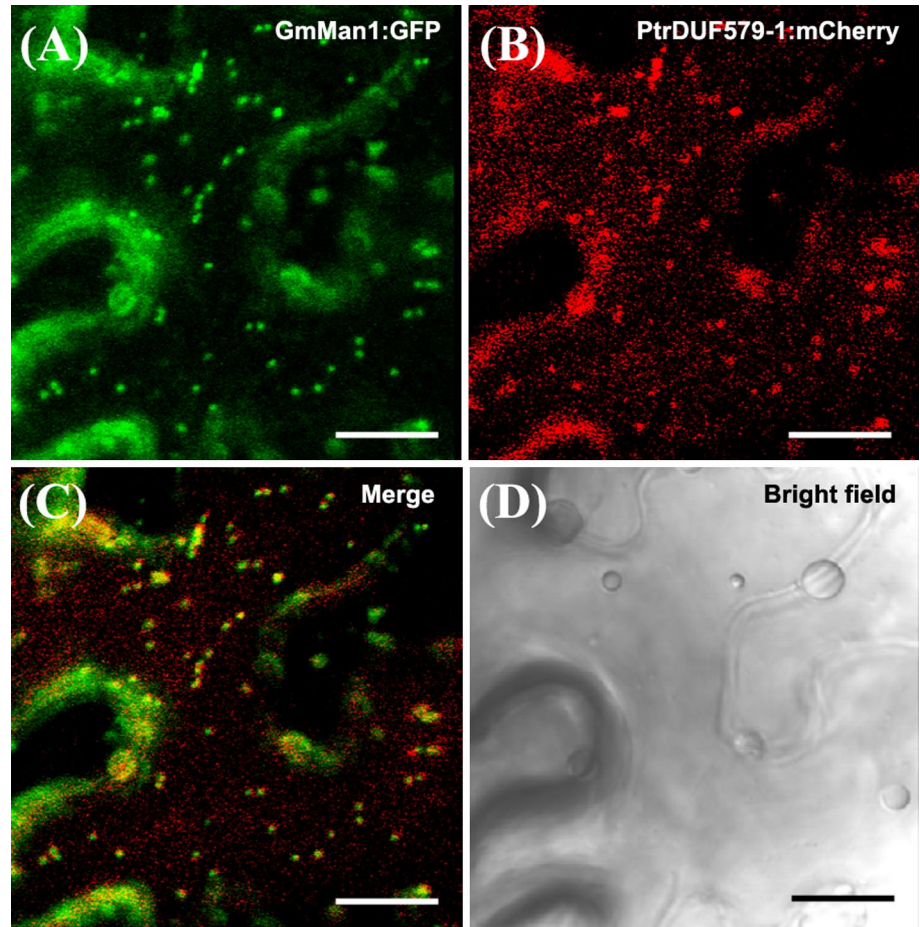
c, f PtrDUF579-3 is localized in xylem cells including fiber, vessel and ray cell. **d, g** PtrDUF579-9 is localized in xylem cells including fiber and ray cell. xy, xylem; ca, cambium; ph, phloem; co, cortex; f, xylem fiber cell; v, vessel cell; r, ray cell. Bars **b, c, d**—20 μm ; **e, f, g**—100 μm

PtrDUF579-2, which was expressed in low levels in wild type, was also repressed (Fig. 5e). However, the expression of *PtrDUF579-3* and *PtrDUF579-9* was not affected in the two transgenic lines (Fig. 5e). The phenotypes of *PtrDUF579-1* suppressed plants could be also attributed to *PtrDUF579-2* down-regulation, since both display similar expression patterns and high sequence identity with each other. The thinner stem observed in *PtrDUF579-1* suppressed plants may reflect changes associated with secondary growth. Stem cross-sections of the transgenic plants were therefore analyzed to examine how the *PtrDUF579-1* gene may affect secondary growth. Transgenic plants displayed normal differentiation of xylem fiber cells and vessel cells in secondary xylem (Fig. 6a–f). However, the cambium zone in the *PtrDUF579-1* suppressed plants was narrower than that in wild type plants (Fig. 6c, d). There were about 3 ± 1 cells per file in the cambium zone of *PtrDUF579-1* suppressed plants versus 5 ± 1 cells per file in wild type plants (Table 1). Consequently, the xylem region of *PtrDUF579-1* suppressed plants was narrower compared to that of wild type plants (Fig. 6e, f). In the 12th internode, 15 ± 5 cells per file were observed in the xylem region of *PtrDUF579-1* suppressed plants, whereas 22 ± 5

cells per file were observed in wild type plants (Table 1). These results indicated suppression of *PtrDUF579-1* affected cambium fusiform initial cells proliferation and secondary xylem growth, resulting in shorter and thinner stems.

