Identification and expression analysis of DREB transcription factor family in pineapple (*Ananas comosus* (L.) Merr.) (#41145)

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

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The *Dehydration Responsive Element Binding* (*DREB*) gene family is one of the most important transcription factor families in plants, which plays a crucial role in regulating plant growth and development as well as response to diverse stresses. Although *DREBs* have been thoroughly characterized in many plant species, the genome-wide identification of *DREB* gene family in pineapple has not been reported yet. In order to analyze the gene and protein properties of *DREB* gene family members in pineapple, a comprehensive genome-wide screening was performed and 20 *AcoDREBs* were obtained. Based on phylogenetic analysis, *DREB* genes were located on 14 pineapple chromosomes and divided into 5 subgroups. *AcoDREBs* within the same phylogenetic group share similar gene structure and domain composition. In addition, gene structural analysis showed that most of the *DREB* genes do not contain introns. *Cis*-element analysis showed that promoter regions of *AcoDREBs* consist with at least one stress response *cis*-element. Expression pattern of *AcoDREB* gene family showed that about 4 genes overexpressed and 6 genes underexpressed in all tissues. Eight *AcoDREB* genes were induced under abiotic stresses. Our study provides new insight for future studies to find out the functions of *DREB* genes in pineapple.

Identification and Expression Analysis of DREB Transcription Factor Family in Pineapple (*Ananas comosus* (L.) Merr.)

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Abstract

The *Dehydration Responsive Element Binding* (*DREB*) gene family is one of the most important transcription factor families in plants, which plays a crucial role in regulating plant growth and development as well as response to diverse stresses. Although *DREBs* have been thoroughly characterized in many plant species, the genome-wide identification of *DREB* gene family in pineapple has not been reported yet. In order to analyze the gene and protein properties of *DREB* gene family members in pineapple, a comprehensive genome-wide screening was performed and 20 *AcoDREBs* were obtained. Based on phylogenetic analysis, *DREB* genes were located on 14 pineapple chromosomes and divided into 5 subgroups. *AcoDREBs* within the same phylogenetic group share similar gene structure and domain composition. In addition, gene structural analysis showed that most of the *DREB* genes do not contain introns. *Cis*-element analysis showed that

promoter regions of *AcoDREBs* consist with at least one stress response *cis*-element. Expression pattern of *AcoDREB* gene family showed that about 4 genes overexpressed and 6 genes underexpressed in all tissues. Eight *AcoDREB* genes were induced under abiotic stresses. Our study provides new insight for future studies to find out the functions of *DREB* genes in pineapple.

Keywords: DREB transcription factors, Pineapple, Gene structure, Expression profiles

Introduction

Abiotic stresses, such as ~~high~~ salinity, drought, and high or low temperatures severely affect growth and development of plants. To drive the plant growth and development against abiotic stresses, plants evolve complex signaling transduction pathways and various mechanisms of abiotic stress tolerance by inducing the expression of plant functional proteins and regulatory proteins. The functional proteins consist of membrane proteins (membrane transporters and water channel proteins), key enzymes (proline, betaine, and sugars, etc.), detoxification enzymes (catalase, superoxide dismutase, ascorbate peroxidase, and glutathione *S*-transferase, etc.), and other proteins for protection of macromolecules (LEA protein, osmotin, antifreeze proteins, and mRNA binding protein, etc.). The regulatory proteins include transcription factors (bZIP, MYC, MYB and DREB, etc.), protein kinases (receptor protein kinase, MAP kinase, CDP kinase, and transcription-regulation protein kinase, etc.), and proteinases (phospholipase C and phosphoesterases, etc.). Among those regulatory proteins, transcription factors (TFs) play pivotal roles in response to abiotic stresses through interacting with *cis*-elements present in the promoter region of specific sets of stress-responsive genes to activate or repress their expression (Agarwal et al. 2006).

Dehydration response element binding (DREB) proteins are essential stress-responsive regulatory factors. Members of the DREB transcription factor family can specifically bind to DRE/CRT *cis*-acting elements to control downstream gene expression, and enhance plant tolerance to abiotic stresses. DREB transcription factor family belongs to the AP2/ERF (ethylene-responsive element binding factors) superfamily of transcription factors. The AP2/ERF superfamily is well known for its APETALA2 (AP2) domain, which consists of about 60 to 70 amino acids. In each AP2 domain, there are two conserved sequence blocks: YRG element and RAYD element. The YRG element consists of 19-22 amino acids, contains the conserved YRG motif, which may be related to the DNA specific binding 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 DREB subfamily compare with other subfamilies has slight but important differences in specific amino acid sites. Valine (Val) at position-14 and Glutamine (Glu) at position-19 are conserved in the DREB subfamily(Sakuma et al. 2002).

In *Arabidopsis thaliana*, the genes of DREB subfamily were divided into 6 groups, named A-1 to A-6 or DREB1 to DREB6 (Sakuma et al. 2002). There are many reports about the function of the A-1 and A-2 group' members. The first identified DREB gene was *AtCBF1* from A-1 subfamily,

which strongly induced by low-temperature. Three genes *AtCBF1*, *AtDREB1A*, and *AtDREB1C* were suggested to have a positive function in low-temperature stress response. A ~~typical~~ member of the DREB1 in sweet potato (*Ipomoea batatas*) *SwDREB1*, showed involvement in plant response to low temperature (Kim et al. 2008). Overexpression of the *ZjDREB1.4* in *Arabidopsis*, showed an increase in 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 increased the cold tolerance of rice plants (Donde et al. 2019). As a whole, DREB1 mainly takes part in the cold stress regulation in plants. According to the previous reported studies of DREB2, the function of the DREB2 is mainly involved in plant responses to drought and salt stress (Liu et al. 1998). The first reported At*DREB2A* and At*DREB2B* were induced by dehydration and high-salt stress (Sakuma et al. 2002). Overexpression of *GmDREB2* in *Arabidopsis* resulted in enhanced tolerance to high-salt stresses and without growth retardation (Chen et al. 2007a). In Sugar cane, overexpression of *EaDREB2* can greatly enhance the tolerance to drought and salinity stress ~~compared to untransformed plants~~ (Augustine et al. 2015). Compared with DREB1 and DREB2, the genes of the DREB3 to DREB6 subgroups have only been reported in recent years. A-4 subgroup gene *ZmDREB4.1* was cloned from maize (*Zea mays*), and it was found to play an important role in the negative regulation of plant growth and development (Li et al. 2018a). A novel A-5 type DREB gene, *ScDREB8*, can improve the salt tolerance in *Arabidopsis* at the seedling stage by up-regulating the expression of downstream stress-related genes (Liang et al. 2017). *CmDREB6* was classed into the DREB6 subgroup, whose overexpression enhanced the tolerance of chrysanthemum to heat stress (Du et al. 2018). The DREB family have been identified in several plant species, including *Arabidopsis thaliana* (Hwang et al. 2012), *Perennial ryegrass* (Xiong and Fei 2006), *Triticum L* (Mondini, Nachit and Pagnotta 2015), *Dendronthema* (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). However, there are no reports of *DREB* genes of pineapple, and the whole genome characteristics of DREB transcription factors in pineapple have not been studied. Therefore, there is an urgent need for a thorough bioinformatic analysis and characterization of *DREB* genes in pineapple genome.

Pineapple, a tropical fruit, has an important economic value. Pineapple is widely grown in tropical and subtropical regions. The cultivation of pineapple is of great significance to the development of local agriculture. With the change of global climate, various abiotic stresses seriously affect the growth of pineapple. In this study, we identified 20 pineapple *DREB* genes and divided into five subgroups. The gene structure, protein structure, protein motifs, gene locations on chromosomes, and expression profiles of *AcoDREB* genes were analyzed. Our results provide novel insights into the stress responses of *DREB* genes and broaden our understanding of the function of *DREB* genes in pineapple.

