

RESEARCH PAPER

Over-expression of a putative poplar glycosyltransferase gene, *PtGT1*, in tobacco increases lignin content and causes early flowering

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Abstract

Family 1 glycosyltransferases catalyse the glycosylation of small molecules and play an important role in maintaining cell homeostasis and regulating plant growth and development. In this study, a putative glycosyltransferase gene of family 1, *PtGT1*, was cloned from poplar (*Populus tomentosa* Carr.). Sequence analysis showed that this gene encodes a protein of 481 amino acid residues with a conserved PSPG box at its C-terminal, suggesting that it is active in the glycosylation of plant secondary products. The *PtGT1* gene was expressed in poplar stems and leaves, with a particularly high expression level in elongating stems. Transgenic tobacco plants ectopically over-expressing *PtGT1* were obtained and phenotypes were analysed. Wiesner and Mäule staining showed that stem xylem of transgenic tobacco plants stained more strongly than controls. Measurement of the Klason lignins showed much higher lignin content in the transgenic lines than in control plants. Furthermore, the ectopic over-expression of *PtGT1* in tobacco resulted in an early flowering phenotype. These findings offer a possible starting point towards better understanding of the function of poplar *PtGT1*, and provide a novel strategy for lignin engineering and flowering control in plants through the genetic manipulation of a poplar glycosyltransferase gene.

Key words: Flowering, glycosyltransferase, lignin, poplar, tobacco.

Introduction

Glycosylation occurs in a wide range of biological processes in plants and is thought to play an important role in the production of a range of plant compounds. Glycosyltransferases (GTs) are enzymes responsible for the glycosylation of plant compounds. Substrate recognition, sequence similarity, and phylogenetic analysis have shown that GTs can be divided into 94 distinct families. Family 1 contains the greatest number of GTs found in plants and most contain a carboxy-terminal consensus sequence termed the ‘plant secondary product glycosyltransferase box’ (PSPG box). Family 1 GTs usually recognize substrates of low-molecular-weight lipophilic compounds and use uridine 5′-diphospho sugars as the sugar donors, and are thus termed UDP-sugar glycosyltransferases (UGTs) (Jones and Vogt, 2001; Bowles *et al.*, 2005; Wang and Hou, 2009). Completion of the

genome sequence analysis of *Arabidopsis thaliana* has allowed comprehensive analysis of the multigene families of glycosyltransferases and the discovery of a large glycosyltransferase superfamily consisting of 119 putative UGT genes (Li *et al.*, 2001).

Although glycosyltransferase activities and glycosylated products have long been known in a variety of plants, the enzymes and genes involved in glycosylation have only recently been isolated, and their roles in plant growth and development are now better understood. In recent years, dozens of glycosyltransferase genes have been identified, and many have been functionally characterized. It is now recognized that the glycosylation of low-molecular-weight compounds in plants, through the addition of a sugar moiety to the acceptors, usually changes acceptors in terms

of their bioactivity, stability, solubility, subcellular localization, and binding properties to other molecules (Bowles *et al.*, 2005). Therefore, glycosyltransferases might have an important role in maintaining cell homeostasis and regulating plant growth, development, and defence responses to stressful environments (Jones and Vogt, 2001; Lim and Bowles, 2004). For example, glycosyltransferases have been shown to play a role in processes such as the synthesis of various secondary metabolites, the modification of hormones, detoxification, and cell wall synthesis (Hou *et al.*, 2004; Wang and Hou, 2009). The study of glycosyltransferases and glycosylation of plant molecules has attracted considerable research interest because understanding the catalytic mechanisms of these enzymes and their physiological roles would be of great significance for *in vitro* design and synthesis of valuable glycosides, and for *in vivo* metabolic engineering of crops for important agronomic traits (Kristensen *et al.*, 2005; Lim, 2005; Weis *et al.*, 2008).

Poplar (*Populus* spp.) is a woody plant of great commercial and ecological value. Its small genome size, ease of transformation, and rapid growth make it an ideal model for other forest trees. Publication of the *Populus trichocarpa* genome offers unique opportunities to characterize glycosyltransferase genes in at least one woody plant model system. Recently, Geisler-Lee *et al.* (2006) identified over 1 600 genes encoding carbohydrate-active enzymes (CAZymes), including glycosyltransferases, in *P. trichocarpa* and found some key differences in metabolism and gene expression of the CAZyme at the genomic scale between it and the herbaceous *Arabidopsis*. Approximately 840 GTs were identified in their study. Interestingly, the GT1 family of secondary metabolite-glycosylating enzymes was the largest and most diverse gene family identified, and much larger in poplar than in *Arabidopsis*, perhaps due to additional specialized functions in processes such as wood formation, dormancy, and longevity. The functions of several individual poplar glycosyltransferase genes have also been characterized. For example, Zhou *et al.* (2007) cloned two poplar glycosyltransferase genes, *PoGT8D* and *PoGT43B* (belonging to the GT8 and GT43 families, respectively) and found that they were associated with the secondary wall and involved in glucuronoxylan biosynthesis. Although the largest GT1 family of poplar has been identified at the genome scale, little is known so far about the impact of individual poplar GT1 family genes on plant growth and development. No association between glycosyltransferase and lignin biosynthesis has yet been reported.