Next, compositions of the cambium cell wall next to the phloem and xylem layers were analyzed. The cambium cell walls scraped from both layers were sequentially extracted by pectinase and xyloglucan endoglucanase, the two hydrolysis fractions were named the PE fraction and XG fraction respectively, and used for monosaccharide composition analysis. Compared to wild type plants, lower arabinose and galactose content was observed in the PE fraction from the phloem side of *PtrDUF579-1* suppressed plants (Fig. 7a). The content of other sugars including methylated fucose and methylated xylose, as well as the content of galaturonic acid (GalA) and glucuronic acid (GlcA) were unchanged (Fig. 7a, b). No changes in sugar content were observed in the XG fraction (Fig. 7c). Consistently lower levels of galactose content were detected in the PE fraction of the cambium cell wall on the xylem side (Fig. 7d). Lower arabinose content was also detected while the other sugars remained unchanged (Fig. 7d). The GlcA content

Fig. 4 Subcellular localization of PtrDUF579-1. PtrDUF579-1 protein was labeled with mCherry and its co-localization with Golgi marker, GmMan1:GFP, was carried out in tobacco leaves. The signal of PtrDUF579-1:mCherry was matched with GmMan1:GFP (a–d). Bars 20 μ m



was slightly decreased in the PE fraction of cambium cell wall on the xylem side, while GalA content was unchanged (Fig. 7e). The sugars content in the XG fraction was also unchanged (Fig. 7f). Furthermore, the sugars content in 4 N KOH fraction remained stable indicating that the secondary cell wall was not affected (Fig. 7g). In summary, changes to the monosaccharide composition in PE fraction from both scrapes suggest cambium cell wall pectin biosynthesis may be affected in *PtrDUF579-1* suppressed plants. Potentially, the effect of *PtrDUF579-1* suppression on cambium cell proliferation may reflect the function of *PtrDUF579-1* in cell wall biosynthesis.

Discussion

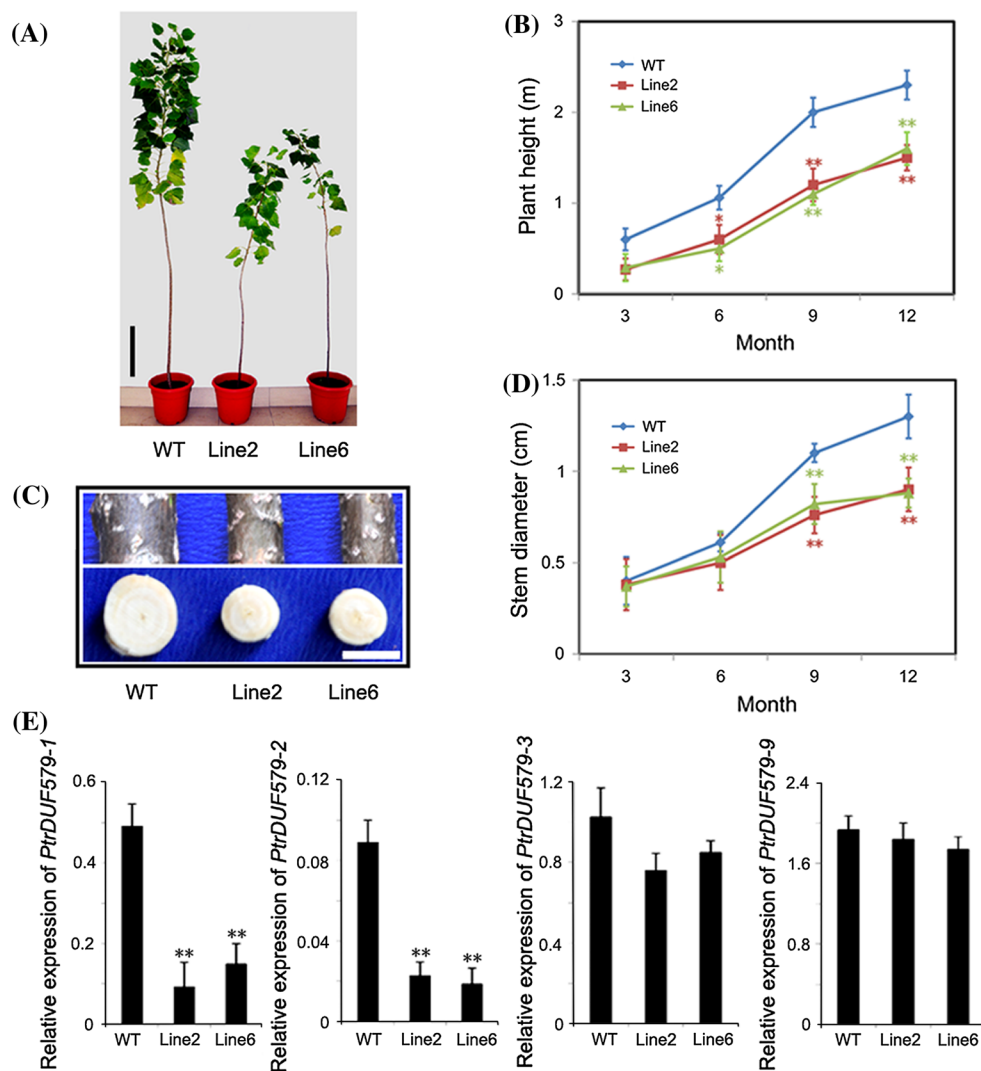
DUF579 family members may play a variety of roles in *Populus*

