A Small Guanosine Triphosphate Binding Protein *PagRabE1b* Promotes Xylem Development in Poplar

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Rab GTPases are the subfamily of the small guanosine triphosphate (GTP)-binding proteins which participated in the regulation of various biological processes. Recent studies have found that plant Rabs play some specific functions. However, the functions of Rabs in xylem development in trees remain unclear. In this study, functional identification of *PagRabE1b* in *Populus* was performed. Quantitative reverse transcription PCR (qRT-PCR) results showed that *PagRabE1b* was highly accumulated in stems, especially in phloem and xylem tissues. Overexpression of *PagRabE1b* in poplar enhanced programmed cell death (PCD) and increased the growth rate and the secondary cell wall (SCW) thickness. Quantitative analysis of monosaccharide content showed that various monosaccharides were significantly increased in secondary xylem tissues of the overexpressed lines. Flow cytometry analysis revealed that the number of apoptotic cells in *PagRabE1b*-OE lines is more than a wild type (WT), which indicated that *PagRabE1b* may play an important role in PCD. Further studies showed that overexpression of *PagRabE1b* increased the expression level of genes involved in SCW biosynthesis, PCD, and autophagy. Collectively, the results suggest that *PagRabE1b* plays a positive role in promoting the xylem development of poplar.

**Keywords:** poplar, small gtp binding protein, *PagRabE1b*, cell wall, wood formation

**INTRODUCTION**

Trees are the most abundant natural sources important for sustainable energy and the sinks of atmospheric carbon dioxide (Zhang et al., 2015; Zhong and Ye, 2015). Wood biomass is mainly composed of the secondary cell wall (SCW) and is widely used in many applications, such as house construction, biofuels, pulping, and paper-making. Because of the substantial economic value of wood, elucidating the molecular regulatory mechanism of wood formation will be useful for the manipulation of wood quality and quantity through molecular breeding.

The development of SCW is a complicated procedure requiring synchronization of several regulatory and metabolic pathways. Previous studies have revealed that the SCW biosynthesis is mainly regulated by transcriptional regulatory networks of NAC and MYB transcription factor (TF) families (Zhong and Ye, 2015). During SCW formation, several NAC TFs are considered as...
master switches and in the top layer of the SCW regulatory network (Xie et al., 2018). A series of additional TFs, such as MYB46 and MYB83, belong to the second layer of a transcriptional network of the SCW formation. In addition, several other MYB TFs in the third layer have been identified as direct targets of MYB46/83 (Zhang et al., 2018a); most of these MYB members positively regulate SCW biosynthesis. SCWs mainly consist of cellulose, hemicelluloses, and lignin. Cellulose taking about 40–50% of wood components is glucose polymers. Hemicelluloses constitute a major part of lignocellulosic biomass, mainly including xylolignans, xylans, mannans, glucomannans, and β-(1→3, 1→4)-glucans (Scheller and Ulvskov, 2010). Lignin is a complex three-dimensional polyphenolic polymer of p-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) lignins (Boerjan et al., 2003). Monolignol biosynthesis is carried out through the common phenylpropanoid pathway, and several central genes involved in lignin synthesis during secondary wall formation in Populus had been well-identified (Wang et al., 2018). Finally, the lignified vessel and fiber cells undergo programmed cell death (PCD) for complete lysis of cell content in the maturation of xylem cells (Courtois-Moreau et al., 2009).

Small GTPases also play important roles in the regulation of cell wall development by monitoring cytoskeletal arrangement and membrane trafficking (Oda and Fukuda, 2014). In the eukaryotic, the Ras subfamily of small GTPases comprises five families: Ras, Rab, Ran, Arf, and Rho (Hall, 1998). Rab and Ran of plants play critical roles in the development of primary and SCWs (Oda and Fukuda, 2014). ROPGEF4 and ROPGAP3 mediate Rho GTPase ROP11 to originate the fundamental patterning of SCWs in xylem cells, and then ROP11 interacts with MIDD1 to provoke local depolymerization of cortical microtubules (Oda and Fukuda, 2012). In Arabidopsis, a boundary of the ROP domain (BDRI) and Wallin (WAL) complex regulates cell wall development. BDRI could recruit WAL to the plasma membrane and then regulate an ROP-act in a pathway to shape pit boundaries (Sugiyama et al., 2019). Rab GTPase has also been shown to play important roles in SCW deposition. Overexpression of a constitutively active mutant of RabG3b (RabG3bCA) stimulated both autophagy and tracheary element formation in Arabidopsis (Kwon et al., 2010) and increased xylem growth due to the stimulation of autophagy during xylem development in Populus (Kwon et al., 2011).