Materials & Methods

Identification of the DREB family members in Pineapple

The total of *Oryza sativa* and *Arabidopsis*'s *DREB* amino acid sequences were obtained from Rice Genome Annotation Project (http://rice.plantbiology.msu.edu/index.shtml) and TAIR (http://www.arabidopsis.org), respectively. To identify the DREB amino acid sequences, we have used the DREB genes of *Arabidopsis* to search pineapple genome with BLAST-P and based on a Hidden Markov Model (HMM), we downloaded the AP2（PF00847）domain as query to execute an hmmer search (https://www.ebi.ac.uk/Tools/hmmer/search/hmmsearch) with parameters set default in pineapple genome. Subsequently, we deleted the redundant sequences and used SMART (http://smart.embl-heidelberg.de/) to verify the completeness and existence of the core domain from these sequences. Finally, the remaining sequences were used for further phylogenetic analysis.

Protein characteristics and Chromosomal localization

The information about gene lengths, number of amino acids, CDS length and chromosome localization of *AcoDREB* genes was collected from Phytozome. The molecular weight and isoelectric points of predicted *AcoDREB* were detected using the ExPASy proteomics server (http://expasy.org/) (Gasteiger et al. 2003). According to the gene's start position and the length of related chromosome, MapChart was used to visualize the 20 *DREB* genes mapped on 25 chromosomes and scaffolds localization.

***Cis*-acting element analysis of *AcoDREB* genes' promoters**

The upstream sequences (2.0 kb) of the *AcoDREB*-coding sequences were retrieved from the Phytozome and then submitted to PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) to identify six regulatory elements, abscisic acid (ABA)-responsive elements, involved in the ABA responsiveness; dehydration-responsive elements (DREs), involved in dehydrated, low-temp and salt stresses; low temperature-responsive elements (LTRE), involved in low-temperature response; TC-rich repeats, involved in defense and stress response; W-boxes, binding site of WRKY transcription factor in defense responses; and MBS, MYB binding site involved in drought-inducibility.

Sequence alignment and Phylogenetic analysis

The CDS sequences of the *AcoDREB* genes were obtained from the Phytozome, imported to DNAMAN for sequence alignment. The phylogenetic tree was constructed by IQ tree with the Maximum likelihood (ML) method (Chernomor, von Haeseler and Minh 2016, Nguyen et al. 2015), the parameters were set default, except for the ultrafast bootstrap option n=1000 (Hoang et al. 2018) after performing multiple sequence alignments using MUSCLE 3.7 (Edgar 2004) with default parameters. Neighbor-joining (NJ) method of MEGA7 (Kumar, Stecher and Tamura 2016) was also used to construct tree and to validate the ML method results.

Gene Structural Analysis and Conserved Motif Identification

The *DREB* genes structure including the number and position of exon and intron were determined using the Gene Structure Display Server (http://gsds.cbi.pku.edu.cn/) (Guo et al. 2007). To analyze the amino-acid sequences of the 20 *AcoDREBs*, MEME (Multiple EM for Motif Elicitation) (http://meme-suite.org/tools/meme) was used, the maximum number of motifs was set to 10, and default options were used.

Plant Material and Growth Conditions

Pineapple (*Ananas comosus*) variety MD2 was provided by Qin Lab, Haixia Institute of Science and Technology, Fujian Agriculture and Forestry University, Fujian, China (~~www.qinlab.net~~). Plant crowns were grown on soil mixture [peat moss:perlite,2:1(v/v)] in plastic pots placed in greenhouse at ~30 °C with light intensity of 60–70 umol m^{-1} s^{-1} photons, under 70% humidity with 16-h light/8-h dark photoperiod (Ali et al. 2017, Rahman et al. 2017).

RNA-Seq for Different Tissues in Pineapple

We used RNA extraction kit (Omega Bio-Tek, Shanghai, China) to extract total RNA from different tissues of pineapple (MD2), including sepal, gynoecium, ovule, petal, and stamen. The collection of different tissues was done following the previous method (Chen et al. 2017). NEBNext Ultra RNA Library Prep Kit for Illumina was used to prepare qualified libraries and then sent for sequencing. The RNA-Seq data of root, leaf, leaf base and leaf tip, flower and fruit at different development stages were collected from Pineapple Genomics Database （http:/ /pineapple.angiosperms.org/pineapple/html/index.html） (Ming et al. 2015). Using TopHatv2.1.1 (Trapnell et al. 2012) with default parameter settings, the trimmed pair-end reads of all tissues were aligned to pineapple genome. Cufflinks v2.2.1 software and Cuffdiffv2.2.1 were used to estimate the FPKM values, then the heatmap of DREB genes expression profiles was generated by pheatmap a packages of R.

~~Plant material and~~ treatments

One-month-old plants in rooting medium were used as the panting material for the stress treatment. Uniform tissue cultured seedlings were obtained from Qin Lab. (Priyadarshani et al. 2018). Seedlings were subjected to the stress treatments including low-temperature (4 ℃), high-temperature (45 ℃), drought (350 mM mannitol) and high salt (150 mM NaCl) conditions. The root and leaf of samples were collected at 6h, 12h, 24h and 48h time period after treatment (Ali et al. 2017). Samples from seedlings those were not subjected to stress treatments were used as a control. Collected samples were immediately stored in liquid nitrogen prior to total RNA extraction (Rahman et al. 2017).

RNA Extraction and Quantitative real-time PCR

Total RNA was extracted using RNA plant extraction Kit (Omega Bio-Tek, Shanghai, China) following manufacturer's protocol. According to the supplier's instructions to use AMV reverse transcriptase (Takara), 1μg of purified total RNA was reverse transcribed to cDNA in a 20 μL

reaction volume (Cai et al. 2019). To determine the relative transcript levels of selected *DREB* genes, real-time PCR was performed with gene specific primers according to the manufacturer's instructions on the Bio-Rad Real-time PCR system (Foster City, CA, USA).The specific primers used in this experiment are given in Supplemental Table S2. The PCR program was set: 95 °C for 30 s; 40 cycles of 95 °C for 5 s and 60 °C for 34 s; 95 °C for 15 s. Three technical replicates and at least three independent biological replicates were performed in each case (Cai et al. 2017, Zhang et al. 2018).

Results

Genome-Wide Identification and Chromosomal location of Pineapple *DREB* genes

To identify *AcoDREB* amino acid sequences, we searched the pineapple genome, based on the *DREB* family in *Arabidopsis*. A total of 20 *DREB* amino acid sequences were obtained from pineapple proteome. According to the gene ID, the candidate genes named *AcoDREB1* to *AcoDREB20* and the amino acid sequences of these genes are shown in Table S1. The characteristics of the 20 *DREB* genes are listed in Table 1, including the gene name, gene ID, gene and amino acids length, pI (Isoelectric points), Mw (Molecular weights). The number of amino acids in 20 *AcoDREB* varied from 149 (*AcoDREB13*) to 463 (*AcoDREB20*). The length of CDS also varied widely from 450 (*AcoDREB13*) to 1392 (*AcoDREB20*) bp. The relative molecular weight of these DREB proteins range from 16316.44 (*AcoDREB13*) to 49311.65 (*AcoDREB20*) kDa, and the predicted isoelectric points varied from 4.71 (*AcoDREB10*) to 9.68 (*AcoDREB07*). According to our mapped results, these 20 *DREB* genes were mapped on fourteen pineapple chromosomes (Fig.1). Among them, Chr2 possesses three *AcoDREB* genes, four chromosomes (Chr3, Chr5, Chr6, and Chr17) contain two genes, and the another nine chromosomes possess one gene in each.

