Genome-wide identification and analysis of cystatin family genes in Sorghum (*Sorghum bicolor* L.)

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To set a systematic study of the Sorghum *cystatins* (*SbCys*) gene family, a comprehensive genome-wide analysis of the *SbCys* family genes was performed by bioinformatics-based methods. In total, 18 *SbCys* genes were identified in Sorghum, which distributed unevenly on chromosomes, and two genes were involved in tandem duplication event. All *SbCys* genes had similar exon/intron structure and motifs, indicating their high evolutionary conservation. Transcriptome analysis showed that 16 *SbCys* genes were expressed in at least one tested tissues, and most genes displayed higher expression levels in reproductive tissues than in vegetable tissues, indicating that the *SbCys* genes participated in the regulation of seed formation. Furthermore, the expressions of 7 *SbCys* genes were induced by *Bipolaris sorghicola* infection, while only 2 genes were responsive to aphid infestation. In addition, quantitative real-time polymerase chain reaction (qRT-PCR) confirmed that 17 *SbCys* genes were induced by one or two abiotic stresses (dehydration, salt shock and ABA stresses). In addition, the interaction network indicated that SbCys proteins were associated with several biological processes, including seed development and stress responses. Notably, the expression of *SbCys4* was up-regulated under biotic and abiotic stresses, suggesting its potential roles in mediating the response of Sorghum to adverse environmental impact. Our results provide new insights into the structural and functional characteristics of *SbCys* gene family, which laid the foundation for better understanding the roles and regulatory mechanism of Sorghum cystatins in seed development and responses to different stress conditions.

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ABSTRACT

To set a systematic study of the Sorghum *cystatins* (*SbCys*) gene family, a comprehensive genome-wide analysis of the *SbCys* family genes was performed by bioinformatics-based methods. In total, 18 *SbCys* genes were identified in Sorghum, which distributed unevenly on chromosomes, and two genes were involved in tandem duplication event. All *SbCys* genes had similar exon/intron structure and motifs, indicating their high evolutionary conservation. Transcriptome analysis showed that 16 *SbCys* genes were expressed in at least one tested tissues, and most genes displayed higher expression levels in reproductive tissues than in vegetable tissues, indicating that the *SbCys* genes participated in the regulation of seed formation. Furthermore, the expressions of 7 *SbCys* genes were induced by *Bipolaris sorghicola* infection, while only 2 genes were responsive to aphid infestation. In addition, quantitative real-time polymerase chain reaction (qRT-PCR) confirmed that 17 *SbCys* genes were induced by one or two abiotic stresses (dehydration, salt shock and ABA stresses). In addition, the interaction network indicated that SbCys proteins were associated with several biological processes, including seed development and stress responses. Notably, the expression of *SbCys4* was up-regulated under biotic and abiotic stresses, suggesting its potential roles in mediating the response of Sorghum to adverse environmental impact. Our results provide new insights into the structural and functional characteristics of *SbCys* gene family, which laid the foundation for better understanding the roles and regulatory mechanism of Sorghum cystatins in seed development and responses to different stress conditions.

INTRODUCTION

Cystatins are competitive and reversible inhibitors of cysteins proteases from families C1A and C13, which have been identified in many plant species (Martinez and Diaz, 2008; Zhao et al. 2014). Based on their primary sequence homology, three signature motifs include a QxVxG reactive site, a tryptophan residue (W) located downstream of the reactive site, and one or two glycine (G) residues in the flexible N terminus of the protein. These three motifs are important for the cystatin inhibitory mechanism (Jenko et al. 2003; Stubbs et al. 1990). In addition, a consensus sequence ([LVI]-[AGT]-[RKE]-[FY]-[AS]-[VI]-x-[EDQV]-[HYFQ]-N) in cystatins is conformed to a predicted secondary α-helix structure (Margis et al. 1998). Most plant cystatins are small proteins with a molecular mass in the 12- to 16-kD range (Margis et al. 1998). Some plant cystatins contain a C-terminal extension that raises their molecular weights up to 23 kDa, are thought to be involved in the inhibition of cysteine protease activities in the peptidase C13 family (Martinez et al. 2007; Martinez and Diaz, 2008).

The principal functions of plant cystatins are related to the regulation of endogenous cysproteases during plant growth and development, senescence, and programmed cell death (Belenghi et al. 2010; Díazmendoza et al. 2014; Zhao et al. 2014). Additionally, Plant cystatins have been used as effective molecules against different pests and pathogens (Martinez et al. 2016). For example, several publications reported the inhibition of recombinant cystatins on the growth of some pests and fungi (Martinez et al. 2005; Lima et al. 2015). Tomato plants over-expressing the wheat cystatin *TaMDC1* displayed a broad stress resistance for bacterial pathogen, and the defense responses were mediated by methyl jasmonate and salicylic acid (Christova et al. 2018). The inhibition of amaranth cystatin on the digestive insect cysteine endopeptidases was observed by Valdés-Rodríguez et al. (2015). Plant cystatins are also involved in the responses to abiotic stresses, such as over-expression of *MpCYS4* in apple delayed natural and stress-induced leaf senescence (Tan et al. 2017). Song et al. (2017) found that the expression of *AtCYS5* was induced by heat stress (HS) and exogenous ABA treatment in germinating seed, furthermore, over expression of *AtCYS5* enhanced HS tolerance in transgenic *Arabidopsis*.

To date, plant cystatin family genes had been well described in several plant species such as *Arabidopsis*, rice, soybean, wheat, and *Populus trichocarpa* (Martinez and Diaz, 2008; Wang et al. 2015; Yuan et al. 2016; Dutt et al. 2016). However, a genome-wide study of cystatins family genes in Sorghum (*Sorghum bicolor* L.) has not yet been performed. Sorghum is the world's fifth biggest crop (after rice, wheat, maize, and barley), belonging to a C4 grass that grows in arid and semi-arid regions (Taylor et al. 2010). Its drought tolerance is a consequence of morphological and anatomical characteristics (i.e., thick leaf wax, deep root system) and physiological responses (i.e., stay-green, osmotic adjustment), is considered as a plant model for drought tolerance in genomic research (Sunita et al. 2011). Recently, the completion of the whole genome assembly of Sorghum (*Sorghum bicolor* L.) makes it possible to identify and analyze cystatin family genes in Sorghum (Paterson et al. 2009). In this study, we aimed to perform a genome-wide identification of *SbCys* family genes in Sorghum and analyze their phylogeny, conserved motifs, structure, *cis*-elements, and expression profile in different tissues. We also explored the expression patterns of *SbCys* genes in response to biotic and abiotic stresses. The results may lay a foundation for further functional analyses of cystatin genes.

MATERIALS AND METHODS

Identification of SbCys family members in Sorghum genome

The identification of SbCys candidates was conducted according to the methods of Lozano et al. (2015) with some modification. The cystatin sequences of *Arabidopsis*, rice, and barely were downloaded from TAIR (http://www.Arabidopsis.org), the Rice Genome Annotation Project (http://rice.plantbiology.msu.edu/index.shtml), and Ensembl database (http://plants.ensembl.org), respectively. The whole-genome sequence of Sorghum was downloaded from Ensembl database (http://plants.ensembl.org). Then predicted proteins from Sorghum genome were scanned using HMMER v3 (http://hmmer.org/) using the Hidden Markov Model (HMM) profile of cystatin (PF00031) from Pfam protein family database (http://pfam.xfam.org/) (Finn et al. 2011). From the proteins obtained using the raw cystatin HMM, a high-quality protein set with a cut-off *e*-value $< 1 \times 10^{-10}$ was aligned and used to construct a Sorghum specific cystatin HMM using hmmbuild from the HMMER v3 suite. Then all proteins with *e*-value < 0.01 were selected by the new Sorghum specific HMM. Cystatin sequences were further filtered based on the closest homolog from *Arabidopsis*, rice and barely using ClustalW and the UNIREF100 sequence database. Proteins without typical domain (Aspartic acid proteinase inhibitor) and reactive site motif (QxVxG) were removed from posterior analysis.

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Sequence alignment, structure analysis, and phylogenetic tree construction

The Multiple Expectation for Motif Elicitation (MEME) program was used to identify conserved motifs shared among SbCys proteins. The parameters of MEME were as follows: maximum number of motifs, 10; optimum width, between 6 and 50; and number of repetitions, any.

The three-dimensional structures of Sorghum cystatins were modelled by the automated SWISS-MODEL program (http://swissmodel.expasy.org/interactive) (Peitsch 1996). The known crystal structure of rice oryzacystatin I (OC-I) (Nagata et al. 2000) and SiCYS (Hu et al. 2015) were used to construct the homology-based models. Structure analysis was conducted by the RasMol

2.7 program (Sayle and Milner-White 1995).

A phylogenetic tree was constructed using MEGA X with the maximum likelihood method according to the Whelan and Goldman + freq. Model. Bootstrap analysis was performed by 1000 replicates with the p-distance model. The phylogenetic tree was visualized and optimized in Figtree (http://tree.bio.ed.ac.uk/software/figtree/).

Transcript structures, chromosomal location and gene duplication

The genomic structure of each *SbCys* gene was derived from the alignment of their coding sequence to their corresponding genome full-length sequence. The diagrams of these *SbCys* genes were drawn by the Gene Structure Display Server (GSDS, http://gsds.cbi.-pku.edu.cn/) (Hu et al. 2014). The chromosomal locations of *SbCys* genes were retrieved from the Sorghum_bicolor_NCBIv3 map. The genes were plotted on chromosomes using the Map Gene2chromosome (MG2C, version 2.0) tool (http://mg2c.iask.in/). Gene duplication events of *SbCys* family genes were investigated according to the following two criteria: (1) the alignment covered > 75% of the longer gene, (2) the aligned region had an identity > 75%, (3) located in less than 100 kb single region or separated by less than five genes (Gu et al. 2002). For microsynteny analysis, the CDS sequence of every cystatin from *Arabidopsis*, barley, rice, and Sorghum was used as the query to search against all other cystatins using NCBI_blast software with *e*-value ≤ $1e^{-10}$. The Circos software was used to display the results of collinearity gene pairs (Krzywinski et al. 2009).

