J. Plant Biol. (2017) 60:129-136
DOI 10.1007/s12374-016-0904-8

ORIGINAL ARTICLE

Identification and Functional Analysis of a Promoter Sequence for Phloem Tissue Specific Gene Expression from *Populus trichocarpa*

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Received: October 21, 2016 / Accepted: November 16, 2016

Abstract Unlike xylem, which is primarily composed of dead cells in vascular bundles, phloem has living cells. It transports organic nutrients and long-distance communication signals to all parts of plants. In this report, we describe a promoter from *Populus trichocarpa* that drives strong gene expression in a phloem tissue-specific manner. First, we identified five candidate genes with strong expression in the developing phloem (DP) tissue from whole-transcriptome gene chip analyses of different tissue types of poplar. The putative promoter sequences of them were isolated and tested for their promoter activity in transgenic Arabidopsis plants. Among them, a promoter of the Potri.001G340200.1 gene (called the *PtrDP3* promoter) was identified that has the strongest activity in phloem tissue. *PtrDP3* promoter activity was found exclusively in phloem cells of the stem and root tissues of transgenic Arabidopsis plants, which was reproduced in the transgenic poplar plants. The phloem-specific activity of the *PtrDP3* promoter was detected as early as in three-day-old seedlings and was not affected by abiotic stresses or exogenously applied plant hormones in transgenic Arabidopsis plants. Promoter deletion analysis identified a 100-bp regulatory region of the *PtrDP3* promoter, which is necessary to drive phloem specific expression. This study provides evidence of a strong phloem-specific promoter that is suitable for phloem-specific biotechnological modifications in plants.

Keywords: Arabidopsis, Biotechnology, Phloem tissue, Poplar, promoter, Tissue-specific

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Introduction

Higher plants evolve phloem tissue in their vasculature for an exquisite long-distance transporting system. Phloem is a complex tissue composed of sieve elements (SEs) and companion cells (CCs) with unique subcellular structures (recent review, De Rybel et al. 2016). SEs serve as transport conduits for delivering not only photoassimilates but also proteins with diverse functions (e.g., signaling, metabolism, and defense), small RNAs, and hormones throughout the whole plant body to regulate many aspects of plant development (Ruiz-Medrano et al. 2001; De Rybel et al. 2016).

Trees represent a huge biomass for many industrial applications and function as an increasingly important carbon sink that can reduce anthropogenic increases of atmospheric carbon dioxide (Somerville 2006). Genetic modifications have been attempted to increase tree biomass for decades (Baucher et al. 2003; Ragauskas et al. 2006; Chen and Dixon 2007; Han et al. 2007; Weng et al. 2008). Promoters that enable targeted expression of transgenes in particular cell/tissue types or developmental stages have recently been isolated and utilized to obtain desired transgenic phenotypes (Ratke et al. 2015; Jeon et al. 2016; Nguyen et al. 2016). This can be done without collateral pleiotropic consequences using a constitutive 35S promoter (No et al. 2000; Coleman et al. 2006, 2008). One example is the developing xylem (DX) tissue-specific expression of *PdGA20ox1*, a key gene in the production of bioactive gibberellins in *Pinus densiflora*, which successfully avoids undesirable phenotypes such as poor root growth and leaf development in transgenic poplars (Park et al. 2015; Jeon et al. 2016). Since the majority of biomass in trees is derived from radial growth of stems as a sink tissue, manipulation of long-distance transport

of carbon metabolites, signaling molecules, and defense-related proteins through phloem tissue is critical.

Although phloem differentiation from (pro)cambial meristem is still elusive, there have been many studies of phloem specific genes and their promoters (Stadler and Sauer 1996; Guo et al. 2004; Noll et al. 2007, 2009; Schneidereit et al. 2008; Ruping et al. 2010; Bucsenez et al. 2012). Among them, a promoter of the Arabidopsis sucrose transporter gene *AtSUC2* restricts expression to the CCs of source tissues (Stadler and Sauer 1996). The *AtSUC2* promoter was found to have two *cis*-elements for HD-ZIP (homeodomain leucine zipper) and DOF (DNA-binding with one finger) transcription factors, which are essential for CC-specific expression (Schneidereit et al. 2008). However, not many SE-specific elements have been described yet.

