

## Rapid report

# Developmental and seasonal expression of *PtaHB1*, a *Populus* gene encoding a class III HD-Zip protein, is closely associated with secondary growth and inversely correlated with the level of microRNA (*miR166*)

Author for correspondence:

Kyung-Hwan Han

Tel: +517 353 4751

Fax: +517 432 1143

Email: hanky@msu.edu

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Jae-Heung Ko<sup>1</sup>, Constantinos Prassinos<sup>1</sup> and Kyung-Hwan Han

Department of Forestry, Michigan State University, 126 Natural Resources, East Lansing, MI 48824-

1222, USA. <sup>1</sup>These authors contributed equally to the paper

**Key words:** homeodomain leucine-zipper (HD-ZIP) protein, microRNA, *miR166*, secondary growth, transcriptome, vascular cambium.

### Summary

- In contrast to our knowledge of the shoot apical meristem, our understanding of cambium meristem differentiation and maintenance is limited. Class III homeodomain leucine-zipper (HD-Zip) proteins have been shown to play a regulatory role in vascular differentiation.
- The hybrid aspen (*Populus tremula* × *Populus alba*) class III HD-Zip transcription factor (*PtaHB1*) and microRNA 166 (*Pta-miR166*) family were cloned from hybrid aspen using a combination of *in silico* and polymerase chain reaction methods. Expression analyses of *PtaHB1* and *Pta-miR166* were performed by Northern blot analysis.
- The expression of *PtaHB1* was closely associated with wood formation and regulated both developmentally and seasonally, with the highest expression during the active growing season. Also, its expression was inversely correlated with the level of *Pta-miR166*. *Pta-miR166*-directed cleavage of *PtaHB1* *in vivo* was confirmed using modified 5'-rapid amplification of cDNA ends (RACE).
- The expression of *Pta-miR166* was much higher in the winter than in the growing seasons, suggesting seasonal and developmental regulation of microRNA in this perennial plant species.

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## Introduction

In woody plant growth, the primary vascular tissues of shoots and roots are replaced by secondary vascular tissues, which are produced by a secondary meristem (Mauseth, 1998). This secondary growth (i.e. wood formation) is a highly ordered developmental process, which involves the patterned division of vascular cambium cells and a subsequent regulated differentiation of cambial derivatives into secondary xylem and phloem tissues. Secondary growth is an important biological process as its product provides necessary mechanical support and a conduit for the long-distance transport of water and nutrients, allowing trees to grow tall and eventually out-compete other herbaceous vegetation for light and nutrient uptake. Woody plant species comprise over 90% of the terrestrial biomass on Earth and play a critical role in the nutrient and carbon cycles of many ecosystems. Although many aspects of primary growth have been extensively elucidated using various model species, our current understanding of plant biology is incomplete due to the lack of knowledge of this fundamentally important aspect of plant development which also has significant practical implications.

Various molecular signals are differentially induced by cell-to-cell contacts and relative cell positions, and are turned on and off in response to environmental cues, nutritional status and/or other long-range stimuli. All of these regulatory steps work by changing the global pattern of gene expression in an individual cell. Thus, the control of cambial activity and derivative differentiation is accomplished by changing the activity of key genes involved in the developmental pathways, which determine the epigenetic state of the vascular cambium.

Homeodomain leucine-zipper (HD-Zip) proteins are unique to plants (Sessa *et al.*, 1993). These proteins have a characteristic leucine-zipper dimerization motif linked to their DNA-binding domain (Sessa *et al.*, 1997), a putative lipid- or steroid-binding StAR-related lipid-transfer (START) domain (Ponting & Aravind, 1999), and a conserved C-terminal domain of unknown function. HD-Zip proteins have been grouped into four classes (Sessa *et al.*, 1994). While class IV HD-Zip proteins have been suggested to be involved in epidermal cell fate (Rerie *et al.*, 1994; Lu *et al.*, 1996), several class III HD-Zip proteins have been suggested to have regulatory roles in vascular differentiation, including REVOLUTA/INTERFASCICULAR FIBERLESS1 (REV) and AtHB-8 (HB, homeobox) in Arabidopsis, and ZeHB-10, -11, -12 and -13 in *Zinnia elegans* (Talbert *et al.*, 1995; Baima *et al.*, 1995, 2001; Zhong & Ye, 1999; Ohashi-Ito *et al.*, 2002; Ohashi-Ito & Fukuda, 2003).

REV is one of five Arabidopsis class III HD-Zip proteins. Loss-of-function mutations induce defects in leaf development and stem cell specification as well as in vascular development and auxin transport (Zhong & Ye, 1999, 2001, 2004; Otsuga *et al.*, 2001). Previous studies on dominant gain-of-function mutations in three Arabidopsis class III HD-Zip genes (*PHABULOSA*, *PHAVOLUTA* and *REVOLUTA*) have

shown that these genes are necessary for meristem formation, adaxial–abaxial patterning and vascular differentiation, and that their expression is tightly controlled by microRNA (*miR165/166*) (McConnell & Barton, 1998; McConnell *et al.*, 2001; Emery *et al.*, 2003; Juarez *et al.*, 2004; Kidner & Martienssen, 2004; Mallory *et al.*, 2004; McHale & Koning, 2004; Kim *et al.*, 2005).

