




The Gibberellic Acid-Stimulated Transcript Gene Family in Moso Bamboo: A Genome-Wide Survey and Expression Profiling During Development and Abiotic Stresses

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Abstract

Gibberellic acid-stimulated transcript (GAST) proteins are widely distributed among plant species and are involved in various aspects of plant growth, plant responses to stress, and hormone crosstalk. However, in moso bamboo (*Phyllostachys edulis*), bioinformatics data and functional information on the GAST family are limited. Here, a total of eight *GAST* genes were first identified in the whole genome of moso bamboo and then clustered into three groups based on phylogenetic analysis. Each clade is well supported by the conserved intron/exon structures and motifs, suggesting that the members within the same subfamily perform similar functions. A number of development-, hormone-, and stress-related elements are randomly distributed in the promoter sequences of *PheGASTs*. Tissue expression analysis indicates that most *PheGASTs* might be associated with moso bamboo flower development and rapid shoot growth. Furthermore, the *PheGASTs* display phytohormone- and stress-inducible expression patterns in response to various circumstances (abscisic acid, gibberellins, cold, drought, and salinity). Among these, *PheGASR1* was assumed to have a distinct regulatory role in rapid shoot growth and abiotic stress responses involving the ABA pathway. Collectively, our results provide extensive insights into the *GAST* gene family, thereby contributing to the screening of additional candidate genes in moso bamboo.

Keywords Moso bamboo · *GAST* gene family · Genome-wide analysis · Tissue development · Hormone crosstalk · Abiotic stress treatment

Introduction

Gibberellic acid-stimulated transcript (GAST) proteins are widely distributed among plant species and comprise three distinct domains: (1) a putative signal peptide of 18–29 residues; (2) a variable region displaying high levels of divergence among family members in terms of amino

acid composition and sequence length; and (3) a C-terminal region of approximately 60 amino acids containing 12 cysteine residues at conserved positions, which is also known as the GASA domain (Aubert et al. 1998). To date, our understanding of the mode of action of GAST proteins is limited, although studies have suggested that the cysteine residues play a central role in their antioxidant activity (Ben-Nissan et al. 2004; Rubinovich and Weiss 2010; Rubinovich et al. 2014).

Most *GAST* genes are regulated by gibberellins (GAs) as well as by other hormones, such as abscisic acid (ABA), auxins (IAA), and brassinosteroids (BRs) (Zhang and Wang 2008; Wang et al. 2009; Liu et al. 2013; Sun et al. 2013; Lee et al. 2015; Jie et al. 2016). The *GAST* genes are also involved in various biological processes, including plant development and abiotic stress responses. For example, in strawberry, *FaGAST* is upregulated in the fruits and roots but downregulated in leaves and stolons, and its overexpression in transgenic *Fragaria vesca* causes a delay in growth and a reduction in fruit size (de la Fuente et al. 2006). Moreover,

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ectopic expression of *FsGASA4* enhances salt, oxidative, and heat stress tolerance during seed germination in transgenic *Arabidopsis thaliana* by increasing salicylic acid (SA) biosynthesis (Alonso-Ramirez et al. 2009). Expression analyses indicate that the spatial and temporal regulation of *GAST* genes is highly specific, each displaying a distinct expression pattern, and their expression is mostly correlated with young tissues and actively growing organs (Zhang and Wang 2008; Zimmermann et al. 2010; Nahirñak et al. 2016). Overall, the *GAST* family participates in hormone signaling pathways that modulate hormonal responses and levels, as well as in a variety of plant developmental processes and environmental responses, thereby suggesting that these play essential functions in plants (Nahirñak et al. 2012).

China has extensive bamboo resources and a large bamboo industry, with a natural bamboo forest area of 6,010,000 ha. The total area of moso bamboo forest accounts for about 74% of the total area of bamboo forests in China, reaching about 4,430,000 ha. The annual felling of moso bamboo is 500 million–6 billion trees, and the output of various bamboo woods is about 4,500,000 tons, thereby providing valuable materials for the development of industrial and agricultural production, as well as cultural events in China (Wu et al. 2015). Additionally, moso bamboo is characterized by a prolonged vegetative phase before it flowers and a rapid shoot growth rate (as fast as 1 m/day at peak growth, reaching a height of 20 m in 45–60 days) (Peng et al. 2013a). GAs are essential endogenous hormones that play vital roles in controlling growth and a wide variety of developmental processes in plants. Treatment of moso bamboo seedlings with exogenous GA₃ induces elongation of the culm internode and an increase in plant height, blade quality, and cell length (Jiang et al. 2016). Furthermore, during the rapid shoot growth phase, the shoot apical meristem (SAM) region produces high amounts of total GA₃, and temporal changes in concentrations of endogenous GA₃ are generally bimodal in different parts of the shoots (bottom, middle, and top) (Gamuyao et al. 2017; Li et al. 2017). A total of 57 genes that are involved in the GA pathway are differentially expressed during rapid shoot growth (Peng et al. 2013b). During the reproductive growth phase, GA metabolism genes related to flower development, such as *GAMYB*, *GID1*, and *GID2*, are upregulated at four different periods of flower development (Gao et al. 2014; Ge et al. 2017). The *GAST* family may act as downstream members of the DELLA protein family in the GA pathway (Zhang and Wang 2008). Our team has been investigating the functional roles of some GA-related genes (unpublished data), but the role of the *GAST* family is still not well understood. The present study performed genome-wide identification of the *GAST* family, followed by detailed bioinformatics analysis. Moreover, expression patterns of the *PheGAST* genes were also investigated during flower and shoot development, as well as

in response to hormones (GA and ABA) and abiotic stresses (salt, drought, and cold). Thus, the goal of this study was to elucidate the role of the *GAST* gene family in moso bamboo tissue development, as well as in response to abiotic stress.

Materials and Methods

Identification of Putative Genes That Encode *GAST* Proteins

The *GAST* genes were searched in the bamboo genome database (BambooGDB, <http://www.bamboogdb.org/>) (Peng et al. 2013a) using known plant *GAST* sequences of a nucleotide database (<https://www.ncbi.nlm.nih.gov/nucleotide>) as query. The aligned result was filtered by searching Conserved Domain Database (<https://www.ncbi.nlm.nih.gov/guide/domains-structures/>). Only the gene models that contained intact GASA domains and hits with *E* values < 0.001 were identified as members of the *PheGAST* family. We also referred to the database of full-length non-chimeric (FLNC) reads (<http://www.forestrylab.org/db/PhePacBio/>), searching putative novel or mis-annotated *GAST* genes. To obtain a more comprehensive overview on *PheGASTs*, the *GAST* genes of *Zea mays*, *Oryza sativa*, *A. thaliana*, and *Brachypodium distachyon* were identified based on previous studies (Zhang and Wang 2008; Zimmermann et al. 2010; Nahirñak et al. 2016) and downloaded from Phytozome v11.0 (<http://www.phytozome.net/>). The sequences of *Petunia hybrida* *GIP1*, wheat *TaGASR7-A1*, and potato *GAST1* were obtained from the protein database (<https://www.ncbi.nlm.nih.gov/protein/308535464>).

