

Endo-1,4-β-glucanase gene involved into the rapid elongation of *Phyllostachys heterocycla* var. *pubescens*

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Abstract

Key message *Endo-1,4-β-glucanase* gene was isolated from *Phyllostachys heterocycla* var. *pubescens* internodes. It is engaged in the culm or stem development by regulation of the biosynthesis of cellulose.

Abstract The cellulase protein *endo-1,4-β-glucanase* is a member in the large glycosyl hydrolase gene family 9 (GH 9). It is widely distributed in plants, animals, microorganisms and plays roles in cell wall metabolism, including cellulose biosynthesis and degradation, modification of cell wall polysaccharides and cell wall loosening during cell elongation. Our previous studies have identified a gene homologous to *endo-1,4-β-glucanase* and found its expression pattern changed significantly during the rapid elongation of *Phyllostachys heterocycla* var. *pubescens* internodes. In this work, we isolated the full length *endo-1,4-β-glucanase* gene from bamboo shoot and named as *Phbeta-1,4-glu*. Further characterization showed that *Phbeta-1,4-glu* belongs to GH 9 family, a conserved family in bamboo with slight variation in intron numbers and positions. Phylogenetic analysis revealed that *P. heterocycle* GH 9 genes exhibit a diverse phylogeny with rice, maize, *Brachypodium*, poplar and *Arabidopsis*. Among them, bamboo GH 9 genes show a closer relationship to ones of

Brachypodium. RT-PCR analysis demonstrated that the expression pattern of *Phbeta-1,4-glu* varied among different tissues of bamboo shoot with a lowest expression level in the tender parts. The function of *Phbeta-1,4-glu* in bamboo's height growth and cellulose content was confirmed via its transformation into the model plant *Arabidopsis*. Functions of *Phbeta-1,4-glu* in bamboo shoot have been analyzed through a combinational methods of gene structure and phylogeny. Its gene expression pattern has been investigated and its function verified. The results confirmed that *Phbeta-1,4-glu* engages in the rapid elongation of bamboo culm from cellulose biosynthesis to cellulose degradation. Current investigations provide valuable information for its functional studies and potential utilization in future.

Keywords *endo-1,4-β-glucanase* · *Phyllostachys heterocycla* var. *pubescens* · Internodes · Phylogenetic relationship · Cellulose

Introduction

As being one of the most significant non-timber forest products in the world, bamboo not only can be used as building materials, it can also be used as food materials. Bamboo fibers possess versatile advantages in biodegradability, fast renewability, high annual biomass yield, high aspect ratio, and high strength to weight ratio, which make them ideal substitutions for man-made fibers. Currently, the latter is dominantly used in the production of fiber-reinforced composites and biorefinery bioethanol, such as bio-ethanol, bio-methane, flavonoids, and functional xylo-oligosaccharides (He et al. 2014; Yu et al. 2014). In China, there are 48 genera and nearly 500 species reported in

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bamboo subfamily (Bambusoideae), among them *Phyllostachys heterocycla* var. *pubescens* accounts for over two thirds of commercially planted bamboo. Bamboo leads a rather striking life history, characterized by a prolonged vegetative phase lasting for decades before heading to the flowering stage (Peng et al. 2013a). What's more, it grows quite quickly during culm elongation, with a maximum rate reaching to 100 cm per day (Ueda 1960).

Anatomical observations indicated that the development of culms is dominated by cell division in the initial stages while by cell elongation in the middle and late stages. The continuously elongated cell length and gradually decreasing numbers of cell nuclei imply that cell division and elongation occur simultaneously, and this finally affects internode elongation (He et al. 2013; Cui et al. 2012). Physiological and biochemical research revealed that the rapid elongation of culms was mainly attributed to intercalary meristem initiation and proliferation, high-level protein synthesis, cellular respiration, cell wall synthesis and hormone regulation (Peng et al. 2013b; Zhou et al. 2011). He et al. (2013) reported that phytohormones such as indole-3-acetic acid (IAA), gibberellic acid (GA3), abscisic acid (ABA) and cytokinin zeatin riboside (ZR) appeared to actively promote culm development. Application of variety of molecular studies, such as cDNA library (Das et al. 2005; Gui et al. 2010; Liu et al. 2012; Sharma et al. 2008; Sungkaew et al. 2009; Zhang et al. 2011), Expressed Sequence Tags (EST) (Wang et al. 2009; Wei et al. 2011), proteomics (Logacheva et al. 2011), draft genome (Li et al. 2011), RNA-seq (Scurlock et al. 2000), transcriptome sequencing (Peng et al. 2013b), have uncovered that the plant hormones, cell cycle regulation, cell wall metabolism and cell morphogenesis genes might cooperate together to regulate the fast growth of bamboo shoots (He et al. 2013). A homologous fragment of *endo-1,4-β-glucanase* gene was reported in *Ph. heterocycla* var. *pubescens* via suppressive subtractive hybridization, the gene expression was found remarkably changed during the rapid elongation of *P. heterocycle* internodes (Zhou et al. 2011).

Endo-1,4-β-glucanases belong to glycosyl hydrolase gene family 9 (GH 9), and are kinds of cellulose proteins widely distributed in animals and plants (Maloney et al. 2012; Sami and Shakoory 2008). Glycoside hydrolases could hydrolyse glycosidic bonds between carbohydrates or between carbohydrates and non-carbohydrate moieties, and collectively exhibiting a wide range of substrate specificities (Urbanowicz et al. 2007). *Endo-1,4-β-glucanases* have been implicated in several aspects of cell wall metabolism in higher plants, including cellulose biosynthesis and degradation, modification of other wall polysaccharides that contain contiguous (1,4)-β-glucosyl residues, and wall loosening during cell elongation

(Buchanan et al. 2012). *Endo-1,4-β-glucanase* gene plays vital role for proper plant height growth. For example, *OsGLU1*, a putative membrane-bound endo-1,4-D-glucanase from rice, affected plant internode elongation (Zhou et al. 2006); *d2003*, a novel allele of Viviparous 8 (VP8), was required for maize internode elongation (Lv et al. 2014); *PtrCel9A6* from *Populus* promoted the cell wall formation (Yu et al 2013). In *Arabidopsis* (*Arabidopsis thaliana*), tomato (*Solanum lycopersicon*) and poplar (*Populus tremuloides*), expression of *endo-1,4-β-glucanase* genes impacted cellulose content of the cell wall (Bhandari et al. 2006; Brummell et al. 1997; Buchanan et al. 2012; Shani et al. 2004, 2006; Zuo et al. 2000). While in light-grown plants, such as in *Brassica napus*, expression of a membrane-anchored *endo-1,4-β-glucanase* was inversely correlated to elongation (Mølhøj et al. 2001).

Although functions of several members of *endo-1,4-β-glucanases* have been studied, phylogenetic relationship among them is still unclear. Its clarification will simplify our understanding towards assigning the functions for other related genes. In the regularly updated database, Carbohydrate-Active Enzymes (CAZY; <http://www.cazy.org>), 133 distinct GH families are listed. They correspond to 14 clans. Here, a clan is illustrated as a group of families sharing a common ancestry and is recognized by significant similarities in tertiary structures together with a conservation for the catalytic residues and catalytic mechanism (Henrissat and Bairoch 1996). It has been reported that GH 9 family can be further divided into three sub-families (GH 9A, GH 9B and GH 9C sub-families) (Henrissat and Bairoch 1993; Urbanowicz et al. 2007). The members of the GH 9A sub-family are membrane bound proteins with a short NH₂-terminal cytosolic domain, a putative single spanning transmembrane helix and a characteristic DAGD motif (Brummell et al. 1997). The putative transmembrane domain was identified by a hydrophobic region of 39 amino acids flanked on each side with four or five charged amino acids (Boyd and Beckwith 1990; Brummell et al. 1997). The COOH-terminus is a proline-rich region, suggesting the possibility for its involving in protein-protein interactions (Kay et al. 2000). The GH 9B sub-family contains proteins with a catalytic domain and a signal peptide at the NH₂-terminus. This peptide may target the protein to the cell wall through the Golgi apparatus or endoplasmic reticulum. While for the GH 9C sub-family, apart from a catalytic domain and an NH₂-terminal signal peptide, the COOH-terminal end has a carbohydrate binding module (CBM) (Henrissat and Bairoch 1993; Buchanan et al. 2012; Urbanowicz et al. 2007).