MicroRNAs (miRNAs) are a class of small (21-nucleotide) noncoding RNA molecules that can regulate gene expression in plants and animals by post-transcriptional repression of protein-coding genes (for a recent review, see Bartel, 2004). miRNAs represent one of the more abundant regulators of gene expression in multicellular organisms (Bartel, 2004). Since the first discovery in *Caenorhabditis elegans* (Lee *et al.*, 1993; Wightman *et al.*, 1993), more than 3322 miRNAs have been reported in various organisms (Griffiths-Jones, 2004). Although over 200 miRNAs have been found in plants (Reinhart *et al.*, 2002; Bonnet *et al.*, 2004; Jones-Rhoades & Bartel, 2004; Wang *et al.*, 2004; Lu *et al.*, 2005), their functions are largely unknown. The accumulation of many plant miRNAs varies at different developmental stages, suggesting that their expression may be spatially and temporally regulated (Llave *et al.*, 2002; Reinhart *et al.*, 2002). The fact that many of the miRNAs are well conserved among land plants indicates conserved functional roles for plant miRNAs (Reinhart *et al.*, 2002; Floyd & Bowman, 2004).

We describe a hybrid aspen (*Populus tremula* L. × *Populus alba* L.) class III HD-Zip gene *PtaHB1* which is homologous to Arabidopsis *REVOLUTA* and whose expression is closely associated with wood formation and regulated developmentally and seasonally. Its transcript level was inversely correlated with that of microRNA (*miR166*). This report provides experimental evidence for seasonal and developmental regulation of a microRNA in a perennial plant species.

## Materials and Methods

### Plant material

The plants used in this study were of the aspen *Populus tremula* L. × *Populus alba* L. INRA clone 717 hybrid. These plants were clonally propagated through tissue culture as described in Han *et al.* (2000). Plants were grown in pots in a growth chamber (16 h : 8 h light : dark photoperiod at 25°C) or in nursery beds on campus at Michigan State University. When the trees reached 1 month of age, the following samples were harvested from the stem: *c.* 1 cm of stem segment directly below the shoot apical meristem (primary growth, PG); a segment between leaf plastochron index (LPI; Larson *et al.*, 1971) 4 and 6 (transition to secondary growth, TS); and a segment between LPI 8 and 11 (secondary growth, SG). A 10-cm stem segment starting from ground level was used to separate bark from xylem by peeling. Leaves in the region of LPI 3–8 were used as the leaf sample after class I and II veins had been removed

with a scalpel. All samples were quickly frozen in liquid nitrogen. Five trees were used in this analysis. For the seasonal growth cycle study, stem samples (LPI 4–6, TS) of 2-year-old hybrid aspen grown in nursery beds were harvested in the middle of March, July, October, and December (three trees per season). For each season, three trees were used for sampling and the samples were pooled for RNA extraction and Northern blot analysis.

### Full-length cloning of *PtaHB1*

Total RNA was isolated from stem tissue of hybrid aspen using the RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA) following the manufacturer's instructions. For reverse transcriptase–polymerase chain reaction (RT-PCR) analysis, 1 µg of total RNA was reverse-transcribed using the Superscript II polymerase (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. A putative full-length *PtaHB1* homolog of *Populus trichocarpa* was computationally cloned by searching the Joint Genome Institute (JGI) *Populus trichocarpa* genome database (<http://genome.jgi-psf.org/Poptr1/Poptr1.home.html>) using the corresponding expressed sequence tag (EST) of *PtaHB1*. Introns and exons were estimated by sequence alignment with other class III HD-Zip genes identified from *Arabidopsis* and *Z. elegans*. PCR primers for the open reading frame (ORF) were designed from the resulting sequence information and used to clone *PtaHB1* from hybrid aspen. 5'- and 3'-rapid amplification of cDNA ends (RACE) (BD SMART RACE cDNA Amplification Kit; Clontech, Palo Alto, CA) was used to obtain the full-length clone and subsequent sequencing analysis was performed to obtain complete sequence information for *PtaHB1*.

### Northern blot analysis

Total RNA was extracted as described in the previous section. For each sample, 20 µg of total RNA was analyzed on a 1% agarose gel containing formaldehyde and then transferred to a nylon membrane (Hybond N+ Amersham, Piscataway, NJ, USA). The probe was purified using the Qiagen PCR purification kit. For labeling, 25 ng of probe was incorporated in the one-tube reaction RediPrime system (Amersham) with the addition of [ $\alpha$ - $^{32}$ P] dCTP (NEN). The hybridization reactions were performed at 42°C for 16–20 h and the membranes were then washed (according to the manufacturer's instructions) and exposed to an X-ray film (X-Omat AR; Kodak).

### 5' RNA ligase-mediated RACE of mRNA to identify the cleavage site

Total RNAs were isolated from stem tissues (LPI 4–6, TS) collected in four different seasons, and mixed in equal amounts. Poly(A)<sup>+</sup> RNA was prepared from mixed total RNAs by

two rounds of purification with the Oligotex mRNA Midi Kit (Qiagen). RNA ligase-mediated 5'-RACE was performed using the GeneRacer kit (Invitrogen Life Technologies, Carlsbad, CA, USA) as described by Kasschau *et al.* (2003). A GeneRacer RNA Oligo adapter was directly ligated to mRNA (150 ng) without further modification. A GeneRacer Oligo dT primer was used to prime cDNA synthesis with reverse transcriptase. Gene-specific 5'-RACE reactions were performed with a GeneRacer 5'-Nested Primer and a gene-specific primer of *PtaHB1* (801R 5'-CCAGAAATCCCGCGCAGGAGCTAGA-3'). The conditions used for this amplification step were the same as those for gene-specific RACE recommended by the manufacturer, with the exception that an extension time of 2.5 min was used. A 5'-RACE product of the expected size (~300 bp) was extracted from the gel, purified, cloned into a pGEM-T vector (Promega, Madison, WI, USA) and sequenced.

