

Genome-wide analysis of AP2/ERF Transcription Factors in sugarcane *Saccharum spontaneum* L. Reveals Functional Divergence During Drought, Salt Stress and Plant Hormones Treatment

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Abstract

Background: APETALA2/ETHYLENE RESPONSIVE FACTOR (AP2/ERF) transcription factors play important roles in plant growth, development, metabolism, as well as in biotic and abiotic stress responses. However, there are few studies concerning *AP2/ERF* genes in sugarcane, which is the most critical sugar and energy crop worldwide.

Results: A total of 218 *AP2/ERF* genes were identified in the *Saccharum spontaneum* genome. Phylogenetic analysis showed that these genes could be divided into four groups, including 43 *AP2s*, 160 *ERFs*, and *Dehydration-responsive element-binding (DREB)* factors, 11 *ABI3/VPs (RAV)* and 4 *Soloist* genes. These genes were unevenly distributed on 32 chromosomes. Analysis of the structural of *SsAP2/ERF* genes showed that 91 *SsAP2/ERFs* lacked introns. Sugarcane and sorghum have a collinear relationship between 168 *SsAP2/ERF* genes and sorghum *AP2/ERF* genes that reflects their similarity. Multiple cis-regulatory elements (CREs) are present in the *SsAP2/ERF* promoter, and many are related to abiotic stresses, suggesting that *SsAP2/ERF* activity could contribute to the adaptation of sugarcane crops to environmental changes. The tissue-specific analysis showed spatiotemporal expression of *SsAP2/ERF* in the stems and leaves of sugarcane at different stages of development. In 10 sugarcane samples, 39 *SsAP2/ERFs* were not expressed at all, whereas 58 *SsAP2/ERFs* were expressed in all samples. Quantitative PCR experiments showed that *SsERF52* expression was up-regulated under salt stress, but suppressed under drought stress. *SsSoloist4* had the most considerable upregulation in response to treatment with the exogenous hormones ABA and GA. Within 3 hours of ABA or PEG6000 treatment, *SsSoloist4* expression was up-regulated, indicating that this gene could play a role in ABA and GA-associated drought stress response mechanisms. Analysis of *AP2/ERF* gene expression patterns under different treatments indicated that *SsAP2/ERF* genes play an important role in drought and salt stress responses of *S. spontaneum*.

Conclusions: In this study, a total of 218 members of the AP2 / ERF superfamily were identified in sugarcane, and their genetic structure, evolution characteristics, and expression patterns were studied and analyzed. The results of this study provide a foundation for future analyses to elucidate the importance of AP2/ERF transcription factors in the function and molecular breeding of sugarcane.

Introduction

Unfavorable environmental conditions can have severe impacts on crop yields, and thus strategies to improve crop survival under adverse conditions are needed [1]. Plants respond to environmental stress via complex regulatory mechanisms that elicit a series of physiological and biochemical responses [2]. Transcription factors play an essential role in converting stress-induced signals into cellular responses. When various abiotic stresses stimulate plants, signaling pathways involving molecules such as abscisic acid and ethylene are activated [3]. This activation is often associated with changes in the expression of transcription factors, which specifically bind to trans-acting elements in promoter regions at downstream target genes. For cis-acting elements, the regulatory effect is executed through the activation or inhibition

of the expression of downstream functional genes [4]. In plants, these two main processes are involved in responses to biotic or abiotic stress that are mediated by various transcription factors. The AP2/EREBP (APETALA2/ethylene response element-binding protein) superfamily comprises a large class of transcription factors in plants. Multiple studies have demonstrated that AP2/ERF transcription factors in plants are important for stress responses, and their expression is regulated by plant hormones [5–7]. In response to stress in plants, expression of AP2/ERF transcription factors is regulated to coordinate growth under stress conditions [8].

AP2/ERF family members contain a highly conserved AP2/ERF DNA binding domain. Based on sequence similarities and the number of AP2/ERF domains, the AP2/ERF superfamily can be divided into four categories: AP2, ERF (Ethylene-responsive factor), RAV (related to ABI3/VP1) and Soloist[9]. In most cases, the AP2 family contains proteins having two AP2/ERF domains that are known to be involved in regulating plant developmental processes [10]. RAV proteins contain two different DNA-binding domains (AP2 and B3), which are regulated by ethylene or brassinosteroid hormones and also are involved in biotic and abiotic stress responses [11, 12]. The ERF family is divided into two subfamilies: ERF and DREB (dewater-responsive element binding). Both ERF and DREB contain only one AP2/ERF domain and are key regulators of plant responses to biotic and abiotic stress[13]. The Soloist group contains only one AP2 conserved domain and forms a separate group based on its significant structural difference from the other AP2/ERF family members. However, there is limited evidence that members of the Soloist group are positive regulators of SA-mediated plant defense against pathogens [14].

AP2/ERF transcription factors have well-documented functions in plant growth and development. For example, in *Arabidopsis thaliana* WIND1 (RAP2.4 ,At1g78080) is involved in controlling cell differentiation that, in turn, affects proliferation, axillary bud growth, and bud branching [15]. The ERF family gene OsEATB in rice limits internode elongation by down-regulating gibberellin biosynthesis genes [16]. In tomatoes [17], grapes [18], Chinese jujube [19], and bananas [20], some AP2/ERF superfamily members are involved in fruit maturation.

AP2/ERF transcription factors also play a crucial role in abiotic stress responses in plants. For example, OsEREBP1 and OsEREBP2 modulate the expression of OsRMC, a negative regulator of rice salt stress [21]. Overexpression of maize ZmDBP3 enhances tolerance of transgenic *Arabidopsis* to drought and cold stress [22]. In contrast, overexpression of the WIN₁ gene from sorghum confers drought resistance to *Arabidopsis* by regulating the biosynthesis of the epidermis [23]. The ERF and DREB families, in particular, contain members that have excellent performance in response to abiotic stress. The DREB protein can specifically bind A/GCCGAC (DRE/CRT) elements related to genes involved in responses to ABA, drought, and low temperature while ERF subfamily members can interact with the core sequence AGCCGCC (GCC-box) of the ethylene response element (ERE). Such binding to ERE elements regulate responses to ethylene response and abiotic stress, and also promotes disease resistance [24]. However, many reports suggest that both *Arabidopsis* ERF and DREB can be combined with DRE/CRT or ERE elements, indicating that they have a potential role in abiotic and biotic stress [25–27]. DREBs belong to the ABA-independent signal transduction pathway and can be divided into two subclasses: DREB1/CBF

and DREB2. DREB1/CBF genes are thought to be involved mainly in the sensation of low temperature, whereas most DREB2 genes participate in responses to water or heat shock stress [28]. However, there is increasing evidence that the stress regulation mediated by the DREB1/CBF and DREB2 genes is species-specific [29–31]. AP2/ERF transcription factors are also likely to be essential mediators of plant resistance, but there are few studies concerning the activity of these genes in sugarcane.

Sugarcane (*Saccharum* spp.) is the world's most important crop for sugar and biofuel [32]. Sugarcane provides 75% and 40% of global sugar and ethanol production [32, 33]. Damage to sugarcane caused by environmental stress thus can have substantial economic impacts [34]. For example, drought stress during sugar cane growth can reduce productivity by between 30% and 70%, and reduce sucrose formation and sucrose recovery by 5% [35]. Genome-wide analysis of the presence of AP2/ERF transcription factors in wild sugarcane *Saccharum spontaneum* species would be necessary for sugarcane resistance research.

In this study, we identified members of the AP2/ERF superfamily in the *S. spontaneum* genome. We also carried out the phylogenetic relationships, gene structure, conserved domains, promoters, chromosomal location distribution, and gene duplication of these genes. The effects of AP2/ERF genes on sugarcane adaptation to environmental changes were analyzed to enhance our understanding of the mechanisms by which AP2/ERF transcription factors modulate abiotic stress responses.

Materials And Methods

Identification and classification of members AP2/ERF superfamily genes in sugarcane

The genome sequences and the sequence information of sugarcane were downloaded from the website of 'S.spontaneum AP85-441 genome' (http://www.life.illinois.edu/ming/downloads/Spontaneum_genome/). The protein sequences of AP2/ERF superfamily genes in rice and *Arabidopsis* were collected from the NCBI (<https://www.ncbi.nlm.nih.gov/>). These proteins were used as query sequences in the local BLAST program (Basic Local Alignment Search Tool) in order to find members of AP2/ERF superfamily genes of the sugarcane genome with the following parameters: expected values $\leq 1E-5$. All BLAST hits were checked and searched for conserved AP2 domains online using the Search Pfam feature of the Pfam (<https://pfam.xfam.org/>) website under default parameters. In addition, the results of Pfam were verified again using the NCBI CDD tool (<https://www.ncbi.nlm.nih.gov/Structure/bwrpsb/bwrpsb.cgi>) and the cutoff set to 0.01.

