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PtrHB7, a class III HD-Zip Gene, Plays a Critical Role in Regulation of Vascular Cambium Differentiation in *Populus*

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ABSTRACT A key question in the secondary growth of trees is how differentiation of the vascular cambium cells is directed to concurrently form two different tissues: xylem or phloem. class III homeodomain-leucine zipper (HD-Zip III) genes are known to play critical roles in the initiation, patterning, and differentiation of the vascular system in the process of primary and secondary growth. However, the mechanism of how these genes control secondary vascular differentiation is unknown. Here, we show that a *Populus* class III HD-Zip gene, *PtrHB7*, was preferentially expressed in cambial zone. *PtrHB7*-suppressed plants displayed significant changes in vascular tissues with a reduction in xylem but increase in phloem. Transcriptional analysis revealed that genes regulating xylem differentiation were down-regulated, whereas genes regulating phloem differentiation were up-regulated. Correspondingly, *PtrHB7* overexpression enhanced differentiation of cambial cells toward xylem cells but inhibited phloem differentiation. *PtrHB7* regulation on cambial cell differentiation was associated with its transcript abundance. Together, the results demonstrated that *PtrHB7* plays a critical role in controlling a balanced differentiation between secondary xylem and phloem tissues in the process of *Populus* secondary growth in a dosage-dependent manner.

Key words: *HD-Zip III*; cambium; xylem; fiber cell; secondary growth; *Populus*.

INTRODUCTION

Different from primary growth which gives rise to elongation growth (at the shoot and root tip), plant secondary growth derives from the vascular cambium and results in girth expansion. During secondary growth in tree species, secondary xylem and phloem tissues develop perennially from the fusiform initials of the vascular cambium through two distinct differentiation pathways. The differentiation occurs via a highly ordered developmental process, which involves division of the vascular cambium, vascular cell differentiation, cell wall thickening, and other biological processes. However, the underlying molecular mechanism that regulates differentiation pathways toward secondary xylem or phloem formation has not been well understood.

Class III homeodomain-leucine zipper (HD-Zip III) transcription factor genes have been studied for playing crucial roles in regulating primary vascular system development in *Arabidopsis*. These genes include *REVOLUTA* (*REV*)/*INTERFASCICULAR FIBERLESS1* (*IFL1*), *PHABULOSA* (*PHB*), *PHAVOLUTA* (*PHV*), *CORONA*/*AtHB15* (*CNA*), and *AtHB8*, which display overlapping, antagonistic, or distinct roles (Talbert et al., 1995; Zhong and Ye, 1999; Emery et al., 2003; Prigge et al., 2005). *REV* plays a role in regulating the growth of apical meristems (Talbert et al., 1995; Ratcliffe et al., 2000), while the same gene, named *IFL1* in another study, is involved in the regulation of vascular tissue formation (Zhong and Ye, 1999). *PHB* and *PHV* play roles in the establishment of leaf polarity and their defects can cause the transformation of abaxial leaf fates into adaxial leaf fates (McConnell and Barton, 1998; McConnell et al., 2001). The *CNA* gene also plays a role in apical embryo patterning by antagonizing *REV* function (Prigge et al., 2005). Loss-of-function analysis of *PHB*, *PHV*, and *REV* indicate that the three genes perform overlapping functions in regulating apical embryo patterning (Emery et al., 2003). Among the five *HD-Zip IIIs*, *AtHB8* is the only

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gene that is not expressed in shoot apical meristem (Prigge et al., 2005); its expression is restricted to procambial cells (Baima et al., 1995). *AtHB8* appears to have lost some of its functions in embryo patterning and meristem size restriction and evolved a more specialized role in vascular differentiation during seed plant evolution (Baima et al., 2001; Prigge and Clark, 2006).

Studies have reported characterization of *HD-Zip III*s in other plant systems. There are also five *HD-Zip III* genes, *OsHB1–OsHB5*, in the genome of rice, a monocot species which develops scattered vascular bundles but lacks secondary growth (Floyd et al., 2006). Investigation of *OsHB1–OsHB4* expression pattern and ectopic expression phenotypes indicate that they are essential for radial pattern formation during embryogenesis and the leaf initiation process in the SAM. The results suggest *OsHB1–OsHB4* have conserved functions with their homolog in *Arabidopsis*. However, *OsHB5* expression emerges much later and fainter at the start of vascular tissue development in embryo (Itoh et al., 2008).

Eight *HD-Zip III* genes were identified in *Populus trichocarpa* (Ko et al., 2006; Cote et al., 2010). The *Populus* homolog of the *Arabidopsis REV* gene, *PRE*, is expressed in shoot apical meristem, cambial zone, and secondary vascular tissue. Overexpression of *PRE* in *Populus* causes abnormal primary and secondary growth, which suggests *PRE* may play a role in the initiation of the cambium and the patterning of secondary vascular tissues (Robischon et al., 2011). In addition, overexpression of *PCN*, a homolog of *CNA* in *Populus*, delayed xylem and phloem fiber differentiation during secondary growth (Du et al., 2011), while knockdown of *PCN* caused abnormal lignification in pith cells. This indicates that *PCN*, similar to its *Arabidopsis* ortholog *CNA*, may negatively regulate secondary vascular cell differentiation (Kim et al., 2005; Du et al., 2011). *AtHB8* in *Arabidopsis* has evolved a more specialized role in vascular differentiation but its function is still not fully characterized. This paper examines the role of *PtrHB7*, an ortholog of the *Arabidopsis AtHB8* gene in the *Populus* lineage, which contains a well-developed secondary vascular system. *PtrHB7* shows preferential expression in cambial zone cells. Down-regulation of *PtrHB7* expression suppresses differentiation from cambial descendant cells to xylem but enhances its differentiation to phloem. Consistently with this observation, a number of xylem-forming genes in *PtrHB7*-suppressed plants are down-regulated while a group of phloem-relative genes are up-regulated. In contrast, *PtrHB7* overexpression enhances xylem differentiation but inhibits phloem differentiation. The degree of the enhancement is directly related to *PtrHB7* transcript levels. Our results demonstrate that *PtrHB7* plays an essential role in controlling the balanced differentiation between secondary xylem and phloem tissues in a dosage-dependent manner during the process of secondary growth in *Populus*.

RESULTS

Evolutionary Linkages of *HD-Zip III* Genes in Plants Undergoing Different Vascular Development

*HD-Zip III*s are plant-specific genes that form a small family in each species. We analyzed them in three different types of plants that each represents a distinct vascular system: *Arabidopsis* (*Arabidopsis thaliana*), rice (*Oryza sativa*), and *Populus* (*Populus trichocarpa*). *Arabidopsis*, a herbaceous species, produces vascular bundles in the process of primary growth and acropetally develops certain secondary vascular cells through the meristematic activity of its interfascicular cambium. Rice, a monocot species, develops scattered vascular bundles but lacks secondary growth. *Populus*, a woody species, is able to perennially develop a secondary vascular system from the vascular cambium meristem. Five *HD-Zip III*s (*REV*, *ATHB14*/*PHB*, *ATHB9*/*PHV*, *ATHB15*/*CNA*, and *ATHB8*) exist in the genome of *Arabidopsis* (Ariel et al., 2007) and the genome of rice (*OsHB1*, *OsHB2*, *OsHB3*, *OsHB4*, and *OsHB5*) (Itoh et al., 2008). In the genome of *Populus*, eight *HD-Zip III* genes are identified, named *PtrHB1* to *PtrHB8*, respectively (Ko et al., 2006; Cote et al., 2010). The eight *Populus HD-Zip III* genes are located on seven different chromosomes and each contains 18 exons (Supplemental Figure 1 and Supplemental Table 1).

