

Genome-Wide Analysis of the *MADS-Box* Gene Family in Foxtail Millet (*Setaria Italica* L.) and Expression Analyses to Reveal Their Role During Drought Stress

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Abstract

MADS-box gene family is a key regulatory factor family, which controls vegetative growth, reproductive development and can be used to mediate abiotic stresses in many plants. However, Knowledge of this gene family is still limited in *Setaria italica*. In the present study, a total of 70 *SitMADS* genes were identified and renamed on the basis of the chromosomal distribution of the *SitMADS* genes. According to gene structure, conserved motif and phylogenetic feature, the 70 *SitMADS*s were classified into type-I (M α , M β , M γ) and type-II (MIKC^C and MIKC^{*}). All of the *SitMADS* genes were randomly distributed on nine chromosomes, and five tandem duplicated genes and 12 pairs of duplicated gene segments were detected in the *SitMADS* genes family. Synteny analysis provided a high homology between *SitMADS* genes and *OsMADS* genes. A *cis*-element analysis inferred that *SitMADS* genes, except for *SitMADS23*, possessed at least one drought stress response and ABA(Absciscic Acid)-induced response *cis*-element. Real-time quantitative PCR analysis was used to detect the expression patterns of *SitMADS* genes in various tissues and demonstrated that the genes responded drought stress and ABA treatments. *SitMADS23*, *SitMADS42*, *SitMADS51*, *SitMADS52*, *SitMADS58* and *SitMADS64* were highly expressed in PEG(Polyethylene glycol) and drought stress, which suggested its important role in drought stress response. *SitMADS51*, *SitMADS63* and *SitMADS64* seemed to be responsive to ABA hormone signaling, suggesting that they were involved in the ABA signaling pathways. This paper provided a deep insight into the evolutionary characteristics of *SitMADS* genes. The results provide comprehensive information for further analyses of the molecular functions of the *MADS-box* gene family in *Setaria italica*.

Introduction

The *MADS* transcription factor is one of the most widely studied transcription factors in eukaryotes, including fungi, animals and plants. *MADS* is derived from the acronym of *MCM1* in *Saccharomyces cerevisiae*, the homologous gene *AGAMOUS*(AG) in *Arabidopsis thaliana*, the homologous gene *DEFICIENS* in *Antirrhinum majus* and serum response factor *SRF* in *homo sapiens*(Honma and Goto 2001; Norman et al. 1988; Sommer et al. 1990; Yanofsky et al. 1990).

Previous researches have illustrated that the *MADS-box* genes have been divided into two different lineages (type-I and type-II). *MADS-box* proteins have four characteristic domains: *MADS*-box domain (M), intervention domain (I), keratin-like domain (K) and C-terminal domain (C)(Theissen et al. 1996; Yang et al. 2003). The *MADS*-box genes usually have a highly conserved N-terminal DNA binding domain consisting of about 58 amino acids, called the M domain, in which the recognition sequence CArG box [CC(A/T)6GG] can bind to DNA and activate downstream gene expression(Riechmann et al. 1996). The M-domain is the most conservative. The I-domain contributes to the specificity and dimerization of DNA binding. The K-domain (K-box) is also conserved and has the characteristics of a spiral coil structure(Fan et al. 1997). The C-domain is least conserved in protein complex formation and transcriptional activation(Cho et al. 1999). Type-I *MADS-box* proteins lack the K domain, while type-II proteins contain these four typical domains(Kaufmann et al. 2005). According to gene replication and phylogenetic analysis, type-II proteins are further divided into three subgroups: M α , M β and M γ , and type-I of *MADS-box*

family members are divided into MIKC^C and MIKC^{*} (De Bodt et al. 2003). Comparing with MIKC^C, MIKC^{*} group members have longer I-domain and less K-domain (Kofuji et al. 2003). Through phylogenetic analysis, MIKC^C group is further divided into 13 distinct gene branches, including AG, AGL2, AGL6, AGL12, AGL15, AGL17, GGM13, DEF/GLO, FLC, STMADS11, SQUA, TM3/SOC1 and TM8 (Becker and Theissen 2003).