Little is known of the functions of DUF579 family proteins during secondary growth. In this study, we identified six pairs of *PtrDUF579* genes in the *Populus* genome and characterized their expression patterns. The six pairs were

found to share a low degree of sequence identity, display diverse expression patterns across various tissues, and are localized in different types of cells. Among the proteins, *PtrDUF579-1* was found to be expressed mainly in cambium zone cells through immunolocalization analysis using highly specific antibodies, while *PtrDUF579-3* and *PtrDUF579-9* are expressed in the developing xylem. A recent study showed that *PtrDUF579-1* expression was detected mainly in xylem cells through in situ hybridization analysis (Yuan et al. 2014). This difference could be due to the detection methods used in two studies. It remains to be investigated why *PtrDUF579-1* specific antibodies did not detect the presence of *PtrDUF579-1* proteins in xylem cells. Our results also revealed that *PtrDUF579* proteins may undergo posttranslational modifications which caused the detection of larger than predicted protein sizes. Further characterization of the modifications to the *PtrDUF579* protein and their impact on the protein's biochemical activity or biological function will enable a better mechanistic understanding of the functions of this gene family. Current results suggest proteins in the *PtrDUF579* family may play distinct roles in various types of cells and tissues in *Populus*.

Fig. 5 Characterization of *PtrDUF579-1* suppressed transgenic plants. **a** Morphology of *PtrDUF579-1* suppressed plants with one year old. Bar 15 cm.

b Plant height of WT and transgenic plants in one year. **c** Bottom stem of *PtrDUF579-1* suppressed plants with one year old. Bar 1 cm. **d** Bottom stem diameter of WT and transgenic plants in one year. **e** Expression of *PtrDUF579-1*, *PtrDUF579-2*, *PtrDUF579-3* and *PtrDUF579-9* in wild type (WT) and transgenic plants, respectively. Each transgenic line was multiplied through cutting propagation for analysis of biological repeats. Significance was determined by Student's *t* test: *, $P < 0.05$. **, $P < 0.01$. The values are mean \pm SE, $n = 5$



Role of *PtrDUF579-1* in vascular cambium division

Tree cambium cells undergo periclinal asymmetric division to produce a higher ratio of xylem precursor cells to phloem precursor cells. Regulation of this process, which is essential during trunk development, is not well understood. The observation that *PtrDUF579-1* is predominantly expressed in cambium zone cells suggests that its function may be related to cambium division or differentiation. Suppression of *PtrDUF579-1* led to a decrease in the number of cambium cells and xylem cell layers and resulted in plants with thinner stems. Meanwhile, no changes were observed in the differentiation of xylem fiber cells and vessel cells in the secondary xylem of *PtrDUF579-1* suppressed plants. These results indicate *PtrDUF579-1* contributes to cambium cell division but not cambium cell differentiation during secondary growth in *Populus*.

Since the *PtrDUF579-1* protein was shown to be localized in the Golgi apparatus where cell wall polysaccharides

are synthesized, *PtrDUF579-1* could affect the biosynthesis of cell wall polysaccharides. Composition analysis of cambium zone cell walls in *PtrDUF579-1* suppressed plants revealed that suppression of *PtrDUF579-1* resulted in changes to the content of arabinose, galactose and GlcA in the PE fraction, potentially by affecting the biosynthesis of pectin polysaccharides. Additionally, it is worthwhile to note that a change in GlcA in xylem PE, but not in phloem PE was observed in *PtrDUF579-1* suppressed plants. This may reflect a distinct effect that *PtrDUF579-1* has on xylem and phloem cell wall biosynthesis in the process of periclinal asymmetric divisions of cambium cells. Arabinose, galactose and GlcA are reported to be present in side chain of Rhamnogalacturonan I (RG-I) and Rhamnogalacturonan II (RG-II) pectin polysaccharides (Atmodjo et al. 2013; Mohnen 2008). *PtrDUF579-1* is a homolog of *Arabidopsis* GXM1 protein (72 % identity) which is reported to catalyze the 4-O-methylation of the GlcA residue of GX. The biochemical activity of four *PtrDUF579*