Phylogenetic analysis of the HD-Zip III proteins across the three species indicates that they can be divided into three groups (Figure 1). In Group I, each of the three species

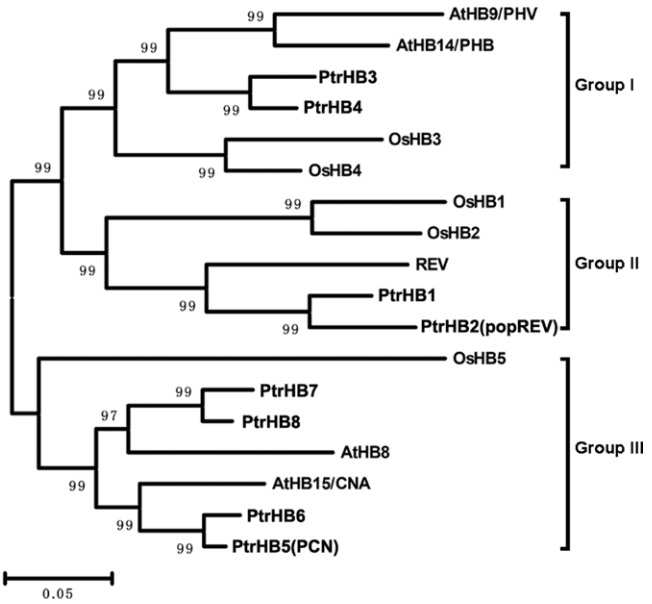

Figure 1. Phylogenetic Tree of the HD-Zip III Proteins from Three Representative Species, *Populus*, *Arabidopsis*, and Rice.
The HD-Zip IIIs are classified into three groups (Groups I, II, and III). The scale bar corresponds to 0.05 amino acid substitutions per position in the sequence. Ptr, *Populus trichocarpa*; At, *Arabidopsis thaliana*; Os, *Oryza sativa*.

contains a pair of duplicated genes: *PtrHB3* and *PtrHB4* in *Populus*, *PHB* and *PHV* in *Arabidopsis*, and *OsHB3* and *OsHB4* in rice. The duplication in this group appears to have occurred after speciation. Group II includes duplicated pairs of genes in *Populus* (*PtrHB1* and *PtrHB2*) and in rice (*OsHB1* and *OsHB2*), but a single *REV* gene in *Arabidopsis*. Overall, the genes in Groups I and II appear to have undergone a similar evolutionary process in the three species in contrast to Group III genes. Group III includes one gene in rice (*OsHB5*), two in *Arabidopsis* (*CNA* and *AtHB8*), and four in *Populus* (*PtrHB5*, *PtrHB6*, *PtrHB7*, and *PtrHB8*). It is worth noting that the gene duplication in Group III took place after the rice–eudicot split. Furthermore, an additional duplication occurred in *Populus* compared to *Arabidopsis*. It is still unclear whether the different duplication events of the *HD-Zip III* genes across the three species resulted in the development of sub-functionalizations associated with the different vascular systems.

Expression of the *HD-Zip III*s in the Process of *Populus* Vascular Development

HD-Zip III gene expression was examined during the development of the secondary vascular system in *Populus*. Cells from cambium, developing xylem, developing phloem, cortex, and mature leaf tissues were collected using laser microdissection according to the method described in our previous study (Song et al., 2010). Expression of *HD-Zip III*s in the collected cell samples was measured using real-time RT–PCR analysis. Results showed that expression of the eight *HD-Zip III*s were detected in various types of tissue cells with different patterns and expression levels (Figure 2). Expression of *PtrHB1* was low in the cambium, developing xylem, and mature leaf. *PtrHB5* and *PtrHB6* were expressed at low levels in the vascular cambium, developing xylem, and cortex tissues. *PtrHB7* and *PtrHB8* were highly expressed in vascular cambium and developing xylem tissue, but expressed at low levels in the cortex and mature leaf tissues. The results suggest that *PtrHB7* and *PtrHB8* are more active in secondary vascular tissue than non-vascular tissue.

PtrHB7 expression in secondary vascular tissue was verified by promoter analysis. A 2.5-kb promoter fragment of *PtrHB7* was cloned and constructed into *GUS* fusion plasmid and transformed into *Populus* to generate promoter–*GUS* transgenic plants. In 26 independent transgenic lines, GUS staining was visualized in 24 lines. Promoter activity was strong in the cambial zone of vascular tissue but not in shoot apical meristem (Figure 3).

Suppression of *PtrHB7* Expression Affects the Process of Secondary Vascular Differentiation

To characterize *PtrHB7* function in detail, an antisense *PtrHB7* was introduced into wild-type *Populus* under the control of the *PtrHB7* promoter or a *35S* promoter. A total of 23 and 13 independent lines for the two kinds of transgenic plants were generated, respectively. These transgenic plants displayed similar stem morphologies but different degrees of curling of their leaf blades. Four independent lines for each type of transgenic plant were selected for further analysis.

Suppression of *PtrHB7* resulted in reduced leaf size and shortened plant heights in *PtrHB7* promoter-controlled transgenics (Figure 4A and 4B). The diameter of the stem was increased and the internode length decreased (Figure 4C, 4D, 4L, and 4M). Compared to the wild-type, the stem of the transgenic plants showed a narrower xylem zone, wider phloem zone (Figure 4E–4H) and wider cambial zone (Figure 4I and 4J). The wider cambial zone indicates delayed differentiation of cambial cells. The extra layers of phloem cells suggest enhanced differentiation from cambial zone cells to secondary phloem, while the development of fewer layers of xylem cells (Figure 4G and 4H) suggest suppression of *PtrHB7* impeded the differentiation of the cambium zone cells into secondary xylem. In stem cross-sections, expansion of the cortex region in the transgenic plant was observed (Figure 4E and 4F), which contributed to wider stem diameter. Leaf traces were more frequently observed (Figure 4E) as a result of shorter stem internodes.

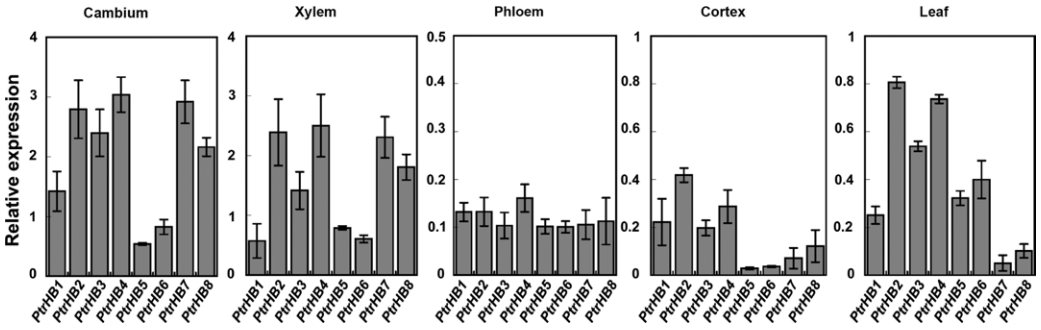

Figure 2. Expression of Eight *Populus HD-Zip III* Genes in Various Types of Differentiating Cells during Secondary Growth.
The cells from various tissues were collected from the cross-sections of the 12th stem internode or the 12th leaf from the top by laser microdissection. DX, developing xylem; Ca, cambium; DP, developing phloem; Co, cortex; Le, mature leaf. The expression level in each sample was normalized using *actin2* as an internal control. The values are means ± SE, $n = 3$.

