Plant Mol Biol Rep (2011) 29:224–231
DOI 10.1007/s11105-010-0222-0

Identification of Differentially Expressed Sequence Tags in Rapidly Elongating *Phyllostachys pubescens* Internodes by Suppressive Subtractive Hybridization

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Published online: 16 June 2010

Abstract Bamboo shoots grow quickly through the rapid elongation of internodes, but the precise molecular mechanisms underlying this process remain unknown. We used a combination of suppressive subtractive hybridization (SSH), dot blotting, sequencing and bioinformatics to identify *Phyllostachys pubescens* genes that are differentially expressed in rapidly elongating vs. static internodes (SIs). We isolated 1020 expressed sequence tags (ESTs) by SSH, 173 of which were shown to be differentially expressed by dot blotting. We then sequenced the 20 ESTs showing the greatest difference in expression, 13 of which were preferentially expressed in elongating internodes and seven in SIs. Functional characterization of the ESTs showed that rapid internode elongation requires meristem initiation and proliferation, high-level protein synthesis, cellular respiration, and cell wall synthesis, as well as the regulation of the activated methyl cycle, gibberellin and brassinosteroid biosynthesis, and their signal transduction pathways.

Keywords *Phyllostachys pubescens* · Intermodal elongation · Suppressive Subtractive Hybridization (SSH) · Expressed Sequence Tags (ESTs)

Abbreviations

SSH	suppressive subtractive hybridization
ESTs	expressed sequence tags
HD-Zip	homeodomain leucine zipper
SNAREs	soluble *N*-ethylmaleimide-sensitive fusion protein attachment protein receptors
ASK	*shaggy*-related protein kinase
SAHH	*S*-adenosyl-L-homocysteine hydrolase
REIs	rapidly elongating internodes
SIs	static internodes

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Introduction

The bamboo subfamily (Bambusoideae) is a division of the grasses (Poaceae), which is further divided into nine subtribes comprising 77 genera and about 1030 species worldwide (Soderstrom and Ellis 1987; Dransfield and Widjaja 1995). In China, there are 48 genera and nearly 500 species, among which *Phyllostachys pubescens* accounts for over two thirds of commercially planted bamboo because it is valued as a source of building material, fodder, and food (Fu 2001). Bamboo generally grows faster than other woody plants, with culm elongation rates of 24.5 cm/day for *Phyllostachys bambusoides*, 9.7 cm/day for *Bambusa oldhamii* (Lin 1958), and >100 cm/day for *P. pubescens* (Ueda 1960; Li et al. 1997).

Internode elongation involves a number of steps including cell division within the intercalary meristem, cell elongation, and lignification (Martin 1988; Morrison et al. 1994). Several genes required for internode elongation have been indentified, such as *ZmREV* (*RLD1*), which is expressed in the adaxial portion of the leaf following the emergence of primordia (Juarez et al. 2004), and *OsGLU1*, which is involved in the synthesis and deposition of fibers in the extending cell walls (Zhou et al. 2006). Hormones such as gibberellin, ethylene, and abscisic acid are also thought to play an important role (Suge 1985; Azuma et al. 1990; Sauter et al. 1995). The precise molecular mechanism of rapid internode elongation in bamboo remains unknown, but the regulation of carbohydrate metabolism is likely to

play a pivotal role because the bamboo shoot has no leaves, and the sheath-covered internodes are etiolated, so energy for growth must be derived from stored carbon sources imported from connected photosynthetic tissues (Liese and Weiner 1995; Okahisa et al. 2006). The respiration rate of *P. pubescens* shoots is also much higher than that of mature culms (Isagi et al. 1997), suggesting there is active metabolism and efficient energy production during elongation. Additionally, several different sucrose synthases play roles during bamboo growth, both in terms of polysaccharide biosynthesis and energy production (Chiu et al. 2006).