Multiple alignments and Phylogenetic analysis of *DREB* Gene Family

Multiple alignments with the AP2 domain of AcoDREBs indicated that 20 AcoDREBs had a highly conserved domain and exhibited the typical characteristics of DREBs (Fig.2). Except for the conserved YRG and RAYD motif, all of them also contained the conserved amino acids in the specific sites. The AP2 domain of DREBs contained conserved Val at position-14 and Glu at position-19. Specifically, Val at position-14 is important in DNA-binding specificity to the DRE, and Glu-19 not as important as Val-14 (Sakuma et al. 2002). According to the sequence alignment results, we found that all the AcoDREBs have conserved Val at position-14, and 11 while Glu at position-19.

To clarify phylogenetic relationships of the *DREBs* in pineapple, the multi-species phylogenetic tree was constructed based on the full-length amino acid sequences of *DREBs* from pineapple, *Arabidopsis* and rice. Specifically, *AT3G57600* and *AT2G40220* (red frame) belong to the *Arabidopsis* groups A-2 and A-3, respectively. Compared with the *AtDREBs*, the pineapple *DREB* genes lack the homologous gene of the A-3 group. Therefore, we divided *AcoDREBs* into five subgroups, Ⅰ to Ⅴ (Fig.3). Among the groups, group Ⅳ has five members. *AcoDREB01*,

AcoDREB02, and *AcoDREB03* were in the same group (group Ⅰ) and *AcoDREB04*, *05*, *06* and *19* were assigned to group Ⅱ. There are four members in group Ⅲ (*AcoDREB07*, *AcoDREB08*, *AcoDREB09* and *AcoDREB10*) and Ⅴ (*AcoDREB16*, *AcoDREB17*, *AcoDREB18*, and *AcoDREB20*）.

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

To study stress-related conservative *cis*-elements distribution pattern of *AcoDREB* genes during stress response, the 2.0-kb upstream sequences from the translation start sites of were analyzed using PlantCARE. We found six abiotic stress response elements namely ABA-responsive elements, DRE, LTRE, TC-rich repeat, MBS and W-box, and displayed in Fig.4. *AcoDREB* genes possessed at least one kind of *cis*-acting regulatory element, which indicated that the expressions of *AcoDREBs* were associated with these abiotic stresses. In total, 9 *AcoDREBs* had one or more LRTEs, meaning a potential low-temperature response under low-temperature conditions. One to eight ABA-responsive elements existed in 16 *AcoDREBs*, and only *AcoDREB09*, *12*, and *17* had TC-rich repeat. MBSs were located in 7 *AcoDREBs*, W-boxes and DREs all appeared in 10 *AcoDREBs.* The results of the *cis*-element analysis showed that the *AcoDREB* genes could respond to various kinds of abiotic stresses.

Gene structure and Conserved motifs in DREB proteins

Structural diversity is very common in duplicated genes and has resulted in the generation of distinct paralogs functionally. To understand the gene structural diversity further, the numbers and positions of exons and introns were determined by comparing the full-length cDNA sequences and the corresponding genomic DNA sequences (Fig.5). The results indicated that most *AcoDREB* genes have no introns. Among these genes, four genes (*AcoDREB18*, *04*, *19*, and *13*) have one intron each, and *AcoDREB05* has three introns. Interestingly, the members of group Ⅱ are different in the number of exons, introns and the length of UTR, which implies that the four paralogs may play different roles in the growth and development of pineapple.

As shown in Fig.6, the distribution of the motifs in *AcoDREBs* are relatively conserved. Motif 1, 2 and 3 are present in all genes and the motifs in different subgroups showed a certain degree of divergences. For example, motif 1, 2, 3, 4 and 9 were harbored by three proteins all in subgroup Ⅰ. Motif 7 was only present in two proteins (*AcoDREB07* and *AcoDREB08*) of subgroup Ⅲ. Motif 4 was only presented in *AcoDREB05* of subgroup Ⅱ. *AcoDREB* members within the same subgroups were generally found to share a common motif composition. The results suggested that these members in the same subfamily might have similar functions (Fig.S1).

Expression profile of *DREB* genes in various tissues at different developmental stages

For understanding of the expression profiles of *AcoDREB* genes in different tissues, we used transcriptome sequencing to discover the expressions of the 20 *AcoDREBs* in nine different tissues, which include root, leaf, flower, fruit, gynoecium, stamen, petal, calyx and ovule. The

expression level of 20 *AcoDREBs* can be divided into four clusters based on ~~the hierarchical cluster~~ (Fig.7). In cluster i, four genes (*AcoDREB05*, *AcoDREB16*, *AcoDREB17*, and *AcoDREB20*) had high expression level in all tissues, implying that those genes play an important role in plant growth, flower and seed development, and others. The expression level of *AcoDREB09* in stamen is lower than in other tissues. The expression level of *AcoDREB19* in root is the lowest, suggesting that it may not take part in the crucial functions of the pineapple root. In addition, the 6 genes (*AcoDREB02*, *AcoDREB04*, *AcoDREB07*, *AcoDREB08*, *AcoDREB10,* and *AcoDREB13*) of cluster iii were expressed in all tissues at very low levels, suggesting that those genes might be expressed under special conditions. In cluster ii and iv, most genes are specifically expressed in certain tissues or stages. Two genes (*AcoDREB01* and *AcoDREB15*) showed higher expression levels only in sepal when compared to other parts, suggesting that these genes may play a positive role in flower organs. *AcoDREB06* was expressed highly in stage 6 of stamen, meaning that this gene might be related to the maturity of the stamen. *AcoDREB18* showed high expression during stamen development. *AcoDREB11* was expressed in ovule, stamen, and gynoecium, suggesting this gene may function widely during the gametophyte development and *AcoDREB03* was highly expressed in 4 different tissues, including root, calyx, petal, and gynoecium. According to previous studies (Azam et al. 2018, Su et al. 2017), different stages of the pineapple reproductive organs were defined. To validate RNA-Seq data, we selected 16 genes (except *AcoDREB04*, *AcoDREB07*, *AcoDREB08* and *AcoDREB13*) to perform quantitative real-time PCR (qRT-PCR) analysis. We found that the expression of selected genes (Fig.8) in the root, leaf and other seven tissues coincide with the RNA-Seq data.

Expression Patterns of *DREB* Genes under Abiotic Stress

To further explore the expression patterns in the *AcoDREB* genes under various abiotic stress conditions including salt, drought, cold and heat, we examined the expression patterns of 8 *AcoDREB* genes (*AcoDREB01,03, 06, 09, 11, 14, 18* and *19*) in '*MD2*' variety of pineapple by qRT-PCR with 3 biological and 3 technical replications. The relative expression level of the *AcoDREB* genes under all stress conditions fluctuated during the 48-h treatment. Cold stress is abiotic stress that also has drastic effects on plant growth and development and causes major loss to crop yield (Cai et al. 2015). In the shoot, most of the *AcoDREB* genes were more sensitive to cold stress than other parts of the plant. Among these genes, 3 (*AcoDREB01*, *AcoDREB06*, and *AcoDREB18*) of them responded rapidly under cold stress and reached the highest level after 6 hours. The two genes (*AcoDREB09* and *AcoDREB19*) reached the highest expression level after 24 hours. The expression levels of *AcoDREB06* were down regulated significantly after subjected to 4 abiotic stresses in the shoot of pineapple. After 48-h, the expression levels of cold stress and drought stress were restored. Salt stress is also major stress, which adversely affects plants and causes a major loss in crop yield. According to the expression analysis of eight genes in roots after 12 hours of 150 mM salt treatment, we found that the expression levels of all genes increased rapidly and reached a maximum after 12 hours. According to the experimental results we found that three genes (*AcoDREB03*, *AcoDREB06*, and *AcoDREB14*) were more sensitive to

cold stress than salt stress, and *AcoDREB03* and *AcoDREB14* reached the highest expression level after 48 hours. Clearly, the *AcoDREB11* gene was sensitive to drought stress, the expression level lowered rapidly compared with the control.

