Starch branching enzymes (SBEs) in banana: genome-wide identification and expression analysis reveal their involvement in fruit development, ripening and regulated responses to abiotic/biotic stresses

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Starch branching enzyme (SBE), which is one of the key enzymes associated with amylopectin biosynthesis, plays important roles in variable biological processes. Despite its importance, SBE is rarely studied in the banana (*Musa acuminata* L.) which is a typical starchy fruit. Here, a family of ten SBE proteins (MaSBE) was firstly identified through genome-wide characterization in *M. acuminata*, which could be clustered into three subfamilies. Systematic transcriptome analysis revealed temporal and spatial expression variations of *MaSBE* genes and differential response patterns under abiotic and biotic stresses in both banana genotypes, Fen Jiao (FJ) and BaXi Jiao (BX). Moreover, *MaSBE2.4* was temporally regulated during fruit development and ripening as well as in response to various abiotic/biotic stresses in both genotypes. Specifically, *MaSBE2.3* expression level was higher in FJ than in BX following cold, salt, and drought stress treatments, and it was specifically induced by fungal infection in BX. Characterization of hormone- and stress-related *cis*-acting elements in the promoters of *MaSBE* genes suggests their multiple biological functions. In conclusion, our study provides new insights into the complex transcriptional characteristics of the *SBE* genes, and demonstrates their crucial roles in improving amylopectin biosynthesis and strengthening stress resistance in banana.

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ABSTRACT Starch branching enzyme (SBE), which is one of the key enzymes associated with amylopectin biosynthesis, plays important roles in variable biological processes. Despite its importance, SBE is rarely studied in the banana (*Musa acuminata* L.) which is a typical starchy fruit. Here, a family of ten SBE proteins (MaSBE) was firstly identified through genome-wide characterization in *M. acuminata*, which could be clustered into three subfamilies. Systematic transcriptome analysis revealed temporal and spatial expression variations of *MaSBE* genes and differential response patterns under abiotic and biotic stresses in both banana genotypes, Fen Jiao (FJ) and BaXi Jiao (BX). Moreover, *MaSBE2.4* was temporally regulated during fruit development and ripening as well as in response to various abiotic/biotic stresses in both genotypes. Specifically, *MaSBE2.3* expression level was higher in FJ than in BX following cold, salt, and drought stress treatments, and it was specifically induced by fungal infection in BX. Characterization of hormone- and stress-related *cis*-acting elements in the promoters of *MaSBE* genes suggests their multiple biological functions. In conclusion, our study provides new insights into the complex transcriptional characteristics of the *SBE* genes, and demonstrates their crucial roles in improving amylopectin biosynthesis and strengthening stress resistance in banana.

Keywords *Musa acuminata*, Starch branching enzyme, Genome-wide characteristics, Fruit development, Abiotic stress, Biotic stress

INTRODUCTION

Plant starch is a major nutrition and calories source in the human diet that consists of two glucan polymers, linear amylose and branched amylopectin. Most crop starches, including those derived from a series of fresh starchy fruits such as banana (*Musa acuminata*), kiwifruit (*Actinidia deliciosa*), and mango (*Magnifera indica*), have an amylose content of approximately 15% - 25% and an amylopectin content of approximately 75% - 85% (*Millan-Testa et al., 2005*; *Nardozza et al., 2013*; *Kaur et al., 2016*; *Miao et al., 2017*; *Wang et al., 2018*). Amylopectin, which is the main component of starch, is primarily synthesized by soluble starch synthases (SSs), starch branching enzymes (SBEs), and debranching enzymes (DBEs) (*Myers et al., 2000*). SBEs, among these three major enzyme classes, control the structure and physical properties of starch granules and influence the yield and quality of plant products (*Li & Gilbert, 2016*; *Pan et al., 2018*).

In particular, SBEs activities determine the branching pattern and polymodal distribution of chain lengths by catalyzing the formation of amylopectin introduction of α-1,6- branch points into α-1,4-linked Glc chains (*Kim & Guiltinan, 1999*; *Tetlow & Emes, 2014*). A variable number of SBE have identified in many plant species through genome wide characterization, including three in *Arabidopsis thaliana* (*Han et al., 2007*), four in rice (*Oryza sativa*) (*Han et al., 2007*), three in maize (*Zea mays*) (*Tetlow & Emes, 2014*), seven in wheat (*Triticum aestivum*) (*Tyagi et al., 2017*), three in barley (*Han et al., 2007*), three in sorghum (*Sorghum bicolor*) (*Tetlow & Emes, 2014*), three in potato (*Solanum tuberosum*) (*Han et al., 2007*), and six in cassava (*Manihot esculenta*) (*Pei et al., 2015*). However, it is generally recognized that SBEs are composed of at least three isoforms including SBEI, SBEII, and SBEIII, all of which have typical alpha amylase domain (PF00128) and a C-terminal all-beta domain (PF02806) (*Burton et al., 1995*; *Han et al., 2007*; *Tyagi et al., 2017*). SBEI transfers relatively longer glucan chains using amylose as a substrate (*Tetlow & Emes, 2014*), while members of the SBEII family prefer to use shorter glucan chains for amylopectin formation and have lower affinity for amylose than SBEI isoforms (*Dumez et al., 2006*; *Tetlow & Emes, 2014*). The functionality of SBEIII remains unclear despite of its activity was found to increase during the grain filling period in wheat (*Kang et al., 2013*). Currently, in addition to starch modifying effects, numerous expression and functional analyses have further indicated that SBEs could play important roles in various plant biological processes, including seed germination, seedling growth, development and maturity, abiotic stress, and disease infections. For instance, a mutant deficient in SBEI activity in maize was found to have reduced kernel germination rate and coleoptile growth (*Xia et al., 2011*). Chestnut (*Castanea mollissima*) *CmSBEI* and *CmSBEII* were identified to play an important role in nut development and cotyledon growth (*Chen et al., 2017*). The expression studies of kidney bean (*Phaseolus vulgaris*) mutants *pvsbe1* and *pvsbe2* suggested *pvsbe1* and *pvsbe2* play a crucial role in mid-stage and late-stage of seed maturation, respectively (*Hamada et al., 2001*). Temporally expression of *SBE1* enhanced the accumulation of amylopectin at approximately 44 d post-pollination in apple (*Han et al., 2007*). In rice, a gene encoding starch branching enzyme, *RBE4*, was found to be expressed in developing seeds (*Mizuno et al., 2001*). Additionally, *OsSBEIIa* expression regulated the properties of sugary endosperm

during development and maturity (*Lee et al., 2017*), while a mutant defective in maize *SBEIIa* showed reduced branching in leaf starch, which led to a severe senescence-like phenotype in maize leaves (*Yandeau-Nelson et al., 2011*). Furthermore, multiple *SBE* gene expression was induced by various stress conditions, including temperature, salt, drought, and plant diseases (*Jiang, Dian &Wu et al., 2003*; *Theerawitaya et al., 2012*; *Pei et al., 2015*). In rice, decreased activity of SBE1 and SBE3 has been shown to increase the amount of long chain amylopectin at high temperature (*Jiang, Dian & Wu et al., 2003*). Under salt stress, *SBEI* and *SBEII* from rice IR29 (salt-sensitive genotype) were up-regulated, suggesting they play a role in the supply of amylopectin or carbohydrate in salt-stressed seedlings of the IR29 cultivar (*Theerawitaya et al., 2012*). Under a mild water deficicy during grain filling, SBE activity was substantially enhanced, resulting in significant increases in wheat grain yield (*Yang et al., 2004*). In cassava, expression of *MeSBE2.2* was regulated by salt, drought, and abscisic acid signals, while *MeSBE3* showed a up-regulation response to drought, salt, and abscisic acid in leaves but not in storage root, implying that they might be important components of the starch biosynthesis pathway under various environmental stresses (*Pei et al., 2015*). Finally, wheat powdery mildew infection was found to significantly depress SBE activity and amylopectin content in wheat grain during grain development (*Li et al., 2017*). All these studies suggest that the *SBE* gene family are not only critical factors of plant growth and development but also important for plant responses to abiotic/biotic stresses.

