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A survey of *Populus* PIN-FORMED family genes reveals their diversified expression patterns

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Abstract

The plant hormone auxin is a key regulator of plant development, and its uneven distribution maintained by polar intercellular auxin transport in plant tissues can trigger a wide range of developmental processes. Although the roles of PIN-FORMED (PIN) proteins in intercellular auxin flow have been extensively characterized in *Arabidopsis*, their roles in woody plants remain unclear. Here, a comprehensive analysis of PIN proteins in *Populus* is presented. Fifteen PINs are encoded in the genome of *Populus*, including four PIN1s, one PIN2, two PIN3s, three PIN5s, three PIN6s, and two PIN8s. Similar to *Arabidopsis* AtPIN proteins, PtPINs share conserved topology and transmembrane domains, and are either plasma membrane- or endoplasmic reticulum-localized. The more diversified expansion of the PIN family in *Populus*, comparing to that in *Arabidopsis*, indicates that some auxin-regulated developmental processes, such as secondary growth, may exhibit unique features in trees. More importantly, different sets of PtoPINs have been found to be strongly expressed in the roots, leaves, and cambium in *Populus*; the dynamic expression patterns of selected PtoPINs were further examined during the regeneration of shoots and roots. This genome-wide analysis of the *Populus* PIN family provides important cues for their potential roles in tree growth and development.

Key words: Cambium, endoplasmic reticulum, PIN family, plasma membrane, *Populus*, shoot and root regeneration.

Introduction

The phytohormone auxin plays crucial roles in regulating plant developmental processes through auxin concentration gradients that occur within tissues (Tanaka et al., 2006; Mravec et al., 2009). Auxin is synthesized primarily in shoots and is distributed directionally to the sites of action (Vanneste and Friml, 2009). To assure that auxin is distributed effectively, higher plants have evolved two transport systems: a long-distance network through the phloem (Tanaka et al., 2006) and a slow cell-to-cell distribution network (Dhonukshe et al., 2007). Cell-to-cell auxin transport builds up and maintains the auxin gradient that provides positional information in a variety of developmental processes, including embryo and root patterning, organ formation, vascular tissue differentiation, growth responses to environmental stimuli, and wood formation (Nilsson et al., 2008; Mravec et al., 2009; Vanneste and Friml, 2009).

Multiple classes of auxin transport proteins participate in cell-to-cell auxin transport. For example, ABCB/PGP proteins are a class of ATP-dependent auxin transport proteins, AUX1/LAX proteins mediate auxin influx to cells, and
PIN-FORMED (PIN) proteins determine the directionality of auxin flow (Wiśniewska et al., 2006; Dhonukshe et al., 2007). Eight PIN proteins (AtPIN1–8) are encoded in the Arabidopsis genome. Except for AtPIN6, whose biological function is unclear, the developmental functions of the other AtPINs have been characterized. The ‘long’ AtPINs control diverse developmental processes, such as the establishment of the apical–basal axis in embryogenesis, organogenesis of organs such as lateral roots and shoot-derived organs, phyllotaxis, root meristem patterning, vascular tissue differentiation and regeneration, and tropisms (Dhonukshe et al., 2007; Mravec et al., 2009). AtPIN5 and AtPIN8 localize to the endoplasmic reticulum (ER) and regulate intracellular auxin homeostasis and metabolism (Mravec et al., 2009; Bosco et al., 2012; Ding et al., 2012).

Populus is a group of economic plant species that are grown as bioenergy feedstocks for lignocellulosic biomass production as well as timber production (Nieminen et al., 2012). With the completion of sequencing of the Populus trichocarpa genome and the development of various genetic, genomic, and biochemical tools, Populus has become a model plant species for studying wood formation, long-term perennial growth and seasonality, flowering, sex determination, and biotic interactions (Jansson and Douglas, 2007). However, few studies of PIN proteins and their roles on Populus growth and development have been reported.

In the present study, an inventory of Populus PIN proteins was generated and a comprehensive analysis of this gene family was performed. These analyses revealed that at least 15 PtPIN gene loci are present in the Populus genome. Although similarly to AtPINs, the gene structure and protein topology of PtPINs are conserved, a more expanded PIN family and more diversified PIN gene expression patterns occur in Populus. Moreover, a set of PtoPINs has been found to be highly dynamic and strongly induced during the regeneration of shoots and adventitious roots (ARs). These data provide useful information toward understanding PIN-mediated auxin signalling and PIN-regulated developmental processes in Populus.