Discussion

With the change of global climate, all kinds of environmental stresses have brought serious harm to the growth of plants and severely inhibited the survival, development finally reducing the yield of crop plants. In the process of growth and development, plants can respond to various environmental stresses through changing the expression of functional genes activated due to the external environment. These stress resistance genes can be roughly divided into two categories: the first group is functional genes that are directly responsible for the production of proteins for stress resistance in plants, such as aquaporin, LEA protein, antioxidant enzyme, etc. The second group is anti-stress genes that encode regulatory proteins, such as transcription factors and protein kinases. Among them, transcription factors can specifically recognize and bind cis-acting elements in promoters, to regulate the transcription expression of downstream genes. There are hundreds of transcription factors found in higher plants, which play an important role in plant reproductive development and physiological metabolism (Liu, Zhang and Chen 2001). Transcription factors are involved in regulating plant growth and development and in response to environmental stress by control a broad range of downstream genes. *AtMYB4* is a transcription factor in the battle against UV (Hemm, Herrmann and Chapple 2001). Transcription factor *GmMYB22* can enhance drought tolerance, salt tolerance, and ABA sensitivity in *Arabidopsis thaliana* (Shan et al. 2012). One class of bZIP proteins, TGA/OBF family members, can interact with NPR1 to involved in the SA defense signaling pathway (Singh, Foley and Onate-Sanchez 2002). The DREB transcription factors contain a conserved AP2/ EREBP domain, which is involved in plant response to external environmental stress. By specific combining with DRE (Dehydration responsive element) *cis*-acting elements, DREB regulates the expression of downstream stress-related genes and improves plant resistance. It was found that the mutation of DRE binding sites could result in the inability of DREB transcription factors to bind (Dubouzet et al. 2003, Liu et al. 1998). And *DREB1A* binding sites had a preference for two DRE sequence between *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 such as *Arabidopsis,* rice, and corn. Until now, there are increasing reports about the function of *DREB* family genes in the process of plant stress tolerance. The *DREB* genes were first cloned from *Arabidopsis* in 1998 (Liu et al. 1998). It was shown that *DREB1* and *DREB2* play trans roles in two separate signal transduction pathways under low-temperature and dehydration conditions (Liu et al. 1998). In *Arabidopsis*, *VuDREB2a* was reported to enhance the ability of plants to drought resistance (Sadhukhan et al. 2014). DREB also protects plants from both biotic and abiotic stresses by regulating some genes responsible for anthocyanin synthesis (Song et al. 2019). In addition, the members of *MaDREB1-MaDREB4* (*Achr9G04630*, *Achr5G280*, *Achr6G32780*, *Achr11G24820*), which are induced by ethylene in bananas (*Musa*

acuminata), play an important role in regulating fruit ripening (Kuang et al. 2017). Therefore, DREB plays a significant role in plant growth and development.

As a tropical fruit crop with important economic value, pineapple has important research value in plant stress tolerance. However, little is known about the *DREB* gene family in pineapple. Here, we performed a series of analyses on pineapple *DREB* genes, including their characteristics like isoelectric point (pI), molecular weight, chromosome location, gene structure and motif, synteny relationships, domain architecture, promoter ~~action~~ elements, and expression profiles under abiotic stress response (Li et al. 2018b). The DREB family was identified in the whole genome of pineapple, and 20 *AcoDREB* genes were ~~finally~~ obtained (Table.1). This condition is similar to that reported for *Phyllostachys pubescens*, which had 27 *DREB* genes (Wu et al. 2015). However, there are significant differences with *Arabidposis* and rice. Both have 57 *DREB* genes (Nakano et al. 2006). The reason for these differences may be due to the loss of some genes in the evolution process of species. The number of amino acid residues of the *DREB* family ranged from 149 (*AcoDREB13*) to 463 (*AcoDREB20*). The average amino acid length is 255, which is very similar to rice and Chinese jujube (Zhang and Li 2018b). The relative molecular weights ranged from 16316.44 to 49311.65 kDa, and the predicted pI varied from 4.71 (*AcoDREB10*) to 9.68 (*AcoDREB07*) (Table.1). The biochemical characteristics of DREB transcription factors were stable in many species. This means that the function of DREB may also be conserved.

To clarify the phylogenetic relationships of the *AcoDREB* gene family, an unrooted phylogenetic tree was constructed based on the multi-alignment of the sequence of DREBs from Pineapple, *Arabidopsis*, and rice. The results showed that *AcoDREB* genes could be grouped into five subgroups, Ⅰ to Ⅴ (Fig.3) according to the cluster analysis and comparison with *Arabidopsis* and rice in evolution. The number of genes per subgroup is 3, 4, 4, 5 and 5, respectively (Fig.3). Furthermore, the DREB family was divided into 6 subcategories (A1, A2, A3, A4, A5, and A6) and A3 has only one gene in *Arabidopsis*. In the current study we found that, *AcoDREB04,* AT2G40220 (A3 Group) and AT3G57600 (A2 Group) were divided into the same branch in the evolutionary tree (Fig.3). According the analysis of sequence and domain, we finally classified *AcoDREB04* into A2 subgroup (Nakano et al. 2006). So, we suggest that there is no A3 subgroup in DREB family of pineapple. These genes were evenly distributed in the five subgroups.

Additionally, the intron-exon structure and core domain analyses of *AcoDREBs* were carried out based on the phylogenetic tree. The introns are very few in the whole DREB family. The maximum number of introns were found in *AcoDREB05*, while the other genes didn't have introns, as reported previously in grapes and jujube (Zhang and Li 2018a, Zhao et al. 2014). According to the results from core domain analysis, we found that all members of *AcoDREB* have three conserved domains (Fig.6). Among the three domains, two of them (YRG and RAYD) form the AP2 structure, which is the feature of the whole DREB family (Okamuro et al. 1997). The AP2 domain of DREBs was found to contain conserved Val at position-14 and Glu at position-19. Specifically, Val at position-14 is important in DNA-binding specificity to the DRE (Sakuma et al. 2002). In this study, we found that all the 20 *AcoDREBs* had conserved Val at position-14, and 11 *AcoDREB*s possessed Glu at position-19 (Fig.2). Except for the conserved

amino acids in the specific sites, all of them also contained the conserved YRG and RAYD motif (Fig.2).

We further studied their expression levels and found that the expression of some *AcoDREB* genes were parallel whit other species. According to the result of RNA-Seq and Q-PCR, *AcoDREB19* is highly expressed in anthers (Fig.7, Fig.8), which is consistent with its rice homologous gene (LOC_Os08g27220) (Davidson et al. 2012). *AcoDREB16* and homologous gene (LOC_Os10g22600) are highly expressed in root. It suggests that homologous genes in different species maybe have similar expression patterns.

