Genome-Wide Study of the *ABI3* Gene Family and Identification of Putative miRNA Targeting *ABI3* Gene in *Oryza Sativa* ssp. Indica

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Research Article

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Abstract

Rice is one of the important cereal crops mainly cultivated in Asia and its productivity is severely affected by drought stress. In response to drought stress, several genes are reported to be up-regulated or down-regulated in plants. Gene expression is negatively regulated by non-coding endogenous microRNAs post-transcriptionally either by mRNA degrading or translational silencing. In the past, single or multiple stress-responsive genes were over-expressed in order to generate drought-tolerant transgenic rice but with very little success. Recently, the development of transgenic plants by over-expressing transcription factors have received much attention because of their ability to regulate several genes. Abscisic Acid Insensitive 3 (ABI3) is a transcription factor, which is known to play a crucial role in mediating plant stress tolerance. Using the Ensembl plants database, we identified 83 putative *OsABI3* genes in *Oryza sativa* Indica. Through *in silico* approach, five potential miRNAs i.e., ath-miR5021, csi-miR3948, osa-miRf11773-akr, osa-miRf12029-akr and ptc-miRf10053-akr that target *OsABI3* genes were identified. Further, the expression of the selected *ABI3* genes were analyzed in rice seedlings exposed to 15% PEG, using the RT-qPCR. In comparison to control, *OsABI3* genes showed relatively enhanced expression when exposed to drought stress treatment. This indicates that *OsABI3* genes may play important role in development and drought stress in rice seedlings.

1 Introduction

Rice is staple food of more than half of the world's population and its productivity as well as quality are severely affected by extreme environmental conditions especially drought stress. The growth and development of rice completely depends on the availability of water in the field during the whole duration of its growth (Waziri et al. 2016). Therefore, sustainable water is required during rice cultivation. Other environmental stresses like, salinity, low temperature, high Cd concentration in soil, excessive application of phosphate fertilizers also greatly affect the production of rice (Sun et al. 2017; Purty et al. 2017).

In order to overcome these problems and ensure growth of rice crop in areas susceptible to these stresses, genetic manipulation of several stress-responsive genes have been used in the past to generate stress tolerant crop species. Recently, several transcription factors such as DREB, LEC1, LEC2, FUSCA 3, PHD finger, WRKY has been reported to play a vital role in stress signal transduction (To et al. 2006; Waziri et al. 2020). One such transcription factor is ABI3 which has been reported in many plants and play crucial role in mediating plant stress tolerance responses. ABI3 is conserved in higher plant species as well as non-angiosperms.

ABI3 is a transcription factor which is the part of protein family which contains B3 domain. There are 4 conserved domains in it, which include three basic and one acidic domain. An acidic A1 and the other three basic B1, B2 and B3 (Bedi et al. 2016). A1 domain is primarily considered to be involved in activation process. The basic conserved domains B1 and B2 possess sequences responsible for nuclear localization, with B2 interacts primarily with transcription factors. The most important transcription factor is ABI5, which has conserved bZIP domain. The second basic conserved domain B2 has been known to cause trans-activation by binding to the conserved G-box element (CACGTG) or to ABREs (Ezcurra et al. 2000; Marella and Quatrano 2007). The most conserved domain which is characteristic to ABI3 protein is B3. Domain B3 along with B2 or alone has been found to interact with many conserved cis-elements that are present in the promoter region of identified seed specific genes (Giraudat et al. 1992; Suzuki et al. 1997).

It has also been shown that in seeds, there are different splice forms of ABI3, formed during their different developmental stages by undergoing alternate splicing. Moreover, its expression is strictly regulated by group of chromatin modifying protein. These chromatin modifying proteins are primarily Chromodomain Helicase DNA Binding Protein 3 (CHD3) like. The regulation is done during the seed germination process, in response to water stress (Perruc et al. 2007). Osmotic and desiccation stress, caused by relative water availability, is an integral part of seed development process and hence involve highly complicated stress tolerance pathways to ensure seed maturation. ABI3, along with bZIP containing ABI5 and other transcription factors specifically expressed in seeds, play major role in expression of genes that are responsible for osmotolerance proteins in seeds.

In plants, many studies have revealed that microRNAs (miRNAs) play a vital post-transcriptional regulatory role in gene expression by targeting mRNA cleavage or translational inhibition (Lee et al. 2004; Singh et al. 2020). Plant miRNAs are

reported to possess important functions in several metabolic and biological pathways such as tissue development and differentiation, biotic and abiotic stress responses, phytohormones signaling, and secondary metabolite production (Khraiwesh et al. 2012; Shriram et al. 2016). Nonetheless, the evolutionary highly conserved nature of an extensive number of miRNAs simplified the process of characterization of novel miRNA orthologs in new plant species through homologs identification (Dezulian et al. 2006).

Knowing the importance of miRNA and their roles in gene regulation, in the present investigation, experiments were designed for genome wide analysis of *ABI3* gene family and *in silico* identification of miRNAs and their potential *ABI3* gene targets in rice through computational approach. Further, we studied the expression of selected *ABI3* genes in rice to elucidate its role under drought stress. Our research will be beneficial in generation of rice varieties tolerant to drought stress and can further ensure the growth of rice crop under water stressed environment as the rising water crisis is hampering the rice crop production in our country.