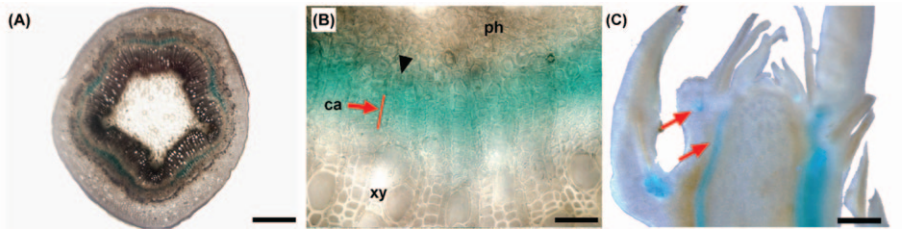

Figure 3. Histochemical Analysis of the GUS Activity during Secondary Growth of the *ProPtrHB7–GUS* Transgenic *Populus*.
GUS staining was observed in the 12th internode **(A)**, the cambial zone **(B)**, and the shoot apical meristem **(C)**. Bar = 500 μm in (A) and (C); bar = 50 μm in (B).

Variations in the leaves of *35S* promoter-controlled plants (Figure 5A) were observed compared to the *PtrHB7* promoter-controlled group (Figure 4A). Mainly, the leaf blades of the plants curled downward, which may be the result of the misregulation of other *HD-Zip IIIs* (Figure 5A and 5E). Other phenotypic changes including shorter plant height (Figure 5A and 5B), wider stem diameter (Figure 5C and 5L), shorter internode length (Figure 5C and 5M), less xylem tissue (Figure 5G and 5H), and wider cambial zone (Figure 5I and 5J) appeared consistent with those observed in *PtrHB7* promoter-controlled transgenic plants (Figure 4). Similar stem phenotypes but different leaf morphologies between the two types of transgenic plants further suggest the *PtrHB7* promoter is active in secondary vascular cambial cells. Transcript analysis of *PtrHB7* showed that levels of expression were significantly lower in both types of promoter-controlled transgenic plants (Figures 4K and 5K). It was also noticed that *PtrHB8* expression was down-regulated in stem tissues due to the high sequence identity between *PtrHB7* and *PtrHB8* (Supplemental Figure 2). Expression of other *HD-Zip III* genes was not obviously affected in the stem with the exception of *PtrHB4* in *35S* promoter-controlled plants (Supplemental Figure 2). Together, the results indicate that suppression of *PtrHB7*/*PtrHB8* expression impedes secondary xylem differentiation.

Overexpression of *PtrHB7* Results in a Gradient of Morphological Changes in *Populus*

To investigate the effect of *PtrHB7* overexpression in *Populus*, a mutation was introduced which allows *PtrHB7* to avoid miRNA-targeted regulation by *miR165/166* (Tang et al., 2003;

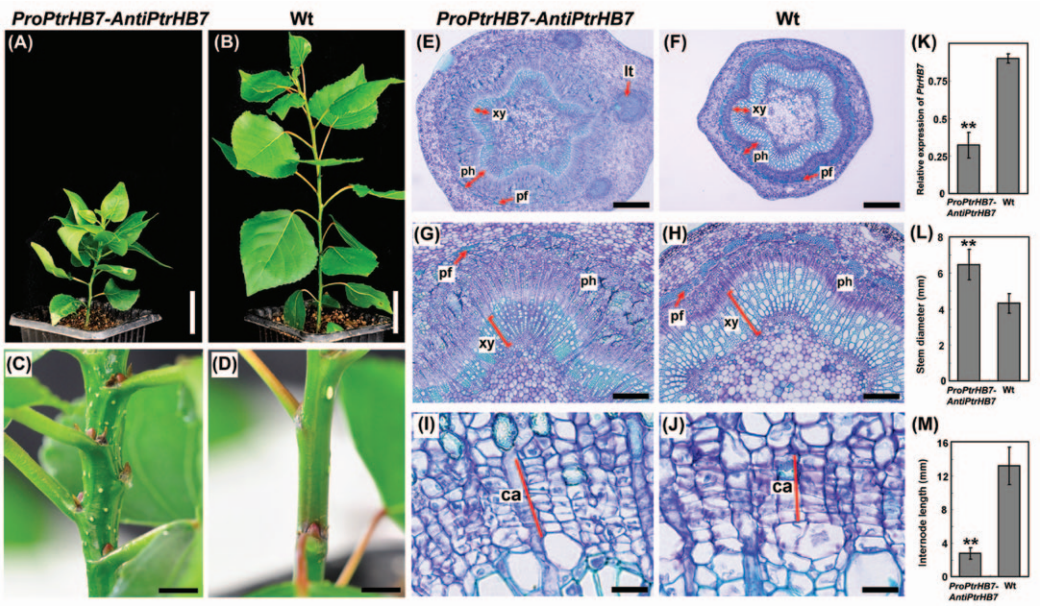

Figure 4. Morphology of the *PtrHB7* Knockdown *Populus* under Control of the *PtrHB7* Promoter.
ProPtrHB7–AntiPtrHB7 transgenic **(A)** and wild-type plants at 2 months old **(B)**. Stem of transgenic **(C)** and wild-type plants **(D)**. Cross-section of transgenic **(E, G, I)** and wild-type plant **(F, H, J)** at internode 12. Bar = 5 cm in (A) and (B); bar = 2 cm in (C) and (D); bar = 500 μm in (E) and (F); bar = 200 μm in (G) and (H); bar = 20 μm in (I) and (J). ca, cambial zone; xy, xylem; ph, phloem; pf, phloem fiber; lt, leaf trace. The *ProPtrHB7–AntiPtrHB7* transgenic plants displayed reduction of xylem tissue ((G) versus (H)) and more cell layers at the cambial zone ((I) versus (J)). Relative expression of *PtrHB7* **(K)**, stem diameter **(L)**, and internode length **(M)** were measured in four independent transgenic and four wild-type plants. Significance as determined by Student's *t*-test: ** $P < 0.001$. The values are means ± SE.