We subjected pineapple seedlings to different abiotic stress conditions to study the expression changes of eight *AcoDREB* genes. Under different abiotic stress conditions, DREB genes showed the ability to response to stress (Torres et al. 2019). However, these genes show different characteristics in shoot and root. The expression patterns of the eight genes were almost identical and induced in the shoot and root under salt stress (Fig.9a). According to the earlier report, A1 subgroup could play an important role in plant response to salt and drought stress in *Arabidopsis* (Yamaguchi-Shinozaki and Shinozaki 2006). According to our analysis *AcoDREB01* and *AcoDREB03* classified as group I, correspond to A1 subgroup in *Arabidopsis thaliana*, showed induced expression level when subjected to salt and drought stress (Fig.9a). The expression of AcoDREB01 and AcoDREB03 were obviously induced in root under salt stress. Under the drought stress, AcoDREB01 and AcoDREB03 were induced in shoot. It indicated that AcoDREB01 and AcoDREB03 may play important roles in root and shoots respectively under salt and drought stress. In addition, it was found that some genes, belonging to the same evolutionary branch, show similar expression patterns under abiotic stress. *AcoDREB01* and *AcoDREB03* mRNA increasingly accumulated and reached its maximum level after 24 hours of treatment in the pineapple leaves (Fig.9). This expression pattern is similar to that in the root, though its highest point is at 12 hours after treatment. Under mannitol treatment, the expression in the leaves was increased, while the expression in roots was reduced (Fig.9b). At the time of sampling, the expression patterns of the two genes were consistent, indicating that the two genes in the same subgroup maybe have similar functions in response to salt and drought stress. The group IV members, the expression level of *AcoDREB11* and *AcoDREB14*, were increased under both salt and cold stress significantly (Fig.9). which is similar with the A5 group member *GmDREB2* (Chen et al. 2007b), indicating that the function is conserved between pineapple and soybean. As the member of the V subgroup, *AcoDREB18* and *AcoDREB19* have the same response with different abiotic stresses. The results showed that the genes of the same subgroup were functionally conserved (Fig.9). Expression of *AcoDREB06* was inhibited in the leaves and increased in the roots. It shows that by enhancing the expression of *AcoDREB06* in plants will help to increase the resistance of roots to different abiotic stresses. On the contrary, *AcoDREB06* may regulate other pathways to respond to plant stress in the case of decreased expression in the shoot (Fig.9), such as HARDY (AT2g36450) can improve drought and salt tolerance by reducing transpiration in (Abogadallah et al. 2011). The RNA-Seq data analysis results showed that *AcoDREB19* was almost not expressed in roots. In our study, the expression of *AcoDREB19*

increased significantly under abiotic stress induction, indicating that *AcoDREB19* may play a key role in plants response to abiotic stress and can enable plants to cope with a variety of adverse environments. In addition, *DREB* genes are also involved in the process of plants responding to biological stress, which will be great significance.

Conclusions

In conclusion, we accomplished the first genome-wide analysis of the DREB TFs family in pineapple and identified 20 genes encoding *DREB* family transcription factors. Further, we conducted a detailed investigation of DREB transcription factors with respect to their structure, characterization, 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 data provide insights into potential functions of pineapple *DREBs*. The results will provide a useful basis for the further understanding of the structure-function relationship of *DREB* transcription factor family members. Further, this study will help in developing strategies for the future improvement of stress tolerance in pineapple.

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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)	PI	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

Figure 1

Figure 1: Chromosomal locations for pineapple *DREB*.

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.

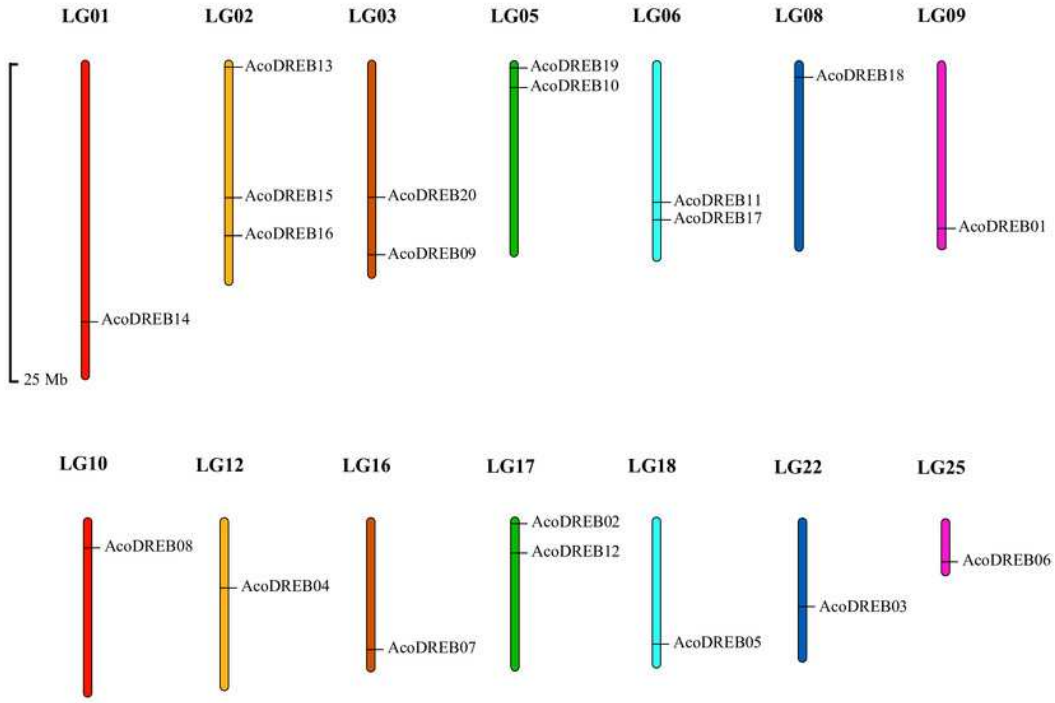

Figure 2

Figure 2: The protein sequence multi-alignment of AP2 domains of *DREB*s from pineapple.

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

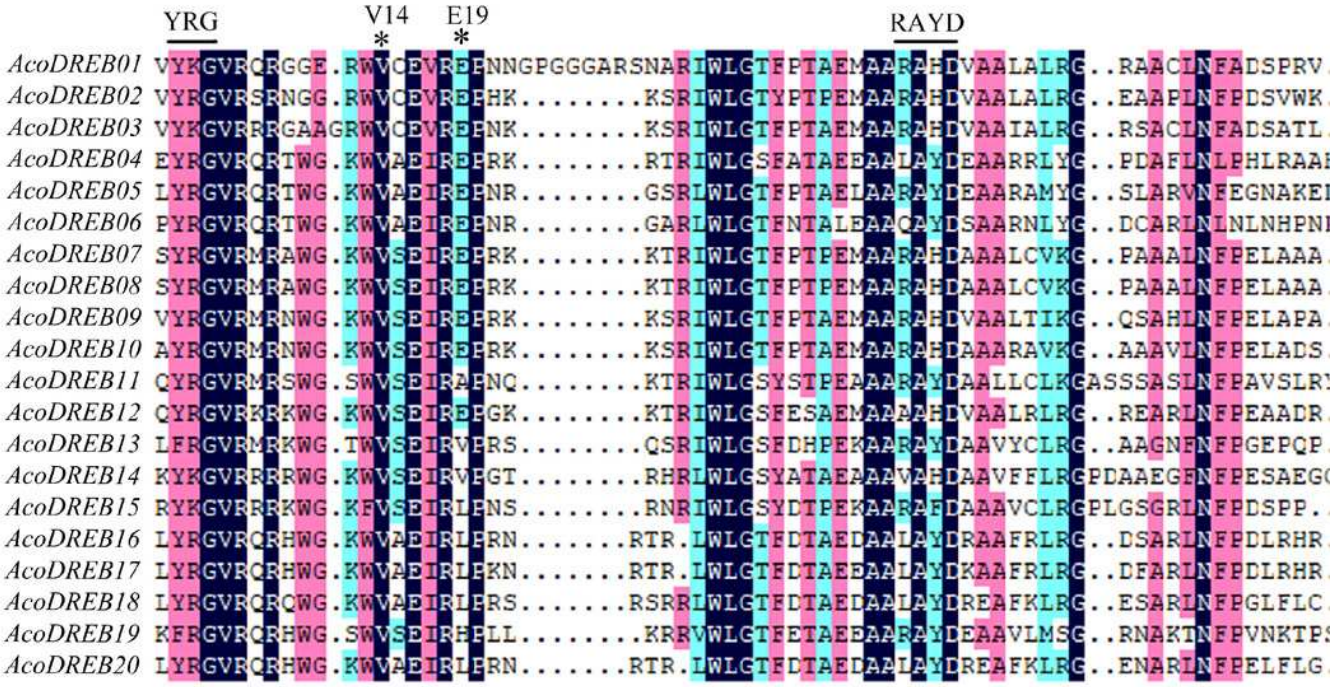

Figure 3

Figure 3: Phylogeny of DREB proteins between 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.