Phylogenetic analysis of sugarcane AP2/ERF genes

Multiple alignments of candidate AP2/ERF genes were performed to explore the phylogenetic relationship of sugarcane AP2/ERF genes using ClustalW [36] with default parameters. The results were used to construct phylogenetic trees by the neighbor-joining method and were then visualized using MEGA 6.0

software [37]. The phylogenetic trees were generated using complete protein sequences with the following parameters: pair-wise deletion, Poisson correction, and 1,000 bootstrap replicate.

Gene structure and conserved motif analyses

Conserved motifs of AP2/ERF proteins were identified using the online tool Multiple Em for Motif Elicitation (MEME) version 5.0.5 [38] (<http://meme-suite.org/tools/meme>) with the following parameters: (1) the number of occurrences of a single motif distributed among the sequences within the model was set to zero or one per sequence; (2) the maximum number of motifs found was set as 25; (3) the optimum motif width was set to ≥ 6 and ≤ 50 ; and (4) motifs with a matched E-value should be below 0.05. Gene structure was investigated using GSDS 2.0 (<http://gsds.cbi.pku.edu.cn>) [39]. We used TBtools software [40] to integrate phylogenetic trees, conserved motifs, and gene structure results.

Chromosomal distribution and duplication analysis of AP2/ERF superfamily genes

The chromosomal distribution information of the identified genes was searched against the reference sugarcane genome database, and the results obtained were visualized using TBtools software. Analysis of gene Duplication events using Multiple collinear scanning toolkits (MCScanX) [41]. The syntenic relationship between the SsAP2/ERF genes and AP2/ERF genes from selected plants was determined by using Dual Synteny Plotter software (<https://github.com/CJ-Chen/TBtools>). The putative duplication events were detected for the AP2/ERF genes. Tandem duplication was identified as two proteins with a similarity of greater than 40% and separated by four or fewer gene loci; others were identified as segmental duplications, separated by more than five genes. Non-synonymous (k_a) and synonymous (k_s) substitution of each duplicated AP2/ERF genes were calculated using KaKs_Calculator 2.0 [42]. The divergence time (T) was calculated by

$T = Ks / (2 \times 6.1 \times 10^{-9}) \times 10^{-6}$ Mya [43]. These results were visualized using TB tools.

Analysis of cis-acting elements of AP2/ERF superfamily genes

Two thousand bp upstream of the transcriptional start site of each SsAP2/ERF gene was selected to inspect potential CREs. They were submitted to the PlantCARE website (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>).

Transcriptome data source and bioinformatic analysis

Sugarcane tissue-specific expression data from Saccharum Genome Database (SGD: <http://sugarcane.zhangjisenlab.cn/sgd/html/index.html>). Data includes leaves and stems of the seedling stage of 35 days and leaves and stems (3, 6, 9) at the early maturity stage of 9 months and at the maturity stage of 12 months. All SsAP2 / ERF FPKMs (Segmentals Per Kilobase of transcript per Million segmental mapped) are used to make heat maps and cluster analysis through TB tools.

Plant materials

The experimental materials were tissue culture seedlings of sugarcane cultivar ROC22. When the tissue culture seedlings were treated with hormones and simulated abiotic stress at the stage of 5–7 leaf, respectively. Hormonal treatment was sprayed with abscisic acid (ABA, 100uM) and gibberellin (GA, 100uM), respectively. Abiotic stress treatment was irrigated with NaCl (250 mM) to simulate salt stress, and PEG6000 (20%) was used to simulate drought stress. Sampling time points are 1, 3, 6, 12, 24, and 72 hours. The collected samples were quickly placed in liquid nitrogen and stored in a -80 ° C refrigerator for subsequent RNA extraction.

Quantitative Real-Time PCR (qRT-PCR) analysis

Each RT-qPCR reaction mixture comprised of 20 µL reaction buffer consisting of 10 µL Taq polymerase (SYBR Premix Ex TaqII; Takara), 2 µL of each forward and reverse primers (2 µM), 2 µL of cDNA and 6 µL of water. The RNA expression level was normalized to the level of 25S-rRNA expression. The amplification was conducted in LightCycler 96 Real-Time PCR System (Roche Lightcycler® 480). A standard thermal profile for SYBR Premix was as followed: cDNA synthesis at 37 °C for 15 min and enzyme inactivation at 85 °C for 5 s. qPCR conditions were: initial denaturation 95 °C for 30 s, denaturation 95 °C for 5 s, annealing and extension 60 °C for 30 s. Transcripts expression levels were calculated with the $2^{-\Delta\Delta Ct}$ method, as previously mentioned in Livak, K. J. and Schmittgen, T. D [44]. Primers used for this analysis are mentioned in Additional file 12.

Data Statistics

All data were analyzed for variance using IBM SPSS Statistics. Statistical methods were used to compare the significance levels of LSD (least significant difference test, LSD) at 0.05.

Results

Identification and classification of AP2/ERF genes

A total of 218 complete AP2/ERF genes were identified in the sugarcane genome database. The identified genes ranged from 416 to 22,786 bp and were predicted to encode proteins having between 127 to 876 amino acids (aa) (Additional file 1). Based on sequence similarities and the number of conserved AP2 domains (Additional file 4), the AP2/ERF genes were divided into four families: AP2, ERF, RAV, and Soloist. The AP2 family had 43 genes, of which 36 have two identical conserved AP2 domains, and 7 have only one conserved AP2 domain (SsAP2-37 to SsAP2-43). Arabidopsis has 4 AP2/ERF genes that contain only one AP2 domain, but these differ from the ERF type and are closer to the AP2 type. These four genes are, therefore, classified as the AP2 family. The phylogenetic tree shows that the branches of these 7 SsAP2/ERF genes aggregate with the AP2 family instead of the ERF family, so they are also classified as AP2 families according to the classification of Arabidopsis. The ERF family had 160 genes that have only one AP2 conserved domain. Among them, 59 and 101 were assigned to DREB (SsDREB1 to SsDREB59) and ERF (SsERF1 to SsERF101) subfamily, respectively. The RAV family comprises 11 members (SsRAV1

to SsRAV11) that contain conserved AP2 and B3 domains. Another four genes (SsSoloist1 to SsSoloist4) also had only one conserved AP2 domain but had less similarity to ERF. Instead, these four genes had higher homology to Arabidopsis At4g13040 and thus were classified into the Soloist. Because no reliable naming method was defined in previous research on the AP2/ERF family, our naming convention is based on the grouping of 218 sequences and their chromosomal positions. The total number of AP2/ERF superfamily candidate genes in sugarcane is higher than that for Arabidopsis (147) and rice (181) [9]. The number of genes in the AP2, RAV, and Soloist subfamilies was double that for both Arabidopsis and rice. However, the number of ERF family members was similar but slightly higher (122 and 145 for Arabidopsis and rice, respectively).

Phylogenetic Analysis

To analyze the evolutionary relationship of the SsAP2/ERF genes, we constructed phylogenetic trees based on the amino acid sequences encoded by these genes (Fig. 1). The phylogenetic tree clusters all of the SsAP2/ERF genes and had four main branches: AP2, ERF, RAV, and Soloist, which are consistent with the results for examination of conserved amino acid sequences encoded by the candidate genes.

According to the classification criteria for Arabidopsis and rice [9], the ERF family was divided into two subfamilies: the DREB subfamily (59 members, divided into groups I, II, III and IV, containing 19, 11, 19 and 10 members, respectively); and the ERF subfamily (101 members divided into groups V, VI, VII, VIII, IX, X, and XIV containing 12, 11, 12, 18, 24, 16 and 8 members, respectively (Fig. 2). Notably, most species lack a XIV group, although Os08g41030 in rice has a conserved domain similar to these eight members of sugarcane and can be classified into the XIV group. Arabidopsis has no genes that are consistent with the XIV group. Thus additional examination is needed to determine whether functional differentiation occurred and the significance of such differentiation.

Gene structure and conserved motif analysis

To characterize the structural diversity of SsAP2/ERF genes, we analyzed the number of introns and exons and the distribution of conserved domains in the coding sequence of a single SsAP2/ERF gene (Additional file 5: Fig. S6). Through gene structure analysis, differences in the AP2, ERF, and RAV subfamilies can be observed, and the results of the conserved domain analysis were consistent with those of previous studies. The number of introns among the different family genes varied markedly (Fig. 3B) and 41, 40, 1, and 9 members of the SsDREB, SsERF, SsAP2, and SsRAV subfamilies, respectively, have no introns. For the SsERF family members, 50.6% have no introns, whereas 97.6% of SsAP2 family members do. Meanwhile, 81.8% of SsRAVs have no introns, but all 4 SsSoloist members do.

