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Populus endo-beta-mannanase PtrMAN6 plays a role in coordinating cell wall remodeling with suppression of secondary wall thickening through generation of oligosaccharide signals

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SUMMARY

Endo-1,4-β-mannanase is known to able to hydrolyze mannan-type polysaccharides in cell wall remodeling, but its function in regulating wall thickening has been little studied. Here we show that a *Populus* endo-1,4-β-mannanase gene, named *PtrMAN6*, suppresses cell wall thickening during xylem differentiation. *PtrMAN6* is expressed specifically in xylem tissue and its encoded protein localizes to developing vessel cells. Overexpression of *PtrMAN6* enhanced wall loosening as well as suppressed secondary wall thickening, whilst knockdown of its expression promoted secondary wall thickening. Transcriptional analysis revealed that *PtrMAN6* overexpression downregulated the transcriptional program of secondary cell wall thickening, whilst *PtrMAN6* knockdown upregulated transcriptional activities toward secondary wall formation. Activity of PtrMAN6 hydrolysis resulted in the generation of oligosaccharide compounds from cell wall polysaccharides. Application of the oligosaccharides resulted in cellular and transcriptional changes that were similar to those found in *PtrMAN6* overexpressed transgenic plants. Overall, our results demonstrated that *PtrMAN6* plays a role in hydrolysis of mannan-type wall polysaccharides to produce oligosaccharides that may serve as signaling molecules to suppress cell wall thickening during wood xylem cell differentiation.

Keywords: mannanase, oligosaccharide, cell wall, xylem, sclerenchyma.

INTRODUCTION

In higher plants, cell walls (CWs) make up the bodily structure and stockpile the majority of photosynthesis-fixed carbon and solar energy. CWs can be generally classified into the primary cell wall (PCW) and secondary cell wall (SCW), which are formed through different processes and are regulated via different pathways. The PCW begins to form along with cell plate assembly during cytokinesis and continues to be modified as the cell expands. However, SCW differentiation occurs only in certain types of cells, such as vessel (or tracheary) elements and fiber cells in vascular tissue. Thus, to develop wall-thickened cells, meristematic cells must receive signals that initiate the cell differentiating and wall thickening programs.

Several plant hormones have been reported to regulate the differentiation of wall-thickened vascular tissue. Auxin plays a role in promoting the differentiation of procambial cells into xylem (Milioni *et al.*, 2001; Moyle *et al.*, 2002), and brassinosteroids (BRs) are required for the later stages of tracheary element (TE) differentiation (Iwasaki and Shibaoka, 1991; Yamamoto *et al.*, 2001). In contrast, cytokinins play an important role in maintaining procambial cell identity and preventing protoxylem specification (Mahonen *et al.*, 2006). Gibberellin has also been shown to stimulate xylem expansion in Arabidopsis hypocotyl (Ragni *et al.*, 2011). Some peptides and oligosaccharides have also been reported to function as extracellular signaling molecules that regulate TE differentiation. Oligosaccharides (Roberts *et al.*, 1997) and sulphated pentapeptides (PSK-α; Matsubayashi *et al.*, 1999) promoted TE differentiation in a zinnia (*Zinnia elegans* L.) culture system. Moreover, xylogen, a proteoglycan-like factor, was found to mediate an inductive cell-cell interaction involved in plant tissue differentiation (Motose *et al.*, 2004). Also in the zinnia xylogenic culture system, a 12-amino acid peptide from the CLAVATA3/ESR-related (CLE) gene family that inhibited TE differentiation was isolated as an extracellular signaling molecule (Ito *et al.*, 2006).

Galactoglucomannan oligosaccharides (GGMOs, with degree of polymerization (DP) 4-8) are considered to be a type of signaling molecule that affects cell differentiation. Exogenous GGMOs affect SCW thickening by regulating the differentiation of protoxylem-like TEs and metaxylem-like TEs in xylogenic cultures of zinnia (Benova-Kakosova *et al.*, 2006). Interactions between GGMOs and auxin have been studied in the process of seed germination in mung bean (Kollarova *et al.*, 2010). GGMOs induced adventitious root growth in the absence of auxin and inhibited adventitious root induction in the presence of auxin (Kollarova *et al.*, 2005). How GGMOs are produced and integrated in the larger signaling network regulating cell differentiating is still unclear.

Mannan-type oligosaccharides are thought to be produced by endo-1,4-β-mannanase (E.C.3.2.1.78) (MAN) that catalyzes the cleavage of β(1–4) bonds in the backbones of mannan polymers (Shallom and Shoham, 2003). Plant *MAN*s are reported to be involved in the seed germination and post-germination process by promoting hydrolysis of mannan-rich endosperm CWs (Bewley *et al.*, 1997; Nonogaki *et al.*, 2000; Gong and Bewley, 2007; Iglesias-Fernandez *et al.*, 2011a,b; Rodriguez-Gacio Mdel *et al.*, 2012). In tomato, *LeMAN1* is expressed in the endosperm of germinated seeds and plays a role in hydrolyzing stored polysaccharides to provide germinating seeds with its energy and carbon source needs (Bewley *et al.*, 1997; Nonogaki *et al.*, 2000). Tomato *LeMAN2* (Nonogaki *et al.*, 2000) and Arabidopsis *AtMAN5*, *AtMAN6* and *AtMAN7* (Iglesias-Fernandez *et al.*, 2011a,b) are involved in the hydrolysis of mannan-rich CWs to allow for radicle emergence and the completion of germination. MAN activity was also detected in CW softening associated with fruit ripening (Bewley *et al.*, 2000; Schroder *et al.*, 2006). Tomato *LeMAN4* is expressed mainly in the skin and outer pericarp of the fruit (Bewley *et al.*, 2000; Banik *et al.*, 2001; Bourgault and Bewley, 2002), and probably contributes to the loosening of the CW through mannan hydrolysis (Bewley *et al.*, 2000) or mannan transglycosylase (Schroder *et al.*, 2006) activity. However, it is unclear whether the mannan-type oligosaccharides produced through MAN hydrolysis play a role in regulating plant development.

In this study, we identified a *Populus* endo-1,4-β-mannanase gene, *PtrMAN6*, that is specifically expressed in xylem tissue and that regulated the transcriptional program governing secondary wall thickening during xylem differentiation. We also present evidence to show that *PtrMAN6* catalyzes the hydrolysis of mannan-type wall polysaccharides to produce GGMOs, which in turn serve as signaling molecules to regulate the transcriptional program of CW thickening.

RESULTS

Expression of *MAN* genes in *Populus trichocarpa*

In our previous study, a MAN protein was identified in the plasma membrane of *Populus* differentiating xylem tissue (Song *et al.*, 2011). Although MAN has been studied for its roles in seed germination and fruit ripening (Bewley *et al.*, 2000; Gong and Bewley, 2007; Iglesias-Fernandez *et al.*, 2011a), the aforementioned result suggests the possibility that MAN plays a yet to be determined role in xylem development.

Eight *MAN*s are predicted in the *Populus trichocarpa* genome (Yuan *et al.*, 2007) and named *PtrMAN1* to *PtrMAN8*, respectively. The MAN protein that was identified in the differentiating xylem plasma membrane is encoded by the *PtrMAN6* gene. When we analyzed the expression of the eight genes, only five gene transcripts (*PtrMAN4*, *PtrMAN5*, *PtrMAN6*, *PtrMAN7* and *PtrMAN8*) were detected in the examined tissues, which included differentiating xylem, differentiating phloem, matured leaf, young leaf and shoot tip. The expression of the five genes was spatially regulated in *P. trichocarpa* (Figure 1). *PtrMAN4*, *PtrMAN6* and *PtrMAN8* were specifically expressed in differentiating xylem tissue that is consistent with the tissue location where the PtrMAN6 protein was detected in our previous study. Meanwhile, *PtrMAN5* was expressed in all tissues examined, while *PtrMAN7* was mainly expressed in young leaf and shoot tip.