### Cloning of *microRNA166* precursors from hybrid aspen and expression analysis

*Populus trichocarpa miR165/166* precursor sequences were predicted by a low-stringency (*E*-value < 10.0) BlastN search against the JGI *Populus trichocarpa* genome database using the precursor sequence of *Arabidopsis miR165alb* as a query. All output sequences were clustered using stackPACK (version 2.2; Electric Genetics, Reston, VA, USA) and the resulting contigs were visually inspected for the presence of the mature *miR165/166* sequence. We named the contigs *Ptr-miR166a-k* (*Ptr* = *P. trichocarpa*) based on the order of clusters from the stackPACK analysis. Secondary structures were predicted using the RNAfold program (Vienna RNA secondary structure server; <http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi>).

Primers for cloning hybrid aspen *miR166* precursors were designed based on the mature *miR166* and its complementary sequence (Supplementary Table S1). Samples of 10 µg of total RNA from stems of hybrid aspen were reverse-transcribed using the 3' primers and a heat-stable reverse transcriptase Transcriptor (Roche, Indianapolis, IN, USA) at a polymerization temperature of 65°C to avoid secondary structure formation. The resulting product was subjected to PCR with a mixture of equal amounts of five 5' primers and two 3' primers [10 pmol (50 µl)<sup>-1</sup> per primer] (Supplementary Table S1). The PCR product was diluted 10 times and subjected to a second PCR with the combinations of all of the primers listed in Supplementary Table S1. The product was analyzed on a 2% agarose gel and the bands were extracted using QIAEXII (Qiagen). Pure bands were cloned in a pGEM-T Easy vector (Promega) and sequenced. We named them *Pta-miR166a-i* (*Pta* = *P. tremula* × *P. alba*) based on the similarity to *Ptr-miR166* members.

MicroRNA Northern blot analysis was carried out as described by Llave *et al.* (2000) with slight modifications. A sample of 10 µg of Trizol-extracted total RNA was separated by 17% polyacrylamide gel electrophoresis (PAGE) along with a

synthetic miR166 21-mer oligonucleotide as a size marker (Ambion, Austin, TX, USA). The RNAs were transferred to a Hybond N+ membrane using a Mini Trans-Blot Cell (Bio-Rad, Hercules, CA, USA) and hybridized at 37°C for 16 h in 7% sodium dodecyl sulfate (SDS), 0.5 M phosphate buffer, pH 7.2, and 10 mM ethylenediaminetetraacetic acid (EDTA) with the probe DNA that was the reverse complement of the mature *miR166* sequence and labeled with [ $\gamma$ -<sup>32</sup>P] ATP by end-labeling with polynucleotide kinase (NEB, Beverly, MA, USA). The membranes were washed twice with 2 × saline sodium citrate (SSC)/0.2% SDS and once with 1 × SSC/0.1% SDS and 0.5 × SSC/0.1% SDS for 20 min at 50°C, and then exposed to an X-ray film (X-Omat AR).

## Results and Discussion

To understand the molecular mechanism that regulates cambium differentiation, it is necessary to identify key transcriptional regulators directing intravascular pattern formation. The existing evidence indicates that class III HD-Zip proteins play regulatory roles in vascular differentiation and xylem formation (Baima *et al.*, 1995; Talbert *et al.*, 1995; Zhong & Ye, 1999; Ohashi-Ito *et al.*, 2002; Ohashi-Ito & Fukuda, 2003; Hawker & Bowman, 2004; Zhong & Ye, 2004). Here, we identified and characterized a *P. tremula* × *P. alba* gene encoding a putative class III HD-Zip transcription factor.

*PtaHB1*, a class III HD-Zip protein, is homologous to *Arabidopsis REVOLUTA*

A full-length cDNA of 3042 bp was cloned by a combination of bioinformatics and PCR amplification from hybrid aspen (*P. tremula* × *P. alba*). It contains an ORF encoding a protein of 843 amino acids (GenBank accession number AY497772). Subsequent sequence analysis revealed that the product of this gene (named *PtaHB1*) is homologous (77% identity of the amino acid sequence) to *Arabidopsis REVOLUTA*, a class III HD-Zip protein (Fig. 1a). The predicted structure of the *PtaHB1* protein contains a START domain (Ponting & Aravind, 1999) as well as a homeodomain and leucine-zipper domain (Fig. 1b). Fig. 1(c) shows the phylogenetic relationship of *PtaHB1* to the class III HD-Zip proteins of *Z. elegans* and *Arabidopsis*. This result suggests that *PtaHB1* may be an ortholog of *Arabidopsis REVOLUTA* and has a close relationship to *ZeHB-11* and *-12*, which were accumulated preferentially in xylem cells (Ohashi-Ito *et al.*, 2002).