Materials and methods

Bioinformatic analysis

BLASTP searches were performed against the Populus trichocarpa genome (release 3.0, http://www.phytozome.net/poplar) using AtPIN protein sequences as queries, and the resulting sequences were used as secondary queries. By removing redundant sequences, transmembrane domains (TMDs) were analysed using the Aramemnon database (http://aramemnon.uni-koeln.de/), with the result providing a consensus transmembrane topology generated from 10 different TMD prediction algorithms (Schwacke et al., 2003). Multiple full-length protein and TMD sequence alignments of AtPIN and PtoPIN proteins were performed using the CLUSTAL X 2.0 software (Thompson et al., 1997). Unrooted phylogenetic trees were constructed with MEGA 4.0 using the neighbour-joining method (Tamura et al., 2007). Genomic structures were determined using the Aramemnon database. PIN topologies were defined using the online HMMTOP tool (http://www.enzim.hu/hmmtop/; Tusnady and Simon, 2001) and visualized by the TMRPres2D software (http://biophysics.biola.edu/TMRPres2D/download.jsp; Spyropoulos et al., 2004). All 15 PtPIN genes were mapped onto the Populus trichocarpa chromosomes. Whole-genome duplication analyses were accomplished as described in Tuskan et al. (2006). MEME version 4.3.0 (http://meme.sdsc.edu; Bailey et al., 2006) was used to elucidate the motifs in the TMDs. MEME was run locally with the following parameters: number of repetitions, any; maximum number of motifs, 20; optimum motif widths, constrained to between 6 and 21 residues.

Plasmids and constructs

The coding sequences of PtoPIN1a, 1c, 2, 3a, 5a, 6a, and 8b without stop codons were amplified from the cDNA of Populus tomentosa.
Carr., and inserted into pEarleyGate101 (ABRC stock DB3-683) to produce 35S::PtoPIN5a-YFP constructs using the Gateway cloning system (Invitrogen). 5′-UTR fragments from PtoPIN2a, PtoPIN5a, PtoPINb, and PtoPIN1d of approximately 3kb were amplified from the genomic DNA of *P. tomentosa* Carr. The primer sequences and promoter lengths are listed in Supplementary Table S1 (available at *JXB* online). The promoter fragments were then inserted into pDONR222.1 and subcloned into pMDC164 to produce 

\[ P_{\text{toPIN2}}:\text{GUS}, P_{\text{toPIN5a}}:\text{GUS}, P_{\text{toPINb}}:\text{GUS}, \text{and } P_{\text{toPIN1d}}:\text{GUS} \]  

constructs using the Gateway cloning system (Invitrogen). At least three independent lines were used for analysis.

**Plant cultivation and transformation**

Wild-type *Arabidopsis thaliana* (accession Colombia, Col-0) plants were grown at 20 °C on soil under a 14/10 day/night cycle. Each 35S::PtoPINs-YFP construct was introduced into the *Agrobacterium tumefaciens* GV3101 by electroporation. *Arabidopsis* transformation was performed using the floral dip method (Clough and Bent, 1998).

Transient expression of PtoPIN5a-YFP, PtoPIN6a-YFP, PtoPINb-YFP, and GFP-HDEL in *Nicotiana benthamiana* leaf lower epidermal cells was performed using *Agrobacterium* transformation as described by Chen et al. (2011). The final OD₆₀₀ for *Agrobacterium* cultures was 0.1 except for GFP-HDEL (OD₆₀₀ = 0.01).

Hybrid poplar trees (*Populus alba × Populus glandulosa*) of clone 84K that was used for transformation were grown at 23–25 °C under a 16/8 day/night cycle with a light intensity of 50 μE m⁻² s⁻¹ provided by cool white fluorescent tubes. Leaf discs from 84K were infected with a culture of *Agrobacterium* harbouring the *P_{\text{toPIN2}}*:GUS, *P_{\text{toPIN5a}}*:GUS, *P_{\text{toPINb}}*:GUS, or *P_{\text{toPIN1d}}*:GUS construct at an OD₆₀₀ of 0.3–0.8. Infected leaf discs were then cocultured in darkness with *Agrobacterium* in the shoot induction medium (SIM: Murashige-Skoog basal medium containing (per litre) 0.5 mg 6-benzylaminopurine and 0.05 mg naphthalene acetic acid) for 3 days at 22 ± 2 °C. The leaf discs were then transferred to the SIM containing (per litre) 3 mg hygromycin and 200 mg Timentin under a 16/8 light/dark regime. After 1 month, individual regenerated shoots were removed and transferred to the root induction medium (RIM: 1/2 Murashige-Skoog medium supplemented with (per litre) 0.05 mg indole-3-butyric acid and 0.02 mg naphthalene acetic acid) containing 3 mg hygromycin and 200 mg Timentin for AR induction.

For shoot induction, leaf discs were cultured on the SIM for 18 days covering six chronological stages (B1–B6), and for AR regeneration, stems were cultured on RIM for 9 days over the chronological stages R1 through R4.