In plants, *MADS*-box proteins participate in the regulation of plant development, including leaf morphogenesis, growth, flower, fruit and seed development (Becker and Theissen 2003; Liu et al. 2008; Tapia-Lopez et al. 2008; Zahn et al. 2006). Not only that, more and more genetic and molecular analysis evidences demonstrate that *MADS*-box genes play an essential role in mediating drought stress. In commercial crops, silencing the *MADS*-box gene *SIMBP8* by RNA interference and found markedly increased the drought resistance of *Lycopersicon esculentum* (Yin et al. 2017). When subjected to drought stress in one or more time point, the expression levels of seven *JcMADS*s significantly elevated in *Jatropha curcas* (Tang et al. 2020). In cereal crops, when the transcription level of *OsMADS26* had down-regulated by RNAi technology, the expression of stress-related genes had up-regulated to improve drought resistance and increase yield in *Oryza sativa* (Khong et al. 2015). *OsMADS23* and *OsMADS25* were induced by mannitol, which may be involved in response to drought stress (Puig et al. 2013). In addition, *OsMADS2*, *OsMADS18*, *OsMADS22*, *OsMADS26*, *OsMADS27*, *OsMADS30* and *OsMADS55* were found to be sensitive to drought stress, and *OsMADS18* may interact with *OsMADS6*, *OsMADS8*, *OsMADS7* and *OsMADS47* under stress conditions (Arora et al. 2007). In *Zea mays*, the *MADS*-box gene *ZMM7-L* was increased significantly under mannitol treatment in maize seedlings (Smaczniak et al. 2012). Recently, *SiMADS51* (*SitMADS50* in this paper) was revealed to be involved in reducing drought stress tolerance in *Setaria italica* (Zhao et al. 2021).

Foxtail millet (*Setaria italica* L.) is both a major global cereal crop and an essential tool in investigating the molecular mechanisms of drought tolerance in cereal crops (Tang et al. 2017). Particularly for countries such as Northern China, foxtail millet is a vital cereal crop rich in protein and minerals. However, global warming, rising temperature and decreasing available water resources have deteriorated drought stress in plants which negatively affect crop production in China. Thus, researchers have been employing molecular biology and genetic approaches to improve foxtail millet yield and quality by enhancing its stress tolerance. Recently, genome-wide identification and analysis has been an effective means to predict the gene function of a large gene family.

The *MADS*-box gene family has been extensively studied in many plant species, such as *Arabidopsis thaliana*, *Oryza sativa*, *Zea mays*, *Triticum aestivum*, *Brachypodium distachyon*, *Sorghum bicolor*, *Fagopyrum tataricum*, *Brassica oleracea*, *Brassica napus*, *Lactuca sativa*, *Nicotiana tabacum*, *Raphanus sativus*, *Citrullus lanatus*, *Lycopersicon esculentum*, *chrysanthemum*, *Jatropha curcas*, and so on (Arora et al. 2007; Bai et al. 2019; Li et al. 2016; Liu et al. 2019; Ning et al. 2019; Parenicova et al. 2003; Saha et al. 2015; Sheng et al. 2019; Tang et al. 2017; Wang et al. 2019; Wang et al. 2019; Wei et al. 2014; Won et al. 2021; Yin et al. 2017; Zhao et al. 2010). However, our understanding of the function of resistance to drought stress in *MADS* gene family in *Setaria italica* is still limited. Thus, we try our best to

systematically study the *MADS-box* gene family in *Setaria italica*, given our current knowledge of its moment in plant drought stress tolerance and the availability of genomic information. In the present study, we identified 70 *MADS* genes (*SitMADSs*) in *Setaria italica* and analyzed their phylogenetic relationship, gene structure, conserved motif, chromosomal distribution gene duplications, phylogenetic features, *cis*-acting element and expression profiles in response to drought and ABA(Absciscic Acid) treatments. Our results provide a bioinformatics analysis and candidate *SitMADS* genes that may be potentially utilized in improving crop resistance to drought stress.