Fig. 6 Suppression of *PtrDUF579-1* affects plant secondary growth. Cross-section shows the secondary vascular tissues in the 12th internode of transgenic plant with six month old (a) and wild type (WT) plant (b). Cambium region in transgenic plant (c) and wild type (WT) plant (d). Secondary xylem region in transgenic plant (e) and WT plant (f). Sections were stained with toluidine blue. ca: cambium; ph: phloem; xy: xylem. Bars a, b—500 μ m; c, d—50 μ m; e, f—200 μ m

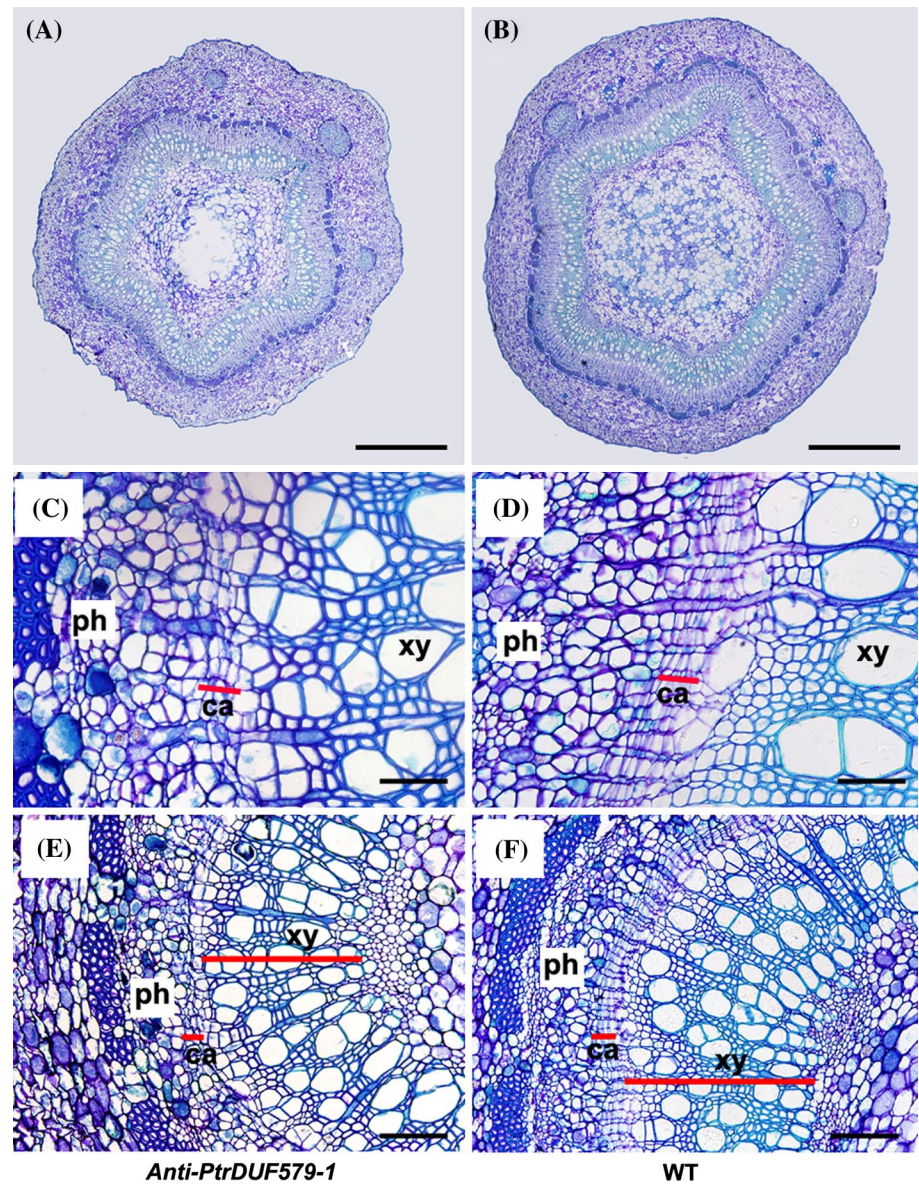


Table 1 Secondary growth phenotypes of *PtrDUF579-1* suppressed *Populus*

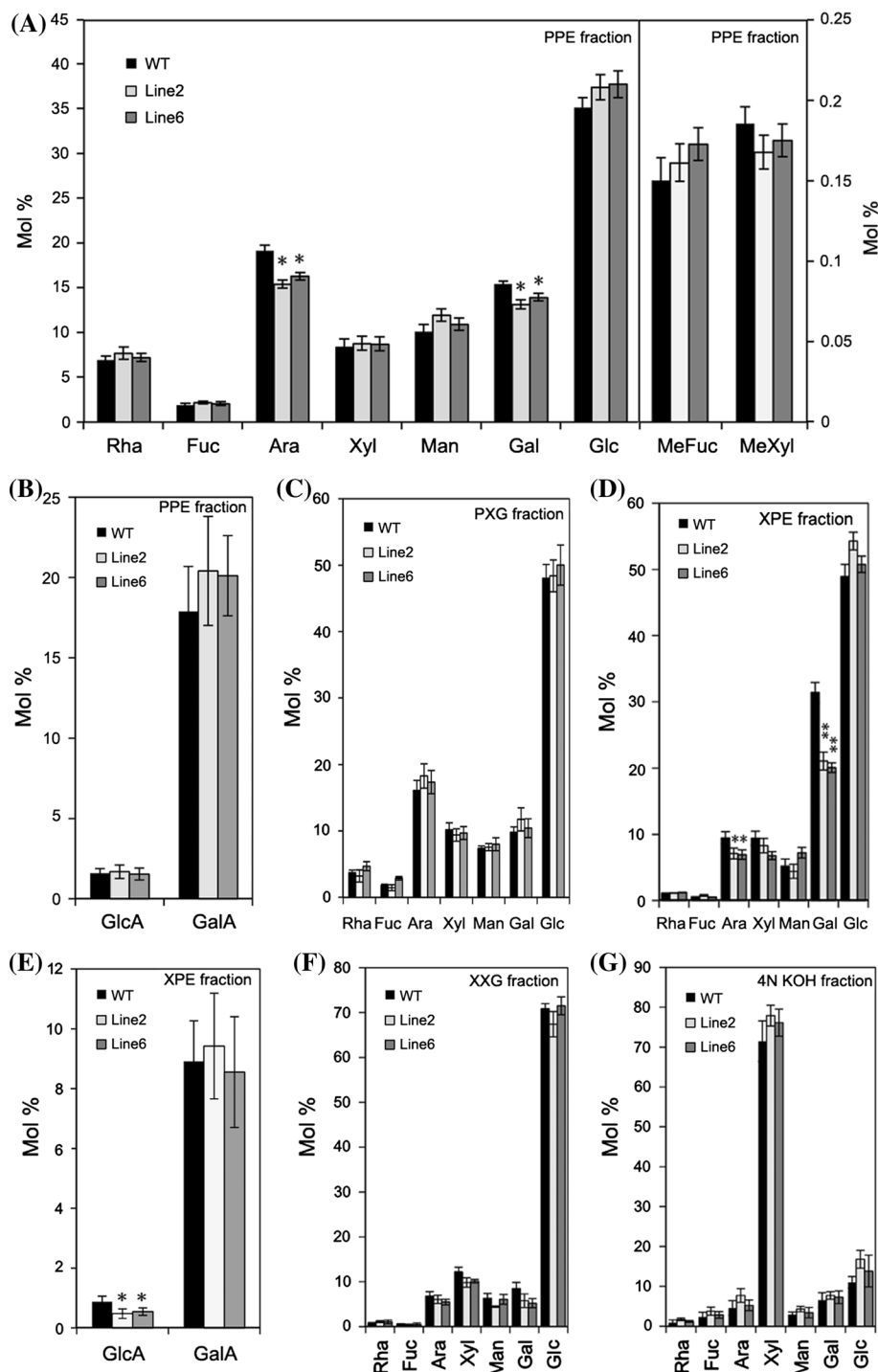
Phenotypes	Line2	Line6	Wild type
Cell layers of cambium zone	2 \pm 1**	3 \pm 1**	5 \pm 1
Number of xylem cell layers per files	17 \pm 3**	15 \pm 5**	22 \pm 5

The secondary growth of 6-month-old plants was examined within the stem internode 12. Statistic data from five transgenic plants of each line are showed as mean \pm SE. Significance was determined by Student's *t* test: ** $P < 0.01$

proteins from Group A, which includes *PtrDUF579-1*, *PtrDUF579-2*, *PtrDUF579-3* and *PtrDUF579-4*, was analyzed using GX as substrate in a recent study. The four DUF579 proteins were detected to catalyze the methylation

of the GlcA residue of GX (Yuan et al. 2014). However, *PtrDUF579-1* and *PtrDUF579-2* displayed a lower substrate affinity and catalytic efficiency compared to *PtrDUF579-3* and *PtrDUF579-4* (Yuan et al. 2014). It is unknown whether *PtrDUF579-1* or *PtrDUF579-2* are able to catalyze the methylation of GlcA residues on other polysaccharides. In this study, *PtrDUF579-1* was found to be predominantly expressed in cambium cells, indicating that *PtrDUF579-1* may be associated with the methylation of polysaccharides in the primary cell wall. 4-O-methyl-GlcA residues has been reported to be associated with RG-I galactan side chain with (1,6)-linkages in the primary cell wall of suspension-cultured sycamore cells (Caffall and Mohnen 2009; Mohnen 2008; An et al. 1994). It is possible that *PtrDUF579-1* contributes to the methylation of GlcA residue in the galactan side chain of RG-I to affect