Phylogenetic Reconstruction

Multiple sequence alignment of the full-length protein sequences of *PheGASTs* was performed using Clustal X 2.1, and neighbor-joining (NJ) and maximum likelihood (ML) phylogenetic trees were constructed using MEGA 7.0 with 1000 bootstrap replicates, respectively. To compare the evolutionary relationship and the predicted functional roles of *PheGASTs*, phylogenetic trees were also constructed using the amino acid sequences of moso bamboo and other species using the same method. To support subgroup designations during phylogenetic analysis, the intron pattern of moso bamboo *GAST* family was displayed using the Gene Structure Display Server (GSDS, <http://gsds.cbi.pku.edu.cn/index.php>) by aligning the cDNA sequences to their corresponding genomic DNA sequences. The conserved motifs of *PheGAST* proteins were defined by Multiple Em for Motif Elicitation (MEME, <http://meme-suite.org/>) (Bailey et al. 2009). The following parameter settings were used: distribution of motifs, zero or one per sequence; maximum

number of motifs to find, 50; minimum width of motif, six; and maximum width of motif, 100. Only motifs with an E value of $< 1e-20$ were retained for further analysis. Subsequently, the MAST program (<http://meme.nbcrl.net/meme/cgi-bin/mast.cgi>) was used to search detected motifs in protein databases (Bailey and Gribskov 1998).

Calculation of Non-synonymous (K_a)/Synonymous (K_s) Values

To further analyze gene duplication events, paralogous and orthologous gene pairs were aligned using Clustal X 2.1 and analyzed using DnaSP software to calculate the non-synonymous (K_a) and synonymous (K_s) substitution rates (Rozas 2009). The divergence time (T) for moso bamboo, rice, and *B. distachyon* was calculated using the following equation: $T = K_s / (2 \times 6.5 \times 10^{-9})$ MYA (Wu et al. 2016). Sliding window analysis of K_a/K_s ratios was performed using SWAKK (<http://ibl.mdanderson.org/swakk/>), with the following parameters: window size 60 and 90 bp.

In Silico Analysis of Cis-Acting Elements of *PheGASTs*

The elements in the promoter fragments (from –1500 bp to the transcription start site) of the *PheGASTs* genes were analyzed using the online program PlantCARE (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>).

Plant Materials and Treatments

Moso bamboo seeds used in our experiments were obtained from Guilin City, Guangxi Province, China. These were germinated on moist germination paper in culture dishes in the dark at room temperature (25 °C), and the seedlings were transferred into a greenhouse at 25 °C under a 16/8-h light/dark cycle as previously described (Chen et al. 2017). These were planted in vermiculite and watered with 1/2 strength Hoagland's nutrient medium once a week. Until 2 months of age, the young seedlings were subject to the hormones and abiotic stresses, and *GAST* gene expression was assayed.

For the hormone treatments, the seedlings were watered with GA₃ (100 μM; Sigma) and ABA (100 μM; Sigma) solution as previously described (Nahirñak et al. 2016), respectively. For low-temperature stress, the plants were transferred to a growth chamber at 4 °C under the same light and photoperiod conditions (16 h light/8 h dark). For salinity and drought stress, the seedlings were watered with 1/2 Hoagland's solution with 200 mM NaCl and 20% polyethylene glycol 6000 (PRG 6000), respectively. The samples at low-temperature and normal environment were also treated simultaneously with 1/2 strength Hoagland's solution. The second or third young leaves were collected at different time points (0, 1, 6, and 9 h for hormone treatments; 0, 3, 12,

48 h, and 6 days for abiotic stress treatments; and 0, 1, 3, 6, 9, 12, 48 h and 6 days for mock-treated samples), with three biological replicates for RNA preparation. All leaf samples were immediately frozen in liquid nitrogen and stored at –80 °C until further analysis.

For tissue-specific analysis, the Illumina RNA-seq data (Peng et al. 2013b; Gao et al. 2014; Li et al. 2017) of developing flowers and shoots of moso bamboo were used for expression profiling of the *PheGASTs*. Four stages of flowering moso bamboo were defined based on the anatomical structure of the floral organs (F1, F2, F3, and F4). At stage F1, the floral buds begin to form. At stage F2, the floral organs gradually mature but do not undergo flowering. At stage F3, the flowers are in full blossom. At stage F4, pistils and stamens have withered, and embryo formation commences (Gao et al. 2014). Seven developmental stages of moso bamboo shoots were selected, including winter shoots (underground period) and six shoot heights (0.5, 1, 3, 6, 9, and 12 m), which were designated as S1, S2, S3, S4, S5, S6, and S7, respectively (Li et al. 2017).

Gene Expression Analysis

Previously generated and processed transcriptome data of *PheGASTs* in developing flowers and shoots were used in transcriptomics analysis. The reads per kilobase of exon model per million mapped reads (RPKM) units representing the expression in developing flowers were obtained from the NIH Short Read Archive (SRA) database (<https://www.ncbi.nlm.nih.gov/sra/?term=SRR1187864>). For developing shoots, the FPKM data of different stages were provided by Li et al. (2017). The RPKM/FPKM values of all *GAST* genes were retrieved and displayed in Table S5 as previously described (Gao et al. 2014; Li et al. 2017).

The expression profiles of *PheGASTs* in different hormones and abiotic stresses treatments were analyzed by quantitative RT-PCR (qRT-PCR). Each 20-μL reaction contained 0.4 μL (10 μM) of each primer, 1.5 μL (30 ng) of cDNA, 7.7 μL H₂O, and 10 μL of SYBR Green I master mix (Roche, Mannheim, Germany), according to the manufacturer's instructions. The qRT-PCR parameters were as follows: 95 °C for 5 min, followed by 45 cycles of 95 °C for 10 s, 60 °C for 10 s, and 72 °C for 20 s. The gene-specific primers were designed using Primer 3.0, and their specificity was checked with BLAST using information provided by the local CDS database that was downloaded from BambooGDB (<http://www.bamboogdb.org/page/microna.jsp>) (Chen et al. 2017). The primers used for qRT-PCR and the melting curves of the PCR products are shown in Online Resources 1 and 2, respectively. The tonoplast intrinsic protein 41 gene (*TIP41*) was used as reference gene under different stress treatments (Fan et al. 2013; Qi et al. 2013; Wu et al. 2015, 2016). To ensure the reliability of the data, another reference

of nucleotide tract-binding protein gene (*NTB*) was also used to validate the expression patterns (Fan et al. 2013; Sun et al. 2016). The qPCR assays were performed with three biological and four technical replicates, and quantitative analysis was performed using the $2^{-\Delta\Delta C_t}$ method, whereas the untreated control (0 h) were normalized to 1 as described elsewhere (Chen et al. 2017). Finally, the statistical analyses were performed using SPSS 19.0 software.

Results

Identification, Scaffold Location, and Structural Analysis of Moso Bamboo *GAST* Genes.

A total of eight *GAST* genes in moso bamboo were finally identified based on homology to members of the GASA family from other dicots or monocots, which were further confirmed by the Conserved Domain Database for a complete GASA domain. Moreover, no novel and mis-annotated genes were detected in the FLNC database (Wang et al. 2017). To obtain a more comprehensive overview on the *PheGAST* genes, we also searched for *GAST* homologs in rice, maize, *A. thaliana*, *B. distachyon* and *P. hybrida* *GIP1*, wheat *TaGASR7-A1*, and potato *GAST1*. The moso bamboo *GAST* genes were named according to their closest rice and maize relatives (*PheGAST1*, *PheGASR2*, *PheGSL9*, *PheGASR1*, and *PheGSL8*), and others were numbered in sequence when no ortholog was available (*PheGAST2*, *PheGAST3*, and *PheGAST4*). All non-redundant *PheGASTs* were mapped and found to be distributed among eight scaffolds of the *P. edulis* genome (Table 1). Each scaffold only harbored one gene, unlike those in maize and rice (Zimmermann et al. 2010).