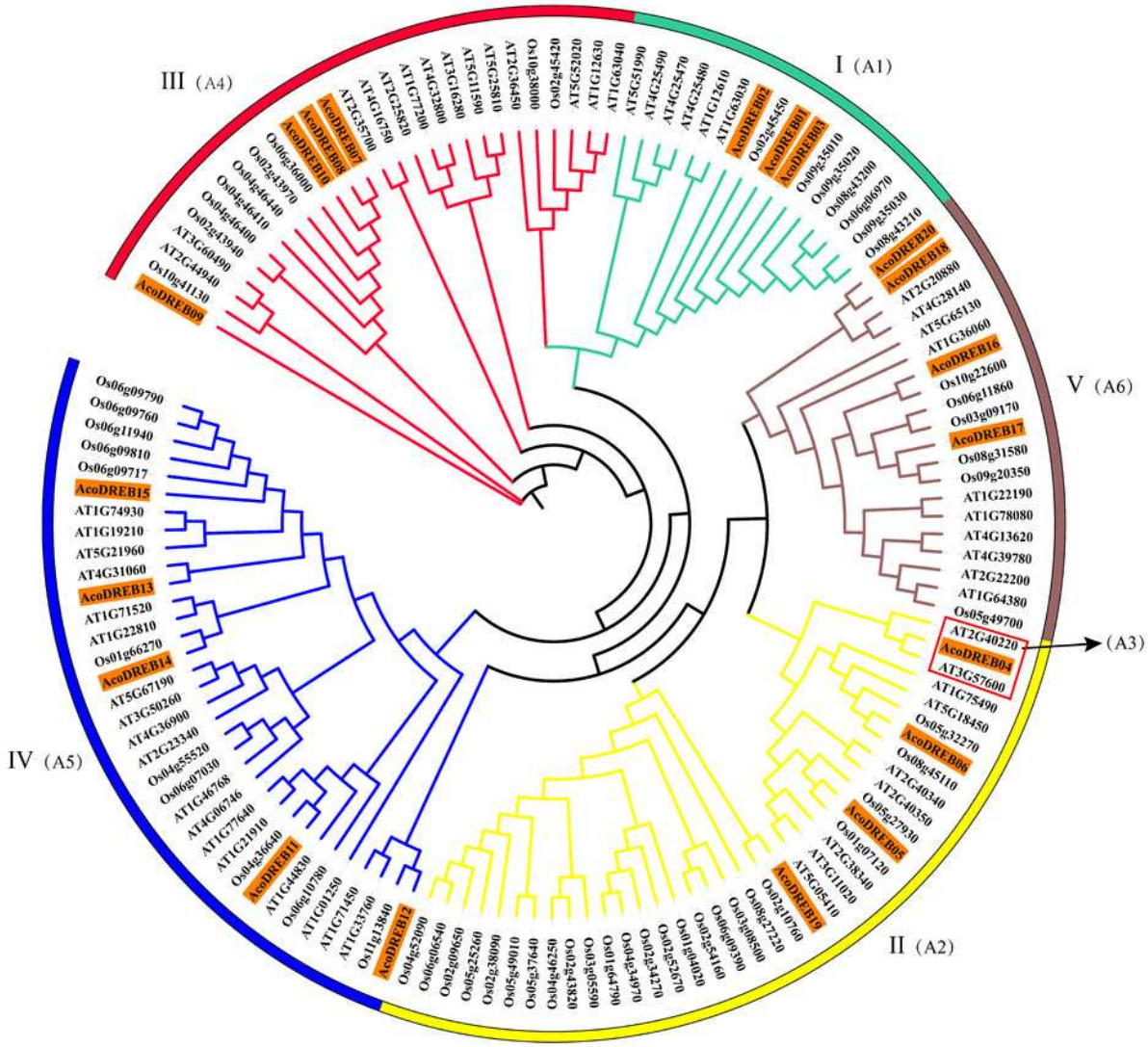

Figure 4

Figure 4: Predicted cis-elements in *AcoDREB* promoters.

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

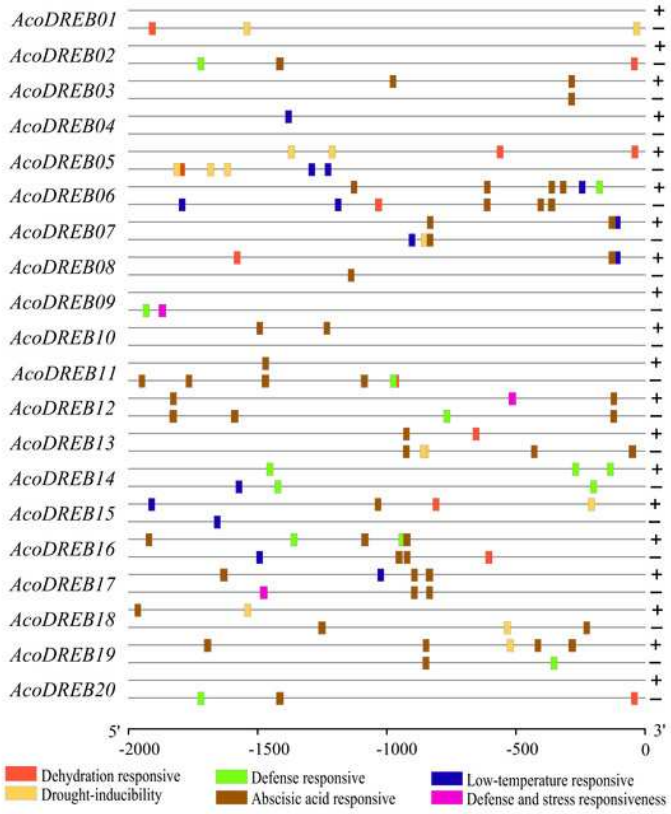

Figure 5

Figure 5 : Exon–intron organization of pineapple DREB genes.