Fig. 7 Suppression of *PtrDUF579-1* affects pectin composition of cambium cell wall at phloem and xylem side. **a** Neutral sugar contents in the phloem PE fraction of wild-type and *PtrDUF579-1* suppressed plants. **b** Uronic acids in the phloem PE fraction of wild-type and *PtrDUF579-1* suppressed plants. **c** Sugar contents in the phloem XG fraction of wild-type and *PtrDUF579-1* suppressed plants. **d** Sugars in xylem PE fraction of wild-type and *PtrDUF579-1* suppressed plants. **e** Uronic acids in xylem PE fraction of wild-type and *PtrDUF579-1* suppressed plants. **f** Sugar contents in the xylem XG fraction of wild-type and *PtrDUF579-1* suppressed plants. **g** Sugar contents in xylem 4 N KOH fraction of wild-type and *PtrDUF579-1* suppressed plants. PPE: phloem side pectin fraction; PXG: phloem side xyloglucan fraction; XPE: xylem side pectin fraction; XXG: xylem side xyloglucan fraction; Each transgenic line was multiplied through cutting propagation for analysis of biological repeats. Significance was determined by Student's *t* test: *, $P < 0.05$. **, $P < 0.01$. The values are mean \pm SE, $n = 3$



RG-I biosynthesis. However the methylation activity of *PtrDUF579-1* on pectin polysaccharides residues remains to be further verified.

In RG-II, fucose and xylose are often methylated and the galacturonan backbones is often modified by methyl esterification which facilitates the formation of hydrophobic pectin gel to stabilize cell wall junction zones (Harholt et al. 2010; Thakur et al. 1997). In this study, the content of

methylated fucose and xylose was found to be unchanged, indicating that *PtrDUF579-1* may not affect the methylation of fucose and xylose. Pectin polysaccharides, which is reported to be synthesized by large protein complex, are one of the major components of the cell plate formed during cell proliferation (Staelin and Moore 1995; Atmodjo et al. 2013). RG-I, RG-II and homogalacturonan (HG) interact with each other through covalent linkages to form

complex pectin structures which are required for cell adhesion and have been reported to affect plant cell proliferation and organ development (Atmodjo et al. 2013; Harholt et al. 2010). Evidence in the literature suggests that mutations which impact the structure of RG I and RG II can have major consequences for plant development. Reduction of pectic arabinan side chain could affect the cell wall integrity of pollen at certain development stages, which suggest pectin arabinan side chains play a role in the remodelling of the pollen cell wall (Cankar et al. 2014). Mutation to *mur1* altered the side chain of RG-II and affected the dimer structure of RGII. The mutant displayed small leaf and dwarfed plant size (O'Neill et al. 2001). Mutations to *NpGUTI* (*glucuronyltransferase 1*), which is required for adding glucuronic acid to RG II, affected cell proliferation during shoot meristem development and organ formation (Iwai et al. 2002). *Quasimodo1* (*qual-1*) mutants showed reduced levels of galacturonic acid in pectin and a disrupted pattern of cell proliferation which led to abnormal cotyledons, leaves, and hypocotyls (Bouton et al. 2002). 4-*O*-methyl-GlcA is also present in arabinogalactan side chains of arabinogalactan proteins (AGP) in plants (Tryfona et al. 2012; Gaspar et al. 2001). Whether PtrDUF579-1 catalyzes the methylation of GlcA residues in arabinogalactan side chains of AGPs, which function in cambium cells remains to be examined. The present study showed that suppression of *PtrDUF579-1* affected pectin biosynthesis and reduced cambium cell proliferation, which in turn led to reduced plant growth. The detail mechanism by which PtrDUF579-1 may impact pectin biosynthesis and its effect on cambium cell activity needs to be further studied.

This study provides an array of evidence which suggest PtrDUF579-1 plays a role in cambium cell proliferation during secondary growth of *Populus* through its involvement in cell wall biosynthesis. The results shed new light on the function of DUF579 family members during vascular cambium proliferation in *Populus*.