In this work, we isolated a putative *endo-1,4-β-glucanase* gene from bamboo shoot, deciphered its structural and physicochemical properties, as well as its phylogenetic relationships with three plants from the grass family (rice,

maize, and *Brachypodium*) and two from dicots (*Arabidopsis*, and *Populus*). By transforming the *Arabidopsis* model plants, we further analysed how much the gene contribute in plant height, cellulose content and cell wall composition.

Results

Structural and physicochemical properties of *Phbeta-1,4-glu*

To analyse the structural and physicochemical properties of *endo-1,4-β-glucanase* in bamboo, the putative gene, named as *Phbeta-1,4-glu*, was cloned from the cDNA library isolated from bamboo shoot. Sequencing results revealed that it has a total length of 1934 bp, with a complete open reading frame from 196 to 1854 bp, encoding a protein with 617 amino acids. The calculated molecular weight for the protein is 68.9 kDa with an isoelectric point of 9.04. Moreover, 21 α -helices (14.09 %), 47 β -sheets (31.54 %), 47 corners (31.54 %) and 34 random coils (22.83 %) were detected from the predicted secondary structure of the protein (Fig. S1). Besides, analysis of the protein sequence revealed that the amino acid residues from 72 to 94 form an N-terminal transmembrane region with 1–71 towards the inner side of the membrane, and 95–617 towards the outside (Fig. S2).

Urbanowicz et al. (2007) proposed that TomCel3 (GenBank ID: U78526), TomCel1 (GenBank ID: U13054) and TomCel8 (GenBank ID: AF098292) were the classic GH 9 sub-family members responding to GH 9A, GH 9B and GH 9C sub-families, respectively, with an unambiguous domain structure. After multiple alignment of *Phbeta-1,4-glu* against these three GH 9 representatives, it revealed that *Phbeta-1,4-glu* shared sequence similarity with TomCel3, a member in GH 9A sub-family (Fig. 1). Domain architecture comparison further claimed *Phbeta-1,4-glu*'s position in GH 9A sub-family (Table 1).

Homology alignment and phylogeny analysis

The whole GH 9 family members of bamboo were retrieved by blasting the cloned *Phbeta-1,4-glu* to the moso bamboo genome. The identified gene numbers are listed as: Ph01001209G0550, Ph01001459G0050, Ph01000332G1080, Ph01000780G0630, Ph01001005G0230, Ph01001262G0150, Ph01000900G0690, Ph01000003G1660, Ph01000609G0140, Ph01000342G0910, Ph01001590G0100, Ph01000060G1680, Ph01003005G0220, Ph01004196G0020, Ph01000060G0530, Ph01001311G0450, Ph01000114G1210, Ph01000092G0890, Ph01000836G0270, Ph010030

78G0310, Ph01000058G1610, Ph01001311G0460, Ph01001406G0160, Ph01001406G0170.

Examination of the intron numbers and positions of GH 9 genes in bamboo manifested that although the number of introns varies in general, the intron positions and exon sizes do not (Fig. 2 middle): five introns and six exons are often found in most of the GH 9 genes. Nevertheless, there are exceptions. Ph01003078G0310 and Ph01000058G1610 contain seven introns and eight exons; Ph01001311G0460 and Ph01001406G0170 have only one intron and two exons. A phylogenetic tree of bamboo GH 9 members was built based on the 28 amino acid sequences, including *Phbeta-1,4-glu* (Fig. 2 left). According to the tree characteristics, the GH 9 family members in bamboo could be divided into three groups, nominated as PhGH9-1, PhGH9-2 and PhGH9-3. Genes with similar number of introns and exons clustered together, with 6/5, 2/1, 5/4 (intron/exon), respectively, indicating that genetic relationships within these three clades are closer than with others. Furthermore, TomCel3, TomCel1 and TomCel8 were all embodied in PhGH9-2. Consequently, domain architectures of these 28 species were speculated on the NCBI website (Fig. 2 right). The results suggested that genes showing closer relationship in phylogenetic tree exhibited the most identical domain structures. The domain architectures of Ph01001209G0550, *Phbeta-1,4-glu*, TomCel3, Ph01001459G0050 and Ph01000332G1080 displayed close relationship and contained an N-terminal cytosolic domain, a transmembrane region and a characteristic DAGD motif. The gene structures are also fairly similar too.

An unrooted parsimonious phylogenetic tree was generated based on GH 9 amino acid sequences of the four species (maize, rice *Brachypodium*, poplar) obtained from PlantGDB website (<http://www.plantgdb.org/PtGDB/>), and the 24 moso bamboo sequences (Fig. 3). According to the tree's dendron topology, four groups were separated, namely GH 9-I, GH 9-II, GH 9-III, GH 9-IV. Interestingly, rice, *Brachypodium*, poplar and *Arabidopsis* occurred in every group. However, what's worth noting was that, in GH 9-I, GH 9-II, GH 9-III, bamboo GH 9 genes were always clustered with rice, maize and *Brachypodium*, but kept a relatively far relationship with poplar and *Arabidopsis*. While in GH 9-IV, there were only one rice (Os05g03840.1), one *Brachypodium* (Bradi2g38360.1), one poplar (PtPOPTR0014s04390.1) and four *Arabidopsis* (AtGH9B12, AtGH9B11, AtGH9B10, AtGH9B9) involved, except bamboo and maize, suggesting mutation or genome duplication event may take place during the evolution history (Paterson et al. 2004, 2009; Salse et al. 2008).