It has been shown that most *SIEVE ELEMENT OCCLUSION* (*SEO*) family genes from diverse plant species, such as *Medicago truncatula*, *Glycine max*, *Solanum phureja*, and *Arabidopsis thaliana*, are specifically expressed in immature SEs (Noll et al. 2007, 2009; Ruping et al. 2010; Bucsenez et al. 2012). Bucsenez et al. (2012) analyzed the SE-specific *Medicago truncatula* SEO-F1 promoter (PMtSEO-F1) by constructing deletion, substitution, and hybrid constructs of the promoter. They found putative *cis*-regulatory elements for SE specific expression.

Here, we describe the isolation and characterization of a promoter sequence of the Potri.001G340200.1 gene (called the *PtrDP3* promoter) from *Populus trichocarpa*. The Potri.001G340200.1 gene encodes a putative protein homologous to Arabidopsis SEO family protein. *PtrDP3* promoter activity was found exclusively in phloem cells in both transgenic Arabidopsis and poplar plants and was not influenced by abiotic stresses or exogenously applied growth regulators. Further promoter deletion analysis identified a 100-bp regulatory region responsible for phloem specific expression.

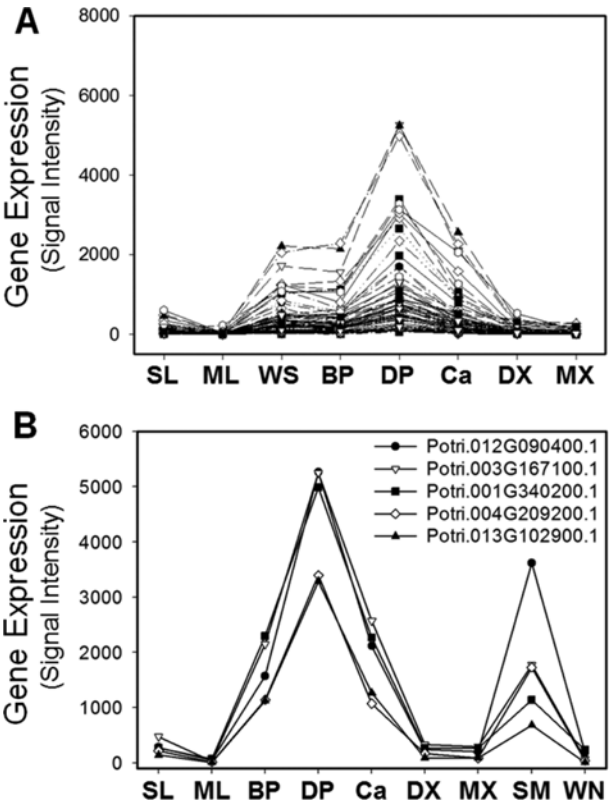

Fig. 1. Selection of the five candidate genes abundantly expressed in DP-tissue. (A) A total of 81 genes were identified by applying the following sequential filters and plotted. First, genes that were up-regulated (>5 fold) in DP compared to both SL and ML were 1,340 genes; second, genes that were up-regulated (>2 fold) in DP compared to BP, Ca, DX and MX narrowed the selection down to 102 genes; third, only genes with 'Presence' calling in DP were included in the final 81 genes. A list of the genes is provided in supplementary Table S1. (B) DP-tissue specific and strong expression of the five selected candidate genes. Tissue specific expression of candidate genes, Potri.012G090400.1 (*PtrDP1*), Potri.003G167100.1 (*PtrDP2*), Potri.001G340200.1 (*PtrDP3*), Potri.004G209200.1 (*PtrDP4*), and Potri.013G102900.1 (*PtrDP5*), are plotted. SL, shoot apical meristem and leaf primordial; ML, mature leaf without major veins; BP, bark and mature phloem; DP, developing phloem; Ca, cambial zone; DX, developing xylem; MX, mature xylem; WS'whole stem' as an internal control (Ko et al. 2012). SM, WN; summer and winter stem, respectively (Ko et al. 2011).

Results and Discussion

Isolation of Promoter Sequences for Phloem Specific Expression

In order to identify phloem-specific and strongly expressed genes, the same strategy that was reported recently was used (Nguyen et al. 2016). In brief, sequential filtering was performed using stem tissue type transcriptome data of Poplar Genome Arrays (Affymetrix), which include bark and mature phloem (BP), developing phloem (DP), cambial zone (Ca), developing xylem (DX), mature xylem (MX) tissue, whole stem (WS), shoot apical meristem and leaf primordia (SL), and mature leaf without major veins (ML) (Ko et al. 2012). As described in Fig. 1A, a total of 81 genes were isolated and further narrowed down to five candidate genes based on their DP-tissue specificity and gene expression level (i.e., Potri.012G090400.1, Potri.003G167100.1, Potri.001G340200.1, Potri.004G209200.1, and Potri.013G102900.1) (Fig. 1B).