The Rab family in plants is categorized into eight subfamilies (RabA-RabH) (Vernoud et al., 2003). We found out that there was a total of 67 PtRab genes in Populus trichocarpa and were grouped into eight subfamilies (Zhang et al., 2018b). Most of the PtRab genes were preferentially expressed in phloem and xylem. During the development of the poplar stem, the majority of PtRab genes were preferentially expressed in the transition region from primary growth to secondary growth. These results suggested that the PtRab genes might participate in the biological processes related to xylem development. In this study, we explored the function of PagRabE1b in xylem development using transgenics overexpressing constitutively active mutant and native PagRabE1b, which revealed that PagRabE1b influenced both SCW biosynthesis and final PCD during wood formation.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Hybrid poplar (Populus alba × Populus glandulosa, Pag) clone 84 K was used for gene transformation. Plants were cultivated for 4 weeks under in vitro conditions and then transplanted to the soil and grown for 12 weeks in a greenhouse (16-h light: 8-h dark, 25°C: 20°C, with relative humidity in the range 50–60%).

Vector Construction and Plant Transformation

PagRabE1b and PagRabE1b (Q74L) (point mutation of Q74 to L in motif 2, a constitutively active form of PagRabE1b) genes were cloned into the pMDC32 plant expression vector and transformed into poplars previously (Zhang et al., 2018b). Two overexpression lines of PagRabE1b and PagRabE1b (Q74L) with high expression levels were selected and named as OE-1 and OE-9 for PagRabE1b and QL-8 and QL-13 for PtRabE1b (Q74L), respectively.

Histological Analyses

The basal stems of wild type (WT), OE-1, OE-9, QL-8, and QL-13 plants were sectioned for histology using a vibratome (VT1000S, Leica, Wetzlar, Germany) to a thickness of 50 µm. Then, the sections were stained with 0.05% toluidine blue (TBO) for 60 s at room temperature, rinsed three times in water, and photographed with an Olympus BX51 microscope (Du et al., 2020).

Wall Thickness Measurement

Secondary cell wall thickness was observed by using confocal laser scanning microscopy (CLSM) and transmission electron microscopy (TEM). The 15th internodes of 2-month-old WT and PagRabE1b transgenic plants were sectioned using a vibratome at a thickness of 50 µm. CLSM pictures were captured with an LSM880 microscope (Zeiss) using 488-nm laser excitation (5% power). TEM observation was implemented as described by Zhao et al. (2020). The 20th internodes from 3-month-old WT and transgenic plants were cut into 2-mm pieces, which were fixed with 2.5% paraformaldehyde and 0.5% glutaraldehyde in PBS (0.1 M, pH 7.4) with vacuum infiltration. The pieces were then washed three times with 0.1 M PBS and further fixed in 1% osmic acid for 2 h at room temperature. Ultrathin sections were made and photographed using an HT-7700 microscope (Hitachi) and a Gatan ORIUS™ SC1000CCD Camera (Gatan Inc., USA). The cell wall thickness of at least 100 cells from three individual plants of each line was measured using ImageJ software and analyzed statistically using Student’s t-test.

Analysis of SCW Composition

The 10th to 20th internodes of 3-month-old WT and PagRabE1b transgenic plants were collected and freeze-dried at −60°C on a vacuum freeze. The dried stems were powdered by ball milling and used to measure alcohol-insoluble residues according to Zhao et al. (2020). The monosaccharide content was analyzed through GC-MS (Agilent, Santa Clara,
CA, USA). Three biological replicates were analyzed for each line.

**Programmed Cell Death Analysis of Stem-Differentiating Xylem Protoplasts**

The *Populus* protoplasts from SDX isolation were carried out as previously described by Lin et al. (2013) and Wang et al. (2020) with minor modifications. Briefly, the debarked stem segments of 3-month-old WT and *PagRabE1b* transgenic poplars were incubated in a cell wall digestion enzyme solution [1.5% (wt/vol) Cellulase R-10 and 0.4% (wt/vol) pectolyase Y-23 in 20-mM MES, 0.6-M mannitol and 20-mM KCl solution, 10-mM CaCl₂, and 0.1% (wt/vol) BSA, pH 5.7] for 40 min in the dark at room temperature. Protoplasts were filtered through a 195-µm filter paper and then resuspended in W5 solution (2-mM MES, 125-mM CaCl₂, and 0.1-M glucose, and 5-mM KCl). The isolated protoplasts were resuspended in a 195-µl pre-diluted binding buffer, and 5-µl Annexin V-FITC solution (Solarbio, Beijing, China) was added, mixed, and incubated for 30 min at room temperature, and then added 1 µl of the 20-µg ml⁻¹ propidium iodide (PI) storage solution. After the addition of another 300-µl binding buffer, the suspended protoplasts were analyzed using a BD Aria SORP cell sorter (BD Biosciences, USA) with 488-nm excitation for FITC and 530 nm for PI. Three independent sets of experiments were performed.

