Identification and expression analysis of the DREB transcription factor family in pineapple (*Ananas comosus* (L.) Merr.)

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Background : Dehydration responsive element-binding (DREB) transcription factors have crucial roles in plant growth and development as well as the responses to different environmental stresses. Although *DREB* genes have been thoroughly characterized in many plant species, genome-wide identification of the *DREB* gene family has not yet been reported in pineapple.

Results: To survey the *DREB* gene family members in pineapple and the predicted proteins, a comprehensive genome-wide screening was performed, and the analysis identified 20 *AcoDREB* genes on 14 pineapple chromosomes. Phylogenetic analysis showed that the *AcoDREB* genes could be divided into five subgroups, and *AcoDREBs* within the same phylogenetic group were found to have similar gene structures and domain compositions. Gene structure analysis showed that most of the *AcoDREB* genes (75%) lacked introns, and the promoter regions of all 20 *AcoDREB* genes had at least one stress response-related *cis*-element. Expression pattern analysis identified four genes with high expression levels and six genes with low expression levels in all analyzed tissues. Under abiotic stress treatment, expression changes were detected for eight selected *AcoDREB* genes.

Conclusions: This report presents the first genome-wide analysis of the DREB transcription factor family in pineapple, and 20 *AcoDREB* genes were identified. Our results showed that *AcoDREB* genes can respond to abiotic stresses (drought, high salt, high- and low-temperature stress). The present findings not only provide preliminary data for future functional analysis of *AcoDREB* genes in pineapple, but may provide useful information for developing new pineapple varieties with high agronomic traits such as resistance to different kind of stress conditions.

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ABSTRACT

Background: Dehydration responsive element-binding (DREB) transcription factors have crucial roles in plant growth and development as well as the responses to different environmental stresses. Although *DREB* genes have been thoroughly characterized in many plant species, genome-wide identification of the *DREB* gene family has not yet been reported in pineapple.
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could be divided into five subgroups, and *AcoDREBs* within the same phylogenetic group were found to have similar gene structures and domain compositions. Gene structure analysis showed that most of the *AcoDREB* genes (75%) lacked introns, and the promoter regions of all 20 *AcoDREB* genes had at least one stress response-related *cis*-element. Expression pattern analysis identified four genes with high expression levels and six genes with low expression levels in all analyzed tissues. Under abiotic stress treatment, expression changes were detected for eight selected *AcoDREB* genes.

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Keywords: pineapple, DREB transcription factors, phylogenetic analysis, expression profiles

INTRODUCTION

Abiotic stresses, such as salinity, drought, and high or low temperatures, severely affect the growth and development of plants. To adapt to these environmental conditions, plants evolved complex signal transduction pathways and various response mechanisms that are induced by specific functional and regulatory proteins. The functional proteins include membrane proteins (transporters and water channel proteins); osmolyte biosynthesis enzymes (to produce proline, betaine, soluble sugars, etc.); detoxification enzymes (catalase, superoxide dismutase, ascorbate peroxidase, glutathione *S*-transferase, etc.); and other proteins that help protect macromolecules (LEA protein, osmotin, antifreeze proteins, mRNA binding protein, etc.). The regulatory proteins include transcription factors (bZIP, MYC, MYB, DREB, etc.); protein kinases (receptor protein kinase, MAP kinase, CDP kinase, transcription-regulation protein kinase, etc.); and proteinases (phospholipase C, phosphoesterases, etc.) (*Agarwal et al. 2006*). Among these regulatory proteins, transcription factors (TFs) play pivotal roles in abiotic stress responses. Specifically, they activate or repress the expression of stress-response genes by recognizing and binding *cis*-elements in the promoters of their targets (*Golldack et al. 2011; Malhotra & Sowdhamini 2014; Agarwal et al. 2017*). They are the key targets for genetic engineering to enhance stress tolerance in valuable crop plants (*Century et al. 2008*).

Dehydration response element-binding (DREB) TFs could response tolerance to abiotic stresses by specifically binding to DRE/CRT *cis*-elements to control downstream gene expression. The DREB TF family belongs to the APETALA2/Ethylene-Responsive Factor (AP2/ERF) superfamily of TFs. The AP2/ERF superfamily is characterized by the AP2 domain, which is about 60-70 amino acids in length and contains two conserved sequence blocks, the YRG element and the RAYD element. The YRG element is 19-22 amino acids long and contains the

conserved YRG motif, which may be related to the DNA-binding specificity of AP2 protein. The RAYD element has a conserved core region that can form an amphipathic α-helix in the AP2 domains (*Okamuro et al. 1997*). The AP2 domain of the DREB subfamily, compared to that of other subfamilies, has important differences at specific amino acid sites. These include the valine (Val) residue at position 14 and glutamine (Glu) at position 19, which are conserved in the DREB subfamily (*Sakuma et al. 2002*).

The DREB subfamily members in *Arabidopsis thaliana* can be divided into six groups, named A-1 to A-6 or DREB1 to DREB6 (*Sakuma et al. 2002*). The functions of A-1 and A-2 members are well characterized. The first identified *DREB* gene was the A-1 member *AtCBF1*, which is strongly induced by low temperature. In addition to the *AtCBF1* gene, *AtDREB1A* and *AtDREB1C* also positively regulate low-temperature stress response (*Jaglo-Ottosen et al. 1998; Liu et al. 1998*). *SwDREB1* from sweet potato (*Ipomoea batatas*) was also found to be involved in the response to low temperature (*Kim et al. 2008*), and overexpression of zoysia grass *ZjDREB1.4* in *Arabidopsis* enhanced tolerance to high and freezing temperature stresses without obvious growth inhibition (*Feng et al. 2019*). In rice, the interaction of *OsDREB1A*, *OsDREB1B*, and *OsDREB1C* with the GCC box was found to enhance the cold tolerance of rice plants (*Donde et al. 2019*). Based on the findings in different species, DREB1 is mainly associated with cold stress regulation. In contrast, DREB2 is mainly associated with the responses to drought and salt stress (*Liu et al. 1998*). *AtDREB2A* and *AtDREB2B* were the first reported A-2 members, and they were found to be induced by dehydration and high-salt stress (*Sakuma et al. 2002*). Overexpression of soybean *GmDREB2* in *Arabidopsis* enhanced tolerance to high-salt stress without growth retardation (*Chen et al. 2007*). In sugarcane, overexpression of *EaDREB2* similarly enhanced the tolerance of plants to drought and salinity stress (*Augustine et al. 2015*). In contrast to A-1 and A-2 subgroup genes, the functions of A-3 to A-6 members are only beginning to be uncovered. The A-4 subgroup gene *ZmDREB4.1* was cloned from maize, and it was associated with the negative regulation of plant growth and development (*Li et al. 2018*). A novel A-5 subgroup gene from desert moss, *ScDREB8*, enhanced the salt tolerance of *Arabidopsis* seedlings by up-regulating the expression of stress-related genes (*Liang et al. 2017*). *CmDREB6* belongs to the DREB6 subgroup, and its overexpression enhanced the tolerance of chrysanthemum to heat stress (*Du et al. 2018*). The plant species in which DREB family genes have been identified include *A. thaliana* (*Hwang et al. 2012*), perennial ryegrass (*Xiong & Fei 2006*), *Triticum* L. (*Mondini et al. 2015*), *Dendranthema* (*Yang et al. 2009*), *Zea mays* (*Qin et al. 2007*), and *Oryza sativa* L. (*Cui et al. 2011; Gumi et al. 2018; Matsukura et al. 2010*). According to previous research in several plant species have proved that the most of *DREB* genes responded to different stress conditions. But, *DREB* genes have not been reported in pineapple (*Ananas comosus* (L.) Merr.), Therefore, we have conducted this study identify *AcoDREB* genes as well as the characteristics of the encoded DREB TFs.

Pineapple is a tropical fruit widely grown in tropical and subtropical regions. After banana and citrus, it is the third most important tropical fruit in world production (*Moyle et al. 2005*). The crop has high economic value, and pineapple cultivation is of great significance to the development of local agriculture. However, the changes in global climate have underscored how different abiotic and biotic stresses critically affect the growth of pineapple (*Mittler 2006; Ray et al. 2013*). The pineapple fruit can be injured under high temperature, the low temperatures can cause diminishing growth, and the plant is also severely damaged by long-term drought. Similarly biotic stresses such as pests, diseases, and weeds also will lead to significant yield loss in pineapple production (*Lobo & Paull 2016*).The DREB family has been reported to participate in diverse biological processes. Therefore, there is considerable interest in identifying the function of the *AcoDREB* genes in pineapple.

In this study, we identified 20 *AcoDREB* genes belonging to five subgroups and analyzed their gene and protein structures, protein motifs, chromosomal distribution, and expression profiles. Our results provide a relatively complete profile of pineapple *DREB* gene family. This constitutes a foundation for further functional analysis of each member, which will undoubtedly facilitate transformation of pineapple via gene-transfer techniques that can then be used for improving pineapple tolerance to abiotic and biotic stress (*Priyadarshani et al. 2019*)

MATERIALS & METHODS

Identification of DREB family members in pineapple

DREB amino acid sequences from *Oryza sativa* and *Arabidopsis* were obtained from the Rice Genome Annotation Project (RGAP, http://rice.plantbiology.msu.edu/index.shtml) (*Kawahara et al. 2013*) and The *Arabidopsis* Information Resource (TAIR, http://www.arabidopsis.org) (*Berardini et al. 2015*), respectively. The DREB sequences from *Arabidopsis* were used as search queries in BLAST-P against the pineapple genome. We also downloaded the AP2 (PF00847) domain as a query to perform a HMMER search (https://www.ebi.ac.uk/Tools/hmmer/search/hmmsearch), a software package that uses profile hidden Markov Models (HMM); the parameters were set to default for the pineapple genome (*Ming et al. 2015*). We eliminated the redundant sequences and used Simple Modular Architecture Research Tool (SMART, http://smart.embl-heidelberg.de/) (*Letunic & Bork 2018*) to verify the existence and completeness of the core domain within the identified sequences. The sequences that remained after these analyses were used for phylogenetic analysis.