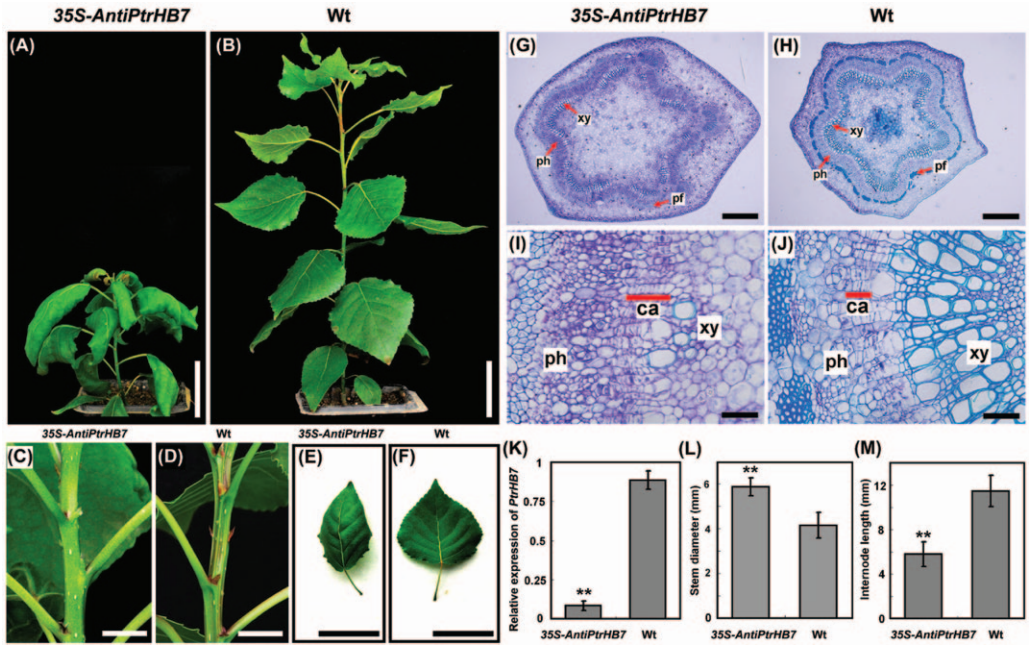

Figure 5. Morphology of the *PtrHB7* Knockdown *Populus* under Control of the *35S* Promoter.
35S-AntiPtrHB7 transgenic **(A)** and wild-type plant **(B)** at 2 months old. Stem and leaf of transgenic **(C, E)** and wild-type plants **(D, F)**. Cross-section of transgenic **(G, I)** and wild-type **(H, J)** plants at internode 10. The *35S-AntiPtrHB7* transgenic plant displayed downward curling leaves, reduction of xylem tissue, more cell layers of cambial zone. Bar = 5 cm in (A)–(F); bar = 500 μm in (G) and (H); bar = 50 μm in (I) and (J). ca, cambial zone; xy, xylem; ph, phloem; pf, phloem fiber. Relative expression of *PtrHB7* **(K)**, stem diameter **(L)**, and internode length **(M)** were measured in four independent lines of *35S-AntiPtrHB7* transgenic and four wild-type plants. Significance as determined by Student's *t*-test: ** $P <^0.001$. The values are means ± SE.

Floyd et al., 2006; Zhang et al., 2006). *PtrHB7* was mutated with five nucleotide changes (named *PtrHB7mt*) without amino acid alternation in the region complementary to *miR166* (Supplemental Figure 3). Under the control of *35S* promoter, the *PtrHB7mt* was introduced into *Populus* through *Agrobacterium*-mediated transformation. Characterization of the morphology of the transformants was carried out from the plantlet regeneration stage to their growth in a greenhouse. Some transformed calli developed needle-shaped leaves during regeneration and had difficulties developing into a normal shoot (Supplemental Figure 4). Transformants that were able to develop into plantlets were shorter and had leaves that curled upward to varying degrees of severity (Figure 6A and 6B). The transgenic plants were divided into two groups according to the severities of their phenotypes, with type I (Figure 6A, 6D, 6G, and 6J) plants showing severe morphological changes and type II displaying relative milder phenotypes (Figure 6B, 6E, 6H, and 6K). The ratio of type I to type II plants was approximately 1:3. For a more detailed morphological analysis, we selected 15 independent transgenic lines of each type and grew them in a greenhouse for 4 months. The stems of both types of transgenic plants could not grow up straight (Figure 6G and 6H). The leaves of the transgenics were smaller and curled upward (Figure 6J and 6K). The observed differences in phenotype are summarized in Table 1. Quantitative characterization of the phenotypes of the two types of transgenic plants also indicated that the morphological alterations in type I plants are more severe than in type II plants. Expression of *PtrHB7* was measured by real-time RT–PCR (Figure 6M). The level of *PtrHB7* transcript was much higher in the transgenic plants than in wild-type. Within the transgenic plants, different levels of *PtrHB7* transcripts were observed between type I and type II plants. Type I plants expressed significantly higher levels of *PtrHB7* than type II plants.

Analysis of vascular structure indicated that *PtrHB7mt* overexpression reduced the size of the vascular cylinder, causing the plants to grow smaller. Significantly, the vascular structure was altered in *PtrHB7mt*-overexpressed plants, which exhibited more xylem cell layers, fewer phloem cell layers, and fewer phloem fiber cells (Table 1 and Figure 7A, 7E, 7D, and 7H). In particular, fewer cell layers were observed in the dividing region of the vascular cambium in *PtrHB7mt*-overexpressed plants (Figure 7B and 7F, and Table 1). Differences in secondary vascular development between type I and type II plants were also observed.

Xylem differentiation was much more enhanced and phloem differentiation suppressed in type I plants (Figure 7C) compared to type II plants (Figure 7G). Ectopic vascular fiber cells, which originated from ectopic cambium in the cortex, were more frequently observed outside of the phloem region in type I than type II plants (Figure 7C and 7G). Cork cambium

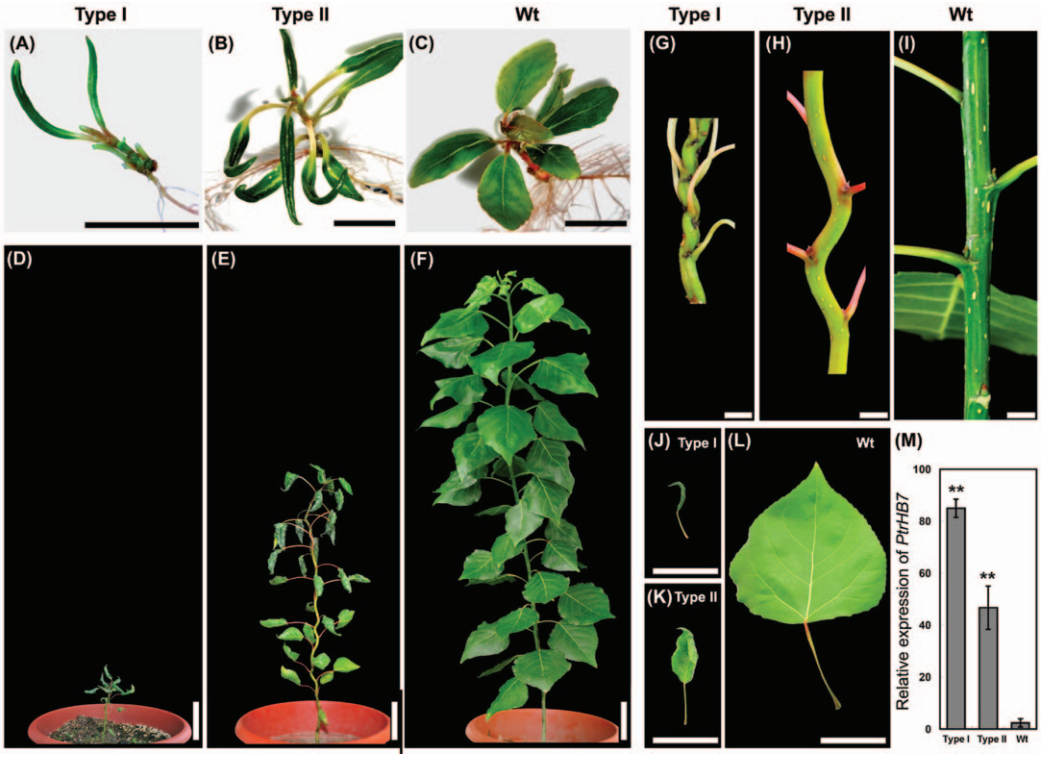

Figure 6. Morphology Alternations in *PtrHB7mt*-Overexpressed *Populus*.
The transgenic plant phenotypes can be classified into two types according to the degrees of morphological changes. Transgenic plants are shown in type I **(A, D, G, J)** and type II **(B, E, H, K)**, compared to wild-type **(C, F, I, L)** at stage of seedling ((A)–(C) bar = 2 cm) and 4 months old ((D)–(F) bar = 5 cm). The morphological changes are shown with stem ((G)–(I) bar = 2 cm) and leaves ((J)–(L) bar = 5 cm) of the 4-month-old plants. *PtrHB7* transcripts were measured in five independent lines transgenic and five wild-type plants **(M)**. Significance as determined by Student's *t*-test: ** $P < 0.001$. The values are means ± SE.