2 Material And Methods

Identification and domain analysis of ABI3 family genes in rice

To identify the candidate ABI3 family genes in the rice genome, we used the three different keywords namely: “Abscisic Acid Insensitive 3”, “ABI3” and Pfam ID “PF02362” to search against the rice genome database using the Ensembl Plant database (http://plants.ensembl.org/Oryza_indica/Info/Index). The amino acid sequences retrieved were used for domain analysis using “Pfam Database” (https://pfam.xfam.org/). The multiple sequence alignment was conducted for all the ABI3 proteins containing B3 domain by using MEGA X program with default parameters (Kumar et al. 2018). The aligned ABI3 proteins sequences were used for construction of phylogenetic trees using Neighbor Joining (NJ) algorithm.

Chromosome mapping of ABI3 family genes

Distribution of ABI3 family genes on the chromosomes was studied using “maptool@oryza” tool (http://viewer.shigen.info/oryzavw/maptool/MapTool.do). The distribution of all ABI3 family genes with conserved B3 domain with their relative positioning was hence deciphered.

Identification of potential miRNAs and their target ABI3 genes

Workflow of the identification and characterization of potential miRNAs, and their target genes is depicted in Fig. 1. A total of 10898 mature miRNA sequences were retrieved from PMRD: Plant micro RNA Database (http://bioinformatics.cau.edu.cn/PMRD/) (Zhang et al. 2010). With identity value 90, CD-HIT-v4.5.4 was used to remove the redundancy in miRNA sequences (Fu et al. 2012). In order to identify miRNA-targeted ABI3 genes of Indica rice, local BLAST was performed using Blast2GO version 5.2 (Conesa et al. 2005). BLASTx analysis (E-value ≤ 1e-10) was performed to remove protein-coding sequences from precursor sequences.

Prediction of the secondary structure of pre-miRNAs

Prediction of the secondary structure was done by using the software MFOLD 3.1 (http://www.bioinfo.rpi.edu/applications/mfold/rna/form1.cgi) (Zuker 2003). The following criteria were used for screening the candidates of potential miRNAs: minimum length of the pre-miRNA to be 60 nt; pre-miRNA should be folded into appropriate stem-loop hairpin secondary structure; mature miRNA sequence should be placed in one arm of the hairpin structure; not > 6 nt mismatches in miRNA/miRNA duplex; No loops or breaks between the miRNA/miRNA duplex; A + U content within 30–70%; Predicted secondary structure should have higher minimal folding free energy index (MFEI) and negative minimal folding free energy (MFE) values (Meyers et al. 2008). The MFE or ΔG (-kcal/mol) values generated from the MFOLD web server of the stem-loop structures were used for calculating the MFE index values using the following formula:

$$MFEI = \frac{(\mathrm{MFE} / \mathrm{Lengthofprecursormi RNAsequence}) \times 100}{(\mathrm{G} + \mathrm{Ccontent})\%}$$

Plant materials and stress treatments

Seeds of *Orzya sativa* Pusa Basmati 1 (PB1) were pre-treated with antifungal agent and surface sterilized with 0.1% $HgCl_2$ prior to germination in hydroponic system for 48h in dark. For further growth, seedlings were geminated under control condition i.e., (25 ± 2°C, 12:12 h light:dark cycle) for 13 days. For drought stress treatment, 15 days old seedlings were treated with 15% PEG for 24 hours. Simultaneously, seedlings maintained in de-ionized water were taken as control. After stress treatment, seedlings were harvested in liquid nitrogen and stored at – 80°C till further use.

RNA isolation and qRT-PCR analysis

Total RNA was isolated from treated as well as untreated seedlings using TRIzol reagent (Invitrogen, USA). Around 2µg total RNA was used for first strand cDNA synthesis by following the manufacturer's protocol (Thermo Scientific, EU). To check the quality of cDNA preparation PCR was done with housekeeping gene actin. The cDNA synthesized were later used as template for expression studies using qRT-PCR. Gene specific primers of seven ABI3 genes (BGIOSGA002753, BGIOSGA002748, BGIOSGA020315, BGIOSGA022468, BGIOSGA012100, BGIOSGA006943, BGIOSGA013136) were designed manually using exon-exon junction region to amplify 200–250 base-pairs PCR products and their specificity were examined through Primer Blast of NCBI. The rice actin 1 gene (LOC_Os03g50885) was used as an internal control to normalize the gene expression level. The qRT-PCR was performed on an AriaMx-Real-Time PCR system (Agilent technologies, USA). The total reaction volume was 10 µl which contained 5µl of 2X KAPA SYBR FAST qPCR Master Mix Universal, 200nM gene specific primers and 0.5µl of cDNA in a total volume of 10µl. The thermal cycle reaction conditions were 95˚C for 3min, followed by 40 cycles of 95˚C at 10 sec and then 57˚C for 30 sec. A melting curve was generated at the end of 40 cycles for analyzing the specificity of each gene. The experiment was conducted with 2 independent biological replicate and three technical replicate for each sample. The relative gene expression of individual gene was calculated via 2^⁻ΔΔCT method (Livak and Schmittgen 2001).

3 Results

Identification and phylogenetic analysis of ABI3 family genes in rice

In the present investigation, using "Abscisic acid insensitive 3", "ABI3", and Pfam ID "PF02362" as keywords, we identified 2, 6 and 83 *ABI3* genes in rice from Ensemble Plant database. Domain analysis was carried out for these 91 ABI3 family genes and the ones not containing complete B3 domain were excluded. Eight genes were eliminated and remaining 83 containing B3 domains were used for further analysis (Supplementary Table 1). As the characteristic domain present in ABI3 family genes is primarily B3 and hence its presence is necessary for its expression in plants. The eliminated genes contained other functional domains but B3 domain was absent, therefore they were excluded for further analysis and only genes showing presence of complete B3 domain were selected for conducting expression studies in rice. Phylogenetic tree analysis revealed that OsABI3 proteins can be divided into eight major groups (Group I to VIII). Group I formed the largest clade with 21 members followed by group III which has 15 members (Fig. 2).