Sequence analysis of the five expressed *Populus* MANs indicated that PtrMAN4 and PtrMAN6 shared 93% sequence identity while other MAN sequences were more divergent, sharing only about 50% sequence identity (Table S1). The highly conserved sequence makes it likely that PtrMAN4 and PtrMAN6 could initiate similar pathways in *Populus*.

Localization of PtrMAN6

The PtrMAN6 protein is predicted to be a secretory protein that contains a signal peptide at its N terminus (Figure S1a; Petersen *et al.*, 2011). To examine the subcellular localization of PtrMAN6, a construct coding for a PtrMAN6: GFP fusion protein was generated under the control of a

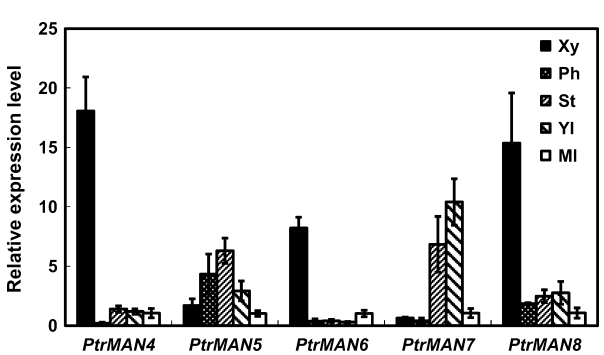

Figure 1. Expression profile of *PtrMAN*s in various *Populus* tissues.
This experiment was performed three times using different batches of plants. Error bars represent the standard error (SE) of three technical replicates using pooled samples of at least three independent plants. Xy, xylem; Ph, phloem; St, shoot tip; Yl, young leaf; Ml, mature leaf.

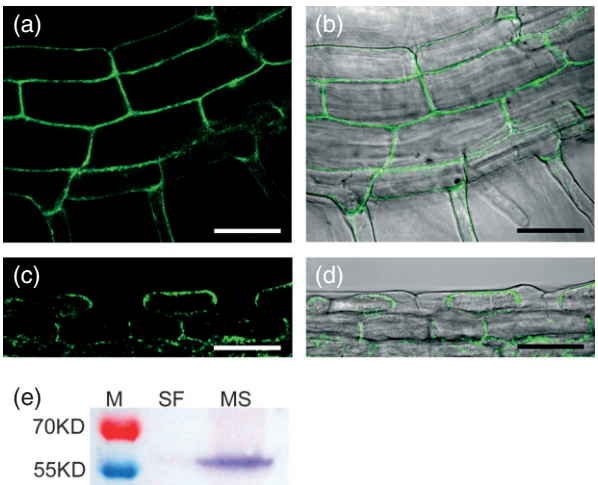

Figure 2. Analysis of PtrMAN6 plasma membrane localization.
(a,b) Stable expression of PtrMAN6:GFP fusion protein in Arabidopsis root cells, showing fluorescent signals on the plasma membrane.
(c,d) Verification of PtrMAN6:GFP fluorescent signals on the plasma membrane by plasmolysis treatment with 30% sucrose.
(a,c) Fluorescent images.
(b,d) Images under bright-field microscopy.
(e) Western blot showing PtrMAN6 is present in the microsomal (MS), not in the soluble fraction (SF). M, molecular mass marker. Scale bar = 50 μm.

cauliflower mosaic virus (CaMV) 35S promoter. The construct was transformed into Arabidopsis and the young roots of the transgenics were used for fluorescence location analysis. Results revealed that PtrMAN6 was specifically localized on the plasma membrane (Figure 2a,b), a finding that was further confirmed by plasmolysis analysis (Figure 2c,d). Western blot analysis detected PtrMAN6 protein in the microsomal but not in the soluble fraction of *Populus* xylem proteins (Figure 2e). However, PtrMAN6 could be partially washed off from the membrane fraction using 100 mM sodium carbonate. This finding suggests that PtrMAN6 may be bound to the membrane as an integral monotopic protein. Transient expression of *PtrMAN6* in onion epidermal cells was carried out to determine which portion of the peptide is responsible for the localization. A full-length PtrMAN6 and the N-terminal sequences of 31 amino acids were both fused with green fluorescent protein (GFP) (Figure S1b). Both the full-length PtrMAN6 and putative signal peptide were able to direct its fused GFP protein onto the plasma membrane (Figure S1c,d) and were compared with the GFP control that showed fluorescent signals in the cytoplasm and nuclei (Figure S1e). Together, these results demonstrate that PtrMAN6 is localized on the plasma membrane by its N-terminal peptide.

In order to determine the cell-type expression of PtrMAN6, antibodies against two unique peptides from PtrMAN6 protein were produced and the antibody specificity in recognizing PtrMAN6 was confirmed (Figure S2a–c). Immunolocalization was carried out using stem and young petiole, of which the sections were hybridized with specific antibodies against PtrMAN6. Highly specific PtrMAN6 signals was detected in the developing vessel cells of xylem tissue (Figure 3), and this result suggested that PtrMAN6 plays a role specifically in vessel element development during *Populus* xylem differentiation.

PtrMAN6 activity, glycosylation and dimerization

As the *PtrMAN6* gene was predicted to code for a putative endo-1,4-beta-mannanase, it was expected to be able to digest azurine cross-linked (AZCL)-galactomannan (Schroder *et al.*, 2006). We first produced recombinant proteins of PtrMAN6 in *E. coli* but its enzymatic activity could not be determined (Figure S2c,d). When we examined both recombinant protein and plant-sourced PtrMAN6 protein by western blot analysis, the latter showed a larger molecular size than the former (Figure S2e). This difference suggests potential post-translational modification of PtrMAN6 that results in a larger molecular size as detected in the western blot. Thus we transformed *Populus* with a CaMV 35S:PtrMAN6 construct in order to produce a large amount of plant-sourced PtrMAN6 protein from non-*PtrMAN6*-expressing tissue.

PtrMAN proteins were extracted from the young leaves of transgenic *Populus* plants that overexpressed *PtrMAN6* (*PtrMAN6* expression is minimal in wild type; Figure 1) and proteins were used to determine its enzymatic activity. A significant amount of PtrMAN6 hydrolysis activity was detected with the protein from the transgenic leaves, while small levels of activity were observed from the wild type (Figure 4). A detailed characterization of the enzyme properties was conducted using plant-sourced proteins. At 37°C, PtrMAN6 was particularly sensitive to pH conditions and exhibited maximum activity at pH 5 (Figure 4a). At its optimal pH, PtrMAN6 showed a temperature optimum of 50°C (Figure 4b). Thus the conditions of pH 5 and 50°C were applied to subsequent measurements of enzyme activity. Under these conditions, the enzyme was examined for its ability to hydrolyze various polysaccharide substrates. Results demonstrated that PtrMAN6 was able to cleave mannan-type polysaccharides including galactoglucomannan (GGM), galactomannan, glucomannan and mannan with the highest activity in digestion of GGM polysaccharides (Figure S2g).