Banana (*M. acuminata*) is one of the world's most popular starch-rich fresh fruit and is the principal staple food in some African countries including Uganda where consumption levels is 0.5 kg per person per day by average and as high as 1 kg per person per day in some regions (*Paul et al., 2017*). In unripe banana fruit, total starch content is approximately 74% - 88% (dry weight, DW), amylopectin content accounts for about 70% - 80% of the total starch content (*D'Hont et al., 2012*; *Miao et al., 2014*; *Miao et al., 2017*). Amylopectin biosynthesis is very important in banana as it is a major component of fruit yield, quality, and economic value. Further, the ability to tolerant or resistant to various abiotic/biotic stresses, such as cold, salt and drought, and various fungal diseases is critical to banana production (*Hu et al., 2016*; *Miao et al., 2017*). Genome-wide investigations of key candidate genes associated with fruit development and multiple stress responses are therefore necessary to improve yield and quality of banana fruit and strengthen stress tolerance in banana (*Asif et al., 2014*; *Hu et al., 2016*; *Miao et al., 2017*). In this report, we identified ten MaSBE proteins from the banana A genome and studied their molecular features including phylogenetic relationships, exon-intron structure, protein motifs, and expression patterns at different developmental stages and under different abiotic/biotic stresses in both banana genotypes. Furthermore, hormone- and stress- related *cis*-elements of *MaSBE* promoters were analyzed *in silico*. This work aims to shed some light on the underlying mechanisms of MaSBE's potential functional roles in banana fruit development, ripening and abiotic/biotic stresses at the genome-wide level.

MATERIALS & METHODS

Plant materials

Two genotype cultivars, BaXi Jiao (*M. acuminata* AAA group cv. Cavendish, BX) and Fen Jiao (*M. acuminata* AAB group cv. Fenjiao, FJ), were applied to comparative analyses. BX is a triploid cultivar with AAA genotype that presents high quality, high yield, long shelf life, but rather poor tolerance to various abiotic stresses. FJ is a triploid cultivar with AAB genotype that shows rapid ripening, good flavor, and high abiotic stress tolerance. Two banana cultivars were planted in the banana plantation (Chinese Academy of Tropical Agricultural Sciences, Danzhou, Hainan, China). Different tissues materials (roots, leaves, and fruits) at 80 days after emergence from the pseudostem (DAF) were used for *SBE* spatial expression analysis. Developing banana fruits of 0 (budding), 20 (cutting flower), and 80 (harvest) DAF were collected for *SBE* temporal expression analysis. BX fruits stored for 0 (peel green), 8 (peel yellowish green), and 14 (peel yellow) day after harvest (DPH) and FJ fruits of 0 (peel green), 3 (peel yellowish green), and 6 (peel yellow) DPH were used for *SBE* ripening expression analysis. Five-leaf stage banana plants were grown under a 16 h light/8 h dark photoperiod at 28 °C, 200 $\mu mol \cdot m^{-2} \cdot s^{-1}$ light intensity, and 70% relative humidity and were selected for abiotic/biotic stress treatments. Twenty four banana plants were irrigated with 300 $mmol \cdot L^{-1}$ NaCl and 200 $mmol \cdot L^{-1}$ mannitol for 7 d for salt and osmotic treatment. Twelve banana plants were maintained at 4 °C for 22 h for cold treatment. Roots of twelve banana seedlings were dipped in *Fusarium oxysporum* f. sp. cubense (Foc) Tropical Race 4 (TR4) spore suspension of 1.5×10^6 condiam/L^{-1} for biotic treatment. Each sample or each treatment contains three biological replicates.

Genome-wide identification and phylogenetic analyses of the MaSBE protein family

SBE family proteins from banana were downloaded from the A-genome (*M. acuminata*, DH-Pahang, 2n = 22) database (http://banana-genome.cirad.fr) (*D'Hont et al., 2012*) . SBE amino acid sequences from *Arabidopsis thaliana*, *Solanum lycopersicum*, *Oryza sativa*, and *Vitis vinifera* sequences were downloaded from the TAIR (http://www.arabidopsis.org), SGN (http://www.sgn.cornell.edu/index.pl), RGAP (http://rice.plantbiology.msu.edu), and GAZE (http://www.genoscope.cns.fr/vitis) databases, respectively. The typically conserved alpha amylase domain (PFAM: PF00128, http://pfam.sanger.ac.uk) and a C-terminal all-beta domain (PFAM: PF02806, http://pfam.sanger.ac.uk) was selected to compare the predicted banana SBE proteins with the HMMER database (http://hmmer.org). Additionally, BLAST was used to analyse the downloaded banana SBEs with all SBEs from *A. thaliana*, *S. lycopersicum*, *O. sativa*, and *V. vinifera* queries. Conserved domain of the predicted banana SBEs was further searched by the conserved domain database (CDD) (http://www.ncbi.nlm.nih.gov/cdd) and PFAM (http://pfam.sanger.ac.uk) database. The accession numbers of banana SBEs were presented in Table S1. A neighbor-joining phylogenetic tree was constructed with the SBE proteins sequences from *M. acuminata*, *A. thaliana*, *O. sativa*, *S. lycopersicum*, and *V. vinifera* by the Clustal X 2.0 and MEGA 5.0 software with bootstrap values for 1,000 replicates (*Hu et al., 2016*).

Characterization of protein motifs and exon-intron structure

The ExPASy tools (http://expasy.org/) were used to predict the molecular mass and isoelectric points of the MaSBE proteins. Motifs of MaSBE proteins were identified and annotated by the MEME software (http://meme-suite.org) and by the InterProScan database (http://www.ebi.ac.uk/Tools/pfa/iprscan). Exon-intron structural features of *MaSBE* genes were analyzed by Gene Structure Display Server (GSDS) software (http://gsds.cbi.pku.edu.cn). 2,000 bp upstream promoter sequences of *MaSBE* genes were downloaded from the banana A-genome database (http://banana-genome.cirad.fr) and was used to predict the transcription start site and *cis*-acting elements by a database (http://www.fruitfly.org/seq_tools/promoter.html) and by the PlantCARE software (http://bioinformatics.psb.ugent.be/webtools/plantcare/html).

RNA extraction, library construction and transcriptomic analysis

Roots, leaves, and fruits at 80 DAF were collected for transcriptome analysis of *MaSBE* genes in different tissues. BX pulps at 0, 20, and 80 DAF, 8 and 14 DPH and FJ pulps at 0, 20, and 80 DAF, 3 and 6 DPH were used for the transcriptome analysis of *MaSBE* genes at different fruit developmental and ripening stages. Leaves or roots at the five-leaf stage derived from banana seedlings that had been treated with low temperature (4°C) for 22 h, 300 mM NaCl for 7 d, 200 mM mannitol for 7 d, or Foc TR4 treatments were selected for transcriptome analysis of of *MaSBE* genes under different abiotic/biotic stresses.