Chromosome mapping of ABI3 family genes

Chromosomal distribution of ABI3 genes was carried out and the results showed wide scattering of the genes throughout the genome of rice plants. The genes are distributed on the 12 chromosomes with maximum probable density on chromosome 1, 3, 4 and 12 and are not randomly equally distributed on all chromosomes (Fig. 3). Genes present on chromosme 1, 3, 4 and 12 are 21, 18, 13 and 11 respectively and other chromosomes showed lesser numbers of genes. Very high probable gene density is indicative of regions conserved for the gene presence in the rice genome during the course of evolution.

Identification of potential miRNAs and their target ABI3 genes

For identification of potential rice miRNA, a total of 10898 plant miRNAs were retrieved from PMRD (Plant micro RNA database)., CD-HIT-v4.5.4 With identity value of 90, was used to remove the redundancy in miRNA sequences. After removing redundant sequences, a set of 5025 miRNA sequences (reference set of miRNA sequences) were analysed for sequence similarity (Local BLAST by using Blast2GO-v5.2) with the *OsABI3* genes assembly (Fig. 1). BLASTx analysis (E-value ≤ 1e-10) showed that out of 6 miRNA identified, sequences of only 5 miRNA i.e., ath-miR5021, csi-miR3948, osa-miRf11773-akr, osa-miRf12029-akr, and ptc-miRf10053-akr were found to be non-coding, while sequences of 1 miRNA i.e., ath-miRf10989-akr was coding for protein, which was not used for further analysis. Further, in order to identify miRNA-targeted *OsABI3* genes, local BLAST was performed using Blast2GO. The potential *ABI3* specific targets of ath-miR5021 was BGIOSGA028020, csi-miR3948 was BGIOSGA027821, osa-miRf11773-akr was BGIOSGA037707, osa-miRf12029-akr was BGIOSGA011738 and ptc-miRf10053-akr was BGIOSGA014119, respectively (Table 1).

Table 1
Identified potential miRNAs and their target ABI3 specific genes in Indica rice.

S. No.	miRNAs	ABI3 specific target genes
1	ath-miR5021	BGIOSGA028020
2	csi-miR3948	BGIOSGA027821
3	osa-miRf11773-akr	BGIOSGA037707
4	osa-miRf12029-akr	BGIOSGA011738
5	ptc-miRf10053-akr	BGIOSGA014119

Prediction of the secondary structure of potential miRNAs

The five non-coding miRNA sequences i.e., ath-miR5021, csi-miR3948, osa-miRf11773-akr, osa-miRf12029-akr, and ptc-miRf10053-akr, were further used for secondary structure analysis including hairpin stem-loop structure using MFOLD version 3.1 (Fig. 4A-E). The putative miRNAs obtained varied in their lengths ranging from 20 to 24 nucleotides. The negative MFE (-ΔG) of the miRNA precursors were also calculated to study the stability of the hairpin stem-loop structure (Table 2). In comparison to the length of miRNAs, the length of putative precursor miRNAs of rice also varied, ranging from 80 to 171 nucleotides. The stability of the secondary hairpin structure of pre-miRNA was determined by MFE (-ΔG). The distribution of G, C, A, and U nucleotides in the pre-miRNA were found to be different, where it ranged from 5-32.02% for A, 18.6-41.25% for U, 22.22–41.86% for G and 7.19–27.9% for C, respectively (Table 3).

Table 2
Determination of minimal free folding energy (MFE) of the identified potential miRNA from Indica rice.

miRNAs	Mature miRNA sequence	ST	Loc	LP	LM	NM	(G + C) %	MFE (ΔG)	AMFE	MFEI
ath-miR5021	UGAGAAGAAGAAGAAGAAAA	+	5′	171	20	1	36.257	42.7	24.971	0.689
csi-miR3948	UGGAGUGGGAGUGGGAGUAGGGUG	+	5′	153	24	1	29.412	27	17.647	0.600
osa-miRf11773-akr	GCCAUUGCCAUGGCCAUGGCCUCG	+	3′	83	24	1	60.241	36.9	44.458	0.738
osa-miRf12029-akr	AUGUUGGCCGCCGCCGCUGCCAU	+	5′	86	23	1	69.767	41.7	48.488	0.695
ptc-miRf10053-akr	CUGUAGUAGUUGCUGCUGCUGC	+	3′	80	22	1	53.750	29.8	37.250	0.693

Table 3
The distribution of A, U, G, and C in the identified pre-miRNAs of Indica rice.

miRNAs	miRNA Family	A%	U%	G%	C%	A/U ratio	G/C ratio	(A + U) %
ath-miR5021	miR5021	26.316	37.427	26.901	9.357	0.703	2.875	63.743
csi-miR3948	miR3948	32.026	38.562	22.222	7.190	0.831	3.091	70.588
osa-miRf11773-akr	miRf11773-akr	16.867	22.892	32.530	27.711	0.737	1.174	39.759
osa-miRf12029-akr	miRf12029-akr	11.628	18.605	41.860	27.907	0.625	1.500	30.233
ptc-miRf10053-akr	miRf10053-akr	5	41.250	33.750	20	0.121	1.688	46.250

Morphological response rice seedlings to drought stress treatments

Rice seedlings subjected to 15% PEG showed chlorosis (loss of chlorophyll) of the shoot region, curling of shoot regions, necrosis of shoot tissue, necrosis of root tissue and stunted growth of both shoot and root (Fig. 5).

