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Molecular cloning, expression analyses and primary evolution studies of *REV*- and *TB1*-like genes in bamboo

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Summary Most cultured bamboos are perennial woody evergreens that reproduce from rhizomes. It is unclear why some rhizome buds develop into aerial bamboo shoots instead of new rhizomes. REVOLUTA (REV)-like Class III homeodomain leucine-zipper (HD-Zip) proteins and TEOSINTE BRANCHED1 (TB1)-like transcription factors have been shown to play regulatory roles in meristem initiation and outgrowth. We cloned and analyzed the bamboo (*Phyllostachys praecox* C.D. Chu & C.S. Chao.) *REV*- (*PpHB1*) and *TB1*-like (*PpTB1*) gene. Gene expression was mainly detected by in situ hybridization. *PpHB1* expression was detected in the tips of lateral buds, on the adaxial portion of the leaf and within the developing procambium, indicating its close correlation to rhizome bud formation and procambial development. *PpTB1* expression was mainly detected on the top of buds at later developmental stages, suggesting it was more likely involved in bud outgrowth. Meristem genes might therefore serve as specific molecular markers of rhizome bud development and could be useful in studies designed to elucidate the mechanisms underlying bamboo shoot development. In addition, meristem genes such as *TB1*-like sequences may be useful in phylogenetic analyses of bamboo species.

Keywords: bamboo evolution, meristem development, Phyllostachys praecox.

Introduction

Bamboo (Bambusoideae) is the largest member of the grass family Poaceae, which includes more than 1000 species distributed throughout the world. Most cultured bamboos, which are vital to the economy of many countries in the tropics and subtropics, are perennial woody evergreens that reproduce from rhizomes. Compared with ordinary grasses, rhizome development in bamboo is complex. A rhizome bud can develop into either a new rhizome or an aerial bamboo shoot, which generally grows to its full height in a single season, making bamboo the fastest growing plant in the world. Depending on species, rhizomes in bamboos may develop monopodially or sympodially (Li et al. 2003).

Previous studies have revealed some of the physiological mechanisms underlying rhizome bud development. High concentrations of gibberellin, cytokinin and auxin are detected in rhizome buds that develop as aerial shoots in *Phyllostachys praecox* C.D. Chu & C.S. Chao (Hu et al. 1996). In *Phyllostachys pubescens* Mazel ex J. Houzeau, high concentrations of auxin and gibberellin are found in the apical tip of growing bamboo shoots (Ding 1997). Detection of hormones in *P. praecox* by enzyme-linked immunosorbent assay (ELISA) shows high auxin concentration is correlated to the outgrowth of the rhizome bud whether as a new rhizome or an aerial shoot, whereas a high cytokinin concentration is present only in rhizome buds that develop as aerial shoots (Huang et al. 2002). However, little is known about the function of phytohormones during rhizome bud development. It is also unclear why some rhizome buds develop into aerial shoots instead of new rhizomes. Molecular markers of meristem development would be of value in studies of the mechanisms underlying rhizome bud development.

Rhizome bud development in bamboo is mainly a process of shoot branching and is similar to the tillering in some species of gramineous plants, including the caespitose bamboos. Some shoot-branching-related genes have been described in model plants such as *Arabidopsis* and rice. These genes are divided into three classes based on whether they affect meristem initiation, meristem outgrowth or both (Ward and Leyser 2004). REVOLUTA/INTERFASCICULAR FIBERLESS1 (REV/IFL1) is one of five *Arabidopsis* HD-Zip III proteins that belong to a large family of transcription factors apparently unique to plants (Zhong and Ye 1999). *REV* homologous genes have been studied in *Zea mays* L. (maize; Juarez et al. 2004), *Zinnia elegans* L. (Ohashi-Ito et al. 2005) and *Populus tremula* L. × *P. alba* L. (Ko et al. 2005) and are involved in meristem initiation, auxin polar flow, adaxial–abaxial patterning and vascular development. TEOSINTE BRANCHED1 (TB1) is a member of the TCP family of DNA-binding

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transcriptional regulators (Cubas et al. 1999, Kosugi et al. 2002). Studies on the maize ZmTB1 and its ortholog rice OsTB1 show that TB1 homologs are involved in meristem outgrowth and contribute greatly to plant morphogenesis (Doebley et al. 1997, Takeda et al. 2003). Thus, studies on *REV*- and *TB1*-like genes of bamboo may help elucidate the molecular mechanisms underlying rhizome bud development.

Phylogenetic reconstructions of *REV*- and *TB1*-like genes may provide insight into bamboo taxonomy because these genes are important in plant morphogenesis and evolution (Lukens et al. 2001, Prigge et al. 2006). The taxonomy of bamboo species is difficult to study because of the absence of flowers, seeds and seedlings. Although about 34 of the world's 88 bamboo genera have been found and described in China, some genus delimitations are controversial (Li et al. 2003). Phylogenetic analysis through application of molecular evolution methods may, in some cases, resolve such taxonomic uncertainties. To date, only internal transcribed spacer (ITS) regions between 26S rDNA and 18S rDNA have been used to study genetic delimitations of woody bamboos (Guo et al. 2001). To overcome the limitations of some sequences, more homologous genes suitable for taxonomic studies of the genus must be identified, and meristem-related genes appear to be good candidates.