Similar to other species (Zhang and Wang 2008; Zimmermann et al. 2010; Nahirñak et al. 2016), most *PheGASTs* contain an individual stretch of 19–29 amino acids at the N terminus and a conserved GASA domain with 12 cysteines (Cys, C) at its C-terminal (Fig. 1a). However, a

base substitution was detected at the first three C residues in *PheGAST2* and the ninth C residue in *PheGASR2*, respectively. For *PheGAST2*, the first C residue was deleted, and the other two positions were substituted to alanine (Ala, A). For *PheGASR2*, the ninth C residue was substituted by glycine (Gly, G).

To better mine the gene structure and conserved motifs among moso bamboo *GASTs*, we constructed NJ and ML phylogenetic trees using amino acid sequences (Fig. 1b and Online Resource 3), respectively. These showed good agreement with the same classification, dividing *PheGASTs* into three subfamilies with at least 50% bootstrap support. Among these, the *PheGASR2* was grouped alone in both phylogenetic trees. Figure 1c shows that all *PheGAST* genes are disrupted by one (*PheGAST2*, *PheGSL8*, *PheGSL9*, and *PheGASR1*) to three (*PheGAST4*) introns. Remarkably, genes in the same subfamily generally show the similar intron pattern; for example, *PheGSL9* and *PheGASR1* contain a single intron (that is, intron 1), whereas *PheGAST1* and *PheGSL8* lack intron 2. Ten conserved motifs were also detected within the *PheGAST* proteins (Fig. 1d), with the exception of a putative signal peptide and GASA domain (motifs 1, 2, 3, and 4), and some *PheGASTs* have the same motif in their variable regions, but the functional roles of these motifs remain unclear.

Phylogenetic Relationship of *PheGAST* Proteins

To investigate the evolutionary relationship among members of the GASA family, NJ and ML phylogenetic trees were constructed based on the full-length protein sequences of all known GASA members in *P. edulis*, *Z. mays*, *O. sativa*, *A. thaliana*, and *B. distachyon*, as well as the *TaGASR7-A1* from wheat, the *GIP1* from a *P. hybrid*, and the *StGAST1* from potato (Fig. 2 and Online Resource 4). The resulting phylogenetic tree is organized into four subfamilies, namely, *PheGASR2* in subfamily 1, *PheGAST1* and *PheGSL8* in

Table 1 Identification of *GAST* gene family members in *P. edulis*

Gene name	BambooGDB assembly name	Scaffolds	Protein length (aa)	Signal peptide (aa)	C-Terminal Cys-rich domain position
<i>PheGAST1</i>	PH01000140G0260	PH01000140	112	YES (26)	51–112
<i>PheGSL8</i>	PH01002500G0080	PH01002500	107	YES (20)	46–107
<i>PheGSL9</i>	PH01000073G1210	PH01000073	94	YES (19)	35–94
<i>PheGAST3</i>	PH01000122G0840	PH01000122	107	YES (28)	48–107
<i>PheGAST4</i>	PH01000534G0860	PH01000534	113	YES (29)	54–113
<i>PheGAST2</i>	PH01000727G0250	PH01000727	81	YES (24)	34–81
<i>PheGASR2</i>	PH01000041G1250	PH01000041	105	YES (24)	45–105
<i>PheGASR1</i>	PH01001477G0260	PH01001477	95	YES (24)	33–95

The putative signal peptide was predicted by the SignalP.v4.0 software

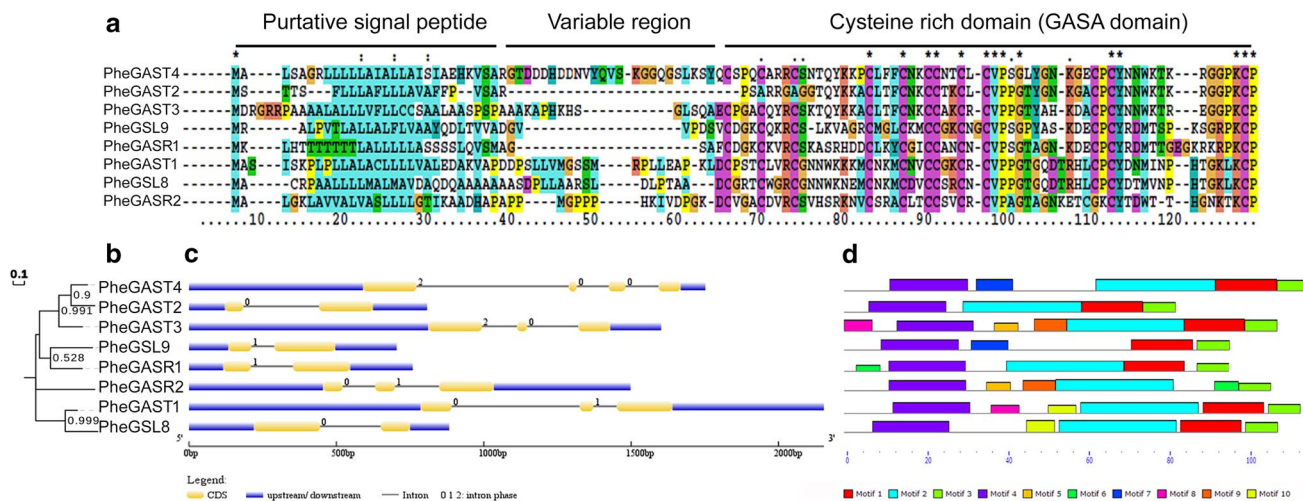


Fig. 1 Sequences alignment and structure analysis of PheGASTs. **a** Multiple alignment of deduced amino acid sequences of PheGAST proteins. Amino acid sequences are aligned using Clustal W software. Color shading shows amino acid identities; the putative signal peptide and conservative cysteine-rich C-terminal domain are labeled. The highly conserve C residues in C-terminal are shaded in purple. **b** Phylogenetic relationships among GAST proteins in moso bamboo. An unrooted phylogenetic tree constructed using the neighbor-joining

method. The branches are reconfirmed by 1000 bootstrap replicates. The bootstrap value of each branch is indicated. **c** Exon–intron organization of the corresponding genes. The exons and introns are represented by yellow boxes and lines, respectively. **d** Distribution of conserved motifs of corresponding genes. Using MEME, the conserved motifs within the protein sequence are represented by boxes. The box size indicates the length of the motif. Gray lines represent the length of the proteins

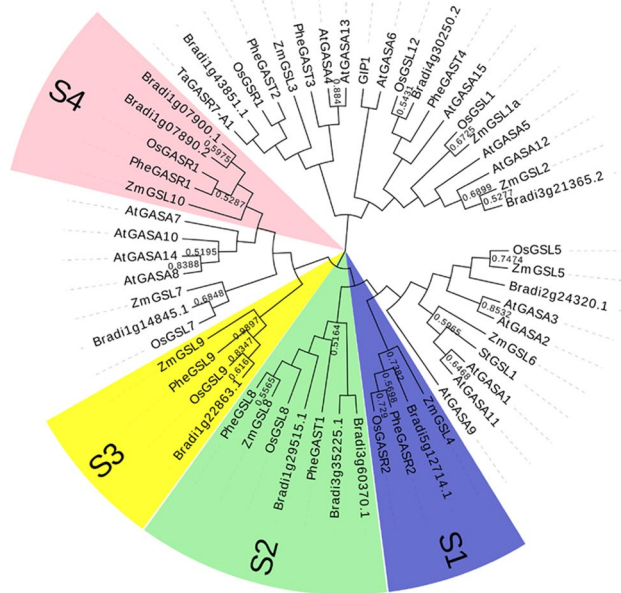


Fig. 2 Phylogenetic analysis of predicted GAST proteins in different species. The unrooted neighbor-joining (NJ) tree was constructed based on the amino acids of moso bamboo (8), *Arabidopsis* (14), rice (9), maize (10), *B. distachyon* (12), *P. hybrida* GIP1, wheat TaGASR7-A1, and potato GAST1 using MEGA7.0 with 1000 bootstrap replicates. Branches with less than 50% bootstrap support are collapsed. The names of the subfamilies (S1, S2, S3, and S4) are shown outside the circle. Branch lines of subtrees are colored based on GAST subfamilies