Confirmation of Phloem-tissue Specific Promoter Activity

To confirm phloem tissue specificity of those five candidate promoters, *in planta*, we have generated transgenic Arabidopsis plants harboring each promoter::GUS construct. Among them, the promoter of the Potri.001G340200.1 gene (*PtrDP3* promoter) was identified as having very specific to phloem

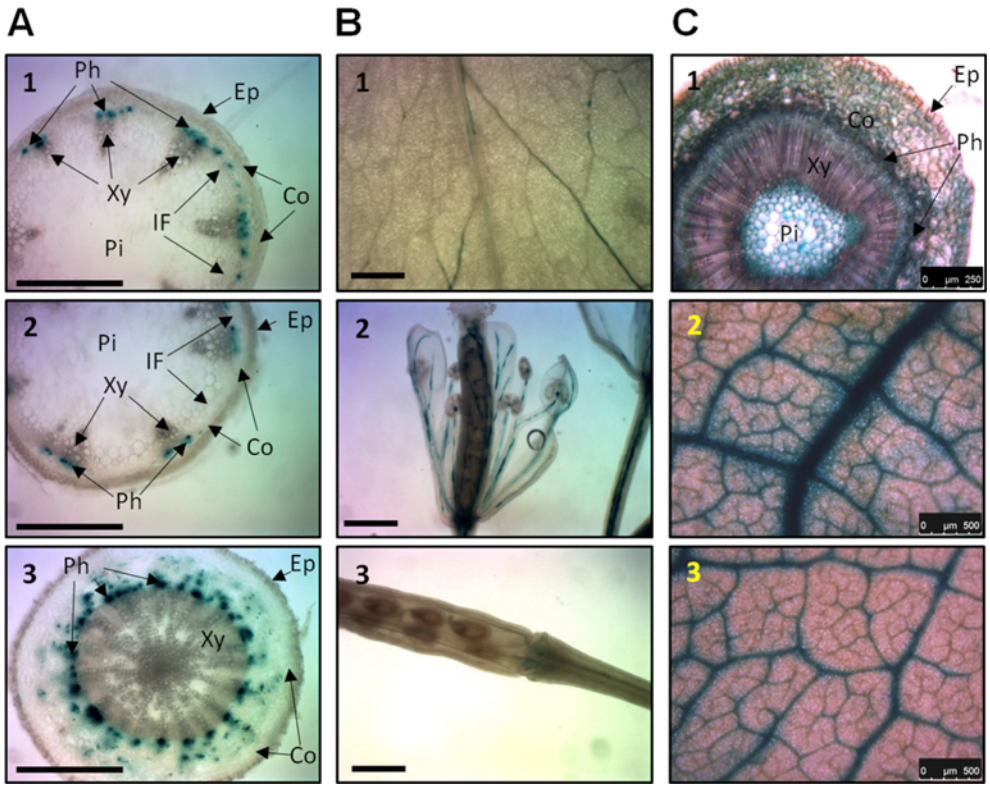

Fig. 2. *PtrDP3* promoter activity in phloem tissue of vascular bundle. (A) Cross-section of stem (#1, #2) and root (#3) of the PtrDP3pro::GUS transgenic Arabidopsis plants; #1 and #2 show lower and middle stem, respectively. (B) Various parts of the PtrDP3pro::GUS transgenic Arabidopsis plant such as leaf (#1), flower (#2), and silique (#3). Scale bars represent 0.5 mm. (C) Cross-section of lower stem (#1) and leaf with major vein (#2) and without major vein (#3) of the PtrDP3pro::GUS transgenic poplar plants. Scale bars are indicated. Ep, epidermis; Co, cortex; IF, interfascicular fiber; Xy, xylem; Ph, phloem; Pi, pith cells. Note that GUS activity is found only in the vascular tissues.