We used MEME to investigate SsAP2/ERF gene diversity further and predicted the presence of 25 conserved motifs in the AP2/ERF family (Fig. 3C). Motif-1 and -2 were present in all SsAP2/ERF protein sequences; Both motif-3 and -4 were available in most members of the AP2, ERF, DREB, and RAV subfamily; Motif-6, -7, -8, -20, and -22 were found only in the AP2 family; Motif-10, -12 and -15 appeared in Group I; Motif-13 appeared in Group XIV, and Motif-9 and -11 were unique to the RAV family. We also

found that Motif-1, -2, and -7 existed in the AP2 conserved domain, whereas Motif-9, -11, and -16 were located in the B3 domain (Fig. 3C). As expected, most close relatives among subfamily members had similar motif compositions, suggesting that AP2/ERF proteins within the same subfamily are functionally identical.

Chromosome distribution

The 218 SsAP2/ERF genes we identified were mapped to 32 chromosomes, and the distribution across the chromosomes varied widely (Fig. 4). Chr2A had the most with 12 genes, whereas Chr6C and Chr8C each had only three genes (Additional file 7). At least one of the 59 SsDREB and 101 SsERF genes could be found on each of the 32 chromosomes, and the four Soloist genes were distributed on four homologous chromosomes, Chr4A, Chr4B, Chr4C, and Chr4D. Members of the SsRAV family were present only on chromosomes 3 and 7. Surprisingly, 50% of the SsAP2/ERF genes localized to one of the four rearrangement chromosomes (SsChr02, SsChr05, SsChr06 and SsChr07) and 9 SsAP2/ERF genes were present in the rearranged region that includes SsChr5 (Sb05S) 57.6–89.1 Mbp, SsChr6 (Sb05L) 54.6–90.6 Mbp, SsChr7 (Sb08S) 62.0–83.3 Mbp, SsChr2 (Sb08L) 98.5–125.9 Mbp [45].

Gene duplication analysis

Multiple studies indicated that gene families evolved due to genome-wide duplication, segmental duplication or tandem duplication, and gene diversification occurred after these duplication events. To explore SsAP2/ERF genes evolution, we studied tandem and segmental repetitive events of these genes using chromosomal information for *S. spontaneum* (Additional file 8). A total of 8 pairs of tandem duplication genes were detected, and of these two pairs were RAV genes, and six ones were ERF genes (Fig. 4). In particular, SRAV4 and SsRAV5, SsRAV5, and SsRAV6 are two tandem duplication gene pairs, and three genes are located on the same chromosome and are adjacent. In addition, 70 pairs of 103 SsAP2/ERF segmental duplication genes were found (Fig. 5). Among these, 53 occurred between alleles, and 17 were between non-alleles. There were four pairs of SsAP2/ERF segmental duplication gene pairs distributed on Chr4 and Chr5. This distribution may be because both Chr4 and Chr5 chromosomes include segments from the ancestral A4 chromosome [45].

We then estimated the time for the divergence of the SsAP2/ERF genes that were predicted to have undergone tandem and segmental duplication based on Ks values (Additional file 8). The divergence time for the 8 SsAP2/ERF tandem duplication pairs ranged from 14.2 to 104.2 million years ago (mya), illustrating that these SsAP2/ERFs arose from recent gene duplication events in *S. spontaneum*. Meanwhile, 64 segmental duplication pairs arose earlier, based on a divergence time that ranged from 4.9 to 89.9 mya, whereas the other 4 segmental duplication pairs were ancient, diverging between 164.7 and 212.1 mya.

Non-synonymous (Ka) and synonymous (Ks) mutation frequencies can be used to assess selection pressure after duplication events, where $Ka/Ks = 1$ indicated neutral selection, $Ka/Ks < 1$ showed purifying selection, and $Ka/Ks > 1$ indicated positive selection of evolution. We calculated Ka/Ks values

for SsAP2/ERF genes in tandem and segmental duplications to detect which selection type promoted AP2/ERF gene family evolution (Additional file 8). The Ka/Ks ratio of tandem duplication gene pairs among AP2/ERF genes ranged from 0.17 to 1.24, with an average of 0.57. Tandem duplication gene pairs having a Ka/Ks ratio < 1 accounted for 89% of the genes tested. The Ka/Ks ratio for segmental duplication gene pairs ranged from 0 to 2.6, with an average of 0.62, and 92% of pairs had Ka/Ks < 1. These results indicate that repetitive SsAP2/ERF genes were under intense purification selection pressure, and the duplication-producing gene had strongly evolved and maintained its functional stability. Meanwhile, nine replicate gene pairs had Ka/Ks > 1, indicating that these genes were subject to positive selection after duplication differentiation.

Evolutionary analysis of SsAP2/ERFs and other plant AP2/ERFs

A syntenic map of five representative species was constructed to examine the evolutionary origin of the AP2/ERF genes in sugarcane (Fig. 6 and Additional file 9). A total of 168 SsAP2/ERF genes were synonymous with genes in sorghum, followed by rice (151), wheat (145), corn (143), and Arabidopsis (19). Orthologous gene pairs were distributed across all SsChrs. Some SsAP2/ERF genes were associated with at least three pairs of corresponding genes (particularly AP2/ERF genes in sugarcane and wheat), suggesting that these genes played an essential role in SsAP2/ERF superfamily evolution. Interestingly, some collinear gene pairs (90 SsAP2/ERF genes) between sugarcane and rice/sorghum/wheat/maize were identified but were absent between sugarcane and Arabidopsis, indicating that these orthologous pairs formed after the divergence of dicotyledonous and monocotyledonous plants.

Analysis of putative cis-regulatory elements (CREs)

CRE is a non-coding DNA sequence that regulates transcription in a defined temporal/spatial expression pattern. CREs are important for understanding expression differences and mutations. We used PlantCare to identify putative CREs of 2,000 bp located on SsAP2/ERF genes (Fig. 7 and Additional file 10). These CREs were classified according to their function, and the number of CRE in each sequence was determined. Many CREs were predicted to respond to different hormone elicitors, such as abscisic acid (ABA), ethylene, salicylic acid (SA), jasmonic acid, methyl jasmonate, gibberellin, cytokinins, and auxins. Of these, ABA-reactive elements were the most prevalent. There were also CREs in genes that respond to various stress stimuli, such as dehydration, salt stress, injury, pathogens, oxidative stress, hypoxia, high osmotic pressure, heat, and cold stress; many were related to dehydration elements. A total of 64 SsAP2/ERF genes were found to contain cis-acting regulatory elements involved in the ethylene-responsive element (ERE), while 182 contained cis-acting elements involved in the abscisic acid response (ABRE). Another 108 SsAP2/ERF genes contained cis-acting elements involved in low-temperature response (LTR) or salicylic acid responsiveness (TCA), whereas 155 contained photoresponsive elements. The various stimuli response elements were unevenly distributed among the families. The promoter region in some AP2/ERF genes contained cis-acting elements for various transcription factors such as

MYB, MYC, and ERE and DRE elements, indicating that there may be a mutual regulatory relationship between transcription factors.

Expression pattern of AP2/ERF genes during sugarcane development

To understand specific spatiotemporal expression patterns of SsAP2/ERF genes, we analyzed the expression profiles of the identified genes in 10 different tissues and at different developmental stages using publicly available gene expression data (Fig. 8,9 and Additional file 11). Among the SsAP2/ERF genes examined, 39 were not expressed in all tissue samples, 58 were expressed in all ten tissue types (FPKM > 0), and 27 were constitutively expressed (FPKM > 2). The 19 genes in DREB group I were all expressed in at least one sample, among which SsDREB17, SsDREB27, and SsDREB28 were highly expressed in all samples, indicating that these group I members are likely essential functional genes. The expression levels of 26 SsAP2/ERF genes increased during leaf maturation, suggesting that they may play an important role in leaf growth and development. The expression of SsDREB, SsERF91, SsERF98, SsERF39, and SsERF99 in stems was much higher than that in leaves in the three developmental stages. Among them, four ERF genes belonged to ERF group VII, indicating that they play an important role in cane stems. Expression of 11 genes, including SsDREB56, SsDREB48, SsERF15, and SsRAV3, as well as SsSoloist1 and SsSoloist2, in the three stages of leaf development, was higher than that in the stems, indicating that these genes play an important role in leaf development. The expression of 10 genes, including SsDREB55, SsERF62, and SsERF54 in different tissues in the pre-mature and mature stages, was higher than that seen in various tissues in the seedling stage. Among these genes, many were in the ERF subfamily, indicating that they play an essential role after the sugarcane plant enters the mature stage.

Expression analyses of SsAP2/ERF genes in response to abiotic stress and hormone treatments

To further confirm whether SsAP2/ERF gene expression was affected by different abiotic stresses and hormone treatments, we examined 12 genes from 5 families and analyzed their expression patterns following different treatments (Fig. 10, 11). Expression of all 12 genes was induced by NaCl, PEG6000, and ABA treatments, and the magnitude of the up-regulation was most significant at 3 hours after treatment. Different treatments had varying degrees of influence on gene expression. For example, except for SsSoloist4, which was down-regulated after 72 hours of NaCl treatment, the expression of the other genes examined was induced by NaCl, and the difference was significantly higher than that seen for the other three treatments. Some genes had opposite expression patterns under different treatments. For example, SsERF52 was down-regulated at one h, six h, and 12 h after PEG6000 treatment but was up-regulated at three h, and the expression levels were significantly lower than that for other genes. Meanwhile, SsERF52 was induced by salt stress at six treatment times, and the increases in expression

were considerably higher than that for other genes, indicating that SsERF52 responded differently to salt stress and drought stress. SsAP2-13 was inhibited by GA treatment but induced by the other three treatments.