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

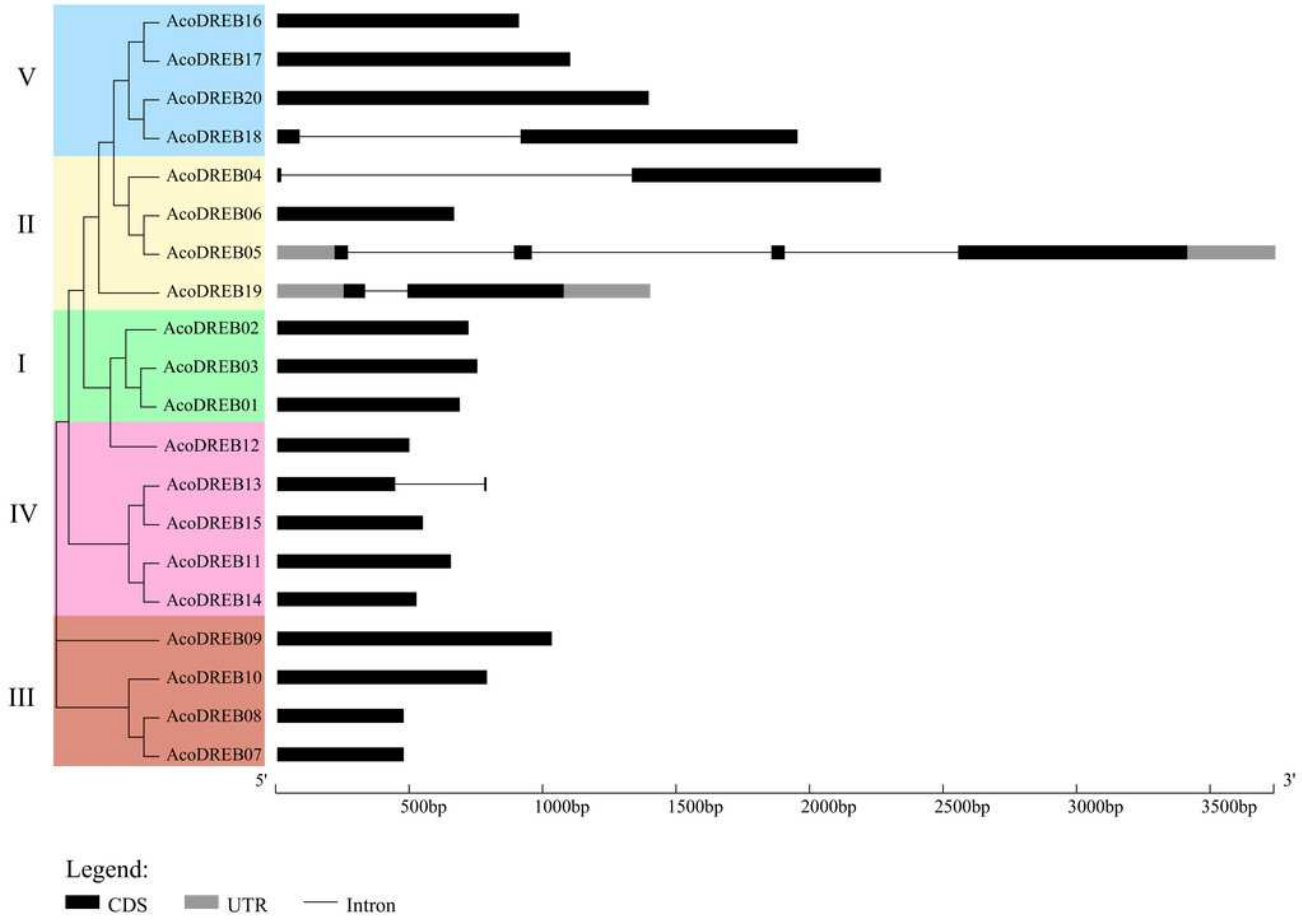

Figure 6

Figure 6: The conserved motifs of *AcoDREB* genes.

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.

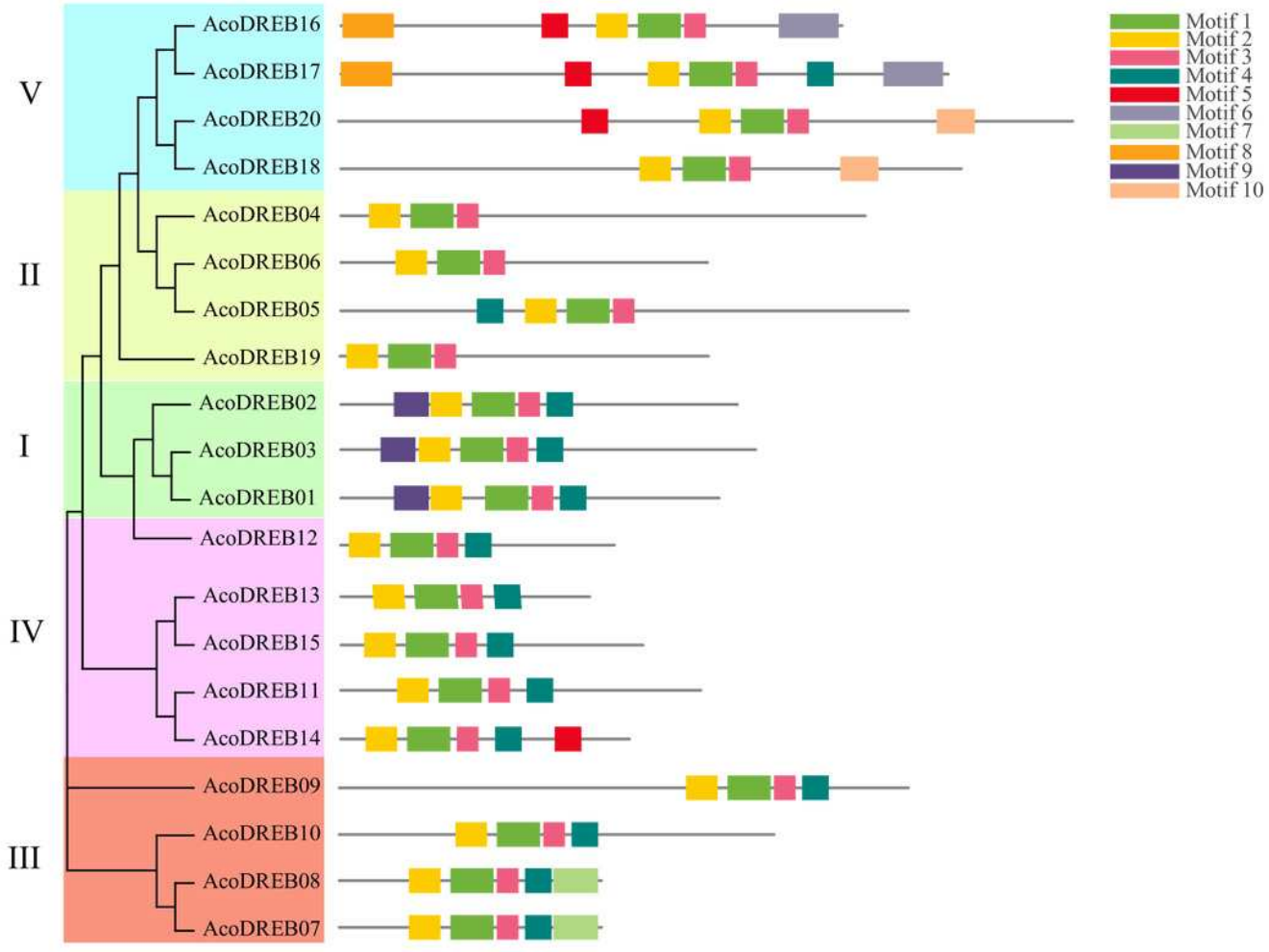

Figure 7

Figure .7: Heat-map of tissue-specific expression profiles of DREB genes in pineapple.

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.