Total RNA was extracted according to the protocol of the plant RNAout Kit (TIANGEN Biotech, Beijing, China), then used to construct RNA-seq libraries by the Beijing Genomics Institute (BGI, Shenzhen, China). A GAII kit (Illumina, San Diego, CA) was used for deep sequencing according to the manufacturer's instructions. The average sequencing depth was 5.34X. In the raw sequence reads, adapter sequences and low quality sequences were removed by the FASTX-toolkit and FastQC, respectively. The clean reads were collected and mapped to the DH-Pahang A-genome (*M. acuminata*, *2n* = 22). Cufflinks were used to construct transcriptome assemblies. Each gene expression level was calculated as fragments per kilobase of exon per million fragments (FPKM). Differentially expressed genes were identified by DEGseq. Each sample had three biological replicates and two technical replicates.

Gene expression analysis by quantitative real-time polymerase chain reaction (qRT-PCR)

Expression patterns of *MaSBEs* in different tissues, at fruit different development and ripening stages, and under abiotic/biotic stresses were further determined by qRT-PCR in a Stratagene Mx3000P detection system (Stratagene, San Diego, CA) with a SYBR® Premix *Ex* Taq™ kit (TaKaRa, Shiga, Japan). Primer pairs were identified by melting curve analysis and agarose gel electrophoresis and its amplification efficiencies ranged from 0.9 to 1.1. These identified primer pairs were used to quantification analysis (Table S2). *MaActin* (accession number: EF672732) and *MaUBQ2* (accession number: HQ853254) were selected as internal

reference genes to normalize the relative expression levels of *MaSBE* genes. The gene expression levels were calculated based on $2^{-\Delta\Delta CT}$ method (*Livak & Schmittgen, 2001*). Each sample contained three replicates.

Statistical analysis methods

All statistical analyses were performed using SPSS (Chicago, IL). Analysis of variance was employed to identify differences based on Student's *t*- tests. Three biological replicates were analyzed for each sample. A $p< 0.05$ (*) and $p< 0.01$ (**) were taken to indicate significance.

RESULTS

Identification and phylogenetic analysis of banana *MaSEB* genes

BLAST and hidden Markov models (HMM) searches were conducted to extensively identify banana *M. acuminata MaSBE* genes using *A. thaliana AtSBE*, *O. sativa OsSBE*, *S. lycopersicum SlSBE*, and *V. vinifera VvSBE* sequences as queries. Ten *MaSBE* genes were isolated from the *M. acuminata* A genome database, which were designated as *MaSBE-1, -2.1, -2.2, -2.3, -2.4, -3.1, -3.2, -3.3, -3.4,* and *-3.5* based on their phylogenetic relationship with *O. sativa OsSBEs*. Conserved domain analysis further confirmed that all predicted MaSBE proteins contained one alpha amylase domain (PF00128) and a C-terminal all-beta domain (PF02806), which are the hallmarks of the SBE protein (Table S3). The size of amino acid residues of the predicted MaSBE proteins varied from 66 (MaSBE3.4) to 908 (MaSBE3.2), with relative molecular masses between 24.796 (MaSBE2.2) and 104.969 (MaSBE3.2) kDa, and isoelectric points ranging from 5.18 (MaSBE1) to 7.74 (MaSBE2.2) (Table S1). The highly variable structures of MaSBEs may imply their potentially different functional roles in different biological processes.

To understand the evolutionary relationships between SBE family proteins, a phylogenetic tree was constructed by aligning ten, three, three, three, and four SEB proteins from *M. acuminata*, *A. thaliana*, *S. lycopersicum*, *O. sativa*, and *V. vinifera,* respectively, using the ClustalX and MEGA5.0 software (Fig. 1). All MaSBE proteins fell into three Groups (I–III) based on the phylogenetic tree. Groups II and III were large, with more than four MaSBE members, whereas Group I contained one MaSBE1 protein, consistent with *S. lycopersicum* and *V. vinifera*. Compared with other plant species, there were five *MaSBE* members in Group III, while there was only one each in *S. lycopersicum* and *V. vinifera*.

Banana *MaSBE* gene structure and conserved motif analysis

The evolutionary analysis within *MaSBE* families was further supported by exon-intron structural divergence using GSDS software analysis (Fig. 2A). The *MaSBE* gene exon-intron organization was clearly different among the members of Group I, II, and III, implying the functional divergence and evolutionary history among these three groups of *SBE* genes.

198 To further study the protein structural diversity and predict the functionality of the MaSBE protein family,
199 ten conserved motifs of MaSBE proteins were identified and annotated by the MEME5.2 software and InterPro
200 database (Fig. 2B, Table S3). Motifs 5 and 6 were annotated as a C-terminal all-beta domain (PF02806) and
201 alpha amylase domain (PF00128), respectively, which is characteristic of the SBE protein family. Specifically,
202 four MaSBE proteins (MaSBE-2.1, -2.2, -2.3, and -2.4) contained motifs 1-10, whereas all MaSBE proteins
203 contained motifs 1-5, 8, and 9.

204 **Expression analysis of *MaSBE* genes in banana different tissues**

205 To study the expression patterns of *MaSBE* genes in different tissues and their potential functions in banana
206 plant growth and fruit development, different banana tissues including roots, leaves, and fruits from both BX
207 and FJ genotypes were selected and used to transcriptional analysis. Three *MaSBE* genes were expressed in the
208 tested tissues in both genotypes (Fig. 3A, Table S4). However, the expression of *MaSBE-1*, *-2.1*, *-2.2*, *-3.1*, -
209 *3.3*, *-3.4*, and *-3.5* was not detected.

210 In BX, *MaSBE2.3* was expressed in all tested tissues, while its highest expression level was found in the
211 fruits (FPKM > 75). *MaSBE2.4* was also highly expressed in the leaves and fruits (FPKM > 24). However,
212 *MaSBE3.2* was expressed at low levels in the roots, leaves, and fruits (FPKM < 0.5).

213 In FJ, three *MaSBEs* expressed in all examined tissues. The highest expression of *MaSBE2.3* was detected
214 in the fruits with FRKM >18. High expression levels of *MaSBE2.4* were also detected in fruits (FRKM > 40)
215 and leaves (FRKM > 70). However, *MaSBE3.2* was barely detectable in any of the tissue types examined
216 (FPKM < 0.3).

217 In all examined tissues, *MaSBE-2.3* and *-2.4* exhibited consistently high levels of expression (FRKM > 20)
218 in leaves and fruits in both genotypes, suggesting their important role in banana leave and fruit development.
219 This is in sharp contrast to the expression of *MaSBE2.3* in roots, which was high (FRKM > 5) in FJ, but low
220 (FRKM < 0.6) in BX. These findings may imply a divergent functional role of these genes in root growth and
221 development in both genotypes. This genotype-based tissue expression analysis requires further functional
222 study of the *MaSBE* gene family in banana.