Unlike model plant species such as *Arabidopsis thaliana* and rice (*Oryza sativa*), there is relatively little genomic information available for bamboo species (Tang 2009). It is therefore necessary to develop tools and resources specific for bamboo in order to gain insight into its unique characteristics, such as rapid internodal growth. Suppressive subtractive hybridization (SSH) allows the construction of a cDNA library enriched for differentially expressed genes, which helps to identify genes that are developmentally regulated or induced/repressed in response to intracellular or external signals. In this study, we used cDNA derived from rapidly elongating internodes (REIs) and static internodes (SIs) of *P. pubescens* to develop an SSH library enriched for genes involved in the elongation of bamboo shoots.

Materials and Methods

Plant Materials and RNA Isolation

We selected a *P. pubescens* culm 1.82 m in length containing 43 visible internodes, which were numbered from the base to the canopy (Fig. 1). Internode 13 was the longest (16.2 cm) and was already lignified, whereas internodes 31, 32, and 33 were shorter (8.1, 7.3, and 6.9 cm, respectively) and were still very soft. This implied that internodes 31, 32, and 33 had the potential for rapid elongation, whereas internode 13 was likely to be static (Li et al. 1997). Wall tissue from internode 13 (static) and pooled wall tissue from REIs 31, 32, and 33 were frozen in liquid nitrogen and stored at −80°C. For RNA extraction, wall tissue was ground in liquid nitrogen and total RNA was isolated using Trizol reagent (Invitrogen, USA) according to the manufacturer's instructions. For each 500 μg of total RNA, 5 μg of poly(A)$^+$ RNA was isolated using the Oligotex mRNA Mini Kit (QIAGEN, USA).

Suppressive Subtractive Hybridization

SSH was carried out using the PCR-Select cDNA Subtraction Kit (Clontech, California, USA) according to the manufacturer's instructions. After digesting the cDNA samples with *Rsa*I, two SSH reactions were performed, one with cDNA derived from REIs as the driver and cDNA from the SIs as the tester, and another with the reverse arrangement. The subtractive products were amplified in two rounds of polymerase chain reaction (PCR) using oligonucleotide primers complementary to the adapters. The first round comprised a 5-min incubation at 95°C followed by 25 cycles at 94°C for 30 s, 66°C for 30 s, and 72°C for 90 s. The second nested PCR comprised 15 cycles at 94°C for 30 s, 66°C for 30 s, and 72°C for 90 s. The final PCR products in the two SSH experiments represented genes expressed predominantly in SIs and REIs, respectively.

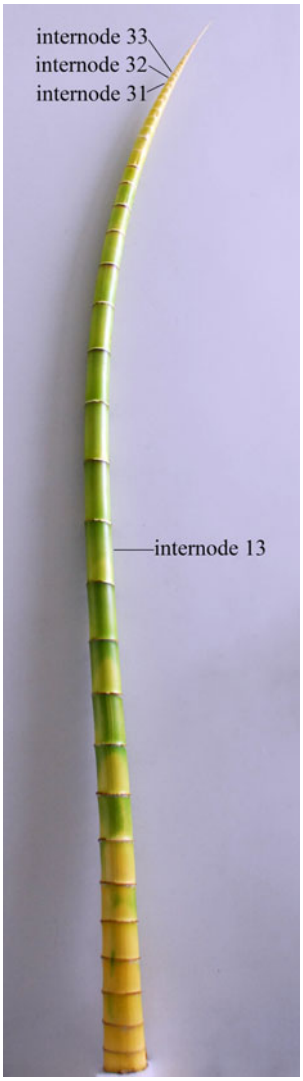

Fig. 1 Internodes used for RNA extraction. Internode 13 was the longest and was static, whereas internodes 31, 32, and 33 were shorter and retained the capacity for rapid elongation. RNA from internode 13 and pooled RNA from internodes 31, 32, and 33 were used as templates in the two reciprocal SSH reactions