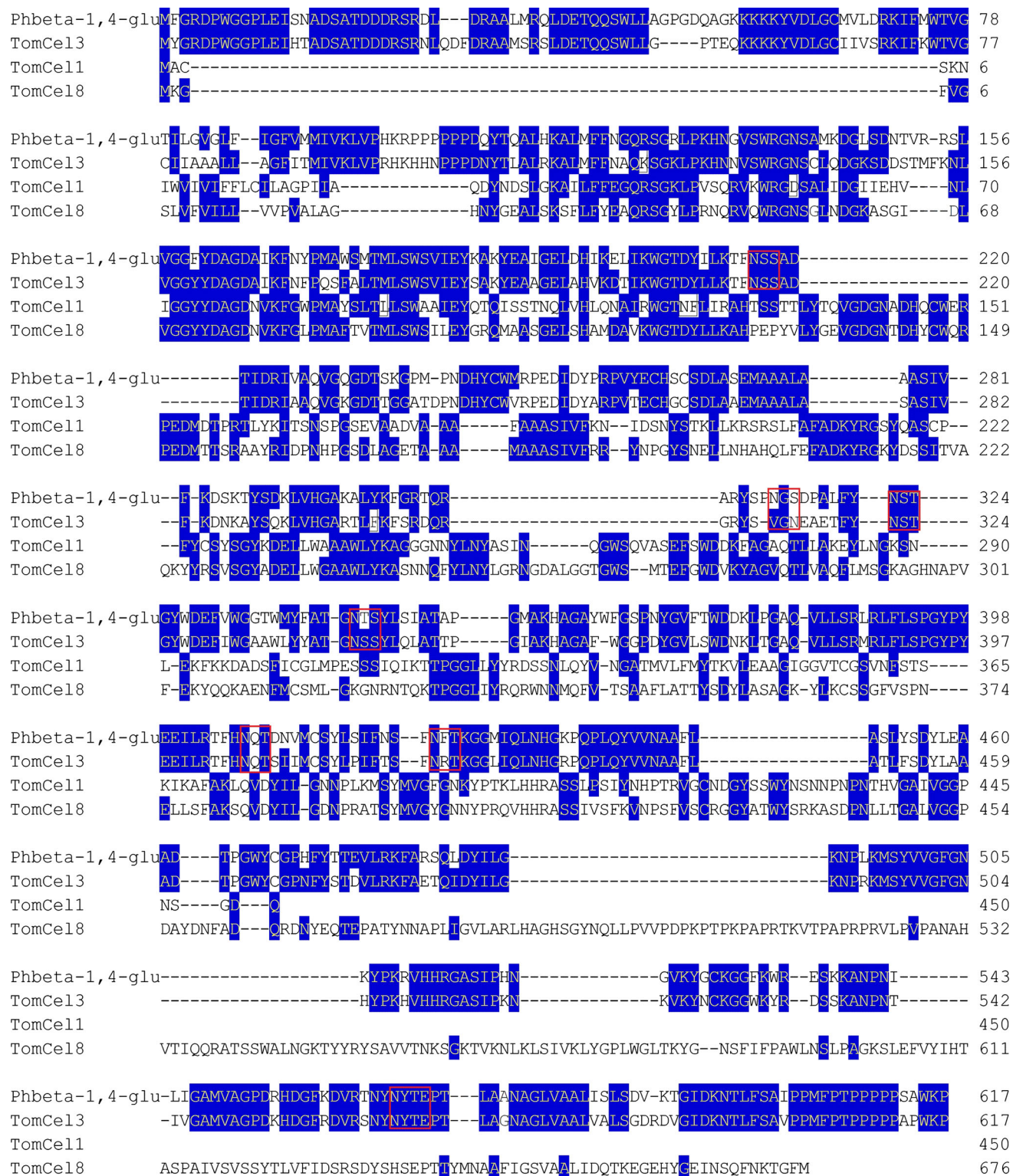

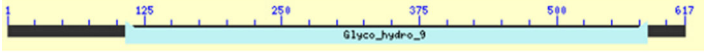
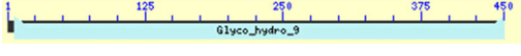
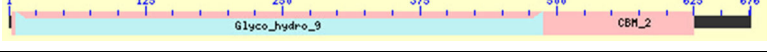


Fig. 1 Multiple sequence alignment of *Phbeta-1,4-glu* with three representatives from the classical GH 9 sub-family, TomCel3, TomCell1 and TomCel8. Amino acid identities are indicated by the blue shade. The potential N-glycosylation site in *Phbeta-1,4-glu* are shown in red boxes

Table 1 Domain architecture of *Phbeta-1,4-glu*, TomCel3, TomCel1 and TomCel8

Current Designation	Domain Architecture
<i>Phbeta-1,4-glu</i>	
TomCel3	
TomCel1	
TomCel8	

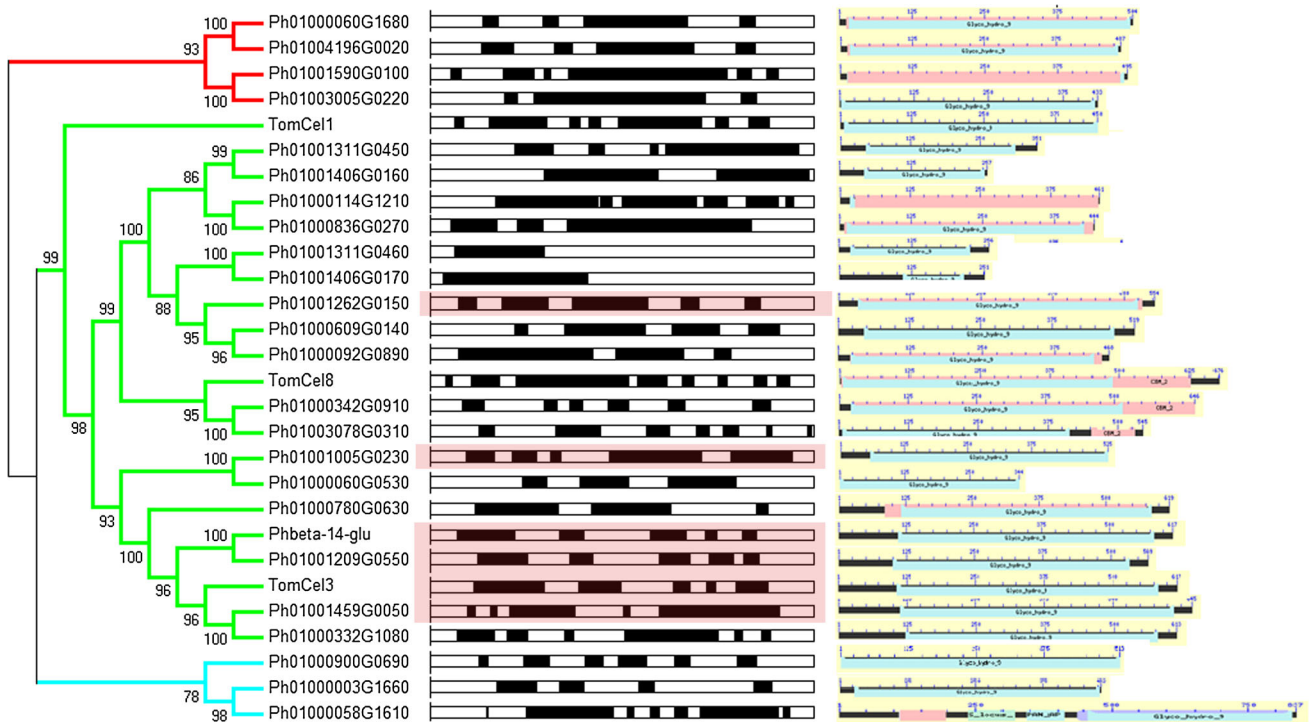


Fig. 2 Phylogenetic tree, gene structures and domain architectures in bamboo and tomato GH 9 members. *Left* Phylogenetic tree built on endo-1,4-β-glucanase amino acid sequence of bamboo and tomato. Clades with *red lines* are grouped into PhGH9-1; clades with *green lines* grouped into PhGH9-2; clades with *blue lines* grouped into

PhGH9-3. *Middle* Gene structures of bamboo and tomato GH 9 members. Introns: *black boxes*; exons: *white boxes*. The *red shades* refer to the conserved exons/intron. *Right* Domain architectures of bamboo and tomato GH 9 members speculated from the NCBI website

Expression pattern of *Phbeta-1,4-glu* in different parts of bamboo

In order to investigate the role of *Phbeta-1,4-glu* in the rapid elongation of *P. heterocycla* culm, the expression pattern of *Phbeta-1,4-glu* gene were measured by real-time fluorescent quantitative PCR (RT-PCR). Previous research uncovered that bamboo with culm height below 1 meter (m) is in the initial or ascending stage, which mainly conducts cell differentiation. Whereas, when the plant

reaches to 6 m or higher, it is in the boosting or terminal stage primarily dominated by cell elongation and fibrocyte addition (He et al. 2013). Samples collected in this paper were in the initial and promoting phase, with height reaching 0.6 and 3.2 m, respectively. Various expression levels of *Phbeta-1,4-glu* among different tissues of bamboo shoot were detected (Fig. 4a, b). For bamboo 1 which was unearthed for seven days (Fig. 4a), it was in the initial stage of bamboo culm development and dominated by cell division. The gene expression level from tender parts to