We consider that *P. praecox*, a typical scattered bamboo that produces edible shoots earlier in spring than any other bamboo species, is a good model in which to study the molecular mechanisms underlying rhizome bud development because numerous physiological studies have been carried out on this species in the past decade. In this paper, we describe the *P. praecox REV*- and *TB1*-like genes, *PpHB1* and *PpTB1*, and the relationships between their expression and rhizome bud formation, procambial development and rhizome bud outgrowth. Our results suggest that these meristem genes provide specific molecular markers of rhizome bud development and could be of value in elucidating bamboo phylogenetics and the physiological mechanisms underlying bamboo shoot development.

Materials and methods

Sampling

Between February and July, we collected shoot, leaf and young floret samples for gene cloning and expression analysis from plants growing in the bamboo botanical garden of Zhejiang Forestry Academy.

RNA isolation

Tissues were ground in liquid nitrogen and total RNA was extracted with TRIZOL Reagent (BBI, Hamilton, ON, Canada) according to the manufacturer's instructions. The RNA samples were treated with DNase I (Promega) at 37 °C for 30 minutes before the RT-PCR experiments. The quality and quantity of total RNA were measured by both electrophoresis and optical absorbency. Only RNA samples with an $A_{260}/A_{280} > 2.0$ were used for RT-PCR.

Gene cloning

PpHB1 was cloned from rhizome buds with the gene-specific primers hbf1: 5′-CAGAGTTCCTC-TCCAAGGCTACAGG-3′ and hbr1: 5′-CAAGTGGCATAATGATCTGGCTCCC-3′ and a BD SMART RACE cDNA amplification kit (Clontech) according to the manufacturer's instructions. *PpTB1* was cloned from bamboo shoots with 3′ RACE and the gene-specific primer tb1f1: 5′-GGAGTCCCATCAGTAAAGC-3′. Cloning of *TB1*-like genes from bamboo genomic DNA was carried out by PCR with primers tb1f1 and tb1r1: 5′-CGCATCCGGTTC-TTCTCCTTGGT-3′. Amplification was performed for 4 min at 94 °C followed by 30 cycles of 30 s at 94 °C, 60 s at 58 °C and 60 s at 72 °C, ending with 5 min at 72 °C. The amplified fragments were ligated into pBluescript, transformed into *E. coli* JM109 and sequenced with an ABI 3730. To ensure the correctness of the sequences, two independent PCRs were carried out and at least two clones were sequenced.

Sequencing and phylogenetic analysis

Sequence alignments were conducted with AlignX of Vector NTI Suite 9.0 with the multiple alignment parameters gap opening penalty 4, gap extension penalty 0.2 and PAM protein mass matrix. Phylogenetic trees of homologous genes were constructed by the neighbor-joining method of MEGA3.1 (Kumar et al. 2004). MBE Toolbox 2.0 based on Matlab 6.5 performed the evolutionary analysis (Cai et al. 2005, Cai et al. 2006).

Southern blot

Southern hybridizations were performed with DIG high-prime DNA labeling and detection starter kit II (Roche) according to the manufacturer's instructions. Genomic DNA was extracted by the CTAB method and digested with *Kpn* I. The partial genomic sequence amplified with hbf2: 5′-ATGGTGGCGG-CGGTGGCGATGC-3′ and hbr2: 5′-GTTCTAGATTCACC-AAATCACAGGCACG-3′ was chosen as the probe sequence of *PpHB1* and the codon DNA sequence (CDS) was chosen as the probe sequence of *PpTB1*. Membranes were washed according to the aforementioned kit.

Semi-quantitative RT-PCR analysis

First, the β-actin homologous gene in bamboo, *PpACT1*, was cloned from *P. praecox* according to the gene from rice. Then a two-step semi-quantitative RT-PCR method was performed to measure gene expression of samples of bamboo shoot, leaf and young floret. Oligo-$(dT)_{15}$ was the primer in the first step of cDNA synthesis and BD PowerScript Reverse Transcriptase (Clontech) was applied according to the manufacturer's instruction. The yield of cDNA was measured based on the PCR signal generated from the internal standard house-keeping gene *PpACT1* amplified from 18 to 25 cycles starting with 0.1 μl of the cDNA solution on a Biometra PCR machine (MJ Research Inc, MA). The volume of each cDNA pool was adjusted to give the same exponential phase PCR signal strength for *PpACT1* after 22 cycles. Gene-specific primers located on either side of the stop codon were designed to detect the expression of each transcript. For *PpHB1*, hb1-f1 (5′- GCAATA-