Four glycosylation sites were predicted in the PtrMAN6 amino acid sequence (Figure S2f). PtrMAN6 from *Populus* xylem tissue displayed a single band with a molecular mass of approximate 58 kDa based on western blot analysis (Figure 4c, Lane 1). After treatment with endoglycosidase H (Endo H_f), PtrMAN6 was detected with a molecular mass of about 50 kDa (Figure 4c, Lane 3), which is consistent with its theoretical molecular mass. The result suggests that plant-sourced PtrMAN6 is modified through N-glycosylation. When plant-sourced PtrMAN6 was treated

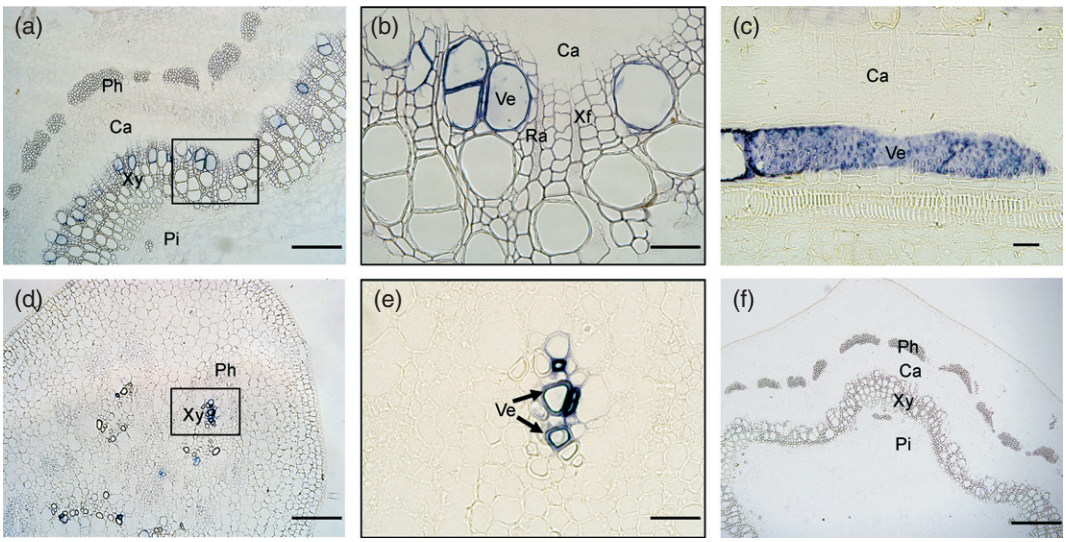

Figure 3. Immunolocalization of PtrMAN6 in developing vessel cells in *Populus*.
(a, b, f) Cross-sections and (c) longitudinal sections of the stem at the sixth internode and (d, e) cross-sections of young petiole were hybridized with anti-PtrMAN6 antibodies (a–e) or preimmune IgG (f). PtrMAN6 is specially localized in developing vessel.
(b,e) High magnification of the frames in (a, d). Xy, xylem; Ph, phloem; Ca, cambium; Pi, pith; Ve, vessel cell; Xf, xylem fiber cell; Ra, ray cell. Scale bars in (a, f) = 200 μm; in (b,c) = 50 μm; in (d) = 100 μm; in (e) = 500 μm.

incompletely with Endo H_f, several bands between 50 and 58 kDa were also observed (Figure 4c; Lane 2), a finding that suggested that PtrMAN6 might contain multiple N-glycosylation sites.

To examine the effect of glycosylation on PtrMAN6 activities, native enzymes extracted from *Populus* differentiating xylem were treated with Endo H_f. PtrMAN6 activity decreased by about 50% after 2 h of Endo H_f treatment, compared with the untreated controls (Figure 4d). These results suggest that N-glycosylation of PtrMAN6 is required for its enzymatic activities in *Populus*. Furthermore, when analyzed by SDS-PAGE and immunoblot under reducing conditions, PtrMAN6 proteins migrated as monomeric proteins (Figure S2a). However, under non-reducing conditions, PtrMAN6 proteins migrated with an apparent molecular mass of approximately twice that of the monomers (Figure 4e). When the proteins were treated with different thiol reductants (β-mercaptoethanol (β-ME) and dithiothreitol (DTT) for 2 h, the fraction of PtrMAN6 monomers increased significantly (Figure 4e). The fraction of PtrMAN6 dimers gradually decreased with increase in the reductant concentration (Figure S2h). This finding suggests that native PtrMAN6 tends to form disulfide-linked homodimers. However, prokaryotic recombinant PtrMAN6 in *E. coli* was not able to dimerize under either non-reducing or reducing conditions (Figure S2i). To examine the effect of dimerization on PtrMAN6 activities, native enzymes were treated with thiol reductants. Compared with the untreated control, PtrMAN6 activity decreased by about 84 and 53% after 2 h of 5% β-ME or 10 mM DTT treatment, respectively (Figure 4f). These results suggest that disulfide-linked dimerization of PtrMAN6 is also required for its enzymatic activities in *Populus*.

Effects of *PtrMAN6* on *Populus* vascular development

To investigate the genetic function of *PtrMAN6*, we transformed *Populus* with constructs that resulted in the overexpression and knockdown of *PtrMAN6*. At least 30 independent transgenic lines were generated for each construct. Three transgenic lines with high expression of the transgene (Figure S3a) were selected and characterized for morphology, wood anatomy and other characteristics. Compared with wild type (WT), overexpression of *PtrMAN6* resulted in softer stems and petioles while the transgenic plants with downregulated expression of *PtrMAN6* gene displayed slightly stronger stems (Figure 5a,b).

Examination of the stem cross-section revealed that the CW thickening in the vascular cells of transgenic plants differed from that in WT. In *PtrMAN6* overexpressed plants, lignin deposition was significantly delayed and reduced in xylem cells. In contrast, lignin deposition occurred earlier and at elevated levels in *PtrMAN6* downregulated plants (Figure 5c,d). It appears that *PtrMAN6* downregulation also affected the wall thickening process in pith cells and fiber cells (Figure 5c). Determination of lignin content further confirmed that overexpression of *PtrMAN6* resulted in lignin reduction (Figure 5e). Crystalline cellulose content in *PtrMAN6*-overexpressed plants was also decreased (Figure 5f). The size of vascular cells (in diameter) in the 12th internode stem sections were measured under ultraviolet (UV) light view (Figure S3b–d) and showed large differences between transgenic and WT plants (Figure 5g).

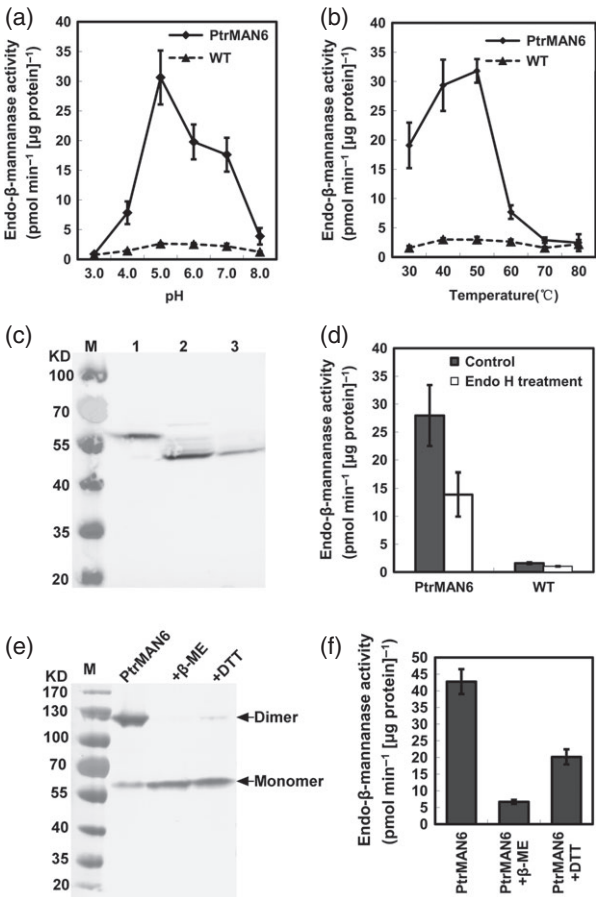

Figure 4. Enzymatic activities of PtrMAN6.
Proteins from the leaves of *PtrMAN6*-overexpressed plants were used for enzyme analysis. WT, wild type plants.
(a) Effect of pH on PtrMAN6 activity.
(b) Effect of temperature on PtrMAN6 activity.
(c) PtrMAN6 treated with endoglycosidase Endo H_f and analyzed by western blot. Lane M, molecular mass standard; Lane 1, untreated protein; Lane 2 and 3, the protein treated with Endo H_f for 30 min and 60 min, respectively.
(d) Effect of Endo H_f treatment on PtrMAN6 activity.
(e) Native PtrMAN6 protein from *Populus* was electrophoresed on 10% SDS-PAGE gels under non-reducing (PtrMAN6) or reducing condition (+β-ME: 5% β-mercaptoethanol; +DTT: 10 mM 1,4-dithiothreitol) and detected by immunoblot with anti-PtrMAN6 IgG. Monomeric and dimeric proteins are indicated. Lane M, molecular mass standard.
(f) Effect of dimerization on PtrMAN6 activity. Error bars represent standard error (SE) of triplicate sample measurements.