Calculation of Ka and Ks

To assess the degree of natural selection on *SbCys* genes, the rate ratio of *Ka* (nonsynonymous substitution rate) to *Ks* (synonymous substitution rate) was calculated using KaKs Calculator 2.0 (Zhang et al. 2006). The Ka/Ks ratio > 1, < 1, or = 1 indicates positive, negative, or neutral evolution, respectively (Yadav et al. 2015).

Promoter analysis of *SbCys* genes

To investigate the *cis*-regulatory elements in a promoter region, the upstream sequences (1.5 kb) of the start codon in each *SbCys* gene were scanned in the PlantCARE database (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) and New PLACE (https://www.dna.affrc.go.jp/PLACE/?action=newplace).

Analysis of interaction networks of the SbCys proteins

The functional interacting network models of SbCys proteins were integrated using the web STRING program (http://string-db.org/) based on an *Arabidopsis* association model; the confidence parameters were set at a 0.40 threshold, the number of interactors was set to five interactors. *Arabidopsis* AtCys proteins were mapped to Sorghum SbCys proteins based on their homologous relationship, and the interaction network of SbCys proteins was drawn by Cytoscape_v3.6.0.

Expression analysis of *SbCys* genes under biotic stresses

The RNA-Seq data used for investigating the expression patterns of *SbCys* genes in various tissues were downloaded from NCBI SRA (Sequence Read Archive) database (ERP024508) (Wang et al. 2018). Root, shoot, and whole organism were collected at 14 days after germination. Embryo, endosperm and pericarp were collected at 20 days after pollination. Pollen samples were collected at booting stage. Inflorescences were collected according to the sizes: 1-5 mm, 5-10 mm, and 1-2 cm. Three biological replicates were performed for each plant tissue. RNA was sequenced using the Illumina HiSeq 2500 system to generate 250 bp pair-end reads.

RNA-seq data of biotic stresses were obtained from two experiments. The first experiment measured the transcriptome response of a resistant Sorghum (*Sorghum bicolor* L. Moench) infected with *Bipolaris sorghicola* (DRP 000986) (Yazawa et al. 2013). RNA samples were collected at 0, 12 and 24 hours post-inoculation with one biological replicate. RNA-seq was run using Illumina technology to give 100-base-pair single-end reads on a HiSeq2000 system. The second study measured changes in the transcriptome of Sorghum leaves infested by sugarcane aphid (Tetreault et al. 2019). The RNA-seq data were downloaded from the NCBI SRA database (SRP162227). In this study, two treatments (infested and control) were arranged and two Sorghum genotypes (resistant cultivar RTx2783 and susceptible cultivar BCK60) were used. Leaf samples were collected from treated and control plants at 5, 10 and 15 days post sugarcane aphid infestation. Three biological replicates were performed for all treatment and time combinations. RNA was sequenced using the Illumina Hiseq 2500 platform to generate 100 bp single end reads. The accession numbers and sample information were listed in Table S1. The differential expression of *SbCys* genes were investigated by Hisat2 (http:/kim-lab.org/), Htseq (http://www.htseq.org/), and DESeq2 (R package) based on the RNA-seq data (Wen, 2017). The $p \leq 0.05$ and $|\text{logFC}| \geq 1.5$ were set as the cut-off criterion.

Plant materials and treatments

Seed of Sorghum (*Sorghum bicolor* L. cv. Jinza 35) were surface sterilized (15 min in 4% NaClO), washed with distilled water several times, and transferred to moist germination paper for 3 days in an incubator at 25 °C. These seedlings were grown in holes of foam floating plastic containers (30 seedlings per container) with constant aeration in Hoagland solution in a growth room with 14 h/30 °C light and 10 h/22 °C dark regime. The nutrient solution was routinely changed every 3 days. At the three-leaf stage (the juvenile phase (Hashimoto et al. 2019)), abiotic stresses including ABA, salinity, and dehydration treatments were initiated according to the procedures described in previous reports (Dugas et al. 2011; Wang et al. 2012; Yan et al.

2017). The plants were transferred quickly to the nutrient solution containing 0.1 mM ABA (dissolved in ethanol), 5 µL ethanol (control for ABA treatment), 250 mM sodium chloride (NaCl), or 15% (W/V) polyethylene glycol (PEG) 6,000. The central part of flag leaves from randomly selected Sorghum plants were harvested respectively at 0, 12 and 24 hours post-treatment per trial, and immediately frozen in liquid nitrogen and stored at -80 ˚C prior to RNA isolation. For each treatment at a given time, three biological replicates were used. The leaf samples of 10 plants came from the same container for one biological replicate. That is, three containers were used for three biological replicates respectively.

RNA extraction and qRT-PCR analysis

Total RNA of 100 mg leaf samples was isolated using the "TaKaRa MiniBEST Plant RNA Extraction" Kit (TaKaRa, Dalian, China) following the manufacturer's instructions. Purity and concentration of RNA samples were evaluated by measuring the A_{260}/A_{230} and A_{260}/A_{280} ratios. In order to digest the genomic DNA, the RNAs were treated with RNase-free DNase I. Reverse transcription was performed according to the kit instruction (Promega, Madison, USA). Primer pairs for qRT-PCR analysis were designed by Primer3Plus program (http://www.bioinformatics.nl), and shown in Table S2. A 20 µl reaction volume containing 0.4 µl of each primer (forward and reverse), 2 µl 10-fold diluted cDNA, 7.2 µl of nuclease-free water and 10 µl of GoTaq® qPCR Master Mix (Perfect Real Time; Promega). PCR reaction included one cycle at 95 ˚C for 3 min, followed by 39 cycles of 95 ˚C for 15 s, 60 ˚C for 30s and 72 ˚C for 20s. The reactions were conducted using CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, Inc.). Three independent biological replicates and two technical replicates of each sample were performed. Gene-specific amplification of both reference and *cystatin* genes were standardized by the presence of a single, dominant peak in the qRT-PCR dissociation curve analyses. All data were analyzed by CFX Manager Software (Bio-Rad Laboratories, Inc.). The efficiency range of the qRT-PCR amplifications for all of the genes tested was between 91% and 100%. The average target (*SbCys*) cT (threshold cycle) values were normalized to reference (*β-actin*) cT values. The fold change between treated sample and control was calculated using the slightly modified $2^{-(\Delta\Delta Ct)}$ method as described by Kebrom et al. (2010). A probability of $p \leq 0.05$ was considered to be significant.

RESULTS

Identification and analysis of *SbCys* genes

To extensively identify all of SbCys family members in Sorghum, we constructed a Sorghum-specific HMM for the SbCys domain to scan Sorghum genome, and 22 gene candidates were identified. After removing the repetitive and/or incomplete sequences, the rest of SbCys sequences were submitted to Pfam (http://pfam.xfam.org/) and SMART (http://smart.embl-heidelberg.de/) to confirm the conserved domain. Finally, a total of 18 non-redundant SbCys proteins were identified and were serially renamed from *SbCys1* to *SbCys17* according to their location and order in chromosomes. Gene names, gene IDs, chromosomal locations, amino acid numbers and protein sequences were listed in Table S3. The average length of these SbCys proteins was 148 amino acid residues and the length mainly centered on the range of 105 to 240 amino acid residues.

Chromosome distribution analysis showed that the number of *SbCys* genes on each chromosome is different (Fig. 1). Chromosome 1 had the greatest number of *SbCys* genes (9 genes), followed by chromosomes 9 and 3 (4 and 3 genes, respectively). Chromosomes 2 and 4 had just one *SbCys* gene, whereas chromosomes 5, 6, 7, 8 and 10 had no *SbCys* genes. Half of *SbCys* genes were distributed on chromosome 1, suggesting that *SbCys* genes may have a chromosomal preference. proposed reason?

Gene structure analysis of *SbCys* genes

The analysis of exon-intron structure can provide significant information about the gene function, organization and evolution of multiple gene families (Xu et al. 2012). Schematic structures of *SbCys* genes from Sorghum were obtained using the GSDS program (Fig. 2). Among the *SbCys* genes, more than half (12, 66.7%) were intronless, three genes (*SbCys11*, *SbCys15*, and *SbCys16*) had one intron, two genes (*SbCys14* and *SbCys17*) had two introns, and one gene (*SbCys10*) had three introns. These six *SbCys* genes with one or more introns were clustered into one clade, suggesting the evolutionary event may effect on the gene structure (Altenhoff et al. 2012).

Sequence alignment, protein motifs analysis, and structural predication of SbCys

Alignments of SbCys sequences were carried out to search for amino acid variants that could lead to differences in their inhibitory capability for cysteine proteases. The results were shown in Fig. 3a. N-terminal and C-terminal extensions with varying lengths that presented in several SbCys proteins were not displayed in the comparison. These predicted structures shared many identical residues including α-helix and the four β-sheets (β2-5) (Fig. 3a). Analysis of conserved motifs of SbCys proteins also revealed that some typical conserved motifs could be detected in most SbCys proteins, such as motif 1, 2, 3, and 4, form a fundamental structural combination (Fig. 3b and 3c). Motif 1 was conserved in the central loop region with a consensus sequence of "QxVxG" and could be detected in most SbCys proteins, which played an important role in the inhibitory capacity of cystatins towards their target cysteine proteases (Meriem et al. 2010). Motif 2 contained a particular consensus sequence ([LVI][GA][RQG][WF]AV) that conformed to a predicted secondary α-helix structure (Martinez et al. 2009). The other two typical motifs for

SbCys proteins, motif 3 (V[WY][EVG]KPW) and motif 4 ([RK]xLxxF), were firstly described in tobacco (Zhao et al. 2014), were also detected in most SbCys proteins, indicating their conserved and common role in both dicots and monocots. Motif 5 existed only in 3 SbCys family members (SbCys5, SbCys8, and SbCys15,). Details of the 5 conserved motifs were shown in Fig. S1.