**RNA isolation and RT-PCR**

Total RNA was extracted from the *P. tomentosa* Carr roots, leaves, stems, cambium with part of the phloem, immature xylem, catkins, pollen, and hormone-treated materials at different stages, using the RNeasy Plant Mini Kit and RNase-free DNase I (Qiagen, Hilden, Germany). First-strand cDNA synthesis was carried out with approximately 1.5 μg RNA using the SuperScript III First-Strand Synthesis System (Life Technologies, Carlsbad, CA, USA) according to the manufacturer’s instructions. RT-PCR primers with melting temperatures of 58–60 °C and amplicon lengths of 150–260 bp were designed using Primer3 software (http://frodo.wi.mit.edu/primer3/input.htm) and their sequences are shown in Supplementary Table S1. The amplified fragments were separated by agarose gel electrophoresis. Real-time qRT-PCR was performed using the SYBR Premix Ex Taq II Kit (TaKaRa Dalian, Dalian, China) on an 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) according to the manufacturer’s instructions.

**Promoter·GUS assay**

Histochemical GUS staining was performed by incubating 2-week-old seedlings and 4-week-old stem sections in 90% cold acetone until all samples were harvested. Each sample was washed three times on ice using a staining buffer containing 50 mM sodium phosphate (pH 7.0), 2 mM potassium ferrocyanide, 2 mM potassium ferricyanide, 10 mM EDTA, and 0.2% (v/v) Triton X-100. The fixed samples were then transferred into the staining solution (staining buffer with 20% (v/v) methanol and 1 mM X-Gluc) and were slowly subjected to vacuum to infiltrate the staining solution into the tissues. After 12 h incubation at 37 °C with gentle agitation, the samples were rinsed in 70% ethanol for imaging.

**Protein localization analysis**

Fluorescence was observed using a LSM 510 confocal laser scanning microscope (Carl Zeiss, Oberkochen, Germany). Tobacco leaves were analysed 3–6 days after infiltration. The roots of 4-day-old *Arabidopsis* seedlings expressing 35S::PtoPIN1a-YFP, 35S::PtoPIN1c-YFP, 35S::PtoPIN2-YFP, 35S::PtoPIN3a-YFP, 35S::PtoPIN5a-YFP, 35S::PtoPIN6a-YFP, or 35S::PtoPIN8b-YFP treated with 50 μM brefeldin A (BFA) for 2h were immersed in 2 μM

![Fig. 2. Subcellular localization of the plasma membrane-localized PIN proteins. (A–E) Confocal images of tobacco leaf epidermis showing colocalization of PtoPIN1a-YFP (A), PtoPIN1c-YFP (B), PtoPIN2-YFP (C), PtoPIN3a-YFP (D), and PtoPIN8b-YFP with FM4-64 (5 min after staining). Bar, 10 μm.](https://academic.oup.com/jxb/article-abstract/65/9/2437/523417)
FM4-64 for 15 min for subcellular localization analysis. For fluorescence imaging of YFP/FM4-64 samples, 488/543-nm excitation and 505–530/585-nm filters were used.

Results

PIN gene family in Populus trichocarpa: identification, phylogeny, and protein structure

Some of the PIN-encoding sequences in the Populus genome had been previously identified and phylogenetically analysed together with other land plants, but a systemic nomenclature for the Populus PINs was not provided (Křeček et al., 2009; Carraro et al., 2012; Forestan et al., 2012). The present study found that there were 16 PIN gene sequences in the Populus trichocarpa genome. However, since the sequences Potri. T058300 and Potri.019G052800 came from the same locus, it is believed that 15 PIN genes were present in the Populus genome. Details of these 15 Populus PIN family genes, including accession numbers and similarities to their Arabidopsis orthologues, are listed in Supplementary Table S2. Based on their phylogenetic relationship with the Arabidopsis PINs (Fig. 1 and Supplementary Fig. S1), it seems that four PIN1s (PtrPIN1a–d), one PIN2 (PtrPIN2), two PIN3s (PtrPIN3a, b), three PIN5s (PtrPIN5a–c), three PIN6s (PtrPIN6a–c), and two PIN8s (PtrPIN8a, b) can be classified in Populus. All PtrPIN genes fall into two branches, the ‘long’ Class I PINs containing PtrPIN1 (four members), PtrPIN2 (one member), PtrPIN3 (two members), and PtrPIN6 (three members), and the ‘short’ Class II PINs containing PtrPIN5 (three members) and PtrPIN8 (two members) (Supplementary Fig. S1). With the exception of AtPIN2, PtrPIN5c, and PtrPIN6c, all other PINs from each subclass share a similar intron/exon organization, including size and the arrangements of introns and exons (Fig. 1, right panel).

Duplicated blocks of the Populus trichocarpa genome were established in a previous study (Tuskan et al., 2006). The mapping of the 15 PtrPINs onto the Populus trichocarpa genome (Supplementary Fig. S2) indicated that the expansion of the PtrPIN family was caused mainly by chromosomal duplication within the genome. There are five pairs of duplicated PIN genes in the Populus trichocarpa genome: PtrPIN1a/PtrPIN1b, PtrPIN1c/PtrPIN1d, PtrPIN3a/PtrPIN3b, PtrPIN6a/PtrPIN6b, and PtrPIN8a/PtrPIN8b.