Dot Blotting

The subtracted cDNAs identified above were purified using the QIAquick PCR Purification Kit (QIAGEN) and inserted into vector pMD18-T (TaKaRa Biotechnology Co., China). After transformation and selection in *Escherichia coli* JM109, 1020 independent colonies were used as PCR

templates. T7prom/SP6 universal primers derived from pUC18T were used as forward and reverse primers to amplify the inserted cDNAs. PCR products were separated by 1% agarose gel electrophoresis, and 0.2 μg of each recovered product was dissolved in 0.3 M NaOH and spotted onto a positively charged nylon membrane (Roche, USA). Two membranes with the same lattice pattern were produced and baked in vacuo at 80°C for 2 h. The membranes were prehybridized for 30 min and then hybridized with denatured DIG-labeled probes for 12 h using the DIG Easy Hyb kit (Roche). Two sets of labeled probes were prepared, one from the forward-subtracted products and one from the reverse-subtracted products described in the preceding section. The probes were labeled using the DIG High Prime DNA Labeling and Detection Starter Kit II (Roche). Posthybridization washes with 0.5× standard saline citrate (SSC), 0.1% sodium dodecyl sulfate (SDS) were followed by incubations in blocking solution, antibody solution, and washing solutions according to the manufacturers' protocols before immunological detection with ready-to-use chloro-5-substituted adamantyl-1,2-dioxetane phosphate and exposure to X-ray film at 20°C for 15 to 20 min. The membranes were scanned using PowerLook 3000, and grayscale images were assessed visually and signal intensities were quantified using TINA 2.09e software (Raytest, Straubenhardt, Germany). Clones corresponding to dots whose signal intensity differed significantly between the REI and SI preparations were selected for further analysis.

Sequencing and Sequence Analysis

The selected cDNAs were sequenced using the BigDye Terminator V3.1 Cycle Sequencing Kit and an ABI 3100-Avant DNA Analyzer (Applied Biosystems, USA). The predicted unigene sequences were used to query the nonredundant nucleotide database at GenBank (http://www.ncbi.nlm.nih.gov/) using BLASTX.

Semiquantitative PCR

First-strand cDNAs were synthesized using mRNA isolated from REIs and SIs, and those showing significantly different expression levels were validated by semiquantitative reverse transcriptase (RT)-PCR, with *actin* mRNA used as a control (Matsui et al. 2004). The primers specific for each gene are listed in Table 1. The PCR conditions were as described above for the colony amplification procedures.

Results

Construction of the Subtracted Library

RNA derived from REIs and SIs was fractionated by agarose gel electrophoresis revealing bands >1 kb in length (Fig. 2a). Other quality indicators such as the 28 *S*:18 *S* intensity ratio (~2:1), A260/A280 (>1.9), and A260/A230 (>1) confirmed that the RNA was suitable for further experiments (Schultz et al. 1994). A 2-μg aliquot of mRNA produced about 1.2 μg of double-stranded cDNA with a size range of 0.5 to 3 kb (Fig. 2b), which was reduced to 0.1 to 2 kb following digestion with *Rsa*I (Fig. 2c). The efficiency of subtraction was confirmed by testing for the abundance of *actin* cDNA before and after subtraction. An *actin* product appeared after 15 PCR cycles for the unsubtracted cDNA and after 35 cycles for the subtracted cDNA (Fig. 2d).

Confirmation of Expressed Sequence Tags with Differential Expression

Differentially expressed cDNA products were cloned in vector pMD-18T and introduced into bacteria. In total, 1020 colonies were isolated. Colony PCR analysis revealed insert fragments >0.25 kb in length (Fig. 3). Dot blots confirmed that 173 genes were differentially