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CGTCCGTAGCGTTG-3′) and hb1-r1(5′-ACATGGATTGC-G -TCGGATGG-3′) were the primers, whereas pt1f726 (5′-G-ACGAGGGAGAAGAACCGGATG-3′), pt1r1107 (5′- GTG-CGGGAGTAGTTCTAATACCGT-3′) and pt1sr1230 (5′- CA-TGCGATGACCAAACCaa-3′) were the primers for two transcripts of *PpTB1*. *PpACT1* was amplified with primers act-f1: 5′-TTCATTGGTATGGAAGCTGCTG-3′ and act-r1: 5′-GT-AGCTTACATGGCAAGGACTTG-3′. Control PCRs were performed on non-reverse-transcribed RNA templates to ensure no DNA contamination influenced the results. For the different primer combinations, amplifications were performed at annealing temperatures recommended by OMIGA 2.0. To ensure the correctness of sequences, amplified fragments were sequenced with an ABI 3730.

Histological analysis and in situ hybridization

Samples were fixed in 4% paraformaldehyde in phosphate buffer, pH 7.0, overnight at 4 °C. Fixed tissues were dehydrated in a graded ethanol series, replaced with xylene, embedded in paraffin and sectioned at 10 µm on a rotary microtome (Leica RM2135). After checking with the aid of a microscope, selected slides were treated as described by Braissant and Wahli (1998). Two templates for riboprobe syntheses were constructed by cloning the CDS of *PpTB1-1* and *PpHB1* into pBluescript (Invitrogen). The antisense and sense RNA probes were separately generated by T3 and T7 RNA polymerase after linearization of the plasmid. Some sections were stained with Ehrlich's hematoxylin for histological analysis.

Results

PpHB1 is a new REVOLUTA-like HD-Zip III homeobox gene

In previous studies, we used a rice gene chip to detect gene expression in rhizome buds before shoot formation in *Phyllostachys praecox*, and identified a series of genes expressed in rhizome buds, six of which were isolated by the RACE method with gene-specific primers of rice. BLAST searches showed that one of the six genes was a new *REVOLUTA*-like (*REV*-like) homeobox gene named *PpHB1*. Subsequent sequence analysis indicated *PpHB1* encoded 838 amino acids and, like other *REV* homologous genes, had a microRNA-165/166 target site. The alignment showed the predicted structure of PpHB1 contained the main characteristics of class III homeodomain leucine-zipper (HD-Zip) proteins (Figure 1). PpHB1 shared the highest identity (93.1%) with OsREV among the known or putative REV homologs and shared 70.4% identity with REV. Although REV homologs have been shown to be associated with the lignification of plants, no obvious differences were observed in the putative amino acid sequences between the herbaceous (*Arabidopsis* and rice) and woody (*Populus* and bamboo) members. Comparative differences between monocot (rice and bamboo) and eudicot (*Arabidopsis* and *Populus*) groups were clear.

PpTB1 is a new TB1-like TCP gene

To isolate the *TB1*-like sequence in *Phyllostachys praecox*, 3′-RACE was performed with the upstream primer targeting the 5′ untranslated region (5′-UTR) of rice. Two cDNAs, 1296 and 1185 bp, were cloned from the bamboo shoot. BLAST searches indicated both cDNAs were *TB1*-like sequences and subsequent sequence analysis showed they contained the same open reading frame (ORF). Thus, they were different transcripts of the same gene (*PpTB1*), and were subsequently named *PpTB1-1* (1296 bp) and *PpTB1-2* (1185 bp). *PpTB1* was a new member of the TCP gene family encoding 349 amino acids with SP, TCP and R domains (Figure 2). Sequence alignments among the known TB1 homologs showed that PpTB1 shared the highest identity (71.7%) with *Danthoniopsis dinteri* DdTB1 at the amino acid level, and 64.7% and 62.6% identity with maize ZmTB1 and rice OsTB1, respectively. The sequence of *PpTB1* was also cloned from genomic DNA with gene-specific primers, indicating that it, like other known *TB1* homologous genes, contained no introns.

Molecular phylogenetic analyses indicate PpHB1 and PpTB1 are similar to their rice homologs

To investigate the relationship of PpHB1 and PpTB1 to known similar sequences, molecular phylogenies were reconstructed among these putative homologs. Phylogenetic analysis of the REV homologs indicated that PpHB1 and putative OsREV were similar and diverged later than other known REV orthologs (Figure 3A). According to the phylogenetic analysis, the REV homologs were clearly separated into monocot and eudicot groups. For the TB1 phylogeny, PpTB1 was similarly grouped with OsTB1, although PpTB1 did not share the highest identity with its rice counterpart among those homologs (Figure 3B). The disagreement between sequence comparison and phylogeny was caused by the complete deletion of gaps and missing data in the phylogenetic reconstruction, indicating that PpTB1 was more similar to OsTB1 in the conserved region.