Materials and methods

Plant growth and transformation

Populus trichocarpa was used for gene cloning and expression analysis. *Populus* (*Populus euramericana* cv. 'Nanlin895') was used for genetic transformation. *Populus* transformation was performed according to the leaf disc inoculation protocol used in our lab (Li et al. 2003). After transgenic plants were verified, they were multiplied through micro-cutting propagation for biological repeats. Young *Populus* and tobacco (*Nicotiana benthamiana*) plants were grown in a phytotron with a

light and dark cycle of 16 h and 8 h at 22 °C, and a light density of 150 $\mu\text{E m}^{-2} \text{s}^{-1}$. *Populus* trees at three-months were moved to a greenhouse with supplementary light of 200 $\mu\text{E m}^{-2} \text{s}^{-1}$.

Sequence analysis of *Populus* and *Arabidopsis* DUF579 family genes

The nucleic acid sequences of DUF579 family genes were identified from the *P. trichocarpa* genome (<http://www.phytozome.net/poplar>). Protein sequence for alignment with *Arabidopsis* DUF579 proteins were deduced using the ClustalW method (<http://www.ebi.ac.uk/Tools/msa/clustalw2>). The phylogenetic relationship of *Populus* and *Arabidopsis* DUF579 family proteins were analyzed by the MEGA5.0 program using neighbor-joining method (Tamura et al. 2011). Bootstrap values were calculated from 1,000 trials. Transmembrane signal peptide was predicted by the TMHMM server (<http://www.cbs.dtu.dk/services/TMHMM>).

Gene expression analysis

Populus trichocarpa tissues were sampled from 6-month-old plants at growing season to analyze the expression profile of the *PtrDUF579* gene. Shoot tip tissues were collected about 5 mm in length from shoot apical buds. After that, young stem (the second internode) was collected. After peeling off the bark of the stem segment (diameter about 1 cm), developing xylem and phloem tissues were collected from the inner surface of the bark and outer surface of the wood with a razor blade. The 3rd to 4th leaves from the tip were collected as young leaves. All samples were immediately frozen in liquid nitrogen for RNA extraction. Total RNA was extracted from various tissues using the modified CTAB method (Chang et al. 1993). Total RNA used for analyzing the expression of *PtrDUF579* genes was extracted from stem segment of the 12th internode of WT and *PtrDUF579-1* suppressed plants. After treatment with RNase-free DNase I to remove DNA contamination, 1 μg of total RNA was reversely transcribed into cDNAs. The cDNAs were then used for gene expression analysis by quantitative PCR. Gene-specific primers were designed to amplify a specific fragment (100–300 bp in length) of the detected genes (Table S2). The primers were examined and verified for their specificity and amplification efficiency. Using the cDNA prepared above as a template, quantitative real-time PCR was performed using Power 2 \times SYBR Real-time PCR premixture (Biotek) on a MyiQ Real-Time PCR Detection System (Bio-Rad, Winston-Salem, NC, USA). The gene expression was normalized against *PtrActin2* expression and analyzed by Delta CT method using three biological replicates from independent plants.

Antibody production

Specific peptides for PtrDUF579 proteins were synthesized and injected into mouse or rabbit to raise antibodies (Abmart, Shanghai, China). Peptides used were “HHV-TYDSKVN” for PtrDUF579-1 “PKAENDDLTNPSSS” and “DEEDQPKPSTPA” for PtrDUF579-3 and “IKPIS-DVLRK” and “RELIASKEQIK” for PtrDUF579-9 (Fig. S1). Following the established immunization protocol, crude mouse ascites or rabbit serum were collected and purified using protein-A/G Sepharose. The purified antibodies were diluted into a concentration of $1 \mu\text{g} \mu\text{l}^{-1}$ for later use.

Western blot analysis

Total protein, microsomal protein and soluble protein from developing xylem and phloem tissues were extracted according to previous protocol (Song et al. 2010). For Western blot analysis, the protein was quantified and boiled in $2 \times$ sample buffer (100 mM Tris-HCl, 2 % sodium dodecylsulfate, SDS, 10 % glycerol, 2 % β -mercaptoethanol and 0.5 % bromophenol blue) and $5 \mu\text{g}$ protein was loaded onto 9 % sodium dodecylsulfate-polyacrylamide electrophoresis (SDS-PAGE) gel. After electrophoresis separation, proteins were transferred onto polyvinylidene difluoride (PVDF) membrane and analyzed as described previously (Song et al. 2010). The first antibodies were diluted 1:1,000 for PtrDUF579-1, 1:500 for PtrDUF579-3, 1:1,000 for PtrDUF579-9 and 1:2,000 for Actin (Abmart). The secondary antibodies (linked with alkaline phosphatase, Santa Cruz, CA, USA) were diluted in 1:5,000.