The predicted three-dimensional structures of the Sorghum cystatins were established using the SWISS-MODEL program based on the known crystal structure of OC-I and SiCYS (Fig. 4). Although these structures were predicted with variable degrees of accuracy, all of Sorghum cystatins shared similar protein structure with rice OC-I (Fig. 4a), excepting SbCys10 that shared similar protein structure with SiCYS (Fig. 4b). In additon, SbCys14 showed a significant variation in its predicted three-dimensional structures, an extra α-helix occurred in the C-terminal region, which probably due to the cystatin contained a C-terminal extension. Two important motifs (the conserve QxVxG motif and W residue) of Sorghum cystatins involved in the interaction with the target cysteine enzymes were also shown in Fig. 4. The predicted structure of SbCys13 showed some distortions in the region of the β2 sheet, probably due to the insertion of a methionine in the first position of the conserved QxVxG motif.

Phylogenetic analysis of *SbCys* genes

Cystatin gene family is highly conserved in both monocots and dicotyledons (Martinez and Diaz, 2008). To investigate the phylogenic relationships of SbCys proteins to other known plant cystatins, a multiple sequence alignment of SbCys sequences to the sequences from *Arabidopsis*, rice, and barley was conducted by the ClustalW program. As showed in Fig. 5, these cystatins were categorized into three groups, including Group I, Group II, and Group III. A total of 21 cystatins were classified to Group I and 6 cystatins from Sorghum. Group II contained 7 cystatins, only one cystatin from Sorghum. The remaining 21 proteins were assigned to Group III and 11 SbCys proteins fell into this group. In addition, some bootstrap values in the phylogenetic tree were low, suggesting that high sequence differentiation in these cystatins occurred. Microsynteny analysis indicated that one orthologous gene pair was identified in the cross of barley and Sorghum, rice and Sorghum, respectively, while no orthologous gene pair between *Arabidopsis* and Sorghum was found (Fig. S2). These data indicated that *SbCys* genes were more closely to those of rice and barley than that of *Arabidopsis*. Interestingly, a pair of *SbCys* genes (*SbCys2-1* and *SbCys2-2*) was confirmed to be tandem duplication in Sorghum (Fig. S2). Analysis of duplicated *SbCys* genes showed that the *Ka*/*Ks* ratios far less than 1, varying from 0.0976 to 0.5679 (Table S4), indicating that negative selection occurred in the duplication event.

Promoter analysis of *SbCys* genes

In order to obtain useful information on the regulatory mechanism of cystatin gene expression, the 1.5 kb upstream sequences from the translation start sites of *SbCys* genes were submitted into PlantCARE database to detect the *cis*-elements. Various putative plant regulatory elements in the promoter region of *SbCys* genes were shown in Fig. 6 and Table S5. Several potential regulatory elements involved in stress-related transcription factor-binding sites were found, including G-box, W-box, TC-rich repeats, MBS, heat shock elements (HSEs), and ABA-response element (ABRE). The identified *SbCys* genes possessed at least 1 stress-response-related *cis*-element, suggesting that the expressions of *SbCys* genes were related to these abiotic stresses. All of *SbCys* genes had one or more G-box with the exception of *SbCys9*, implying that these *SbCys* genes could be induced by light stress. 14 *SbCys* genes possessed MBS element, ABRE element was found in 12 *SbCys* genes, and HSE element was located in 10 *SbCys* genes. TC-rich repeats and W-boxes were located in 8 genes, respectively. In addition, Skn-1_motif was conserved in the promoter regions of most *SbCys* genes, indicating these genes were associated with the regulation of seed storage protein gene expression (Strömvik and Fauteux, 2009). The high diversity of the *cis*-acting elements suggested that these *SbCys* genes might have a wide range of functional roles and could be involved in multiple stress responses and growth and development progress (Zhang et al. 2008).

Protein interaction network of SbCys proteins

In this study, the interactions of the SbCys proteins were investigated in an *Arabidopsis* association model using STRING software. As shown in Fig. 7, the interaction network of cystatins showed a complex functional relationship. AtCys2 (corresponding to SbCys12) interacted with stress related proteins (AT1G56280, AT3G19580, AT5G67450, and AtCys1) and growth and development related proteins (AT1G63100 and AT5G04340), AtCys1 (corresponding to SbCys11, 15, 16, and 17) interacted with some vacuolar-processing enzyme which involved in processing of vacuolar seed protein precursors into the mature forms, and AtCys5 (corresponding to SbCys1, 2-1, 3, 4, 5, 6, 7, 8, 9, and 13) interacted with several lipid-transfer proteins (AT1G07747, AT1G52415, AT2G16592, AT3G29152, and AT4G12825). The results suggested that cystatins might be associated with many biological processes by protein interactions, such as pollen development, stress responses, and seed maturation (Wang et al. 2012).

Expression profile of *SbCys* genes in different Sorghum tissues

To obtain the spatial and temporal expression patterns of all *SbCys* genes, RNA-seq data (ERP024508) were downloaded to explore the expression levels of *SbCys* genes in different tissues including root, stem, whole organism, pollen, endosperm, embryo, inflorescence (1-5mm, 1-10mm, and 1-2cm), and pericarp. As shown in Fig. 8 and S3, most *SbCys* genes were expressed in one tissue at least, except for *SbCys13*, which were barely expressed in any tissue. The expression patterns of *SbCys* genes in reproductive tissues were significantly difference from vegetable tissues. Such as *SbCys2-1*, *SbCys3*, *SbCys4*, *SbCys5*, *SbCys7*, *SbCys9*, *SbCys12*, and *SbCys17* showed relatively higher expression levels in reproductive tissues including pollen, endosperm, embryo, and pericarp than in vegetable tissues, while the expression of *SbCys7* and *SbCys15* were higher in vegetable tissues than in reproductive tissues. It was worth noting that majority of *SbCys* genes had lower expression levels during inflorescence development excepting for *SbCys17* which displayed higher expression pattern.

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Expression of *SbCys* genes under biotic stresses

To gain insight into the potential roles of *SbCys* genes in response to *Bipolaris sorghicola* infection and sugarcane aphid infestation, their relative expression patterns were investigated by using the public transcription data from NCBI SRA database (DRP000986 and SRP162227, respectively). As shown in Fig. 9 and 10, the expression patterns of *SbCys* genes were different under the two biotic stresses. In response to *Bipolaris sorghicola* infection, seven *SbCys* genes were induced and only 2 genes (*SbCys12* and *SbCys13*) were suppressed in infected Sorghum leaves compared with control (Fig. 9a). However, under aphid infestation, four *SbCys* genes (*SbCys4*, *SbCys10*, *SbCys11*, and *SbCys14*) were up-regulated and 3 genes (*SbCys1*, *SbCys3*, and *SbCys17*) were down-regulated relative to control in susceptible Sorghum line (BCK60). In resistant Sorghum line (RTx2783), only two *SbCys* genes (*SbCys4* and *SbCys11*) were induced, and the rest were barely expressed in Sorghum leaves with aphid infection (Fig. 9b and 10). These results might suggest that *SbCys* genes played different roles in responding to pathogen infection and aphid infestation.

Expression profiling of *SbCys* genes under abiotic stresses

We also investigated the expression of *SbCys* genes in response to various abiotic stresses including dehydration, salt shock, and ABA (Fig. 11). Under dehydration stress, seven *SbCys* genes (*SbCys4*, *SbCys5*, *SbCys6*, *SbCys9*, *SbCys10*, *SbCys11*, and *SbCys17*) were induced to present a significant up-regulation from 0 to 24 h, while the expressions of *SbCys2-1*, *SbCys12*, *SbCys15*, and *SbCys16* were decreased. Furthermore, the expressions of 4 *SbCys* genes (*SbCys1*, *SbCys3*, *SbCys8*, and *SbCys14*) displayed an up-down trend from 0 h to 24 h (Fig. 11a). With salt shock treatment, the expressions of *SbCys2-1*, *SbCys3*, *SbCys4*, *SbCys8*, *SbCys10*, and *SbCys11* were significantly up-regulated at all treatment time points, whereas *SbCys16* showed a significant down-regulated trend (Fig. 11b). In addition, *SbCys6*, *SbCys13 SbCys14*, *SbCys15*, and *SbCys17* showed up and down expression trends, but *SbCys5* displayed down and up expression pattern (Fig. 11b). After exogenous ABA treatment, the expressions of 4 *SbCys* genes (*SbCys2-2*, *SbCys3*, *SbCys4*, and *SbCys7*) were significantly up-regulated at all time points, but 9 genes (*SbCys1*, *SbCys2-1*, *SbCys5*, *SbCys8*, *SbCys10*, *SbCys11*, *SbCys13*, *SbCys14*, and *SbCys17*) were down-regulated. Additionally, *SbCys12*, *SbCys15*, and *SbCys16* displayed an up-down expression trends (Fig. 11c). Interestingly, all *SbCys* genes were up-regulated in response to one or two stresses except *SbCys4* that was significantly induced under dehydration, salt shock and ABA stresses, suggesting that SbCys4 might play an important role in response to different stress responses.