AtPIN proteins were predicted to have 10 TMDs flanking both sides of the central hydrophilic loop (Barbez et al., 2012; Viaene et al., 2013). An in-silico prediction of PtrPINs showed a similar topology to that of their Arabidopsis PIN orthologues (Supplementary Fig. S3). All PIN proteins from Populus and Arabidopsis share the same TMD–hydrophilic loop–TMD structure, and even the numbers of TMDs in both the N- and C-terminal regions are conserved. For example, the ‘long’ PIN AtPIN1 and PtrPIN1a–d were predicted to have an identical topology (Supplementary Fig. S3A–E). Supplementary Fig. S3I and J showed the same topology for short AtPIN5 and PtrPIN5a. A comparison of the predicted TMDs among all PtrPINs (Supplementary Fig. S3R and S) also confirmed that the amino acid sequence of each TMD is conserved regardless of the class and subclass.
Subcellular localization of PtoPINs

PINs were classified into ER- and plasma membrane-localized proteins in Arabidopsis (Křeček et al., 2009). The subcellular localization of seven selected PtoPINs, representing each paralogous pair and subclass was analyzed by transient expression of YFP-fused PtoPINs in N. benthamiana leaf epidermal cells. In N. benthamiana, epidermal cells transiently expressing PtoPIN1a-YFP, PtoPIN1c-YFP, PtoPIN2-YFP, PtoPIN3a-YFP, or PtoPIN8b-YFP (Fig. 2), the fusion proteins were found to be targeted to the plasma membrane as indicated by colocalization with FM4-64 within 5 min of the onset of staining. In N. benthamiana epidermal cells transiently expressing PtoPIN5a-YFP and PtoPIN6a-YFP, the fusion proteins were showing network-like reticular structures. To determine the identity of these network-like reticular structures, transient coexpression of PtoPIN5a-YFP or PtoPIN6a-YFP with the well-known luminal ER marker GFP-HDEL was performed. Coexpression analysis showed strong colocalization of PtoPIN5a-YFP and PtoPIN6a-YFP with GFP-HDEL in lower epidermal cells of N. benthamiana leaves (Fig. 3A–F). In addition to the ER-localization, PtoPIN6a-YFP was also presented in the plasma membrane of cells, as indicated by FM4-64, when the optical plane was focused on the middle part of the cell (Fig. 3G–I).

The plasma membrane localization of PtoPINs was further confirmed by BFA treatment. When transgenic Arabidopsis expressing PtoPIN1a-YFP, PtoPIN1c-YFP, PtoPIN2-YFP, PtoPIN3a-YFP, PtoPIN8b-YFP, or PtoPIN6a-YFP was treated with BFA, in root epidermal cells these fusion proteins were found to be presented in the punctate structures, which can be also marked by internalized FM4-64 (Fig. 4A–E and G). In contrast, in transgenic Arabidopsis expressing PtoPIN5a-YFP, the fusion protein was absent from BFA bodies (Fig. 4F). In Arabidopsis root cells, the BFA treatment produces so-called

![Fig. 4. The BFA effect on PtoPIN trafficking in ectopic expressed Arabidopsis.](https://academic.oup.com/jxb/article-abstract/65/9/2437/523417)
BFA compartments (which can be recognized by following FM4-64 internalization) where the recycling of some endocytic proteins from endosomes is inhibited (Grebe et al., 2003; Chow et al., 2008). The relocation of PtoPIN1α-YFP, PtoPIN1c-YFP, PtoPIN2-YFP, PtoPIN3α-YFP, PtoPIN8b-YFP, or PtoPIN6α-YFP in the punctate structure marked by FM4-64 confirmed that these PtoPINs (but not PtoPIN5α-YFP) are targeted to the plasma membrane.

Expression of PtoPINs in various tissues and organs

Gene expression patterns can provide useful insights into the physiological functions of the corresponding proteins. The expression patterns of PIN genes (PtoPIN) in the roots, leaves, stems, cambium zone, immature xylem, catkins, and pollen of P. tomentosa Carr. were analysed by semiquantitative RT-PCR (Fig. 5). PtoPIN1c, PtoPIN2, and PtoPIN5α were only expressed in the roots. PtoPIN3α was only expressed in the leaves. PtoPIN3b, 5b, and 5c were enriched in the leaves. The expression levels of PtoPIN1α, 1b, 1d, 6α, and 6b in the cambium zone were stronger than in the other tissues. Among them, PtoPIN1d, 6α, and 6b were found to be expressed mainly in the cambium zone. While the seven PtoPINs PtoPIN1α, 1b, 1d, 5b, 5c, 8a, and 8b were ubiquitously expressed, it is noteworthy that PtoPIN8α and 8b were highly transcribed in the catkins and pollen, where PtoPIN5c was also present. In accordance with this, the RNA sequence data further confirmed the expression pattern of PINs in Populus (Supplementary Fig. S4).