Table 1 Primers used for semiquantitative RT-PCR

EST name	Forward primer	Reverse primer
REfa-1	ATCACCTGGGTAACAATGGG	AAGAAGGGCTGACGCTGTGG
REce-2	CCGAGGTACGGCCTGTCTCCTT	TCGATCTGCGCCCGGTGAAT
Repro-4	GTTGATTCGGCAGGTGAGTT	TCGTTCGTCTGACTTGGGTAT
REen-6	CCACTACCTTCACTAACCCTTCGG	AAGAACAGGTCAAGGCATCAGG
REsu-7	ACTGTTGTCCGTTGGCTTCTG	CTGGTGGTTACTTCGTCTGGTCT
REme-8	AACTGTTGTCCGTTGGCTTCT	GTCCTGGTGGTTACTTCGTCTG
REwa-10	ACCGTGTCGCCGGTGTAGAACC	CCAAGCCGACTCCACCGACCT
STmo-1	ATACACCATCCCATTCCCTCC	GGCAACCTGCATGTTCTCACA
STga-2	CCAGACATTATTGCCCTTTGAG	GCCTGTTTGCTTTGCGAGTA
STde-3	CGGTCCCAATCAGAGCAATCAC	CGGGCAGGTACTGCATCTCG
Actin (AB114677)	GCAATCCAGGCAGTTCTTTCC	CAGCGGCTTCCATACCAATC

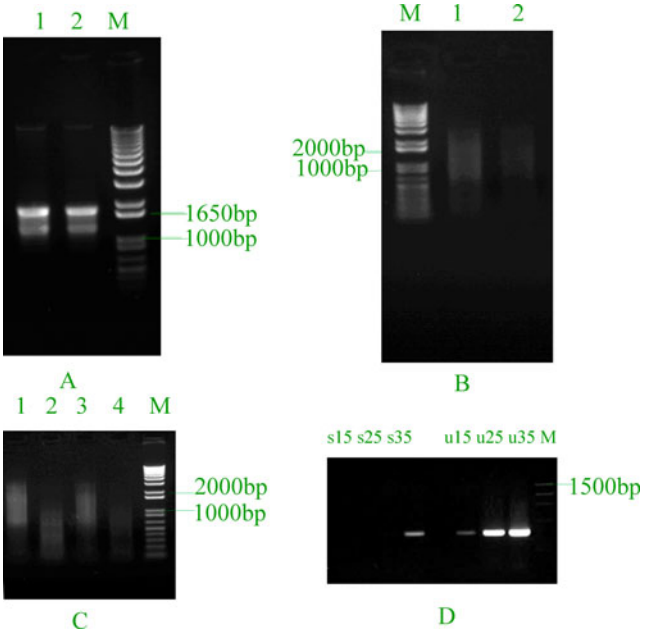

Fig. 2 Agarose gel electrophoresis of RNA and DNA from REIs and SIs. **a** RNA. Lane 1: REIs. Lane 2: SIs. Lane M: 1 kb plus DNA ladder (Invitrogen). **b** cDNA. Lane 1: REIs. Lane 2: SIs. Lane M: 1 kb plus DNA ladder. **c** Comparison of intact cDNA and cDNA digested with *Rsa*I. 1: REIs. 2: SIs. Lane M: 1 kb plus DNA ladder. **d** Reduction of *actin* abundance by PCR-Select subtraction. PCR was performed with the *actin* forward and reverse primers (Table 1) using the subtracted cDNA (lanes 1–3) and the unsubtracted cDNA (lanes 5–7) as the template. Lanes 1–3: 15, 25, 35 cycles. Lanes 5–7: 15, 25, 35 cycles. Lane M: 100-bp DNA ladder (Biobasic Inc, USA)

expressed, 126 predominantly expressed in REIs, and 47 in SIs. This included 20 genes with a tenfold difference in signal intensity between the samples (a REI/SI signal intensity ratio of ≥10 or ≤0.1 based on dot blotting results), 13 of which were expressed predominantly in REIs and seven of which were expressed predominantly in SIs (Table 2). The expression profiles of ten of the genes were validated by semiquantitative RT-PCR, generating results that were approximately consistent with the dot blot experiments (Table 2 and Fig. 4).