PpHB1 and PpTB1 may be single-copy genes in the bamboo genome

To test whether *PpHB1* and *PpTB1* exist as a multigene family or as a single copy gene in *Phyllostachys praecox*, Southern blots were performed on the genomic DNA using the PpHB1 and PpTB1 probes, respectively (Figure 4A). One strong band and one comparatively weak band were detected for the *PpHB1* hybridization, indicating it may be a single-copy gene but with several paralogs in the genome. For the *PpTB1* hybridization, only one strongly hybridizing fragment was detected. The *PpTB1* sequence could be acquired by PCR amplification of the genome and direct sequencing of the PCR product, further suggesting it is unlikely to have paralogous sequences in *P. praecox*.

PpHB1 and PpTB1 have different expression patterns during bamboo development

To differentiate the expression characteristics of *PpHB1* and *PpTB1*, RT-PCR and in situ hybridization were performed. Semi-quantitative RT-PCR indicated that the expression patterns of *PpHB1* and *PpTB1* differed (Figure 4B). *PpHB1* was

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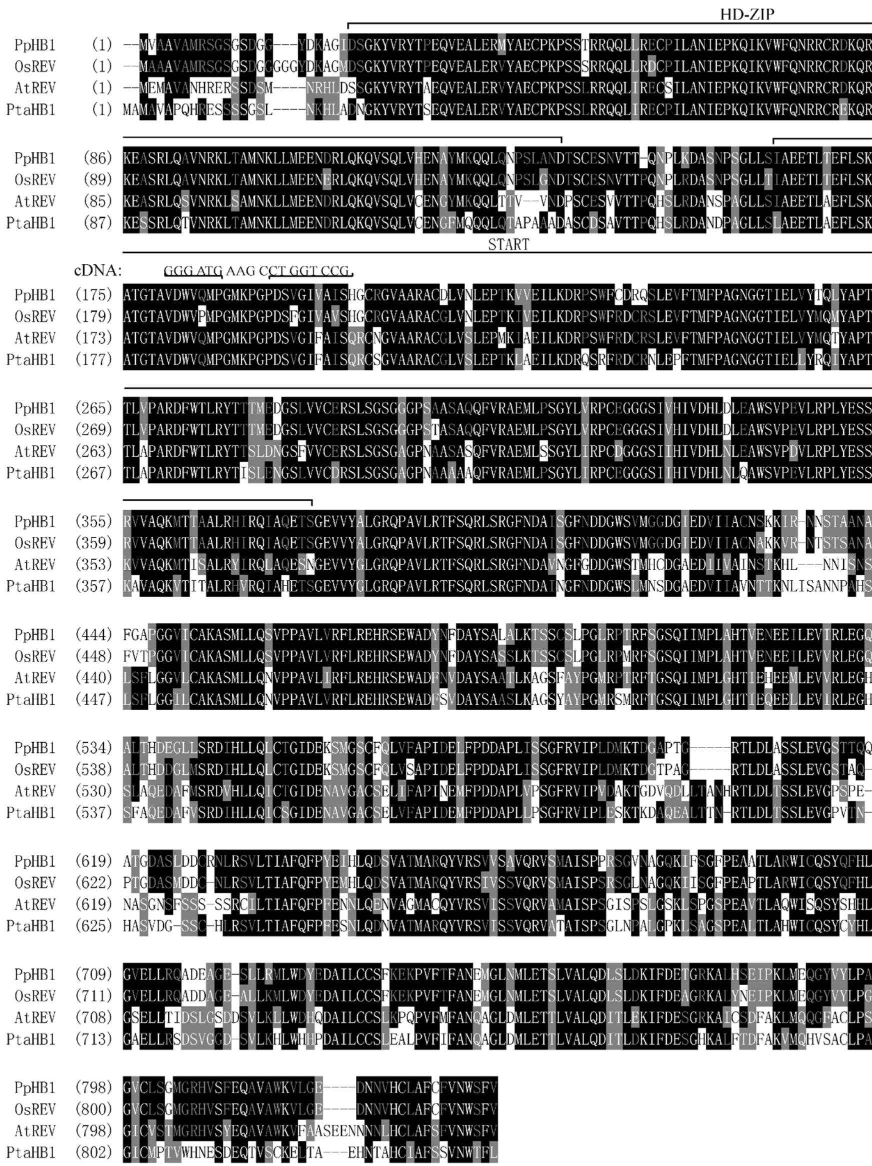

Figure 1. Alignment of four REV (AtREV) homologs from two herbaceous (*Arabidopsis* and rice) and two woody (*Phyllostachys praecox* and hybrid aspen) plants. Sequence alignment of the deduced amino acids of *PpHB1* (DQ842222), *OsREV* (NM_197030), *AtREV* (*REVOLUTA*, AF188994) and *PtaHB1* (AY497772) was conducted with AlignX. Regions of identity (white letter with black background), conservation (gray letter with black background) and similarity (white letter with gray background) are indicated. The HD-ZIP and START domains were predicted according to REV. The microRNA165/166 target sequence is indicated as a cDNA sequence above the corresponding amino acid sequence.