tissue with strong activity as well in transgenic Arabidopsis plants (data not shown). *PtrDP3* promoter activity was exclusively found in phloem tissue of the stem and root cross sections of *PtrDP3*pro::GUS transgenic Arabidopsis plants (Fig. 2A). Interestingly, some GUS signals were detected in the interfascicular region of lower stem section (#1 of Fig. 2A). These signals might come from secondary phloem tissues developed by vascular cambium activity. It has been known that vascular cambium is developed in rosette level stems of fully matured Arabidopsis. Accordingly, there were no such signals in middle stem in #2 of Fig. 2A. Likewise, the GUS signal was only detected in vascular tissue of other plant organs, such as the flower, leaf, and silique (Fig. 2B). To verify *PtrDP3* promoter activity, transgenic poplar plants expressing the *PtrDP3*pro::GUS construct were generated. GUS expression was specifically found in the phloem region of the stem and major veins of *PtrDP3*pro::GUS transgenic poplar plant leaves (Fig. 2C). The Potri.001G340200.1 gene encodes a putative protein of 722 amino acids homologous to Arabidopsis SIEVE ELEMENT OCCLUSION-RELATED 1 (AtSEOR1; At3g01680) with 52% identity (Suppl. Fig. S1 and S2). *AtSEOR1* is required for Arabidopsis P-protein filament formation in phloem sieve elements (Ruping et al. 2010; Froelich et al. 2011). The expression profile of the SEO (SIEVE ELEMENT OCCLUSION) gene family has been specifically reported in the sieve tube element of phloem tissue in many plant species, including *Arabidopsis thaliana*, *Medicago truncatula*, and *Glycine max* (Ruping et al. 2010; Froelich et al. 2011; Anstead et al. 2012). Thus, the phloem tissue-specific activity of the *PtrDP3* promoter is consistent with the SEO gene family and expected to be SE-specific.

In addition, *PtrDP3* promoter activity during plant developmental stages (i.e., temporal activity) was examined by evaluating GUS activity after germination of *PtrDP3*pro:: GUS transgenic Arabidopsis plants. *PtrDP3* promoter activity was observed as early as in three-day-old seedlings. The activity was highly specific and strong in vascular tissues of root, hypocotyl, cotyledon, and leaves (Fig. 3).

PtrDP3 Promoter Activity was Not Influenced by Various Stimuli

Plants survive by adjusting their growth and development to their environmental stimuli. To investigate whether *PtrDP3*

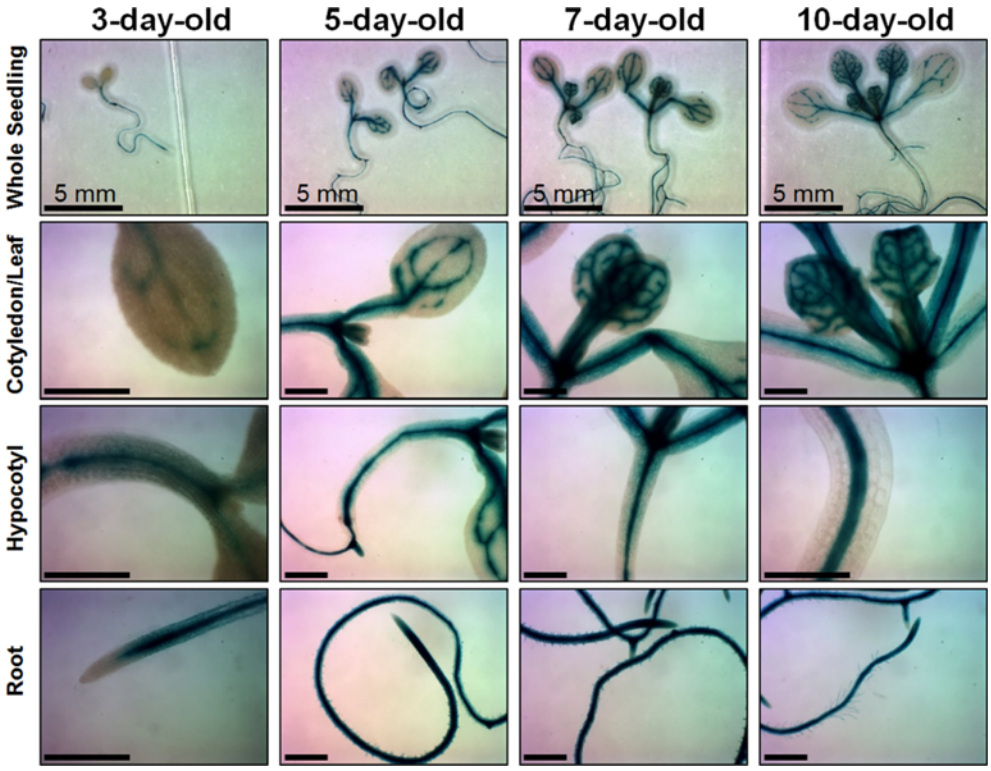

Fig. 3. *PtrDP3* promoter activity in developmental stages of transgenic Arabidopsis plants. GUS activity driven by *PtrDP3* promoter was visualized during early seedling development of PtrDP3pro::GUS transgenic Arabidopsis plants. Note that strong GUS activities exclusively found in vascular tissues from 3-day-old plants. Scale bars represent 0.5 mm except indicated otherwise.