In this study, the cloning of a putative GT1 family glycosyltransferase gene, *PtGT1*, from poplar is reported and its over-expression in tobacco analysed. It was found that the over-expression of *PtGT1* in tobacco substantially increases lignin content. Moreover, the expression of *PtGT1* also results in an early flowering phenotype. Our data suggest a possible starting point for deeper studies into the function of poplar PtGT1, and a novel approach to lignin engineering and flowering control in plants using a genetically modified poplar glycosyltransferase gene.

Materials and methods

Plant materials and growth conditions

Plant materials used in this study were Chinese white poplar (*Populus tomentosa* Carr.) and tobacco (*Nicotiana tabacum* Linn, Wisconsin 38). Their tissue cultures and regenerated plantlets were maintained in a culture room at 23±2 °C under a 14/10 h light/dark photoperiod, and a light intensity of 60 μmol·m⁻² s⁻¹. Plants grown in flowerpots in soil–Perlite mixtures were maintained in a greenhouse at 25±2 °C under a 16/8 h light/ dark photoperiod and a light intensity of 100 μmol·m⁻² s⁻¹.

Isolation of poplar GT cDNA

Populus trichocarpa was used as a bridge to obtain the *P. tomentosa* PCR primers for the amplification of the full-length glycosyltransferase gene *PtGT1* because its genome had already been sequenced. The *P. trichocarpa* genome database (<http://genome.jgi-psf.org/Poptr1/Poptr1.home.html>) was first searched for glycosyltransferase homologues of *Arabidopsis thaliana* UGT72E1-E3 cDNA, which were already known to glucosylate several phenylpropanoids (Lim *et al.*, 2001) and were used as reference sequences in this study. Two primers were designed according to the *P. trichocarpa* homologues obtained: a forward primer 5'-ATAG-GATCCATGCAAAAACACAAAACCTCA-3' with a *Bam*HI cloning site at the 5' end; and a reverse primer 5'-ATACCCGGGCTAGG-CACCTTGAGCCTTTG-3' with a *Sma*I cloning site at the 5' end. *Populus tomentosa* total RNA was extracted from its young shoots using the Trizol method, and this RNA was used in an RT-PCR reaction using the primer pairs mentioned above. The amplified products of three separate RT-PCRs were cloned into appropriate intermediate vectors and sequenced. After comparing the sequence results to determine the consistent sequences, the clone containing the correct GT cDNA sequences of *P. tomentosa* was selected. *PtGT1* cDNA sequences were deposited in GenBank under the accession number HM776516.

Sequence analysis of *PtGT1*

The conserved protein domain sequences of poplar PtGT1 and *Arabidopsis* UGT72E1-E3 were obtained from the Proteomics Server of the Expert Protein Analysis system (ExPASy) of the Swiss Institute of Bioinformatics (<http://cn.expasy.org>; (Gasteiger *et al.*, 2003) using their cDNA sequences. The amino acid sequences of PtGT1 and UGT72E1-E3 were aligned using the ClustalX program and the online website (http://www.ch.embnet.org/software/BOX_form.html). Genetic distance matrices were obtained from the alignments using ClustalX 2 and Neighbor-Joining trees constructed with bootstrap sampling of 1000 replications using MEGA 4.0 programs (Zhou *et al.*, 2006, 2007; An *et al.*, 2010). The sequences of all the published *Arabidopsis* family 1 GTs used in the phylogenetic tree were obtained from the Carbohydrate-active enzymes database (http://www.cazy.org/GT1_eukaryota.html) and the NCBI database (Aspeborg *et al.*, 2005)

Expression pattern analysis of *PtGT1* in poplar (*Populus tomentosa* Carr.)

For the expression pattern analyses of *PtGT1*, 2-month-old seedlings regenerated from poplar tissue culture were cut into six parts: young leaves, mature leaves, top stems (rapidly elongating stems), middle stems (elongating stems near cessation), bottom stems (non-elongating stems), and roots. RNA was isolated from each of the six parts using the same method as described in the section relating to RT-PCR analysis of the transgenic tobacco plants. The RT-PCR reactions were repeated three times with identical results. The expression level of the 18S rRNA reference gene (primers: 5'-CTGCCCGTTGCTCTGATGATTCA-3' and 5'-CCTTGGATGTGGTAGCCGTTTCT-3') was used as an internal control.

Construction of the expression vector

The *PtGT1* cDNA was cut out from the intermediate plasmid using the *Bam*HI and *Sma*I restriction endonucleases. Plant expression plasmid pBI121 was cut with *Sac*I, blunted and then cut again with *Bam*HI. The *PtGT1* cDNA was inserted into the pBI121 plasmid, behind the CaMV 35S promoter, to produce the recombinant expression vector pBI121-PtGT1. The recombinant plasmid was then confirmed by appropriate restriction endonuclease digestion and transferred to tobacco using the *Agrobacterium*-mediated method (Li *et al.*, 1992).