Expression patterns of four PtoPIN genes

To further confirm the expression patterns of the PtoPIN genes, two root-specific and two generally expressed PtoPINs were selected for analysis using a promoter::GUS assay. Plants containing the transgenes P_PtoPIN2::GUS, P_PtoPIN6::GUS, P_PtoPIN1b::GUS, and P_PtoPIN5α::GUS were analysed. PtoPIN2 and PtoPIN5α were found to be root-specific in a semiquantitative expression analysis (Fig. 5). GUS assays confirmed that P_PtoPIN2::GUS and P_PtoPIN6::GUS transgenes were expressed mainly in the roots and lateral root tips (Fig. 6A–F). Although these two root-specific PtoPIN promoters showed restrained activity, mainly in the root-tip region, closer observation revealed that their expression patterns in the root tips did not overlap totally. The GUS staining of plants containing the P_PtoPIN1b::GUS transgene was largely restricted to the tip region of the root (Fig. 6E, F), whereas the staining of plants containing the P_PtoPIN5α::GUS transgene comprised the entire root-tip region but was stronger in the area behind the tip of the root (Fig. 6B).

Based on the semiquantitative expression analysis, PtoPIN1b and PtoPIN1d were generally expressed in all of the tested tissues (Fig. 5). GUS staining of samplings expressing P_PtoPIN1b::GUS and P_PtoPIN1d::GUS confirmed the general expression patterns of these genes (Fig. 6G–P). Closer investigation indicated that the expression of PtoPIN1b was more concentrated in the cambial zone in stem sections and shoot-tip regions, including young leaves that were not fully expanded (Fig. 6G,H), than PtoPIN1d. In contrast, the expression of PtoPIN1d was most evident in veins, stems, and middle tissues of roots (Fig. 6L–P).

Dynamic expression of PtoPINs during shoot and AR regeneration

Shoot and AR regeneration using leaf or stem explants is one of the best tools for the rapid propagation and genetic manipulation of Populus. These processes comprise very important biological events, including re-establishment of the shoot apical meristem (SAM) and root apical meristem (RAM), and lead to the final leaf and root differentiation, as determined by the formation and maintenance of polarized auxin gradients. Shoot regeneration comprises several successive stages, including the B1 preinduction stage (Supplementary Fig. S5A), the B2 callus induction stage (Supplementary Fig. S5B), the B3 callus expansion stage (Supplementary Fig. S5C), the B4 callus transition stage (Supplementary Fig. S5D), the B5 shoot regeneration stage (Supplementary Fig. S5E), and the B6 shoot growth stage (Supplementary Fig. S5F). The dynamic expression of 15 PtoPINs during the process of shoot induction was examined using quantitative real-time PCR (Fig. 7A). The expression levels of all Populus PIN genes were changed at least 2-fold, and the expression levels of PtoPIN1α, PtoPIN1b,
PIN family genes in *Populus* | 2443

*PtoPIN1c*, *PtoPIN1d*, *PtoPIN6a*, and *PtoPIN8b* were changed over 5-fold during the regeneration of shoots. The *PtoPIN1a* and *PtoPIN1b* transcript levels were increased at the B2 callus induction stage and then reduced in the B3 to B6 stages, suggesting that they are involved in the callus induction and formation. *PtoPIN1c* and *PtoPIN1d* were the most strongly induced...
during shoot regeneration. These genes were induced gradually at the B2 and B3 stages and peaked at the B4 and B5 stages, suggesting that they may have a polar auxin transport function during the induction and regeneration of the vascular system. Although the expression levels of PtoPIN3a, PtoPIN3b, and PtoPIN5a were low, their transcripts were downregulated in all shoot regeneration stages.

AR induction consists of the R1 preinduction stage (Supplementary Fig. S5G), the R2 callus regeneration stage (Supplementary Fig. S5H), the R3 AR emergence stage (Supplementary Fig. S5I), and the R4 AR elongation stage (Supplementary Fig. S5J). The expression levels of all 15 PtoPINs were examined using real-time PCR during the process of AR induction (Fig. 7B). Among the 15 PtoPIN genes, the three root-specific PIN genes PtoPIN1c, PtoPIN2, and PtoPIN5a showed significant induction. The expression of PtoPIN1c was induced and maintained at the 20-fold level in the R2, R3, and R4 stages compared with the R1 stage. PtoPIN2 was strongly induced only at the R3 and R4 stages. PtoPIN5a showed slight induction at the R2 stage and strong induction at the R3 and R4 stages.