**Gene Expression Analysis**

The apex, stems, and roots were harvested from 8-week-old poplars and were sampled for quantitative reverse transcription PCR (qRT-PCR). For sampling, the xylem and phloem–cambium tissues from stems of 8-week-old poplar were debarked and scraped with a sterile double-edged razor blade from the outer of the debarked stem or the inner surface of the peeled bark. To examine the expression levels of marker genes involved in xylem development by qRT-PCR, 2-month-old WT and *PagRabE1b* transgenic poplars were debarked, and developing xylems were scraped. Total RNA was extracted from all the samples using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) and genomic DNA was eliminated with the on-column treatment of RNase-free DNase I (Qiagen, Hilden, Germany). First-strand complementary DNA (cDNA) was synthesized with about 1-µg RNA using the SuperScript III reverse transcription kit (Invitrogen) according to the instruction of the manufacturer. Primers with annealing temperatures of 58–60°C and amplification lengths of 100–250 bp were designed using Primer3 software (http://primer3.ut.ee/). All primers were listed in Supplementary Table 1. qRT-PCR was carried out using KAPA SYBR FAST qPCR master mixture on Roche LightCycler 480 (Roche Applied Science, Penzberg, Upper Bavaria, Germany) according to the instructions of the manufacturer. All experiments were performed in three biological replicates and three technical replicates. The *PagActin* gene (Potri.001G309500) was used as the internal control (Wang et al., 2016; Zhao et al., 2020).

**RESULTS**

**Expression Pattern of PagRabE1b**

To investigate the expression pattern of *PagRabE1b*, qRT-PCR was used to analyze the gene expression in tissues collected from the apex, stems, and roots. The results revealed that *PagRabE1b* expression could be detected in all the tissues and was high in xylem and phloem–cambium (Figure 1A). A similar expression pattern was also observed by analysis of the AspWood database (http://aspwood.popgenie.org) (Sundell et al., 2017), which showed that *PagRabE1b* is highly expressed in expanding xylem (Figure 1B). These results suggested a potential role of *PagRabE1b* in xylem development in poplar.

**Overexpression of PagRabE1b Promotes Xylem Development in Transgenic Poplar**

Mutation of particular residues of small GTPases can generate constitutively active forms, which can be used to explore Rab functions. Overexpression of constitutively active RabG3b stimulated autophagy and increased xylem development in *Arabidopsis* and *Populus* (Kwon et al., 2010, 2011). To elucidate the function of *PagRabE1b* in xylem development, transgenic poplars with *PagRabE1b* and *PagRabE1b* (Q74L) overexpression were generated under the control of the CaMV 35S promoter. Two independently transgenic lines (OE-1, OE-9) with higher expression of *PagRabE1b* and two lines with overexpressing a constitutively active form of *PagRabE1b* (Q74L) (QL-8, QL-13) (Zhang et al., 2016) were selected for further analysis (Figures 2A,B). All WT and transgenic lines were grown in the greenhouse under the same environmental conditions. Compared with the WT, 2-month-old transgenic plants of *PagRabE1b* and *PagRabE1b* (Q74L) exhibited rapid growth and development (Figure 2A). Quantitative measurement of the growth rate and basal stem diameter showed that *PagRabE1b* transgenic plants had a 20.3–28.9% increase in the growth rate and an 11.8–26.5% increase in stem width compared with wild-type plants (Figures 2C,D). To examine whether the xylem development was altered in *PagRabE1b* transgenic poplars, cross-sections of basal stems from 3-month-old WT and *PagRabE1b* transgenic lines were analyzed. Compared with the WT, xylem width was significantly increased in all *PagRabE1b* overexpression lines (Figure 3A). Quantitative analysis showed that the xylem radial width was increased by 27–50% in *PagRabE1b* transgenic plants compared with WT plants (Figure 3B). All these results suggested that *PagRabE1b* may play critical roles in regulating poplar secondary growth and xylem development.