In addition, morphological differences of adventitious root development in calli were observed in the *PtrMAN6*-overexpressed plants (Figure S3f) but not in transformed controls (Figure S3e). Together, these results suggest that *PtrMAN6*, in addition to functioning as an enzyme to digest mannan-type polysaccharides resulting in a relaxation of the CW, is also involved in regulating other biological events during CW thickening.

Monosaccharide composition in the transgenic plants was determined. As shown in Table 1, the neutral sugars content in transgenic plants was changed dramatically. The content of mannose and xylose was significantly lower in *PtrMAN6*-overexpressed plants. In the examined transgenic lines, mannose content decreased by 33 and 36%; xylose content was reduced by 18 and 24%. These two sugars are the main components of mannan and xylan, which are abundantly localized in the SCWs of dicots (Handford *et al.*, 2003; Scheller and Ulvskov, 2010). In contrast, the levels of other sugars such as fucose, arabinose and rhamnose were increased significantly. These sugars are relatively more abundant in primary wall hemicellulose and pectin. In *PtrMAN6*-suppressed plants, changes in sugar content that were the opposite of those in *PtrMAN6*-overexpressed plants were observed. Together, these data suggest that secondary wall formation is repressed in *PtrMAN6*-overexpressed plants but accelerated in *PtrMAN6*-suppressed plants.

Transcriptional program regulated by *PtrMAN6* expression

It is known that several transcription factors, such as wood-associated NAC domain transcription factors *WND*s (*WND1A* to *WND6A* and *WND1B* to *WND6B* in *Populus*), MYB3(v-myb avian myeloblastosis viral oncogene homolog), *MYB20* and *MYB28* are key regulators that dictate the transcriptional program toward xylem cell differentiation, CW thickening and lignin biosynthesis (Zhong and Ye, 2009; McCarthy *et al.*, 2010; Zhong *et al.*, 2010; Ohtani *et al.*, 2011). To examine how *PtrMAN6* is involved in the suppression of CW thickening, we examined the expression of the key transcriptional factors in transgenic and WT plants. Overexpression of *PtrMAN6* downregulated the expression of transcription factors including *WND1A*, *WND2A*, *WND3A*, *WND4A*, *WND5A*, WND6A, *MYB3*, *MYB20* and *MYB28* (Figure 6a). Furthermore, several other SCW-related genes, such as *CesA8*, *GT43B*, *C3H1* and *CAD4* genes, in *Populus* were also downregulated in *PtrMAN6*-overexpressed plants (Figure 6a). On the other hand, suppression of *PtrMAN6* upregulated the transcriptional activities of these genes (Figure 6b). These results indicated that upregulation of *PtrMAN6* suppresses the transcriptional program that regulates xylem cell differentiation and CW thickening.

Regulation of *PtrMAN6* may be mediated by oligosaccharides

To understand how PtrMAN6 hydrolysis is implicated in the regulation of transcriptional activities during vascular cell development, we examined the products from *Populus* GGM hydrolyzed by PtrMAN6. GGM was extracted from *Populus* xylem tissue and digested by plant-sourced PtrMAN6 enzyme. The products were examined by high pressure liquid chromotography/quadruple time of flight mass spectrometry (HPLC/QTOF-MS). In digestions of 8–24 h, the same oligosaccharide products were identified (Figure 7; Figure S4), whilst no such products were detected in the reaction with heat-inactivated PtrMAN6

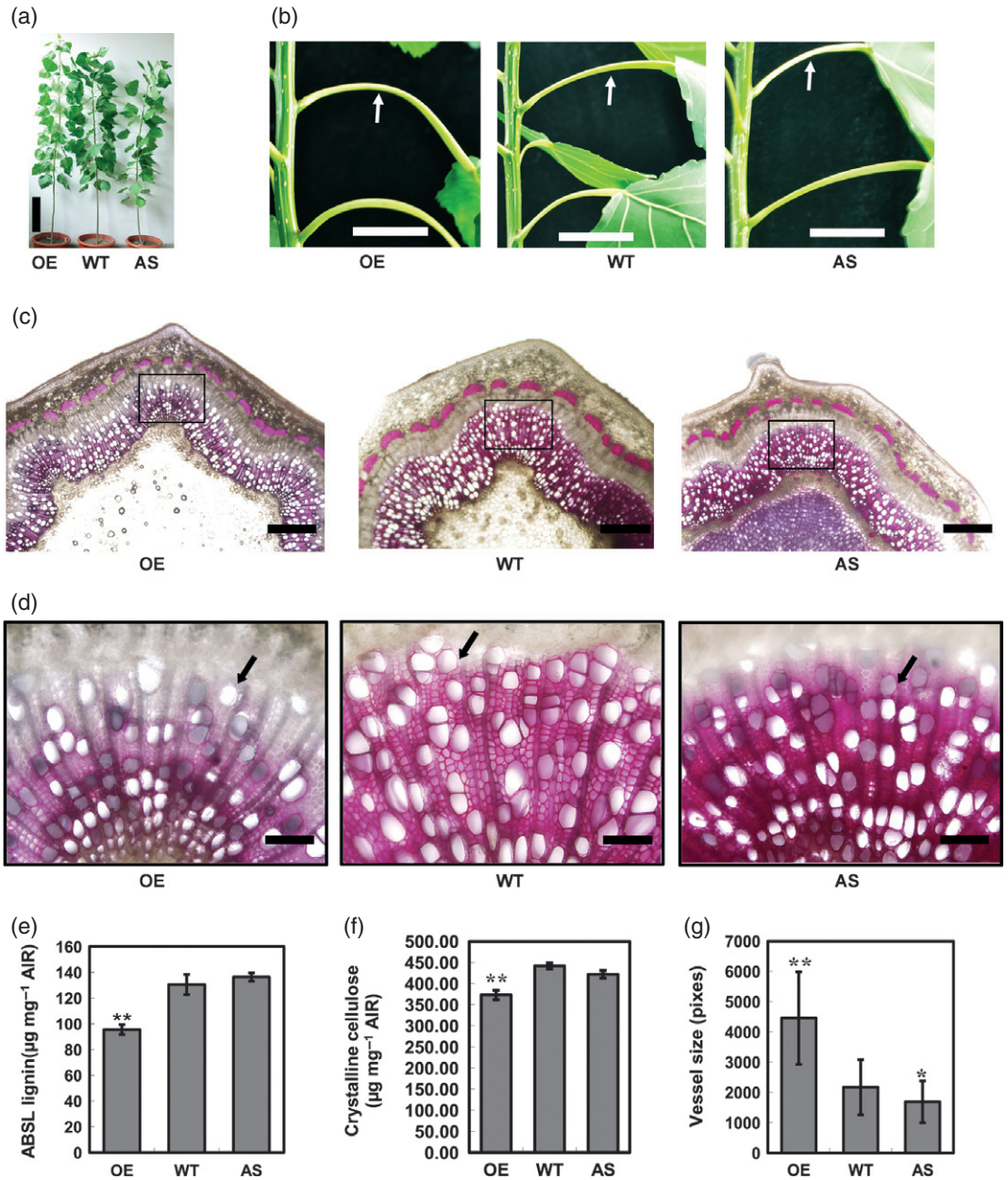

Figure 5. Phenotype of *PtrMAN6*-overexpressed and *PtrMAN6*-suppressed transgenic *Populus*.
(a) Representative *PtrMAN6*-overexpressed plant (OE, left), wild type plant (WT, middle) and *PtrMAN6*-suppressed plant (AS, right).
(b) Partial shoots of the plants in (a). Arrow indicates the petiole of the14th leaf from the tip.
(c) Cross-sections of the 14th internode stem showing weaker lignin deposition in the xylem cell walls (CWs) of OE plants (left) and stronger lignin deposition in the xylem CWs as well as the pith CWs of the AS plants (right) compared with the WT (middle).
(d) High magnification of the framed zones in (c). Arrow indicates vessel cells.
(e) Lignin content of wood tissue in 1-year-old plants.
(f) Crystalline cellulose content.
(g) Size of vessel cells measured in the stem sections at the 12th internode. Scale bars in (a) = 20 cm; in (b) = 3 cm; in (c) = 500 μm; in (d) = 100 μm.

enzymes. The identified GGMOs had a DP range from 2–7. We next investigated how the GGMO molecules affected plant growth and vascular cell differentiation.