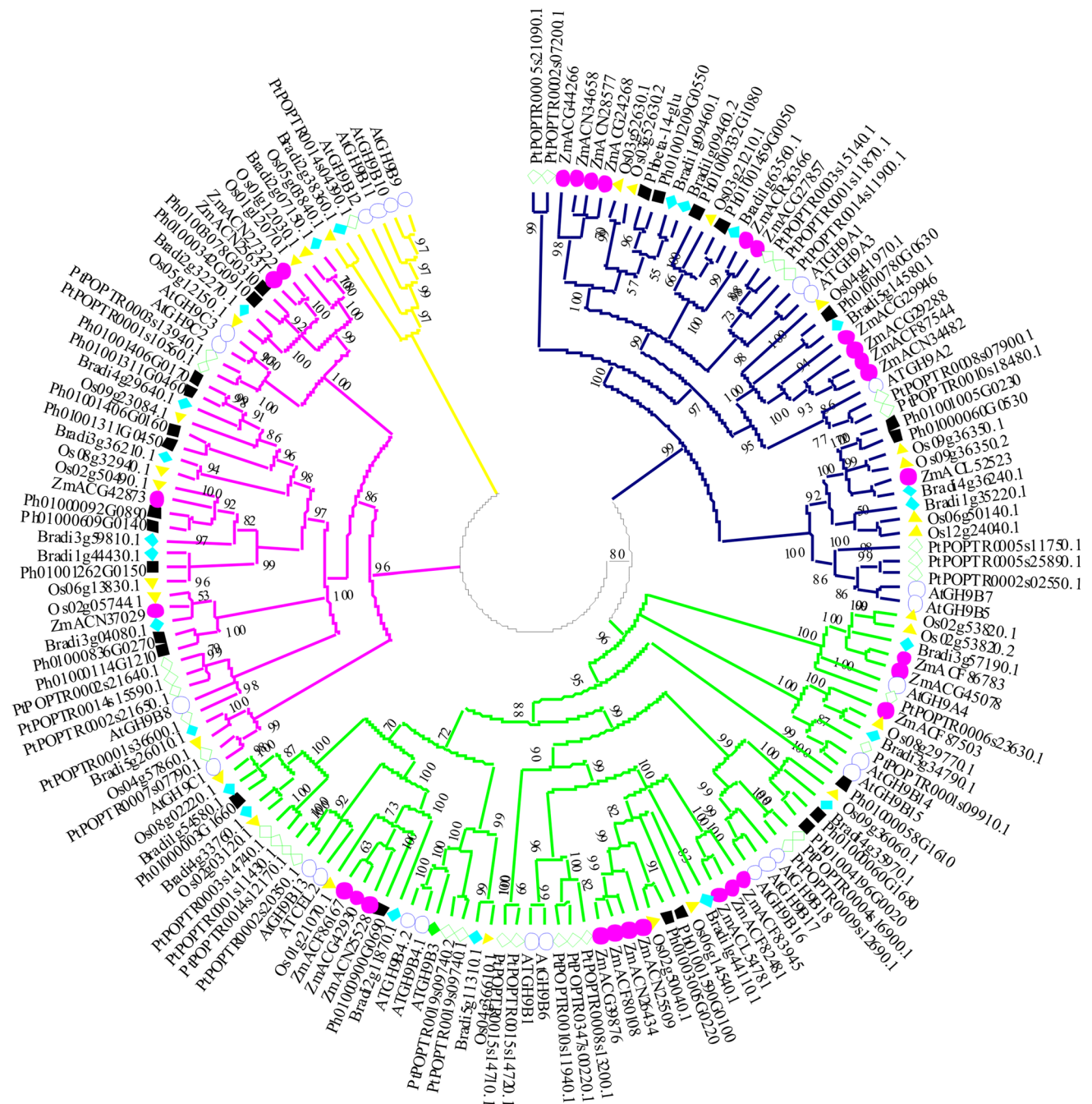


Fig. 3 Unrooted phylogenetic tree of four grasses (bamboo, maize, rice and Brachypodium) and two dicots (poplar and Arabidopsis) based on GH 9 gene amino acid sequences. GH 9-I: mazarine lines; GH 9-II: green lines; GH 9-III: pink lines; GH 9-IV: yellow lines.

Bamboo: black square; Brachypodium: blue prism; rice: yellow triangle; maize: pink circle; poplar: green empty prism; Arabidopsis: blue empty circle

mature parts increased progressively with the lowest expression level in Phin1-1 and the highest in Phin1-11. Within the longest node both above and below ground (node 3), three portions were separated (top, middle and basal nodes). The expression results showed that *Phbeta-1,4-glu* in middle internode (Phin1-5; Phin1-10) was lowest in expression level while in top (Phin1-3 and Phin1-4;

Phin1-8 and Phin1-9) and basal (Phin1-6 and Phin1-7; Phin1-11 and Phin1-12) portions, the expression were a little higher, with the highest in basal node underground. The top and basal nodes of the longest node both above and below ground (node 3) were further divided into two parts: the ring and the internal compartment of a node. They exhibited distinct expression results in above node and

underground parts. For the longest node above ground, the node internal compartments possessed higher expression level than rings. On the contrary, the internal compartments of nodes in the underground portions reflected lower expression level than the rings. On the whole, the expression level of *Phbeta-1,4-glu* increased from top to basal node.

While for bamboo 2 unearthed for 20 days (Fig. 4b), it demonstrated the same trend as with bamboo 1. The expression level increased from morphology apex to basal culm and the middle internode possessed lower expression level than the top and basal nodes within an internode. What's more, *Phbeta-1,4-glu* in rings exhibited lower expression level than the internal compartment parts. Hence, it was obvious that there were variances in different portions of bamboo culm, which seems to be related with the mature degree of tissues, performing as the more tender the bamboo tissues, the lower the expression level.

Functional verification of *Phbeta-1,4-glu* in transgenic plants

T3 Arabidopsis plants with *Phbeta-1,4-glu* transformed were used to study the function of the gene. Plant height was measured and recorded when the flower stalks initiated. The results showed that, transgenic plants exhibited significant difference with an average height of 32.13 cm compared to 25.76 cm in wild type plants, implying that *Phbeta-1,4-glu* may engage in the growth of stem length ($P < 0.01$, Table 2). The raw stem materials were collected from transgenic and wild-type plants for cellulose content and cell wall structure analysis. In the transgenic plants, the mean cellulose content was lower than the wild type plant although with low support (the mean cellulose content: $(23.86 \pm 13.21) \%$ (mean value \pm standard deviation) in transgenic plant; $(40.74 \pm 28.04) \%$ in wild type ones, Table S1). In addition, cells in transgenic plants were noticeably elongated than wild-type ones (Fig. S3), e.g, cells of the transgenic were almost three times longer than WT plants, suggesting that *Phbeta-1,4-glu* participated in cell prolongation.

Discussion

Phbeta-1,4-glu is a member of GH 9

Base on the sequence available on line (<http://www.cazy.org/>), a total of 133 classes of glycosyl hydrolase gene family were released (Henrissat 1991; Henrissat and Bairoch 1993, 1996). GH 9 was one among the family members, mainly occupied by *endo-1,4-β-glucanases* (Cantarel et al. 2009), which had been further classified

into three sub-families (GH 9A, GH 9B and GH 9C) based on the variations of protein sequences (Urbanowicz et al. 2007). The predicted secondary structure elements of *Phbeta-1,4-glu* uncovered 21 α -helices, 47 β -sheets, 47 corners and 34 random coils (Fig. S1). In addition, the deduced *Phbeta-1,4-glu* protein lacks a typical eukaryotic signal peptide sequence but instead has a highly charged region at the N-terminus (21 of the first 58 amino acid residues being charged), followed by a predominantly hydrophobic region of 39 amino acid residues. This hydrophobic domain is flanked on the N-terminal side by four consecutive, positively charged amino acid residues and on the C-terminal side by five consecutive positively charged residues. This featured charge distribution indicates the existence of a membrane-spanning domain (Boyd and Beckwith 1990). Concurrently, the C terminus of *Phbeta-1,4-glu* is rich in proline (nine out of the last 16 amino acids), a characteristic of the linker regions between different domains in microbial celluloses (Beguín 1990). Additionally, the assumed protein possesses seven potential N-glycosylation sites (N-X-S/T) (Fig. 1) (Brummell et al. 1997). All of the results suggested that *Phbeta-1,4-glu* is a member in GH 9A sub-family. Multiple sequence alignment (Fig. 1) and domain architecture (Table 1) analysis further confirmed that *Phbeta-1,4-glu* has the similar property as members in GH 9A sub-family.

Plant *endo-1,4-β-glucanases* hydrolyses cell wall polysaccharides that contain contiguous β -1,4-glucosyl residues in the chains, such as xyloglucans and β -(1,3; 1,4)-glucans, and clearly functions in cell wall degradation. Yet, there is a great deal of evidence pointing to an additional and important role for these enzymes in cellulose synthesis during cell growth (Buchanan et al. 2012; Mølhøj et al. 2001; Zhou et al. 2011). An example is that *Phbeta-1,4-glu* might take part in the rapid elongation of bamboo culms through synthesis of cellulose, as well as cell wall degradation.