Quantitative RT- PCR analysis of selected OsABI3s **genes expression in response to drought stress**

The response of selected *OsABI3* genes under drought stress was studied using RT-qPCR. Expression analysis showed up-regulation of all the seven selected *ABI3* genes under drought stress treatment (Fig. 6). Genes with accession number BGIOSGA020315, BGIOSGA006943 and BGIOSGA013136 showed significant up-regulation in their expression. Under drought stress treatment BGIOSGA013136 showed more than 7-fold increase in the expression. There was not much change in the expression of remaining *ABI3* genes in response to drought stress treatment

4 Discussion

Plant responses to abiotic stresses are highly complex and involve expression of a large number of genes encoding stress related proteins and enzymes working in biosynthetic pathways of osmoprotectants and other stress-related metabolites. In the quest to find genetic factors working in concert with abiotic stress-signaling pathways, various transcription factors have been identified. The studies conducted earlier have shown that transcription factor ABI3 proteins play very important roles in growth and development of plants. However, the regulatory roles of ABI3 family genes in mediating plant stress responses in rice varieties have rarely been studied. Hence, in the present investigation, we performed genome wide analysis to identify rice *ABI3* gene family as well as studied its expression in response to drought stress. B3 domain is crucial for the activity of ABI3 protein, hence B3 domain containing sequences were selected for the further analysis as. Previously, presence of B3 domain in ABI3 protein were also reported in several plant species like Arabidopsis, maize, tomato and non-angiosperms like *Physcomitrella* (Finkelstein and Rock 2002; Bassel et al. 2006; Khandelwal et al. 2010). Transcription factor ABI3 protein was reported to be evolutionary divergent. In the present study, the phylogenetic tree showed that the rice ABI3 family showed divergent evolution as the phylogenetic tree analysis showed 8 major groups. The rice ABI3 family genes showed marked diversity in protein sequence length, from 400 amino acid to 9400 amino acid. Investigation of functional domains and conserved motifs further showed ABI3 family genes show large diversity in their sequences and along with this certain specific domains and motifs are characteristic only to some of the ABI3 family genes. These results are in accordance with previous results that suggested diversity of rice ABI3 family genes. The diversity in structures of rice ABI3 family genes might contribute to the specific function of these genes.

In addition to regulating plant growth and maturation during different stages, ABI3 was demonstrated to regulate stress responses. However, the expression of *ABI3* genes can be controlled by small RNAs, including microRNAs (miRNAs) and endogenous small-interfering RNAs (endo-siRNAs) (Axtell et al. 2007). It has been shown that miRNAs play important roles in gene regulation in response to abiotic stress treatment in plants. In the present study, we identified the potential five potential miRNAs i.e., ath-miR5021, csi-miR3948, osa-miRf11773-akr, osa-miRf12029-akr and ptc-miRf10053-akr that target *OsABI3* genes. Expression analysis showed the differential responses of selected *ABI3* genes when exposed to drought stresses.

Expression of three *OsABI3* genes i.e., BGIOSGA020315, BGIOSGA006943 and BGIOSGA013136 was up-regulated in contrast with that shown by seedling maintained under controlled conditions. This difference indicates that *ABI3* genes respond to drought stress conditions in rice seedlings. The role of *ABI3* has been postulated to maintain the expression of ABA-inducible genes in recovery stages during rehydration (Khandelwal et al. 2010). In *P. patens*, deletion mutants of *ABI3* are not able to recover from desiccation despite being treated with ABA, indicating that *ABI3* is required for *P. patens* vegetative tissues to survive desiccation (Xia et al. 2016).

5 Conclusion

ABI3 family of transcription factors play very crucial role in many physiological pathways for eg. the interaction of plant hormones like auxin- ABA in mediating several morphological as well as physiological systems and regulating seed dormancy. In addition, ABI3 is reported to plays important role in mediating drought stress responses. In the present study, we identified total of 83 *OsABI3* genes and phylogenetic analysis showed the divergent evolution, and expression of selected genes indicated it response to drought stress treatment. Despite the known gene regulatory activities of miRNAs triggered by abiotic stress, most of their functions have yet to be analyzed. Therefore, it will be interesting to further elucidate the function of five miRNAs targeting *OsABI3* gene identified in the present study.

Declarations

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Author Contributions: RSP designed the research project. AV and DKS performed computational work and data analysis. RSP, SC and NC contributed in paper preparation. All the authors have read and agreed to publish the version of the manuscript.

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Declaration of Competing Interest The authors declare that there are no conflicts of interest.

Consent to Participate (Ethics) The authors declare their consent to participate in the work submitted.

Consent to Publish (Ethics) The authors declare their consent to publish the work submitted.