First, we treated the *Populus* stem vascular cells with GGMOs (see experiment description in Experimental Procedures and in Figure S5). Before treatment, the vascular tissue contained approximate 1–7 layers of xylem cells (Figure 8a). After 1 week of treatment, about seven extra layers of new xylem cells developed. Compared with the control (Figure 8b,e), the CW thickening process in the newly formed cells was suppressed and lignification was inhibited after treatment with GGMO (Figure 8c,f; Figure S5d),

Table 1 Monosaccharide composition of cell wall residues

Residues	Rhamnose	Fucose	Arabinose	Xylose	Mannose	Galactose	Glucose
WT	6.59 ± 0.13	1.66 ± 0.05	13.35 ± 0.93	216.93 ± 7.01	17.45 ± 0.87	13.34 ± 0.43	37.47 ± 1.04
*PtrMAN6*AS Line 5	5.60 ± 0.20**	1.22 ± 0.04**	6.91 ± 0.59**	280.55 ± 7.32**	19.87 ± 0.76*	9.59 ± 0.41**	34.69 ± 1.12
*PtrMAN6*AS Line 6	5.19 ± 0.19**	1.16 ± 0.03**	6.56 ± 0.38**	288.67 ± 5.23**	20.19 ± 0.14**	8.85 ± 0.37**	34.44 ± 0.66*
*PtrMAN6*OE Line 1	9.72 ± 0.13**	3.2 ± 0.07**	35.96 ± 0.96**	165.69 ± 1.83**	11.83 ± 0.28**	31.53 ± 0.61**	45.94 ± 0.87**
*PtrMAN6*OE Line 2	8.78 ± 0.15**	2.69 ± 0.04**	24.56 ± 0.60**	178.64 ± 5.29**	11.32 ± 0.29**	22.20 ± 0.49**	38.32 ± 1.12

AIR: Alcohol-insoluble residues. Value: mean (μg mg^{-1} AIR) ± standard error (SE) of four replicates.
*$P < 0.05$ and **$P < 0.01$, respectively, in Student's *t*-test.

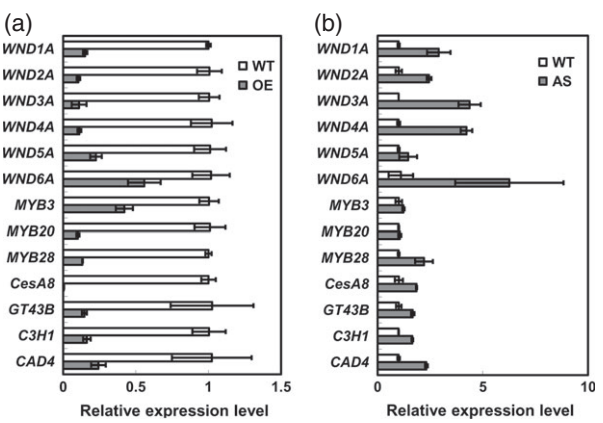

Figure 6. Expression of secondary wall-associated genes in transgenic plants.
(a) Expression of secondary wall-associated genes in *PtrMAN6*-overexpressed plants.
(b) Expression of secondary wall-associated genes in *PtrMAN6*-suppressed plants. Error bars indicate SE of three technical replicates using pooled samples of three independent plants. This experiment was performed three times using different batches of plants.

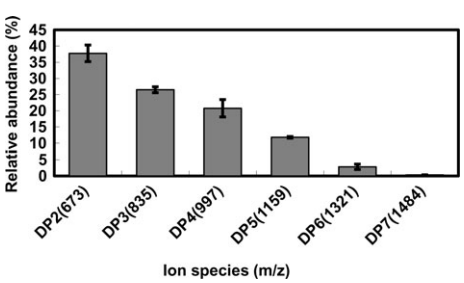

Figure 7. Relative abundance of identified oligosaccharides. DP, degree of polymerization; number: m/z ratio.

a finding that suggested that GGMO treatment suppressed CW thickening during xylem development. Meanwhile, we also investigated the transcript levels of genes associated with cell wall thickening and found that GGMO treatment downregulated the expression of several wall thickening-associated transcriptional factors, such as *WND*s, *MYB3*, *MYB28*, and also genes associated with the lignin biosynthesis pathway (Figure 8d). In this case, GGMO treatment had a similar effect on xylem CW thickening as the changes that occurred in *PtrMAN6*-overexpressed plants. This finding presents the distinct possibility that the regulatory role of *PtrMAN6* may be mediated through its catalytic products, GGMOs, that function as signaling molecules to regulate the transcriptional program of CW thickening.

Second, GGMOs were used to treat the leaf discs from WT and *PtrMAN6*-overexpressed plant for callus induction during transformation (Figure S6). Adventitious root growth, a phenomenon observed during transformation (Figure S3), was recorded following different treatments. At the callus stage, no adventitious root growth was observed in the untreated WT (Figure S6a). Treatment with GGMOs resulted in adventitious root growth in about 50% of the calli, and was similar to the frequency observed with the *PtrMAN6*-overexpressed lines (Figure S6b–d). Overall, MAN-produced oligosaccharides were sufficient to induce phenotypes similar to those caused by *PtrMAN6* overexpression. These results further point to the role that mannan-derived oligosaccharides produced by PtrMAN6 plays as signaling molecules to suppress plant SCW thickening during xylem development.

DISCUSSION

Endo-1,4-beta-mannanase, localized on the plasma membrane, can hydrolyze GGM to produce GGMOs

GGMOs, a group of mannan-derived oligosaccharides, have been reported to act as extracellular signal molecules and regulate xylary cell differentiation (Benova-Kakosova *et al.*, 2006; Richterova-Kucerova *et al.*, 2012). In xylogenic cultures of zinnia, the application of GGMOs is able to increase cell population density and decrease the ratio of protoxylem-like to metaxylem-like TEs (Benova-Kakosova *et al.*, 2006). In mung bean seedlings, GGMOs enhance cell elongation and delay xylem maturation during primary root growth (Richterova-Kucerova *et al.*, 2012). The two studies indicate that GGMOs play a role in regulation of the process of cell growth and differentiation. However, there is little evidence as to how they are produced in plants. Here, we present a body of evidence that supports the role of PtrMAN6, an endo-1,4- beta-mannanase from