promoter activity was affected by these stimuli, the 10-day-old seedlings of *PtrDP3*pro::GUS transgenic Arabidopsis were treated with various abiotic stresses (e.g., cold, drought, salt and osmotic stress) and plant growth regulators (e.g., auxin, cytokinin, and abscisic acid (ABA)), respectively. Our histochemical GUS assay showed that the *PtrDP3* promoter activity was not changed significantly to those stimuli (Fig. 4A). These results were further confirmed by quantitative analysis of GUS activity (Fig. 4B). These facts suggest that the *PtrDP3* promoter is genuinely phloem tissue-specific and thus can be utilized to drive genes-of-interest in phloem tissue regardless of developmental or environmental changes. However, Srivastava et al. (2014) reported that the promoter of *PsSEOF1* (one of SEO family gene from *Pisum sativum*) could drive the expression GUS reporter gene in response to abiotic stress and hormonal treatments in *Agrobacterium*-mediated transient assay using tobacco leaves. Thus, it might be plausible that the stress responsible promoter activities can be different in each member of the SEO gene family. There are more than ten members of SEO gene family in poplar (Phytozome v.11 (https://phytozome.jgi.doe.gov/pz/portal.html#), e.g., Potri.001G340200.1, Potri.001G340300.1, Potri.001G340400.1, Potri.001G340500.1 , Potri.001G340600.1, Potri.010G050300.1, Potri.010G050500.1, Potri.010G050600.1, Potri.010G050800.1, Potri.008G183200.1, Potri.017G071000.1).

Identification of Regulatory Region of PtrDP3 Promoter for Phloem Specific Expression

To identify regulatory regions of the *PtrDP3* promoter for phloem tissue specific expression, a series of promoter deletion analysis was performed. Resulting DNA fragments of the *PtrDP3* promoter (e.g., *PtrDP3-P1* to *PtrDP3-P6*; Fig. 5A) were fused to the GUS reporter gene and used to generate transgenic Arabidopsis plants. GUS activity was observed in each transgenic Arabidopsis (Fig. 5B, Supplemental Fig. S3). Our results showed that a 100 bp sequence from -300 to -200 (from ATG) may be responsible for phloem tissue-specific activity of the *PtrDP3* promoter because *PtrDP3-P4* (-300 ~ ATG) has clear vascular tissue-specific GUS activity while *PtrDP3-P5* (-200 ~ ATG) and *PtrDP3-P6* (-100 ~ ATG) does not (Fig. 5B, Supplemental Fig. S3). Phloem tissue-specific GUS activity of *PtrDP3-P4* was further confirmed in the cross-section of stem tissue of transgenic Arabidopsis plants (Supplemental Fig. S4). Unfortunately, we could not find any conserved *cis*-acting elements in the 100 bp sequence except a motif (5'-TTCTTCCTCG-3') (Fig. 5C), which is similar to PSE box5 of the SE-specific *Medicago truncatula* SEO-F1 promoter (Bucsenez et al. 2012). Thus, more detailed analysis is required to identify *cis*-acting element(s) driving SE-specific gene expression in poplar.