Results

Identification and Characterization of MADS-box Proteins in *Setaria italica*

A total of 512 genes encoding MADS-box protein were compared in nine representative species(*Volvox carteri*, *Micromonas pusilla* CCMP 1545, *Arabidopsis thaliana*, *Ananas comosus*, *Oryza sativa*, *Brachypodium distachyon*, *Sorghum bicolor*, *Zea mays* and *Setaria italica*) and the number of gene members in different subgroups was also calculated (Online Resource F1 and S1). In *Setaria italica*, 89 putative MADS-domain proteins were identified by HMMER(Potter et al. 2018). Then, the MADS-domain protein sequences encoded by non-representative transcripts were excluded. The remaining sequences were checked for the existence of complete MADS-domains using CDD, Pfam and SMART. A total of 70 sequences were confirmed as foxtail millet MADS-box proteins and renamed as being *SitMADS* genes (*SitMADS1* to *SitMADS70*) based on their chromosomal locations (Online Resource S2). The length of *SitMADS* proteins varied from 61 (*SitMADS70*) to 477 (*SitMADS17*) amino acids. The molecular weights ranged from 6.97 KDa (*SitMADS70*) to 53.18 KDa (*SitMADS1*). The predicted isoelectric point values of *SitMADS* proteins were between 4.41 (*SitMADS17*) to 10.65 (*SitMADS70*).

Gene structure and conserved motif distribution of *SitMADS* genes

The exon-intron organizations of all the identified *SitMADS* genes were examined to gain more insight into the evolution of the *MADS* family in *Setaria italica*. The phylogenetic tree and gene structure were integrated together as shown in Online Resource F2, The number of introns varied from 0 to 11. Type-
□ *SitMADS* genes, with and without one intron accounted for 92%, which showed that the genes within the same subgroup had similar genetic organization.

To study the structural features of the *SitMADS* proteins, the MEME(Multiple Em for Motif Elicitation) program was used to identify the conserved motifs of *SitMADS* proteins(Bailey et al. 2009). Meanwhile, the predicted motifs were annotated. A total of four conserved motifs were predicted in 70 *SitMADS* proteins (Online Resource F2). As expected,□typical MADS-box domains were found in all *SitMADS*

proteins. Most type-Ⅱ *SitMADS* proteins contained a specific conserved K domain which were lacked in all type-I *SitMADS* genes.

Phylogenetic analysis of *SitMADS-box* gene family

To analyze the classification and evolutionary relationships among the *SitMADS* members, we constructed a phylogenetic tree based on the protein sequences in *Setaria italica*, *Oryza sativa* and *Arabidopsis thaliana* (Fig. 1). Phylogenetic analysis of the 70 *SitMADS*s together with 108 *AtMADS*s and 72 *OsMADS*s showed that the *SitMADS* family could be classified into 12 distinct groups, according to the classification in *Arabidopsis thaliana* (Parenicova et al. 2003), namely subfamilies MIKC^C (39), MIKC* (4), Ma (18), Mβ (5) and My (4). Totally 56% of the 70 *SitMADS* genes belongs to the MIKC^C-type genes, which could be further divided into 8 subfamilies, including the SOC1, SEP, AP3/PI, ANR1, A, AGL-12, C/D and TT16 subgroup.

Chromosome distribution and syntenic analysis of *SitMADS* genes

To understand the genomic distribution and gene duplication of *SitMADS* genes, 70 *SitMADS* genes were distributed unevenly throughout the nine chromosomes in *Setaria italica* (Fig. 2A). Among them, the chromosome 5 contained the largest number of *SitMADS* genes (13), while only two *SitMADS* genes were localized on chromosome 8. The majority of *SitMADS* genes were located on the proximate or the distal ends of foxtail millet chromosomes.