Discussion

As one of the largest gene families in plants, the AP2/ERF family plays an important role in multiple physiological and biochemical processes by regulating the expression of genes that participate in response to various stress conditions in Arabidopsis, rice [9], corn [46], poplar [47], and grapes [18]. Still, there is limited information concerning the regulation and structure of these genes in sugarcane. In this study, we examined whole-genome data for the wild sugarcane species *S. spontaneum* to identify genes encoding AP2/ERF family transcription factors and analyze their roles based on published expression data for *S. spontaneum* tissue and qRT-PCR results.

Genome-wide analysis of sugarcane identified 218 SsAP2/ERF genes, which is higher than that in rice (164) [48], wheat (117) [49], and Arabidopsis (145) [48], but fewer than that for corn (292) [46]. The genome size varies in different plants, rice (466 Mb) 4.94 Gb (wheat), 125 Mb (Arabidopsis), 2.3 Gb (corn) and 3.36 Gb (*S. spontaneum*), indicating that the number of AP2/ERF superfamily members is relatively stable and does not have an absolute correlation with genome size. However, since AP85-441 ($1n = 4x = 32$) used to sequence the *S. spontaneum* genome was haploid and produced from the culture of the octoploid SES208, the number of AP2/ERF genes in octoploid *S. spontaneum* could be much higher than 218 [45]. Gene duplication plays an important role in gene family amplification to generate gene clusters or hotspots via tandem repetitive and segmental duplication to produce homologous genes that expand the total number of genes. Segmental repetitive events found in 104 SsAP2/ERF genes also validated this possibility. *S. spontaneum* has undergone two Whole Genome Duplication (WGD) events overtime in which its homologous chromosomes underwent duplication from one to two and then to four [45]. SsAP2/ERF gene repeats likely occurred during these two WGDs. We also found evidence that purification selection is the main driving force behind the evolution of the SsAP2/ERF gene family. By evaluating the gene structure of AP2/ERF TF, all SsAP2 and SsSoloist genes had introns, whereas 50.6% and 83.3% of SsERF and SsRAV genes, respectively, had no introns. Loss of introns in genes after segmental duplication occurred more rapidly than the rate of intron acquisition [50]. Also, some studies showed that the number and distribution of introns are related to plant evolution [51], such that introns may have been lost from ERF and RAV family genes during the evolution of higher plants. From these results, ERF and RAV gene differentiation might occur later in *S. spontaneum* evolution.

A syntenic analysis of AP2/ERF was carried out to discover the evolutionary relationship of the SsAP2/ERF genes in five monocotyledonous plants (*O. sativa*, *S. bicolor*, *T. aestivum*, *Z. mays*, *S. spontaneum*) and one dicotyledonous plant (*A. thaliana*). In this analysis, SsAP2/ERF genes had higher homology with AP2/ERF genes from the four kinds of grass and less homology with Arabidopsis. There were more homologous genes between sugarcane and wheat, likely due to the larger genome size and gene number in wheat [52]. By analyzing the homology of SsAP2/ERF and *S. bicolor* AP2/ERF genes, we

found that 168 SsAP2/ERF genes likely existed before the number of *S. spontaneum* chromosomes was reduced from 10 to 8. In addition, 63%-70% of identified SsAP2/ERF genes were homologous in sorghum (168), rice (147), corn (139), and wheat (145). This finding indicated that the AP2/ERF family in different plants might have evolved from a common ancestor.

Due to the plasticity of the AP2/ERF genes and the specificity of individual family members, members of this family are likely to be important targets for genetic engineering and breeding to improve crops [53]. The study of gene expression patterns is important for the prediction of gene function prediction. Analysis of tissue- and growth stage-specific expression showed that SsAP2/ERFs are widely expressed in sugarcane at the seedling stage as well as in leaves during early maturity and the mature stage and in different stem segments, indicating that these genes play important roles in sugarcane growth and development. DREB family genes in Group I were highly expressed in leaves at all development stages and in three stem segments, suggesting that these genes may be involved across the entire development process of sugarcane leaves and stems. The expression levels of 29 SsAP2/ERF genes in stems during the seedling stage were lower than those in the early maturity stage and mature stage. Among them, 28 were members of the DREB and ERF family, which may be related to sugar accumulation in the sugarcane stem and is consistent with the results of previous studies for other plants. For example, compared with wild rice, transgenic rice carrying the ERF protein TSRF1 had 30–60% increases in proline and soluble sugar content that together could enhance the osmotic drought tolerance of rice [54].

Earlier studies of AP2/ERF family genes in other plants emphasized their role in response to hormonal and abiotic stresses. OsERE1BP1 attenuates disease caused by Xoo and confers drought and submergence tolerance in transgenic rice [55]. Induction of the AP2/ERF transcription factor CRL5 promoted crown root initiation by inhibiting cytokinin signaling [56]. In the present study, the expression patterns of 12 SsAP2/ERF genes in response to NaCl, PEG6000, ABA, and GA treatments suggested that these genes have essential roles in abiotic stress and hormone responses in sugarcane. qRT-PCR verification revealed that the expression of these 12 SsAP2/ERF genes distributed across the five subfamilies was significantly induced by NaCl treatment, similar to findings for other species, including the increase in expression of the AP2/ERF family gene CAP2 in chickpeas and soybeans exposed to salt stress [57]. In tobacco, GmERF7 expression can increase salt tolerance [58], whereas the pepper pathogen-induced transcription factor RAV₁ plays a vital role in bacterial salt stress tolerance [59]. Here we showed that the expression of SsERF52 was up-regulated by up to 15-fold after NaCl treatment. This gene has the highest homology with the wheat salt-responsive gene TaERF4 [60]. Like TaERF4 in wheat, SsERF52 expression was not induced by exogenous abscisic acid (ABA). Overexpression of the Arabidopsis AP2/ERF gene HARDY improves drought tolerance by reducing the transpiration of transgenic *Trifolium alexandrinum* L [61]. Drought stress induces expression of the DREB OsAP21 in rice [62], and here we found that expression of 12 SsAP2/ERF genes was induced 3 hr after PEG6000 treatment. Interestingly, the expression level of SsERF52 after PEG6000 treatment was opposite that after NaCl treatment, with inhibition of gene expression at several time points. Multiple lines of evidence indicate that genes expressed during drought and stress responses in plants partially overlap. For

example, transgenic plants that overexpress Arabidopsis OsMYB3R-2 have enhanced cold tolerance, drought tolerance, and salt tolerance [63]. SsERF52 was related to insensitivity to exogenous ABA treatment. Increasing amounts of evidence show that AP2/ERF genes are regulated by hormones such as ABA and GA, and thus these genes play an important role in hormone signaling networks[64]. In the present study, promoter analysis indicated that the promoter regions of most SsAP2/ERF genes contain multiple cis-acting elements that are related to ABA responses. qRT-PCR analysis of the 12 selected SsAP2/ERF genes showed that, except for SsERF52, expression of these genes was induced by ABA. AP2 / ERF genes play an active role in ABA-mediated stress response, a key hormone that regulates abiotic stress responses (including drought, salinity, cold, and heat stress) [64]. For example, ANT [65] in Arabidopsis is induced by ABA to upregulate genes containing DRE and ABRE elements. ANT is homologous to SsAP2-21, indicating that SsAP2/ERF genes may regulate abiotic stress through ABA-dependent pathways. After exogenous GA treatment, the expression levels of SsRAV4, SsRAV11, SsDREB25, and SsAP2-25 were not affected, but GA induced the expression of other genes. Our research indicates that some SsAP2/ERF genes are differentially expressed under various abiotic stresses and hormone treatments, suggesting that this gene family is essential for environmental adaptation. In particular, the expression patterns of some SsAP2/ERF genes and their homologs varied. For example, expression of the SsSoloist4 gene was induced to varying degrees by the four stress treatments, and these expression patterns differed from those for its Arabidopsis homolog At4g13040, which is an active regulator of disease defenses and is induced by SA treatment. However, the expression of this gene is inhibited by low temperature and salt stress. Given the antagonistic roles of SA and ABA in plant defense, At4g13040 expression may have opposing effects in response to ABA and SA treatment. SsSoloist4 expression was up-regulated after 3 hours of ABA treatment, indicating that the regulation mechanism of this gene likely differs from that for At4g13040 under abiotic stress.

The results of this study provide a comprehensive analysis of the AP2/ERF family in sugarcane and an examination of the relationship between the activity of members of this gene family and abiotic stress responses. Our results can provide a foundation for the identification of novel candidate AP2/ERF genes as targets for genetic engineering of novel sugarcane germplasm that will produce plants having enhanced tolerance of abiotic stresses.