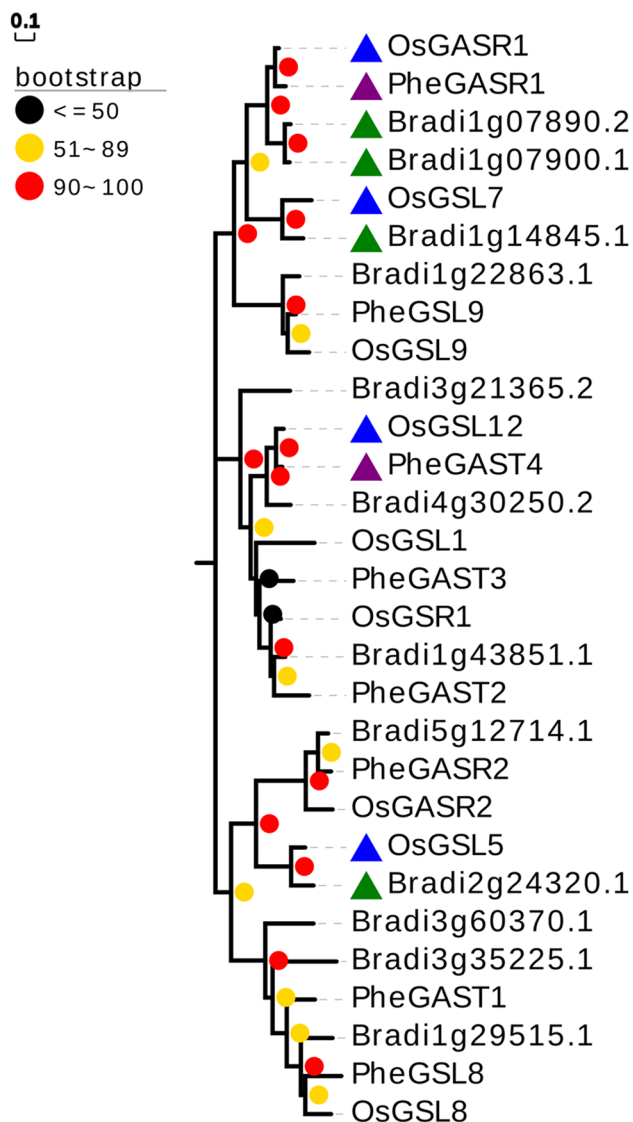
subfamily 2, PheGSL9 in subfamily 3, and PheGASR1 in subfamily 4, with PheGAST2, PheGAST3, and PheGAST4 forming a subfamily specific to moso bamboo (Fig. 1). The subfamilies 1–4 comprise *P. edulis*, *Z. mays*, *O. sativa*, and *B. distachyon*, forming a distinct branch and are thus likely to represent monocot-specific functions. Similar to ZmGASTs (Zimmermann et al. 2010), it was also difficult to assign the PheGASTs to distinct homologs from *A. thaliana*, indicating that some functional roles might have been lost after the divergence of monocots and dicots.

Identification of GAST Orthologs and Paralogs in Moso Bamboo, Rice, and *B. distachyon*

Peng et al. (2013a) earlier showed that bamboo, *B. distachyon*, and rice from the BEP clade (Bambusoideae, Ehrhartoideae, and Pooideae) are more closely related than those belonging to the Panicoideae clade (maize, sorghum, and foxtail millet) (Peng et al. 2013a). To better understand the evolutionary relationship of the members of the GAST gene family of this branch, the time of gene duplication and environmental selection pressure of the three species were further investigated. Phylogenetic reconstruction and BLASTP analysis identified two paralogous gene pairs (*Phe–Phe*) and two orthologous gene pairs (*Os–Phe*). Moreover, two orthologous gene pairs (*Os–Bd*) were also observed (Table 2; Fig. 3). Bootstrap support for the internal nodes was relatively lower, which was mainly due to the variable N-terminal region of the GASTs.

Table 2 Divergence between paralogous and orthologous gene pairs

Gene pairs	Gene 1	Gene 2	K_s	K_a	K_a/K_s	Divergent time (MYA)
Phe–Phe	PheGAST4	PheGAST2	0.55	0.74	1.34	42.2
Phe–Phe	PheGAST1	PheGSL8	0.75	0.63	0.84	57.5
Phe–Os	PheGAST4	OsGSL12	0.72	0.7	0.97	55.5
Phe–Os	PheGASR1	OsGASR1	0.38	0.28	0.73	29.25
Os–Bd	OsGSL7	Bradi1g14845.1	0.74	0.81	1.1	56.5
Os–Bd	OsGSL5	Bradi2g24320.1	0.45	0.57	1.28	34.3

**Fig. 3** Duplication and orthologous relationship among *PheGASTs*, *OsGASTs*, and *BdGASTs*. The paralogous and orthologous pairs with high similarity (red circle) are labeled by triangles of different colors: purple for moso bamboo, blue for rice, and green for *B. distachyon*

Subsequently, K_a/K_s ratios were assessed to verify Darwinian positive selection (Table 2). Interestingly, the K_a/K_s ratios of the two *Phe–Phe* pairs differed, with K_a/K_s above 1 for *PheGAST4/PheGAST2* and K_a/K_s below 1 for *PheGAST1/PheGSL8*. The K_s values of *PheGAST4/PheGAST2* and *PheGAST1/PheGSL8* were 0.55 and 0.75, respectively, indicating that their divergence time was about 42 and 57 million years ago (MYA), respectively. Additionally, the K_s values of the orthologous pairs of *Phe–Os* were both less than 1, and their divergence time was about 29 and 55 MYA, with a mean of 42 MYA. Afterwards, the divergence time for the *Os–Bd* pairs was also calculated based on the K_s values, with a mean of about 40 MYA.

Furthermore, sliding window analysis of the K_a/K_s ratios was conducted for pairwise comparison (Fig. 4a). The motifs representing a cleavable signal sequence (motif 4) and the conserved C-terminal region with a GAST domain (motifs 1, 2, and 3) were described, respectively (Fig. 4b). Regardless of the paralogous pairs of *Phe–Phe* or orthologous pairs of *Os–Phe*, the K_a/K_s ratios were less than 1. It gradually decreased from the N-terminal to the C-terminal, and roughly remained the same at the motif 2 region.