Identification of Differentially Expressed Sequence Tags

The 20 candidate expressed sequence tags (ESTs) were sequenced (GenBank: GT917652–GT917660 and GT917662–GT917672), and BLASTX analysis revealed that 13 of the sequences matched genes with known functions (83–94% identity), three matched genes with unknown functions (REun-12, REun-13, and Stun-4; 86–89% identity), and the remaining four were orphan genes, lacking homology to any other sequence in GenBank (REun-3, STun-5, STun-6, and Stun-7). The 13 ESTs matching sequences with known functions included ten that were predominantly expressed in REIs and three predominantly expressed in SIs. The genes represented a number of different physiological and biochemical functions including meristem initiation and proliferation (REfa-1), cell growth and development (REce-2), protein metabolism (REpro-4, REpro-5), cellular respiration (REen-6), sugar metabolism (REsu-7), methylation (REmet-8, REmet-9), cell wall structure (REwa-10, REwa-11), hormone metabolism (STga-2), and signaling (STmo-1).

Discussion

The rapid growth of bamboo shoots is one of the most distinctive characteristics of bamboo development in comparison to other woody plants, but little is known about the molecular mechanisms underpinning this process. We used SSH to generate a library enriched for genes that are differentially expressed in rapidly elongating vs. SIs. Among 173 clones identified by dot blotting, we sequenced 20 ESTs whose expression level differed significantly between the two tissues, 13 of which were homologous to genes with a known function. The other seven were either orphans or homologous to genes whose functions have not been determined.

Previous studies have implicated several physiological processes in internode elongation. One of the earliest events is the initiation and proliferation of meristem tissue, which is regulated by REVOLUTA (REV)-like class III homeodomain leucine zipper (HD-Zip) proteins (Otsuga et al. 2001), such as ZmREV (RLD1) in maize (Juarez et al. 2004). REV proteins are also associated with fiber deposition and wood formation (Ohashi-Ito et al. 2005), e.g., LFL1 in *A. thaliana* (Zhong and Ye 1999) and PtaHB1 in aspen (Ko et al. 2005). We found that REfa-1, a homolog of the rice *NIF3* gene encoding a REV protein, is up-regulated in REIs, suggesting that high-level expression of this *REV* gene in bamboo may be required for meristem initiation and fiber deposition. In *Pleione praecox*, the *REV* homolog *PpHB1* is also expressed within the developing procambium, suggesting it may underlie the extensive

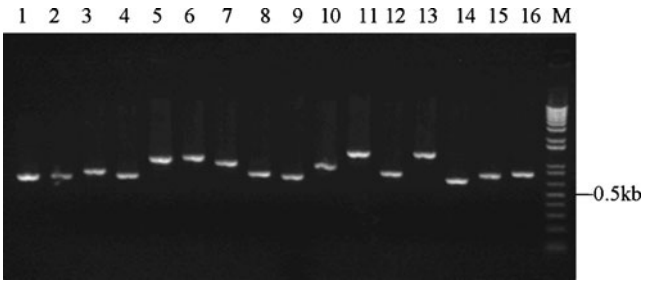

Fig. 3 Detection of inserted fragments by colony PCR analysis. Lanes 1–16 represent 16 randomly selected colonies. Lane M: 1 kb plus DNA ladder (Invitrogen)