Immunolocalization

The 12th internodes of *Populus* stem were embedded and sliced into $10 \mu\text{m}$ -thick sections for immunolocalization according to previous protocol (Song et al. 2010). The first antibodies were diluted in 1:200 for PtrDUF579-1, 1:100 for PtrDUF579-3 and 1:200 for PtrDUF579-9. The secondary antibodies were diluted in 1:1,000. After color development, the sections were gradually dehydrated with alcohols, cleared with xylene and observed under an OLYMPUS BX51 light microscope (Olympus, NY, USA).

Subcellular localization and colocalization experiment

PtrDUF579-1 gene was fused with *mCherry* gene and inserted into pm-rk binary vector after removing *PIP2A* gene (Nelson et al. 2007) (see primer sequences for constructs in Table S2). The constructs of G-gk (Nelson et al. 2007) were used for Golgi identification. *PtrDUF579-1* constructs and G-gk were then transformed

into *Agrobacterium*, and co-injected into the young leaves of one-month old tobacco. After cultivation for 2 days, the leaves were observed under laser confocal scanning microscopy (LCSM) (LSM510 META, ZEISS, Germany) with excitation and detection wavelength as following: 488 and 505–530 nm for GFP, 543 and 560–615 nm for mCherry.

Gene constructs and genetic transformation

The full coding sequences of *PtrDUF579-1* were amplified and cloned from stem cDNA of *P. trichocarpa*. To construct antisense vector, the coding sequences of *PtrDUF579-1* were reversely inserted into pBI121 binary vector under a constitutive CaMV35S promoter. The constructs were transferred into *Populus* by *Agrobacterium* mediated transformation according to the protocol adopted in our lab (Li et al. 2003). Secondary growth was examined with *Populus* stems (12th internode) which were cut into 3 mm length and fixed in FAA solution (formaldehyde: glacial acetic acid: ethanol = 1:1:18) overnight. After being dehydrated in an ethanol concentration series (50, 70, 80, 90, 95, 100 %, each step for 1 h), ethanol was sequentially replaced with ethanol: xylene (1:1) for 30 min at room temperature, then with pure xylene and xylene : paraffin (1:1) for 1 h at 70 °C, respectively, and finally replaced with fresh paraffin for 3 h at 70 °C. After the paraffin replacement was repeated three time, stem segments were embedded into paraffin. The $10\text{-}\mu\text{m}$ sections were obtained with a Leica RM2235 rotary microtome (Leica Microsystems, Germany). After removal of paraffin, sections were stained with 0.05 % toluidine blue and analyzed using an OLYMPUS BX51 light microscope.

Chemical analysis of cell wall components

The cambium cells on the sides of the phloem and xylem from 1-year-old trees grown in green house were collected by gentle scraping with a razor blade and immediately frozen in liquid nitrogen. The collected cells were then ground into a fine powder to prepare alcohol insoluble residues (AIR) according to (Foster et al. 2010). After de-starched procedure and iodine test, a total of 50 mg AIRs were subsequently digested by 30 U of pectinase from *Aspergillus aculeatus* (P2611, Sigma, MO, USA) which contains endopolygalacturonase (EPG) and pectin methylesterase (PME) in 0.1 M NaOAc buffer (pH 5.0) at 37 °C overnight. PE fractions were collected after the suspensions were passed through a cut-off ultrafiltration tube (10 kDa, Millipore). The rest of the pellets were then digested with 30 U of xyloglucan specific endo-1,4- β -glucanase from *Paenibacillus* sp.(E-XEGP, Megazyme, Ireland) in 0.1 M NaOAc buffer (pH5.0) at 37 °C overnight. XG fractions

were collected with a procedure as described above. The neutral sugar content of the PE fractions, XG fractions and 4 N KOH fractions were determined as described (Li et al. 2009). The uronic acids were determined according to (Lliveras-Tenorio et al. 2012) with modifications. Briefly, PE fractions were hydrolyzed with 2 M trifluoroacetic acid (TFA) at 120 °C for 1.5 h. The produced monosaccharides were then dried in a rotatory evaporator and subjected to mercaptalation with 30 μ l of ethanethiol/TFA (2/1, v/v) at room temperature for 20 min. For silylation, 80 μ l of *N,O*-bis(trimethylsilyl) trifluoroacetamide (BSTFA) was added to the mercaptalation mixture and incubated at 60 °C for 20 min. After drying with a nitrogen flow, 50 μ l of BSTFA-trimethylchlorosilane (TMCS) (99:1) and 50 μ l of pyridine were added and kept at 60 °C for 90 min. The final reaction mixture was dried with nitrogen flow, reconstituted in 100 μ l of hexane and 2 μ l of them were subjected to GC-MS analysis. The sugars and uronic acids were separated by HP-5 column, and detected by Agilent 5975 inert MSD system (Agilent, Santa Clara, CA, USA).

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Conflict of interest No conflict of interest declared.

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