223 **Expression analysis of *MaSBE* genes at different development and ripening stages in banana fruit**

224 To investigate the *MaSBE* temporal expression profiles in banana, fruits of BX and FJ collected at 0, 20, and
225 80 DAF, BX collected 8 and 14 DPH, FJ collected 3 and 6 DPH were subjected to transcriptional analysis
226 (Fig. 3B, Table S5). With the exception of *MaSBE-1*, *-2.1*, *-2.2*, *-3.1*, *-3.3*, *-3.4*, and *-3.5*, three *MaSBE* genes
227 showed expression at different fruit development and postharvest ripening stages in these both genotypes.

In BX, *MaSBE2.4* exhibited high expression (FPKM > 24) throughout the entire fruit development and ripening process. The *MaSBE2.3* gene also showed high level of transcript accumulation at all developmental stages tested (FPKM > 29), but relatively lower expressions at 14 DPH (FPKM < 5.0). In FJ, *MaSBE-2.4* and *-2.3* exhibited similarly high level of expression (FPKM > 20) at all the tested fruit development and ripening stages compared to BX. *MaSBE3.2* gene was detected in low transcriptional abundance (FRKM < 0.9) at 0, 20, and 80 DAF, and BX collected 8 and 14 DPH, FJ collected 3 and 6 DPH, respectively, during fruit development and ripening in both BX and FJ. In addition, *MaSBE2.3* was showed much higher expression (FPKM > 76) at 80 DAF in BX than in FJ (FPKM < 19) at 80 DAF. These results suggest a significantly different expressional response of *MaSBE2.3* at late (80 DAF) stage of fruit development.

Expression patterns of banana *MaSBE* genes under cold, salt, and osmotic stresses

Changes in *MaSBEs* expression patterns in BX and FJ in response to cold, salt, and osmotic treatments were analyzed by transcriptomic analysis. Three *MaSBE* genes were identified as inducible in banana leaves by three abiotic stress treatments (Fig. 4A, Table S6).

In BX, *MaSBE2.4* expression was strongly up-regulated (FPKM > 42) under all three abiotic stresses, whereas *MaSBE2.3* was only moderately upregulated under cold and salt stresses (FPKM > 2.0) and *MaSBE3.2* was down-regulated by all three abiotic treatments (FPKM < 0.7). In FJ, *MaSBE2.4* showed similar expression patterns that were significantly induced (FPKM > 60) by cold, salt, and osmotic stresses. The expression of *MaSBE2.3* was moderately induced (FPKM > 3.7) by all three abiotic stress treatments. *MaSBE3.2* expression was down-regulated (FPKM < 0.42) in FJ by all three abiotic stresses, which was consistent with BX. In addition, *MaSBE2.3* showed much higher expression (FPKM > 6.7) in FJ, compared to BX (FPKM < 0.7) under osmotic stress treatment, suggesting genotypic variations in response to osmotic treatment.

Expression patterns of banana *MaSBEs* in response to Foc TR4 infection

To understand the potential functional roles of *MaSBE* genes in defense against fungal diseases in banana, *MaSBE* expression patterns were further detected in the roots of BX and FJ plants at 0 and 2 DPI with Panama disease (Foc TR4). Three of the ten *MaSBE* genes exhibited transcriptional changes after the fungal infection in both the BX and FJ genotypes (Fig. 4B and Table S7).

Three *MaSBE* genes, including *MaSBE-2.3*, *-2.4*, and *-3.2*, showed significantly altered expression by Foc TR4 infection. In BX, *MaSBE-2.3* and *-2.4* exhibited 4.9- and 2.1- fold increases in sequence abundance at 2 DPI, respectively. In FJ, *MaSBE-2.3* and *-2.4* were down-regulated by 2.1- and 1.46- fold, respectively. These results suggest a significantly different transcriptional variation of *MaSBE2.3* and *MaSBE2.4* for Foc TR4 infection.

Identification of the differentially expressed *MaSBEs* using qRT-PCR technology

RNA-seq analysis indicated that *MaSBE-2.3*, *-2.4*, and *-3.2* exhibited different expression levels in different organs, or at different fruit development and ripening stages, or under different stress treatments in BX or FJ. This feature was also verified by qRT-PCR technology. Based on the normalization, all of the tested *MaSBEs* with the exception of *MaSBE3.2* in FJ fruit, in mature fruits of FJ at 3DPH, in BX under salt stress, and in BX at 2DPI exhibited consistent expression patterns as those indicated by RNA-seq analysis (Fig. 5). Moreover, the correlation coefficients ranged from 0.8320 to 0.9999 between RNA-seq and qRT-PCR data (Table S8). These results indicate that RNA-seq analysis supplied suitable expression results for both banana genotypes.

In silico identification of hormone-related and stress-related *cis*-acting elements in the promoters of *MaSBE* genes

Promoters are molecular switches that initiate gene expression, and studies of the *cis*-acting elements in promoter sequences have been used as a useful tool for investigating regulatory mechanisms of gene expression and gene function (*Batra et al., 2017*; *Kim & Guiltinan, 1999*). Four hormone-related (ABA, auxin, gibberellin, and MeJA) and seven abiotic/biotic stress-related (anaerobic, cold, drought, fungal, heat, salicylic acid, and defense) *cis*-acting elements were identified in the promoters of these *MaSBE* genes (Table 1). No less than eight hormone- or stress-related *cis*-acting elements were presented in each of the three expressed *MaSBE* gene promoters (*MaSBE-2.3*, *-2.4*, and *-3.2*), which was more than the number found in those that did not express *MaSBE* genes, suggesting that these three *MaSBEs* may play important functional roles in the regulation of fruit development, ripening, and abiotic/biotic stress responses by a series of hormone- and stress-related *cis*-acting elements.

DISCUSSION

Despite the economic and social importance of bananas, there have been relatively fewer studies of bananas compared to other crops, especially in term of fruit development and response to environmental stress (*Miao et al., 2017*). SBE is a key enzyme in amylopectin synthetic pathway that has also been found to be involved in the regulation of seed germination, seedling growth and development, and in response to several abiotic/biotic stresses in many crops (*Hamada et al., 2001*; *Xia et al., 2011*; *Pei et al., 2015*; *Chen et al., 2017*; *Li et al., 2017*). In this study, we identified ten *MaSBE* genes through a genome-wide search of *M. acuminata*. Ten members could be divided into three distinct groups based on their subunit sizes and phylogenetic relationships. These results are consistent with the *SBEs* classification in other higher plants such as *A. thaliana*, *O. sativa*, and *Populus trichocarpa* (*Han et al., 2007*). The phylogenetic patterns were further identified by gene structure and conserved motif analyses. The three groups in *MaSBEs* are distinct in motif organization. It was found that Group II (*MaSBE-2.1*, *-2.2*, *-2.3*, and *-2.4*) contains ten motifs, Group III (*MaSBE-3.1*, *-3.2*, *-3.3*, *-3.4*, and *-3.5*) contains eight motifs, and Group I contains seven motifs. Such structural features of *MaSBEs* have also been presented in other plant species, such as *M. esculenta* (*Pei et al.,*

2015). Moreover, these three types of subfamilies differ in their exon-intron organization, as previously observed for *Malus* × *domestica* (*Han et al., 2007*).