activity was also enhanced (Figure 7D). Here, the results indicated that *PtrHB7* plays a role in initiating cambium activity and in balancing the differentiation between xylem and phloem tissues, which is essential for normal secondary growth in trees. *PtrHB7* transcript abundance is correlated with the degree severity of the *PtrHB7* phenotype. Another morphological change in *PtrHB7mt*-overexpressed plants was that the stem was curved in a wave shape (Figure 6G–6I). Cross-sections within an internode (upper, middle, and lower parts) (Supplemental Figure 5) indicated that unbalanced xylem differentiation in the vascular cylinder of *PtrHB7mt*-overexpressed plants caused the wavy-stem phenotype. Abnormal xylem formation was observed opposite leaf traces, suggesting that they may interrupt the effect of *PtrHB7mt* overexpression.

Table 1. Phenotypes of *PtrHB7*-Overexpressed *Populus*.

Phenotypes	Type I	Type II	Wild-type
Stem diameter (mm)	3.25 ± 0.414	4.90 ± 0.764	6.40 ± 0.828
Internode length (mm)	8.6 ± 2.7	79.2 ± 18.9	101.9 ± 29.6
Mature leaf length (mm)	25.4 ± 3.6	47.7 ± 3.7	107.4 ± 10.8
Cell layers of cambial zone	2 ± 1	4 ± 1	5 ± 1
Number of xylem cell layers per files	28 ± 3	20 ± 2	16 ± 3

The phenotypes were recorded in the 4-month-old plants or within the stem internode 12. The statistic data from 15 independent transgenic plants are shown as means ± SE.

We employed a heterologous system to analyze gain-of-function mutants. *35S-PtrHB7mt* was transformed into *Arabidopsis*. About 40% of the transgenics showed apparent morphology changes including abnormal growth, reduced plant size, and smaller leaves (Supplemental Figure 6). Transgenic plants which showed severe changes in phenotype and delayed bolting were classified as type I (Supplemental Figure 6A, about 10% of transformants), and plants which showed relatively milder phenotype and normal bolting time were classified as type II (Supplemental Figure 6B, about 30% of transformants). Development of normal inflorescence stems were delayed in type I plants which had small leaves which strongly curled upward. Xylem cells in type I plants

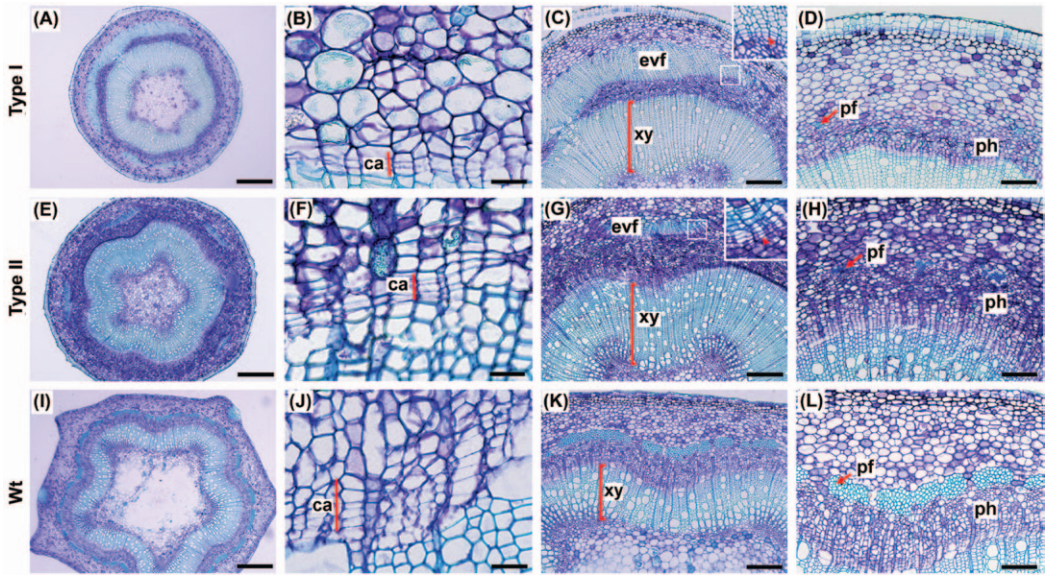

Figure 7. Enhancement of Xylem Differentiation by *PtrHB7* Overexpression.
Cross-section of the 12th internode shows enhancement of xylem differentiation and suppression of phloem differentiation in type I **(A–D)** and type II **(E–H)** transgenic plants compared to wild-type **(I–L)**. Enlarged view of the framed ectopic vascular tissues in (C, G) with ectopic cambial cells indicated by red head. The cambial zone at the 12th internode ((B), (F), and (J) bar = 20 μm, cambial cell layers indicated by red line). Xylem region ((C), (G), and (K) bar = 200 μm). Phloem region ((D), (H), and (L) bar = 100 μm). ca, cambial zone; xy, xylem; pf, phloem fiber; evf, ectopic vascular fiber.

were overwhelmingly developed and enclosed phloem cells (Supplemental Figure 6E and 6H). Type II plants displayed similar phenotypes to type I plants but with relatively milder severity. More xylem cells developed but the normal xylem–phloem patterning was not affected (Supplemental Figure 6F and 6I). We examined *PtrHB7* transcript abundance and found the expression level to be correlated with the severity of the phenotype (Supplemental Figure 6D). The higher the expression of *PtrHB7*, the more severe morphological changes it caused. These results provide additional evidence to suggest that *PtrHB7* regulates xylem differentiation in a dose-dependent manner.

Overexpression of *PtrHB7* Caused the Transcriptional Program to Favor Xylem Development