Conserved and differentiated phylogeny of *endo-1,4-β-glucanase* gene

Twenty-four bamboo amino acid sequences were identified to be homology to *Phbeta-1,4-glu* through Blast. The phylogenetic tree was built with reference to the three tomato sequences (TomCel3, TomCel1 and TomCel8). Clade structure disclosed three clusters with *Phbeta-1,4-glu* embodied in PhGH9-2. Although there were variations in the number of exons/introns, the retention of exon sizes and intron positions among those orthologues were in general detectable from the phylogenetic tree, suggesting that numbers and positions of the introns among species were relatively conserved, especially for the closely related species (Buchanan et al. 2012; Chen 2010). For instance,

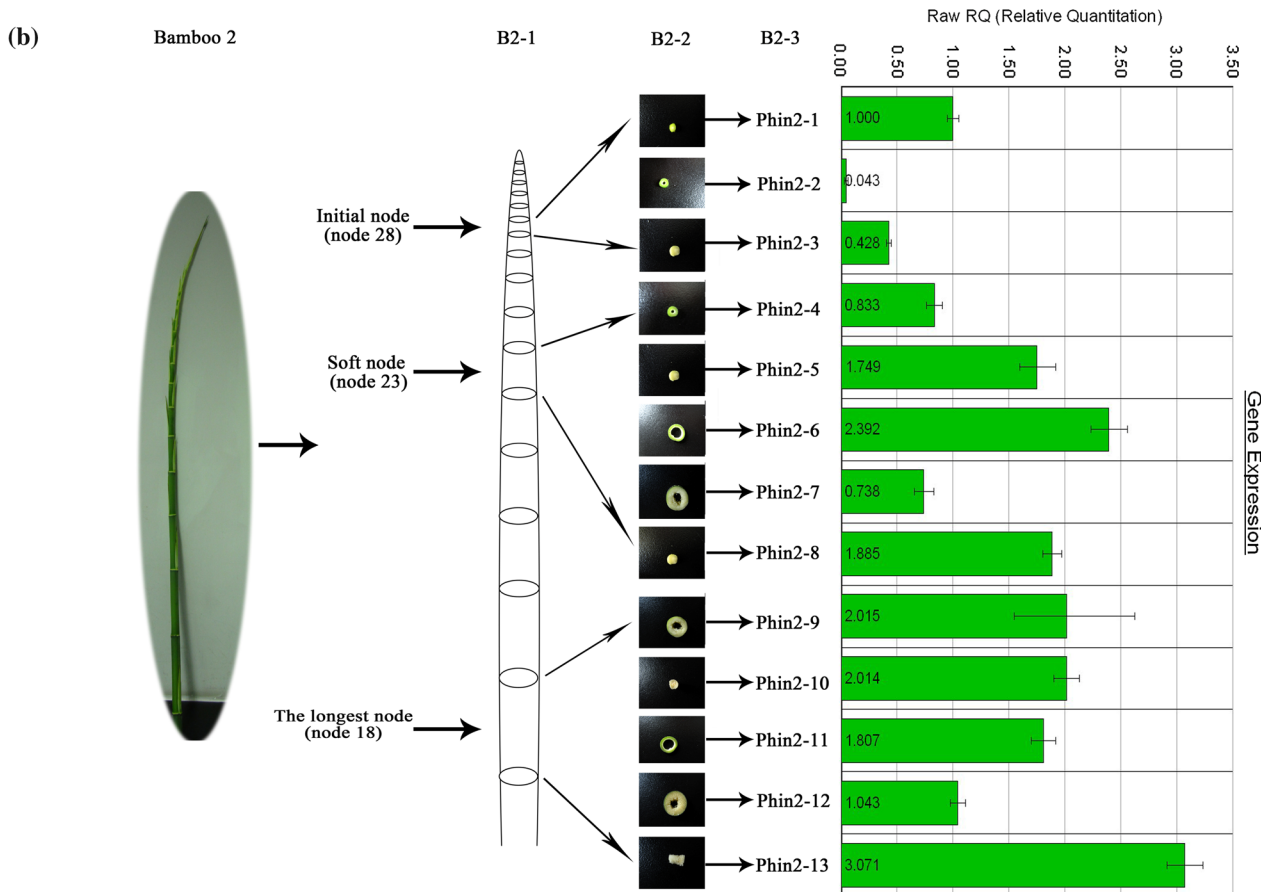
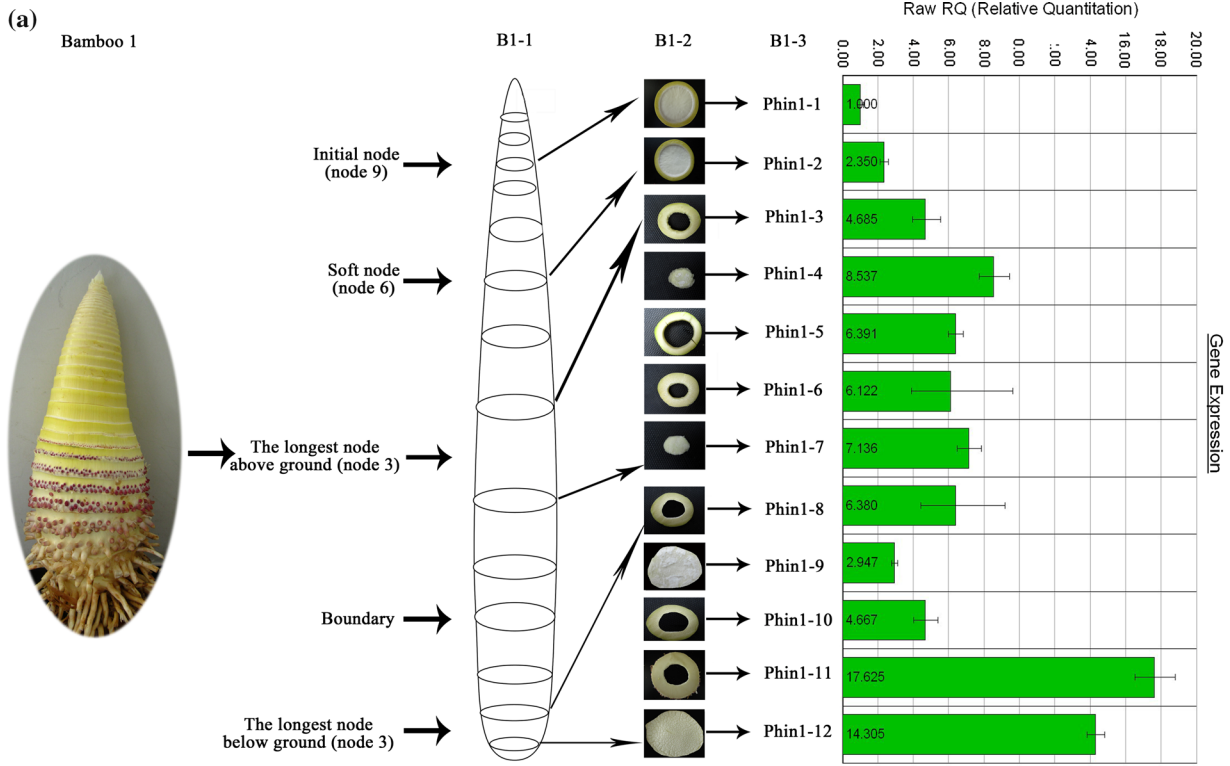


Fig. 4 Two bamboo shoot samples and expression level of *Phbeta-1,4-glu* gene. **a** Bamboo 1 unearthed for 7 days. B1-1: the magnified model for bamboo 1. Above the boundary are the parts unearthed, below it the underground parts. Four nodes were collected. The two longest parts were further divided into five segments as showed in B1-2. B1-3: the names of different parts of bamboo nodes and internodes. Phin1-1 the initial node on the morphology top of shoot; Phin1-2 the soft node next to the morphology top; Phin1-3 to Phin1-7 have the longest nodes on the ground and Phin1-8 to Phin1-12 have the longest node below ground. These two longest nodes were further divided into five parts. Phin1-3 and Phin1-8 rings of nodes above the internode; Phin1-4 and Phin1-9: internal compartment of nodes above the internode; Phin1-5 and Phin1-10 the internodes; Phin1-6 and Phin1-11 rings of nodes below the internode; Phin1-7 and Phin1-12 internal compartment of nodes below internode. **b** Bamboo 2 was unearthed for 20 days. B2-1: the magnified model for bamboo 2 with nodes below the longest internodes omitted. Three nodes were collected and divided into three or five segments as showed in B2-2. B2-3: the names of different parts of bamboo nodes and internodes. Phin2-1: the node above the initial internode; Phin2-2: the initial internode on the morphology top of shoot; Phin2-3: the node below the initial internode; Phin2-4 to Phin2-8 belong to the soft internode/node parts and Phin2-9 to Phin2-13 are the longest internode/node, which has been divided into five parts as described in bamboo 1. *Bar charts* on the *right-side* of **a** and **b** are the expression of *Phbeta-1,4-glu* in different parts of the bamboo shoots

Phbeta-1,4-glu and Ph01001209G0550, Ph01001311G0460 and Ph01001406G0170, Ph01000060G1680 and Ph01004196G0020 were clustered together with the same number of introns and exons. Alternatively, the fact that other GH 9 family members in bamboo behaved a differentiation in the ratio of exons/introns might derive from gene variation. Buchanan et al. (2012) found that loss and/or gain of *endo-1,4-β-glucanase* genes happened during evolutionary process, and the transcription was conserved among orthologous genes across the grass family. On the other hand, when an intron is lost, the number of bases for the resultant single exon is equal to the sum of the two exons in the orthologous or homologous genes if there have been no insertions or deletions within the gene sequence (Buchanan et al. 2012). However, the percentage of total introns in *endo-1,4-β-glucanase* genes varied from 33 to 77 %, implying that gene gain/loss or gene fragment insertion/deletion has taken place in bamboo GH 9 family members. This may be induced by the inconsistent intron evolution rate (Carmel et al. 2007; Gazave et al. 2007; Igea et al. 2010; Rodova et al. 2003; Soria-Carrasco et al. 2007).