DISCUSSION

Plant cystatins are a group of intrinsic small proteins, whose members play important roles in diverse biological processes and stress responses (Martinez et al. 2016; Meriem et al. 2010). Recently, a large number of sequence data from different plant species have been uploaded in

GenBank, which provide convenience for us to describe their characteristics, and several cystatins families have been identified from plants, such as rice, soybean and wheat (Wang et al. 2015; Dutt et al. 2016; Yuan et al. 2016). However, little is known about cystatin family in Sorghum. In the present study, we identified 18 *SbCys* genes from Sorghum genome. The number was less than that of *B. distachyon* genome, where 25 *BdCys* members were identified (Subburaj et al. 2017). The 18 members in Sorghum was a larger number than found in rice (11 genes) and *Arabidopsis* (7 genes) (Wang et al. 2015), but was similar to soybean (20 members) (Yuan et al. 2016). The difference on the cystatin number might reflect the adaptation of plants to environment.

The identified *SbCys* genes were unevenly distributed on chromosomes 1, 2, 3, 4, and 9, and half of them were distributed on chromosome 1 (Fig. 1), suggesting that *SbCys* genes had a chromosomal preference. The uneven distribution of *cystatin* genes in chromosomes was also found in *B. distachyon* genome that the highest number of *BdCys* genes located in chromosome 1 (Subburaj et al. 2017). The phnomenon of chromosomal preference was also observed in *Oryza sativa* genome, but most *OsCys* genes were dispersed over two chromosomes, chromosome 1 and 3 (Wang et al. 2015). Furthermore, several tandem duplication events occurred at chromosomes 1 of *B. distachyon* genome (Subburaj et al. 2017). Two tandem duplication events (*OsCys4*/*OsCys5* and *OsCys6*/*OsCys7*) were found among *OsCys* genes, and existed in chromosomes 1 and 3 (Wang et al. 2015). One tandem duplication event (*SbCys2-1*/*SbCys2-2*) occurred among *SbCys* genes at chromosome 1 (Fig. S2). The tandem duplication events might cause the distinct distribution patterns of *cystatin* genes on the chromosomes (Li et al. 2017).

Eighteen *SbCys* genes were divided into three groups based on phylogenetic analysis (Fig. 5). Some conserved motifs among SbCys proteins had been identified by the alignment of the amino acid sequences (Fig. 3). However, the conservation was accompanied with differences in some important amino acids, indicating that SbCys family members might undergo a complex evolutionary history, which would have a significant influence on their respective functions (Abraham et al. 2006). For example, QxVxG motif, could directly enter and interact with the active site of targeted enzymes, were conserved in all SbCys proteins with the exceptions of 5 cystatins (SbCys1, SbCys6, SbCys8, SbCys9, and SbCys13) that were partially modified by the insertion or variation in important residues (Fig. 3a). Furthermore, three SbCys proteins (SbCys8, SbCys9, and SbCys13) showed significant variations with other Sorghum cystatins in their predicted three-dimensional structures (Fig. 4). The variations in vital amino acid residues might result in the change in cystatin inhibitory action (Melo et al. 2003). In addition, two novel motifs, motif 3 (V[WY][EVG]KPW) and motif 4 ([RK]xLxxF), firstly described in tobacco (Zhao et al. 2014), were also identified in the C-terminalin of many SbCys proteins. The contribution of the two new motifs to cystatin inhibitory action needs to be further studied.

During past decades, plant cystatins were reported to play essential roles in inhibiting endogenous and exogenous cysteine proteases activities during seed development (Gaddour et al. 2001; Kiyosaki et al. 2007). In the present study, as revealed by RNA-seq data analysis (Fig. 8 and S3), the expression levels of several *SbCys* family genes were higher in reproductive tissues than in vegetable tissues, which were consistent with the reports that most cystatins were specifically expressed in developing seeds and played a role in seed development (Dutt et al. 2010; Zhao et al. 2014). Moreover, promoter analysis showed that the highly expressed *SbCys* genes in reproductive tissues possessed endosperm expression related *cis*-elements (Skn-1 and GCN4_motif) (Fig. 6 and Table S5). Our protein interaction prediction results also showed that several SbCys proteins could interact with many functional proteins (Fig. 7), implying these cystatins were involved in regulating the gene expression of cereal grain storage proteins (Diaz-Mendoza et al. 2016).

Plant cystatins are involved in various biotic stress responses and probably act as defense proteins against pests and pathogen infection (Meriem et al. 2010). At present, some cystatins with insecticidal activity have been isolated from barley, corn, tomato and papaya etc. (Alvarez-Alfageme et al. 2007; Goulet et al. 2008; Kiggundu et al. 2010), and several cystatins having antifungal activities were also isolated from taro, cacao, and wheat (Christova et al. 2006; Pirovani et al. 2010; Chen et al. 2014). Although studies on insecticidal and antifungal activity of plant cystatins have been well established in vitro, the knowledge about their roles in plants in response to biotic stresses is limited. To explore the properties of *SbCys* genes responding to pest and pathogen infection, we conducted the analysis on the expression patterns of *SbCys* genes. The results showed that the expressions of most *cystatin* genes were induced during *Bipolaris sorghicola* infection, suggesting these cystatins played functions in inhibiting exogenous cysteine proteases secreted by pathogens to infect plant tissues (Fig. 9a). Interestingly, for sugarcane arthropods infestation, only two genes (*SbCys4* and *SbCys11*) were up-regulated significantly in susceptible and resistant Sorghum lines (Fig. 9b and 10), the expressions of the rest genes were no obvious change or were down-regulated. These differential expression patterns between *SbCys* genes might suggest that some of them had evolved to inhibit specific cysteine proteinases. The exact roles of these *SbCys* genes in insecticidal and antifungal activity in vivo are worthy to be explored in the further study.

Another characteristic of cystatin genes is that they are involved in various abiotic stress responses in different plant species, such as rice, barley, and maize (Gaddour et al. 2001; Massonneau et al. 2005; Huang et al. 2007). In *Arabidopsis*, the expression levels of *AtCYS1* and

AtCYS2 were enhanced by high temperature and wounding stresses (Hwang et al. 2010). *AtCYSa* and *AtCYSb* were also induced by different abiotic stresses such as salt, drought, oxidation and cold stresses (Zhang et al. 2008). Velasco-Arroyo et al. (2018) reported that the silence of barley *HvCPI-2* and *HvCPI-4* specifically modified leaf responses to drought stress. Wang et al. (2015) observed the significant change in the expression levels of several rice *OsCYS* genes under cold, drought, salt, and hormone treatments. In the present study, most *SbCys* genes were found to have positive or negative responses to dehydration, salt shock, and ABA stresses. Moreover, the interaction results showed that most cystatins could interact with stresses-related proteins, implying that the cystatins played critical roles in response to diverse stress conditions. Notably, the expression of *SbCys4* was significantly up-regulated under three stress conditions (Fig. 11), suggesting a specific role of SbCys4 in responding to various stress conditions. Promoter analysis indicated that stress-related *cis*-elements were widespread in the promoter region of these cystatin genes (Table S5), and *SbCys4* possessed plenty of stress-related *cis*-elements, including G-box, ABRE, HSE, MBS and TC-rich repeats. These results provide an effective reference for the functional verification of the *SbCys* family genes under abiotic stresses.

CONCLUSIONS

In the current study, we identified 18 *SbCys* family genes in Sorghum genome through a genome-wide survey. The chromosomal localization, conserved protein domain, gene structure, the phylogenetic relationship, as well as the interaction network of these *SbCys* genes was systematically analyzed, revealing special characteristics of *SbCys* family genes in Sorghum. The identified *SbCys* genes displayed an uneven distribution in Sorghum chromosomes. All *SbCys* genes shared similar exon/intron organization and conserved motifs. Phylogenetic analysis suggested that Sorghum cystatins had higher homology with monocotyledon than dicotyledon. The variation of amino acids in Sorghum cystatin critical active sites suggested that they might undergo a complex evolutionary process and possess structural and functional divergence. The expression profile of *SbCys* genes in different tissues indicated that most *SbCys* genes were involved in tissue growth and development. Changes in the expression of *SbCys* genes under biotic and abiotic stresses indicated that many *SbCys* genes played important roles in response to unfavorable growth conditions. It was noting that the expression of *SbCys4* was significantly enhanced under biotic and abiotic stresses, suggesting its unique role in mediating the response of Sorghum to adverse environment conditions.

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Figure 1

Chromosome localization of *SbCys* genes.

Chromosome number is indicated at the top of each bar. The size of chromosome was labeled on the left of the figure.