Overexpression of *PtrHB7* in *Arabidopsis* significantly enhanced xylem development. To investigate the transcriptional cascade initiated by the heterologous expression of *PtrHB7*, RNA was isolated from the homozygous lines of *PtrHB7*-transgenic plants (type II) and from wild-type *Arabidopsis* for RNA-seq analysis (Marioni et al., 2008; Wang et al., 2009) via the standard BGI experimental pipeline (http://en.genomics.cn/). A total of 5 206 821 and 5 967 738 cDNA reads were detected in the stems of *PtrHB7*-overexpressed and wild-type plants; 93.23% and 95.42% of them, respectively, were mapped to the annotated *Arabidopsis* genome. The mapping analysis resulted in the detection of 22 456 and 21 423 genes in the two samples, respectively. Analysis of transcript abundance showed that 3733 genes (about 14% of the total detected genes) were up-regulated and 1567 genes (about 7% of total detected genes) down-regulated in the *PtrHB7* transgenic plants (Supplemental Figure 7A and Supplemental Tables 2 and 3). Functionally, the regulated genes can be classified into six categories: signal transduction, cell wall biosynthesis, programmed cell death, metabolism, unknown function, and unclassified (Supplemental Figure 7B). A number of genes that are known to play significant roles during vascular development and cell wall formation are regulated as a result of *PtrHB7* overexpression (Supplemental Table 4). This was further confirmed by real-time RT–PCR analysis of the transcripts in the transgenic *Arabidopsis* (Supplemental Figure 8), suggesting that ectopic expression of *PtrHB7* in *Arabidopsis* caused a transcriptional cascade toward xylem formation. Particularly, a battery of transcription factor (TF) genes believed to participate in regulating xylem differentiation and secondary cell wall formation were up-regulated such as *SND1* (Zhong et al., 2006), *NST1*, *NST2* (Zhong et al., 2007b), *VND7* (Yamaguchi et al., 2008), and *MYB20*, *43*, *46*, *52*, *85*, and *103*, *KNAT7*, and *SND3* (Zhong et al., 2007a, 2008; Zhou et al., 2009). A number of other TFs that have roles in regulating cambial cell maintenance or phloem differentiation were down-regulated or unaffected, such as *BP*/*KNAT1* (Venglat et al., 2002), *KAN1* (Kerstetter et al., 2001; Emery et al., 2003; Ilegems et al., 2010), *CLE41* (Etchells and Turner, 2010), and *APL* (Bonke et al., 2003).

A number of genes responsible for cellulose, hemicellulose, and lignin biosynthesis were also up-regulated, such as *CesA4*, *7*, *8*, *10* (Taylor et al., 2003), *TBL3* (Bischoff et al.,

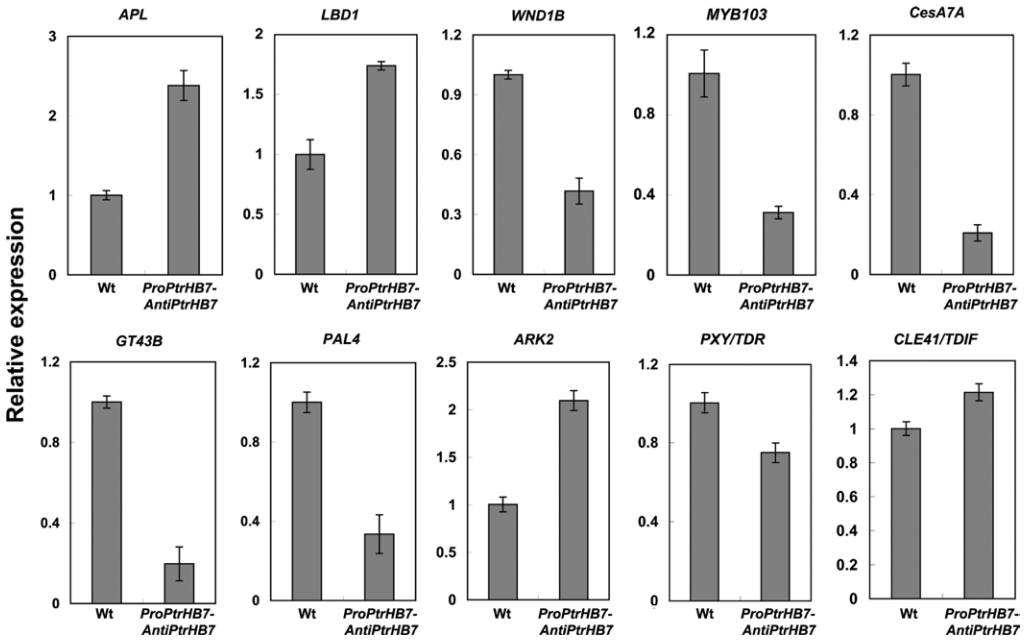

Figure 8. Expression of the Vascular Development-Related Genes in the PtrHB7-Suppressed Plants.
The expression was measured in the 12th stem internode by real-time RT–PCR. The expression level in each sample was normalized using actin2 as an internal control. The values are means ± SE, $n = 3$.

2010), and *COBL4* (Brown et al., 2005) for cellulose biosynthesis, *IRX8* (Persson et al., 2007), *IRX9* (Pena et al., 2007), *IRX10* (Brown et al., 2009), *IRX14* (Wu et al., 2010), *IRX15* (Jensen et al., 2011), and *PARVUS* (Lee et al., 2007) for hemicellulose biosynthesis, and *PAL1*, *4CL1*, *HCT*, and *AtCAD5* for lignin biosynthesis. Two xylem peptidase genes, *XCP2* and *XSP1* (Zhao et al., 2000), were also up-regulated.

Expression of the vascular development-associated genes was also investigated in transgenic *Populus*. Transcripts in the stem vascular tissues of *ProPtrHB7-AntiPtrHB7* plants were measured by real-time RT–PCR analysis (Figure 8). Genes responsible for phloem and xylem differentiation were differentially regulated in *PtrHB7*-suppressed plants. TFs for phloem differentiation that were up-regulated include *LBD1* (Yordanov et al., 2010) and *Populus* homolog *APL*. Genes responsible for xylem differentiation that were down-regulated include TF genes *WND1B* (Zhong et al., 2011) and the homolog of *Arabidopsis MYB103*. Genes responsible for cellulose, xylan, and lignin biosynthesis were also down-regulated, including *CesA7A*, *GT43B*, and *PAL4*. Meanwhile, the expression level of *Populus ARBORKNOX2* (*ARK2*) increased in *PtrHB7*-suppressed plants, which was reported to play a role in maintaining the cambial cell population (Du et al., 2009; Cano-Delgado et al., 2010). Furthermore, the *Populus* homolog of the *PXY/TDR* gene was down-regulated and the homolog of *CLE41/TDIF* was up-regulated in *PtrHB7* down-regulated plants. This observation is consistent with reports that CLE41 regulates xylem–phloem patterning by suppressing the differentiation of vascular stem cells into xylem cells through the receptor kinase, PXY (Fisher and Turner, 2007; Hirakawa et al., 2008). Transcriptional analysis of *PtrHB7* down-regulated *Populus* demonstrate *PtrHB7* differentially regulates the transcriptional programs that guide secondary xylem and phloem differentiation from vascular cambial cells.

Together, results from transcriptional analysis of both *PtrHB7* knockdown *Populus* and overexpressed *Arabidopsis* demonstrate that *PtrHB7* plays an important role in the formation of the vascular system through regulating the particular transcriptional programs that guide the balanced differentiation between xylem and phloem tissues.

DISCUSSION

PtrHB7 Plays a Positive Role in Promoting Vascular Cambium Differentiation during Secondary Growth in *Populus*

Secondary growth, derived from cambium activity, produces more xylem and less phloem during wood formation (Spicer and Groover, 2010). How the regulation of cambial cell differentiation is coordinated during secondary growth is still unclear. *HD-Zip III* genes are known to play critical roles in regulating primary xylem and phloem development (Zhong and Ye, 1999; Emery et al., 2003; Prigge et al., 2005). Among eight *Populus HD-Zip IIIs*, *popREV* is reported to play a role in cambium initiation and *PCN* plays a negative role in cambium differentiation during secondary growth (Du et al., 2011; Robischon et al., 2011). The involvement of other members of the *Populus HD-Zip III* family during secondary vascular development remains unknown.