Plant transformation and transgenic plant regeneration

Leaf discs from 6-week-old sterile tobacco seedlings were prepared for the transformation experiment. Leaf discs were immersed for 20 min in a suspension of recombinant *A. tumefaciens* LBA4404 containing plant expression vector pBI121-PtGT1, and were then dried on sterile filter paper. The leaf discs were then co-cultured with recombinant *A. tumefaciens* LBA4404 for 2 d on a solid MS medium (Murashige and Skoog, 1962) in the dark. The leaf discs were then transferred to a differentiation medium (a solid MS medium with 1 mg l⁻¹ 6-BA, 0.1 mg l⁻¹ IAA, 500 mg l⁻¹ cephalosporins, and 100 mg l⁻¹ kanamycin). Once the buds of the T₀ generation had grown sufficiently, they were transferred to a root-inducing medium (1/2 solid MS medium with 500 mg l⁻¹ cephalosporins and 100 mg l⁻¹ kanamycin). T₀ plants with developed root systems were transferred to flowerpots and grown in a greenhouse until T₁ seed set. T₂ homozygous transgenic plants were used for the transgene expression analysis and further experiments.

Identification of the transgenic tobacco plants by PCR and RT-PCR

A total of 100 mg of fresh leaves from wild-type (WT) and T₂ homozygous transgenic tobacco plants were used for DNA extraction using the method described by Yoshimura *et al.* (2004). About 100 ng of their genomic DNA was used to amplify the target gene *PtGT1* using PCR. The PtGT1-F primer (5'-CGACAACCAACCCTACGAAT-3') and the PtGT1-R primer (5'-GAGGAACCACCTTTACTGCT-3') were used in the PCR with the following thermal regime: an initial 94 °C for 5 min; followed by 31 cycles of 94 °C for 50 s, 56 °C for 50 s, 72 °C for 1 min; with a final 72 °C for 10 min. Agarose electrophoresis (1% agarose) was performed and the amplified bands photographed under UV light.

For the RT-PCR analysis, total RNA was isolated using Trizol (Invitrogen, Auckland, New Zealand) according to the manufacturer's instructions. Approximately 30 µg of the total RNA was treated with RNase-free DNase. RNA concentration and quality were assessed photometrically (Biophotometer; Eppendorf, Hamburg, Germany) and by gel electrophoresis. Approximately 3 µg of total RNA was utilized to synthesize single-strand cDNA using reverse transcriptase (Superscript III, Invitrogen) and oligo-DT18 primers, according to the manufacturer's instructions. The first strand cDNA was diluted 1:10 ddH₂O, and 2 µl of the diluted cDNA was used as a template for RT-PCR analysis. The RT-PCR system was performed in a total volume of 20 µl, with 2 µl of 10× PCR buffer, 1 µl of 2.5 mM dNTPs, and 1 µl of 10 µM PtGT1-F/R primer mentioned above, and 1 unit *Taq* DNA polymerase. PCR conditions were the same as used previously for PCR analysis.

Histochemical staining

In order to examine the lignified cell walls in stems, the transgenic and WT tobacco plants were grown at the same conditions for ~2 months. The second internodes of stems (from ground level) were excised, the bark removed, and the internodes hand-cut into 20–30 µm thick slices, and subjected to histochemical analysis.

Wiesner staining was performed by incubating sections in 1% phloroglucinol (w/v) in 6 mol l⁻¹ HCl for 5 min, and the sections observed under a dissecting microscope (Pomar *et al.*, 2002; Weng *et al.*, 2010). For Mäule staining, hand-cut stem sections were soaked in 1% KMnO₄ for 5 min, then rinsed with water, destained in 30% HCl, washed with water, mounted in concentrated NH₄OH, and examined under a dissecting microscope (Atanassova *et al.*, 1995; Weng *et al.*, 2010).

Assay of Klason lignin content

The second internodes of stems (from ground level) of transgenic and WT tobacco plants grown in the same conditions for approximately 2 months, were excised, the bark removed, and the internodes then cut into thin sections and put into an 80 °C oven. The dried stem materials were ground into a fine powder, extracted four times in methanol and dried. Then 200 mg of the extract was mixed with 5 ml of 72% (w/w) sulphuric acid at 30 °C and hydrolysed for 1 h. The hydrolysate was diluted to 4% sulphur by the addition of water and then cooked for 1 h in boiling water. The solid residue was filtered through a glass filter. Finally, the sample was washed, dried at 80 °C overnight and then weighed. The lignin content was measured and expressed as a percentage of the original weight of cell wall residue (Dence, 1992).

Phenotype analysis of transgenic tobacco plants

Fifteen transgenic plants from each line were grown alongside WT plants in the same conditions in a greenhouse. The date and number of leaves on each plant were recorded when each one flowered.

Data collection and statistical analysis

Data presented are the means ±SE of at least three independent experiments, with at least 10 plant samples per genotype. The experimental data were analysed by one-way ANOVA; the comparisons between the mean values of transgenics and WT were evaluated by the least-significant difference test at *P* < 0.01 (indicated with double stars above the bars in the figures). Statistical analyses were performed using SPSS/PC ver. 14.0 software.