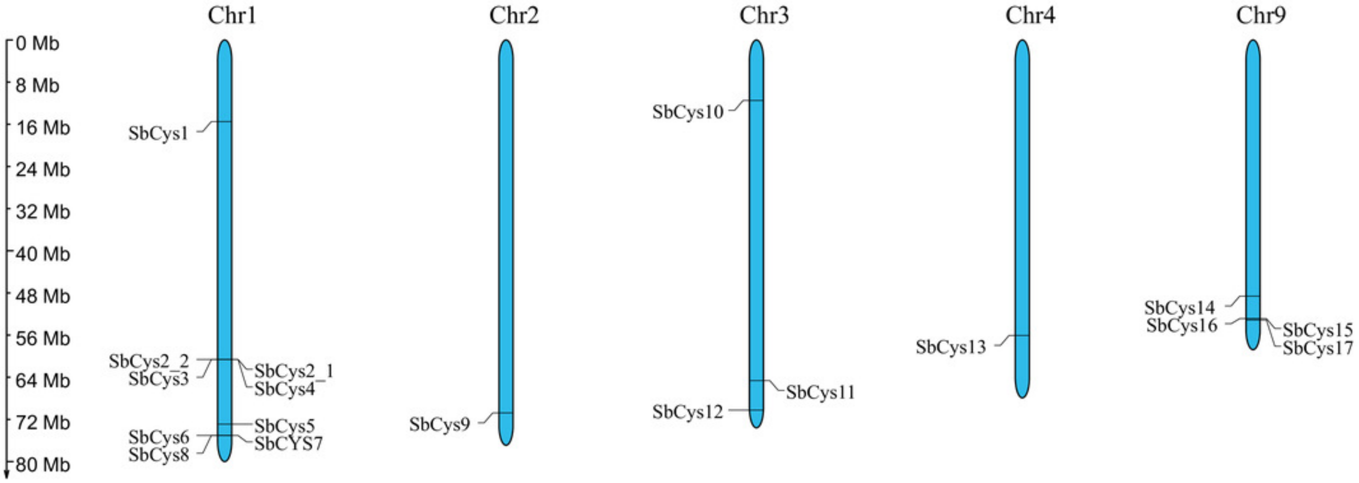


Figure 2

Phylogenetic relationship and gene structure of *SbCys* genes.

A phylogenetic tree was constructed using MEGA X by the maximum likelihood method with 1000 bootstrap replicates. Exon/intron structures were identified by online tool GSDS. Lengths of exons and introns of each *SbCys* genes were exhibited proportionally. Exons and introns are shown by blue bars and black horizontal lines, respectively.

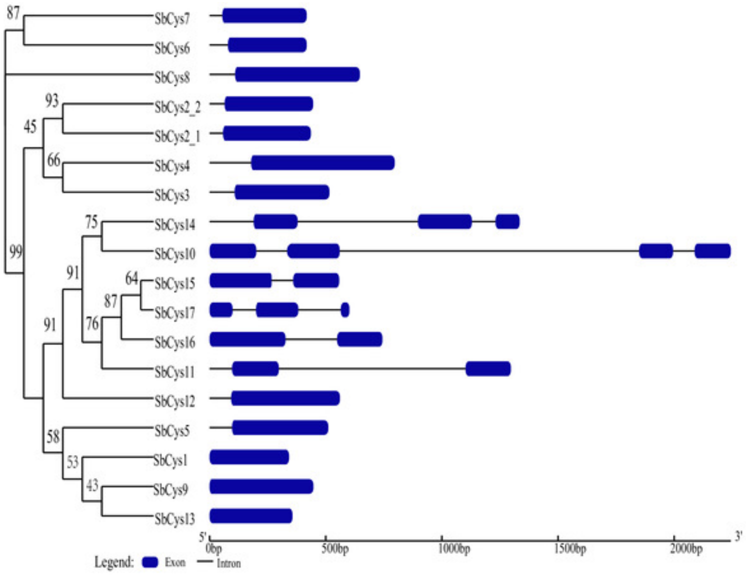


Figure 3

The amino acid alignment and conserved motifs distribution of SbCys.

(A) The locations of the secondary structures (α-helix and β-sheets) were included. The main cystatin conserved motifs are in black boxes. The strong and weak conservative changes in amino acids are marked by dark gray and light gray font, respectively. (B) The motifs were identified by MEME. Each motif was represented by one color box. (C) Conserved protein motif 1 (QxVxG), motif 2 (LARFAV and G-residue), motif 3 (W-residue), motif 4 ([RK]xLxxF), and motif 5(P-residue) presented in the variable region of cystatin genes.

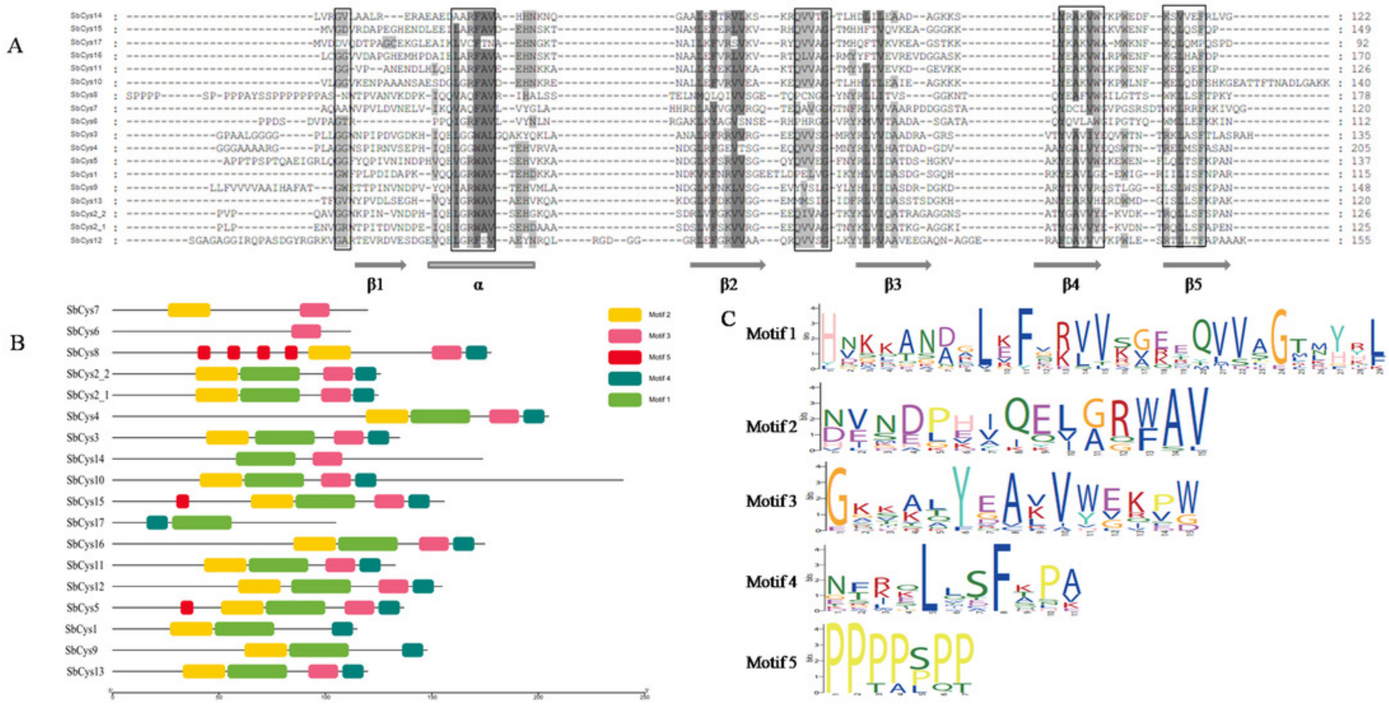


Figure 4

The three-dimensional structure prediction of Sorghum cystatins.

(A) The three-dimensional structures of SbCys proteins were predicted using the automated SWISS-MODEL program with OC-I as a template. (B) The three-dimensional structure of SbCys10 was predicted using the automated SWISS-MODEL program with SiCYS as a template. Two important motifs involved in the interaction with the target enzymes are indicated: the reactive site (asterisks) and W residue (crosses).

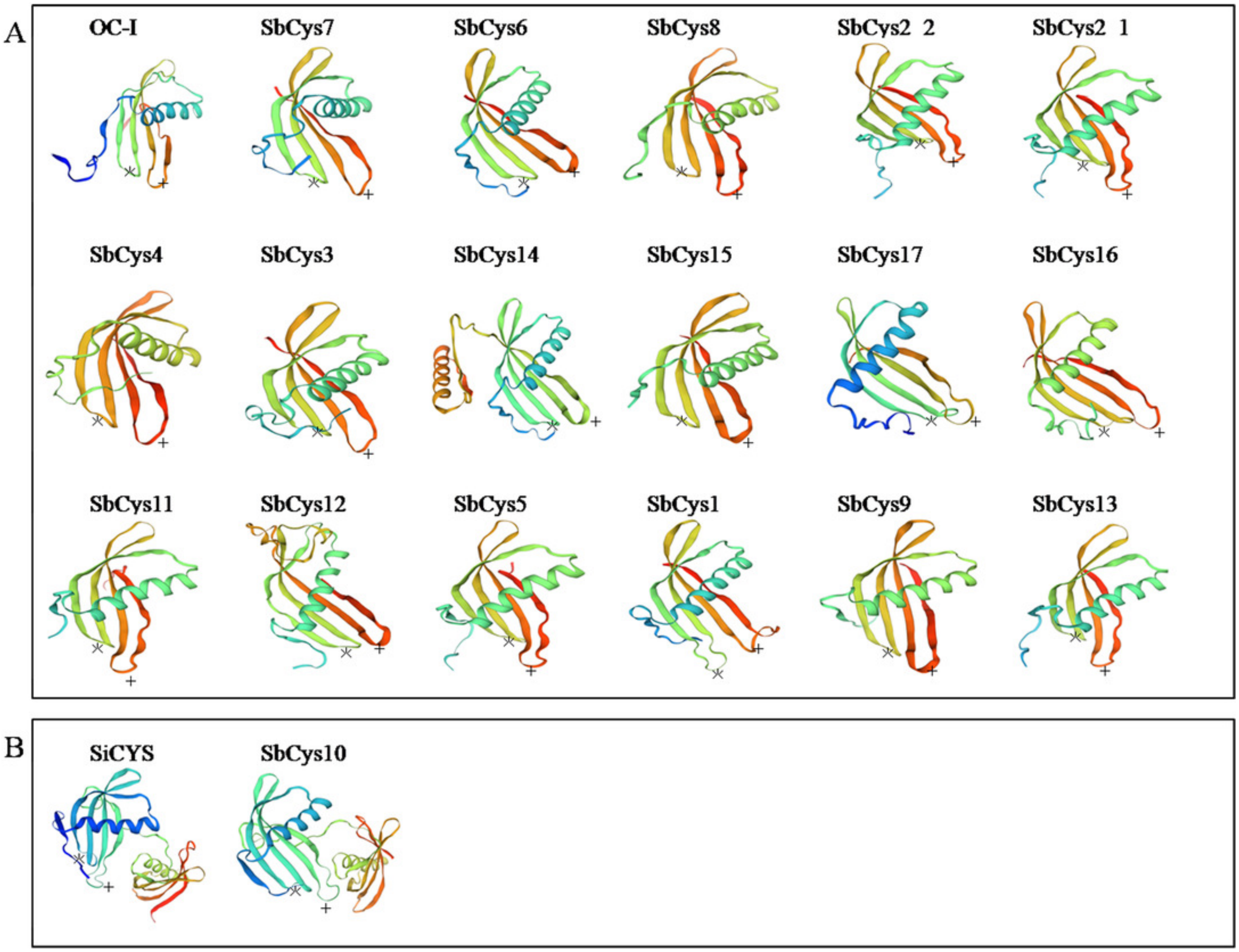


Figure 5

Phylogenetic relationships of the cystatins from *Arabidopsis*, rice, barley and Sorghum.