**PagRabE1b Overexpression Affects SCW Deposition**

Several studies have suggested that RabGTPase has positive roles in the controlling of SCW development. Therefore, we first examined the SCW structure by lignin autofluorescence using CLSM. The results showed that lignification and SCW deposition were increased in *PagRabE1b* transgenic plants than in WT (Supplementary Figure 1). Then, transverse-sections of the 20th internode of 3-month-old transgenic plants were used to...
FIGURE 1 | Expression patterns of PagRabE1b in poplar. (A) Quantitative reverse transcription PCR (qRT-PCR) was carried out to analyze the gene expression levels of PagRabE1b in different tissues of poplar. The actin gene was used as an internal control. (B) Expression profiles of PagRabE1b among developing phloem, cambium, and developing xylem. Data were derived from the AspWood database (http://aspwood.popgenie.org) (Sundell et al., 2017). A, apex; S, stem; Xy, xylem; Ph, phloem; Ca, cambium; R, root.

FIGURE 2 | Growth phenotypes of PagRabE1b transgenic poplars compared with wild type (WT). (A) A photograph of WT and PagRabE1b overexpression lines. Bar = 10 cm; (B) qRT-PCR analyses of the transcript levels of PagRabE1b in WT, OE-1, OE-9, QL-8, and QL-13 lines. (C,D) The growth rate and basal diameter of 3-month-old WT, OE-1, OE-9, QL-8, and QL-13 lines. The growth rate and diameter: means ± SD of six clonally propagated plants. Student’s t-test; *P < 0.05; **P < 0.01.

examine the SCW structure using TEM. The results showed that the cell walls of xylem fiber cells were thicker in all PagRabE1b overexpression plants than in WT plants (Figure 3C). Statistical analysis showed that the SCW thickness of xylem fibers was increased by 10.2, 23.5, 17.65, and 13.1 in OE-1, OE-9, QL-8, and QL-13 plants, respectively (Figure 3D).

The differences in SCW thickness may indicate changes in cell wall composition, and therefore, we determined the chemical components of the SCWs using the stem of wild-type and transgenic plants. Overexpression of PagRabE1b appeared to have a remarkable effect on monosaccharide contents. The contents of glucose and galactose were significantly increased in all transgenic plants compared with WT. Among these, the content of glucose was increased by 36.1–170% in PagRabE1b and PagRabE1b (Q74L) overexpression lines than in WT. In addition, the contents of fructose, arabinose,
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**FIGURE 3** | Xylem development was increased by *PagRabE1b* overexpression. (A) Transverse sections of the basal stems of 3-month-old WT, OE-1, OE-9, QL-8, and QL-13 plants. Bar = 200 µm. (B) Transmission electron microscopy (TEM) examination of the cell wall thickness of xylem fiber cells in 3-month-old WT, OE-1, OE-9, QL-8, and QL-13. Bar = 5 µm. (C) Statistical analysis of xylem width in the stems of WT, OE-1, OE-9, QL-8, and QL-13 lines. (D) Statistical analysis of the cell wall thickness of WT, OE-1, OE-9, QL-8, and QL-13 lines. Student’s t-test; *P < 0.05; **P < 0.01.

mannose, xylose, and galactose were significantly increased in the *PagRabE1b* or *PagRabE1b* (Q74L) transgenic lines compared with WT (Figure 4). These results suggest that *PagRabE1b* positively regulates secondary wall biosynthesis in *Populus*.

**PagRabE1b Promotes the PCD Process in Xylem Formation**

Programmed cell death is a pivotal step in xylem formation and is required for the complete maturation of both TEs and fiber cells during xylem development. To investigate the differences of PCD between *PagRabE1b* transgenic and WT plants, protoplasts were isolated from the SDX and analyzed by flow cytometry. Loss of plasma membrane irregularity is one of the earliest characteristics in PCD with exposing phospholipid phosphatidylserine (PS) to the exterior cellular environment. FITC-conjugated Annexin V has a high affinity with PS and acts as a sensitive probe for flow cytometric analysis of the early stage of PCD (O’brien et al., 1998). PI is commonly used to detect dead cells in a population since it is not permeant to live cells. Flow cytometry assay showed that the proportion of apoptotic cells was significantly different in WT and *PagRabE1b* transgenic plants. WT plants contained the lowest percentage (34.3%) of FITC positive cells, while *PagRabE1b*OE-9 and *PagRabE1b*QL-8 plants had the higher percentage (56.0 and 45.9%, respectively), of FITC positive cells (Figure 5). Similar to the FITC staining, *PagRabE1b*OE-9, and *PagRabE1b*QL-8 plants displayed a significant increase in PI-positive cells (41.5 and 34.8%, respectively), compared with WT plants (20.5%) (Figure 5). This result indicates that *PagRabE1b* has a positive role in PCD during xylem development.