Protein characteristics and chromosomal localization

For each of the putative *AcoDREB* genes, the gene length, amino acid number, CDS length, and chromosome position were collected from the Pineapple Genomics Database (PGD, http://pineapple.angiosperms.org/pineapple/html/index.html) (*Xu et al. 2018*). The molecular weights and isoelectric points of the putative proteins were predicted using the ExPASy proteomics server (http://expasy.org/) (*Gasteiger et al. 2003*). Based on the start positions of the

genes and the lengths of the corresponding chromosomes, MapChart (*Voorrips 2002*) was used to visualize the 20 *AcoDREB* genes mapped onto the 25 pineapple chromosomes and scaffold sequences.

Cis-element analysis of _AcoDREB_ gene promoters

The 2 kb upstream sequences of the *AcoDREB* genes were retrieved from the Pineapple Genomics Database then submitted to Plant *Cis*-Acting Regulatory Element (PlantCARE, http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) (*Lescot et al. 2002*) to detect the presence of the following six regulatory elements (*Sazegari et al. 2015*): abscisic acid (ABA)-responsive elements (ABREs; ACGTG/TC), involved in ABA responsiveness (*Yamaguchi-Shinozaki & Shinozaki 1993*); dehydration-responsive elements (DREs; A/GCCGAC), involved in plant responses to dehydration, low temperature, and salt stress (*Narusaka et al. 2003*); low temperature-responsive elements (LTREs; CCGAA), involved in low-temperature response (*Roy Choudhury et al. 2008*); TC-rich repeats (G/ATTCTCT), involved in defense and stress response (*Diaz-De-Leon et al. 1993*); W-boxes (TGACC/T), binding site of WRKY TFs in defense responses (*Jiang et al. 2017*); and MBS (TAACTG), MYB binding site involved in drought-inducibility (*Urao et al. 1993*).

Sequence alignment and phylogenetic analysis

The CDS sequences of the *AcoDREB* genes were obtained from the Pineapple Genomics Database then imported into DNAMAN Version 9 for sequence alignment (*Wang 2016*). The phylogenetic tree was constructed by IQ tree using the maximum likelihood (ML) method (*Chernomor et al. 2016; Nguyen et al. 2015*). For this analysis, the parameters were set to default, except that the ultrafast bootstrap option was set to n=1000 (*Hoang et al. 2018*) after performing multiple sequence alignments using MUSCLE 3.7 (*Edgar 2004*) with default parameters. To validate the ML results, the neighbor-joining (NJ) method was used to construct a tree with MEGA7 (*Kumar et al. 2016*).

Gene structure analysis and conserved motif identification

The *DREB* gene structures, including the numbers and positions of exons and introns, were determined using the Gene Structure Display Server (GSDS, http://gsds.cbi.pku.edu.cn/) (*Guo et al. 2007*). Multiple EM for Motif Elicitation (MEME, http://meme-suite.org/tools/meme) was used to analyze the amino acid sequences of the 20 AcoDREBs; the maximum number of motifs was set to 10, and default options were used (*Bailey et al. 2009*).

Plant material and growth conditions

Pineapple (*Ananas comosus*) variety MD2 was provided by the Qin Lab (Haixia Institute of Science and Technology, Fujian Agriculture and Forestry University, Fujian, China) (*Priyadarshani et al. 2018*). Plants were grown on soil mixture [2:1 (v/v) peat moss:perlite] in

plastic pots in a greenhouse under the following conditions: ~30 °C, 60-70 μmol photons m^{-1} s^{-1} light intensity, 70% humidity, and a 16-h light/8-h dark photoperiod.

RNA-Seq for different pineapple tissues

We used an RNA extraction kit (Omega Bio-Tek, Shanghai, China) to extract total RNA from the following MD2 pineapple tissues: calyx, gynoecium, ovule, petal, and stamen. The different tissues were collected according to previously described methods (*Chen et al. 2017*). NEBNext Ultra RNA Library Prep Kit for Illumina was used for library preparation prior to sequencing. RNA-Seq data for root, leaf, leaf base, leaf tip, flower, and fruit at different development stages were collected from the Pineapple Genomics Database (*Ming et al. 2015*). Using TopHat v2.1.1 (*Trapnell et al. 2012*) with default parameters, the trimmed paired-end reads of all tissues were aligned to the pineapple genome. Cufflinks v2.2.1 and Cuffdiff v2.2.1 were used to estimate the FPKM values. The heatmap showing the *AcoDREB* gene expression profiles was generated using the pheatmap package in R (*Galili et al. 2018*).

Stress treatments

One-month-old plants in rooting medium were used as the planting material for the stress treatment analyses. Uniform tissue-cultured seedlings were obtained from the Qin Lab (*Priyadarshani et al. 2018*). Seedlings were subjected to the following stress treatments: low temperature (4 ℃), high temperature (45 ℃), drought (350 mM mannitol), and high salt (150 mM NaCl). Root and leaf tissues were collected at 6, 12, 24, and 48 hours after treatment. For the control, samples were obtained from seedlings that were not subjected to stress treatments. The collected samples were immediately stored in liquid nitrogen prior to total RNA extraction (*Rahman et al. 2017*).

Quantitative real-time PCR and data analysis

Total RNA was extracted using the Plant RNA Kit (Omega Bio-Tek, Shanghai, China) according to the manufacturer's protocol. The RNA concentrations ranged from 100 to 500 ng/μl, and the OD260/OD280 ratios ranged from 1.8 to 2.0. According to the supplier's instructions for AMV reverse transcriptase (Takara), 1 μg of purified total RNA was reverse transcribed into cDNA in a 20 μL reaction volume (*Cai et al. 2019*). To quantify the relative transcript levels of selected *DREB* genes, real-time PCR was performed with gene-specific primers on the Bio-Rad Real-time PCR system (Foster City, CA, USA) according to the manufacturer's instructions. The gene-specific primers used for this analysis are listed in Supplemental Table S1. The PCR program was as follows: 95 °C for 30 s; 40 cycles of 95 °C for 5 s and 60 °C for 34 s; and 95 °C for 15 s. For all tested genes, three technical replicates and at least three independent biological replicates were performed (*Cai et al. 2017; Zhang et al. 2018*). Relative expression was calculated using the $2^{-\Delta\Delta Ct}$(*Century et al. 2008*). Data were analyzed by using one-way analysis of variance (ANOVA) at a significant level of $*P< 0.05$ and $**P< 0.01$ (Table S2).

RESULTS

Genome-wide identification and chromosomal locations of pineapple *DREB* genes

Using *Arabidopsis* DREB amino acid sequences as search queries in BLAST, 20 DREB amino acid sequences were obtained from the pineapple proteome. The corresponding genes were named *AcoDREB1* to *AcoDREB20* (Table S3), and the amino acid sequences are listed in Table S4. Table 1 lists the following information for the 20 genes: gene name, gene ID, nucleotide and amino acid lengths, and the predicted isoelectric point (pI) and molecular weight (Mw) of the encoded protein. The protein lengths ranged from 149 (*AcoDREB13*) to 463 (*AcoDREB20*) amino acids, and the CDS lengths ranged from 450 (*AcoDREB13*) to 1392 (*AcoDREB20*) bp. The predicted protein molecular weights ranged from 16316.44 (*AcoDREB13*) to 49311.65 (*AcoDREB20*) Da, and the predicted isoelectric points ranged from 4.71 (*AcoDREB10*) to 9.68 (*AcoDREB07*) (Table S5). The 20 *AcoDREB* genes mapped to 14 pineapple chromosomes (Fig. 1), with three genes on Chr2 and two genes each on Chr3, Chr5, Chr6, and Chr17. Nine other chromosomes each contained one *AcoDREB* gene.

Multiple sequence alignment and phylogenetic analysis of the DREB family

Multiple sequence alignment of the AcoDREB AP2 domains indicated that the domain is highly conserved among the 20 AcoDREBs and has the typical characteristics observed in other DREB proteins (Fig. 2). Beyond the conserved YRG and RAYD motifs, all 20 AP2 domain sequences had a Val residue at position 14 (Val-14), and 11 sequences had a Glu residue at position 19 (Glu-19). In terms of DREB binding to DRE *cis*-acting elements, Val-14 is more important than Glu-19 (*Sakuma et al. 2002*).

To clarify the phylogenetic relationships of the DREB family members in pineapple, a multi-species phylogenetic tree was constructed using the full-length amino acid sequences of DREBs from pineapple, *Arabidopsis* (Table S6), and rice (Table S7). In Fig. 3, it should be noted that *AT3G57600* and *AT2G40220* (red frame) belong to the *Arabidopsis* subgroups A-2 and A-3, respectively. Because none of the pineapple *DREB* genes were found to be A-3 subgroup homologs, we divided the *AcoDREBs* into five subgroups, Ⅰ to Ⅴ (Fig. 3). *AcoDREB01*, *02*, and *03* belong to group Ⅰ. There are four members in group Ⅱ (*AcoDREB04*, *05*, *06*, and *19*), group III (*AcoDREB07*, *08*, *09*, and *10*), and group Ⅴ (*AcoDREB16*, *17*, *18*, and *20*). Group IV is the largest, with five members.