Promoter Analysis of *PheGASTs*

Although the exact role played by the Snakin/GAST proteins remains elusive, these have been associated with different aspects of plant development and stress tolerance (Nahirniak et al. 2016). To identify the *cis*-acting elements in the promoters of moso bamboo *GAST* genes, we searched the 1.5-kb upstream sequences from the translation start sites using the PlantCARE database. Hormone-, stress-, and development-related elements were randomly distributed upstream of the *PheGASTs* (Fig. 5a, b), which may be responsible for the wide range of functions of this protein family. Among these, the jasmonic acid (CGTCA-motif)-, abscisic acid (ABRE)-, gibberellin (P-box and GARE-motif)-responsive elements were found within the promoter regions of 6, 6, and 3 *PheGASTs*, respectively. These were all present within the promoters of four genes, namely, *PheGASR1*, *PheGASR2*, *PheGAST1*, and

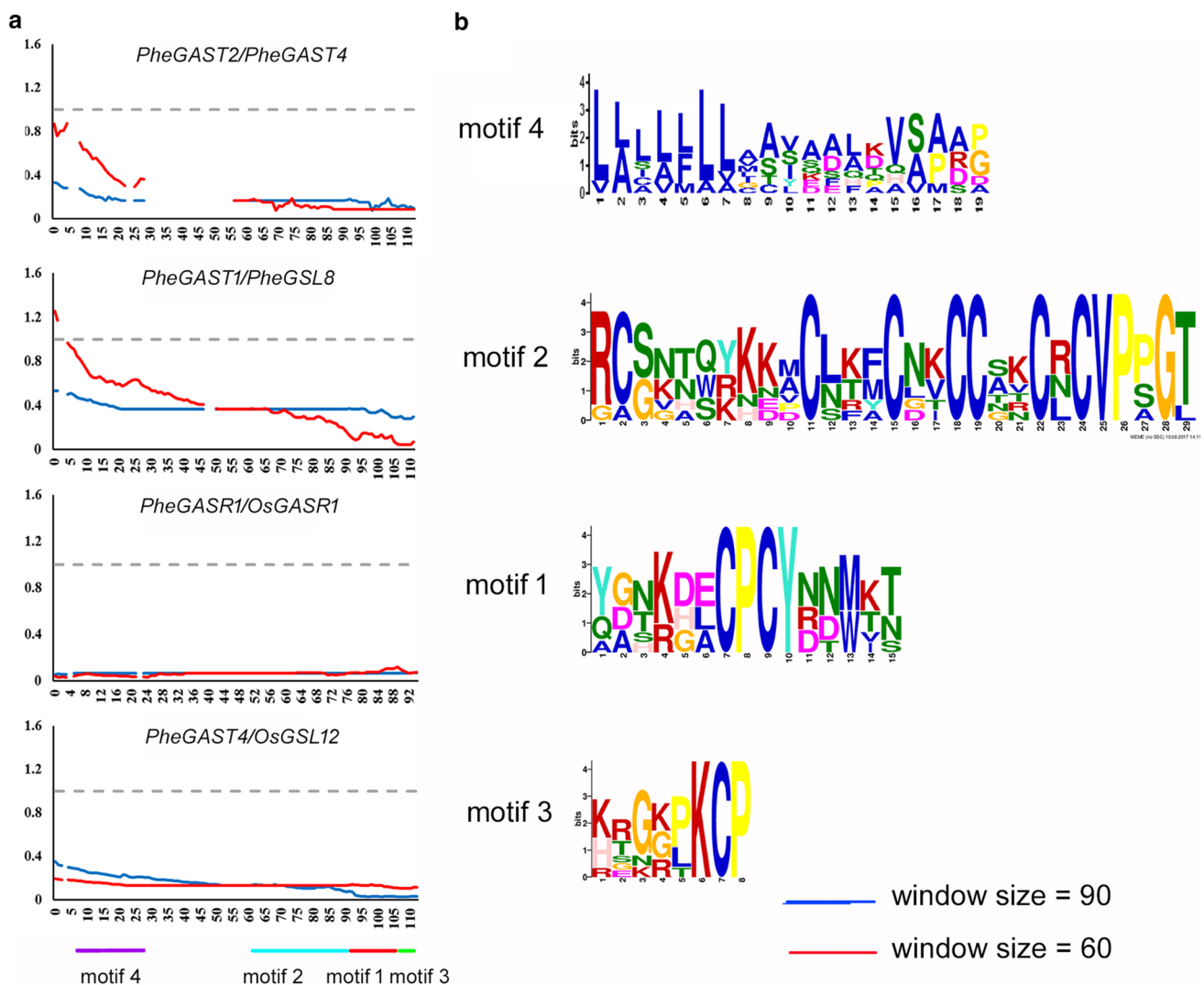


Fig. 4 Sliding window plots of representative duplicated *PheGAST* genes and orthologous of *Phe-Os*. **a** The characterized motifs are noted by different colored lines. The window sizes were 90 and 60 bp

PheGAST4. The element involved in anoxic-specific inducibility (GC-motif) was abundant and mainly distributed upstream of the four genes, whereas the other stress-related elements such as defense- and stress-responsive elements (TC-rich repeats), heat-responsive elements (HSE), low-temperature-responsive elements (LTR), and drought-inducible elements (MBS) were also detected. Moreover, with the exception of *PheGAST2*, all the other *PheGASTs* have at least three stress-related elements within their promoters. Additionally, five *PheGAST* promoters also had binding sites for transcription factors that were related to tissue development, such as the elements involved in seed-specific regulation (RY-element), endosperm expression (Skn-1_motif), and meristem-specific activation (CCG TCC-box).

in length, respectively. **b** The logo of each motif. The motifs, numbered 1–4, are displayed in different colored lines

Expression Patterns of *PheGAST* Genes During Shoot and Flower Development

We analyzed the expression patterns of all *PheGAST* genes in the flowers and shoots based on publicly available RNA-seq data (Peng et al. 2013b; Li et al. 2017) and a heatmap was generated (Fig. 6 and Online Resource 5). We also selected four genes from different groups (*PheGAST2*, *PheGSL8*, *PheGASR1*, and *PheGASR2*) to verify transcripts levels using qPCR (Online Resource 6). These genes exhibit similar expression patterns similar to previous RNA-seq data, thereby suggesting that our transcriptome results are highly reliable. It shows that all *PheGAST* genes present similar expression patterns during flower development in moso bamboo (Fig. 6a). These are upregulated during the earlier stages (F1 and F2) and

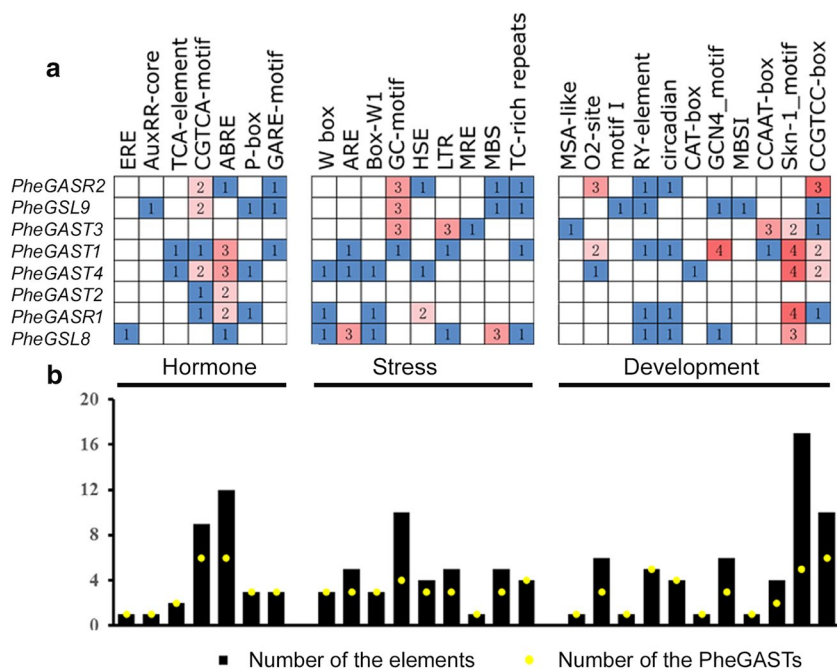


Fig. 5 The *cis*-elements distributed in the promoters of *PheGASTs*. **a** The number of *cis*-acting elements in the promoter region of each *PheGAST* gene. Annotation of *cis*-elements: *ERE* ethylene-responsive element, *AuxRR-core* auxin-responsive element, *TCA-element* involved in salicylic acid responsiveness, *CGTCA-motif* involved in MeJA responsiveness, *ABRE* involved in abscisic acid responsiveness, *GARE-motif* and *P-box* gibberellin-responsive element, *W box* binds WRKY type transcription factors and wounding and pathogen responsiveness. *ARE* essential for the anaerobic induction, *Box-W1* fungal elicitor responsive element, *GC-motif* enhancer-like element involved in anoxic-specific inducibility, *HSE* *cis*-acting element involved in heat stress responsiveness, *LTR* involved in low-temperature responsiveness, *MRE*, *MBS*, and *MYB* binding sites involved