The phylogenetic tree was constructed by MEGA X with the maximum likelihood method. The numbers at the nodes indicate the bootstrap values. Gene names with black, red, and blue represented Group I, Group II, and Group III, respectively.

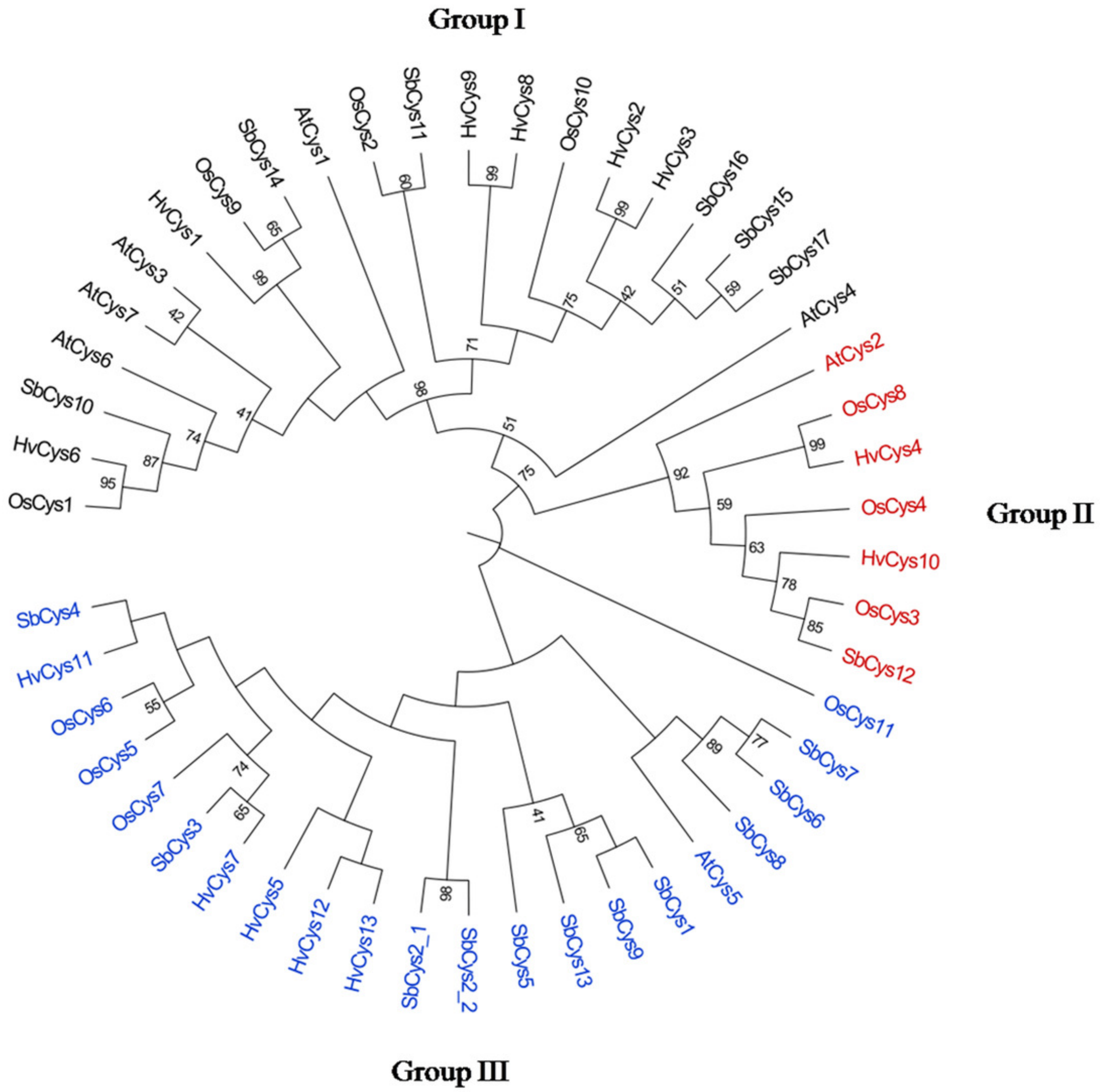

Group I
Group II
Group III
OsCys1
HvCys6
SbCys10
AtCys6
AtCys7
AtCys3
HvCys1
OsCys9
SbCys14
AtCys1
OsCys2
SbCys11
HvCys9
HvCys8
OsCys10
HvCys2
HvCys3
SbCys16
SbCys15
SbCys17
AtCys4
AtCys2
OsCys8
HvCys4
OsCys4
HvCys10
OsCys3
SbCys12
OsCys11
SbCys7
SbCys6
SbCys8
AtCys5
SbCys1
SbCys9
SbCys13
SbCys5
SbCys2_2
SbCys2_1
HvCys13
HvCys12
HvCys5
HvCys7
SbCys3
OsCys7
OsCys5
OsCys6
HvCys11
SbCys4
95
87
74
41
42
99
65
60
99
98
71
75
99
42
51
59
51
75
92
99
59
63
78
85
89
77
65
41
98
74
65
55

Figure 6

The distribution of *cis*-elements in the 1.5 kb upstream promoter regions of *SbCys* genes.

The *cis*-elements in the promoter region of *SbCys* genes were predicted using PlantCARE database (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/). Different *cis*-elements were represented by different shapes and colors.

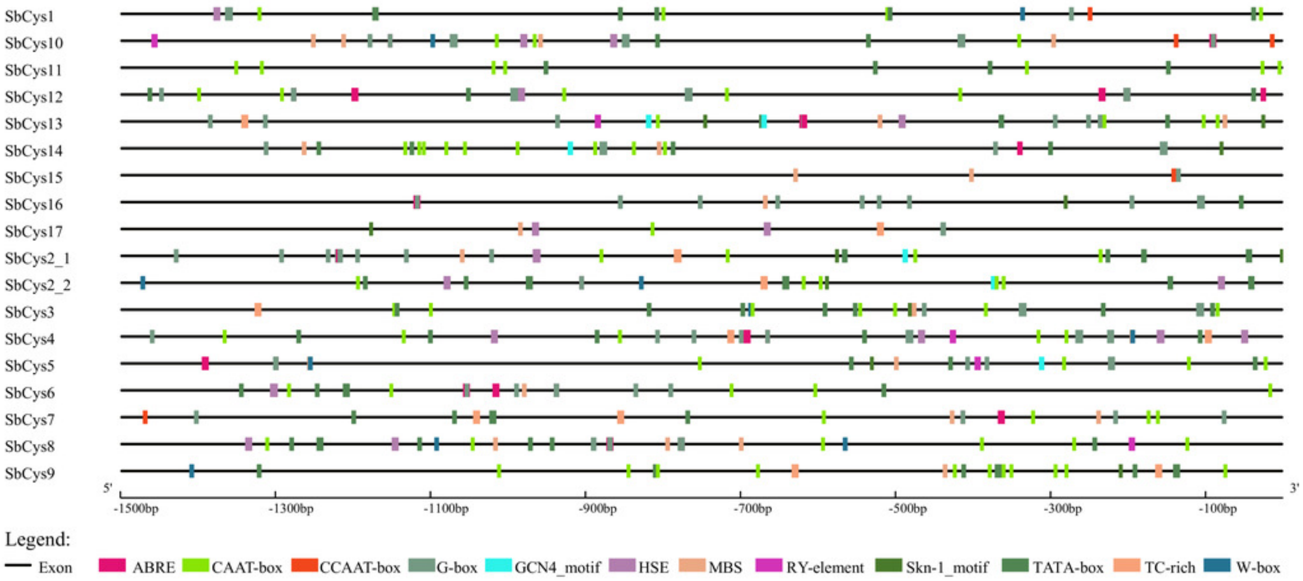


Figure 7

The interaction networks of SbCys proteins according to the orthologs in *Arabidopsis*.

Functional interacting network models were integrated using the STRING tool, and the confidence parameters were set at a 0.40 threshold. Homologous genes in Sorghum and *Arabidopsis* are shown in black and red, respectively.