Conclusions

This study comprehensively analyzed the supergene family of sugarcane AP2 / ERF. The identified 218 AP2 / ERF superfamily genes were classified in detail, and their evolutionary characteristics, expression patterns in different sugarcane tissues and growth stages, and their response to abiotic stress and hormones were studied. These results provide valuable resources for a better understanding of the biological role of the sugarcane AP2 / ERF genes.

Abbreviations

S. spontaneum: *Saccharum spontaneum* L.; *SsAP2/ERF*: sugarcane AP2/ERF; *SsChr*: *Saccharum spontaneum* chromosome; *FPKM*: Segmentals Per Kilobase of transcript per Million segmental mapped; *A4*: Ancestral chromosome A4; *ABA*: abscisic acid; *GA*: gibberellins; *PEG*: Polyethylene Glycol; *SA*: salicylic acid; *Xoo*: *Xanthomonas oryzae* pv. *Oryzae*

Declarations

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Authors' contributions

PTL and ZC performed the experiments. CHH, PPL, GQH, LNX, ZHD analyzed the data. PTL wrote the manuscript. XWZ, YZ, and MQZ designed the study. All authors have read and approved the final manuscript.

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Availability of data and materials

All data analyzed during this study are included in this article and its.

Ethics approval and consent to participate

The sugarcane materials used in the experiment were supplied by the sugarcane clonal germplasm repository of Guangxi University. These plant materials are widely used all over the world, and no permits are required for the collection of plant samples. This article did not contain any studies with human participants or animals and did not involve any endangered or protected species.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

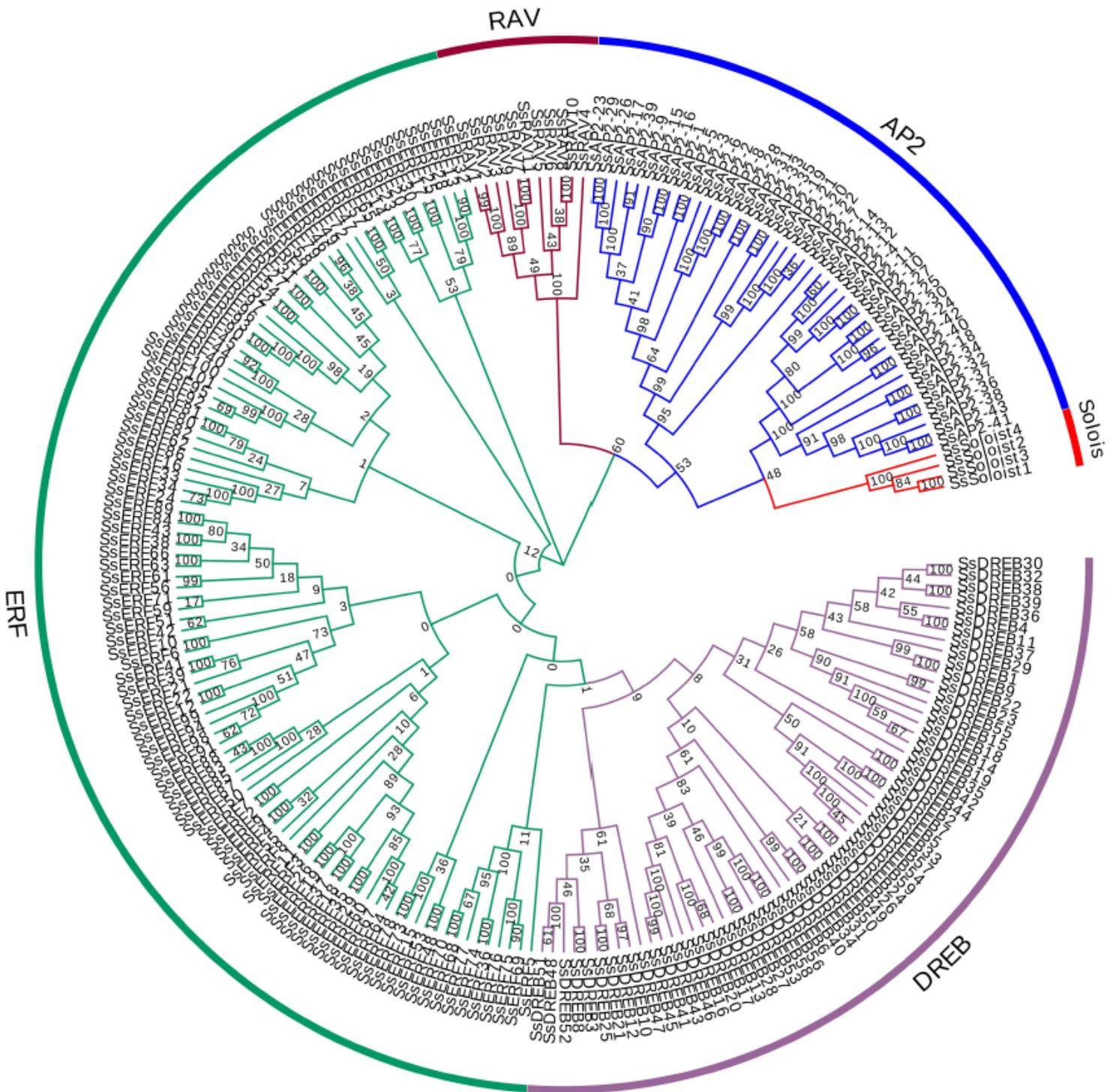


Figure 1

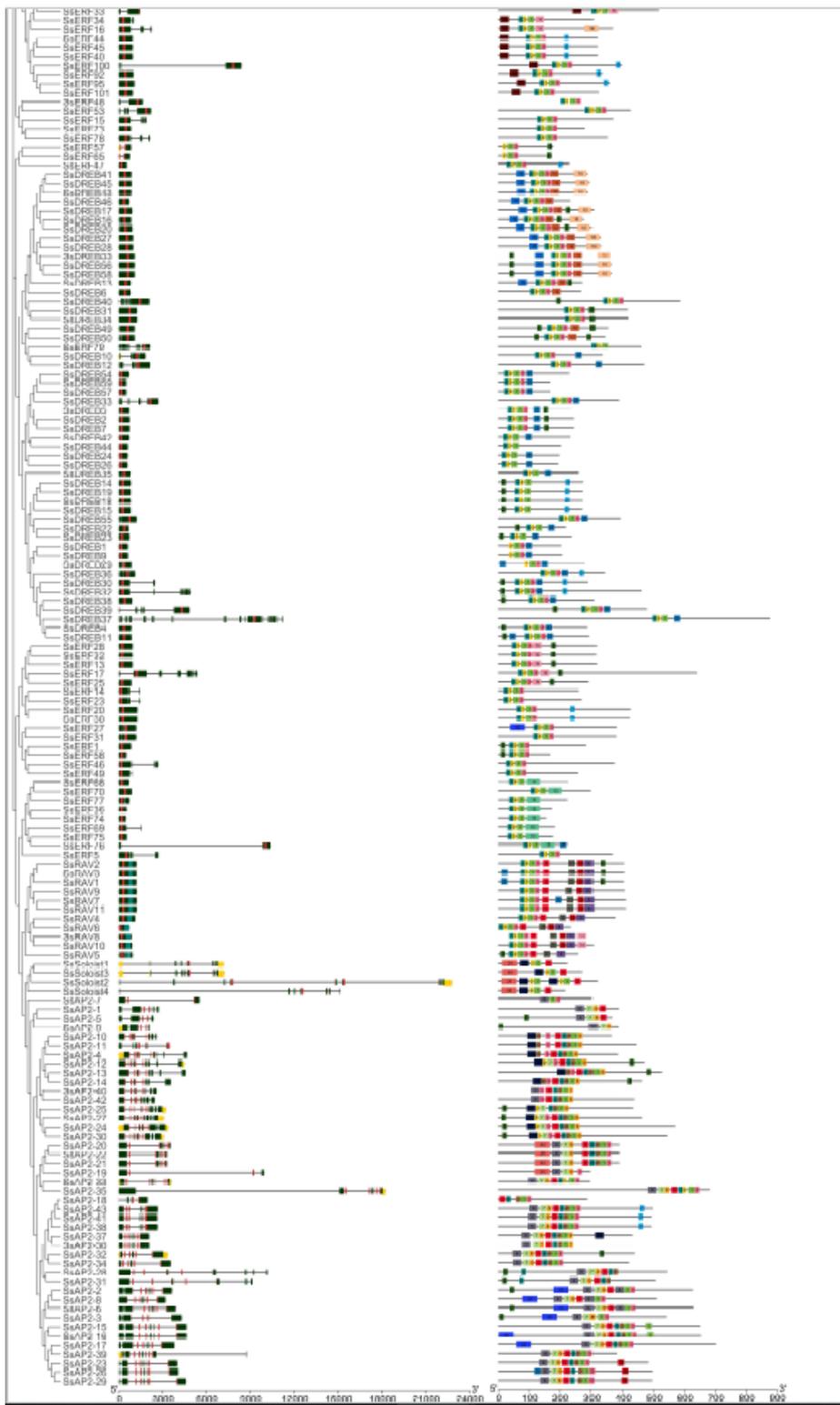


Figure 3

Phylogenetic relationships, gene structure, and architecture of conserved protein motifs in AP2/ERF genes from sugarcane. (A) The phylogenetic tree was constructed based on the full-length sequences of sugarcane AP2/ERF proteins using MEGA7 software. (B) Exon-intron structure of sugarcane AP2/ERF genes. Yellow boxes indicate untranslated 5- and 3- regions; green boxes indicate exons; gray lines indicate introns. The AP2 domains are highlighted by red boxes and B3 domain by blue boxes. (C) The