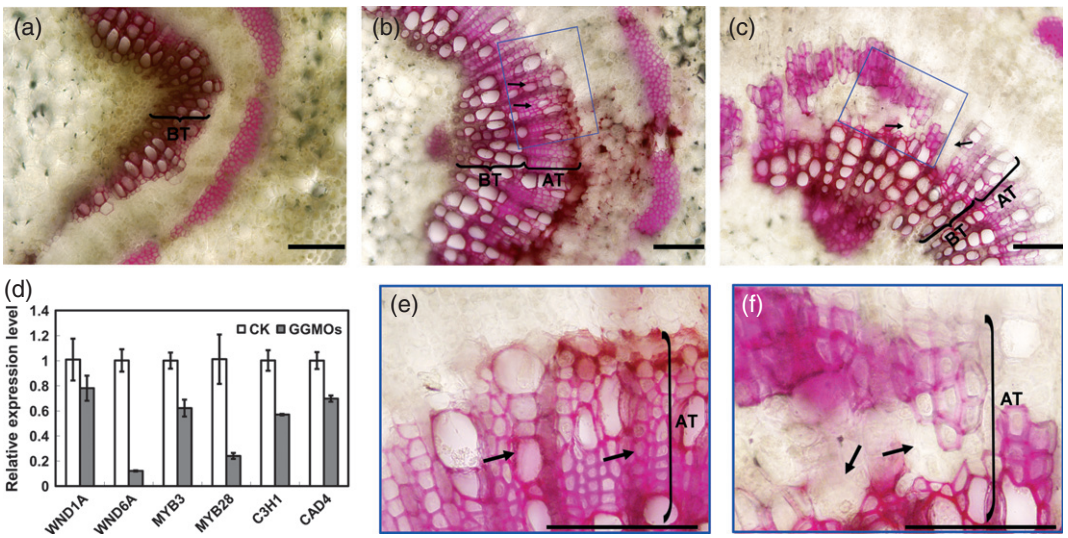

Figure 8. Oligosaccharide effect on wall thickening in xylem tissue.
(a) Lignin deposition in the 6th internode of the stem before galactoglucomannan oligosaccharide (GGMO) treatment.
(b) Lignin deposition in the same internode after 1-week treatment with buffer as control.
(c) Lignin deposition in the same internode after 1-week treatment with 0.4 mg ml^{-1} GGMO solution.
(d) Expression of secondary wall-associated genes in the xylem after GGMO treatment.
(e,f) High magnification of the framed zones in (b) and (c), showing lignin deposition in xylem before treatment (BT) and after treatment (AT). Arrows in (b,c,e,f) indicate the new xylem zone formed after treatment. Scale bar = 100 μm.

poplar, in the production of GGMO molecules that act as signals to suppress CW thickening. The results provide new insights into the signaling networks that direct the transcriptional program for SCW formation during xylem differentiation.

Overexpression of *PtrMAN6* in *Populus* leaves produced active proteins that displayed strong hydrolyzing activity toward mannan-type polysaccharides but minor activity toward other polysaccharides. PtrMAN6 enzyme used GGM as a preferred substrate. When the GGM isolated from *Populus* xylem CWs was hydrolyzed, GGMO molecules were detected and showed biological activity in suppression of cell wall thickening when applied to developing xylem tissue. Further characterization of the oligosaccharide linkage structure would be a next study toward a full elucidation of the mechanisms underlying the GGMO signaling activity.

We found that PtrMAN6 is a glycoprotein and undergoes N-glycosylation that is required for its enzymatic activities. When expressed in a plant system, native PtrMAN6 forms a disulfide-linked homodimer that is essential for its enzymatic activities. However, prokaryotic recombinant PtrMAN6 protein cannot form a dimer under both non-reducing and reducing conditions – a finding that may explain why no enzymatic activity was detected with the prokaryotic recombinant PtrMAN6 protein. In previous studies, MAN activities have been detected using prokaryotic recombination proteins (Bourgault and Bewley, 2002; Schroder *et al.*, 2006). It has not been reported that the N-glycosylation modification and disulfide-linked homodimer are needed for MAN activity in plants (Rodriguez-Gacio Mdel *et al.*, 2012). Our results revealed that PtrMAN6 displays different characteristics when compared with the MAN members in other plants (Rodriguez-Gacio Mdel *et al.*, 2012), suggesting that *PtrMAN6* may play a new function during xylem development.

*PtrMAN*s play a role in coordinating cell wall remodeling, with suppression of SCW formation during xylem differentiation

The hydrolysis of CW mannan-type polysaccharides by MAN is believed to be a necessary biochemical step during seed germination (Bewley *et al.*, 1997; Nonogaki *et al.*, 2000; Gong and Bewley, 2007; Ren *et al.*, 2008; Iglesias-Fernandez *et al.*, 2011a,b), fruit ripening (Bewley *et al.*, 2000; Bourgault *et al.*, 2005) and flower development (Filichkin *et al.*, 2004). In those studies, MAN enzyme is localized in CWs where it hydrolyzes mannan-type polysaccharides (Bewley *et al.*, 2000; Rodriguez-Gacio Mdel *et al.*, 2012). PtrMAN6 is localized on the plasma membrane, probably with its catalytic domain on the non-cytosolic side, and digests CW mannan-type polysaccharides to loosen the CW as well as produce oligosaccharide molecules. The different subcellular location could be due to the N-terminal structure as, compared with other reported MAN members, PtrMAN6 has a rather different N-terminal sequence structure that may be responsible for its membrane location.

During seed germination, fruit ripening and flower development, MANs mainly function in wall loosening.

However, during xylem development, PtrMANs display rather different characteristics. In addition to wall loosening, here we show that MANs are also involved in xylem differentiation and may play a crucial role in suppressing SCW formation during xylem differentiation.

Overexpression of *PtrMAN6* in *Populus* suppressed lignin deposition, while downregulation of *PtrMAN6* accelerated lignin deposition in xylem tissue. Consistent with this result, the *PtrMAN6*-overexpressed transgenics contained less xylose and mannose, which are the main monosaccharides for SCW hemicellulose (Scheller and Ulvskov, 2010), when compared with WT plants. Conversely, the *PtrMAN6*-suppressed transgenics were more enriched in secondary wall-related monosaccharides. This evidence suggests that *PtrMAN6* not only functions in the digestion of polysaccharides, which allows for the relaxing of CW in the process of cell expansion, but could also suppress the thickening of the SCW in xylem tissue.

Gene expression analysis showed that three of the five *PtrMAN* genes are highly expressed in xylem tissue, a finding that is consistent with the expression patterns obtained from the PlaNet consortium database (Mutwil *et al.*, 2011). Interestingly, immunolocalization revealed that PtrMAN6 was specifically localized in xylem vessel cell, but not in other xylem cells such as fiber, ray, parenchyma cell, and cambium cells. Whether expression of the other two *PtrMAN*s has different cell-type specificity need to be further investigated. It is possible that the three *PtrMAN*s could be associated with different cell types in xylem tissue. This suggestion. though, does not rule out the potential for different *PtrMAN* members to carry out a similar biochemical function of digestion to produce oligosaccharides. Actually, the fiber cell wall thickening in the transgenics with *PtrMAN6* antisense gene expression was also affected. This result could be due to sequence similarity among *PtrMAN*s (such as *PtrMAN 6* and *PtrMAN4*). The antisense *PtrMAN6* might have affected other *PtrMAN* expression in various types of xylem cells.

To form secondary walls, a set of transcriptional programs needs to be launched in certain type of cells (Cano-Delgado *et al.*, 2010; Ohashi-Ito *et al.*, 2010). In this study, we show that *PtrMAN6* is involved in the transcriptional program that regulates SCW formation. Transcription factors regulated by *PtrMAN6* include *WND*s, which are considered the master switches for SCW formation (Zhong *et al.*, 2010; Ohtani *et al.*, 2011). Overexpression of *PtrMAN6* downregulated *WND* expression, while suppression of *PtrMAN6* upregulated the *WND* transcription factor genes. Several *WND*s, *MYB3* and *MYB20*, are considered to be main components in the transcriptional networks that direct SCW formation in poplar (McCarthy *et al.*, 2010). *MYB28* is thought to be a switch that regulates lignin biosynthesis (Zhong and Ye, 2009). Expression of these genes involved in lignin, xylan and cellulose biosynthesis is downregulated when *PtrMAN6* is overexpressed. Here, the evidence supports the role that *MAN* plays in negatively regulating SCW formation during xylem development.