Table 2 *P. pubescens* ESTs selected for sequencing

Name*	Size (bp)	Homologous genes	Identity (%)	Functional classification	Expression level		GenBank accession no.[ED4]
					Rapid elongation internodes	SIs	
REfa-1^{s}	357	*O. sativa* *REVOLUTA*-like HD-Zip III homeobox gene	89	Meristem initiation and development			GT917652
REce-2^{s}	432	*Zea mays* SNARE 11 gene	83	Cell growths and development			GT917665
REpro-4^{s}	963	*O. sativa* 25S ribosomal RNA gene	89	Protein metabolism			GT917655
REpro-5	256	*O. sativa* serine carboxypeptidase gene (S10)	92	Protein metabolism			GT917658
REen-6^{s}	302	*Populus tremuloides* mitochondrial lipoamide dehydrogenase gene (LPD1)	89	Cellular respiration			GT917659
REsu-7^{s}	579	*O. sativa* starch-branching enzyme I gene	88	Sugar metabolism			GT917660
REmet-8^{s}	484	*O. sativa* methyltransferase family gene (DUF248)	87	Methylation			GT917662
REmet-9	567	*Triticum aestivum* adenosylhomocysteinase gene	94	Methylation			GT917657
REwa-10^{s}	465	*Z. mays* cell wall glycoprotein gene	87	Cell wall structure			GT917663
REwa-11	430	*O. sativa* *endo*-1,4-β-glucanase gene	91	Cell wall structure			GT917653
REun-12	360	*O. sativa* Os04g0244800 mRNA with unknown function	89				GT917664
REun-13	432	*O. sativa* Os06g0514100 with unknown function	86				GT917670
REun-3	255	None					GT917656
STmo-1^{s}	285	*O. sativa* shaggy-related protein kinase gene (ASK-eta)	93	Hormone metabolism and signal transduction			GT917667
STga-2^{s}	412	*O. sativa* gibberellin 2-oxidase	83				GT917671
STde-3^{s}	355	*Z. mays* cysteine protease 1	89	Defense system			GT917654
STun-4	595	*O. sativa* Os01g0784500 with unknown function.	89				GT917669
STun-5	730	None					GT917672
STun-6	459	None					GT917668
STun-7	397	None					GT917666

Superscript S refers to ESTs selected for semiquantitative RT-PCR analysis

* Expression levels established by dot blotting

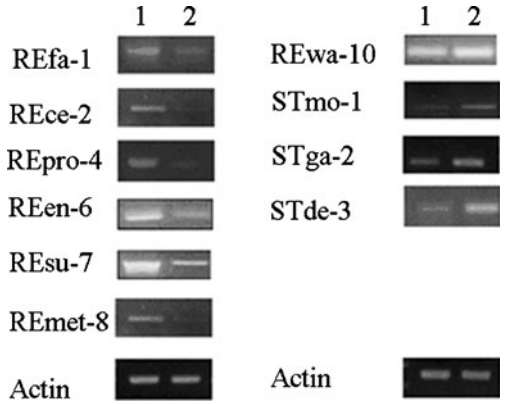

Fig. 4 Semiquantitative RT-PCR analysis of ten candidate genes to confirm differential expression (with *actin* as the control). Lane 1: cDNA template from REIs. Lane 2: cDNA template from SIs

lignification of bamboo shoots (Peng et al. 2007). After initiation, rapid but tightly regulated cell growth and division within the meristem precedes cell elongation and lignification. The SNARE superfamily (soluble *N*-ethylmaleimide-sensitive fusion protein attachment protein receptors; Sutter et al. 2006; Lipka et al. 2007) plays an important role in this process, particularly SNARE 11, a component of the cell plate membrane fusion machinery involved in membrane trafficking and fusion during cytokinesis (Zheng et al. 2002). The identification of REce-2 as a SNARE 11 homolog indicates that rapid but correctly regulated cell division is critical in the early stages of bamboo shoot growth.

The rapid growth in bamboo shoots also requires very efficient protein synthesis and recycling, so it is unsurprising that two of the differentially expressed ESTs (REpro-4 and REpro-5) correspond to genes involved in protein metabolism. REpro-4 corresponds to the 25 *S* ribosomal RNA gene. Its high-level expression in growing bamboo shoots indicates that the number of ribosomes may become a limiting factor during culm elongation. REpro-5 is homologous to a rice S10 serine carboxypeptidase gene. Increasing the availability of the protease may increase the plant's capacity for C-terminal processing, protein turnover, and recycling (Remington and Breddam 1994; Mortensen et al. 1999). In addition to the raw materials necessary for shoot elongation, a large amount of energy is also required. We identified REen-5 as a homolog of the aspen mitochondrial lipoamide dehydrogenase (LPD1) gene, which encodes a pivotal component of several supraenzymatic complexes involved in carbohydrate and energy metabolism. LPD1 is often associated with the Gly decarboxylase complex, which ultimately provides ATP (Williams 1992; Vettakkorumakankav and Patel 1996). The high-level expression of REen-5 in REIs shows that active cellular respiration is required for internode elongation (Lutziger and Oliver 2001), consistent with the much higher respiration rate observed in *P. pubescens* shoots compared to mature culms (Isagi et al. 1997).