Results

The cloning and sequence analysis of the *PtGT1* gene

In order to clone the glucosyltransferase from *P. tomentosa*, *P. trichocarpa* was used as a bridge, because of the availability of its genome sequences, to obtain the homologues of *Arabidopsis thaliana* UGT72E1-E3 cDNA. According to the *P. trichocarpa* homologues, the glucosyltransferase gene, *PtGT1*, was isolated from *P. tomentosa* (Fig. 1). After sequencing, the open reading frame of *PtGT1* was found to be 1 446 bp long, and encoded a putative protein of 481 amino acid residues. *Arabidopsis* UGT72E1-E3 is a small cluster of three closely related genes encoding glucosyltransferases shown to glucosylate several phenylpropanoids *in vitro*, including monolignols, hydroxycinnamic acids, and hydroxycinnamic aldehydes. In this study, the deduced amino acid sequences of *PtGT1* were aligned with UGT72E1-E3 and it was found that PtGT1 exhibits 56%, 52%, and 51% identity and 75%, 73%, and 73% similarity to the *Arabidopsis* UGT72E2, UGT72E3, and UGT72E1 proteins, respectively. The conserved PSPG box for secondary metabolite glycosyltransferases was found in the carboxy-terminal domain of

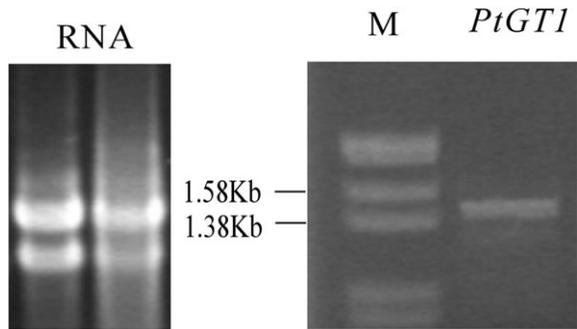


Fig. 1. The cloning of *PtGT1*. The RNA was isolated from poplar (*Populus tomentosa* Carr.) (Left) and the *PtGT1* cDNA was obtained (Right). M: DNA molecular weight marker.

the PtGT1 protein (marked by asterisks in Fig. 2A), suggesting that PtGT1 is a putative secondary metabolite glycosyltransferase. PtGT1 sequences were deposited in GenBank under the accession number HM776516. A phylogenetic comparison was made between PtGT1 and all 40 published *Arabidopsis* family 1 GTs, and the poplar PtGT1 was found to be located on a unique branch with *Arabidopsis* UGT72E1-E3 (Fig. 2B). This suggests that the poplar PtGT1 might recognize the same substrates as *Arabidopsis* UGT72E1-E3. However, when the PtGT1 enzymatic activity was examined *in vitro*, PtGT1 did not show the same activity towards monolignols and other phenylpropanoid compounds as *Arabidopsis* UGT72E1-E3 (data not shown), suggesting that the substrate specificities can not be predicted based on sequence similarities only.

The expression pattern of the poplar *PtGT1* gene

To examine the expression pattern of the poplar *PtGT1* gene, RNA was isolated from younger leaves, older leaves, upper stems (elongating stems), middle stems, lower stems (non-elongating stems), and roots from 2-month-old poplar and the relative expression levels assessed using RT-PCR (Fig. 3). Experimental results showed that upper stems contained higher levels of *PtGT1* transcripts than the other tissues, indicating that *PtGT1* is highly expressed where stems are elongating and secondary cell walls are being laid down. *PtGT1* was expressed at lower levels in leaves and other parts of the stems, and was undetectable in roots.

Over-expression of the poplar *PtGT1* gene in tobacco plants

In order to obtain information on the effects of constitutive over-expression of PtGT1 on plant growth and development, the *PtGT1* gene was first integrated into tobacco plants. The T₂ homozygous transgenic tobacco plants were identified using PCR of their genomic DNA. The expected fragment was amplified in transgenic lines but not in the non-transformed plants (Fig. 4A).

To assess the expression of the *PtGT1* gene in transgenic tobacco plants, 7-week-old transgenic seedlings were subjected to RT-PCR analysis. Two lines, T₄ and T₇, showed relatively high levels of *PtGT1* transcripts (Fig. 4B) showing

that poplar *PtGT1* had been transferred into the tobacco genome and was highly expressed in the transgenic tobacco seedlings. The T₄ and T₇ lines were therefore chosen for further investigation.

Lignin staining and Klason lignin content analysis of transgenic tobacco plants

Because the *PtGT1* gene is more highly expressed in elongating stems than in other poplar tissues, there was a need to examine the possible impacts of the *PtGT1* gene on stem growth and development in transgenic plants. Although the stems of transgenic plants showed no obvious phenotypic characters, lignin deposition was higher in the transgenic tobacco plants. Wiesner and Mäule staining of stem cross-sections showed that transgenic tobacco plants produced more intensive xylem tissue staining than WT (Fig. 5A, B), suggesting that the transgenic tobacco plants have higher total lignin than WT. To verify this finding further, the total lignin contents of transgenic and WT tobacco stem tissues were quantified by Klason analysis (Fig. 6). Two transgenic lines (T₄ and T₇) had significantly higher Klason lignin contents than WT, consistent with the histochemical staining results and showing that PtGT1 over-expression impacts on lignin content in tobacco. The total cellulose contents of stems were also measured in this study, but no substantial differences were found between the total cellulose contents of transgenic and WT tobacco plants (data not shown).

Flowering phenotype of transgenic tobacco plants

Transgenic tobacco lines and WT plants were grown in a greenhouse to observe their phenotypes. Surprisingly, it was found that the transgenic tobacco lines bolted earlier than WT plants (Fig. 7). The leaf numbers and days to flowering of the transgenic tobacco plants and WT were recorded. Leaf numbers of the WT plants were approximately 1.5 times those of the transgenic plants at bolting (Fig. 8A), and the number of days to WT flowering were nearly twice those of the transgenic plants (Fig. 8B). The transgenic plants showed almost no differences in leaf shape or size.