Stress-related *cis*-elements in *AcoDREB* promoters

Considering the potential involvement of *AcoDREB* genes in stress responses, we investigated the distribution pattern of stress-related conserved *cis*-elements in their promoter regions (2 kb region upstream of the transcription start site) using PlantCARE (Table S8). The data for six abiotic stress response elements, ABRE, DRE, LTRE, TC-rich repeat, MBS, and W-box, are shown in Fig. 4. All of the *AcoDREB* genes possessed at least one kind of *cis*-acting regulatory element, indicating that *AcoDREB* expression is associated with the abiotic stresses. Nine

AcoDREBs had one or more LTREs, which are associated with the response to low-temperature conditions. Sixteen *AcoDREBs* had one to eight ABA-responsive elements, and only *AcoDREB09*, *12*, and *17* had the TC-rich repeat element. Seven *AcoDREBs* had the MBS element, while W-boxes and DREs both occurred in 10 *AcoDREBs*. Overall, the results of the *cis*-element analysis indicate that *AcoDREB* genes can respond to different kinds of abiotic stress.

AcoDREB gene structure and conserved motifs in the encoded proteins

Structural diversity is very common among duplicated genes, and it may result in the evolution of functionally distinct paralogs. To analyze the *AcoDREB* gene structures, the exon and intron numbers and positions were determined by comparing the full-length cDNA sequences to the corresponding genomic DNA sequences (Fig. 5). The results showed that 75% of the *AcoDREB* genes (15/20) lacked introns. Four genes (*AcoDREB18*, *04*, *19*, and *13*) had one intron each, and *AcoDREB05* had three introns. Interestingly, the members of group Ⅱ differed in terms of exon and intron number as well as UTR length, which suggests that these four paralogs may have different roles in pineapple growth and development.

As shown in Fig. 6, the distribution of the motifs in AcoDREB proteins was relatively conserved. Motifs 1, 2, and 3 were present in all genes, but the motifs in different subgroups indicated some degree of divergence among them. For example, the three subgroup Ⅰ members contained motifs 4, 5, and 9 in addition to motifs 1, 2, and 3. Motif 7 was only present in two subgroup Ⅲ proteins (AcoDREB07 and AcoDREB08), and motif 4 was only present in AcoDREB05 of subgroup Ⅱ. Generally, members within the same subgroup had similar motif compositions, indicating that they might have similar functions (Fig. S1).

AcoDREB gene expression profiles in different tissues at different developmental stages

The different stages of pineapple reproductive organs were defined according to previous studies (*Azam et al. 2018; Su et al. 2017*). We used transcriptome sequencing data and to analyze the expression patterns of the 20 *AcoDREB* genes in nine different tissues: root, leaf, flower, fruit, gynoecium, stamen, petal, calyx, and ovule (Fig. 7; Table S9). At the same time, we used quantitative real-time PCR (qRT-PCR) to verify the results of RNA-seq. All *AcoDREB* genes except 4 low-expression genes (*AcoDREB04*, *07*, *08*, and *13*) were selected for qRT-PCR analysis in seven tissues. The results obtained were consistent with RNA-Seq expression data of these genes (Fig. 8, Table S10).

Clustering analysis show that the expression patterns of the 20 genes can be divided them into four clusters (Fig. 7). Of the six genes in cluster Ⅰ, four (*AcoDREB05*, *16*, *17*, and *20*) were highly expressed in all tissues, indicating that they may have important roles throughout plant growth. The expression level of *AcoDREB09* was lower in stamens than in other tissues, and *AcoDREB19* had the lowest expression in roots, suggesting that these particular cluster I genes

may not be critical for the development of these respective tissues. The six genes in cluster III (*AcoDREB02*, *04*, *07*, *08*, *10,* and *13*) had very low expression levels in all tissues, suggesting that these genes might only be expressed under special conditions. Most of the genes in clusters II and IV had tissue- or stage-specific expression patterns. For example, *AcoDREB01* and *AcoDREB15* had higher expression specifically in calyxes, suggesting that they may have a positive role in floral organ development. The higher expression of *AcoDREB06* in stage 6 stamens suggests a potential link to stamen maturity. *AcoDREB18* was more highly expressed during stamen development. *AcoDREB11* was expressed in the ovule, stamen, and gynoecium tissues, suggesting this gene may function widely during gametophyte development. *AcoDREB03* was more highly expressed in the four following tissues: root, calyx, petal, and gynoecium.

AcoDREB gene expression under abiotic stress

We also analyzed *AcoDREB* gene expression under various abiotic stress conditions, including salt, drought, cold, and heat. Specifically, we examined the expression patterns of eight *AcoDREB* genes (*AcoDREB01*, *03*, *06*, *09*, *11*, *14*, *18*, and *19*) in the '*MD2*' variety of pineapple using qRT-PCR with three biological and three technical replicates (Fig. 9; Table S2). Under all stress conditions, the relative transcript levels of the *AcoDREB* genes fluctuated during the 48 hour analysis period.

We subjected pineapple plants to salt stress using 150 mM NaCl. The expression of all eight genes increased rapidly in the roots and reached a maximum level after 12 hours. In the shoots, five genes had maximal expression at 12 hours, and two genes had maximal expression at 6 hours. *AcoDREB06* expression in shoots decreased after salt treatment. The dynamic responses of the *AcoDREB* genes after NaCl treatment suggest that they have vital roles in salt stress response (Fig. 9 A-H).

To analyze the response to drought stress, we used 350 mM mannitol treatment. In the shoots, six genes (*AcoDREB01*, *03*, *11*, *14*, *18*, and *19*) were down-regulated after 12 hours. *AcoDREB09* was extremely sensitive to drought stress, and its expression level quickly reached a maximum level at 6 hours after treatment. Except for *AcoDREB06*, the expression levels of the analyzed genes did not change as much in the roots as they did in the shoots. Compared to the control, *AcoDREB03* and *AcoDREB11* were rapidly down-regulated in the roots. These expression pattern changes after mannitol treatment indicate a vital role of *AcoDREB* genes in response to drought conditions (Fig. 9 I-P).

Cold stress is another abiotic stress that drastically affects plant growth and development and causes major crop yield losses (*Cai et al. 2015*). The gene expression levels in the roots and shoots were equally affected by cold treatment. In particular, three genes (*AcoDREB01*, *03*, and *18*) responded rapidly to cold treatment, and their expression levels in the shoots peaked at 6

hours. Two genes (*AcoDREB09* and *AcoDREB19*) reached their maximum expression levels in the shoots after 48 hours (Fig. 9 Q-X).

To analyze the effects of heat stress, the plants were subjected to 45 °C temperature. In the shoots, the majority of the analyzed genes were initially down-regulated then subsequently up-regulated. *AcoDREB03* was the only gene that was up-regulated in the shoots during the first 12 hours. In the roots, the expression levels of four genes (*AcoDREB01*, *09*, *11*, and *18*) gradually increased and peaked at 48 hours. The expression levels of two genes (*AcoDREB03* and *AcoDREB14*) decreased rapidly after exposure to high temperature stress. Unlike the other genes, the expression of *AcoDREB06* in the roots peaked at 12 hours. Collectively, these results suggest the involvement of *AcoDREB* genes in the response to heat stress in pineapple (Fig. 9 Y-FF).

DISCUSSION

The changes in global climate have drawn attention to the detrimental effects of environmental stress on the growth and survival of plants along with crop yield (*Chinnusamy et al. 2004; Mittler 2006; Suzuki et al. 2014*). Throughout their development, plants respond to different environmental stresses through the altered expression of functional genes that are activated by certain stress conditions. These stress resistance genes can be roughly divided into two categories. The first group includes functional genes directly responsible for the production of important stress resistance proteins, such as aquaporin, LEA protein, and antioxidant enzymes. The second group includes genes that encode regulatory proteins, such as TFs and protein kinases. By recognizing and binding specific promoter *cis*-elements, TFs regulate the transcription of downstream genes. There are hundreds of TFs in higher plants, and they have important roles in plant reproductive development and physiological metabolism (*Liu et al. 2001*). In response to environmental stress, TFs regulate plant growth and development by controlling a broad range of downstream genes. The AtMYB4 TF is important against UV (*Hemm et al. 2001*). Transgenic expression of *GmMYB22* in *Arabidopsis* was found to enhance drought tolerance, salt tolerance, and ABA sensitivity (*Shan et al. 2012*). One class of bZIP proteins, the TGA/OBF family members, can interact with NPR1 in the SA defense signaling pathway (*Singh et al. 2002*). The DREB TFs contain a conserved AP2/EREBP domain, which is involved in the response to external environmental stress. DREBs regulate stress-related genes to enhance plant resistance, and they are known to specifically interact with DRE *cis*-elements based on experiments in which mutated DRE binding sites led to abolished DREB TF binding (*Dubouzet et al. 2003; Liu et al. 1998*). Other experiments dissected the preferential binding of DREB1A to two DRE sequences in *Arabidopsis* and *Oryza sativa* (*Dubouzet et al. 2003; Sakuma et al. 2002*).