highly expressed in both bamboo shoots and young florets, but was not expressed in leaves. *PpTB1s* including *PpTB1-1* and *PpTB1-2* transcripts were expressed not only in bamboo shoots and young florets, but also in leaves. Gene expression detected by different combinations of primers indicated that *PpTB1-1* was expressed less in leaf tissue than in the other tissues, whereas *PpTB1s* were expressed more in leaf tissue than in the other tissues. The expression patterns of *PpHB1* and *PpTB1* during the process of rhizome bud development were detected by in situ hybridization (Figure 5). *PpHB1* was highly expressed in the tips of lateral buds at different developmental stages. Its expression was also detected in the adaxial portion of the leaf and within the developing procambium, indicating that it likely preceded the establishment of meristems in the leaf axils. *PpTB1* expression was detected only in the uppermost portion of buds in the later developmental stages, suggesting it may be involved in bud outgrowth.

TB1-like genes are potentially useful in the phylogenetic analysis of bamboo species

The presence of polymorphism is critical for the successful application of homologous genes in the study of bamboo phylogeny and taxonomy. The high conservation between PpHB1 and OsREV suggests that REV homologs are unsuitable for phylogenetic analysis of bamboo species. As a first step to assessing the usefulness of *TB1*-like genes in bamboo phylogeny, an additional four *TB1*-like sequences, *DeTB1* (Accession No. DQ842224), *PfTB1* (DQ842225), *YnTB1* (DQ910763) and *PaTB1* (DQ910764) were cloned from the genomes of two pluricaespitose (*Pleioblastus amarus* P.C. Keng and *Pleioblastus fortunei* Nakai) and two caespitose (*Yushania niitakayamensis* P.C. Keng and *Dendrocalamopsis edulis* P.C. Keng) bamboos. Differences in these homologous genes were then analyzed to test whether there had been some positive selection in the sequences during evolution. Based on the

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Figure 2. Alignment of some known TB1(ZmTB1) homologs. Sequence alignment of the deduced amino acids of *PpTB1* (DQ842222), *PgTB1* (AY631857), *OsTB1* (AY286002) and *ZmTB1*(U94494) was conducted with AlignX. Regions of identity, conservation and similarity are indicated as described in Figure 1. The SP, TCP and R domains were predicted according to the ZmTB1.

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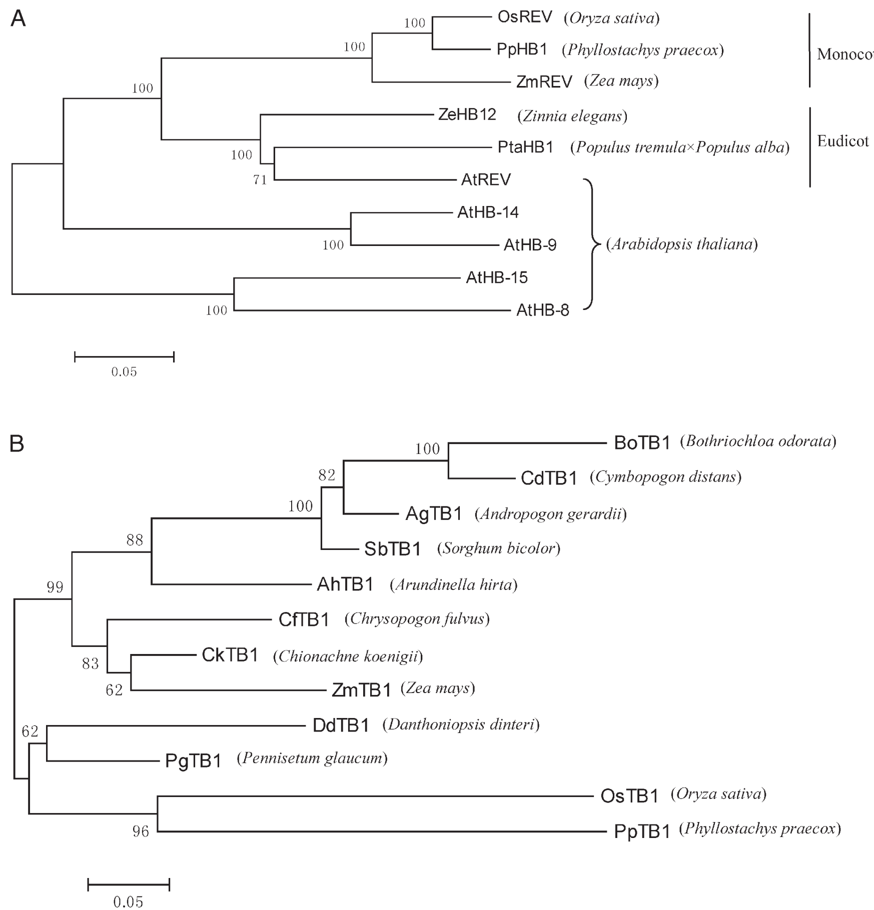