Discussion

In previous research, three glycosyltransferase genes *UGT72E1-E3* were identified as involved in sinapate and monolignol metabolism in *Arabidopsis* (Lim et al., 2001; Lanot et al., 2006, 2008). To obtain their homologues from *P. tomentosa*, *P. trichocarpa* genome sequences were used as a bridge to design the PCR primers for amplifying the full-length of glycosyltransferase gene *PtGT1* of *P. tomentosa* (because of the availability of its genome sequence). The PtGT1 amino acid sequences were deduced according to the obtained cDNA sequences. In its carboxy-terminal domain, a 44-amino acid consensus was found, called the PSPG box, a characteristic of family 1 GTs, indicating that the *PtGT1*

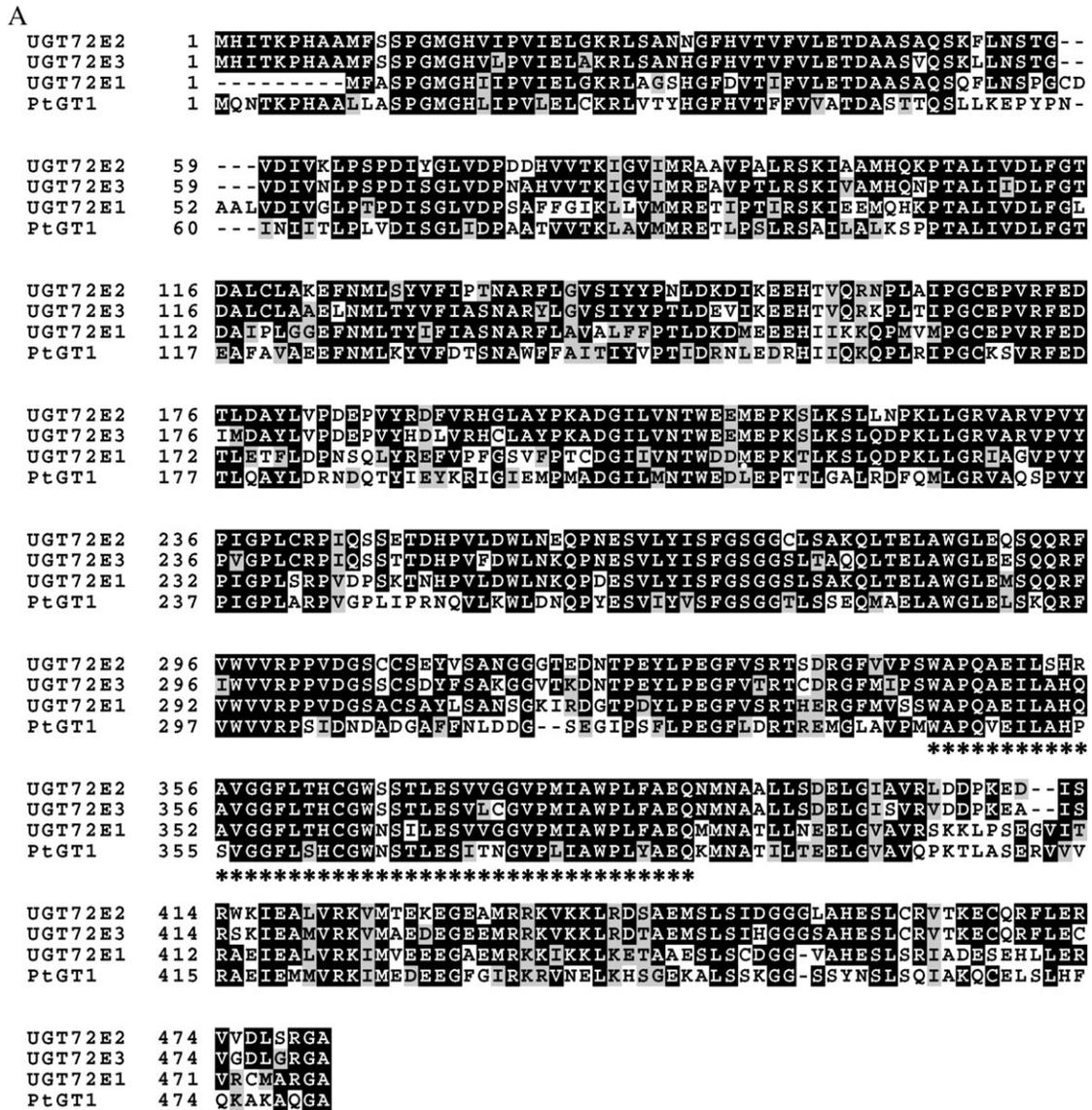


Fig. 2. The sequence analysis of *PtGT1* cDNA. (A) Amino acid sequence alignment of poplar *PtGT1* and *Arabidopsis* UGT72E1-E3. The numbers shown on the left of each sequence are the positions of amino acid residues in the corresponding proteins. Gaps (marked with dashes) were introduced to maximize the sequence alignment. Identical and similar amino acid residues are shaded with black and grey, respectively. The PSPG box is marked by asterisks. (B) Neighbor-Joining tree representing the relationships of *PtGT1* and all 40 published *Arabidopsis* family 1 GTs. Bootstrap values are shown in percentages at the nodes. The GT sequences were retrieved from the Carbohydrate-active enzymes database (http://www.cazy.org/GT1_eukaryota.html) and the NCBI database. The GenBank accession numbers for each sequence are: UGT71B1 (AEE76548.1); UGT71B2 (AAK50110.1); UGT71B6 (AEE76551.1); UGT71B8 (AEE76553.1); UGT71C1 (AAC35226.1); UGT71C3 (AAF82195.1); UGT71C4 (AAG18592.1); UGT72E1 (AAK83619.1); UGT72E2 (AAL32714.1); UGT72E3 (AAC26233.1); UGT73B1 (AAL57652.1); UGT73B2 (AAK59668.1); UGT73B3 (AAL32831.1); UGT73B4 (AAP37678.1); UGT73C1 (AAD20151.1); UGT73C5 (AAD20156.1); UGT73C6 (AAD20155.1); UGT74B1 (AAC00570.1); UGT74D1 (AAD32297.1); UGT74E2 (AAD30627.1); UGT74F1 (AAB64022.1); UGT74F2 (AAB64024.1); UGT75B1 (AAF79730.1); UGT75B2 (AAF79732.1); UGT75C1 (AAL32667.1); UGT75D1 (AAB58497.1); UGT76C1 (AAP21281.1); UGT76C2 (AAK73975.1); UGT78D1 (AAF19756.1); UGT78D2 (AAL61932.1); UGT84A1 (AAN72025.1); UGT84A2 (AAM13998.1); UGT84A3 (AAL15277.1); UGT84A4 (AAS99717.1); UGT84B1 (AAB87119.1); UGT84B2 (AAB87106.1); UGT85A1 (AAF18537.1); UGT89A2 (ABH04465.1); UGT89B1 (AEE35520.1); UGT89C1 (AAF80123.1).

gene is most probably a glycosyltransferase gene active in the glycosylation of plant secondary metabolites (Paquette *et al.*, 2003). Because no data on poplar family 1 glycosyltransferases have been available up to now, a phylogenetic tree was constructed using only *PtGT1* and the 40 family 1

glycosyltransferases of *Arabidopsis* so far identified. *PtGT1* was located on the phylogenetic tree in a unique clade with UGT72E1-E3. This may indicate that *PtGT1* is involved in the glycosylation of phenylpropanoids (including monolignols) similar to UGT72E1-E3. However, our *in vitro*