in light responsiveness and stress inducibility, *TC-rich repeats* involved in defense and stress responsiveness, *MSA-like* *cis*-acting element involved in cell cycle regulation, *O2-site* *cis*-acting regulatory element involved in zein metabolism regulation, *motif I*, *cis*-acting regulatory element root specific, *RY-element* *cis*-acting regulatory element involved in seed-specific regulation, *circadian* involved in circadian control, *CAT-box* related to meristem expression, *Skn-1 motif* and *GCN4_motif* *cis*-regulatory element involved in endosperm expression, *MBS* MYB-binding site involved in flavonoid biosynthetic gene regulation, *CCAAT-box* MYBHv1-binding site, *CCGTCC-box* related to meristem-specific activation. **b** The number of *cis*-elements and the *PheGAST* genes containing *cis*-acting elements

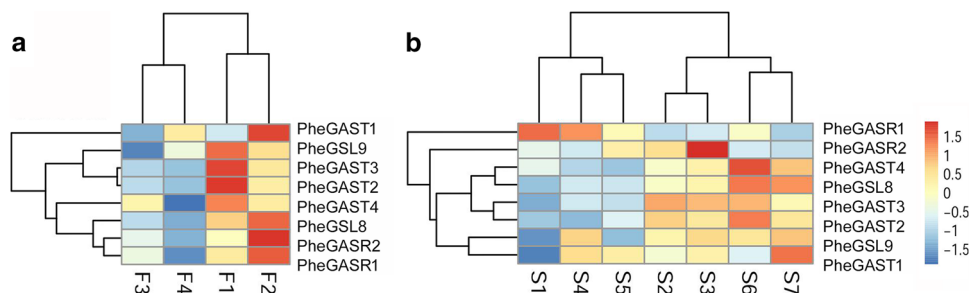


Fig. 6 Expression pattern of *PheGASTs* genes in developing flowers and shoots of moso bamboo. **a** The expression profile of *PheGASTs* in different stages of flowering, *F1* The floral bud formation stage. *F2* The inflorescence growing stage. *F3* The flowers with both pistils and

stamens emerging from glumes at bloom stage. *F4* The flowers are withered. **b** The expression profile of *PheGASTs* at different shoot growth stages. *S1–S7* represent winter bamboo shoot and different heights of shoots (50, 100, 300, 600, 900, and 1200 cm)

downregulated in the later stages (*F3* and *F4*). Among these, the expression level of *PheGASR2* and *PheGSL8* decreases by more than 10-fold in the *F4* period, and *PheGAST2* is hardly detected in the last stage (Table S5). For shoot growth, most *PheGAST* genes show twin peaks

during the early stages (*S2* and *S3*) and late stages (*S6*, *S7*), respectively (Fig. 6b). Interestingly, *PheGASR1* is highly expressed throughout the whole growth process of the bamboo shoots (FPKM > 60), which peaks at periods *S1* and *S4*, respectively (Fig. 6b).

Regulation of the *PheGAST* Genes Expression

As earlier mentioned, most *GAST* genes are regulated by plant hormones and external factors. Here, *in silico* analyses also revealed that the upstream sequences of most *PheGAST* genes carry a variety of hormones and stresses response elements (Fig. 5). Compared to the untreated samples, the expression of the *PheGAST* genes significantly changes with hormone and abiotic stress treatment (Figs. 7, 8). We

initially analyzed the effect of GA₃ and ABA on the expression pattern of the *PheGASTs* (Fig. 7). We found that all *PheGASTs*, except for *PheGAST3*, are upregulated by GA₃ treatment. Some show similar expression profiles (*PheGAST1*, *PheGAST4*, and *PheGSL8*), which were induced by exposure to GA₃ for 1 and 6 h and repressed after 9 h. However, the paralogous pair *PheGSL9* and *PheGASR1* presents double peaks and is upregulated by 1 and 9 h of GA₃ treatment, respectively. For ABA treatment, five *PheGAST*

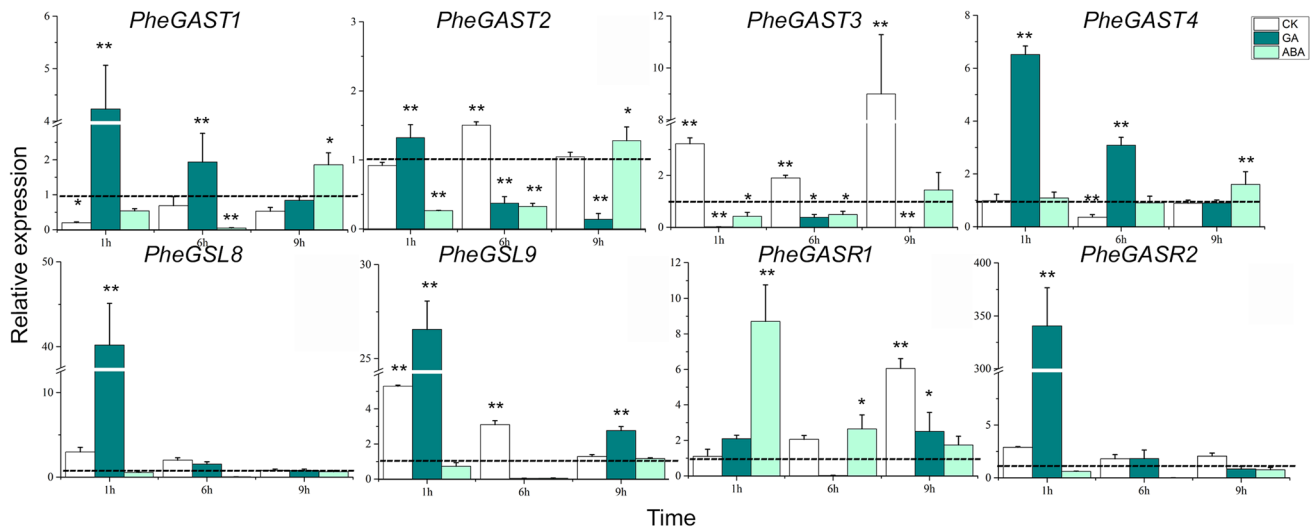


Fig. 7 Expression analysis of the *PheGAST* genes in the leaves of moso bamboo under mock, GA, and ABA treatments. Bars represent standard deviations (SDs) of three technical replicates. *Represents a significant difference relative to the control (0 h). The X axes show

the time points of GA and ABA treatments for each gene (0–9 h). The Y axes show the relative expression levels (fold-change). The control expression at 0 h was standardized by 1, which is represented by broken lines. The *TIP41* gene is used as reference