Because promoter-GUS fusion can provide a better resolution in gene expression analysis, the dynamics of PtoPIN2 and PtoPIN5a were followed using PtoPIN2::GUS and PtoPIN5a::GUS. As indicated in Fig. 8, during the regeneration of roots, auxin response, as indicated by DR5-GUS, was gradually concentrated and focused on certain points within the generated callus from where the roots will be regenerated (Fig. 8A, R2 and R3). In the R4 stage (Supplementary Fig. S5J), the auxin maximum was found in the tip region of regenerated roots (Fig. 8A, R4). In the R1 stage (Supplementary Fig. S5G), the activity of the PtoPIN2 as well as PtoPIN5a promoters was not detected (Fig. 8B, C, R1). However, their activity started to be detectable but not yet concentrated in the R2 stage (Fig. 8B, C, R2, and Supplementary Fig. S5H). In the R3 stage (Supplementary Fig. S5I), the focused activity of the PtoPIN2 as well as PtoPIN5a promoters was clearly visible in the callus generated (Fig. 8B, C, R3). In the R4 stage (Supplementary Fig. S5J), the activity of the PtoPIN2 promoter was restricted in a region behind the root tip (Fig. 8B, R4), while the activity of the PtoPIN5a promoter was found in the root-tip region (Fig. 8C, R4), suggesting their different activity in root development.

**Discussion**

The fundamental features of PIN proteins are conserved across species

The *Populus trichocarpa* genome contains 15 PIN gene loci, which is greater than the number of PINs in rice (11; Wang et al., 2009), maize (14; Forestan et al., 2012), tomato (10; Pattison and Catalá, 2012), grape (10; Forestan et al., 2012), and eucalyptus (10). Except for *PtrPIN5c* and *PtrPIN6c*, each *PtrPIN* has at least one close homologue in the *Arabidopsis* genome. Five pairs of *PtrPINs* are located on duplicated segments, indicating that these pairs come from large-scale duplication events that occurred in the *Populus* genome (Tuskan et al., 2006). *PtrPIN2*, *PtrPIN5c*, and *PtrPIN6c* are also located on duplicated blocks but lack the corresponding duplicates, suggesting that after segmental duplication occurred, genome reorganization (rearrangements) led to the deletion of the corresponding homologue. The highly conserved distribution of exons and introns between *AtPINs* and *PtrPINs* indicates that the structure of the PIN-family genes remained unchanged when the *Populus* and *Arabidopsis* lineages diverged 100–120 million years ago (Tuskan et al., 2006).

The overall predicted topology of the PIN proteins is preserved in *Populus*. Both the TMDs located at the N- and C-termini of *PtrPINs* and the divergent central hydrophilic loop are found in the long PINs such as *PtrPINs*, *OsPINs* (Wang et al., 2009), *ZmPINs* (Forestan et al., 2012), *SiPINs* (Pattison and Catalá, 2012), and *AtPINs* (Paponov et al., 2005). *PtrPIN5a*, *PtrPIN5b*, *PtrPIN8a*, and *PtrPIN8b* lack a full central loop domain, as in *AtPIN5* and *AtPIN8*. Each parallel TMD is highly conserved in *AtPINs* and *PtrPINs* of both the long and short PIN proteins. These results support the hypothesis that all *PIN* genes in higher plants diverged from a single ancestral sequence (Paponov et al., 2005). The highly conserved *PtrPIN* topology suggests that *PtrPINs* share similar functions with their *Arabidopsis* counterparts.
The subcellular localization of PIN proteins determines the direction of auxin flow (Křeček et al., 2009). Proteins of the PtrPIN1, PtrPIN2, PtrPIN3, and PtrPIN8 as well as the PtrPIN6 subclasses are targeted to the plasma membrane, which may mediate cell-to-cell auxin flow and contributes to auxin gradients. Members of the PtrPIN5 as well as the PtrPIN6 subclass localize to the ER, which may function in the regulation of subcellular auxin homeostasis.

Protein function can be further specified in terms of temporally and spatially regulated expression. Similarly to AtPIN1, AtPIN2, AtPIN4, and AtPIN6 (Table 1), the expression patterns of PtoPIN1a, PtoPIN1b, PtoPIN1d, PtoPIN2, PtoPIN3a, PtoPIN3b, PtoPIN6a, and PtoPIN6b are conserved. PtoPIN1a, PtoPIN1b, PtoPIN1d, and members of the PtoPIN6 subclass are expressed ubiquitously in Populus, with the majority of the expression in the cambial and xylem tissues, suggesting that these PINs are required and play essential roles as polar auxin transporters in cambium differentiation. This expression pattern is similar to that of AtPIN1 and AtPIN6 (Table 1). These two subclasses have undergone evolutionary convergence, resulting in the coexpression of AtPIN1 and AtPIN6 under differing growth conditions and developmental stages in Arabidopsis; they have been suggested to perform complementary functions at least at the organ level (Paponov et al., 2005). Therefore, these five PtoPINs from the PtoPIN1 and PtoPIN6 subclasses may coordinately exert their functions mainly during the establishment and maintenance of vascular auxin gradients. PtoPIN2 is primarily expressed in a root-tip region reminiscent of the elongation zone of root tips, similar to AtPIN2, which is involved in root gravitropism (Müller et al., 1998) and lateral organ development (Benková et al., 2003). SiPIN2 and ZmPIN2 are also predominantly expressed in the roots (Forestan et al., 2012; Pattison and Catalá, 2012). Overexpression of PtoPIN2 from Populus tremula × Populus alba (called PtPIN9 in a previous report; Gou et al., 2010) can significantly increase the lateral root formation (Gou et al., 2010), providing evidence that PtoPIN2 play important roles in auxin signalling and regulation in plant development. PtoPIN3a and 3b are transcribed mainly in the leaves. As shown in Fig. 1, PtoPIN3a and PtoPIN3b share an orthologous relationship with the ancestor of AtPIN3, AtPIN4, and AtPIN7. Similarly, AtPIN4 is highly expressed in leaves, including cauline leaves, rosette leaves, and cotyledons (Friml et al., 2002a). Furthermore, a shrunken-leaf morphology has been observed in the Populus lines overexpressing PtoPIN3a (data not shown). Taken all together, it is thought that PtoPIN3a is mainly involved in the Populus leaf development.