The *SBE* family genes have been reported to associate with the fruit development and ripening process in some plant species, including *Malus* × *domestica* (*Han et al., 2007*), *Castanea mollissima* (*Chen et al., 2017*), and *Phaseolus vulgaris* (*Hamada et al., 2001*). In *M.* × *domestica*, *SBE1* was found to express in low levels during the early stages of fruit development, and reached the highest expression during the middle stages of fruit development, implying regulatory control of SBE transcripts activity during apple fruit development (*Han et al., 2007*). In chestnut (*C. mollissima*), *CmSBEI* and *CmSBEII* were involved in starch synthesis and nut development (*Chen et al., 2017*). In kidney bean (*P. vulgaris*), *SBE1* and *SBE2* expression were closely related to amylopectin synthesis and seed maturation (*Hamada et al., 2001*). In the current study, we found that three *MaSBEs* (*MaSBE-2.3*, *-2.4*, and *-3.2*) were expressed at different fruit developmental and ripening stages in both the BX and FJ varieties. The expressed *MaSBE2.4* gene was highly expressed (FPKM > 20) (Fig. 3), implying that *MaSBE2.4* may play an important role in fruit development and ripening in banana. We also found that *MaSBE2.3* showed specifically higher expression levels in early-stage ripening fruit, in contrast to *MaSBE2.4* which showed high expression levels throughout fruit development and ripening. Interestingly, expression patterns of some *MaSBE* genes varied greatly at a certain stage between genotypes. For example, expression level of *MaSBE3.2* in the late stage of banana fruit development was four- fold higher in BX than in FJ. BX is known to have high quality and high yield and long shelf life features compared to the FJ genotype (*Hu et al., 2016*), implying that the increase in *MaSBE* expression in fruit development process augments amylopectin biosynthesis ability, thereby enhancing the quality and yield of banana fruit. The finding is consistent with the results of previous studies suggesting that the banana A-genome contains more genes that are important for fruit yield and quality and could be potentially used as targets in breeding programs (*Hu et al., 2016*; *Miao et al., 2017*).

Bananas are frequently affected or destroyed by multiple abiotic stresses, and can suffer heavy yield and quality losses in response to cold, salt, or drought conditions (*Hu et al., 2016*). In the present study, three *MaSBEs* exhibited transcriptional changes in response to three abiotic stresses (Fig. 4). Here, we demonstrated for the first time that *MaSBE* genes show diverse responses to various abiotic stresses. These findings are consistent with the previously reports of regulation of *SBE* expression by temperature, salt, and drought stresses in *O. sativa* (*Jiang, Dian & Wu et al., 2003*), *T. aestivum* (*Yang et al., 2004*), and *M. esculenta* (*Pei et al., 2015*). Of particular interest, comparative transcriptome analysis clearly demonstrated that the expression of *MaSBE2.3* was significantly higher in FJ than in BX under drought treatment (Fig. 4). It has been previously reported that banana B-genome harbors more genes that are important for enhancing abiotic stress resistance (*Vanhove et al., 2012*). FJ with AAB genotype was found to show higher level of tolerance to abiotic stresses in comparison to banana genotypes containing A-genome alone (*Hu et al., 2016*).

Banana production was seriously threatened or even destroyed by fungal disease caused by Foc TR4 (*Wang et al., 2012*). In wheat, the powdery mildew infection was found to directly decrease SBE activity (*Li et al., 2017*). Similarly, *SBE* down-regulation in leaves leading to decreased amylopectin biosynthesis as a result of leaf blight infection has also been reported in wheat (*Li et al., 2017*). In addition, interactions between host plants and biotrophic pathogens were found to be related to the alteration in starch content in plant tissues (*Horst et al., 2008*). In the current study, the transcriptional change of *MaSBE* genes in response to Foc TR4 infection may suggest their possible role in response to fungal infections in banana. Furthermore, the finding that *MaSBE-2.3* and *-2.4* genes were up-regulated to a greater degree by Foc TR4 infection in BX than in FJ may imply that banana A-genome is more sensitive to biotic stress in banana.

In maize (*Z. mays*), two critical positive *cis* elements of *SBE1* gene promoter, which are positioned at from -314 to -295 and from -284 to -255, were reported to be crucial for and the regulation of amylopectin biosynthesis in suspension-cultured maize endosperm cells (*Kim & Guiltinan, 1999*). Recently, some *cis*-acting elements were found in the cassava *MeSBE* promoters, which are associated with stress response or endosperm and shoot-specific expression (*Pei et al., 2015*). In this study, we identified four hormone- and seven stress-related *cis*-acting elements in the *MaSBE* promoters. Hence the genome-wide identification and transcriptome analysis of *SBE* family genes in banana are likely to play a crucial role in the regulation of multiple biological processes including development and in response to abiotic/biotic stresses.

CONCLUSION

To the best of our knowledge, this is the first study to present a genome-wide analysis of a family of *SBE* genes in banana. These genes could be classified into three groups. Spatial and temporal expression profiles of *MaSBEs* in the two genotype banana cultivars revealed that *MaSBEs* may have different functions during fruit development and ripening. *MaSBEs* expression patterns in response to abiotic stress may reveal their involvements in stress regulation, especially in the B genome-containing banana genotypes. Furthermore, the identification of hormone- and stress-related *cis*-acting elements in *MaSBE* promoters provides further evidence for their potential involvements in fruit development, ripening, and environmental stresses. These results has enriched our understanding of the genome structure and potential functionality of *SBE* genes in banana, and also provide a foundation for the genetic improvement of banana fruit yield, fruit quality, and resistance to multiple abiotic/biotic stresses.

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ADDITIONAL INFORMATION AND DECLARATIONS

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Competing Interests

The authors declare there are no competing interests.

Author Contributions

- Hongxia Miao and Peiguang Sun performed the experiments, analyzed the data, wrote the paper, prepared figures and /or tables, reviewed drafts of the paper.
- Hongxia Miao, Peiguang Sun and Luhua Liu contributed reagents/materials/analysis tools, reviewed drafts of the paper.
- Zhiqiang Jin and Biyu Xu conceived and designed the experiments, analyzed the data, reviewed drafts of the paper.

Supplementary Information

Table S1. Characteristics of SBE proteins in banana.

Table S2. The primer sequences used for qRT-PCR.

Table S3. Conserved amino acid motifs and functional annotation of banana MaSBE proteins

Table S4. Expression data of *MaSBE* genes in different tissues of BX and FJ bananas. Asterisks indicate significant difference between the BX and FJ (*p<0.05; **p<0.01).

Table S5. Expression data of *MaSBE* genes in different stages of fruit development and ripening in BX and FJ bananas. Asterisks indicate significant difference between the BX and FJ (*p<0.05; **p<0.01).

Table S6. Expression data of *MaSBE* genes after various abiotic stress treatments in BX and FJ bananas. Asterisks indicate significant difference between the BX and FJ (*p<0.05; **p<0.01).

Table S7. Expression data of *MaSBE* genes after fungal infection in BX and FJ bananas. Asterisks indicate significant difference between the BX and FJ (*p<0.05; **p<0.01).

Table S8. Correlation analysis between RNA-seq data and qRT-PCR data.

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Figure 1

Figure 1 Phylogenetic analysis of the SBEs from Arabidopsis, rice, tomato, grape, and banana.

The Neighbor-joining tree was drawn using MEGA5.0 with 1000 bootstraps. Three subgroups were identified and classified as Group I, II, and III.