Several studies have elucidated the functions and evolutionary history of *DREB* genes in many plant species including *Arabidopsis,* rice, and maize. There have also been a growing number of findings about the functions of *DREB* genes in stress response processes. *DREB* genes were first

cloned in *Arabidopsis* in 1998 (*Liu et al. 1998*), and the study showed that *DREB1* and *DREB2* are involved in two separate signal transduction pathways under low-temperature and dehydration conditions (*Liu et al. 1998*). In *Arabidopsis*, the expression of *VuDREB2a* from legume cowpea was found to enhance drought resistance (*Sadhukhan et al. 2014*). DREBs also protect plants from both biotic and abiotic stresses by regulating some genes responsible for anthocyanin biosynthesis (*Song et al. 2019*). In addition, the members *MaDREB1–MaDREB4* (*Achr9G04630*, *Achr5G280*, *Achr6G32780*, and *Achr11G24820*) are induced by ethylene in bananas (*Musa acuminata*) and regulate fruit ripening (*Kuang et al. 2017*). These examples from diverse plant species indicate that DREBs contribute significantly to plant growth and development.

Considering its high economic value, pineapple production would benefit tremendously from an improved understanding of the stress tolerance mechanisms in this species. There was limited information about the *DREB* gene family in pineapple, so we identified the pineapple *DREB* genes and gathered the following information: the predicted isoelectric points (*pI*) and molecular weights of the encoded proteins, chromosome location, gene structure and motif, phylogenetic relationships, domain architecture, promoter *cis*-elements, and expression profiles under abiotic stresses.

The present analysis identified 20 *AcoDREB* genes (Table 1), which is fewer than the number of *DREB* genes in other monocots. For example, there are 57 *OsDREB* (*Rashid et al. 2012; Nakano et al. 2006*) (*Oryza sativa*), 51 *ZmDREB* (*Du et al. 2014*) (*Zea mays*), 52 *SbDREB* (*Yan et al. 2013*) (*Sorghum bicolor*), and 27 *PeDREB* (*Wu et al. 2015*) (*Phyllostachys pubescens*) genes. The lower number in pineapple suggests that some genes may have been lost during the evolution of these species. In terms of amino acid length, the predicted AcoDREB proteins ranged from 149 (*AcoDREB13*) to 463 (*AcoDREB20*) amino acids. The average length was 255 amino acids, which is very similar to that in rice and Chinese jujube (*Zhang & Li 2018*). The predicted molecular weights (Mw) ranged from 16.32 (*AcoDREB13*) to 49.3 (*AcoDREB20*) kDa, and the predicted pI values ranged from 4.71 (*AcoDREB10*) to 9.68 (*AcoDREB07*) (Table 1). The ranges reported in other species include the following: 12.13–59.27 kDa Mw and 4.6–10.64 pI in pepper (*Capsicum annuum* L.) (*Jin et al. 2018*) and 17.6–36.3 kDa Mw and 4.5–11.07 pI in moso bamboo (*Phyllostachys edulis*)(*Wu et al. 2015*). Thus, the predicted Mw and pI ranges in pineapple are roughly similar to those reported in other species, indicating some degree of conservation in terms of the biochemical characteristics and functions of DREB TFs in plants. Accordingly, we can make informed hypotheses about the characteristics and functions of DREBs that can be tested in future studies.

To investigate the phylogenetic relationships of the *AcoDREB* gene family, an unrooted phylogenetic tree was constructed based on multiple sequence alignment of DREB amino acid sequences from pineapple, *Arabidopsis*, and rice. The comparative analysis classified the

AcoDREB genes into five subgroups (Fig. 3), and the numbers of genes in subgroups I to V were 3, 4, 4, 5, and 4, respectively (Fig. 3). In *Arabidopsis*, the *DREB* genes can be divided into six subgroups (A1–A6), with only one gene in the A3 subgroup. In the current study, *AcoDREB04, AT2G40220* (A3 subgroup), and *AT3G57600* (A2 subgroup) were on the same branch of the phylogenetic tree (Fig. 3), but we ultimately grouped *AcoDREB04* with the A2 subgroup based on sequence and domain analysis (*Nakano et al. 2006*). As a result, there were no *AcoDREB* genes that grouped together with the A3 subgroup, which may have been lost during the evolution of these species.

Subsequent analysis of the intron-exon structure of *AcoDREB* genes revealed a generally low number of introns. The maximum number of introns was three in *AcoDREB05*, while many of the other genes lacked introns, which is consistent with previous reports in grape and jujube (*Zhao et al. 2014; Zhang & Li 2018*). Some studies proposed that introns can delay regulatory responses. To response to various tresses timely, genes must be rapidly activated, which would be assisted by a compact gene structure with less introns (*Jeffares et al. 2008*). The 8 genes were quickly induced under salt stress (Fig. 9), which may approve the above standpoints in other research. Core domain analysis identified three conserved domains among the predicted AcoDREB proteins (Fig. 6). Two of these domains (YRG and RAYD) form the AP2 structure, which is the characteristic feature of the entire DREB family (*Okamuro et al. 1997*). The DREB AP2 domain also contained a conserved Val residue at position 14 (Val-14) and a Glu residue at position 19 (Glu-19). Val-14 is important for the specific binding of DREBs to the DRE element (*Sakuma et al. 2002*), and it was detected in all 20 AcoDREB sequences. Glu-19 was present in 11 of the AcoDREB sequences (Fig. 2).

The expression patterns of some *AcoDREB* genes resembled the expression patterns of *DREB* genes in other species. The RNA-Seq and qRT-PCR data showed that *AcoDREB19* is highly expressed in anthers (Fig. 7, Fig. 8), which is consistent with the expression of its homolog in rice (LOC_Os08g27220) (*Davidson et al. 2012*). Similarly, *AcoDREB16* and its homolog in rice (LOC_Os10g22600) are both highly expressed in roots. These examples show that homologous *DREB* genes in different species may have similar expression patterns. *OsDREB2A*, a member of the DREB transcription factors in rice, was involved in the abiotic stress response enhancing the salt stress tolerance in overexpression in rice without changing the nutritional composition (*Mallikarjuna et al. 2011*), while not changed the whole nutritional composition (*Cornwell 2014; Cho et al. 2016*). Our results of expression analysis showed that overexpression of *AcoDREBs* in pineapple could help to develop new pineapple varieties with tolerance to abiotic stresses. Furthermore, we found that*AcoDREB05*, *16*, and *17* displayed high expression level in fruit (Fig. 7), indicating that they might play an important role in fruit development. Similarly, these genes possible to be used to change the fruit quality through molecular breeding.

DREB genes are responsive to stress conditions (*Torres et al. 2019*), with different expression characteristics in shoots and roots. We therefore quantified the transcript levels of eight *AcoDREB* genes in pineapple seedlings subjected to different abiotic stress conditions. Under salt stress, the expression patterns of the eight genes were highly similar, and the genes were induced in both shoots and roots (Fig. 9 A-H). It was previously reported that A1 subgroup members have important roles in the response to salt and drought stress in *Arabidopsis* (*Yamaguchi-Shinozaki & Shinozaki 2006*). In our analysis *AcoDREB01* and *AcoDREB03* from subgroup I, which had induced expression in pineapple plants subjected to salt and drought stress (Fig. 9 A-B, I-J). Specifically, the expression of *AcoDREB01* and *AcoDREB03* in roots was clearly induced under salt stress, and both were induced in shoots under drought stress conditions. Therefore, these genes may have important roles in roots and shoots under salt and drought stress, respectively. These two subgroups I genes also had similar expression patterns at the time of sampling, indicating that they may function similarly in response to salt and drought stress. Previous studies showed that *ScDREB10* was up-regulated after NaCl (150 mM) treatment and that *ScDREB10* overexpression enhanced salt stress tolerance in *Arabidopsis* seedlings (*Li et al. 2019; Li et al. 2016*). We therefore speculate that *AcoDREB01* and *AcoDREB03* may similarly promote stress tolerance under salt and drought stress.

Subgroup IV members *AcoDREB11* and *AcoDREB14* both had increased expression under salt treatment and cold stress (Fig. 9). These expression changes are similar to those of the A5 subgroup member *GmDREB2* (*Chen et al. 2007*), suggesting functional conservation of these homologs in pineapple and soybean. At the same time, which provides an example of the functional conservation of genes within the same subgroup. Under different abiotic stresses, *AcoDREB06* expression decreased in the leaves and increased in the roots, indicating that enhanced expression of this gene could potentially improve the resistance of roots to different abiotic stresses. On the other hand, the decreased expression of *AcoDREB06* in shoots suggests that it may also regulate other pathways to respond to plant stress (Fig. 9). *AcoDREB06* may improve drought and salt tolerance by reducing transpiration, similarly to the *Arabidopsis* gene *HARDY* (*AT2G36450*) (*Abogadallah et al. 2011*). The RNA-Seq data indicated that *AcoDREB19* had very low expression in roots. In our study, *AcoDREB19* expression increased significantly under different abiotic stresses, indicating that this gene may play a key role in abiotic stress response by enabling plants to cope with a variety of adverse environments.

Compared with the predicted *cis*-elements in *AcoDREB* promoters and expression analysis (Fig. 4 and Fig. 9), the 8 genes we selected were mostly accord with the results. At the same time, TC-rich and W-boxes elements were found in 4 genes (*AcoDREB01, 06*, *09*, *11*, and *19*) promoters. We speculate that these 4 genes may play a key role in resistance to pineapple diseases (*Hubert et al. 2014; Calderon-Arguedas et al. 2015*). This also provides information to develop disease-resistant seedlings of pineapple through breeding.

CONCLUSIONS

The present analysis identified 20 *AcoDREB* genes in pineapple, and we collected information about their gene structures and expression profiles under various abiotic stresses. To the best of our knowledge, this report is the first genome-wide analysis of *DREB* genes in pineapple, and our results showed that *AcoDREB* genes can respond to different abiotic stresses (drought, high salt, high- and low-temperature stress). The findings therefore not only provide preliminary data for future functional analysis of *AcoDREB* genes in pineapple, but may provide useful information to develop new pineapple varieties, and improve agronomic traits.