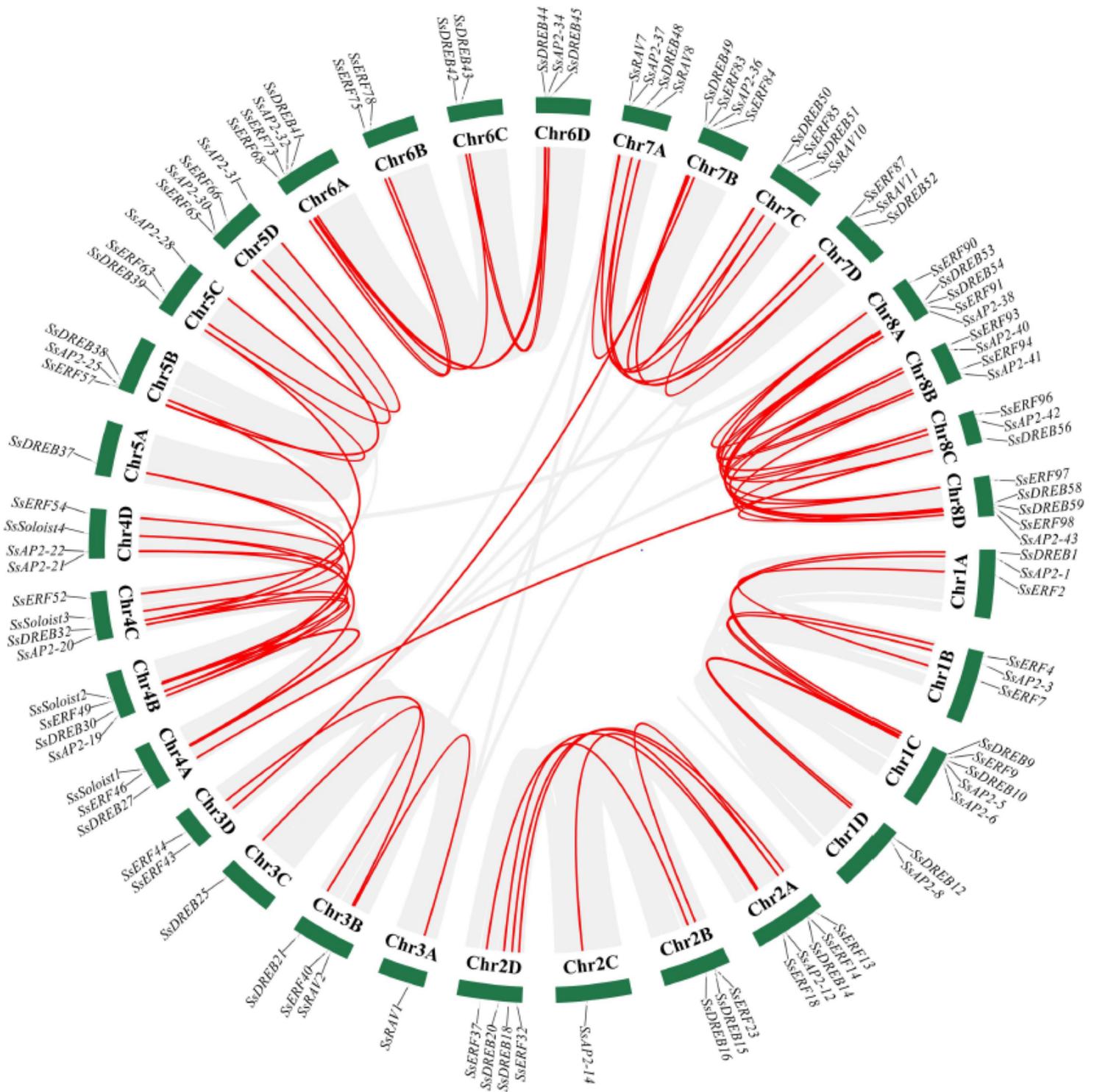


Figure 5

Schematic representations for the interchromosomal relationships of sugarcane AP2/ERF genes. Gray lines indicate all syntenic blocks in the sugarcane genome, and the red lines indicate duplicated AP2/ERF gene pairs.

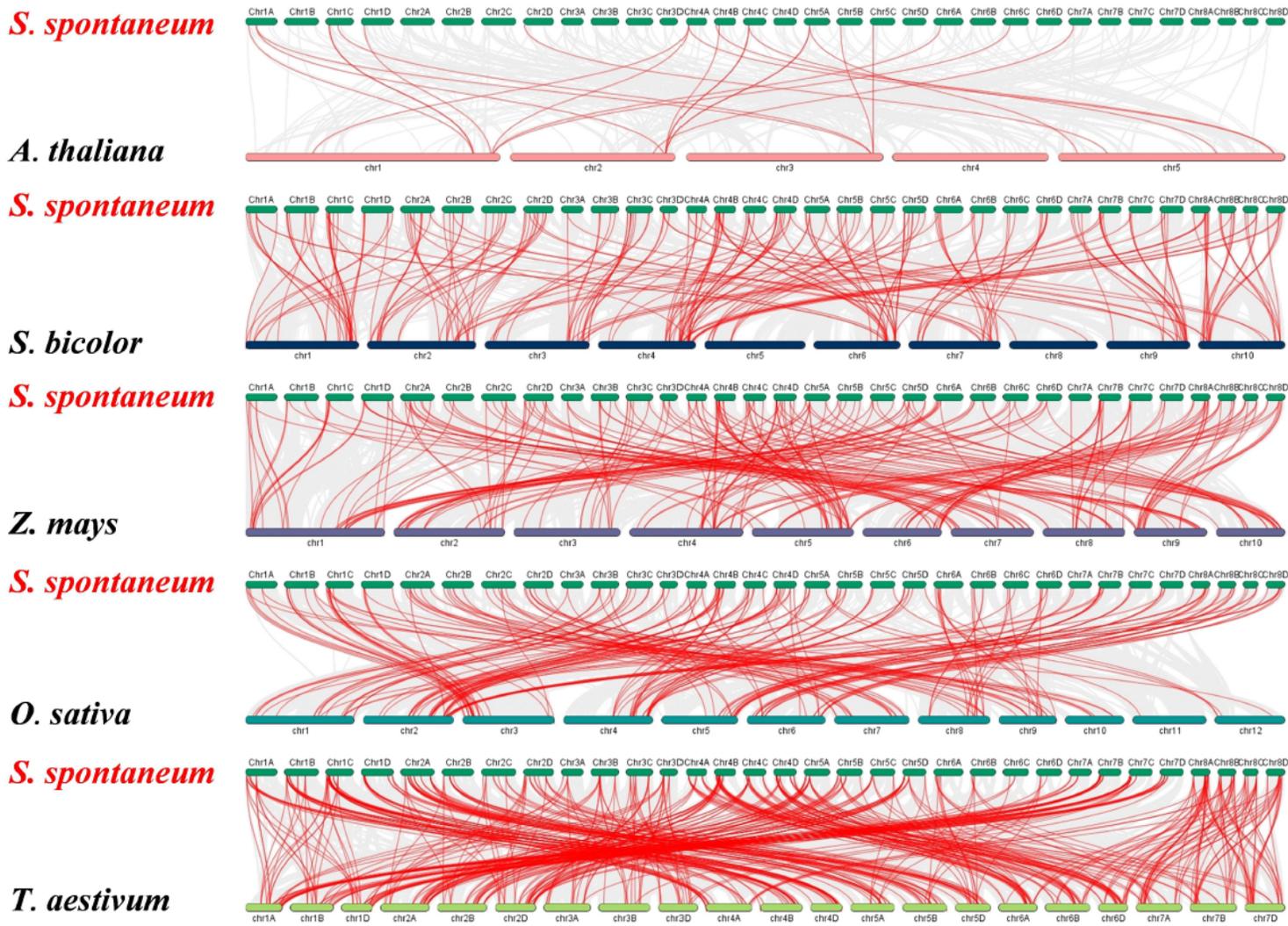


Figure 6

Synteny analysis of AP2/ERF genes between sugarcane and five representative plant species. Gray lines in the background indicate the collinear blocks within sugarcane and other plant genomes, while the red lines highlight the syntenic AP2/ERF gene pairs. The specie names with the prefixes ‘S. Spontaneum’, ‘A. thaliana’, ‘O. sativa’, ‘S. bicolor’, ‘T. aestivum’ and ‘Z. mays’ indicate *Saccharum Spontaneum*, *Arabidopsis thaliana*, *Oryza sativa*, *Sorghum bicolor*, *Triticum aestivum* and *Zea mays*, respectively