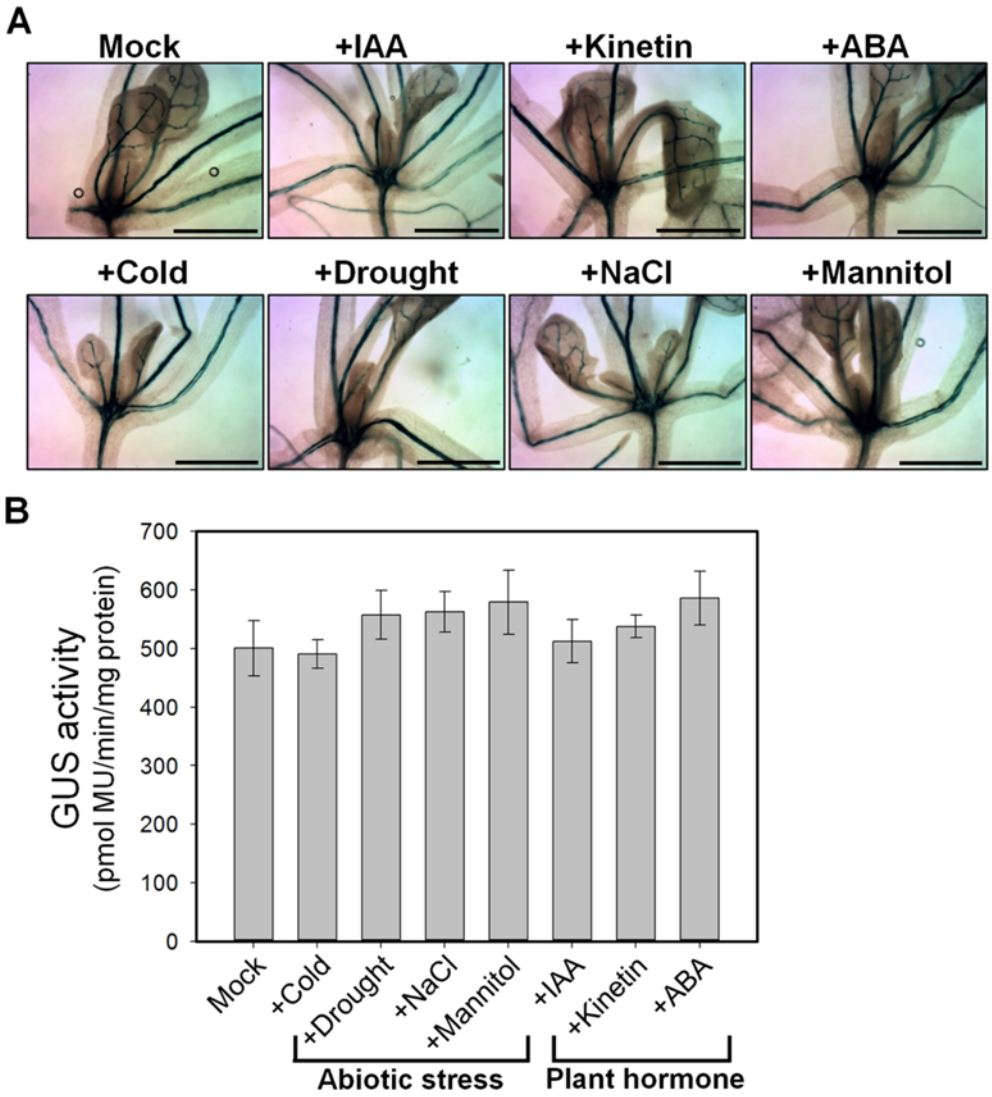

Fig. 4. *PtrDP3* promoter activity was not influenced by various stimuli. (A) *PtrDP3* promoter activity by various plant hormones and abiotic stresses. The indicated plant hormones and abiotic stresses were treated to ten-day-old PtrDP3pro::GUS transgenic Arabidopsis plants and promoter activity was visualized by GUS staining (see, Materials and methods). (B) Quantification of *PtrDP3* promoter activity of the abiotic stress and plant hormones treatments. Quantitative measurements of GUS activity was performed following both abiotic stresses and plant hormone treatments to the transgenic Arabidopsis plants (PtrDP3pro::GUS). Value of GUS activity was presented as pmoles MU/min/mg protein. Error bars indicate S.D of three biological replications. Note that error bars of samples are overlapped with control.

Prospects

Agriculture and forestry are now facing the unprecedented challenges of increasing human populations and global climate changes. A growing human population demands more food and energy from plant/woody biomass resources, while climate changes are expected to limit plant productivity by causing weather extremes and reducing water availability (Karp and Richter 2011; Polle et al. 2013; 2014 UN Report on World's Forests). Recent advances of novel tools in plant biology, including next generation sequencing (NGS) and genome editing technology (e.g., ZFN, TALEN, CRISPR/Cas9) provide opportunities to improve the growth and development of woody perennial plants through more sophisticated molecular breeding. Many genes critical to control the quantity and quality of woody biomass and engineering technologies to facilitate wood utilization have been reported (Harfouche et al. 2011; Polle et al. 2013). Appropriate promoters that drive transgene expression in a highly controlled manner (e.g. developmental- or tissue-specific expression) are required to efficiently utilize these genetic resources. Here, we described the identification and characterization of a potential promoter that is specific for phloem tissue using transgenic Arabidopsis and poplar plants expressing promoter::GUS constructs. This promoter, especially a 100-bp regulatory region, can be effectively used to specifically