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Table 1(on next page)

Table 1: The *DREB* gene family in pineapple

1

Table 1 The *DREB* gene family in pineapple

Gene ID	Gene Name	Chromosomal localization		Amino acids length (aa)	Gene length (bp)	CDS length (bp)	Isoelectric points (pI)	Molecular weights (Mw)
Aco000059	*AcoDREB04*	LG12:5065638-5067899	12	315	2262	948	4.91	33745.45
Aco001190	*AcoDREB16*	LG02:13530546-13531451	2	301	906	906	5.66	33079.93
Aco001600	*AcoDREB05*	LG18:9400576-9404316	18	341	3741	1026	5.05	38147.27
Aco002673	*AcoDREB11*	LG06:10539056-10539706	6	216	651	651	5.22	22927.24
Aco002824	*AcoDREB17*	LG06:11885237-11886334	6	365	1098	1098	5.63	38918.03
Aco003376	*AcoDREB12*	LG17:2435249-2435743	17	164	495	495	5.79	18210.66
Aco006004	*AcoDREB07*	LG16:9780663-9781136	16	157	474	474	9.68	16405.64
Aco007650	*AcoDREB18*	LG08:962022-963979	8	373	1958	1122	9.07	40044.4
Aco008968	*AcoDREB01*	LG09:12532806-12533489	9	227	684	684	6.9	24126.78
Aco009985	*AcoDREB08*	LG10:1992629-1993102	10	157	474	474	9.68	16405.64
Aco010173	*AcoDREB06*	LG25:3102765-3103427	25	220	663	663	5.24	24212.82
Aco012243	*AcoDREB13*	LG02:73387-74171	2	149	785	450	9.63	16316.44
Aco012835	*AcoDREB09*	LG03:15051238-15052266	3	342	1029	1029	8.68	36712.72
Aco014268	*AcoDREB19*	LG05:128578-129975	5	221	1398	666	8.56	24115.21
Aco015162	*AcoDREB10*	LG05:1705173-1705958	5	261	786	786	4.71	27636.53
Aco016346	*AcoDREB20*	LG03:10461754-10463145	3	463	1392	1392	5.56	49311.65
Aco016696	*AcoDREB02*	LG17:191641-192357	17	238	717	717	7.66	26104.49
Aco018023	*AcoDREB14*	LG01:20359723-20360244	1	173	522	522	5.81	19023.86
Aco018980	*AcoDREB15*	LG02:10499315-10499860	2	181	546	546	9.65	19006.18
Aco022517	*AcoDREB03*	LG22:6333171-6333920	22	249	750	750	4.98	25951.31

2

3

4

Figure 1

Figure 1: Locations of *AcoDREB* genes on the pineapple chromosomes.

The chromosome number is indicated above each bar and the length of the bar represents the size of the chromosome in pineapple. Gene start point is shown on chromosome. The figure was generated using MapChart.

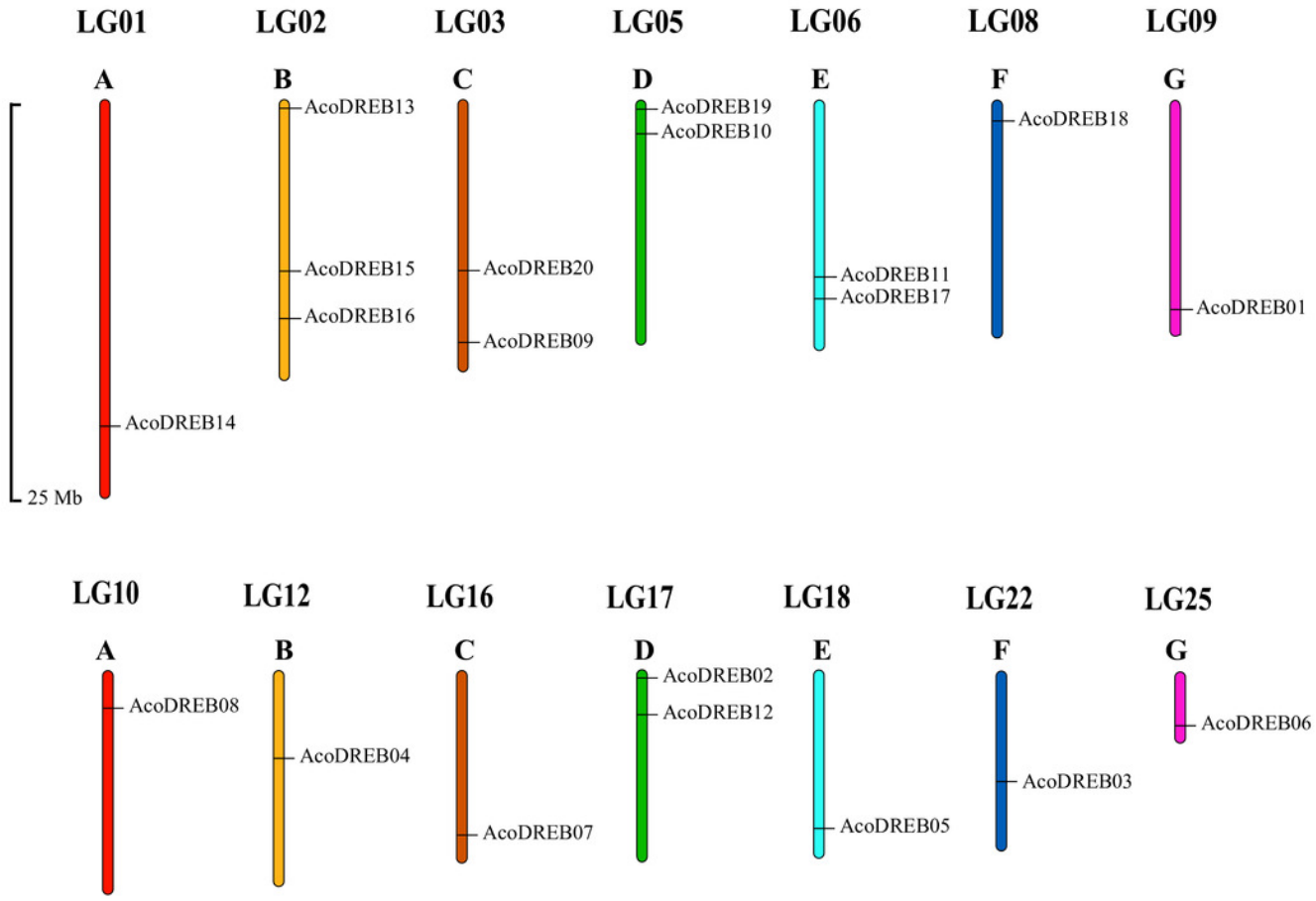

Figure 2

Figure 2: Multiple sequence alignment of the AP2 domain of AcoDREB proteins.

The alignment was performed using the DNAMAN. Conserved V14, E19, YRG and RAYD motifs are highlighted by the asterisks and lines.

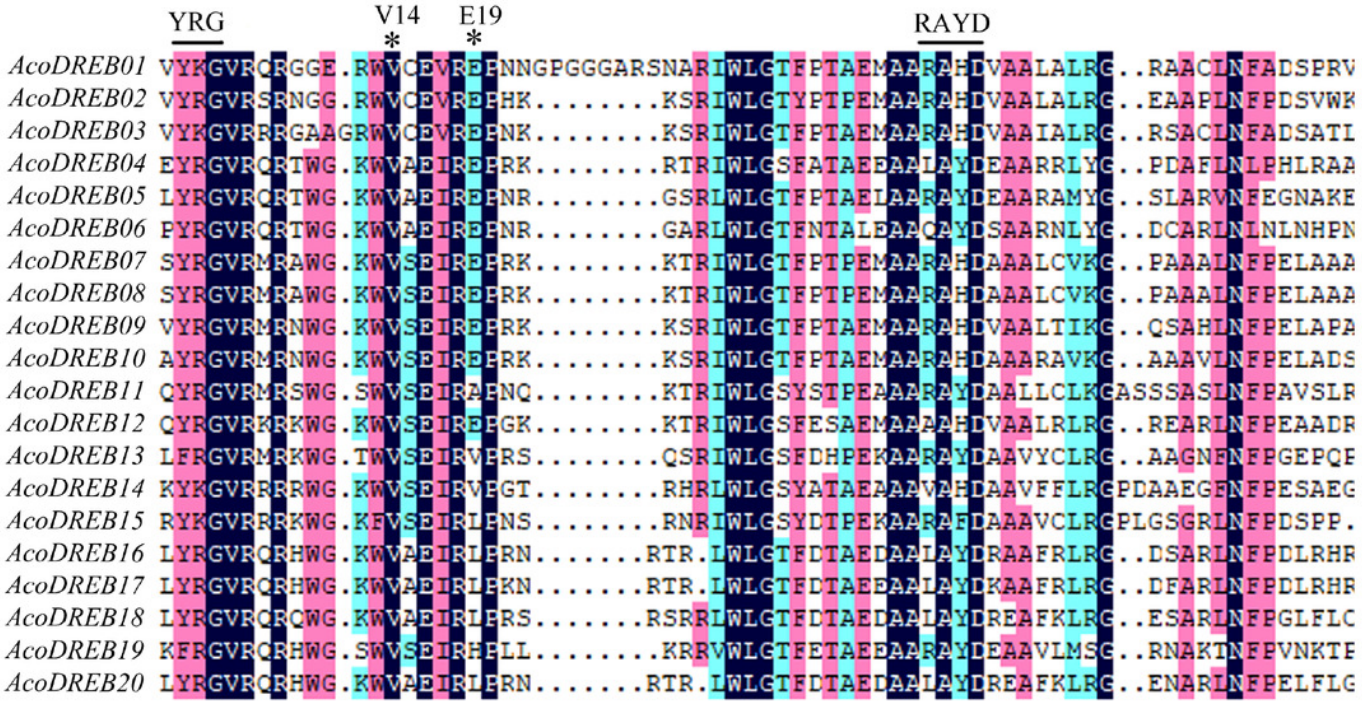

Figure 3

Figure 3: Phylogenetic analysis of DREB proteins in pineapple (Aco), *Arabidopsis*, and rice.