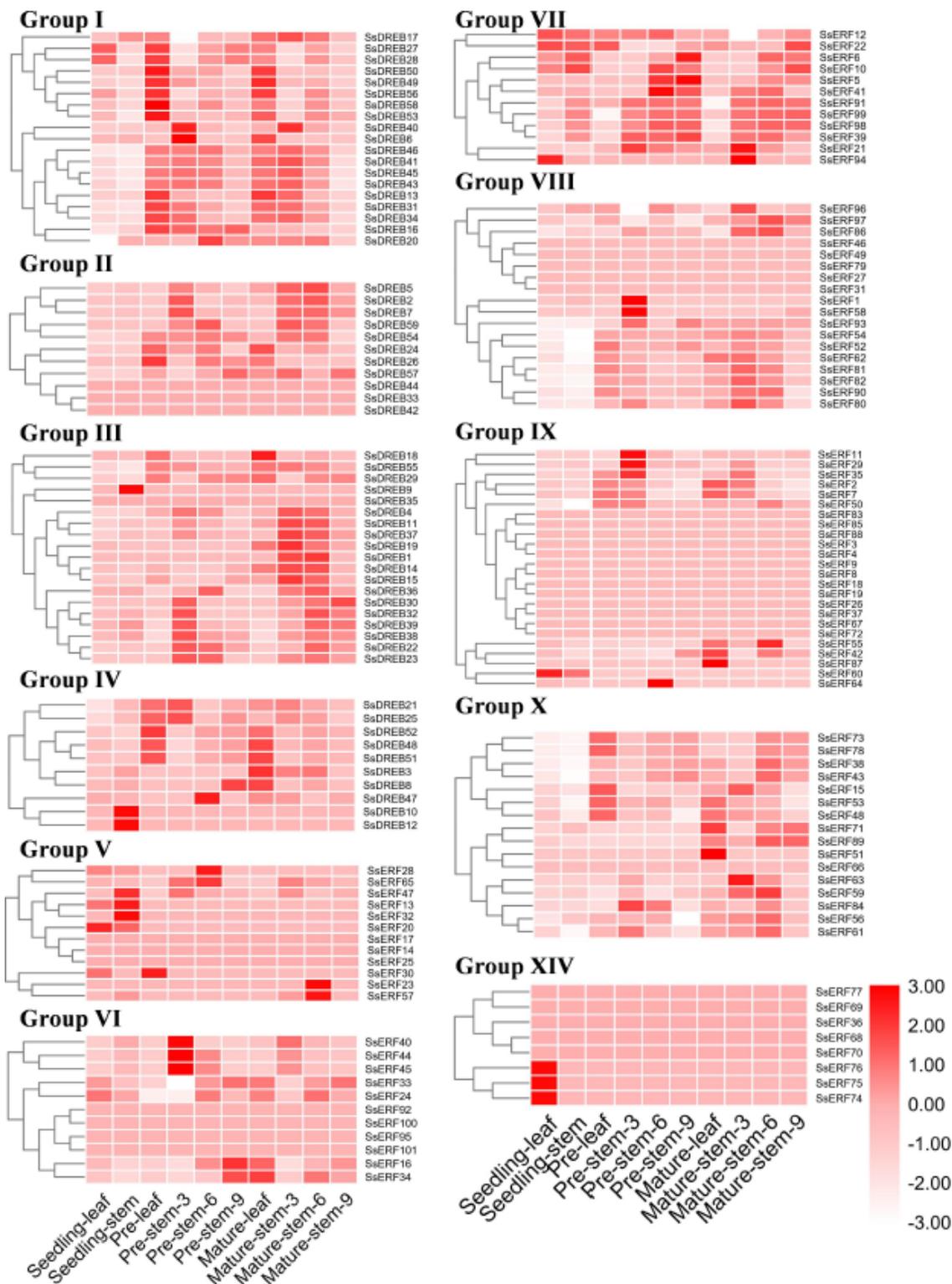


Figure 8

Expression profile of sugarcane ERF and DREB genes, subdivided into groups (I-XIV). Thermal map hierarchical clusters of SsERF and SsDREB genes expression profiles in leaves and stems at the seedling stage, leaves and stems at the early maturity stage (3, 6, 9), leaves, and stems at the mature stage (3, 6, 9). Based on gene expression profiles in transcriptome data, clusters were generated using the Pearson

clustering algorithm. For each line, after z-score-normalized transformation, white and red correspond to low expression and high expression, respectively.

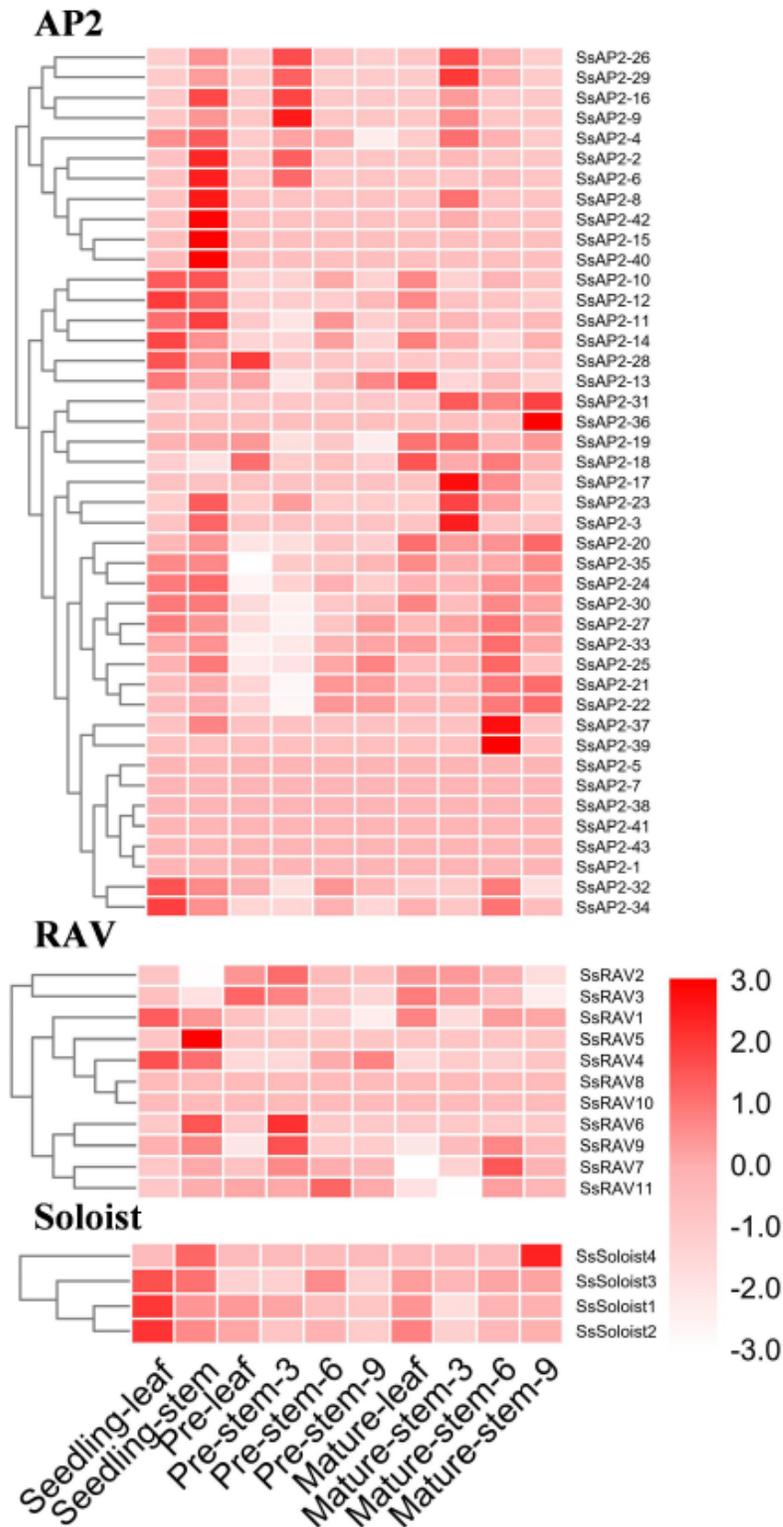


Figure 9

Expression profiles of the sugarcane AP2, RAV, Soloist genes. Thermal map hierarchical clusters of SsAP2, SsRAV, SsSoloist genes expression patterns in leaves and stems at the seedling stage, leaves and stems at the early maturity stage (3, 6, 9), leaves and stems at the mature stage (3, 6, 9). Based on gene

expression profiles in transcriptome data, clusters were generated using the Pearson clustering algorithm. For each line, after z-score-normalized transformation, white and red correspond to low expression and high expression, respectively.