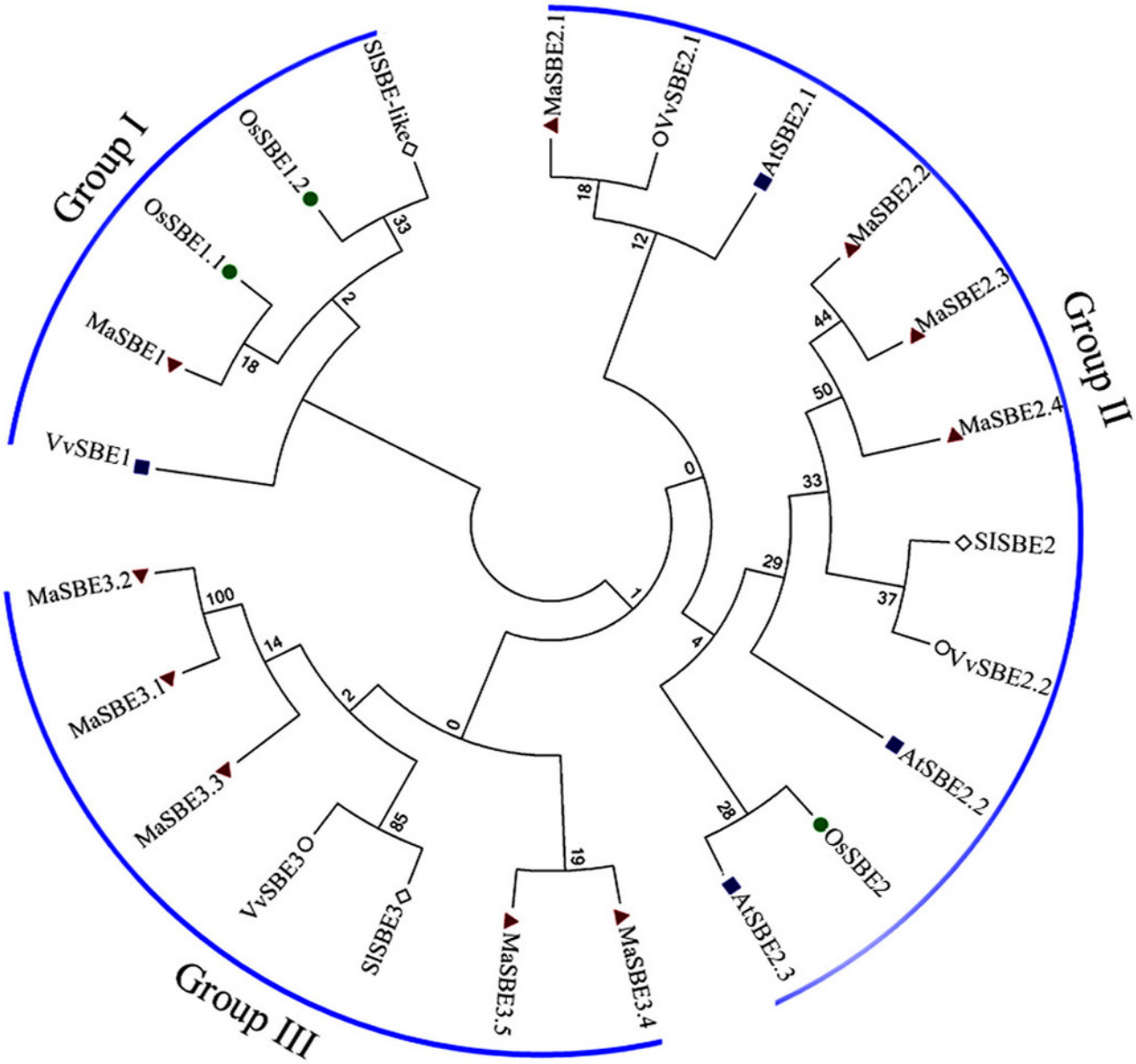

Figure 2

Figure 2 Gene structure (A), phylogenetic and motif (B) analyses of MaSBE proteins.

Exon-intron structure analyses were performed using the Gene Structure Display Server database. Blue boxes indicate upstream/downstream; yellow boxes indicate exons; black lines indicate introns. All proteins were identified by MEME database with the complete amino acid sequences of each MaSBE identified. MaSBEs were classified into Group I, II, and III subgroups based on their phylogenetic relationship.

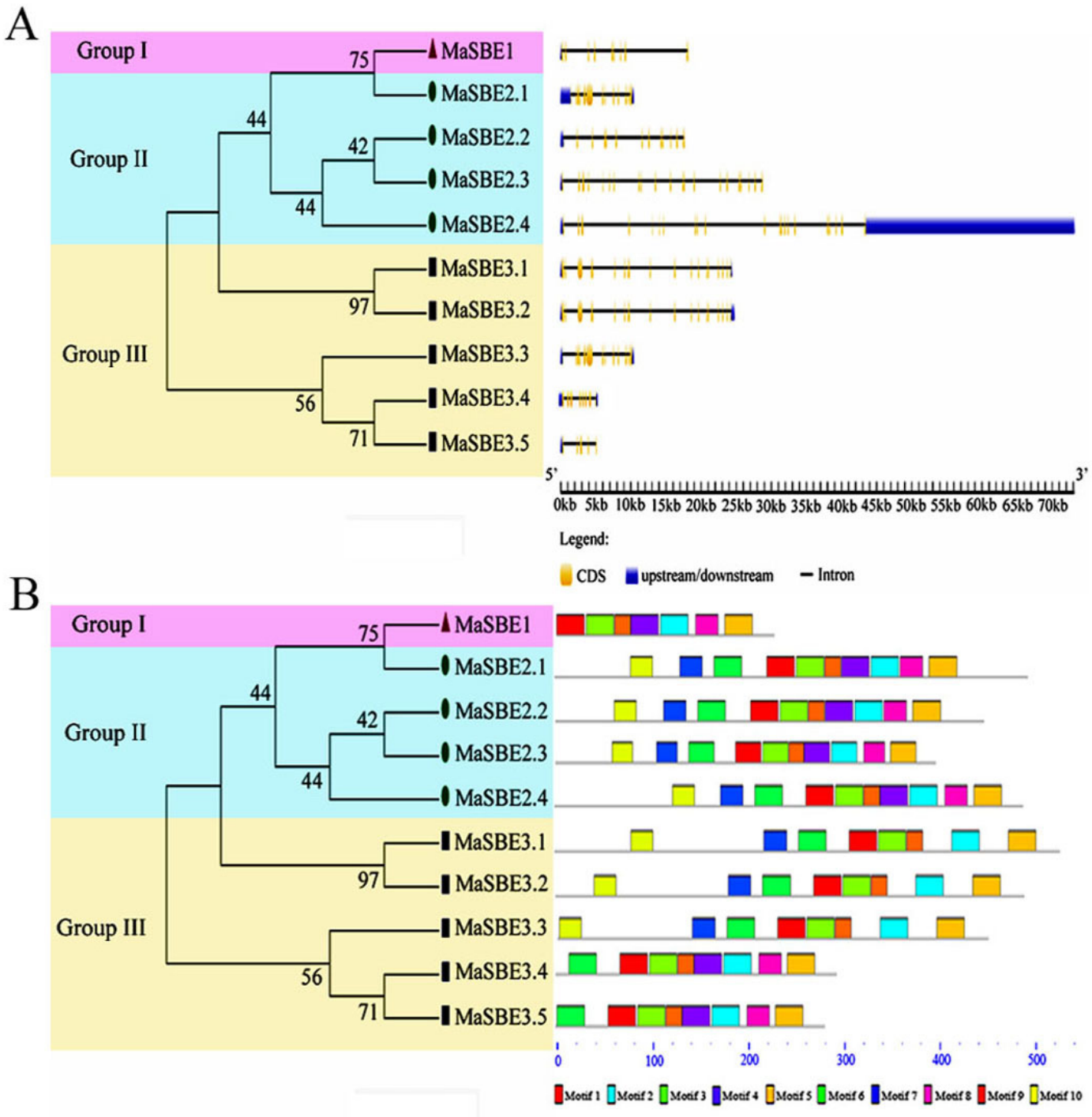

Figure 3

Figure 3 Expression of *MaSBEs* in different tissues (A) and different stages of fruit development and ripening (B) in BX and FJ banana varieties.