The proteins are classified into five groups: I, II, III, IV and V. Classification of *Arabidopsis* by Sakuma et al. (2002) is indicated in parentheses. Different individual subfamilies were shown by different color.

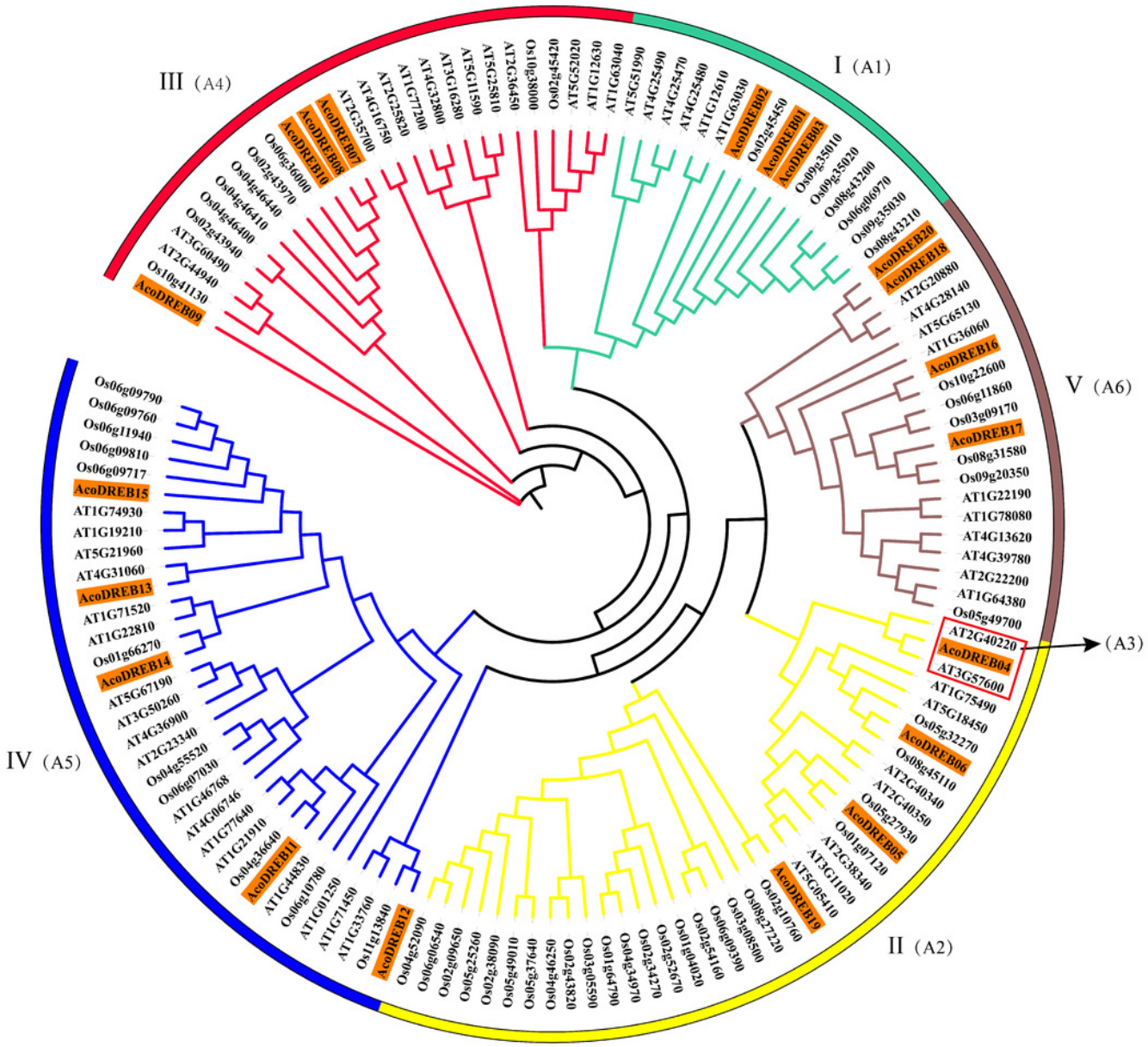
I (A1)
II (A2)
III (A4)
IV (A5)
V (A6)
(A3)
AcoDREB02
AcoDREB01
AcoDREB03
AcoDREB20
AcoDREB18
AcoDREB16
AcoDREB17
AcoDREB04
AcoDREB06
AcoDREB05
AcoDREB19
AcoDREB12
AcoDREB11
AcoDREB13
AcoDREB14
AcoDREB15
AcoDREB09
AcoDREB10
AcoDREB08
AcoDREB07
AT5G11590
AT5G25810
AT2G36450
Os10g38000
Os02g45420
AT5G52020
AT1G12630
AT1G63040
AT5G51990
AT4G25490
AT4G25470
AT4G25480
AT1G12610
AT1G63030
Os02g45450
Os09g35010
Os09g35020
Os08g43200
Os06g06970
Os09g35030
Os08g43210
AT2G20880
AT4G28140
AT5G65130
AT1G36060
Os10g22600
Os06g11860
Os03g09170
Os08g31580
Os09g20350
AT1G22190
AT1G78080
AT4G13620
AT4G39780
AT2G22200
AT1G64380
Os05g49700
AT2G40220
AT3G57600
AT1G75490
AT5G18450
Os05g32270
Os08g45110
AT2G40340
AT2G40350
Os05g27930
Os01g07120
AT2G38340
AT3G11020
AT5G05410
Os02g10760
Os08g27220
Os03g08500
Os06g09390
Os02g54160
Os01g04020
Os02g52670
Os02g34270
Os04g34970
Os01g64790
Os03g05590
Os02g43820
Os04g46250
Os05g37640
Os05g49010
Os02g38090
Os02g09650
Os05g25260
Os06g06540
Os04g52090
Os11g13840
AT1G71450
AT1G01250
Os06g10780
AT1G44830
Os04g36640
AT1G21910
AT1G77640
AT4G06746
AT1G46768
Os06g07030
Os04g55520
AT2G23340
AT4G36900
AT3G50260
AT5G67190
Os01g66270
AT1G22810
AT1G71520
AT4G31060
AT5G21960
AT1G19210
AT1G74930
Os06g09717
Os06g09810
Os06g11940
Os06g09760
Os06g09790
AT2G44940
AT3G60490
Os02g43940
Os04g46400
Os04g46410
Os04g46440
Os02g43970
Os06g36000
AT2G35700
AT4G16750
AT1G77200
AT1G25820
AT4G32800
AT3G16280

Figure 4

Figure 4: Predicted *cis*-elements in *AcoDREB* promoter regions.

Promoter sequences (−2000 bp) of 20 *AcoDREB* were analyzed by PlantCARE. The upstream length to the translation start site can be inferred according to the scale at the bottom.