Figure 3. Phylogeny reconstructions of some REV and TB1-like sequences. A. Phylogeny reconstruction was based on the full deduced amino acids of *PpHB1*, *OsREV*, *AtREV*, *PtaHB1*, *ZmREV* (*RLD1*, AY501430), *ZeHB12* (AB084382), *AtHB-8* (NM_119441), *AtHB-9* (*PHAVOLUTA/PHV*, Y10922), *AtHB-14* (*PHABULOSA/PHB*, Y11122) and *AtHB-15* (NM_179464). B. Phylogeny reconstruction was based on the SP, TCP and R domains of deduced amino acids of *PpTB1*, *OsTB1*, *ZmTB1*, *PgTB1, DdTB1* (AF322134), *CkTB1* (AF322142), *CfTB1* (AF322120), *AhTB1* (AF322131), *SbTB1* (AF322132), *AgTB1* (AF322119), *CdTB1* (AF322129) and *BoTB1* (AF322137).

types of rhizome branching and vascular structure, *D. edulis* is a model species of the most primitive genus in Bambusoideae, *Phyllostachys praecox* belongs to the most advanced bamboo genus and *P. amarus* exhibits typical intergradation (Li et al. 2003). The sequence comparisons of *TB1* homologous genes among these species are shown in Figure 6. A d_N/d_S (nonsynonymous substitution rate/synonymous substitutions rate) value that is significantly greater than 1.0 is considered strong evidence for positive selection. Sliding-window analysis showed that nonsynonymous substitution occurred less fre-

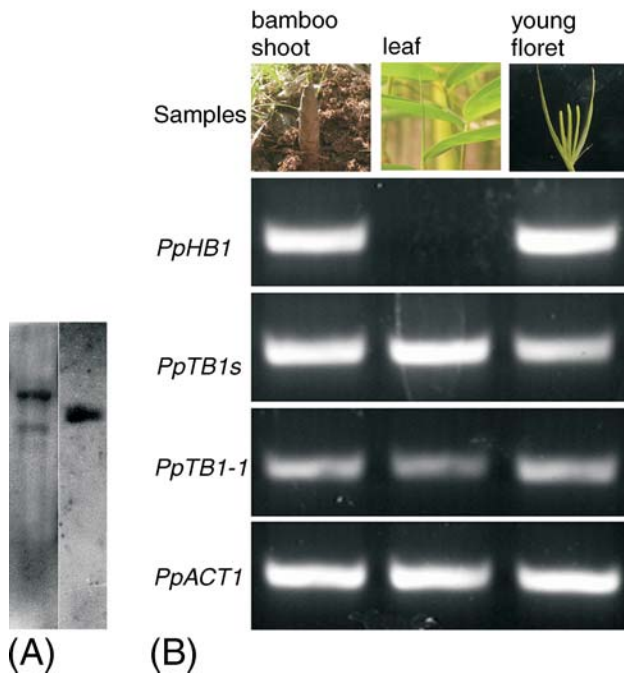

Figure 4. (A) Southern blot of genomic DNA from *Phyllostachys praecox* restriction digested with *Kpn* I and hybridized with Dig-labeled probes of *PpHB1* (left) and *PpTB1* (right) genes. (B) Semi-quantitative RT-PCR analysis of *PpHB1*, *PpTB1s* and *PpTB1-1* in samples of bamboo shoot, leaf and young floret. *PpHB1* was amplified for 30 cycles. *PpTB1s* was amplified with pt1f726 and pt1r1107 for 32 cycles , and *PpTB1-1* was amplified with pt1f726 and pt1sr-1230 for 32 cycles. The control actin was amplified for 26 cycles.

quently (per site) than synonymous substitution through the entire sequence, indicating less probability of positive selection in the *TB1* homologous genes of these species. A similar result was achieved when *PaTB1* and *DeTB1* were replaced with *PfTB1* and *YnTB*, respectively. Synonymous substitution proves to be more suitable for the phylogenetic analysis of close species than nonsynonymous substitutions because it is less likely to be affected by natural selection (Nei and Kumar 2000). Although few sequences have been cloned and analyzed, bamboo *TB1*-like genes showed considerable synonymous substitution, indicating the potential for using *TB1*-like sequences in the phylogenetic reconstruction of bamboo species.

Discussion

Different roles of PpHB1 and PpTB1 in bamboo meristem development

The *REV* gene in *Arabidopsis*, the first and most characterized among the *REV* homologous genes, regulates the relative growth of apical and non-apical meristems, and is necessary for apical meristem initiation (Talbert et al. 1995). *REV* is expressed at the earliest stages of lateral meristems formation and acts at lateral positions to activate the expressions of known meristem regulators (Otsuga et al. 2001). In maize, *ZmREV* (*RLD1*) is expressed at the tip of the shoot apical meristem (SAM) and in a band from the center of the SAM to the site of leaf initiation, which persists during primordial development in the vasculature and on the adaxial side near the margins (Juarez et al. 2004). In bamboo, microscopic analysis showed that the inner structure of the rhizome bud (Figure 5B) was quite different from that of the bamboo shoot (Figure 5C). A rhizome bud comprises a single meristem, whereas a bud giving rise to an aerial shoot consists of an embryonic shoot with many lateral buds, indicating that formation of an aerial shoot from a rhizome bud involves the initiation of many new meristems. The *PpHB1* expression pattern resembles those of *REV* and *RLD1*, suggesting that the *REV* homologous gene in bamboo is probably important in meristem initiation and adaxial–abaxial patterning. Thus, the *REV* homologous gene in bamboo may be chosen as a molecular marker for the detection of bamboo shoot formation.