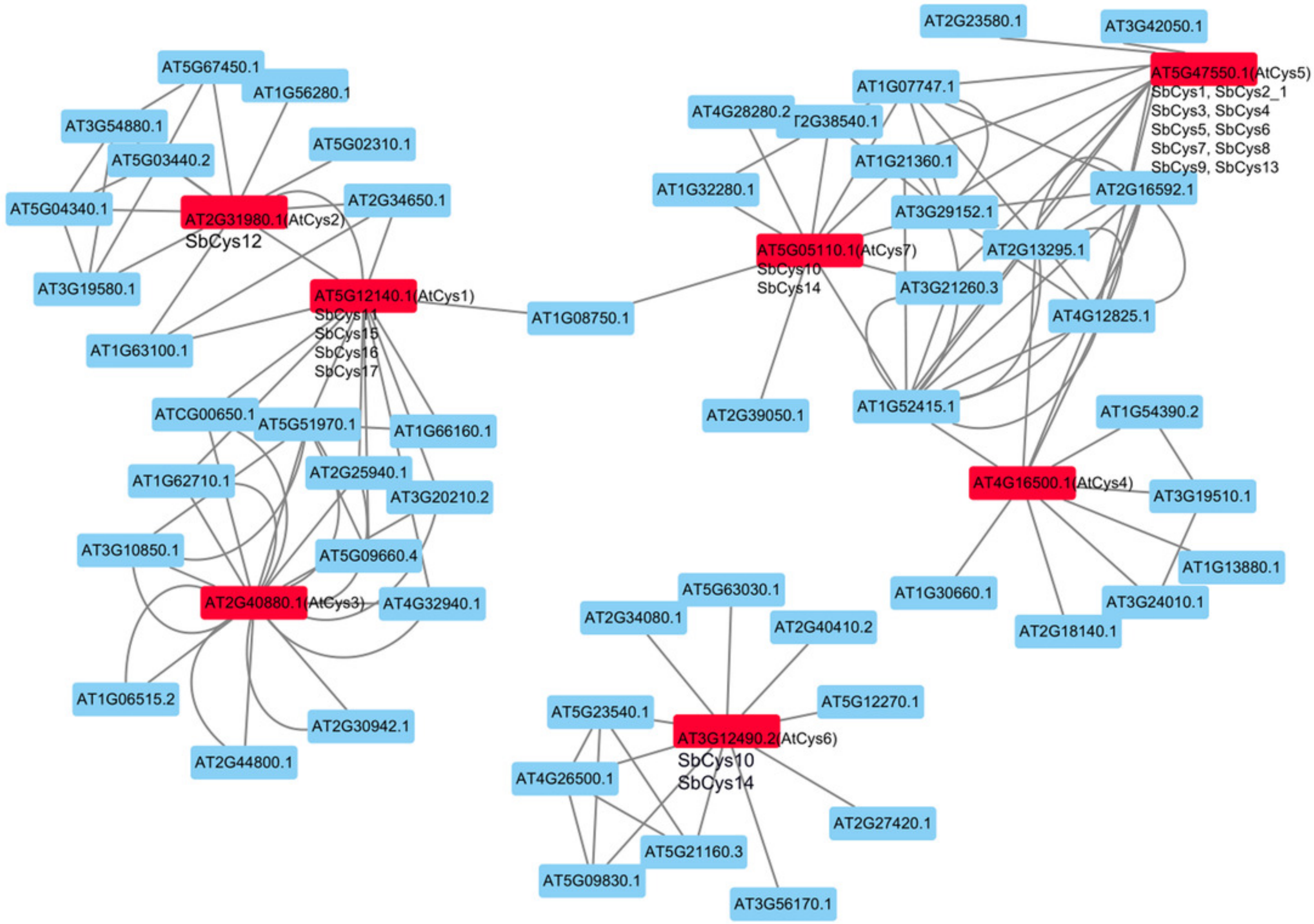


Figure 8

Hierarchical clustering of the expression profiles of *SbCys* genes in different tissues .

Different tissues are exhibited below each column. Root, shoot, and whole organism belonged to vegetable tissues were collected at 14 days after Sorghum seed germination. Reproductive tissues included embryo , endosperm and pericarp were collected at 20 days after pollination; pollens at booting stage; Inflorescences based on sizes: 1-5 mm, 5-10 mm, and 1-2 cm. Log transform data was used to create the heatmap. The scale bar represented the fold change (color figure online). Blue blocks represented the lower expression level and red blocks represented the higher expression level.

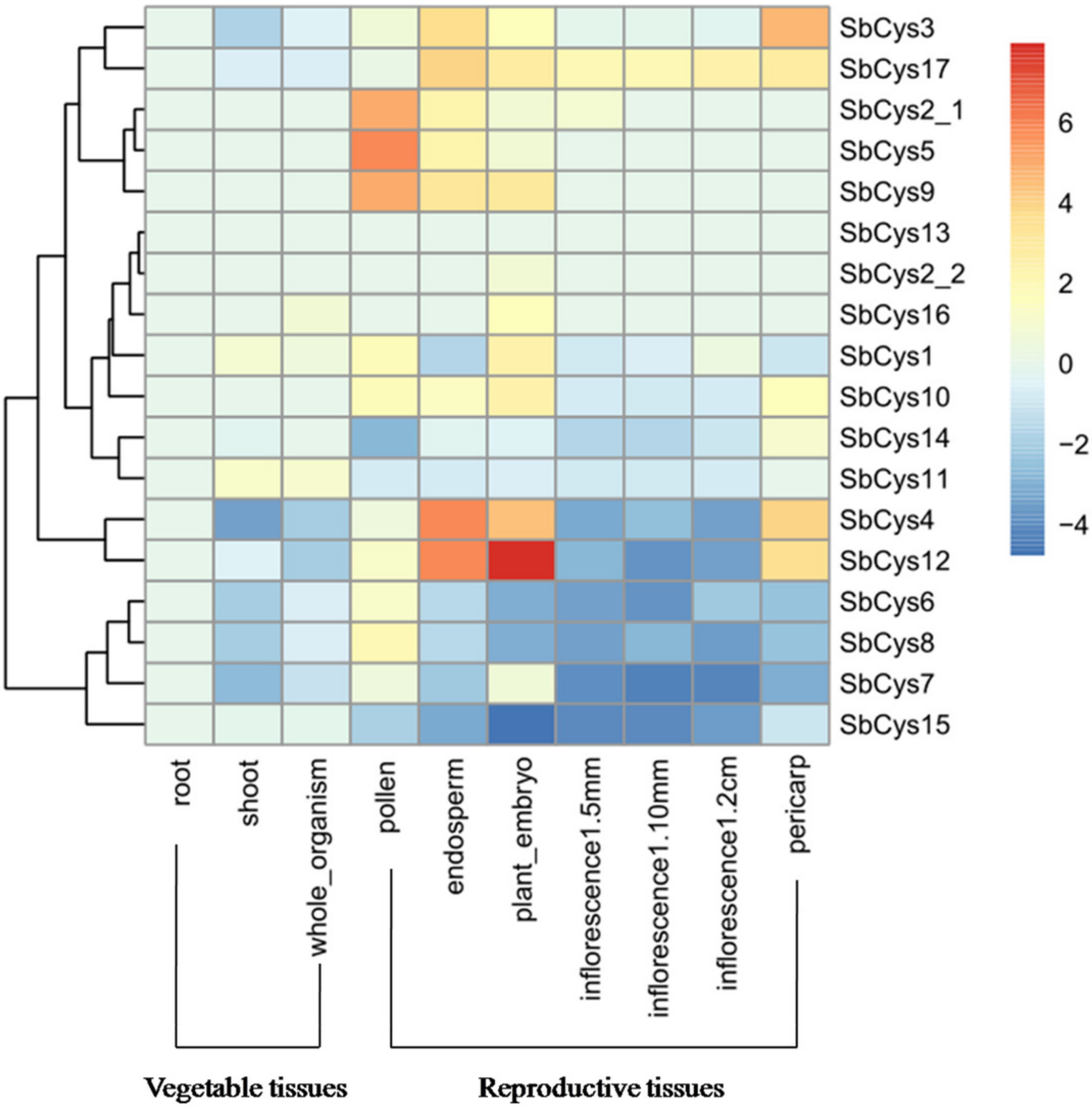

SbCys3
SbCys17
SbCys2_1
SbCys5
SbCys9
SbCys13
SbCys2_2
SbCys16
SbCys1
SbCys10
SbCys14
SbCys11
SbCys4
SbCys12
SbCys6
SbCys8
SbCys7
SbCys15
6
4
2
0
−2
−4
root
shoot
whole_organism
pollen
endosperm
plant_embryo
inflorescence1.5mm
inflorescence1.10mm
inflorescence1.2cm
pericarp
Vegetable tissues
Reproductive tissues

Figure 9

Hierarchical clustering of the expression profiles of *SbCys* genes under biotic stresses.

(A) The expression changes in *SbCys* genes at 0, 12, and 24 hours with *Bipolaris sorghicola* infection. (B) The expression changes of *SbCys* genes at 5, 10, 15 days with sugarcane aphid infestation. Log transform data was used to create the heatmap. The scale bar represents the fold change (color figure online). Blue blocks indicate low expression and red blocks indicate high expression (color figure online).