**Expression Analysis of Xylem Development-Related Genes**

Since manipulation of *PagRabE1b* expression resulted in changes in SCW thickness and PCD of xylem cells, we examined the expression of key genes involved in wood formation by qRT-PCR. As expected, xylem differentiation- and secondary wall
regulation-related NAC and MYB genes, such as SND1-A1 (ortholog of AtSND1), VND6-C1 (AtVND6), MYB21 (AtMYB46 and AtMYB83), MYB031 (AtMYB69), MYB090 (AtMYB52 and AtMYB54), MYB127 (AtMYB67), and MYB128 (AtMYB103), were upregulated (Figure 6, Supplementary Figure 2). SCW synthesis-related genes containing homologs of Arabidopsis CESA4, CESA7, CESA8, LAC4, and LAC17 were also upregulated. Additionally, we also examined the expression levels of PCD-related genes (Peroxidase, VPE, MC9ATG8d1, ATG8f2, and ATG8i) (Kwon et al., 2011) (Figure 6, Supplementary Figure 2). All these genes were upregulated in OE-1, OE-9, QL-8, and QL-13 plants. These results suggest that PagRabE1b overexpression can activate the expression of wood formation-related genes in transgenic plants.

**DISCUSSION**

Wood formation is a complicated biological process, including the division and differentiation of the vascular cambium, cell elongation, SCW deposition, and PCD. Although the transcriptional regulatory network was outlined on the SCW synthesis of xylem cells (Zhang et al., 2018a), the precise regulatory mechanisms of xylem cell differentiation during xylem formation remain elusive. Previous research suggests that small GTPases may activate autophagy during wood formation (Kwon et al., 2011), other than regulate SCW development in the xylem of higher plants (Oda and Fukuda, 2014). Therefore, in this study, we further investigated the function of PagRabE1b in xylem development in poplar.

Eight of the 11 ROP genes in Arabidopsis were expressed in xylem tissues (Winter et al., 2007). In poplar, more than half of the PtRab genes were highly expressed in phloem and xylem (Zhang et al., 2018b). In this study, the qRT-PCR analysis showed that PagRabE1b was highly expressed in xylem and phloem-cambium (Figure 1). Similarly, the Eucalyptus ROP GTPase gene was found preferentially expressed in the cambium area and developing xylem (Foucart et al., 2009). Since cambium includes newly differentiating xylem cells (close to developing xylem) with
higher expression than other cell types as shown in Figure 1, we propose that the involvement of PagRabE1b gene in the process of xylem development.

To investigate the role of RabE1b in woody plants, transgenic poplars overexpressing PagRabE1b were generated. The overexpression of PagRabE1b leads to an increase in both plant length and stem thickness in poplar (Figure 2). These phenotypic alterations are similar to that with Arabidopsis RabG3b overexpression (Kwon et al., 2011). In contrast, the reduced plant size and drastically altered leaf morphology were observed in the Arabidopsis RabE1d downregulated transgenic plants (Speth et al., 2009). In addition, virus-induced gene silencing of NbRabE1 caused various phenotypes, such as growth retardation, premature senescence, and aberrant leaf development (Ahn et al., 2013). These results prove that Rab GTPase plays a pivotal role in plant growth and development.

Phenotypic examination revealed that overexpression of PagRabE1b enhanced SCW thickness of fiber cells in poplar stems (Figure 3). Chemical components measurement showed an increase in cell wall constituents in the stems of transgenic

FIGURE 6 | Expression analysis of xylem development-related genes in WT, OE-1, OE-9, QL-8, and QL-13 lines. The poplar actin gene was used as an internal control. Results are means ± SD of three biological replicates. Student’s t-test; **P < 0.01.
promotes xylem formation. The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author/s.

**AUTHOR CONTRIBUTIONS**

S-TZ conceived and designed the experiments. Y-LL, L-JW, YL, Y-HG, and YC performed the experiments. Y-LL and S-TZ wrote the manuscript. All authors contributed to the article and approved the submitted version.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.686024/full#supplementary-material

**Supplementary Figure 1** | The microscopic images by confocal laser scanning microscopy (CLSM) of Rab transgenic poplars and wild type (WT). (A,B) WT; (C) OE-1; (D) OE-9; (E) QL-8; (F) QL-13.

**Supplementary Figure 2** | Quantitative reverse transcription PCR (qRT-PCR) analysis of xylem development-related genes in WT and transgenic lines. The poplar act in the gene was used as an internal control. Results are means ± SD of three biological replicates. Student’s t-test; *P < 0.05; **P < 0.01.

**Supplementary Table 1** | List of primers used in this study.

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

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