To determine the copy number of the *PtaHB1* gene in the hybrid aspen genome, we performed genomic Southern blot analysis. With high-stringency conditions, we could detect a single band, but three or more bands were detected at lower stringency (Supplementary Fig. S1). This result indicates that *PtaHB1* may be a single-copy gene but have a couple of paralogs in the genome, as in *Arabidopsis* and *Z. elegans*. In fact, we have found a total of eight putative class III HD-zip

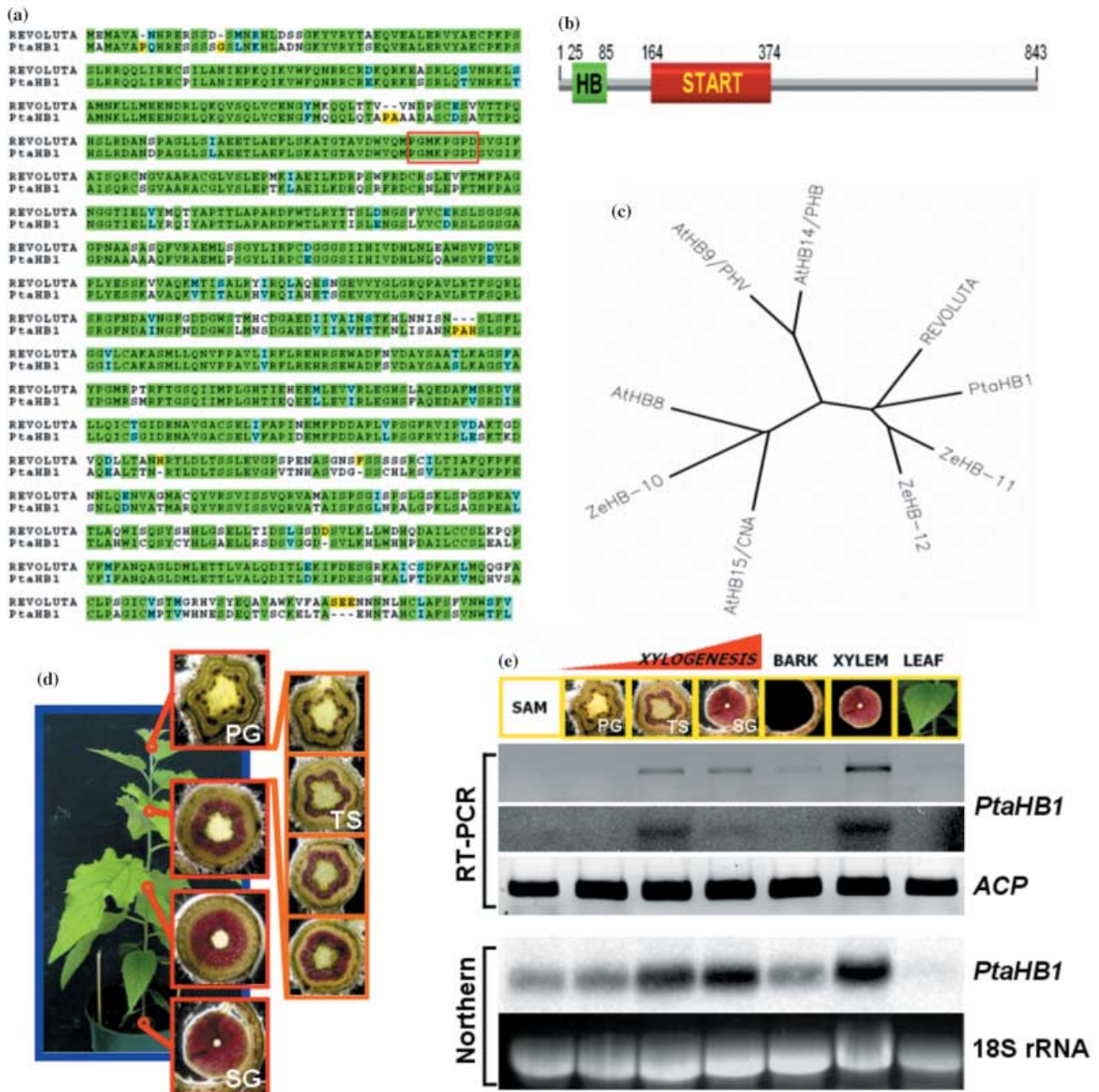
proteins in *P. trichocarpa* by searching the JGI *Populus* Genome database (<http://genome.jgi-psf.org/Poptr1/Poptr1.home.html>). We named these genes *PtrHB1* through *PtrHB8* (*P. trichocarpa* HB protein; accession numbers AY919616–AY919623) (see Supplementary Fig. S2). Phylogenetic analysis with known class III HD-Zip proteins showed that the eight *PtrHBs* were divided into roughly four groups. Interestingly, each group contained two homologous *PtrHBs*, implying a recent gene duplication event in this species.

Tissue-specific expression of the eight *PtrHBs* was investigated using the public Web-based EST database (POPULUSDB; <http://www.populus.db.umu.se/index.html>), which provides digital expression profiles for 18 different tissues that comprise the majority of differentiated organs (Sterky *et al.*, 2004). *PtrHB1* and *2* were expressed only in the cambium and wood cell death tissue. *PtrHB3* and *4* were only found in cambium tissue. *PtrHB5–8* were found in cambium and tension wood tissue. These expression patterns suggest that *PtrHBs* may function during cambium differentiation and wood formation.