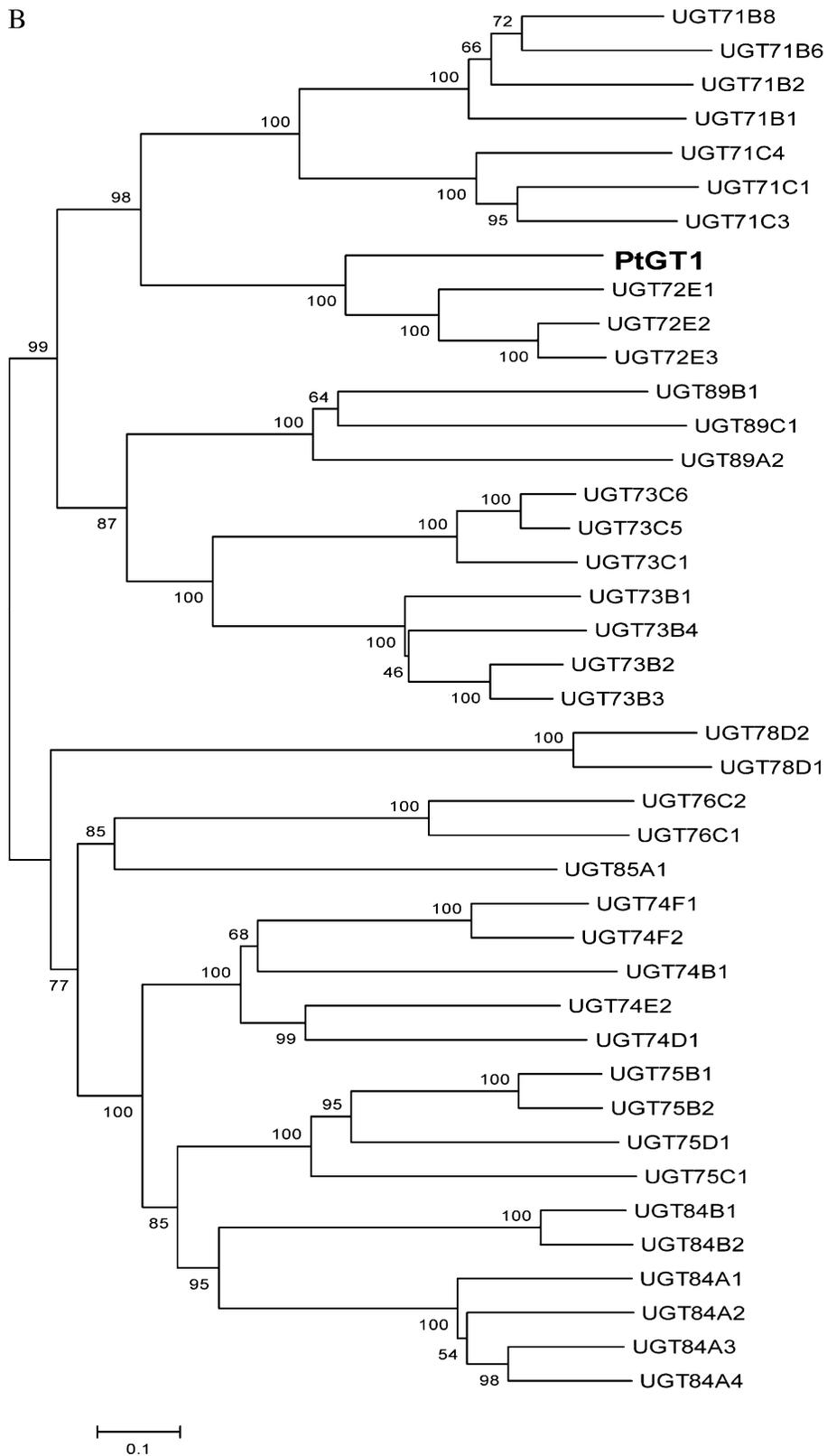


Fig. 2. Continued

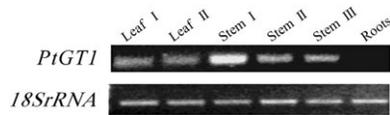


Fig. 3. RT-PCR analysis of the expression of *PtGT1* gene in different poplar tissues. The expression level of the 18S rRNA gene was used as an internal control. Leaf I was young and at the expanding stage, whereas leaf II was the older leaves. Stem I and stem III were from the top (elongating) and bottom (non-elongating) parts of the stems, respectively. Stem II was from the middle part between stem I and stem III.

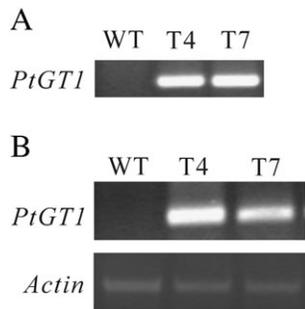


Fig. 4. The analysis results of PCR and RT-PCR for *PtGT1* cDNA in transgenic tobacco plants and the wild type. (A) DNA PCR in wild type (WT) and transgenic T₄ and T₇ tobacco plants. (B) RT-PCR in wild type and transgenic T₄ and T₇ tobacco plants. The expression level of the *Actin* gene was used as an internal control. Data are identical from two independent biological replicates.

biochemical assays indicated that the PtGT1 recombinant enzyme does not recognize phenylpropanoids as substrates. It is therefore likely that PtGT1 participates in a metabolite pathway in poplar distinct from its *Arabidopsis* homologues, UGT72E1-E3.