What's noteworthy is that the three reference genes, TomCel3, TomCel1 and TomCel8, were all embedded in PhGH9-2. In the case of TomCel3, it exhibited closer relationship with *Phbeta-1,4-glu*, Ph01001209G0550, Ph01001459G0050 and Ph01000332G1080. The phylogenetic tree, the gene structure, as well as the domain architecture testified this observation. This illustrated that all the five genes belong to sub-family GH 9A. While for

Table 2 Analysis of height variation in T3 transgenic plants and wild type ones

Source of variation	SS	df	MS	F value	F _{0,01}
Treat	1988.95	1	1988.95	-13.19**	9.33
Error	-1809.37	12	-150.78		
Total variation	179.58	13			

SS sum of squares, df degrees of freedom, MS mean square

** Significantly difference

TomCel1, it performed as a sister group with all the other members in PhGH9-2. Previous study revealed a complex phylogenetic relationship in GH 9B sub-family, showing up as that parts of GH 9B members can be grouped with GH 9A sub-family members and some are embodied in GH 9C sub-family (Buchanan et al. 2012). In addition, the structure of *endo-1,4-β-glucanase* genes from clade B1 indicated that they belong to sub-family GH 9B. However, they were derived from the GH 9A sub-family base on the phylogenetic perspective (Buchanan et al. 2012). Although it is difficult to determine the relationship between GH 9B and other two GH 9 sub-families, TomCel8 demonstrated a closer relationship with two bamboo *endo-1,4-β-glucanase* genes, Ph01000342G0910 and Ph01003078G0310. Despite the ratio of exons/introns is different from each other for the three genes, they all contain an N-terminal signal peptide, a catalytic domain, as well as a carbohydrate binding domain, confirming that they are members in GH 9C sub-family. Apart from the genes discussed above, other *endo-1,4-β-glucanase* genes in bamboo GH 9 family exhibit variations in intron/exon or domain structures. Hence, *endo-1,4-β-glucanase* genes in bamboo were somewhat conserved with clearly identification of GH 9A and GH 9B sub-family members. Moreover, any of the phylogenetic, gene structure or protein architecture analyse alone is insufficient to identify the relationship among the gene sub-family, multi-analysis is necessary, at least in current case.

The number of genes in GH 9 family varies from bamboo, maize, rice, Brachypodium, Arabidopsis and poplar within the range of 23–33. Phylogenetic analysis revealed variation in clade structure between grasses and Arabidopsis, indicating a variation in the level of gene loss and gain during evolution (Buchanan et al. 2012; Peng et al. 2010), as well as the diversity of the *endo-1,4-β-glucanase* genes in grasses and dicots. In the GH 9-I, GH 9-II and GH 9-III groups, bamboo *endo-1,4-β-glucanase* genes were always clustered with rice, maize. They are most close to Brachypodium, but relatively far from poplar and Arabidopsis. While GH 9-IV group includes only seven members, one from rice (Os05g03840.1), one from Brachypodium (Bradi2g38360.1), one from poplar

(PtPOPTR0014s04390.1) and four from Arabidopsis (AtGH9B12, AtGH9B11, AtGH9B10, AtGH9B9). Previous phylogenetic studies of the chloroplast and nuclear genes in grass family reached a consensus that bamboo and rice were sister groups (Group et al. 2001; Kellogg 2001). However, a recent study conducted by Peng et al. (2010) argued that bamboo and Brachypodium were more closely related (Castresana 2002). Interestingly, the phylogenetic trees generated by Maximum Likelihood or Bayesian Inference in this study were inconsistent with Neighbor Joining method regarding to the relationships between rice, bamboo and Brachypodium based on the putative orthologous genes (Peng et al. 2010). These conflicting might be derived from the rapid diversification of grasses. Buchanan et al. (2012) speculated that the amino acid substitutions of *endo-1,4- β -glucanase* occurred after the divergence of monocots from dicots, but before the separation of the grasses. In addition, it was estimated that around 50 million years ago (Ma), the antecedents of the cereals, such as Brachypodium and rice, were separated from the maize (Kellogg 1998; Salse et al. 2008). Hence, it becomes clear that *endo-1,4- β -glucanase* genes in dicots and monocots differentiates distinctly, resulting in a far phylogenetic relationship between them. This was in accord with the phylogeny of GH 9-I, GH 9-II and GH 9-III.

Notwithstanding all the species in Poaceae experienced varying degrees of genomic structure and quantity variation, *in silico* mapping of *endo-1,4- β -glucanase* genes in grasses indicated that these genes are broadly distributed across the genomes (Buchanan et al. 2012). For example, the maize genome underwent a doubling of chromosome number by introduction of a foreign genome, or allotetraploid event followed by non-homologous recombination of the genome and subsequent reversion to the diploid state which happened within 10–4.8 Ma (Gaut and Doebley 1997; Paterson et al. 2004; Swigoňová et al. 2004). Nevertheless, *endo-1,4- β -glucanase* genes were found on every chromosome in maize except chromosome 3 (Buchanan et al. 2012). Consequently, it is relatively easier to analyse the phylogeny of *endo-1,4- β -glucanase* genes in Arabidopsis, poplar and maize, but challenging to resolve the relationships among bamboo, rice and Brachypodium due to their short divergence time or inadequate resolving power with genetic markers. In addition, as discussed in previous paragraph, *Phbeta-1,4-glu*, Ph01001209G0550, Ph01001459G0050 and Ph01000332G1080 could be applied as indicators for GH 9A subfamily, Ph01000342G0910 and Ph01003078G0310 for sub-family GH 9C. We found that GH 9-I consists mainly of GH 9A and GH 9B sub-family members, GH 9-II and GH 9-IV are primarily composed of GH 9B sub-family members, while GH 9-III is made up of GH 9C members. Here, we suggested that *endo-1,4- β -glucanase* gene could be used as

genetic marker in resolving phylogeny and evolutionary process on the level of Class, Order or Family.

Expression of *Phbeta-1,4-glu* in different parts of bamboo shoot

Bamboo is one of the fastest growing lignocellulose-abundant plants on Earth. It can reach a final height of 5–20 m in a single growing season of 2–4 months owing to the rapid elongation of internodes (Magel et al. 2005). The growth of bamboo culms follows the basal-apical order, meanwhile multi-internodes elongate simultaneously though in a different rate (Cui et al. 2012; Xiong et al. 1980; Jiang 2002). Four growth phases in bamboo culms have been pronounced: the initial, ascending, boosting and terminal stages (He et al. 2013). The initial stage was almost dominated by intercalary meristem conducting cell division. The intercalary meristem is derived from the retention of apical meristem or primary meristem (He et al. 2013; Xiong et al. 1980; Jiang 2002). Its cells have been characterized to be rectangular shape, thin walls, large nucleus, abundant of chromatin, dense in cytoplasm, free of accumulated starches. However, they have a higher degree of vacuolization, mainly for horizontal cell division to add cells along the longitudinal direction (Deng et al. 1988). The rapid elongation of bamboo culm was primarily attributed to the activity of intercalary meristem, including cell division, proliferation and elongation (Deng et al. 1988). Bamboos utilized in this study were in the stage of initiation and boosting with their height reaching 0.6 and 3.2 m, respectively. In each bamboo, *Phbeta-1,4-glu* exhibited distinct expressive abundance in different nodes or even different position within a single node. In bamboo 1, the gene expressive abundance increased sequentially from apical to basal parts with a lowest level in Phin1-1 and a highest in Phin1-11. As Phin1-1 is extremely close to the apical node and tenderer than any other sampling nodes, it was found fully filled with intercalary meristem which conducts cell division rather than gene expression (He et al. 2013; Dong 2007). As a result, *Phbeta-1,4-glu* in Phin1-1 displays a lowest expression level. However, for the longest nodes on or under the ground (node 3), intercalary meristem in the upper internodes gradually stopped dividing but were still active in the cell differentiation, and gradually the activity was limited to the base internodes. At this time, a little higher expression level of *Phbeta-1,4-glu* could be detected in the upper internodes (Phin1-3, Phin1-4) where more celluloses have been synthesized, polysaccharides accumulated, and parenchymal cells and fibre cells generated (He et al. 2013; Lao et al. 2013; Xiong et al. 1980). However, as the base internodes (Phin1-6, Phin1-7) are so closely adjacent to the next internode (not sampled) that the expression of *Phbeta-1,4-glu* may also be influenced and increased by the next internode. This was particularly