Fig. 8. GUS staining of tissues from transgenic poplars during adventitious root regeneration. DR5::GUS (A), PPtoPIN2::GUS (B), and PPtoPIN5a::GUS (C) show different patterns of each promoter activities at all four stages.
Table 1. Summary of the expression patterns of AtPINs and PtrPINs

<table>
<thead>
<tr>
<th>References</th>
<th>PtoPIN</th>
<th>Expression pattern</th>
<th>AtPIN</th>
<th>Expression pattern</th>
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<tr>
<td>Gälweiler et al. (1998);</td>
<td>PtoPIN1a</td>
<td>Root, leaf, stem, cambium, xylem, catkins</td>
<td>AtPIN1</td>
<td>Cotyledons, flowers, roots, rosette leaves, seedlings, inflorescence axes, siliques, cambial and xylem tissues, embryogenesis</td>
</tr>
<tr>
<td>Vieten et al. (2005)</td>
<td>PtoPIN1b</td>
<td>Root, leaf, stem, cambium, xylem, catkins</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>PtoPIN1c</td>
<td>Root, pollen</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>PtoPIN1d</td>
<td>Root, leaf, stem, cambium, xylem</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Müller et al. (1998);</td>
<td>PtoPIN2</td>
<td>Root</td>
<td>AtPIN2</td>
<td>Root-tip elongation zone, embryogenesis</td>
</tr>
<tr>
<td>Vieten et al. (2005)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Friml et al. (2002b);</td>
<td>PtoPIN3a</td>
<td>Leaf, stem</td>
<td>AtPIN3</td>
<td>Gravity-sensing tissues, hypocotyl and stem starch sheath cells, root-tip pericycle and columella cell, embryogenesis</td>
</tr>
<tr>
<td>Vieten et al. (2005)</td>
<td>PtoPIN3b</td>
<td>Root, leaf, stem, cambium, xylem, catkins, pollen</td>
<td>AtPIN4</td>
<td>Roots, cauline leaves, inflorescence, rosette leaves, flowers, cotyledons, embryogenesis</td>
</tr>
<tr>
<td>Friml et al. (2002a);</td>
<td>PtoPIN5a</td>
<td>Root</td>
<td>AtPIN5</td>
<td>Hypocotyl, cotyledon vasculature, pericycle, endodermis of main root, weakly in the root tip, whole inflorescence, rosette leaves- mainly in the vasculature and moderately in mesophyll cells, guard cells in cotyledons</td>
</tr>
<tr>
<td>Vieten et al. (2005)</td>
<td>PtoPIN5b</td>
<td>Leaf, stem</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>PtoPIN5c</td>
<td>Leaf, stem</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>PtoPIN6a</td>
<td>Root, leaf, stem, cambium, xylem</td>
<td>AtPIN6</td>
<td>Coexpressed with AtPIN1</td>
</tr>
<tr>
<td></td>
<td>PtoPIN6b</td>
<td>Root, leaf, stem, cambium, xylem</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>PtoPIN6c</td>
<td>Root, leaf, stem</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Paponov et al. (2005)</td>
<td>PtoPIN8a</td>
<td>Root, leaf, stem, cambium, xylem, catkins</td>
<td>AtPIN8</td>
<td>Male gametophyte</td>
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<td>PtoPIN8b</td>
<td>Root, leaf, stem, cambium, catkins, pollen</td>
<td></td>
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<td>Bosco et al. (2012);</td>
<td>PtoPIN9a</td>
<td>Root, leaf, stem, cambium, catkins, pollen</td>
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</tr>
<tr>
<td>Ding et al. (2012)</td>
<td>PtoPIN9b</td>
<td>Root, leaf, stem, cambium, catkins, pollen</td>
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</table>

PtoPIN1b in the cambium polar auxin transport, based on the evolutionary relationship and expression pattern analysis. The expression of PttPIN1d from Populus tremula × tremuloides (called PttPIN2 in a previous report; Schrader, 2003) is continuously maintained in the vascular cambium through a constant lateral distribution of auxin (Schrader, 2003). PtoPIN1c is expressed specifically in the roots, and tomato and maize also contain a PIN1 homologue that is expressed mainly in the roots (Forestan et al., 2012; Pattison and Catalá, 2012). The diverse evolution of root-preferential and cambial-concentrated PtoPIN genes suggests that the function of PIN1 is required in many different types of cells and tissues in Populus.