Although the earliest mutation in the *REV* gene was found to affect meristem initiation, the *REV* gene in *Arabidopsis* was first cloned as an *IFL1* gene whose function is necessary for fiber formation (Zhong and Ye 1999). The *ifl1* mutations cause a reduction in auxin polar transport in the interfascicular and vascular regions, leading to alterations in the differentiation of interfascicular fibers and secondary xylem (Zhong and Ye 2001). Based on the microscopic examinations of several *rev* mutants, Lev-Yadun et al. (2005) found that the total lack of the wavy band of fibers is in many cases a result of poorly lignified secondary walls. Recently, the *REV* homologous gene *PtaHB1* was isolated from hybrid aspen, and its expression was found to be closely associated with wood formation and regulated both developmentally and seasonally with the highest expression during the active growing season (Ko et al. 2005). In bamboo, *PpHB1* is also expressed in many isolated procambia, suggesting it may be correlated to the high lignification of bamboo (Figure 5). Based on this result, we compared the differences in REV homologs between herbaceous and woody plants. However, we found no differences between the sequences of herbaceous and woody plant REV homologs (Figure 1). Thus, we recommend that attention be focused on expression patterns in future comparative studies of herbaceous and woody plant REV homologs.

Compared with *REV* homologous genes, *TB1*-like genes are less conserved and, to date, have been identified only in the Poaceae. *ZmTB1* is expressed in maize axillary meristems and in stamens of ear primordia, consistent with a function of growth suppression in these tissues (Hubbard et al. 2002). Expression of *OsTB1*, as examined with a putative promoter–glucuronidase (GUS) gene fusion, is observed throughout the axillary bud, and transgenic rice plants overexpressing *OsTB1* exhibit markedly reduced lateral branching with propagation of axillary buds being unaffected (Takeda et al. 2003). The expression of *SbTB1* in sorghum is similar to that of *ZmTB1* and *OsTB1* (Kebrom et al. 2006). In bamboo, although the propagation of many axillary buds is observed during aerial shoot development, the buds do not extend until the shoot has reached almost its full length, indicating strong apical dominance. The level of expression of *PpTB1* in the tip of the

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Figure 5. Expression of *PpREV* and *PpTB1* during rhizome bud development. The RNA in situ hybridizations with *PpHB1* and *PpTB1* antisense probes were performed on rhizome (A), rhizome bud (B) and an aerial shoot-forming rhizome bud (C). Frames D, E and F are longitudinal sections of sites indicated by white arrows from A, B and C, respectively. The upper-right corner in F indicates complex shoot branching on an aerial shoot-forming rhizome bud. Expression of *PpHB1* in rhizome (G, J) and bamboo shoot (H, K). Expression of *PpTB1* in bamboo shoot (I, L). *Phyllostachys praecox* (scattered bamboo), *Dendrocalamopsis edulis* (caespitose bamboo) and *Pleioblastus fortunei* (pluricaespitose bamboo) are indicated in M, N and O, respectively. A–C, bar = 2 cm. D–L, bar = 100 μm.

axillary bud coincides with the stage of bamboo shoot development, suggesting that *PpTB1* may play an important role in the suppression of bud outgrowth. In addition, we isolated two transcripts of *PpTB1* that had different expression levels. Because the sequence length and characteristics of 3′-UTR may affect transcript translation (Mbongolo Mbella et al. 2000), the two transcripts we found suggest that there is regulation of *TB1*-like genes at the translational level.

Potential value of REV- and TB1-like genes to the understanding of bamboo evolution

Data on molecular phylogeny are valuable for the phylogenetic analysis of bamboo species because of the lack of taxon-specific morphological characters in bamboo. To deduce the phylogeny of many bamboo species, considerable polymorphism of the sequences among bamboo species is required. The rapid concerted evolution in the ITS region makes it a good molecular target for the delimitation of some woody bamboo species (Guo et al. 2001). Although *REV*-like genes are too conserved, *TB1*-like genes may be useful for the phylogenetic reconstruction of bamboo species. Nevertheless, more *TB1*-like sequences need to be isolated from bamboo species before an accurate phylogeny of all bamboo species can be constructed.