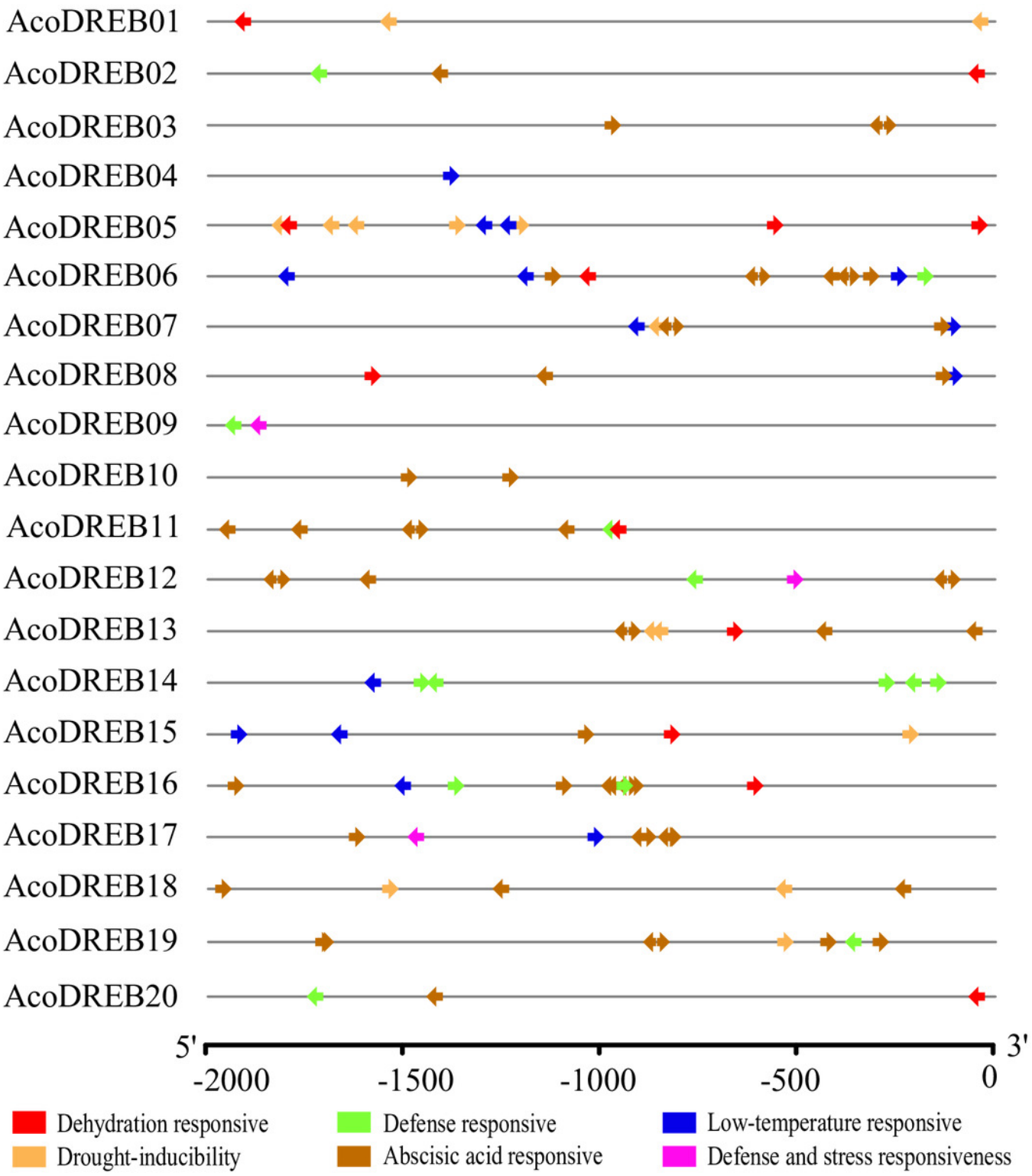
AcoDREB01
AcoDREB02
AcoDREB03
AcoDREB04
AcoDREB05
AcoDREB06
AcoDREB07
AcoDREB08
AcoDREB09
AcoDREB10
AcoDREB11
AcoDREB12
AcoDREB13
AcoDREB14
AcoDREB15
AcoDREB16
AcoDREB17
AcoDREB18
AcoDREB19
AcoDREB20
5'
3'
-2000
-1500
-1000
-500
0
Dehydration responsive
Drought-inducibility
Defense responsive
Abscisic acid responsive
Low-temperature responsive
Defense and stress responsiveness

Figure 5

Figure 5 : Exon–intron organization of *AcoDREB* genes.

Black bars indicates exon (CDS), Gray bars indicated UTR while plain lines showing introns.

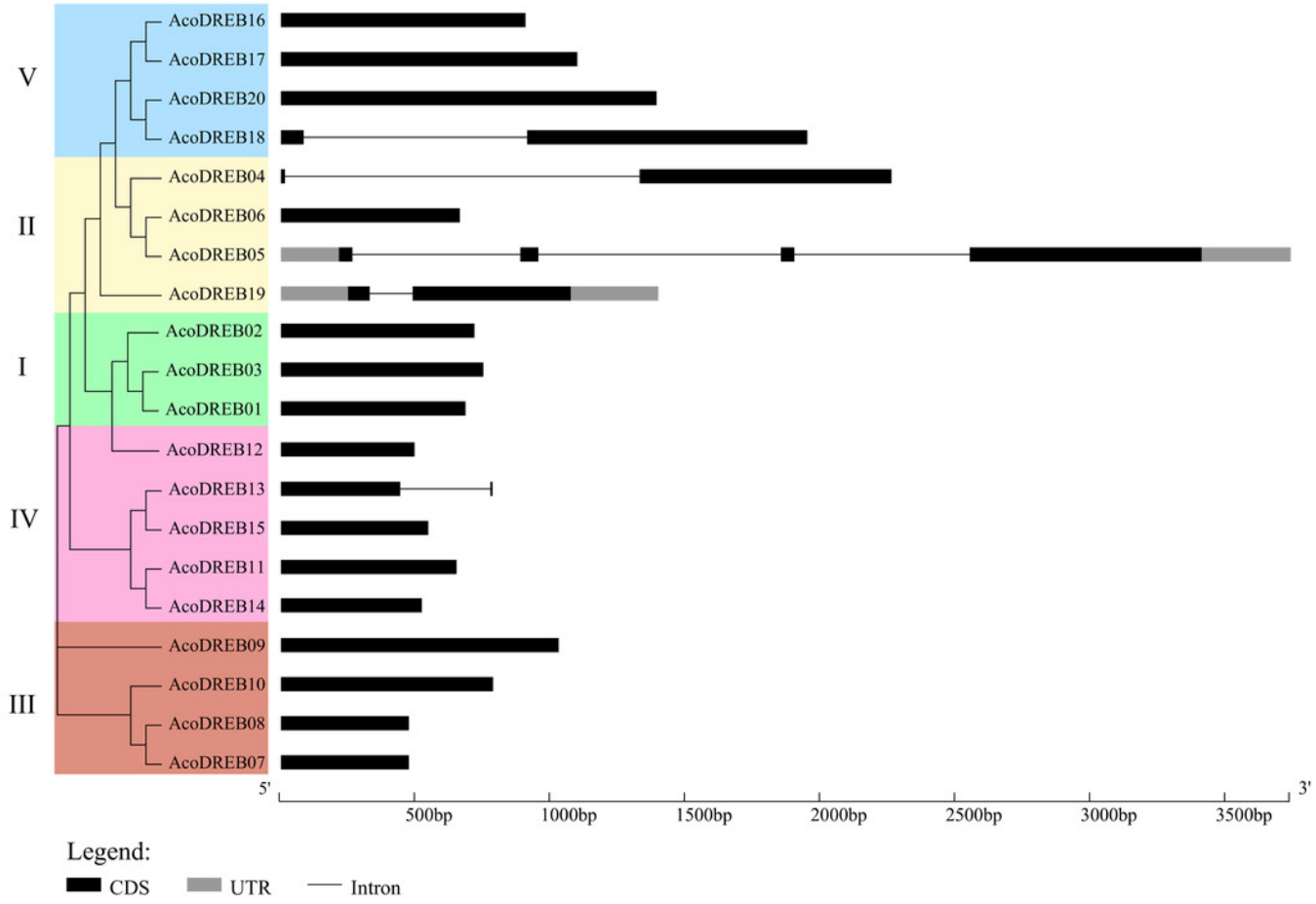

Figure 6

Figure 6: The conserved motifs of the predicted AcoDREB proteins.

The conserved motifs in the AcoDREB proteins were identified with MEME software. Grey lines denote the non-conserved sequences, and each motif is indicated by a colored box numbered on the right. The length of motifs in each protein was presented proportionally.

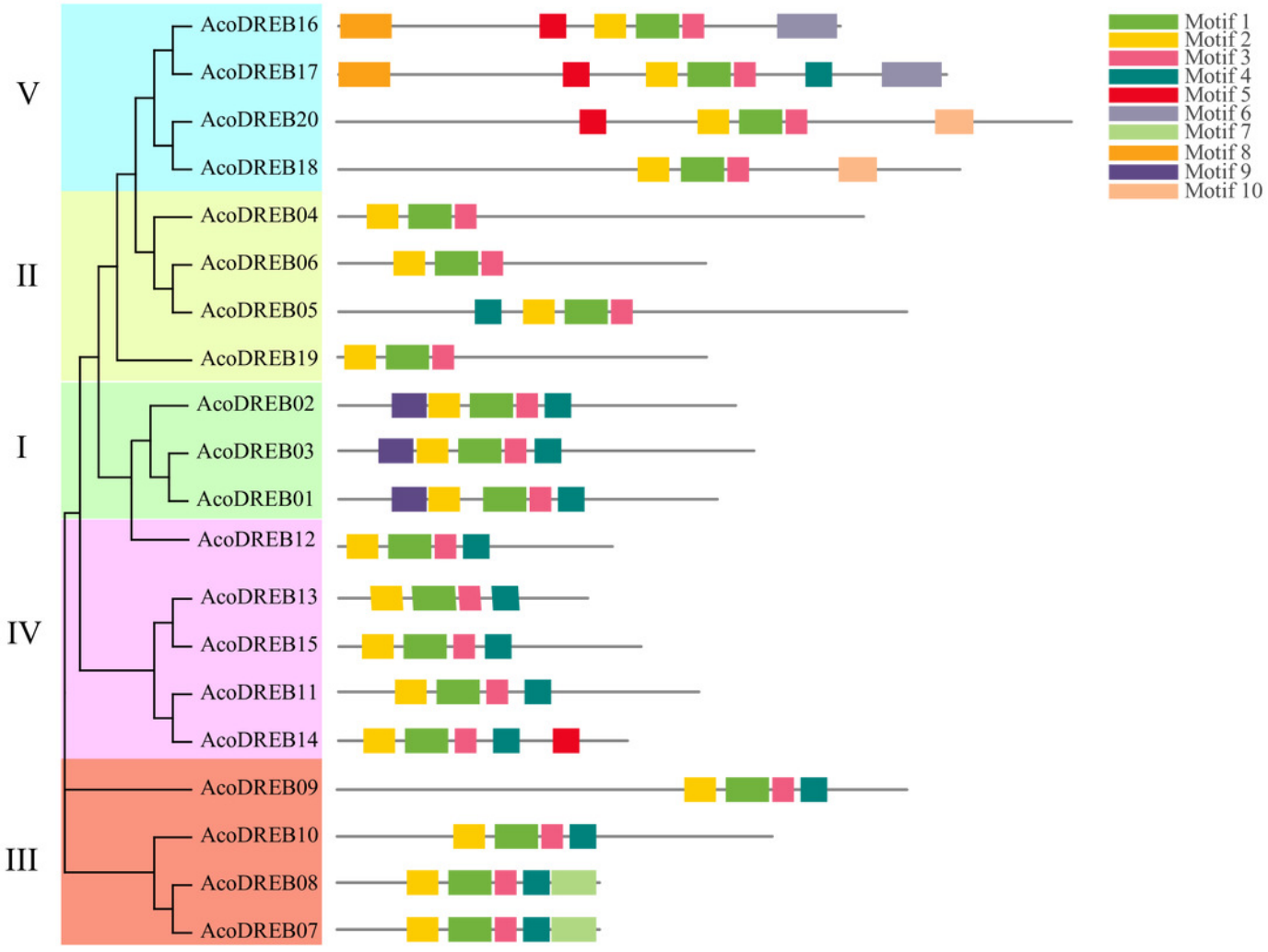

Figure 7

Figure 7: Heatmap showing the expression levels of *AcoDREB* genes in different pineapple tissues.