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Figures

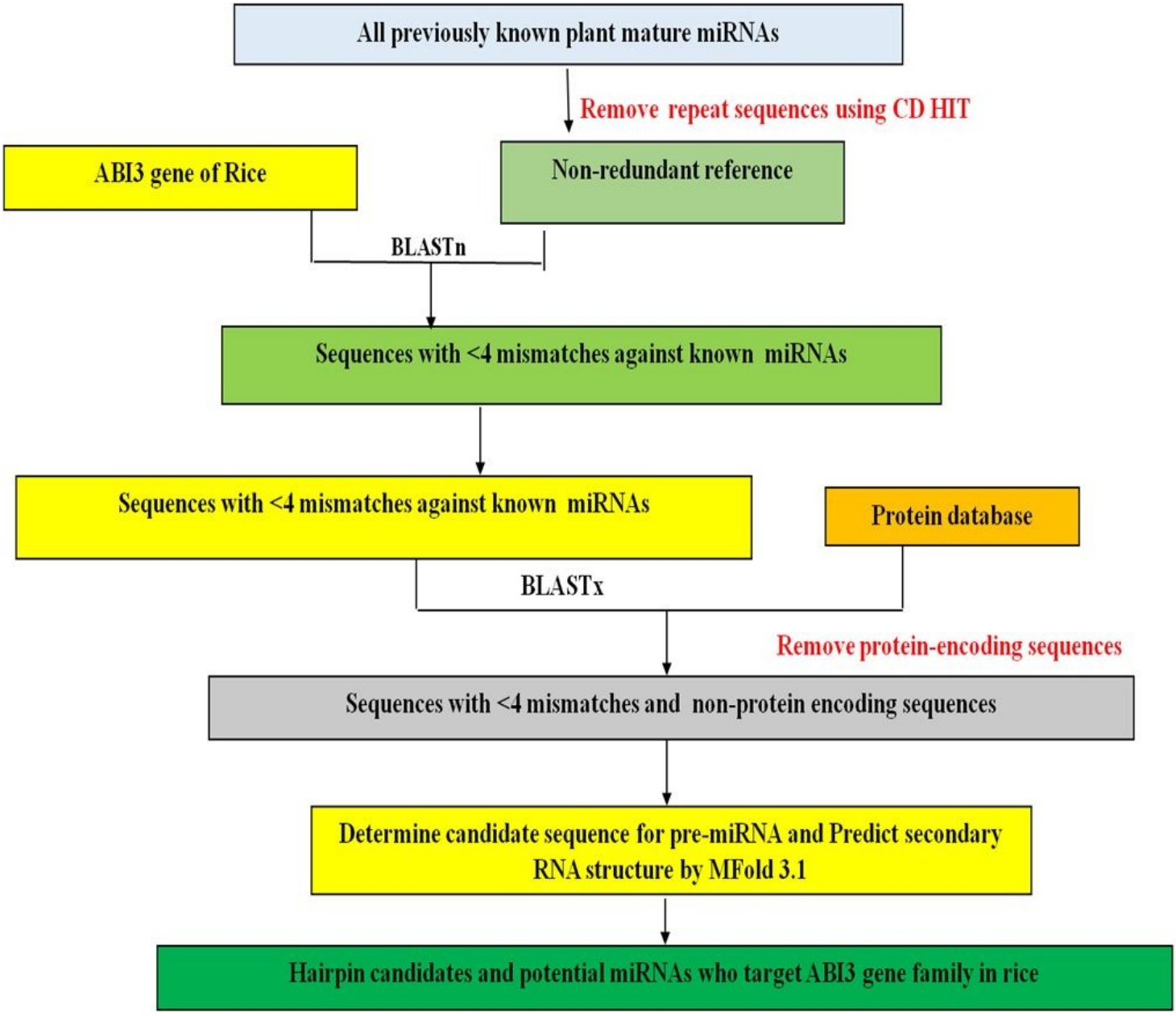

Figure 1

Phylogenetic tree constructed using MEGA X program. Amino acid sequences of all the 83 OsABI3 proteins were used for tree construction.