Lignin is mainly present in secondary thickened plant cell walls and is composed of aromatic polymers resulting from the oxidative coupling of three major monolignols: coniferyl alcohol, sinapyl alcohol, and coumaryl alcohol (Boerjan *et al.*, 2003; Ralph *et al.*, 2006; Vanholme *et al.*, 2010). The main biosynthetic pathways of the monolignols and lignin have been well described (Baucher *et al.*, 2003; Boerjan *et al.*, 2003; Chen and Dixon, 2007; Vanholme *et al.*, 2008, 2010; Grabber *et al.*, 2010; Novaes *et al.*, 2010; Rahantamalala *et al.*, 2010). Monolignol and lignin contents can be altered using metabolic engineering based on the genetic modification of lignin biosynthetic pathways. For example, many genes in the monolignol biosynthetic pathway, such as PAL, C4H, 4CL, HCT, C3H, CCoAMT, CCR, CAD, influence lignin contents when their expression levels are altered (Baucher *et al.*, 2003; Boerjan *et al.*, 2003; Hoffmann *et al.*, 2004; Sibout *et al.*, 2005; Wagner *et al.*, 2007; Mir Derikvand *et al.*, 2008). Glycosylation is a key mechanism in the metabolic homeostasis of plant cells (Bowles *et al.*, 2005). However, the impact on lignin biosynthesis of a glycosyltransferase, responsible for the glycosylation of plant secondary metabolites, has not been reported to date. In this study, it is shown that a putative

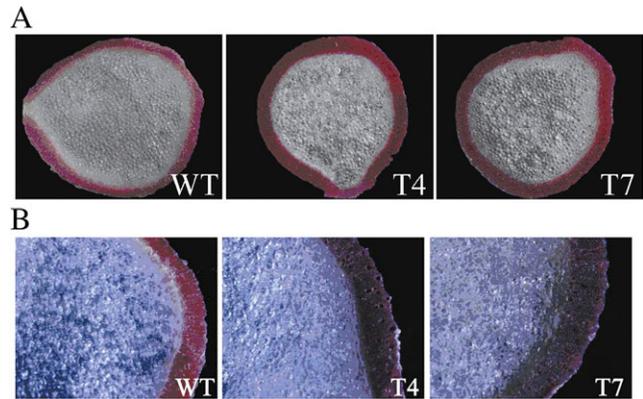


Fig. 5. Wiesner staining (A) and Mäule staining (B) of 2-month-old tobacco stems. WT, wild type; T₄, transgenic line T₄; T₇, transgenic line T₇.