There exist widely inconsistent patterns of evolution among the characteristics of bamboo lineages. Finding explanations for these apparent contradictions at the molecular level will likely be helpful in understanding bamboo evolution. For example, the sympodial rhizome is thought to be more primitive than the monopodial rhizome, but a more advanced type of inflorescence branching is found in bamboo species with sympodial rhizomes, which challenges the reconstruction of bamboo phylogeny. Because meristem development is a key

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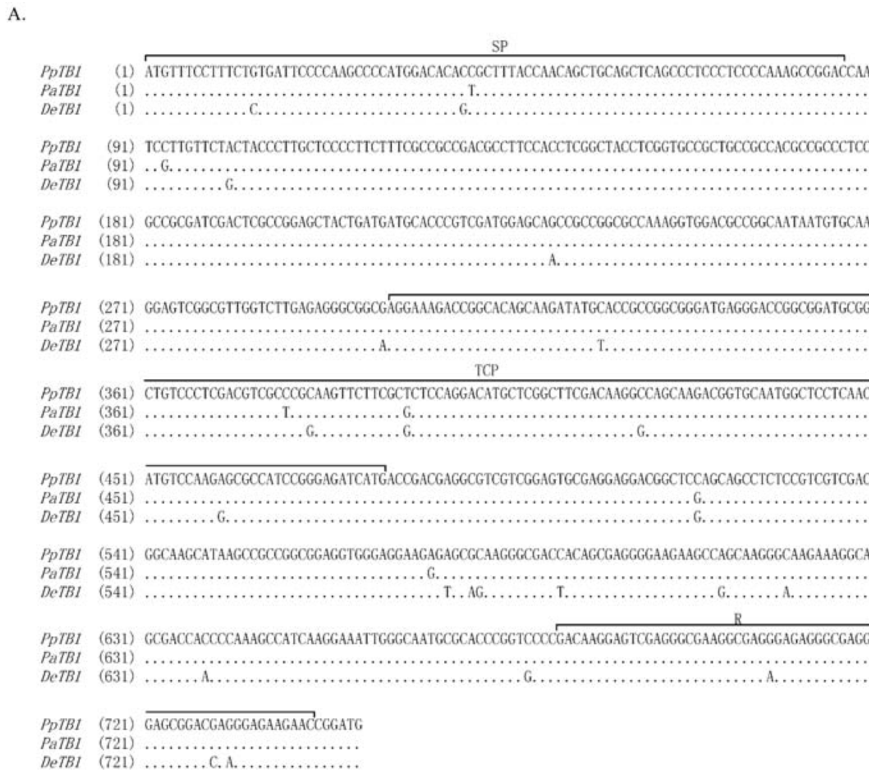

B.

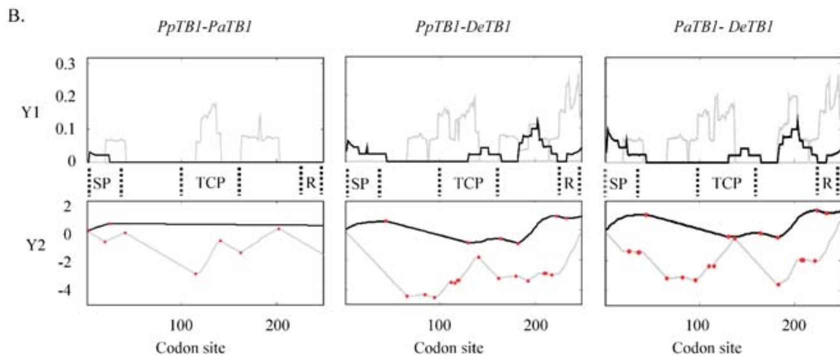

Figure 6. Alignment of partial CDSs of *PpTB1*, *PaTB1* (DQ910764) and *DeTB1* (DQ842224) is shown in (A). Sliding-window analysis of Ks and Ka from different combinations of the three genes is shown in (B). The sequence sites identical with those of *PpTB1* in the alignment (A) are indicated by dot. SP, TCP and R here refer to the corresponding DNA sequences of the conserved domains of TB1-like proteins. Y1 and Y2 in (B) denote substitution number per site and Z′ transformed substitution number per site, respectively. The gray and black lines in sliding window are applied to the synonymous and nonsynonymous substitutions, respectively. Window size equals 20 codons.

step in plant growth and organ formation, a change in some meristem regulatory genes may be sufficient for the evolution of novel morphologies. *TB1* in maize has proved to be one such gene and its function in morphological evolution was identified by the change in its expression pattern (Doebley et al. 1997). This effect of *TB1* on evolution seems to be related to the gene's regulatory region rather than to its protein-coding region (Wang et al. 1999). Analyses of genetic loci and genome synteny suggest that *OsTB1* is the counterpart of maize *TB1* (Takeda et al. 2003). Given the similar sequence characteristics and expression patterns of *TB1*-like genes, a mechanism similar to that found in maize could explain the morphological evolution of other gramineous plants, including bamboo. Perhaps a similar mechanism is associated with the *REV*-like genes. Comparisons of regulatory regions and expression patterns of *REV*- and *TB1*-like genes among different bamboo species may provide useful information for future phylogenetic analyses of these species.

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