The heat map with clustering was created based on the FPKM value of the *MaSBEs*.

Differences in gene expression changes are shown in color as the scale.

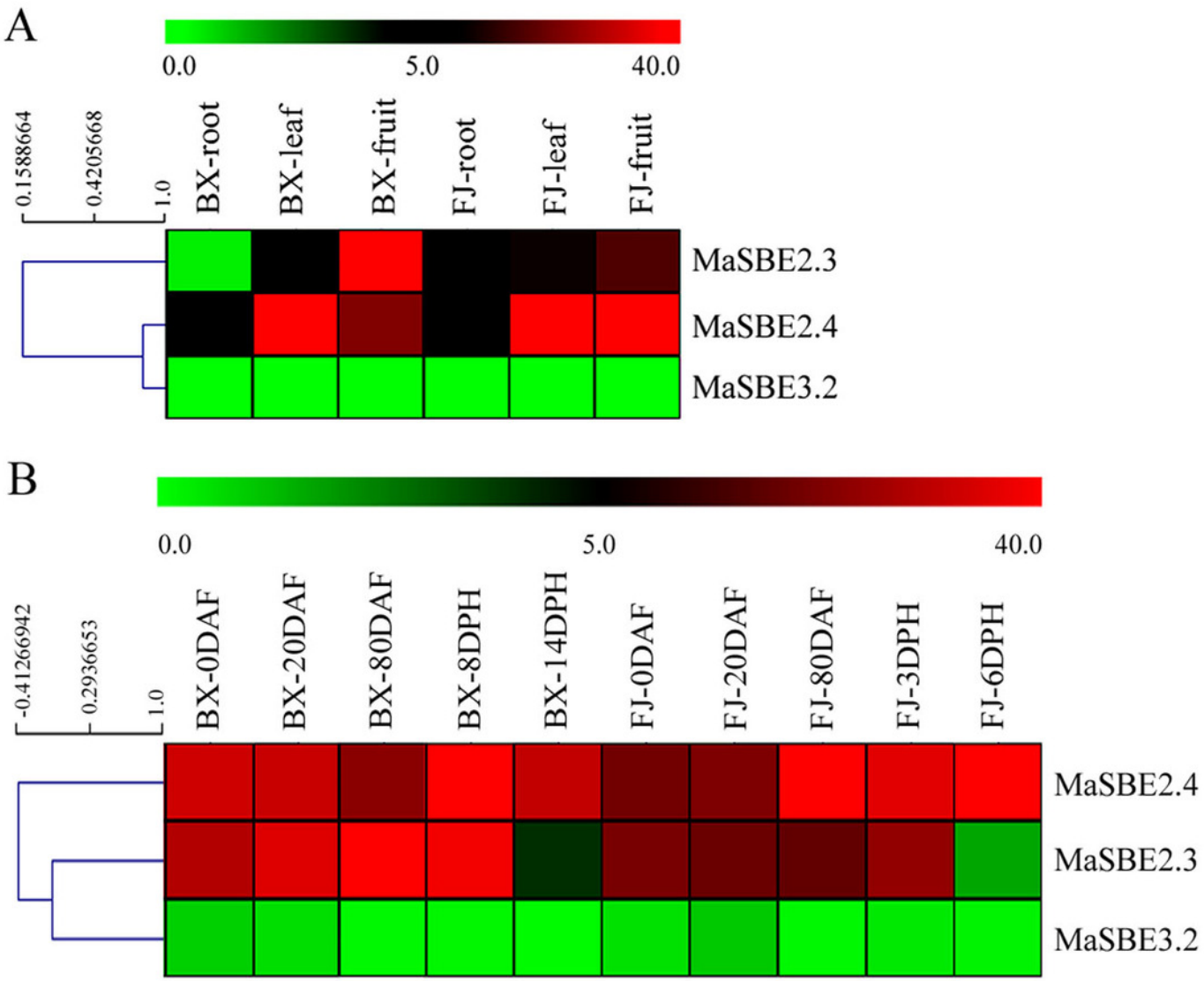

Figure 4

Figure 4 Expression of *MaSBEs* in response to cold, salt, osmotic stresses (A), and fungal disease (B) in BX and FJ banana varieties.

The heat map with clustering was created based on the FPKM value of the *MaSBEs*.

Differences in gene expression are shown in color in the green-red scale.