AtPIN5 shows broad expression in Arabidopsis. In Populus, PtoPIN5a is expressed mainly in the roots, whereas PtoPIN5b and PtoPIN5c are almost leaf-specific. SiPIN10 and ZmPIN5a, homologues of AtPIN5 in tomato and maize, respectively, are expressed mainly in the roots (Forestan et al., 2012; Pattison and Catalá, 2012), which shows that PIN5 is evolved and specialized in some species. The expression of nearly half of PtoPINs (PtoPIN1a, 1b, 3b, 5b, 5c, 8a and 8b) is detected in the catkins, and PtoPIN8a and PtoPIN8b are highly expressed in the catkins and pollen. The expression patterns of PtoPIN8a and PtoPIN8b are different from that of AtPIN8, which is the only one that is predominantly expressed in pollen and weakly expressed in most sporophytic tissues and which represents the most ancestral form of PIN auxin transporters (Ding et al., 2012). Furthermore, AtPIN8 is localized in the ER and its function in the regulation of intracellular auxin homoeostasis is antagonistic to AtPIN5 (Ding et al., 2012). The plasma membrane localization of PtoPIN8b and the widespread expression of PtoPIN8s may indicate that PtoPINs act differently from AtPIN8 and their functions are not restricted to gametophyte generation. This is not a surprise as the development of Populus trees is more complicated than that of Arabidopsis.

Specific PtoPINs are involved in re-establishing the SAM and RAM

Shoot and AR induction proceed through the formation of new auxin gradients. The corresponding PtoPINs should be regulated to maintain cell-to-cell auxin flow. In Arabidopsis, the SAM auxin concentration gradient is maintained by AtPIN1 (Petrasek and Friml, 2009). Induction of new shoot meristems and somatic embryogenesis requires the PIN1-mediated polar auxin transport and establishment of SAM (Gordon et al., 2007; Su et al., 2009). In the Populus shoot regeneration process, members of the PtoPIN1 subclass are upregulated at least 5-fold, suggesting that these PtoPIN1s
play essential roles, such as the re-establishment of the auxin gradient during this process. In addition to PtoPIN1s, other PtoPINs such as PtoPIN5s, PtoPIN6s, and PtoPIN8s exhibit significant induction during this process. How all these players coordinate shoot induction remains elusive.

The RAM auxin concentration gradient is maintained primarily by AtPIN1 and AtPIN2 in Arabidopsis (Petrasek and Friml, 2009). OsPIN1b plays an important role in AR emergence in rice (Xu, 2005; Wang et al., 2009). PtoPIN1c, PtoPIN2, and PtoPIN5a are the only three PIN genes that are upregulated during the Populus AR induction (Fig. 7B). PtoPIN1c is induced starting from the R2 callus stage, suggesting that an auxin gradient is initiated during the callus induction stage. PtoPIN2 and PtoPIN5a are strongly induced starting from the R3 AR emergence stage, which suggests that these two genes are required to fine tune the auxin regulation in later AR regeneration stages. It is worth mentioning that, in the R4 stage, PtoPIN2 is expressed in a region behind the root tip, while PtoPIN5a is restricted in the tip region of roots. This suggests that PtoPIN2 and PtoPIN5a act differently in the regeneration of roots in Populus.

In summary, although the fundamental features of PIN proteins—such as their sequence, gene structure, and protein topology—are conserved between Populus and Arabidopsis, the Populus PIN family shows differentiation and diversification of expression patterns compared with its Arabidopsis counterparts. Considering the vigorous secondary growth and long-distance transport of water, minerals, and signalling materials mediated by the cambium activity and the vascular systems, the expansion and differentiation of the PIN family in Populus are likely necessary. Different sets of PINs were found to be induced during shoot and AR regeneration in Populus, and their induction was shown to be dynamic at different stages of these processes (Fig. 7 and Supplementary Fig. S5). However, the precise coordination of PINs during the development of woody plants, especially in the xylem and phloem differentiation that occurs during secondary growth, requires further investigation.

**Supplementary material**

Supplementary data are available at JXB online.

**Supplementary Fig. S1.** Phylogenetic analysis of the PIN family genes from Arabidopsis and Populus trichocarpa using full-length protein sequences.

**Supplementary Fig. S2.** Genomic localization of the full-length Populus PIN genes.

**Supplementary Fig. S3.** Topology and conserved domain analysis of the PIN family proteins.

**Supplementary Fig. S4.** Heat map showing the expression of 15 PtrPIN genes in different tissues.

**Supplementary Fig. S5.** Shoot and AR regeneration processes.

**Supplementary Table S1.** Primer sequences for PtoPIN2, PtoPIN5a, PtoPIN1b, and PtoPIN1d promoters and semiquantitative PCR.

**Supplementary Table S2.** Summary of the Populus PIN-FORMED gene family.

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**References**