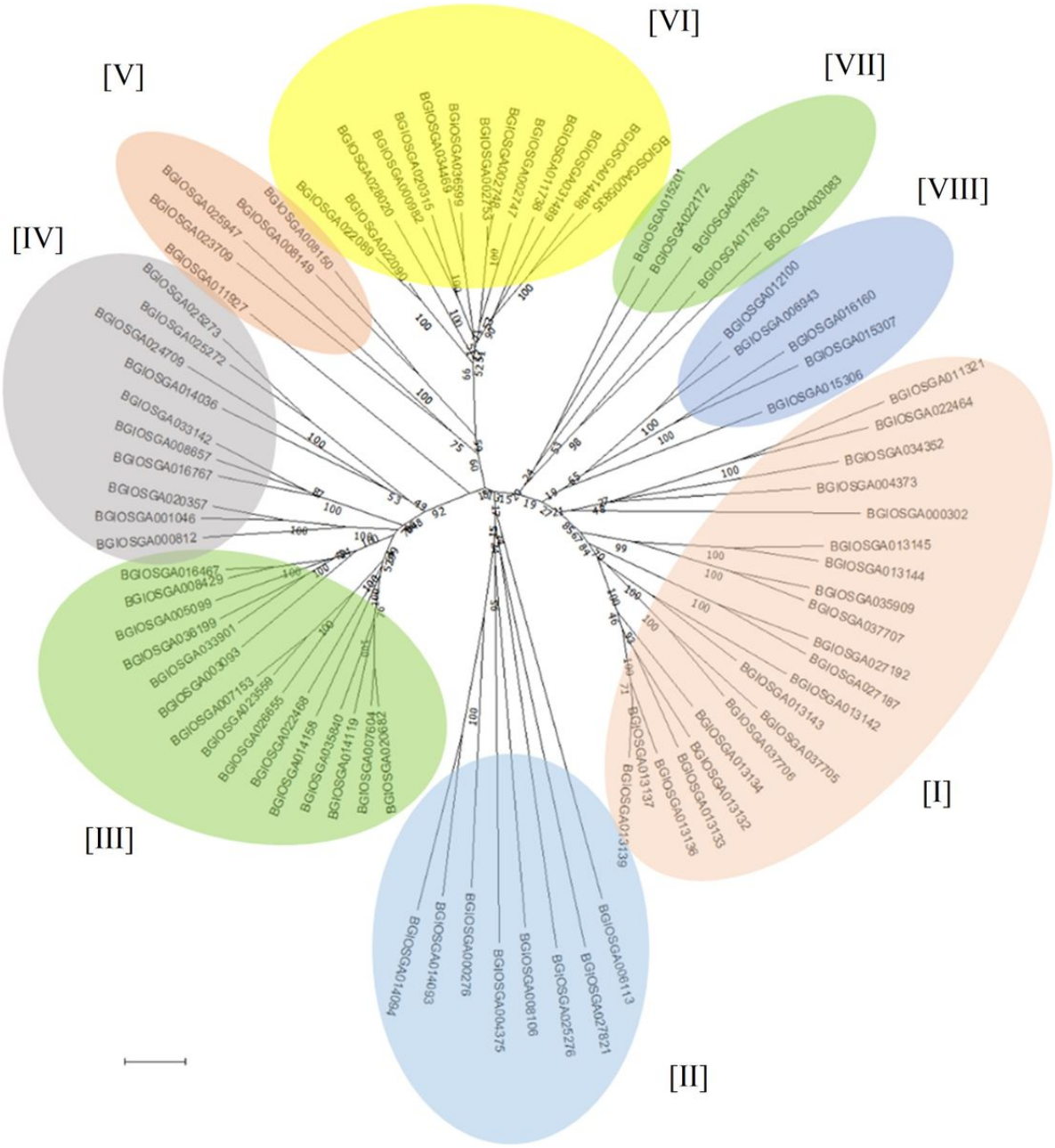

Figure 2

Distribution of ABI3 genes on different chromosomes of rice genome.

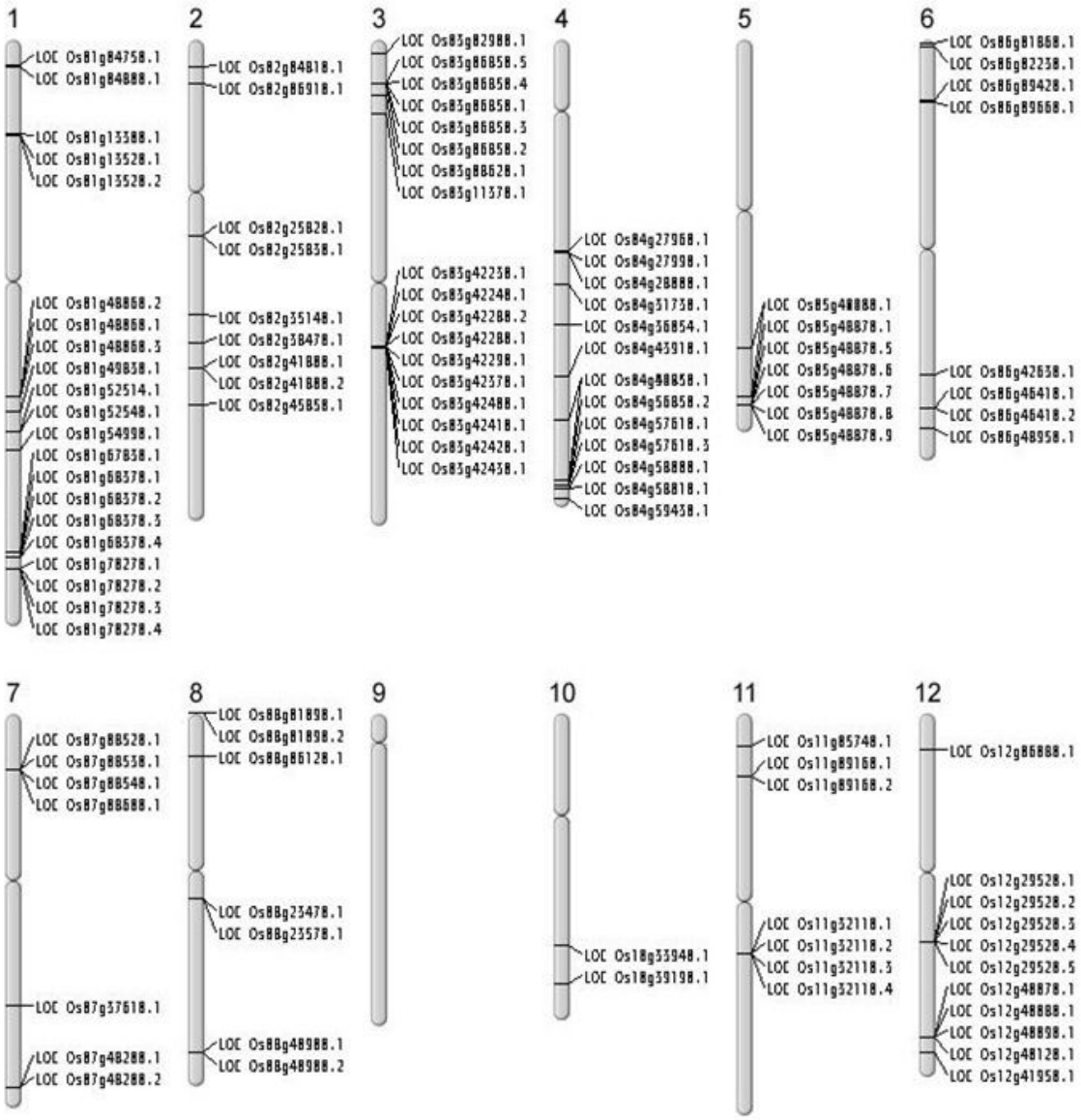

Figure 3

Workflow of the identification and characterization of potential miRNAs and their target ABI3 genes in Oryza sativa Indica Group.