evident in the underground parts (Phin1-11, Phin1-12) which have a relative expression quantity of 31.930 (Fig. 4a). When the intercalary meristem in the base internode stopped differentiation, cells in this part were mature while cells in the top internode became highly aging. In this case, the gene was more actively expressed in the internodes than in the nodes. Previous studies suggested that the aging of the cells in nodes is more serious than in the internodes, which consolidating bamboo's mechanical ability (Xiong et al. 1980). Consequently, the expression difference of *Phbeta-1,4-glu* in top and basal internodes were concealed. The results were consistent with that of He et al. (2013), who also split one internode into three portions, top, middle and basal, respectively.

The expression levels in the ring and in the internal compartment within a node were compared. The internal compartments (Phin1-4, Phin1-7) appeared higher than the rings (Phin1-3, Phin1-6) regardless of the top node or the basal node when they were on the ground. The observation was reversed when the samples were from underground. During the development of shoot, it firstly differentiated as parts of the screw-shaped structure, and then gradually formed nodes and internodes (Feng 2010). In the node, the pith-ring meristem stretched into the central along the epidermis and then acrossed the pith, finally formed the internal of a node, in other words, the forming of internal node was later than the ring (Feng 2010). For the underground parts, cells in ring were more mature than internals, therefore resulting in a higher gene expression. There is no doubt why the gene in the internal compartments of a node expresses higher than in rings.

It has been reported that different portions of the same plant may be at different stages resulting from the sequential basal-apical elongation of the culm internodes (Jiang 2002). Hence, for plants in the boosting stage, such as the case in bamboo 2, the growth of the basal nodes/internodes might be in the descending stage, while the top nodes/internodes might be in the initial or ascending stage. Interestingly, within an internode, the intercalary meristem in the top section differentiated firstly and then withdrew into basal section, behaving as an opposite order of culm elongation. That's why the expression of *Phbeta-1,4-glu* in bamboo 2 demonstrated the same trend as in bamboo 1. In conclusion, our results verified the basal-apical development model for bamboo height elongation. Furthermore, we found that the expressive abundance of *Phbeta-1,4-glu* was not only concerned with the development stage of bamboo shoot, but also with the mature degree of the tissues.

***Phbeta-1,4-glu* played a role in transgenic plant's height and cellulose content**

Endo-1,4-β-glucanases are kinds of cellulose that takes part in cell wall metabolism in higher plants (Buchanan

et al. 2012). It has been reported that mutation of *Korrigan* (*KOR*) in *Arabidopsis* caused dwarfism, resulting in reduction of cell elongation and cellulose content (Zuo et al. 2000). This is because *KOR* is a kind of GA synthesis gene, and the mutation of this gene causes a defective GA synthesis leading to dwarf phenotype (Wu et al. 2014). Nicol et al. (1998) noticed that *Korrigan* mutant cells were enlarged, walls significantly thicker and loosen when compared with the wild-type. Moreover, when the mutant transformed with a complementing genomic fragment, its height was recovered. Using a heat inducible *AtKor1* mutant, *acw1*, Sato et al. reported similar findings (Sato et al. 2001). Cellulose content defects have also been observed in the *prc1* (Fagard et al. 2000) and *kob1* (Pagant et al. 2002). Both mutants exhibited cell expansion defects and dwarf phenotype, suggesting that cellulose synthesis actively contributes to the control of cell elongation. However, in our study, the height and cell size of T3 transgenic plants elongated notably than the WT plants, while the cellulose content was somewhat decreased. This may owe to the transformation of *Phbeta-1,4-glu*, which functions not only on cellulose biosynthesis, but also on degradation. That is, on one hand, augment of *Phbeta-1,4-glu* expression quantity accelerates the hydrolysis of glycosidic bonds between carbohydrates (Delmer and Amor 1995; Du and Jing 2010; Li et al. 2004; Wu et al. 2010; Zhang et al. 2007), resulting in a reduced cellulose content; on the other hand, to maintain a regular metabolism, the decreased cellulose level stimulates *Phbeta-1,4-glu* to proceed cellulose biosynthesis in turn (Buchanan et al. 2012) and thus facilitates the stem elongation, leading to an ever-increasing plant height. Moreover, different selection pressure may occur because both *Arabidopsis* and rice are herbs with plant height range from 78 to 170 cm (Kovi et al. 2011; Lee et al. 2014). Whereas bamboo is a kind of special herb with stem highly lignified and the plant height can reach to approximately 20 m. Therefore, *Phbeta-1,4-glu* is not only involved in the cellulose biosynthesis, but also promotes the fast elongation for the bamboo.

In addition, mechanical changes in the transgenic plants observed in this study might contribute to the stem strength. Although we were unable to provide a direct evidence, it is well known that cellulose content correlates with the mechanical strength. Appenzeller et al. (2004) argued that there was a strong correlation ($r^2 = 0.85$) between intermodal flexural stem strength and cellulose content of the internode, namely increasing cellulose concentration in the cell wall might improve the mechanical strength of the stem. Moreover, Buchanan (2011) suggested that cellulose is an important determinant of stem strength that increases with the cellulose content. Our continuous efforts may reveal the relationship between the cellulose content and the fibre strength for bamboo,

therefore prompt its utilization as an industrial and food material.

Materials and methods

Plant materials and RNA isolation

Two shoots and one culm of *P. pubescens* were collected in the bamboo gardens located in Zhejiang Agriculture and Forestry University, Zhejiang Province, China. The shoot collected on April 12, 2012, was 0.20 m in height with a ground diameter of 0.07 m. The other shoot (bamboo 1) collected on April 11, 2013, was 0.60 m in height with a ground diameter of 0.73 m (Fig. 4a). There were 27 visible nodes in bamboo 1 counted from the ground boundary to the canopy and six visible nodes on the underground parts. Bamboo 2, also collected on April 11, 2013, was 3.20 m in height with a ground diameter of 0.73 m (Fig. 4b). This one had 35 visible nodes. Internode 18 was the longest one (13 cm) and had lignified, whereas internodes 23 and 28 were shorter (6.5, and 2 cm, respectively) and soft. RNA extraction was performed on all the samples. Tissues that used for real-time fluorescent quantitative PCR (RT-PCR) were further divided into different segments as show in Fig. 4. After division, each sample was frozen in liquid nitrogen and stored at -80°C . Total RNA was isolated using RNeasy Plant Mini Kit (QIAGEN, German) according to the manufacturer's instructions. The cDNA was synthesized by Invitrogen SuperScript IIITM Kit (Invitrogen, USA). The reaction system consisted of 1 μg total RNA, 1 μl Oliga (dT) (20 μM), 1 μl 10 mM dNTP, 5 μl ddH₂O and was kept at 65 $^{\circ}\text{C}$ for 5 min and subsequently in ice-bath for more than 1 min. The 2 μl 10 \times RT buffer, 4 μl MgCl₂ (25 μM), 2 μl 0.1 M DTT, 1 μl RNase out, 1 μl SS III RT were added, followed by 50 $^{\circ}\text{C}$ extending for 50 min; 85 $^{\circ}\text{C}$ for 5 min. The 1 μl RNase H was added before the final extending in 37 $^{\circ}\text{C}$ for 20 min, the cDNA productions were diluted tenfolds for further experiments.