Expression of *PtaHB1* is closely associated with secondary growth in the stem

We investigated the tissue- and organ-specific expression of the *PtaHB1* gene using vertical stem segments of hybrid aspen, which represent a gradient of developmental stages with regard to secondary growth (i.e. PG, TS and SG), and three different tissues (bark, xylem and leaf) (Prassinis *et al.*, 2005). The TS sample is from a stem region where vascular cambium has emerged and started producing secondary tissues, whereas the SG sample is from a region where secondary xylem and phloem fibers are heavily lignified, and secondary xylem increases rapidly, restraining the pith area (Fig. 1d). The shoot apical meristem (SAM), including parts of the leaf primordia, was also used in this analysis. Leaf tissues, which have no secondary growth, were used as a negative control for this experiment. It should be noted that leaf veins belonging to classes I and II were removed as sources of genes involved in vascular development (Fig. 1e). The transcript level of *PtaHB1* began to increase in the stem segment where primary-to-secondary growth transition occurs, and showed the greatest accumulation in the secondary xylem tissue (Fig. 3b). In a semiquantitative RT-PCR using gene-specific primers, *PtaHB1* transcript was not detected in the tissues that had no secondary growth (e.g. SAM, PG and leaf) (Fig. 3b). However, in a Northern blot analysis using the entire *PtaHB1* sequence as a probe, low signal levels were detected in SAM and PG. This result suggests that there may be paralogs expressed in those tissues.

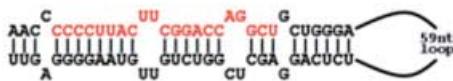
Together with *in situ* hybridization data, which showed preferential accumulation of *PtaHB1* transcripts in the xylem side of the vascular cambium (data not shown), these findings suggest that it is probable that *PtaHB1* may have a role in vascular cambium development of perennial woody plants



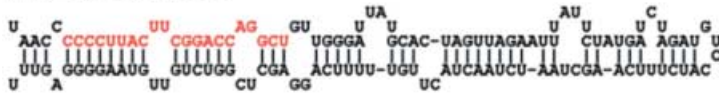
**Fig. 1** Molecular cloning and expression analysis of the *Populus tremula* × *Populus alba* (hybrid aspen) gene *PtaHB1*. (a) Alignment of amino acid sequences with Arabidopsis *REVOLUTA*. The red box indicates the target sites of the microRNA *miR165/166*. (b) Predicted protein structure. HB, homeobox; START, STAR-related lipid-transfer. (c) Phylogenetic analysis of *PtaHB1* with class III HD-Zip proteins identified from Arabidopsis and *Zinnia elegans*. GenBank accession numbers are *AthB14/PHABULOSA* (AAC16263), *AthB9/PHAVOLUTA* (NP\_174337), *AthB8* (NP\_195014), *AthB15/CNA* (AJ439449), *ZeHB-11* (AB084381), *ZeHB-12* (AB084382), *ZeHB-10* (AB084380), *REVOLUTA* (AAF42938), and *PtaHB1* (AY497772). (d) Sectioning of 1-month-old hybrid aspen trees for sample collection. PG, only primary growth; TS, transition to secondary growth; SG, secondary growth. (e) Semiquantitative reverse transcriptase–polymerase chain reaction (RT-PCR) and Northern blot analysis for expression of *PtaHB1* in secondary xylem formation and in different tissues. RT-PCR was performed using a gene-specific primer pair of *PtaHB1*. PCR amplifications were carried out using first-strand cDNA with 30 cycles (upper panel). The lower panel shows the Southern blot using *PtaHB1* as a probe after 10 cycles of RT-PCR. The acyl carrier protein (ACP; accession number CF216383) was used for RT-template/PCR control. This gene was found to be expressed similarly in all the tissues (Prassinis et al., 2005). Northern blot analysis was performed with 20 µg of total RNA extracted from the tissues indicated above (SAM, PG, TS, SG, bark, xylem, leaf) and hybridized with a <sup>32</sup>P-labeled *PtaHB1* cDNA probe. Ethidium bromide-stained ribosomal RNA served as a loading control.

(a)

## Ptr-miR166a



## Ptr-miR166b



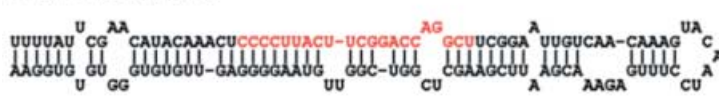
## Ptr-miR166d



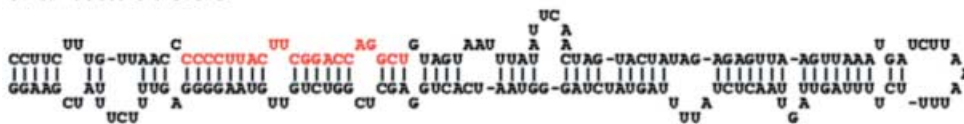
## Ptr-miR166e



## Ptr-miR166f

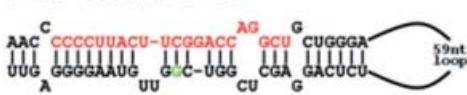


## Ptr-miR166c

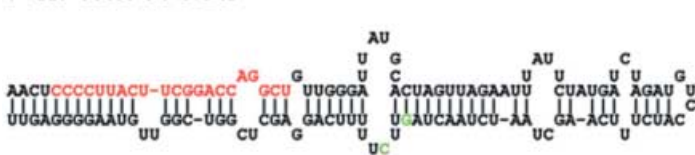


(b)