RNA-Seq expression level can be understood using the givenscale and roman numbers on right-side shows clusters based on gene expression. O, S and G represent ovule, stamen and gynoecium, respectively.

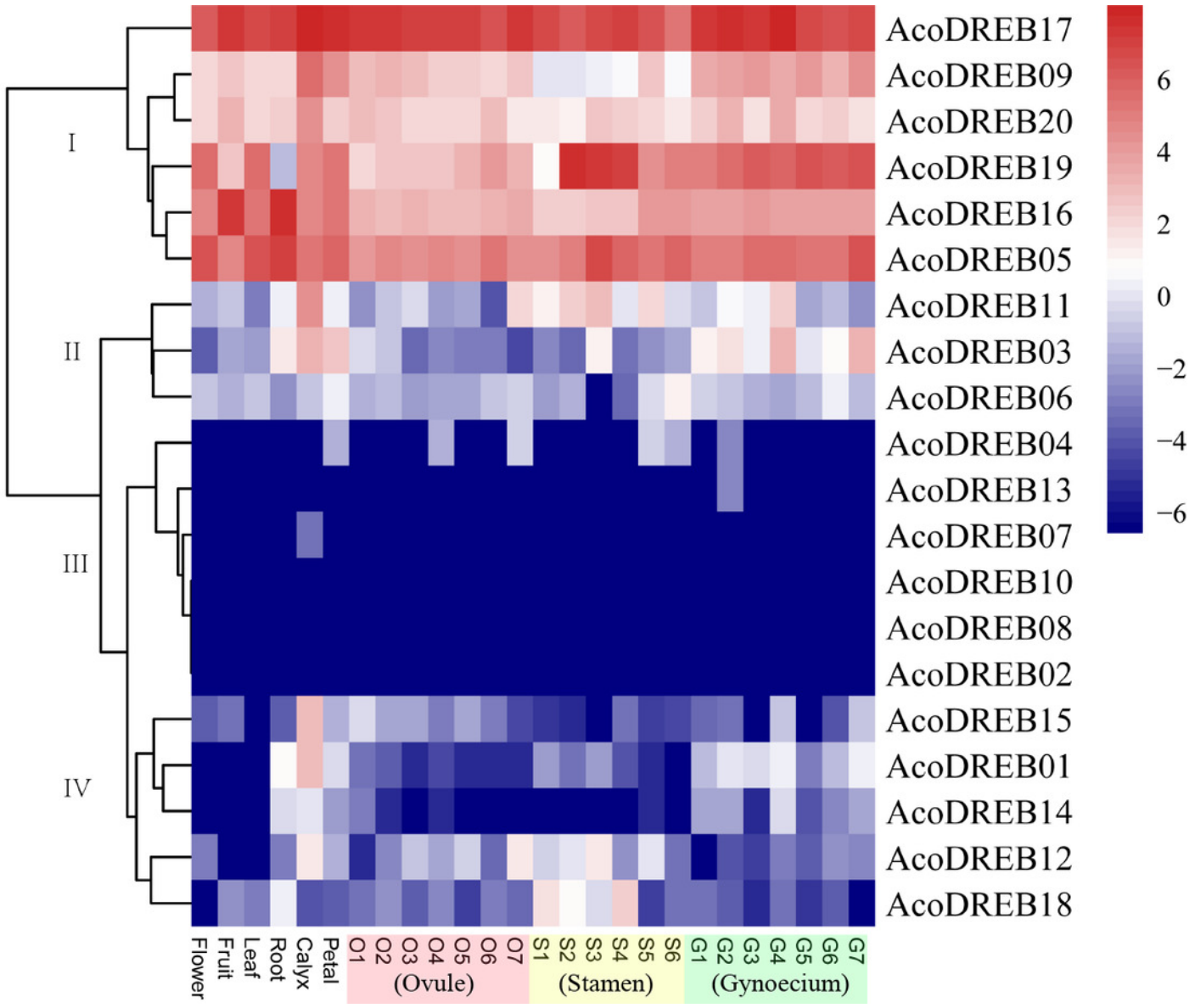

Figure 8

Figure 8: The expression profiles of *AcoDREB* genes in nine tissues validated by qRT-PCR.

Validation of 16 genes at nine different tissues through qRT-PCR. Heat-map was constructed from relative gene expression in different tissues (qRT-PCR) data.

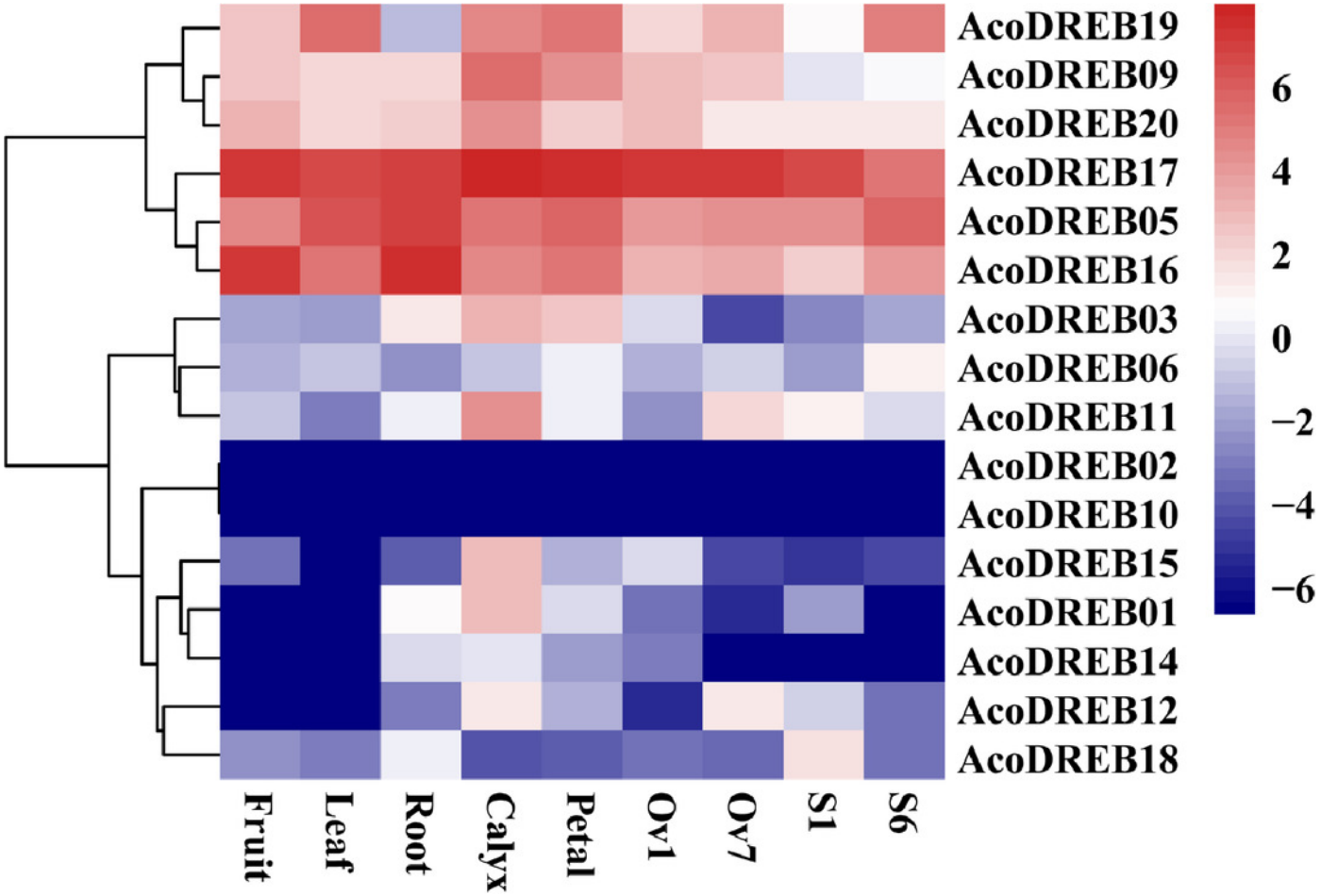

Figure 9

Figure 9: qRT-PCR expression analysis of eight selected *AcoDREB* genes in response to different abiotic stress treatments.

A-H high salt (150 mM NaCl); I-P drought (350 Mm Mannitol); Q-X chilling, exposure to 4 ℃; Y-FF high temperature, exposure to 45 ℃. Mean expression value was calculated from 3 independent replicates. Error bars indicate the standard deviation. Data are presented as mean ± standard deviation (SD). Asterisks on top of the bars indicating statistically significant differences between the stress and counterpart controls (*P< 0.05, **P< 0.01).