The *MAN* regulatory function is mediated through oligosaccharide molecules

MAN, acting as a hydrolase enzyme, is able to suppress SCW formation. What is the mechanism behind this regulation? In previous studies, GGMOs – the degraded products from mannan-type polysaccharides – have been isolated from wood (Dey, 1978, 1980), kiwifruit (Schroder *et al.*, 2001), and tobacco cell cultures (Sims *et al.*, 1997). As signaling molecules, GGMOs have been shown to play a wide range of biological activities in the regulation of cell differentiation (Auxtova *et al.*, 1995; Benova-Kakosova *et al.*, 2006). In this study, results demonstrated that the active PtrMAN enzyme was able to hydrolyze xylem CW GGM to produce GGMOs, which in turn was active in regulation of SCW thickening of xylem tissue. *PtrMAN6* suppression caused early SCW thickening. Overexpression of *PtrMAN6*, which was able to enhance GGMO production, resulted in delayed SCW thickening during xylem differentiation, and had the same result as the application of exogenous oligosaccharides. Thus, both *PtrMAN6* expression regulation and exogenous oligosaccharide application altered the transcriptional activity of secondary wall thickening in a similar manner, a finding that suggested that GGMOs may act as signaling molecules to mediate the regulatory function of *PtrMAN6*.

Together, this study supports the model presented in Figure 9. The *MAN* genes are expressed specifically in

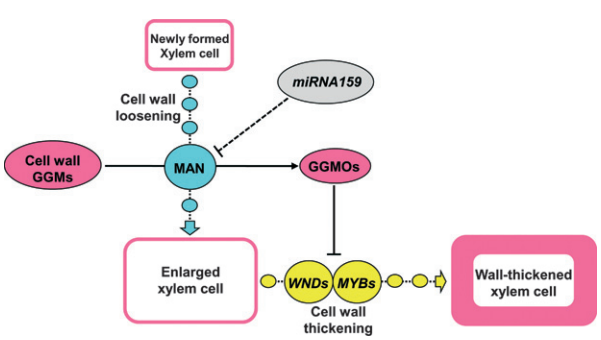

Figure 9. A proposed model for the role of endo-1,4-β-mannanase (MAN) in the regulation of secondary cell wall (SCW) formation.
Xylem differentiation involves cell expansion and cell wall (CW) thickening, which require many factors to loosen cell walls and commence wall thickening program. MAN enzyme, an integral monotopic protein with its catalytic domain on the non-cytosolic side, plays a function in hydrolyzing cell wall CW galactoglucomannan (GGM). This function would result in cell wall loosening and generation of galactoglucomannan oligosaccharides (GGMOs) that serve as signal molecules to modulate the transcriptional program of cell wall thickening. *miRNA159* expression may negatively regulate expression of *PtrMAN* that contains a *miRNA159* target sequence (Lu *et al.*, 2005). *MYB,* v-myb avian myeloblastosis viral oncogene homolog in *Populus*; *WND*, wood-associated NAC domain transcription factor.

xylem cells at the stage of cell expansion. The *MAN* genes display cell-type specificity and may perform similar biochemical function in different cell types. In this study, *PtrMAN6* showed vessel cell specificity. MAN protein is found in plasma membranes localized with its catalytic domain to hydrolyze CW mannan-type polysaccharides. The products of GGMOs are able to function as signaling molecules to modulate the transcriptional program of SCW thickening. GGMOs inhibit transcriptional activity of genes such as *WNDs*, *MYB3*, *MYB20* and *MYB28*, which are critical players in the transcriptional networks governing SCW thickening. This model provides a new 'lens' to understand the regulation of SCW thickening during xylem differentiation. However, further study is needed to decipher the more detailed mechanisms of the model. For example, how is the GGMO signal perceived and transmitted across the plasma membrane? Conversely, precise regulation of *MAN* expression is required in order to accurately direct SCW thickening. The *PtrMAN6* gene contains a target sequence of *miRNA159* that is expressed specifically in developing xylem (Lu *et al.*, 2005), therefore could miRNA play a role in regulation of *MAN* expression in developing xylem? Although further evidence will aid in producing a fuller picture of the process, the results of the current study provide a set of new insights to understand the signaling pathways that suppress the SCW thickening process during xylem cell differentiation.

EXPERIMENTAL PROCEDURES

Plant material

Populus trees in this study were grown in a phytotron for the first 3 months and then moved to a greenhouse. *P. trichocarpa* was used for gene cloning and expression analysis. *Populus* × *euramericana* cv.*'Nanlin895'* was used for genetic transformation according to the protocol used in our laboratory (Li *et al.*, 2003). Arabidopsis (Columbia) was grown in a phytotron with a light and dark cycle of 16 h and 8 h at 22°C and used for genetic transformation in accordance wth previously described methods (Clough and Bent, 1998).

Molecular cloning, constructs and expression of *PtrMAN* genes

PtrMAN sequences were retrieved from the poplar genome database (http://www.phytozome.net/poplar). Total RNA was isolated from various tissues and treated with RNase-free DNase I to remove DNA contamination, in accordance with a previously established protocol (Gui *et al.*, 2011). The full coding sequence of *PtrMAN6* was cloned by RT-PCR and deposited into the NCBI database. To explore the genetic effects of *PtrMAN6* on *Populus* plants, *PtrMAN6* cDNA was subcloned into a binary pBI121 vector in both sense and antisense orientations under the control of the CaMV 35S promoter. To investigate the subcellular localization of *PtrMAN6* in onion epidermal cells, full length and partial cDNA were subcloned into a pA7 vector (Voelker *et al.*, 2006) in frame fusion with GFP under the control of the CaMV 35S promoter. After the sequence of the constructs were confirmed, the above pA7 constructs were bombarded directly into onion epidermal cell and the other constructs were mobilized into *Agrobacterium tumefaciens* strain GV3101 for transformation of *Populus* and Arabidopsis. Prokaryotic protein expression was carried out via a previously established protocol (Gui *et al.*, 2011).The sequences of primers used in this study are listed in Table S2. For real-time quantitative PCR measurement, primers (Table S2) were designed to amplify a specific fragment (100–300 bp in length) from the target genes. Gene expression values were normalized using the *Populus* ACTIN2 gene as a reference.

Antibody production

Two PtrMAN6-specific peptides (EQFKTMVEEVDNH, residues 37–49; ELNDVEEDEWL, residues 61–71), were synthesized and used to raise polyclonal antibody in rabbits (Abmart, Shanghai, China, http://www.ab-mart.com.cn/). Crude antisera were purified using a protein-A Sepharose CI-4B column. Anti-GFP, anti-His and anti-actin monoclonal antibodies were purchased from Abmart.

MAN activity assay

Samples were ground in liquid nitrogen to a fine power and homogenized at 4°C in 1.5 vol of extraction buffer that contained 1 M sodium acetate buffer (pH 5.0), 10 mM ethylenediaminetetraacetic acid (EDTA), 10 mM sodium azide, and 3 mM phenylmethanesulfonyl fluoride (PMSF). The homogenate was centrifuged at 10 000 ***g*** for 30 min at 4°C. The supernatant was further purified and concentrated through a 10 000 M_r cut-off filter. The protein concentration was measured by bicinchoninic acid (BCA) Reagent (Tiangen Biotech, Beijing, China, http://www.tiangen.com/) using bovine serum albumin (BSA) as a standard. MAN activity was determined following the protocol of Iglesias-Fernandez and Matilla (2009) with some modifications. Briefly, 200 μl of reaction mixture that contained 100 μl of 1% (w/v), in 0.1 M sodium acetate buffer (pH 5.0) AZC L-galactomannan (Megazyme, Wicklow, Ireland, http://www.megazyme.com/) and 20 μg of extracted enzyme protein or BSA. After 2 h, the reaction mixture was centrifuged at 12 000 ***g*** for 5 min and measured at an absorbance of 590 nm. The enzyme activity of samples was calculated using commercial endo-β-mannanase (E-BMANN, Megazyme) as a reference according to the manufacturer's instructions. PtrMAN6 activity was examined with various substrates including ivory nut mannan (Megazyme), konjac glucomannan (Megazyme), carob galactomannan (low viscosity, Megazyme), *Populus* galactoglucomannan (extracted GGM), tamarind seed xyloglucan (amyloid, Megazyme), birchwood xylan (Sigma-Aldrich, www.sigmaaldrich.com/) and carboxyl methyl cellulose (Sigma-Aldrich). Next, 20 μg of extracted enzyme protein was incubated with 0.5 ml substrates at 2 g L^{-1} in 0.1 M sodium acetate buffer at pH 5.0. After 2-h incubation at 50°C, the reaction was stopped by the addition of equal volume of 3,5-dinitrosalicylic acid (DNS) reagent (Miller, 1959). One unit of mannan endo-1,4-β -mannanase activity was defined as the amount of enzyme that released 1 μ mole of mannose equivalents in 2 h under the condition.