In this study, *PtrHB7* was found to have overlapping and distinct functions from that of *popREV* during secondary growth. The expression of *PtrHB7* is different from that of *popREV*. *PtrHB7* is primarily and stably expressed in vascular cambium zone cells whereas *popREV* is irregularly expressed in vascular cambium and developing xylem (Robischon et al., 2011). The different expression patterns likely reflect the different roles they play during secondary growth. Overexpression of *PtrHB7* leads to ectopic cambium formation in the cortex which indicates that *PtrHB7* plays a role in initiating cambium activity. The same phenomenon is observed in *popREV*-overexpressed plants, indicating *PtrHB7* and *popREV* have overlapping functions during cambium initiation. However, the phenotype of *popREV*-overexpressed plants is more severe than that of plants overexpressing *PtrHB7*. Overexpression of *popREV* induced the proliferation of ectopic cambium that dramatically altered the patterning of secondary vascular tissue, whereas the secondary vascular patterning in *PtrHB7*-overexpressed plants remained unchanged. Furthermore, overexpression of *popREV* led to the growth of callus on the surface of the stem, but this phenotype was not observed in *PtrHB7*-overexpressed plants. The phenotype of *popREV*-overexpressed plants is unstable, which is consistent with its irregular expression in cambium and developing xylem. In contrast, the phenotype of *PtrHB7*-overexpressed plants is stable, consistently with its stable expression in cambium. The roles of *popREV* in cambium may be associated with the transition from primary growth to secondary growth. Expression of *popREV* is weak during primary growth, becomes strongest in the cambium during the primary–secondary growth transition, and decreases during secondary growth. The role of *popREV* during the primary–secondary growth transition is also supported by the phenotype of *popREV*-overexpressed plants. Subtle defects were observed in internodes containing primary vascular growth but very strong phenotypes observed in older internodes that have already transitioned to secondary growth (Robischon et al., 2011). The stable expression of *PtrHB7* and stable phenotypes of *PtrHB7*-mis-regulated plants indicates *PtrHB7* plays critical roles in regulating cambium cell activity during secondary growth.

Although studies of *popREV* point to its important role in cambium initiation, how *popREV* regulates cambium cell differentiation is not fully understood (Robischon et al., 2011). In this study, alterations of *PtrHB7* expression affected vascular cambial cell differentiation. More layers of cells were observed in the cambial zone in *PtrHB7*-suppressed plants and fewer layers in *PtrHB7*-overexpressed plants, suggesting *PtrHB7* may regulate the differentiation of cambium daughter cells. Suppression of *PtrHB7* significantly altered the vascular differentiation process, resulting in unbalanced phloem–xylem development. *PtrHB7* suppression inhibited xylem differentiation but enhanced phloem differentiation during stem secondary growth. Consistently with this, expression of the genes involved in xylem development were down-regulated and expression of the genes in phloem development and cambium cell maintenance was up-regulated in *PtrHB7*-suppressed plants. The phenotype observed as a result of *PtrHB7* overexpression stands in contrast to the effect of overexpressing *PCN*, which results in delayed xylem development (Du et al., 2011). Thus, *PtrHB7* may antagonize *PCN* during secondary growth. Together, these results demonstrate that *PtrHB7* positively regulates cambium cell proliferation and plays a role in guiding the balanced differentiation of vascular cambium cell into secondary xylem and phloem during wood formation.

The Function of *PtrHB7* Is Dependent on Its Transcript Dosage

PtrHB7 is a key component in the complex pathways regulating xylem differentiation. Our results provide evidence to support the observation that *PtrHB7* regulation is related to its transcript abundance. In transgenic plants, down-regulation of *PtrHB7* expression suppressed xylem differentiation and resulted in less xylem formation. Conversely, overexpression of *PtrHB7* at increasingly higher levels of *PtrHB7* transcripts caused correspondingly more severe phenotypic changes and excessive xylem differentiation. Expression of *PtrHB7* at appropriate levels is therefore important for the development of a normal vascular system. Heterologous expression of *PtrHB7* in *Arabidopsis* further supports the suggestion that *PtrHB7* regulates xylem differentiation in a dosage-dependent manner. *HD-Zip III* genes are involved in different processes during vascular development, which are finely tuned to coordinate the development of the plant vascular system (Prigge et al., 2005; Byrne, 2006; Floyd et al., 2006). The tuning of *HD-Zip III* regulated processes is likely realized through transcript dosage control mechanisms involving redundant *HD-Zip III* gene organization and/or microRNA regulation. In *Arabidopsis* root vascular development, *miR165/166* acts to degrade its target mRNA, which in turn controls the level of *HD-Zip III* transcript to determine xylem cell types (Carlsbecker et al., 2010). MicroRNAs are likely to play a critical role in modulating the transcriptional dosage of *PtrHB7* for normal vascular development.

Potential Link between Evolution of *HD-Zip III* Genes and the Development of the Vascular System in Land Plants

HD-Zip III genes are a class of homeodomain-leucine zipper TFs specific to plants. This gene family underwent diversification during seed plant evolution (Prigge and Clark, 2006). After vascular plants evolved from its bryophyte-like ancestors over 400 Myr ago (Friedman and Cook, 2000), a number of duplication events occurred in the *HD-Zip III* gene family which resulted in divergent clades during the evolution of vascular plants. In this study, analysis of *Arabidopsis*, rice, and *Populus* genomes indicates that Group I and II *HD-Zip III*s show similar duplication events across the three species. However,

the duplications of Group III *HD-Zip III*s (*AtHB8* lineage) are different and may have occurred in *Arabidopsis* and *Populus* only after the monocot–eudicot split about 140–150 Myr ago (Chaw et al., 2004). Furthermore, the Group III *HD-Zip III*s underwent another duplication in *Populus* approximately 8–13 Myr ago prior to the radiation of the genus *Populus* (Sterck et al., 2005; Tuskan et al., 2006).

Evidence indicates that the *HD-Zip III*s in vascular plants are divergent from those in non-vascular plants. For example, the *rev* mutant of *Arabidopsis* is unable to be complemented by its moss homolog *PpHB10* (Prigge and Clark, 2006). *HD-Zip III*s likely played a role in regulating vascular tissue differentiation, embryo patterning (including vascular initiation and pattering), and meristem size restriction in ancestral vascular plants. However, its roles in embryo patterning and meristem size restriction were subsequently lost in the *AtHB8* lineage (Prigge and Clark, 2006), which would be consistent with the lack of *PtrHB7* expression in the shoot apical meristem in *Populus*. *HD-Zip III*s in the *AtHB8* lineage appears to have undergone different duplication events in the three species which resulted in the evolution of three different types of vascular system. Knockout of *AtHB8* do not result in any vascular defects in *Arabidopsis*, which indicated *AtHB8* is not essential for vascular system formation, likely due to functional redundancy with other *HD-Zip III* genes (Baima et al., 2001). Here, knockdown of *PtrHB7* affected secondary vascular tissue differentiation, indicating *PtrHB7* has a more dedicated function in *Populus* compared to its counterpart in *Arabidopsis*. These results imply that *PtrHB7* function has evolved a more specific role in regulating vascular differentiation during secondary growth in woody plants.

METHODS

Plant Materials and Growth Conditions

Populus trichocarpa (the same clone used for *Populus* genome sequencing, genotype Nisqually-1) was grown in a phytotron and used for gene cloning and expression analysis in this study. *Populus* × *euramericana* cv. '*Nanlin895*' was used for genetic transformation analysis. After transgenic plants were generated, screened, and verified, they were grown in a phytotron for 4 months before detailed characterization. *Populus* and *Arabidopsis* were grown in a phytotron with a light and dark cycle of 16 h and 8 h at 22°C.