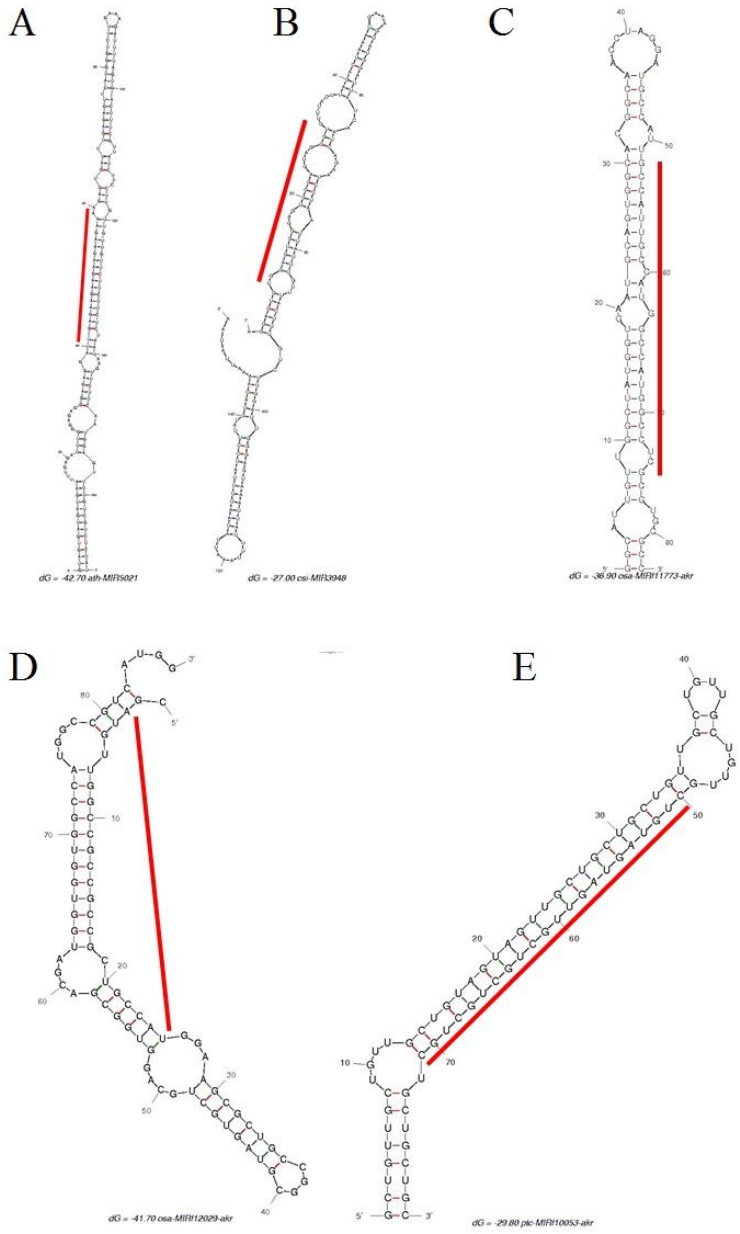

Figure 4

Mature and precursor sequences and the predicted stem-loop structures of identified miRNAs in Oryza sativa Indica Group- (A) ath-miR5021, (B) csi-miR3948, (C) osa-miRf11773-akr, (D) osa-miRf12029-akr and (E) ptc-miRf10053-akr. The mature miRNAs are indicated with bold red line.