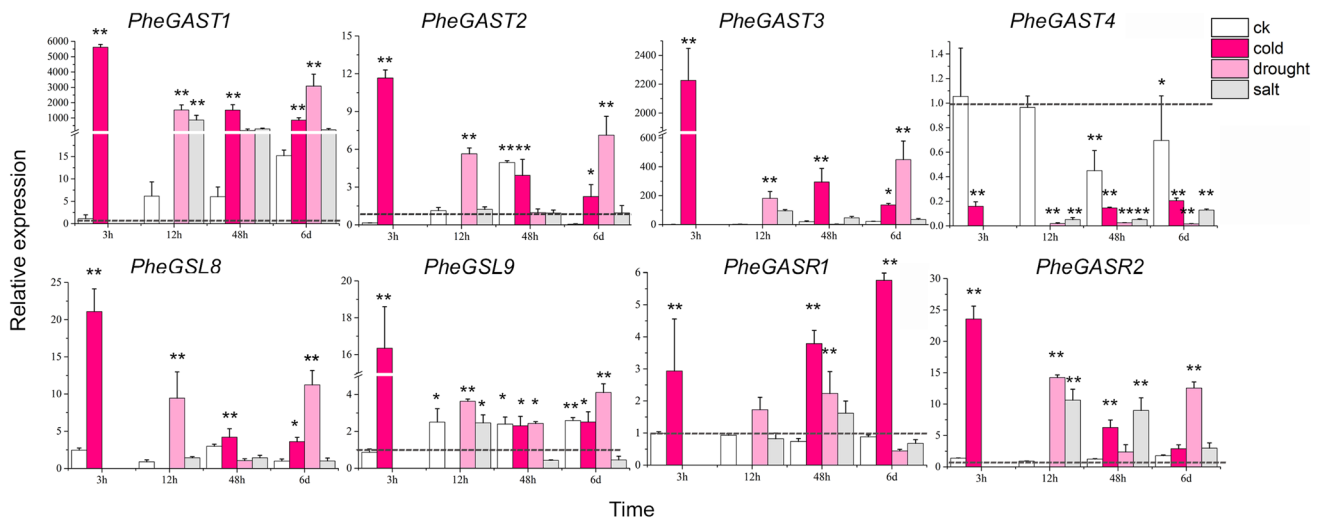


Fig. 8 Expression analysis of the *PheGAST* genes in the leaves of moso bamboo under mock and different abiotic stress treatments. Bars represent standard deviations (SDs) of three technical replicates. *Represents a significant difference relative to the control (0 h). The

X axes show the time points of cold, drought, and salt treatments for each gene (0–6 days). The Y axes show the relative expression levels (fold-change). The control expression at 0 h was standardized by 1, which is represented by red lines. The *TIP41* gene is used as reference

genes were upregulated, and most of their expressions were initially downregulated (1 and 6 h) and then upregulated after 9 h of exposure. Interestingly, *PheGASR1* showed a specific expression pattern. It was significantly induced at 1 h after ABA treatment and upregulated during the rest of the time points.

We further analyzed the expression profiles of the *PheGASTs* during different abiotic stress conditions (that is, salt, drought, and cold; Figs. 8, 9). Except for *PheGAST4*, the expression of all *PheGAST* genes was induced by at least one stress treatment to varying degrees (Fig. 8). We found most *PheGASTs* (*PheGAST1*, *PheGAST2*, *PheGAST3*, *PheGSL8*, and *PheGASR2*) share a similar expression pattern regardless of cold, drought, or salt condition (Fig. 9). For example, these were upregulated at 3 h, but gradually downregulated after 48 h and 6 days of cold treatment (Fig. 9a). With drought treatment, these genes were initially upregulated (after 3 h), subsequently rapidly downregulated (48 h), and then significantly upregulated again after 6 days (Fig. 9b). Their expression was downregulated with salt treatment (Fig. 9c). However, *PheGASR1* exhibits a unique expression pattern under all stress treatments; it is upregulated under low temperature and downregulated under drought conditions. It also peaked at 48 h after salt treatment. This characteristic response model under abiotic stress distinguishes *PheGASR1* from the other *PheGAST* genes.

Additionally, all *PheGAST* genes were validated using another reference gene, *NTB*. The expression levels of some genes varied, but the similar expression patterns were observed with hormone and stress treatment (Online Resources 7 and 8). The results on the DEGs further strengthens the data generated using *TIP41*.

Discussion

In the present study, a total of eight *PheGAST* genes were identified and characterized for the first time from the moso bamboo genome database (Peng et al. 2013a; Zhao et al. 2014). Recently, Wang et al. (2017) used a combination of single-molecule long-read sequencing technology and polyadenylation site sequencing (PAS-seq) to reannotate the bamboo genome, resulting in the correction of 2241 mis-annotated genes and identification of 8091 previously unannotated loci, significantly improving current annotations (Wang et al. 2017). We further consulted and searched for *GAST* genes based on the FLNC reads database; however, no novel or erroneous genes were identified. Therefore, at least eight *PheGAST* genes were finally established, forming a *GAST* family in moso bamboo, and their details are described here for the first time, including their gene structure, phylogeny, conserved motifs, promoter *cis*-elements, and regulation of expression.

All moso bamboo *GAST* family members have structural characteristics typical of secreted polypeptide molecules. Each putative protein contains an N-terminal hydrophobic region consisting of 18–29 amino acids that could act as a cleavable signal sequence (Nahirňak et al. 2016). Downstream of the putative signal peptide, a variable region and a conserved C-terminal domain were defined. Generally, the cysteines of the C-terminus are essential to their biochemical activity and are responsible for their protein structure (Zimmermann et al. 2010; Liu et al. 2013; Nahirňak et al. 2016). Interestingly, different mutations in the moso bamboo *PheGAST2* and *PheGASR2* domains were detected (Fig. 1a). Previous studies have suggested that these proteins play a role in reactive oxygen species scavenging due to the presence of these redox-active cysteines (Wigoda et al. 2006; Rubinovich and Weiss 2010). In plants and bacteria, the replacement of four of the conserved cysteines with alanine residues in *AtGASA4* results in the inhibition of antioxidant

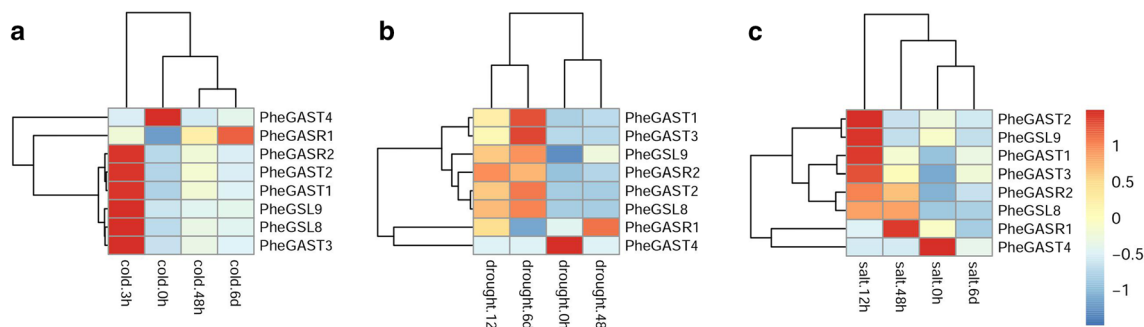


Fig. 9 The expression pattern of the *PheGAST* genes in the leaves of moso bamboo under different abiotic stress treatments. **a** The expression profile of *PheGASTs* during cold treatment. **b** The expression

profile of *PheGASTs* during drought treatment. **c** The expression profile of *PheGASTs* during salt treatment

activity (Rubinovich and Weiss 2010). This suggests that the redox activity of *PheGASR2*, particularly *PheGAST2*, is probably limited by mutations; however, other factors influencing this particular biological activity remain unclear.