Cloning of the *Populus* class III *HD-Zip* cDNAs and Sequence Analysis

Based on information from the *Populus* genome (www.phytozome.net/poplar), the coding sequences of *PtrHB1* to *PtrHB8* cDNAs were PCR-amplified and cloned from a cDNA library constructed from the developing xylem tissue of *Populus trichocarpa*. The cloned cDNA sequences showed a few single nucleotide discrepancies but no changes in amino acid sequence compared to the genome information. Alignment of their deduced protein sequence was performed using the ClustalW method (www.ebi.ac.uk/Tools/msa/clustalw2/). The phylogenetic relationship of the *HD-Zip III*s from different species was analyzed by MEGA 3.1 using the neighbor-joining and UPGMA method (Lin and Nei, 1991). Bootstrap values were calculated from 1000 trials.

Gene Constructs and Genetic Transformation

Mutations at the miRNA165/166 target site were introduced by PCR amplification to construct the *PtrHB7* overexpression construct. Two overlapping primers, PtrHB7mt-S and PtrHB7mt-A (Supplemental Table 5), which mismatched the miRNA binding sites without changing the protein coding sequences, were used to generate mutations in the *PtrHB7* cDNA sequence. The mutated *PtrHB7* cDNA was confirmed by sequencing and used to construct the overexpression vector under a constitutive *35S* promoter. Meanwhile, a partial fragment of *PtrHB7* cDNA was constructed in the antisense direction to suppress its expression under a constitutive *35S* promoter and specific *PtrHB7* promoter, respectively. The constructs were mobilized into *Populus* × *euramericana* cv.'*Nanlin895*' by *Agrobacterium*-mediated transformation according to the protocol adopted in our lab (Li et al., 2003). *Arabidopsis* (Columbia) was transformed using flower-dip method (Chung et al., 2000). The homozygous lines of the *Arabidopsis* transformation were screened and selected for further analysis.

Collection of Specific Tissue Cells and Determination of *HD-Zip III* Expression

Cells of cambium, developing xylem, developing phloem, mature leaf, and cortex tissues were collected in the cross-sections of the 12th stem internode or 12th leaf from the top (fully expanded stage) by laser microdissection using a Veritas Microdissection System (Arcturus Bioscience, www.arctur.com) as previously described (Song et al., 2010). Approximately 500 cells were collected in each sample and the collection time was controlled to <30 min. After the cells were harvested, total RNA were isolated using a PicoPure RNA isolation kit (Arcturus Bio-science, www.arctur.com/). RNA quality was determined using an RNA 6000 Pico Assay kit (Agilent Technologies, www.chem.agilent.com/) coupled with an Agilent 2100 Bioanalyzer (Agilent Technologies, www.chem.agilent.com/) according to the manufacturer's instructions. The RNAs were amplified using a TargetAmp two-round aRNA amplification kit (Epicentre Biotechnologies, www.epibio.com/) to generate cDNA for subsequent analysis.

The cDNA was used to measure *PtrHB* gene expression using real-time RT–PCR analysis. Specific primers were designed to amplify a fragment of 100–300 bp in length from each *PtrHB* genes. The specificity and amplification efficiency of the primers for each of the eight *PtrHB* genes were examined and selected (Supplemental Table 5). Real-time RT–PCR was performed using SYBR Green® Real Time PCR Master Mix QPK212

(TOYOBO, www.bio-toyobo.cn/) and an iQ5™ Real-Time PCR Detection System (Bio-Rad, www.bio-rad.com/) according to the manufacturer's instructions. The expression level in each sample was normalized using *actin2* as an internal control.

For transgenic plants, the total RNA was isolated from the 12th internodes (*Populus*) using the modified CTAB method (Richards et al., 2001) or from stem (*Arabidopsis*) using Trizol (Invitrogen, http://zh.invitrogen.com/). After it was treated with DNase I, the total RNA was used for first-strand cDNA synthesis (TAKARA, www.takara.com.cn/) and then for real-time RT–PCR analysis of the transcript expression levels with *actin2* being used as an internal control. The transcript analysis data was taken as the averages of three biological replicates from independent plants.

GUS Staining and Analysis

Stem sections were hand-cut and then incubated in 90% acetone(v/v) for 10 min on ice, and incubated in GUS stain solution (100 mM sodium phosphate (pH 7.0), 10 mM EDTA, 0.5 mM ferricyanide, 0.5 mM ferrocyanide, 0.1% tritonX-100, 20% methanol, and 2 mM X-Gluc) at 37°C for 2 h. Following staining, sections were cleared by 95% ethanol and preserved in 75% ethanol and photographed.

Microscopic Analysis

Three-millimeter segments of *Populus* stem were taken at different internodes and 1 mm *Arabidopsis* (50-day-old) inflorescence stems were taken at a height of 2 cm from the bottom of the plants. The samples were fixed in formaldehyde-acetic acid solution (formaldehyde:glacial acetic acid:ethanol [1:1:18] for 24 h at 4°C, dehydrated in graded ethanol series, and embedded into paraplast; 10-μm sections were obtained using a Leica RM2235 rotary microtome (Leica, www.leica-microsystems.com/products). The sections were stained with toluidine blue and observed using an OLYMPUS BX51 light microscope (Olympus, www.olympus-global.com/).

RNA Sequencing Analysis

Total RNA was isolated from the inflorescence stems of the transgenic *Arabidopsis* (type II) and wild-type (40 d) using Trizol reagents (Invitrogen, http://zh.invitrogen.com/). After the total RNA quality was verified, the mRNA was purified by using the oligo-dT magnetic beads and interrupted into short fragments (about 200 bp) in the fragmentation buffer. Then first-strand cDNA was synthesized by random hexamer–primer using the mRNA fragments as templates. After the second-strand cDNA was synthesized, the double-strand cDNA was purified with a QiaQuick PCR extraction kit (QIAGEN, www.qiagen.com/Products/) and washed with elution buffer for end repair and single nucleotide 'A' (adenine) addition. After sequencing adaptors were ligated, the fragments were purified by agarose gel electrophoresis and sequenced via Illumina HiSeq™ 2000 with a read length of 50 bp (Illumina, www.illumina.com/systems/). The raw sequence data were analyzed using Illumina HiSeq™ 2000 software. The raw reads were filtered to generate clean reads which were then mapped to the *Arabidopsis* genome using SOAPalibner/soap2 (Li et al., 2009). Reads with no more than two bases of mismatch were used for alignment. Gene annotation was on the basis of the *Arabidopsis* genome database (ftp://ftp.*Arabidopsis*.org/home/tair/Genes/TAIR10_genome_release/). The transcript level was calculated using the RPKM (Reads Per kb per Million reads) method (Mortazavi et al., 2008). The expression difference between two samples was examined with the threshold *P*-value in multiple tests (Audic and Claverie, 1997; Benjamini et al., 2001). The differentially expressed genes were screened according to the following standard: 1≤log2 ratio (RPKM of PtrHB7/RPKM of Wt)≤–1 and FDR (False Discovery Rate) ≤0.001.

SUPPLEMENTARY DATA

Supplementary Data are available at *Molecular Plant Online.*

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SUMMARY

The molecular mechanisms of the plant secondary growth, which gives rise to girth expansion in trees, have been little understood. In this study, function analysis of *Populus* class III HD-Zip genes reveals that *PtrHB7* plays a crucial role in regulation of the secondary growth through balancing secondary xylem and phloem differentiation in a dosage-dependent manner.