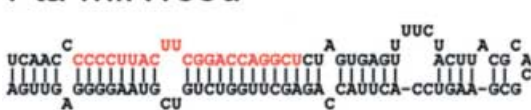
## Pta-miR166a



## Pta-miR166b



## Pta-miR166d



## Pta-miR166e



## Ptr-miR166g



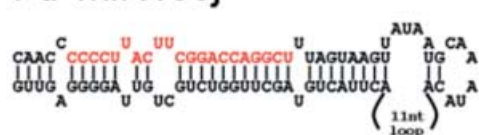
## Ptr-miR166h



## Ptr-miR166i



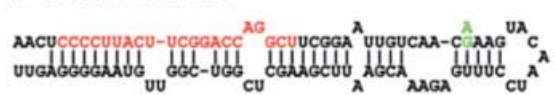
## Ptr-miR166j



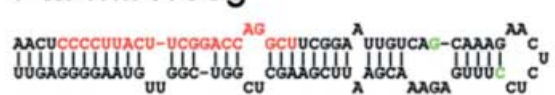
## Ptr-miR166k



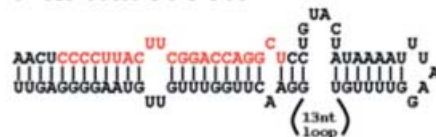
## Pta-miR166f



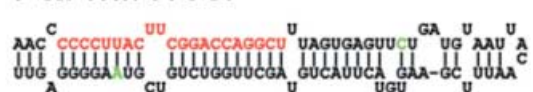
## Pta-miR166g



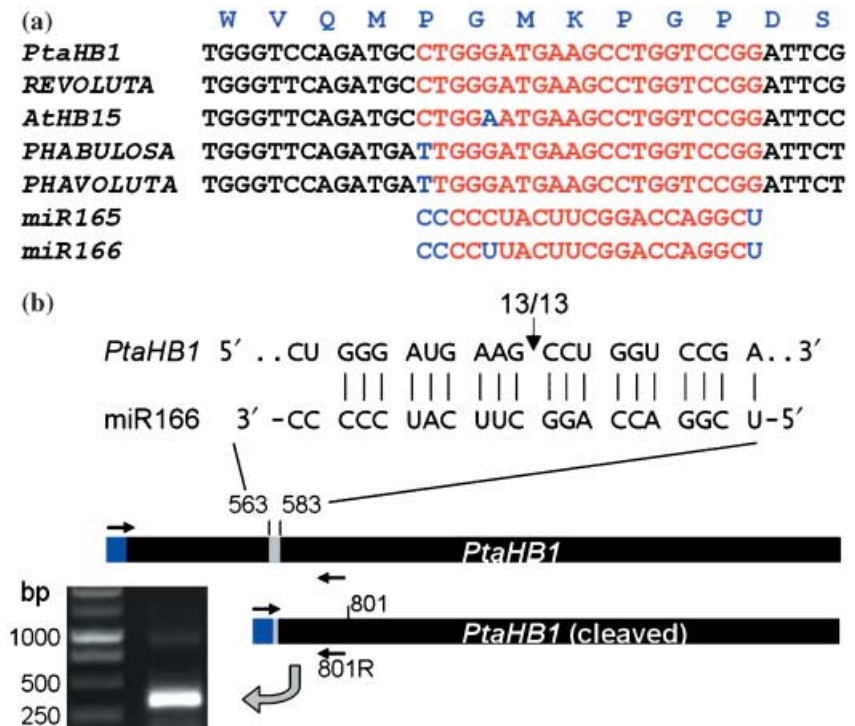
## Pta-miR166h



## Pta-miR166i



**Fig. 3** Conserved microRNA-binding site and experimental verification of the microRNA *miR166*-directed cleavage of the *Populus tremula* × *Populus alba* (hybrid aspen) *PtaHB1* (HB, homeobox). (a) Alignment of sequences with Arabidopsis class III homeodomain leucine-zipper (HD-Zip) genes. The region complementary to the Arabidopsis *miR165/166* microRNA is in red. Blue lettering indicates mismatched nucleotides. Amino acid sequences are indicated by single-letter codes (dark blue). (b) *miR166* cleavage site of *PtaHB1* determined by modified 5' RNA ligase-mediated rapid amplification of cDNA ends (RACE). Thick black lines represent the intact open reading frame (ORF) and the cleaved ORF of *PtaHB1*, respectively. The internal gray region shows the *miR166*-binding site with the nucleotide positions, and the terminal blue box indicates the GeneRacer RNA Oligo adapter (see Materials and Methods). Positions of the nested primers used for 5'-RACE are indicated by horizontal arrows. 801R is a gene-specific primer of *PtaHB1*. The top strand depicts a *miR166* complementary site and the bottom strand depicts *miR166*. The arrow indicates the 5'-termini of *miR166*-guided cleavage products as identified by cloned 5'-RACE products shown in agarose gels, with the frequency of clones shown.



through a function in establishing the polarity of the vasculature within the stem, as in the case of Arabidopsis REVO-LUTA (Emery *et al.*, 2003). Further genetic and biochemical studies using transgenic *Populus* with modified expression of *PtaHB1* will be necessary to determine the exact roles of *PtaHB1* in secondary growth.

### Cloning of hybrid aspen *miR165/166* family genes

Post-transcriptional gene silencing through the direct cleavage of class III HD-Zip genes by *miR165/166* has been well documented in plants (McConnell & Barton, 1998; McConnell *et al.*, 2001; Emery *et al.*, 2003; Juarez *et al.*, 2004; Kidner & Martienssen, 2004; Mallory *et al.*, 2004; McHale & Koning, 2004; Zhong & Ye, 2004; Kim *et al.*, 2005).