Western blot, immunolocalization and PtrMAN glycosylation and dimerization analysis

Proteins were separated on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels. For non-reducing conditions, samples were mixed with an equal volume of 2 × loading buffer (0.1 M Tris–HCl (pH 6.8), 4% SDS, 0.2% bromophenol blue, 20% glycerol). For reducing conditions, samples were mixed with equal volume of 2 × loading buffer supplemented with 0.5–5% β-mercaptoethanol (β-ME) or 10 mM 1,4-dithiothreitol (DTT). Unless noted, all SDS-PAGE were performed under reducing conditions. All samples were boiled for 5 min

prior to electrophoresis. Western blot analysis and immunolocalization were performed according to methods detailed in previous studies (Song *et al.*, 2010). Sodium carbonate treatment was performed as described previously (Fujiki *et al.*, 1982). For PtrMAN glycosylation analysis, extracted PtrMAN protein was first denatured at 100°C for 10 min. Endoglycosidase H_f (Endo H_f, New England Biolabs, http://www.neb-china.com/) was incubated with the denatured proteins at 37°C for 30 min or 1 h according to the manufacturer's instructions. Then, the molecular size of the proteins was estimated by western blot. Native proteins treated by Endo H_f at 37°C for 2 h were analyzed directly for enzyme activity. The same proteins incubated without Endo H_f in the same Endo H_f buffer for the same period were used as a control. For PtrMAN6 dimerization analysis, native proteins incubated with/without 5% β-ME or 10 mM DTT at 37°C for 2 h were analyzed for enzyme activity.

CW composition and vessel size analysis

Wood tissue from 1-year-old *Populus* was used to prepare alcohol-insoluble residues (AIRs) of the CWs. Analysis of monosaccharide composition and crystalline cellulose content was conducted as described previously (Xiong *et al.*, 2010). Lignin content was determined as Foster *et al.* (2010) and lignin deposition was stained with 1% phloroglucinol (w/v) in 12% HCl for 5 min and immediately observed under a light microscope. For measurement of vessel size, the 12th internode stem was fixed and cross-sectioned as described (Hong *et al.*, 2010). Sections were observed under a UV fluorescence microscope equipped with the Image J program for area measurement. Data from 10 sections in each of three line plants were collected and analyzed statistically using Student's *t*-test.

GGMO analysis

Crude galactoglucomannan (GGM) was extracted from *Populus* xylem tissue as described previously (Auxtova *et al.*, 1995). The GGM (1 ml of 0.2% (w/v) in sodium acetate–acetic acid (NaAc–HAc) buffer, pH 5.0) was then treated with the extracted MAN protein (5 μg) at 50°C for various time periods (8, 16 and 24 h). After centrifugation at 13 000 ***g*** for 10 min, the supernatant was passed through a 10 000 M_r cut-off filter and dried in a vacuum evaporator. Then the hydrolyzed products were derivatized with 0.5 M 3-methyl-1-phenyl-2-pyrazoline-5-one (PMP, Sigma-Aldrich) as described (Honda *et al.*, 1989).

The derivatives were analyzed using an Agilent 6520 series LC 1200 MS 6520 QTOF system (Agilent, http://www.home.agilent.com/agilent/home.jspx?cmpid=4542&lc=chi&cc=CN) packed with a Zorbax Extend-C_{18} column (3.0 × 50 mm, 1.8 μm, Agilent). Next, 3 μl of analyte was injected with a constant mobile phase flow rate of 0.3 ml min^{-1}. The mobile phase consisted of 10 mM ammonium acetate in H_2O (A) and 20 mM ammonium acetate in acetonitrile (B) using a gradient elution of 22, 30 and 80% buffer B by a linear increase from 0, 5 and 10 min. The diode array detector (DAD) was set at 214 nm for monitoring and the TOF mass spectrometer was set as scan range from 150–3000 at 160 V and radio frequency (RF) at 750 V in positive scan mode at 4 GHz resolution. The temperature of dry gas of electrospray ionization (ESI) was set at 350°C with holding flow at 9 L min^{-1}. Relative quantification of oligosaccharides was calculated by integrating the peak area of m/z 673.27, 835.32, 997.38, 1159.48, 1321.48 and 1483.54 as qualifiers that are derivatives from GGMOs and PMP.

GGMO effect assay

Crude GGM (20 mg) was digested with plant-sourced PtrMAN6 protein at 50°C for 12 h. The reaction mixture was passed through a 10 000 M_r cut-off filter to remove proteins and polysaccharides. GGMOs content was determined according to the 3,5-dinitrosalicylic acid (DNS) method (Miller, 1959) and diluted to 0.4 mg ml^{-1} in 0.1 M NaAc buffer. To investigate the GGMO effect on xylem CW thickening, 3-month-old *Populus* trees were selected and treated with 0.4 mg ml^{-1} GGMOs in 0.1 M NaAc buffer or 0.1 M NaAc buffer only (control). Treatment of the developing xylem tissue was carried out as follows: the bark of the stem at the 6th internode from the top was gently peeled back about 0.5 cm. A small cotton ball soaked with GGMO solution was inserted in and sealed with Parafilm. After 1 week, the treated developing xylem tissue was examined for cell thickening morphology and gene expression. At least three trees were used for each treatment. In addition, filter-sterilized GGMOs were applied to the solid medium at 2-week subculture intervals to investigate the effect of the treatment on tissue culture. Three groups of experiments (leaf disc explants from WT cultured in the medium with or without 50 mg L^{-1}, leaf dics from PtrMAN6 overexpressed plants cultured in the medium without GGMOs) were carried out for tissue culture observation.

Sequence information

The GenBank accession numbers for the poplar genes studied in this article are *PtrMAN4* (XM_002309155), *PtrMAN5* (XM_002310780), *PtrMAN6* (XM_002323644, JX840449), *PtrMAN7* (XM_002327649), *PtrMAN8* (XM_002330651), *C3H1* (XM_002308824), *CAD4* (EU603306), *CesA8* (XM_002316779), *GT43B* (JF518935), *WND1A* (HQ215847, XM_002317023), *WND2A* (HQ215849), *WND3A* (XM_002322362), *WND4A* (XM_002329829), *WND5A* (XM_002310261), *WND6A* (XM_002327206), *MYB3* (XM_002299908), *MYB20* (XM_002313267), *MYB28* (XM_002307154), *ACT2* (XM_002298674).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Analysis of PtrMAN6 plasma membrane localization.

Figure S2. Characterization of PtrMAN6 N-glycosylation and disulfide-linked dimerization.

Figure S3. Phenotypes of *PtrMAN6*-overexpression transformation.

Figure S4. Oligosaccharides from DP2 to DP7 were identified by HPLC-QTOF-MS analysis.

Figure S5. GGMO effects on *Populus* xylem CW thickening.

Figure S6. Effect of GGMOs and *PtrMAN6* overexpression on tissue culture.

Table S1. Protein sequence identity of PtrMAN members.

Table S2. List of primers used in this study.

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