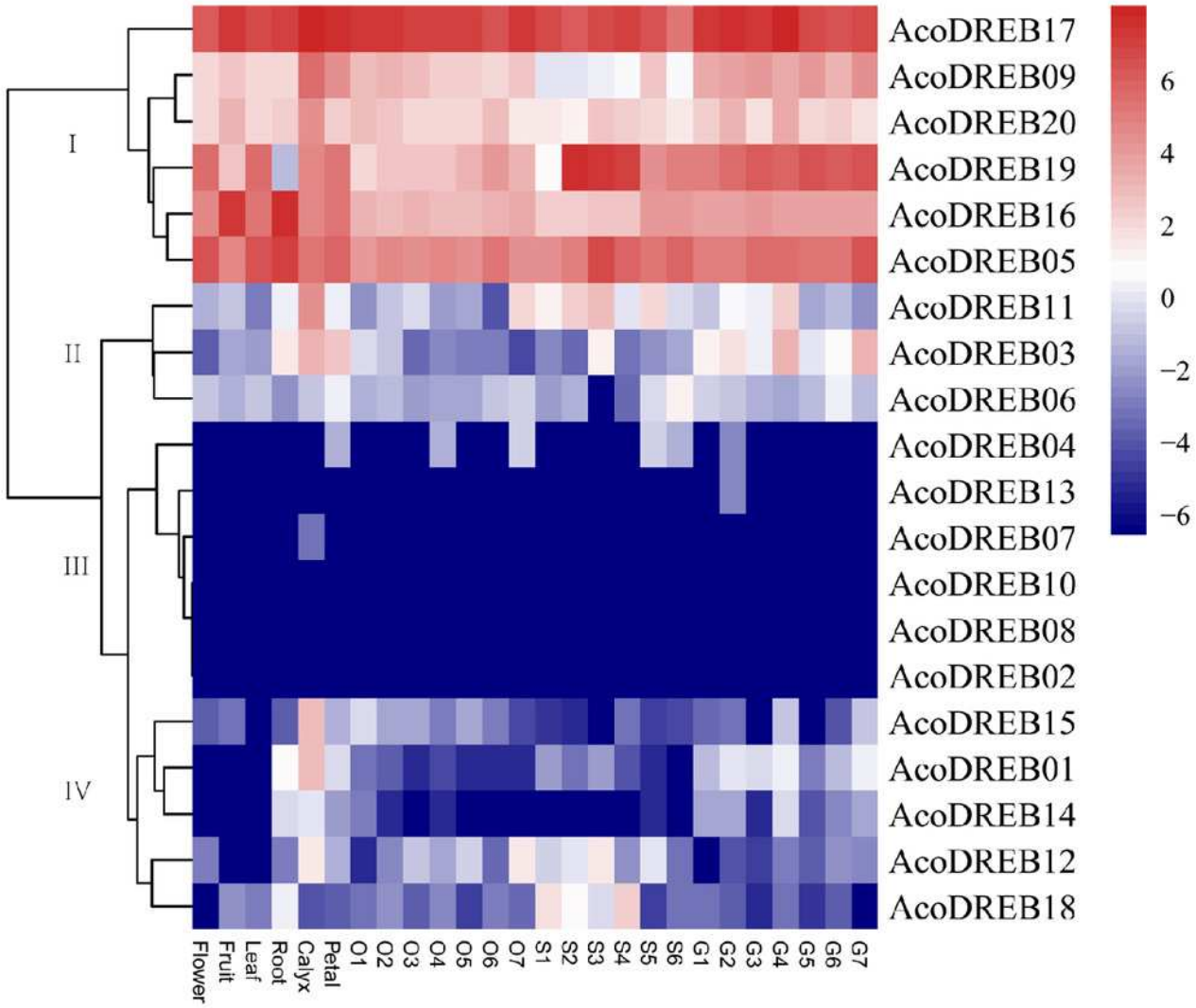

Figure 8

Figure.8: Heat-map for validation of *DREB* genes.

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.

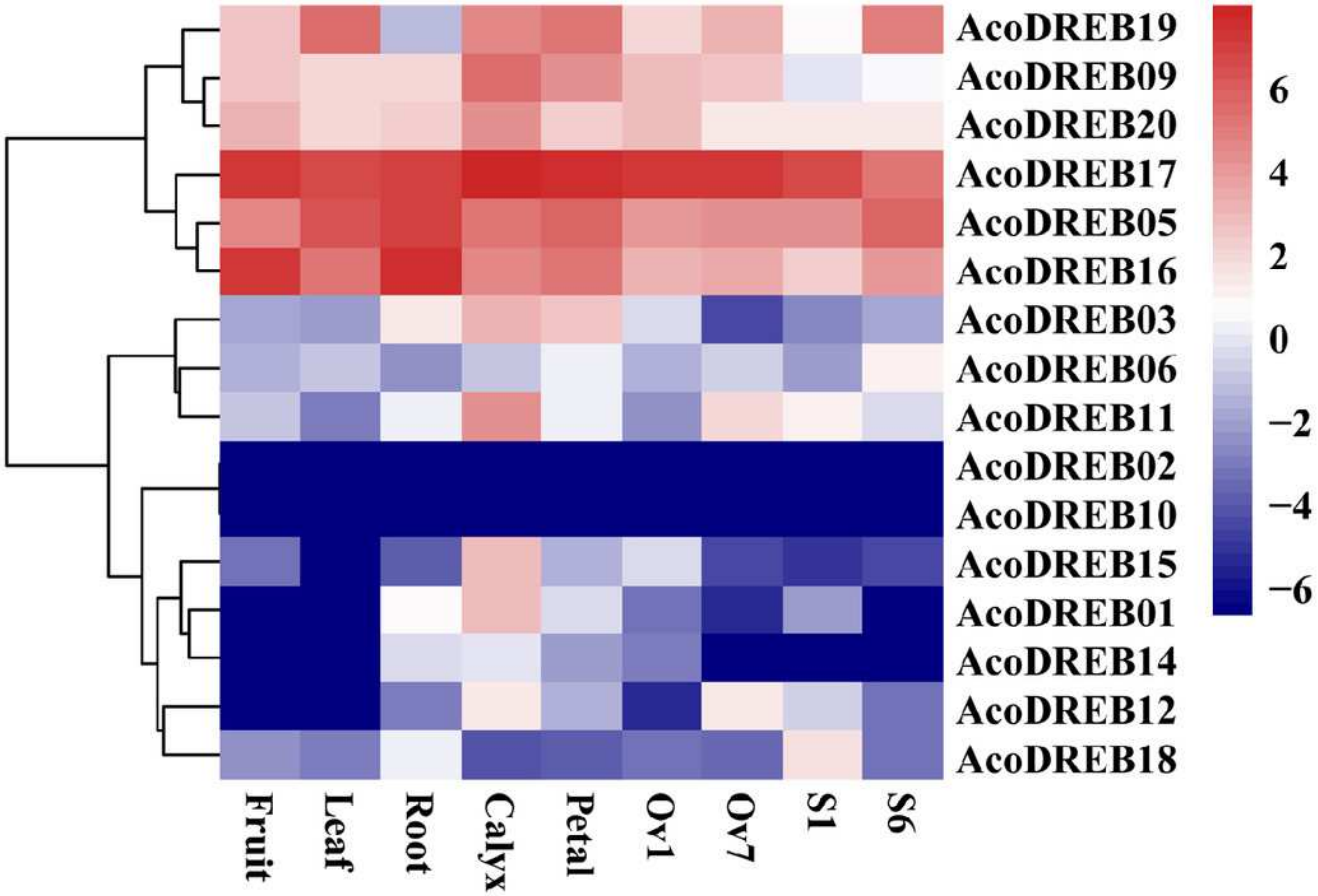

Figure 9

Figure 9: Quantitative real-time PCR of 8 selected *AcoDREB* genes in response to various abiotic stress treatments.

A high salt (150 mM NaCl); B drought (350 Mm Mannitol); C chilling, exposure to 4 ℃; D high temperature, exposure to 45 ℃. Mean expression value was calculated from 3 independent replicates. Vertical bars indicate the standard deviation.