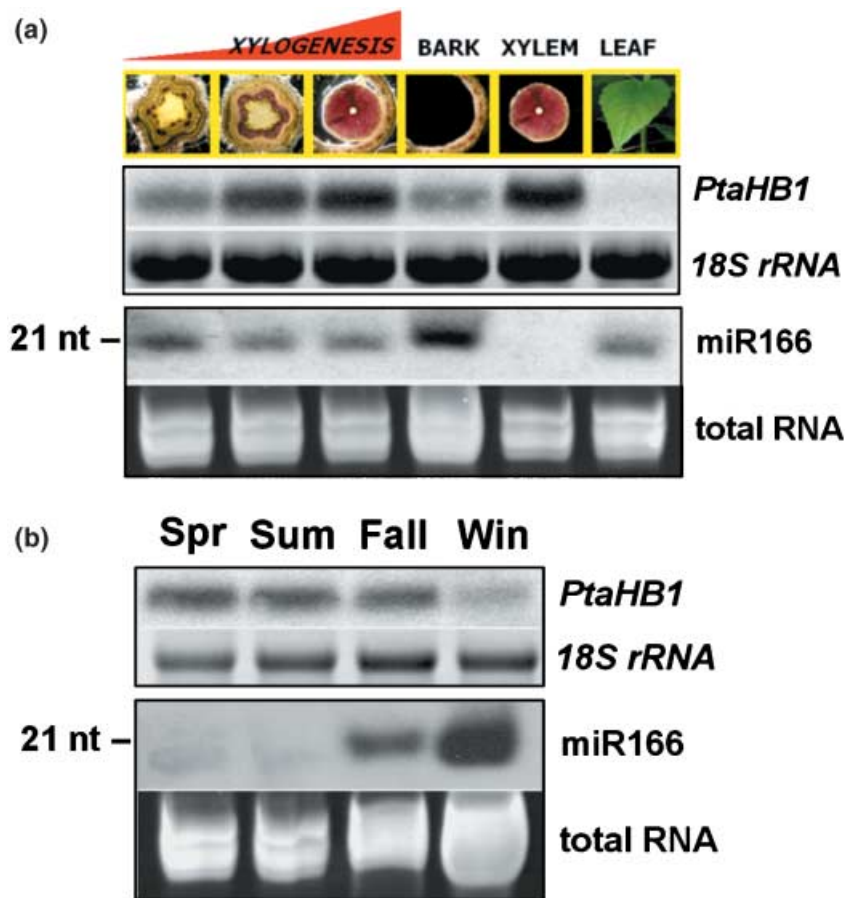
In order to clone the *miR165/166* family genes in hybrid aspen, we performed computational analysis to identify *P. trichocarpa* *miR166* precursor sequences (*Ptr-miR166*; see Materials and Methods). This analysis successfully identified 11 members of the *miR166* family (*Ptr-miR166a-k*; Fig. 2a). Using the sequence information for the *Ptr-miR166* genes, we cloned and sequenced the precursors of eight members of the *miR166* family from hybrid aspen (*Pta-miR166*; Fig. 2b). We

could not clone the homologs of *Ptr-miR166c*, *Ptr-miR166j* and *Ptr-miR166k*, possibly because of low expression in the tissues we used for total RNA extraction. The sequences of *miR166* family members were almost identical between the two species, with less than three nucleotide changes per gene (Fig. 2b). All of the genes had a mature *miR166* sequence identical to that of Arabidopsis, while the precursor sequence length and predicted stem-loop structure varied (Fig. 2). Interestingly, the *P. trichocarpa* genome, as with the rice genome, does not contain any *miR165* precursor sequence.

### *Pta-miR166*-directed cleavage of *PtaHB1* *in vivo*

The predominant mode of microRNA-mediated gene silencing in plants is thought to be the direct cleavage of target mRNAs by its binding to the coding region with near-perfect complementarity (Llave *et al.*, 2002; Reinhart *et al.*, 2002; Carrington & Ambros, 2003; Tang *et al.*, 2003). Recently, Floyd & Bowman (2004) suggested that the suppressional regulation of class III HD-Zip genes by *miR165/166* may be conserved among homologous sequences from all lineages of land plants, including bryophytes, lycopods, ferns and seed plants.

**Fig. 2** Identification of the *Populus tremula* × *Populus alba* (hybrid aspen) microRNA *miR166* family. (a) Predicted stem-loop structures of *Populus trichocarpa* *Ptr-miR166* precursors identified from *P. trichocarpa* by computational analysis. (b) Predicted stem-loop structures of *Pta-miR166* precursors cloned from hybrid aspen. Nucleotides in red indicate the sequence of mature *miR166*. Nucleotide changes in each member between the two species are shown in green. The Vienna RNA Secondary Structure Prediction (Vienna RNA secondary structure server; <http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi>) was used.