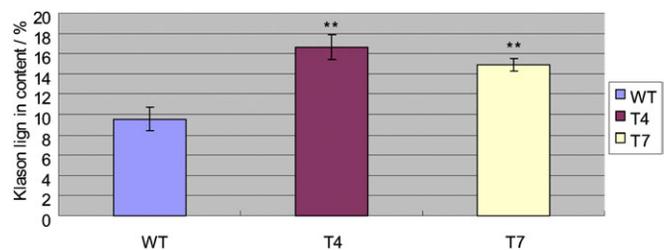


Fig. 6. Klason lignin content analysis of 2-month-old tobacco stems. WT, wild type; T₄, transgenic line T₄; T₇, transgenic line T₇. Error bars represent standard deviations based on assays of biological duplicates. Double stars above the bars represent a very significant difference compared with the wild type.

glycosyltransferase, *PtGT1*, increased the lignin content when constitutively over-expressed in tobacco plants, suggesting the significance of glycosyltransferase *PtGT1* in increasing lignin contents in angiosperms. Our findings may provide a novel strategy for lignin engineering in woody, and other, plants through the genetic manipulation of glycosyltransferase(s) such as *PtGT1*, increasing their value in industry and agriculture. Interestingly, the PtGT1 recombinant enzyme does not recognize the precursors of lignin, including monolignols, as substrates, and the transgenic tobacco plants which over-express the corresponding cDNA do not contain higher levels of monolignol glycosides (data not shown). It is, therefore, suggested that the impact of PtGT1 on lignin biosynthesis is not directly through the glycosylation of monolignols, and further research is needed to understand these molecular mechanisms better.

In addition to its effect on lignin content, the *PtGT1* gene appears to influence flowering time when ectopically expressed in tobacco. Time of flowering is crucial for plants to ensure successful reproduction. The molecular mechanisms of flowering in *A. thaliana*, a model organism, have been intensively studied, and four pathways have been shown to control the development of flowers: the photoperiod pathway, vernalization pathway, autonomous pathway, and gibberellin



Fig. 7. The flowering phenotype of transgenic tobacco plants and the wild type. The transgenic tobacco plants bolted earlier than the wild type. WT, wild-type tobacco plants; T4, transgenic tobacco plant T₄; T7, transgenic tobacco plant T₇.

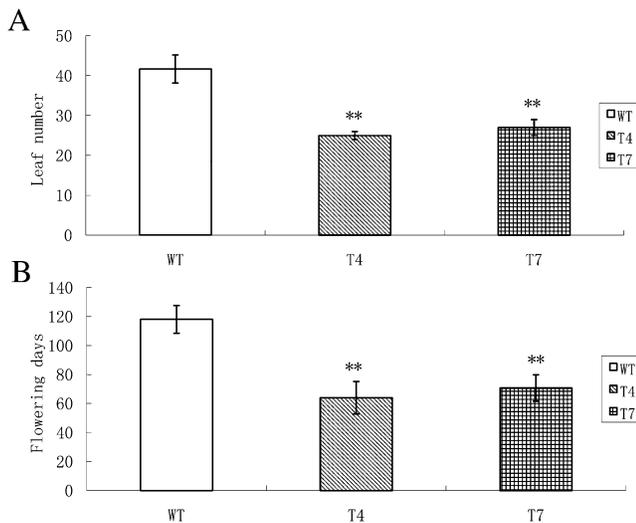


Fig. 8. The leaf numbers (A) and days (B) up to flowering of tobacco plants. WT, wild-type tobacco plants; T4, transgenic tobacco plant T₄; T7, transgenic tobacco plant T₇. Error bars represent standard deviations based on assays of biological duplicates. Double stars above the bars represent very significant difference compared with wild type.

pathway (Simpson, 2002). By contrast, very few genetic studies have been carried out on the flowering of poplar. Recently, An *et al.* (2010) reported that an inverted repeat *PtLFY* fragment (*PtLFY-IR*) of *P. tomentosa* effectively prevented flowering in transgenic tobacco plants. The ectopic expression of a poplar *APETALA3*-like gene in tobacco caused early flowering and fast growth (An *et al.*, 2011). In *Arabidopsis*, several studies have reported that altered UGT levels can impact on hormone homeostasis and compromise the control of flowering. For example, increasing the expression of UGT84B1, an *Arabidopsis* glucosyltransferase of indole-3-acetic acid, in transgenic lines increased the number of bolts from the rosette and the degree of branching (Jackson *et al.*, 2002). Mutants unable to express the UDP-glucose:thiohydroximate *S*-glucosyltransferase, UGT74B1, accumulated more indole-3-acetic acid, flowered later, and were partially sterile (Grubb *et al.*, 2004). UGT73C5 is another *Arabidopsis* glycosyltransferase responsible for the

glucosylation of brassinosteroids, and its over-expression resulted in dramatic changes in flowering time and flower development (Poppenberger *et al.*, 2005). In poplar plants, however, the impact of glycosyltransferases on flowering time has not been studied. In this study, it was found that ectopic expression of a putative poplar glycosyltransferase gene, *PtGT1*, in tobacco caused early flowering, and provides a starting point for the further study of the function of the *PtGT1* gene in the control of flowering time. The observed changes in lignin content are likely to be associated with changes in flowering time. Shadle *et al.* (2007) reported that down-regulation of hydroxycinnamoyl CoA:shikimate hydroxycinnamoyl transferase in transgenic alfalfa resulted in greatly reduced lignin content and a delay in flowering. The early flowering phenotype observed in this study, and the delayed flowering found in previous work (Shadle *et al.*, 2007) provides the possibility that engineered changes in lignin quantity may allow beneficial changes in reproductive development.

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