Clone and sequence analysis of *Phyllostachys pubescens* endo-1,4- β -glucanases gene

The gene fragment identified from previous research (Zhou et al. 2011) was blasted against the bamboo databank and the full length of *endo-1,4- β -glucanases* gene was acquired. Primers were designed base on the full length gene sequence (Table 3). PCR was carried out in a volume of 20 μl containing 10 \times PCR buffer 2.0 μl , MgCl₂ (25 μM) 1.2 μl , dNTP (2.5 mM) 3.2 μl , and primers with each of 0.4 μl (20 μM ; Forward primer: GLY-5; Reverse primer: GLY-3). The primer sequences are listed in Table 3. TakaRa rTaq (5 U/ μl) 0.2 μl , cDNA 2.0 μl , ddH₂O 10.6 μl were included in the reaction. Amplification was performed in a T1 thermocycler (Biometra, Germany) using the following conditions: 1 cycle, 94 $^{\circ}\text{C}$, 5 min; 23 cycles of 94 $^{\circ}\text{C}$, 30 s, 55 $^{\circ}\text{C}$, 30 s, and 72 $^{\circ}\text{C}$, 1 min; 1 cycle, 72 $^{\circ}\text{C}$, 4 min. PCR products were visualized by electrophoresis on a 1 % agarose gel and extracted using an SanPrep Gel Extraction Kit (Sangon Biotech, China). Purified PCR products were cloned into plasmids using the pGEM-T vector system (Promega, USA). Five to 20 clones were selected for each amplification product and cultured to isolate the plasmids. Positive clones were confirmed by colony PCR. After successful identification of the *endo-1,4- β -glucanases* gene through sequencing by Sangon Biotech (Shanghai) Co., Ltd, China, the plasmid with correct sequence was nominated as A1.

Sequences were edited on Seqman and EditSeq program of DNASTar 5 software. Searching for homologous sequences was conducted by BLAST comparison (at NCBI site). Physicochemical property of the protein was predicted by Protean (<http://genome.cbs.dtu.dk/services/TMHMM/>). Online tools were used to predict the transmembrane region of the protein. Domain architectures of *endo-1,4- β -glucanases* protein was conducted on NCBI (<http://www.ncbi.nlm.nih.gov/Structure/lexington/lexington.cgi>). Excel was used for the analysis of gene intron and exon proportion. ClustalX 1.81 software was utilized to align the amino acid sequence between bamboo and other

Table 3 Primers used for both PCR and RT-PCR amplification

Primer	Primer sequence (5'–3')	Description
GLY-5	ATGTTTCGGGAGGGACCCGTGG	GLY-5, GLY-3 were used for amplification of <i>P. pubescens</i> endo- β -1,4-glucanase gene
GLY-3	TTTGTGGCCTTTGCTGTACC	
GLY-Ba-5	CGGGATCCCAGATGTTTCGGGAGGGACCCGTGG	GLY-Ba-5, GLY-Ba-5 were used for amplification of <i>P. pubescens</i> endo- β -1,4-glucanase gene with restriction enzyme cutting sites (BamH and Xba I) added
GLY-Xb-3	GCTCTAGAGCTTTGTGGCCTTTGCTGTACC	
GLY-RT-5	ATCGAGTACAAGGCCAAGTAT	GLY-RT-5, GLY-RT-3 were used for RT-PCR
GLY-RT-3	CGGTCTCATCCAGCAGTAG	

GH 9 family members (Thompson et al. 1997). Phylogenetic tree was constructed using Maximum Likelihood method by MEGA 5.1 software (Tamura et al. 2011).

Transgene construction and plant transformation

PCR amplification was conducted on A1 with new set of primers GLY-Ba-5 and GLY-Xb-3 applied for the purpose of transgene construction (Table 3): 2.0 μ l 10 \times PCR buffer, 1.2 μ l MgCl₂ (25 μ M), 1.6 μ l dNTP (2.5 mM), 0.4 μ l primer (20 μ M) for each, 0.2 μ l TakaRa rTaq (5 U/ μ l), 2.0 μ l A1 plasmid, 12.2 μ l ddH₂O. Amplification was performed with the following conditions: 1 cycle, 94 $^{\circ}$ C, 3 min; 25 cycles, 94 $^{\circ}$ C, 30 s, 55 $^{\circ}$ C, 30 s, 72 $^{\circ}$ C, 1 min; 1 cycle, 72 $^{\circ}$ C, 10 min; 4 $^{\circ}$ C for the end. PCR products were visualized by 1 % agarose gel electrophoresis with ethidium bromide staining and UV illumination. Amplified products were purified through SanPrep Gel Extraction Kit (Sangon Biotech China). The purified PCR products and plasmid (pCAMBIA 1301) were digested by restriction enzyme XbaI and BamHI (NEB, USA) and collected using Mini Elute PCR Purification Kit (Sangon Biotech, China). Plasmid segments were purified by SanPrep Gel Extraction Kit (Sangon Biotech, China) through 1 % agarose gel electrophoresis. Both segments were ligated and the mixture was transformed into *Escherichia coli*. Once succeed, the objective gene was cloned and electro-transformed into *Agrobacterium tumefaciens* strain EHA105.

The transformed *Agrobacterium* strain was used to infect the wild type *Arabidopsis thaliana* plants via the floral dipping method (Clough and Bent 1998). Plants with *Phbeta-1,4-glu* transformed were selected by 1/2 MS medium containing 100 mg/l hygromycin. After cultivation for two generations, the third generation homozygous transgenic plants (T3) were used for paraffin section, cellulose content and plant height determination (Chen and Sun 2010).

Real-time fluorescent quantitative PCR

Total RNA from different parts of bamboo shoots were extracted according to Fig. 4. After PCR verification, each RNA was reverse-transcribed into cDNA. RT-PCR were conducted on 7500 system using SDS software, with β -actin 3 as the reference gene and GLY-RT as the primers (Table 3). Reaction system (20 μ l) contained: 1 μ l cDNA, 0.4 μ l of each primer (10 μ M); 10 μ l 2 \times comSYBR qPCR Mix; 8.2 μ l ddH₂O. Amplification conditions were as followed: 1 cycle, 95 $^{\circ}$ C for 1 min; 40 cycles, 95 $^{\circ}$ C for 15 s, 60 $^{\circ}$ C for 33 s. After each cycle, a read was recorded to build the dissociation curve. Following reaction conditions were applied: 95 $^{\circ}$ C for 15 s; 60 $^{\circ}$ C for 1 min; 95 $^{\circ}$ C

for 15 s; 60 $^{\circ}$ C for 15 s. Three repeats were performed for each of the samples.

Paraffin section

WT *A. thaliana* and T3 transgenic plants after 50 days growth were selected for a traditional paraffin section. Intact leaf and stem sections were stained with safranin-fast green contrast-stain method (Li 1996). Olympus BX 60 fluorescent microscope was used for cell structure analysing and picture capturing.

Measurement of cellulose content and plant height

Plant height was measured and recorded when the flower stalks initiated. Cellulose content was assayed according to Mohr's method (He 2009). Stem materials of T3 transgenic and WT plants were collected with roots, leaves and buds discarded when the flower stalks initiated. The raw materials were firstly dried at 105 $^{\circ}$ C for half an hour and then transformed to 65 $^{\circ}$ C for 2 days. The dried cell wall materials were weighed (n), grinded into powder and then incubated for 25 min in a mixture of ethylic-nitric acid (1:10, v/v) in a boiling-water bath. The samples were centrifuged in 4800 rpm for 10 min and washed with sterile water for three times. The sediments were collected and extracted in 0.5 N vitriol-potassium dichromate mixtures for 10 min in a boiling water bath. The 0.1 N Mohr's salt was used for titration (b) and the extractant was added with 3–5 drops of phenanthroline as an indicator. The titration was stopped when the solution colour turned into bronzing. A single 10 ml 0.5 N vitriol-potassium dichromate mixture was taken as a titration control (a). For the titer of Mohr's salt (k), it was acquired by the titration of 25 ml 0.1 N potassium dichromate with Mohr's salt until the colour become bronzing. Cellulose content (x) were calculated according to the formula: $x = 0.675 \times k(a - b)/n$ (detailed information about a , b , n and k were listed above). Six samples were taken from WT and transgenic plants.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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