**Fig. 4** Expression of *Populus tremula* × *Populus alba* (hybrid aspen) *Pta-miR166* and *PtaHB1* (HB, homeobox). (a) Accumulation of *Pta-miR166* and *PtaHB1* mRNA in secondary xylem formation and in different tissues. Total RNA was isolated from the tissues indicated above (PG, TS, SG, bark, xylem, leaf). For Northern blot analysis, 20 µg of total RNA was used and probed by the *PtaHB1* gene. Expression of *Pta-miR166* was analyzed in a 17% polyacrylamide gel using small RNA enriched total RNA. (b) Seasonal expression of *Pta-miR166* and *PtaHB1*. Spr, spring; Sum, summer; Win, winter. Stem samples (transition to secondary growth; TS) were harvested seasonally indicated above (Spr, Sum, Fall, Win) and used for RNA gel blot analysis. Note the coexistence of *Pta-miR166* and *PtaHB1* transcripts in the TS and secondary growth (SG) samples, which is likely a result of the co-occurrence of bark and xylem in these samples.

The *PtaHB1* gene carries a conserved *miR165/166* target sequence found in Arabidopsis class III HD-Zip genes (Fig. 3a), suggesting that it may also be regulated by *miR165/166*. To verify whether *miR166* can direct cleavage of *PtaHB1*

*in vivo*, we isolated total RNA from hybrid aspen and performed the modified 5'-RACE (see Materials and Methods). The 5'-RACE of *PtaHB1* with a GeneRacer 5'-Nested Primer and a gene-specific primer of *PtaHB1* (801R) yielded a distinct band of the predicted size (~300 bp) on an agarose gel (Fig. 3b). Sequence analysis of this band revealed that the cleavage of the target sequence occurred at the 10th nucleotide of the *miR166* locus (Fig. 3b).

Transcript level of *Pta-miR166* is inversely correlated with that of *PtaHB1*

To predict the functional relevance of *Pta-miR166* and its target *PtaHB1*, we examined the transcript levels of *Pta-miR166* and *PtaHB1* by using vertical stem segments and various tissues as described in Materials and Methods. We detected miRNA signals (21-nucleotide) by Northern blot analysis using a probe complementary to the *miR166* sequence (Fig. 4a). It has been determined that the accumulation of plant microRNAs

is spatially and temporally regulated at different developmental stages (Llave *et al.*, 2002; Reinhart *et al.*, 2002). Consistent with the regulatory mechanism of *miR165/166*, the transcript level of *Pta-miR166* was inversely correlated with that of

*PtaHB1* (Fig. 4a). These data suggest that the differential expression of *PtaHB1* during secondary growth is most likely a result of the negative regulation by *Pta-miR166*. Considering its xylem tissue-preferential expression pattern, *PtaHB1* might control the polar differentiation of the cambium in favor of xylem differentiation. Expression of *Pta-miR166* in the bark, but not in the xylem, further supports the control of bark–xylem differentiation in a manner similar to the adaxial–abaxial control of leaf polarity described by Emery *et al.* (2003).

The activity of the cambial meristem is seasonally modulated (Savidge, 1988; Guglielmino *et al.*, 1997; Utsumi *et al.*, 2003; Mwangi *et al.*, 2005). The expression of *PtaHB1* was higher during the growing seasons (i.e. spring and summer) when compared with the winter season (Fig. 4b). Surprisingly, we found that the expression of *Pta-miR166* started to increase in the fall and was dramatically up-regulated in the winter season (Fig. 4b). These results were reproduced successfully by using samples from the following year (data not shown). To the best of our knowledge, this is the first evidence of seasonal

regulation of a microRNA during the annual growth cycle of perennial plants.

Because of their long life cycles, trees growing in temperate climates have an adaptive mechanism for winter survival, which involves seasonal alternation between active shoot growth and vegetative dormancy (Jordy, 2004). Seasonal regulation of cambial meristem activity is a critical component of the annual growth cycle. While it is of great scientific and economic significance, our understanding of the molecular biology of the annual growth cycle remains rudimentary at best. Here, we provide evidence that *Pta-miR166* is regulated not only developmentally but also seasonally in hybrid aspen, thus implicating *miR166* as one of the regulatory components in the molecular circuitry that controls the annual growth cycle.

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## Supplementary material

The following supplementary material is available for this article online.

**Fig. S1** Genomic Southern blot analysis for the determination of *Populus tremula* × *Populus alba* (hybrid aspen) *PtaHB1* gene copy number. (a) Moderate-stringency hybridization and washing conditions. At least four bands appeared in the *EcoRI* lane. (b) High-stringency hybridization and washing conditions. Note the single strong band in the *EcoRI* lane.

**Fig. S2** Putative class III homeodomain leucine-zipper (HD-Zip) proteins identified from *Populus trichocarpa*. (a) Amino acid sequence alignments of *P. trichocarpa* class III HD-Zip proteins (*PtrHB1*–*PtrHB8*). (b) Phylogenetic analysis of *PtrHBs* with class III HD-Zip proteins identified from *Arabidopsis* and *Zinnia elegans*. Genebank accession numbers are *AtHB14/PHABULOSA* (AAC16263), *AtHB9/PHAVOLUTA* (NP\_174337), *AtHB8* (NP\_195014), *AtHB15/CNA* (AJ439449), *ZeHB-11* (AB084381), *ZeHB-12* (AB084382), *ZeHB-10* (AB084380), *REVOLUTA* (AAF42938), *PtaHB1* (AY497772), *PtrHB1* (AY919616), *PtrHB2* (AY919617), *PtrHB3* (AY919618), *PtrHB4* (AY919619), *PtrHB5* (AY919620), *PtrHB6* (AY919621), *PtrHB7* (AY919622), and *PtrHB8* (AY919623).

**Table S1** Primers for cloning the microRNA *mir166* precursor genes

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