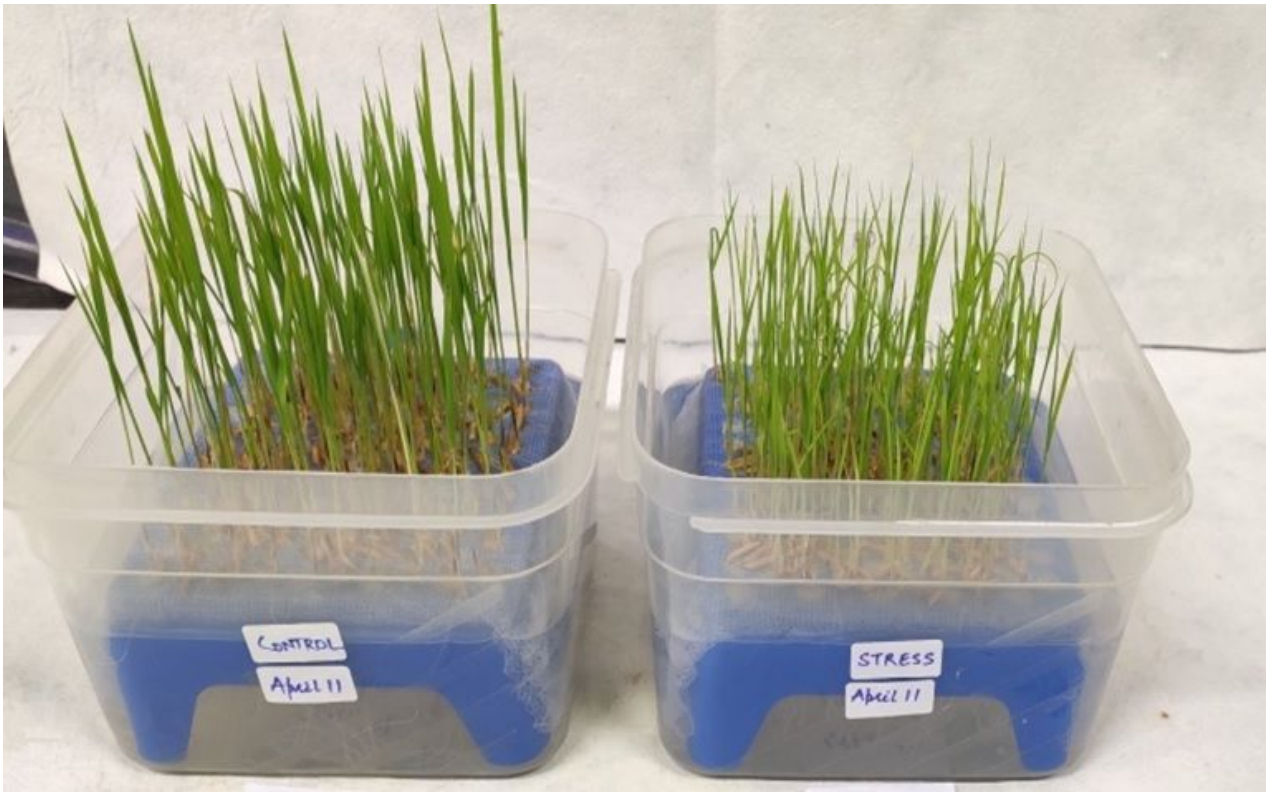

Figure 5

Morphological response of 15 days old rice seedlings to drought stress.

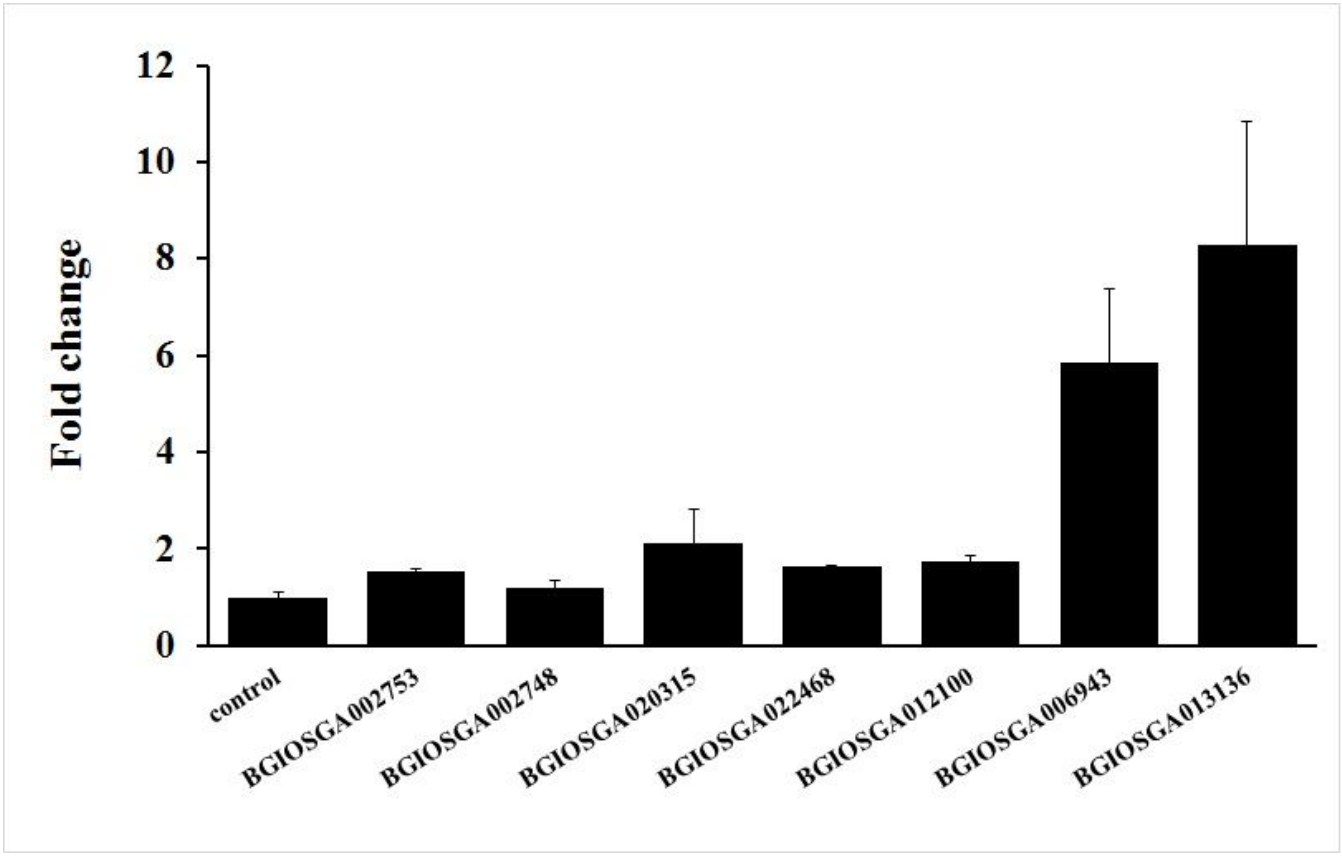

Figure 6

Loading [MathJax]/jax/output/CommonHTML/jax.js s under drought stress.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryTable1.docx