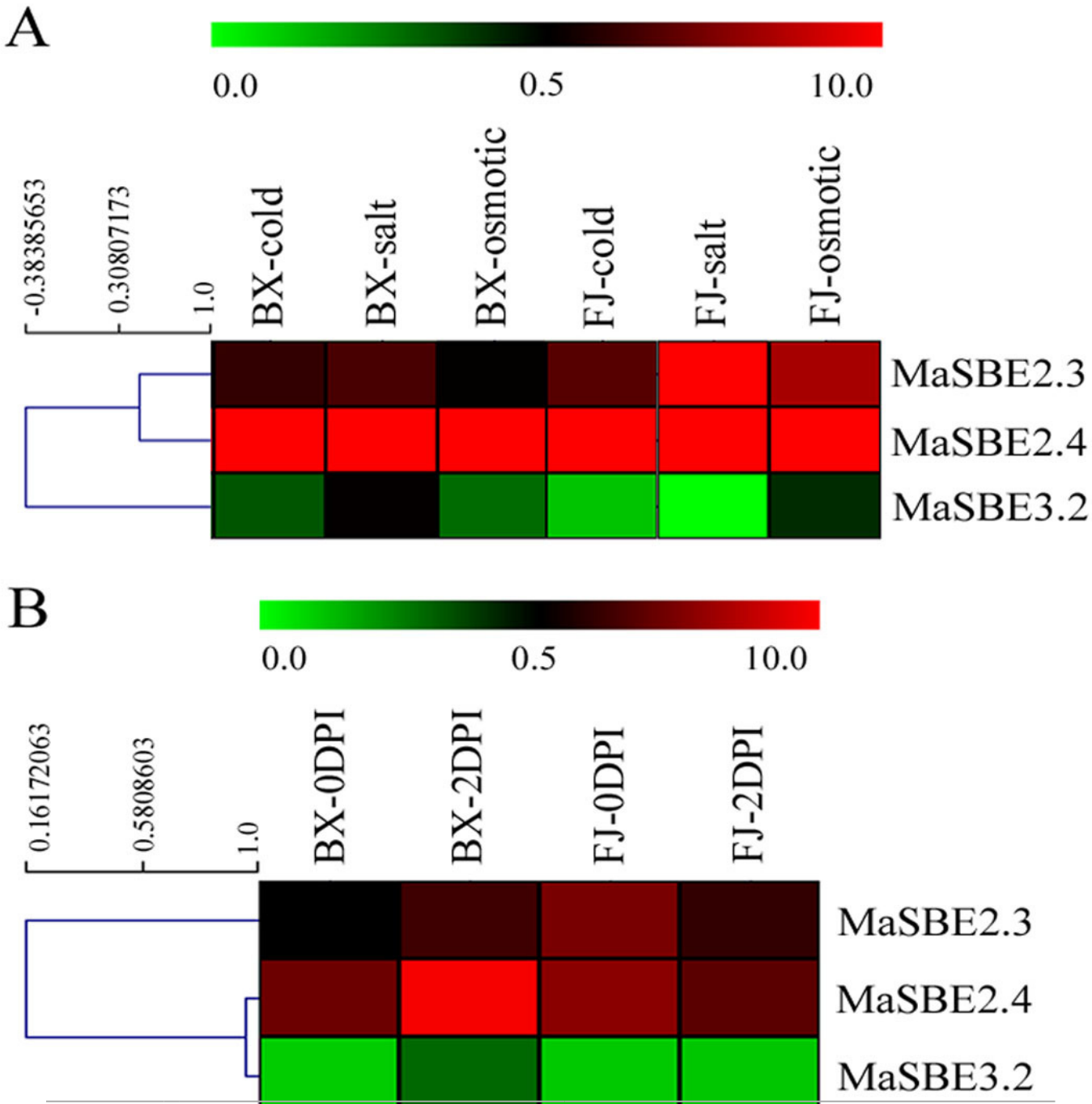

Figure 5

Figure 5 Relative expression of *MaSBEs* in the banana varieties, BX and FJ, by qRT-PCR.

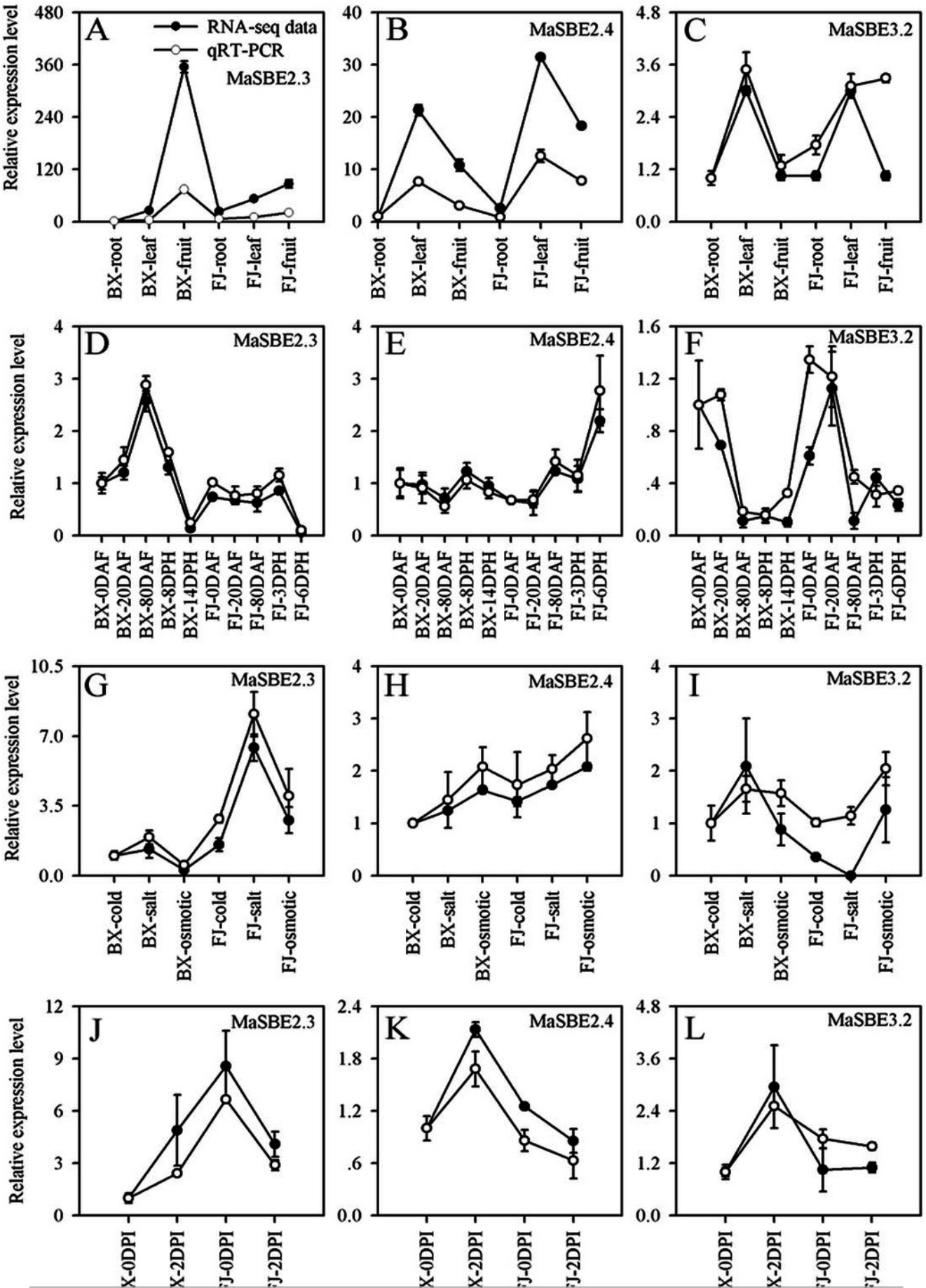

Table 1(on next page)

Table 1 Kinds and numbers of the known hormone-related and stress-related elements found in the upstream regions of *MaSBE* genes.

1

Element	ABRE (ABA)	ARE (Anaerobic)	AuxRR (Auxin)	Box-W1 (Fungal)	CGTCA-motif (MeJA)	GARE (Gibberellin)	HSE (Heat)	LTR (Cold)	MBS (Drought)	TCA-element (Salicylic acid)	TC-rich repeats (Defense)	Total
MaSBE1	0	1	0	0	0	0	0	0	1	0	0	2
MaSBE2.1	2	3	0	1	0	0	0	0	0	0	0	6
MaSBE2.2	0	1	1	0	1	0	0	1	1	0	0	5
MaSBE2.3	2	0	0	0	2	1	2	0	1	2	0	10
MaSBE2.4	3	1	0	0	3	0	1	1	0	0	1	10
MaSBE3.1	0	1	0	1	1	1	0	0	0	0	0	4
MaSBE3.2	0	3	0	1	3	1	0	0	0	0	0	8
MaSBE3.3	0	2	0	2	0	0	0	0	0	1	1	6
MaSBE3.4	0	2	0	1	0	1	0	0	0	0	1	5
MaSBE3.5	0	0	0	0	0	0	1	1	1	0	0	3

2 ABRE, ABA responsive element; ARE, anaerobic responsive element; AuxRR, auxin responsive element; Box-W1, fungal elicitor responsive element; CGTCA-
3 motif, MeJA responsive element; GARE, gibberellins responsive element; HSE, heat stress responsive element; LTR, low temperature responsive element; MBS,
4 MYB binding site involved in drought induction; TCA-element, salicylic acid responsive element; TC-rich repeats, defense responsive elements.