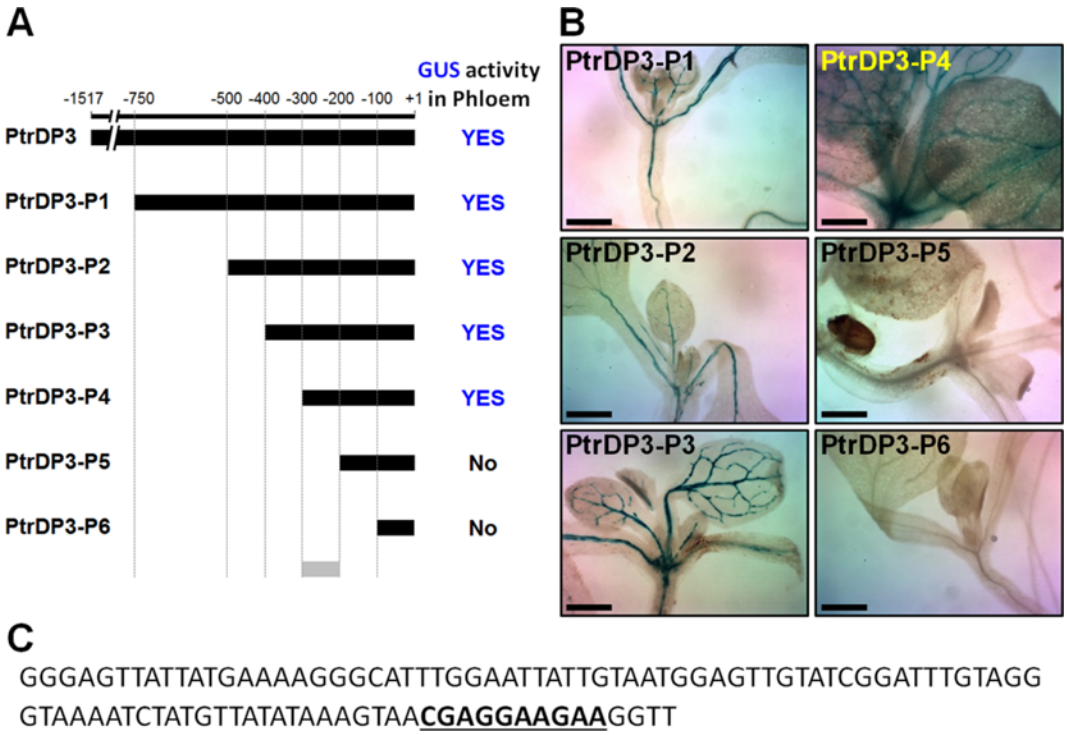

Fig. 5. *PtrDP3* promoter deletion analysis to identify a regulatory region for phloem specific expression. (A) Schematic diagram of *PtrDP3* promoter constructs (PtrDP3-P1 ~ PtrDP3-P6) with sequential 5'-deletion. PtrDP3-P1: -750~+1, PtrDP3-P2: -500~+1, PtrDP3-P3: -400~+1, PtrDP3-P4: -300~+1, PtrDP3-P5: -200~+1, PtrDP3-P6: -100~+1. The gray box in the bottom indicates a sequence ('100 bp') may be responsible for DP-tissue specific expression. (B) GUS activity driven by each 5'-deleted PtrDP3 promoter (indicated) was visualized using ten-day-old transgenic Arabidopsis plants. (C) Nucleotide sequence of the '100 bp' region. A motif (complementary to 'CGAGGAAGAA') similar to PSE box5 is shown in bold letters with underline.

express transgenes (e.g., transporter of carbon metabolites, signaling molecules, and defense-related proteins) in phloem tissue while avoiding unintended pleiotropic side effects frequently caused by the 35S promoter.

Materials and Methods

Plant Materials and Growth Conditions

Arabidopsis thaliana (Col-0) and hybrid poplar (*Populus alba* × *P. tremula* var. *glandulosa*) were used to generate transgenic plants. Arabidopsis was grown on soil or MS-agar medium (MS; M0404, Sigma-Aldrich, St. Louis, MO) with 2% sucrose in a growth room (14 hrs light/10 hrs dark) at 25°C. Hybrid poplar were cultivated on MS-agar medium containing 0.2 mg/L IBA (Indole-3-butyric acid) in a growth chamber (16 hrs light/8 hrs dark) at 25°C.