Similar to the findings of a previous study (Peng et al. 2013a), phylogenetic reconstruction reveals a higher degree of homology among moso bamboo, rice, and *B. distachyon* GAST family proteins. Selection plays an important role in shaping gene families, resulting in different evolutionary patterns among gene families or even within a single gene family (Zhang et al. 2016). K_a/K_s ratios reflect the selection pressure that is experienced by a gene pair. A K_a/K_s ratio of less than 1 is indicative of purifying or negative selection, $K_a/K_s = 1$ suggests neutral selection, and K_a/K_s greater than 1 reflects positive selection (Lynch and Conery 2000). The findings of the present study indicate that the evolution of the *PheGAST* family may have initially involved positive selection, followed by purifying selection. Additionally, Peng et al. (2013a) reported a putative recent whole genome duplication (WGD) event occurring 7–12 MYA based on analyzing the evolutionary history of *P. edulis*, and the estimated times for the divergence of bamboo from *B. distachyon* and rice are approximately 46.9 and 48.6 MYA, respectively (Peng et al. 2013a). The time of division for *GAST* genes in rice and moso bamboo was established at a mean of around 42 MYA, suggesting that the *PheGAST* family underwent further evolution after its divergence from rice. However, the duplication event involving *GASTs* in moso bamboo occurred between around 42 and 57 MYA, long before the recent WGD event, indicating that the recent WGD event is not the main reason for the expansion of the *PheGAST* family in moso bamboo (Peng et al. 2013a). Together, we propose that the *PheGAST* family in moso bamboo completed its expansion following the divergence of other grasses at about 50 MYA, and the recent WGD event is not the primary cause of the duplication of *PheGAST* genes (Kellogg 1998; Yu et al. 2002; Guyot and Keller 2004; Gaut 2010).

Moso bamboo is famous for its prolonged vegetative growth period and rapid shoot growth rates. Plant hormones, including IAA, cytokinins (CK), ABA, and GAs (Peng et al. 2013b; Gao et al. 2014; Ge et al. 2017; Gamuyao et al. 2017; Li et al. 2017), and their related genes are essential to these developmental processes. *GAST* proteins act as integrators of internal and environmental cues and participate in hormone homeostasis to modulate plant development and stress tolerance. These are also involved in plant developmental processes and stress responses that regulate redox homeostasis through their conserved cysteine-rich domains (Székely 2009; Almasia et al. 2010; Nahirňak et al. 2012; Sun et al. 2013). Therefore, we examined the expression patterns of *PheGASTs* in moso bamboo flowers and in shoot growth processes. For flower development, all *PheGAST*

genes are upregulated during the early stages (F1 and F2) and downregulated during the later stages (F3 and F4). A number of *GAST* genes are correlated with young tissues and actively growing flowers, including *AtGASA1*, 4, 6, and 10 in *A. thaliana* (Aubert et al. 1998; Trapalis et al. 2017), whereas *GASA4* and *GASA6* also affect flowering time (Jie et al. 2016). This indicates that *PheGAST* genes, particularly *PheGAST2*, *PheGSL8*, and *PheGASR2*, which are highly expressed at F1 or F2 (RPKM value > 100), might be associated with floral meristem differentiation and are probably involved in a GA-signaling pathway in regulating flowering time in moso bamboo. However, additional investigations verifying our hypothesis are warranted. Our team has been investigating the phenomenon of rapid shoot growth for several years, accumulating valuable information on spatiotemporal changes in morphology, the production of endogenous hormones, and differentially expressed genes (DEGs) (Peng et al. 2013b; Li et al. 2016, 2017). Endogenous hormones involved in moso bamboo shoot growth display a wide range of variations in expression levels (Li et al. 2017). IAA and BR contents increase from S1 and continue to increase with growth, and the expression level of IAA remains high from S5 to S7 (> 150 ng/g FW). The temporal changes of GA_3 were generally bimodal, occurring as double peaks at the early (S2 and S3) and late (S6) stages (Li et al. 2017). The inhibitory factor ABA is upregulated in S1 (> 150 ng/g FW), transiently decreases, and then increases again from S5 to S7. In our study, the expression patterns of most *PheGASTs* show twin peaks that coincide with variations in endogenous GA_3 levels (Li et al. 2017), thereby suggesting that shoot growth involves a GA-dependent pathway. Gene expression analysis of *PheGASTs* in response to hormones (GA and ABA) further supports our viewpoint. We found that most *PheGASTs* respond to GA_3 signaling in a significant and earlier (1 h) manner. Although these are also upregulated by ABA signaling, induction was observed later (after 9 h of treatment). A similar complex regulation using hormones has been previously reported. For example, the expression of the potato *Snakin-2* gene in shoot apices was induced by GA after 12 and 24 h of treatment and is repressed by GA 24 h after treatment in leaves. In addition, *Snakin-2* gene expression in the shoot apices was induced after 12 h of ABA treatment (Nahirňak et al. 2016). Comparatively, it is more important to evaluate the regulation of *PheGASA* genes at earlier time points because if these are early responsive genes, then more drastic expression changes might be observed. Thus, *PheGSL8*, *PheGSL9*, and *PheGASR2* are upregulated by more than dozens or hundreds of times after 1 h of GA_3 treatment. We also noticed that *PheGAST3* expression was not induced by GA treatment; however, this may be an earlier response factor that was induced before 1 h of treatment. In summary, the results of the present study suggest that GA signaling plays a direct regulatory role in

early responsive *GAST* genes, possibly involving moso bamboo tissue growth and development. Finally, we also investigated the effect of abiotic stresses on the expression of *GAST* genes and observed that all *PheGAST* genes, except for *PheGAST4*, are significantly upregulated in moso bamboo seedlings when subjected to stress, particularly *PheGAST1* and *PheGAST3*. These results also coincide with the participation of the *GAST* genes in biotic or abiotic stress tolerance (Ko et al. 2007; Alonso-Ramirez et al. 2009; Sun et al. 2013; Nahirňak et al. 2016), thereby suggesting their possible positive roles in moso bamboo stress resistance.

The present study assessed the expression pattern of the *PheGASR1* gene in response to ABA signaling as well as in relation to shoot growth and abiotic stress resistance. Our findings may be used in discriminating it from other *GAST* genes. During shoot development, the *PheGASR1* gene shows its highest expression levels at the S1 stage; it then subsequently decreases and peaks again at the S4 stage and remains upregulated until S6 (FPKM > 100). Previous research has shown that the expression pattern of *PheGASR1* is apparently regulated by both endogenous ABA and GA₃ (Li et al. 2017). Moreover, the *PheGASR1* gene rapidly responds to ABA treatment and is upregulated at least eightfold higher after 1 h of ABA treatment. The *PheGASR1* gene also exhibits a distinct expression pattern in response to abiotic stresses, particularly under cold and drought treatment. Phylogenetic reconstruction suggests that rice *OsGASR1* is the ortholog of *PheGASR1* (Fig. 2). *OsGASR1* is related to cell proliferation in meristem and panicle development (Furukawa et al. 2006); moreover, it plays important role in seedling growth as well as in salt and ABA stress resistance (Lee et al. 2015, 2017). The function of *OsGASR1* homologs have also been functionally investigated in other species, such as *GhGAST3* in cotton and *TaGASR1* in wheat, which are involved in fiber elongation and heat stress resistance, respectively (Liu et al. 2013; Zhang et al. 2017). More importantly, *PheGASR1* might be involved in regulating the morphological development of rods of moso bamboo and its mutant lines (unpublished data), which is rarely studied in other species. Above all, the distinct expression pattern and related literature indicate the involvement of *PheGASR1* in growth and stress responses through both GA and ABA pathways. However, the correlation between the transcript regulation and protein expression levels remains unclear. Finally, although our study only indicates the particular regulatory role of *PheGASR1* under limited hormones and stresses treatments, it lays the foundation for future functional research investigations.

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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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