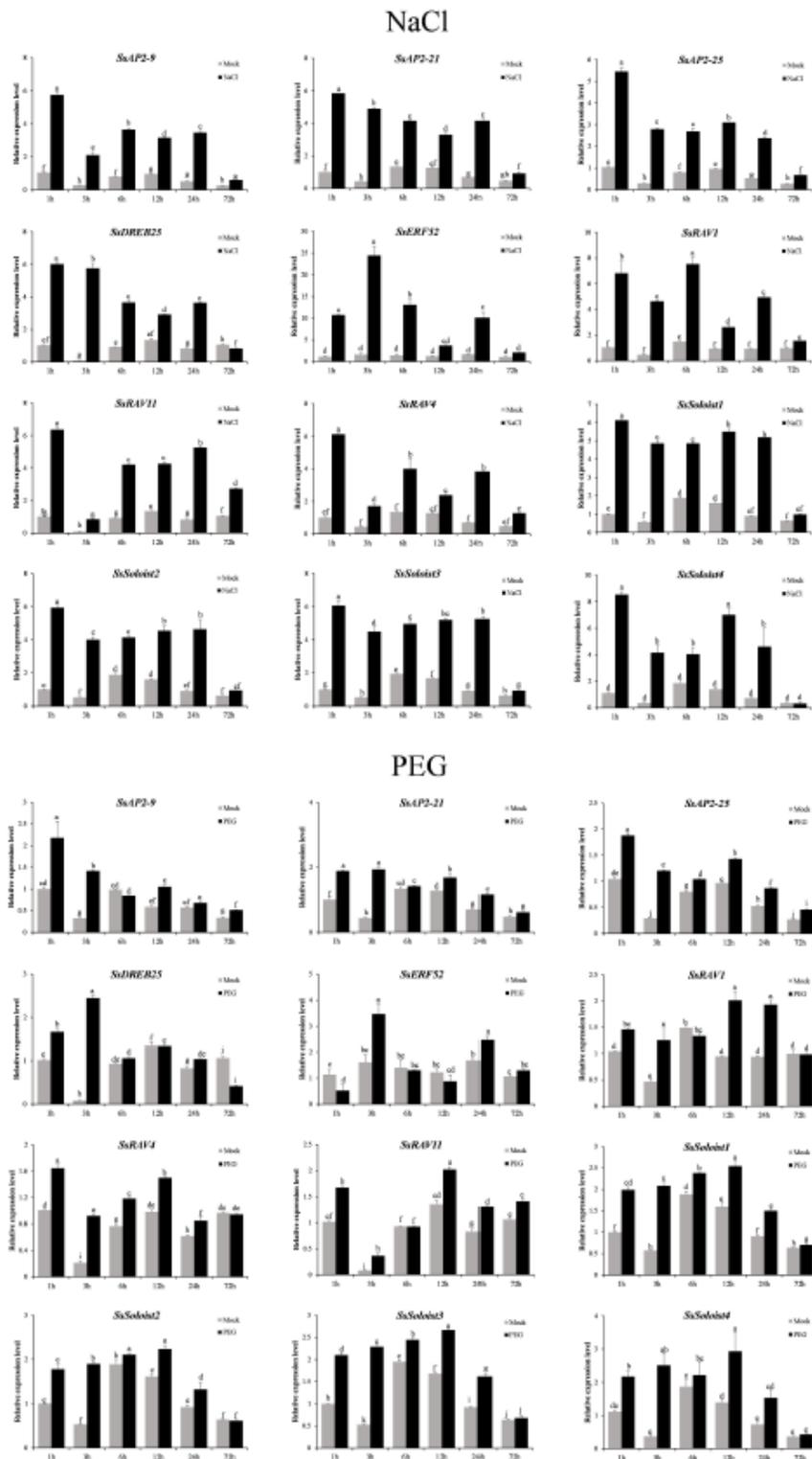


Figure 10

Expression profiles of 12 selected SsAP2/ERF genes in response to various abiotic stress treatments. 25s-RNA gene was used as the internal control and to normalize expression data. Relative transcript

abundance is normalized relative to S mock (1 hour untreated control group) treatment. Error bars represent the standard deviation of the mean. For each gene, different lowercase letters indicate significant differences among mean values (one-way ANOVA with Duncan's multiple range test; $P < 0.05$). The results are based on three replicates in three independent experiments.

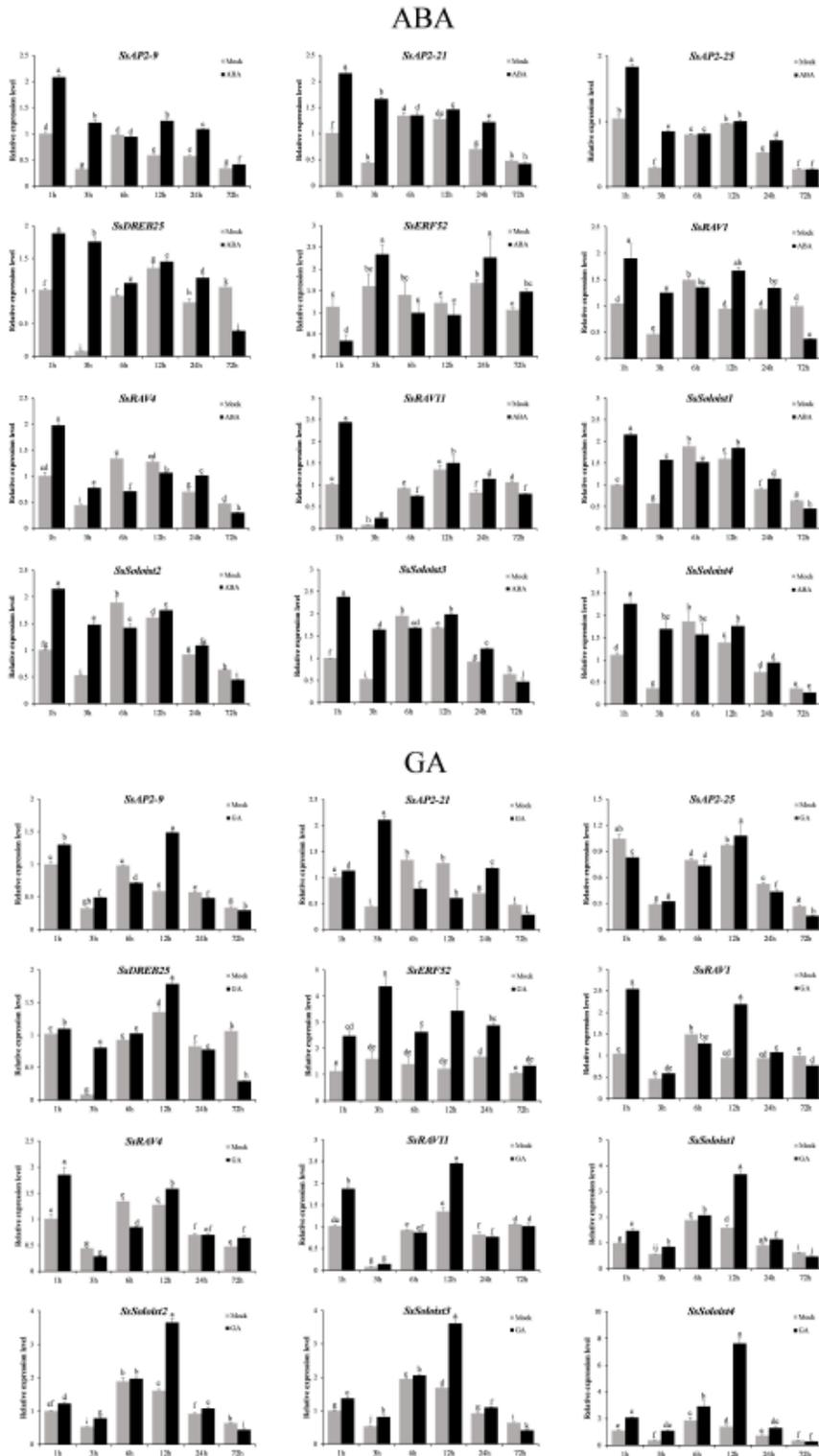


Figure 11

Expression profiles of 12 selected SsAP2/ERF genes in response to various hormone treatments. 25s-RNA gene was used as the internal control and to normalize expression data. Relative transcript abundance is normalized relative to S mock (1 hour untreated control group) treatment. Error bars represent the standard deviation of the mean. For each gene, different lowercase letters indicate significant differences among mean values (one-way ANOVA with Duncan's multiple range test; $P < 0.05$). The results were based on three replicates in three independent experiments.

Supplementary Files

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