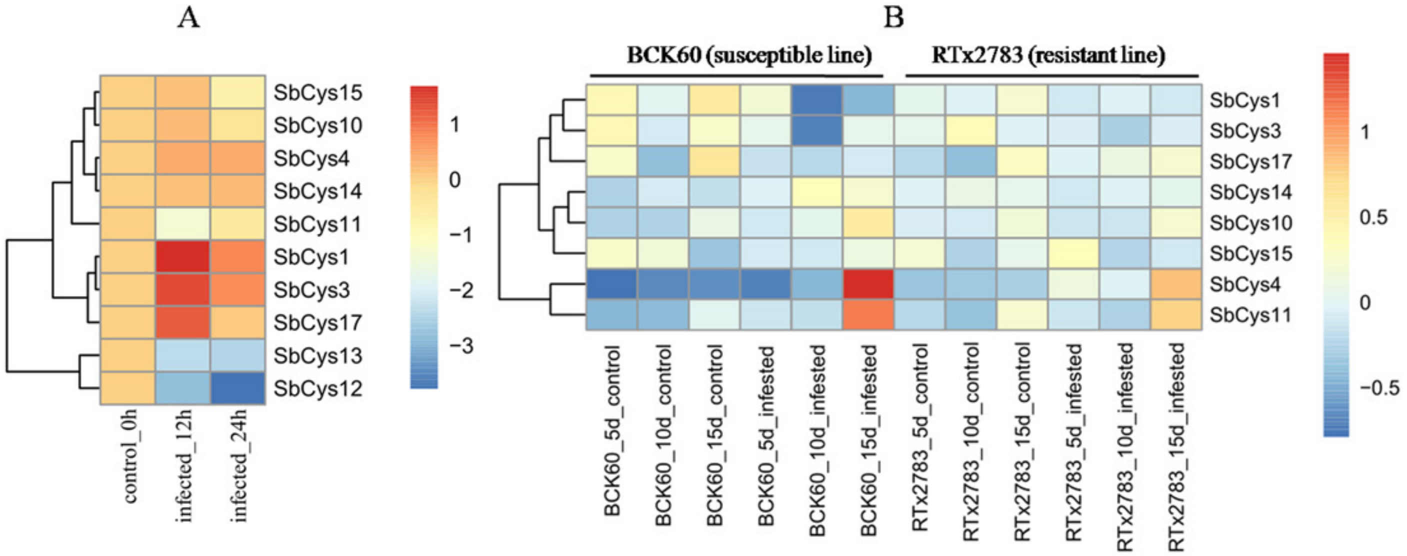


Figure 10

Expression profiles of *SbCys* genes at 5, 10, and 15 days with sugarcane aphid infection.

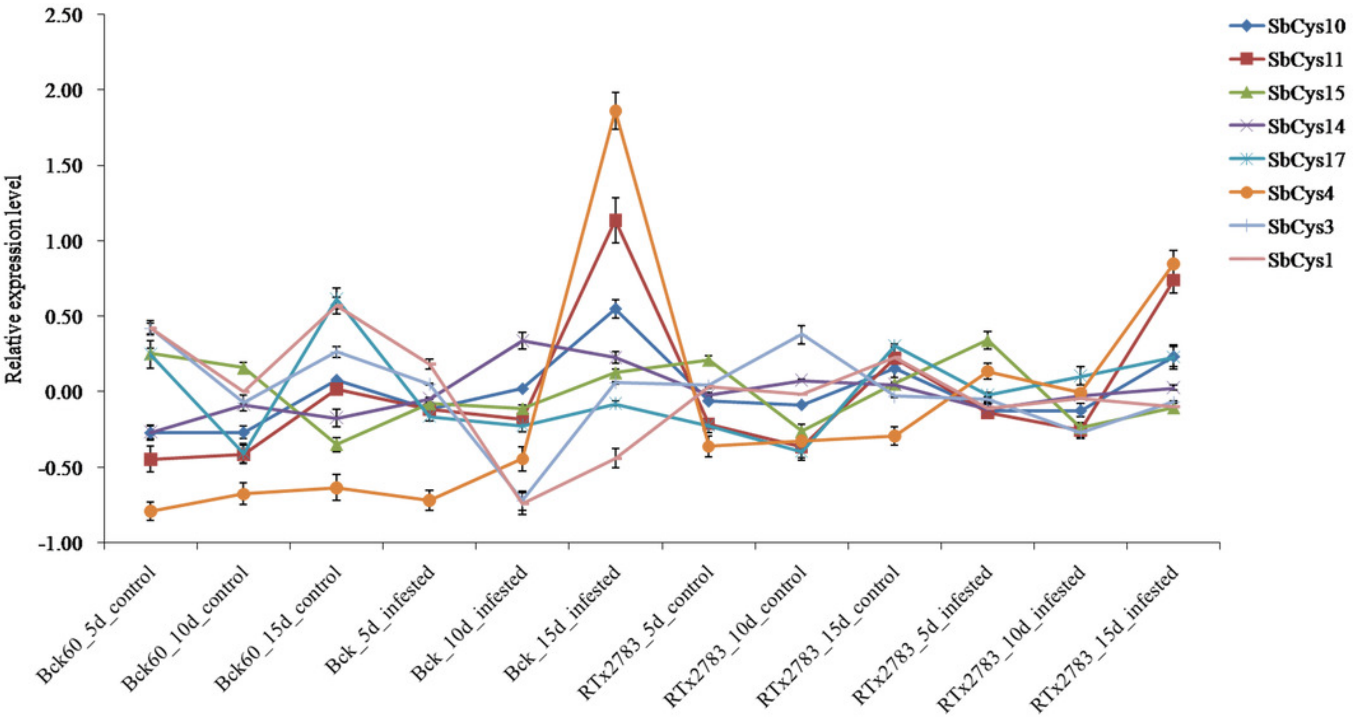
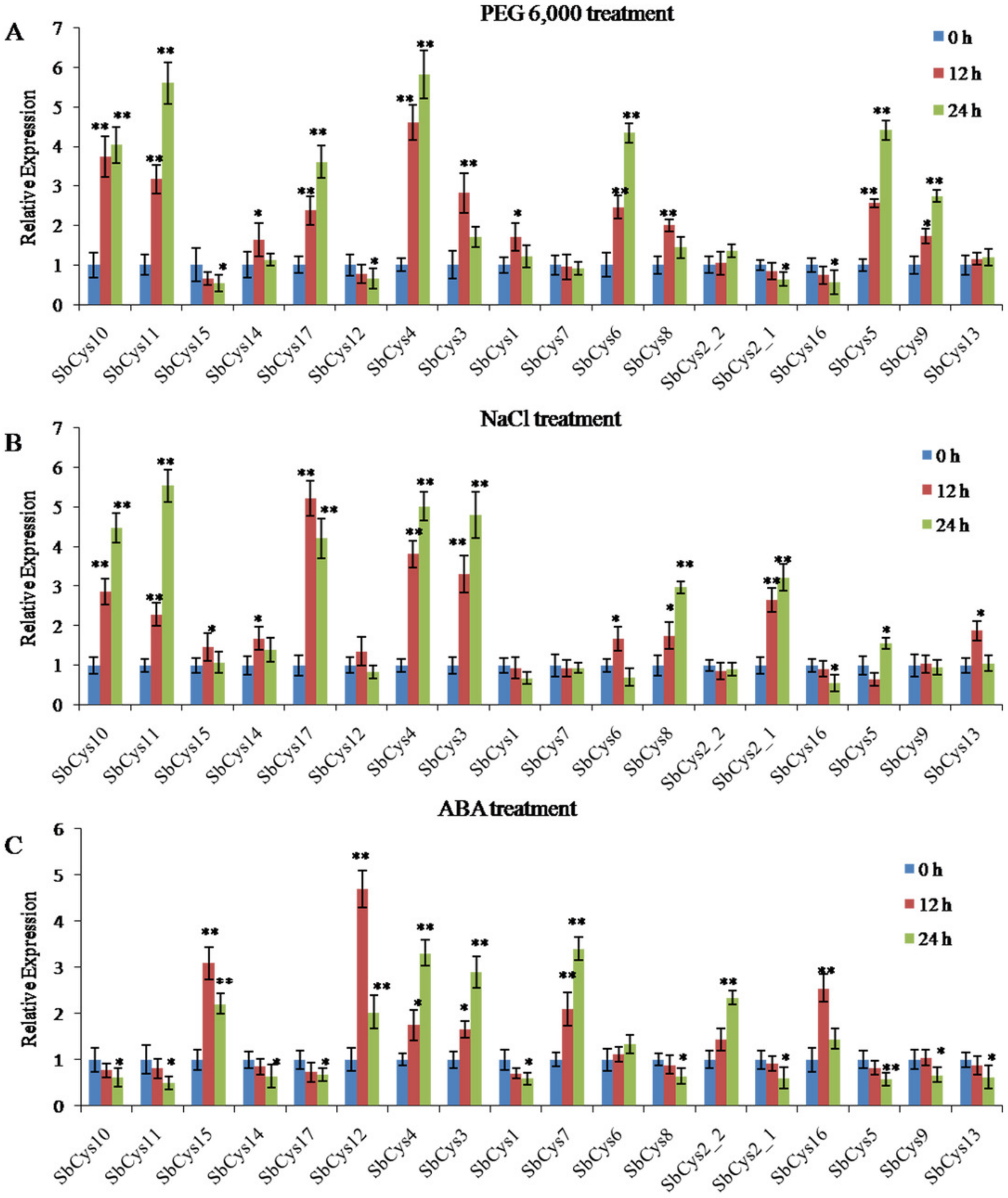


Figure 11

Expression patterns of *SbCys* genes under (A) dehydration (PEG 6,000) treatment, (B) salt shock (NaCl) treatment, and (C) ABA treatment.

qRT-PCR was used to investigate the expression levels of each *SbCys* gene. To visualize the relative expression levels data, 0 h at each treatment was normalized as “1”. * indicated significant differences in comparison with the control at $p \leq 0.05$. ** indicated significant differences in comparison with the control at $p \leq 0.01$.


A
PEG 6,000 treatment
B
NaCl treatment
C
ABA treatment
Relative Expression
0 h
12 h
24 h
0
1
2
3
4
5
6
7
*
**
SbCys10
SbCys11
SbCys15
SbCys14
SbCys17
SbCys12
SbCys4
SbCys3
SbCys1
SbCys7
SbCys6
SbCys8
SbCys2_2
SbCys2_1
SbCys16
SbCys5
SbCys9
SbCys13