Cell elongation is dependent on the laying down of fibers in the nascent cell walls. Wang et al. (2010) detected the high-level expression of starch-branching enzyme I in bamboo buds, and likewise, we found that REsu-7, which is expressed preferentially in REIs, is homologous to starch-branching enzyme genes. We found that REwa-10 was homologous to a maize cell wall hydroxyproline-rich glycoprotein gene (*HRGP*), whereas REwa-11 was homologous to the rice *endo*-β-1, 4-D-glucanase gene (*OsGLU1*), both of which were involved in the synthesis of cell wall components (Stiefel et al. 1990; Zhou et al. 2006). *HRGP* is expressed at sites of early vascular differentiation in embryos, coleoptiles, leaves, hypocotyls and both primary and lateral roots, as well as at much lower levels throughout developing maize plants (Stiefel et al. 1990). Loss of function mutations in the *OsGLU1* gene result in rice plants with shorter and narrower cells than wild-type plants (Zhou et al. 2006). The slightly elevated expression of REwa-10 and REwa-11 in REIs may facilitate the synthesis and deposition of fibers in the extending cell walls.

Two further ESTs up-regulated in REIs (REmet-8 and REmet-9) were found to be homologous to a putative rice methyltransferase gene (DUF248) and a wheat *S*-adenosyl-L-homocysteine hydrolase (SAHH) gene. Methyltransferases are predominantly part of the activated methyl cycle, providing key precursors in amino acid and nucleotide biosynthesis (Shu et al. 2006). SAHH is a key enzyme involved in DNA methylation (Tanaka et al. 1997), which is essential for normal plant development (Chan et al. 2005). Both genes would need to be expressed more strongly in REIs to facilitate the increase in cell number and biomass that occurs during rapid internode elongation.

Genes that affect plant height are often found to encode enzymes involved in hormone metabolism or components of the corresponding signaling pathways (Peng et al. 1997, 1999). We identified two ESTs (STmo-1 and STga-2) predominantly expressed in SIs. STmo-1 was homologous to a rice *shaggy*-related protein kinase (ASK-eta), which is a negative regulator of brassinosteroid signaling (Clouse and Sasse 1998; Li and Nam 2002), whereas STga-2 was homologous to rice GA 2-oxidase, whose role is to inactivate gibberellins by 2β-hydroxylation (Pimenta and Lange 2006). The preferential expression of these ESTs in SIs suggested that rapid stem elongation in bamboo requires the derepression of a negative regulatory system.

In conclusion, we identified 20 promising candidates from a panel of >173 ESTs that were differentially expressed in rapidly elongating vs. SIs in bamboo, using a

combination of SSH, dot blotting, and semiquantitative RT-PCR for verification. Thirteen of the sequences could be assigned putative functions on the basis of homology searching, revealing the involvement of proteins involved in meristem initiation and proliferation, protein biosynthesis, cellular respiration, the regulation of methylation as part of the activated methyl cycle, starch accumulation, cell wall synthesis, hormone synthesis, and signal transaction. These data provide insight into the molecular mechanisms underlying the rapid internode elongation that characterizes the growth of bamboo shoots.

Acknowledgements This work was supported by a special grant from the National Natural Science Foundation of China (no. 30371181) and the Natural Science Foundation of Zhejiang Province (nos. Y3080002 and R303420).

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