Production of Promoter::GUS Transgenic Plants

Approximately 1.5 kb of genomic DNA upstream of the start codon of putative DP-tissue specific genes was isolated by PCR (named *PtrDP1* ~ *PtrDP5* promoter) and inserted upstream of the *GUS* gene in pBGWFS7 or pKGWFS7 vectors using Gateway cloning (Invitrogen, Carlsbad, CA) to produce *PtrDP* promoter::GUS constructs. For a sequential 5'-deletion analysis of *PtrDP3* promoter, PCR amplification of each promoter sequences (i.e., *PtrDP3-P1*: -750~+1, *PtrDP3-P2*: -500~+1, *PtrDP3-P3*: -400~+1, *PtrDP3-P4*: -300~+1, *PtrDP3-P5*: -200~+1, and *PtrDP3-P6*: -100~+1) were performed using the *PtrDP3* promoter as a template and cloned to pBGWFS7 vector as described. *Agrobacterium*-mediated genetic transformation of Arabidopsis and poplar was performed by the floral-dip (Clough and Bent, 1998) or callus-mediated method (Choi et al. 2005), respectively. All constructs used in this study were verified by DNA sequencing. Promoters and primers used for amplification are listed in suppl. Table S2.

Histochemical GUS Staining of Transgenic Plants and Quantification of GUS Activity

Histochemical GUS staining and quantification of GUS activity of transgenic plants was performed as described by Jefferson et al. (1987) with slight modifications as described in Nguyen et al. (2016). For GUS staining, samples were incubated at 37°C for 18-24 hrs in GUS reaction buffer and visualized after removing chlorophyll by rinsing with 70% ethanol. For quantification of GUS activity, fluorogenic reaction was performed in 100 ml of MUG substrate buffer by adding 10 ml of supernatant of GUS-extracted samples in the dark at 37°C for 20 min. After reaction stop (adding 890 ml of 200 mM Na_2CO_3), liberated 4-methylumbelliferone (MU) was calculated from relative fluorescence units (TKO 100, Hoefer Scientific, San Francisco, CA) and a MU (M1381, Sigma-Aldrich, St. Louis, MO) standard curve. Final GUS activity values were recorded as pmoles MU/min/mg protein.

Treatment with Abiotic Stresses and Plant Hormones

Abiotic stresses (e.g., 150 mM NaCl, 300 mM mannitol, drought and cold) and plant hormones (e.g., 1 mM IAA, 1 mM ABA, and 1 mM

kinetin) were treated as described in Nguyen et al. (2016). In brief, ten-day-old transgenic Arabidopsis plants grown on 1/2 MS agar media were transferred to liquid 1/2 MS media for 6 hrs before treatment. Salt or osmotic stresses were applied by incubating seedlings in a liquid 1/2 MS media containing either 150 mM of NaCl or 300 mM mannitol, respectively, at room temperature (RT) for 12 hrs. Drought stress was treated by drying seedlings on a clean bench for 15 min and then returning them to liquid 1/2 MS media at RT for 12 hrs. Cold stress was imposed by incubating seedlings in liquid 1/2 MS media at 4°C for 12 hrs. Treatment with plant hormones was performed by incubating seedlings in liquid 1/2 MS media containing either 1 mM IAA (Indole-3-acetic acid) or 1 mM ABA or 1 mM kinetin at RT for 12 hrs.

Acknowledgements

This work was funded by a grant to J.-H. Ko by the Korea Forest Service (S111213L080110) and by a grant from the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (NRF-2015R1D1A1A01060807).

Author Contributions

V.P. Nguyen and J.-H. Ko: conceived the project. V.P. Nguyen, J.-S. Cho, J.-H. Lee, M.-H. Kim, Y.-I. Choi, E.-J. Park, W.-C. Kim, S. Hwang, K.-H. Han, J.-H. Ko: performed the experiments and analyzed the data. V.P. Nguyen and J.-H. Ko: wrote the paper.

Supporting Information

Fig. S1. Genetic information of Potri.001G340200.1 gene.

Fig. S2. Potri.001G340200.1 gene encodes a putative protein homologous to Arabidopsis SIEVE ELEMENT OCCLUSION-RELATED 1 (AtSEOR1).

Fig. S3. GUS activities of sequential 5'-deleted *PtrDP3* promoters in transgenic Arabidopsis plants.

Fig. S4. *PtrDP3-P4* promoter activity in transgenic Arabidopsis plants.

Table S1. List of genes abundantly expressed in DP tissue of poplar

Table S2. Primers used in this study

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