There was no positive correlation between the chromosome length and the number of *SitMADS* genes. Gene replication events, including segmental and tandem duplications, were crucial events for the expansion of the gene family in the process of evolution. The chromosomal regions of two or more genes within the 200kb range were defined as tandem duplications (Holub 2001). Two tandem duplicated clusters (*SitMADS19-SitMADS20* and *SitMADS53-SitMADS54-SitMADS55*) formed two gene clusters that were detected on chromosome 3 and 6, respectively (Fig. 2A). Interestingly, the five tandem duplicated genes all belong to the type-Ⅱ. 12 segmental duplication events with 20 *SitMADS* genes were indicated in Fig. 2B. A total of 24 (34.3%) *SitMADS*s were duplicated genes. The result suggested that segmental duplication events may be more important than tandem duplication in the expansion of the *SitMADS* gene family in *Setaria italica*.

The Ka, Ks, and Ka/Ks values of the homologs were calculated using a KaKs_calculator (Online Resource S3). Three pairs of segmental duplication genes were found to have been subject to purifying selection pressure during evolution and negative purifying selection was present.

For better understanding the evolution of *SitMADS* gene family, we constructed three comparative syntenic maps of foxtail millet associated with three representative species, including *Arabidopsis*

thaliana, *Oryza sativa* and *Zea mays*. The numbers of orthologous gene pairs between *Arabidopsis*, rice and maize, were 4, 37 and 23 (Fig. 3, Online Resource S4). Between foxtail millet and rice genes, *SitMADS21*, *SitMADS35*, *SitMADS41*, *SitMADS46*, *SitMADS53* and *SitMADS59* were found to be associated with two syntenic gene pairs, which implied that these genes may have played an important role of *MADS* gene family during evolution.

Expression Profiles of *SitMADS* Genes in Different Tissues

To predict the possible functions of *SitMADS* genes, we further detected the expression profiles of 10 selected *SitMADS* genes in seedlings, roots, leaves, stems and panicles by RT-qPCR (Real-time quantitative PCR) including *SitMADS6* (ANR1), *SitMADS9* (ANR1), *SitMADS23* (Ma), *SitMADS42* (Mβ), *SitMADS46* (AP3/PI), *SitMADS51* (ANR1), *SitMADS52* (MIKC*), *SitMADS58* (ANR1), *SitMADS63* (SEP) and *SitMADS64* (A). The results revealed that ten *SitMADS* genes were highly expressed in roots relative to seedling tissues (Fig. 4). In addition, compared to gene expression in seedling, *SitMADS42*, *SitMADS46* and *SitMADS64* also had relatively high transcriptional activity in leaf tissue, *SitMADS46*, *SitMADS63* and *SitMADS64* had relatively high transcript abundance in the panicle (Fig. 4). These results implied the differential roles of ten genes in tissue development.

Cis-acting regulatory elements in *SitMADS* promoters

For further analyze the role of *SitMADS* in plant resistance to stress, the region about 2000 bp upstream of the start codon (ATG) was used here to analyze *cis*-acting regulatory elements. The *cis*-acting element in the *SitMADS* gene promoter could be divided into three categories according to their functional annotations including light-response, biotic and abiotic stress-response and plant hormones-response (Online Resource F3 and S5). The results showed that many abiotic stress (*WUN-motif*, *GC-motif*, *ARE*, *TC-rich repeats*, *MBS*) related elements participate in regulating gene expression, such as *SitMADS6*, *SitMADS9*, *SitMADS23*, *SitMADS42*, *SitMADS46*, *SitMADS51*, *SitMADS52*, *SitMADS58*, *SitMADS63* and *SitMADS64*. In addition, *ABRE* (abscisic acid response element), which is involved in the response to ABA (Abscisic Acid), were detected in *SitMADS51*, *SitMADS63* and *SitMADS64*. These elements were thought to play important roles in responses to drought stress.

Expression patterns of *SitMADS* genes in response to different treatments

To investigate whether the expression of *SitMADS* genes were influenced by drought stresses and related signaling at the transcriptional level, 10 *SitMADS* genes (*SitMADS6*, *SitMADS9*, *SitMADS23*, *SitMADS42*, *SitMADS46*, *SitMADS51*, *SitMADS52*, *SitMADS58*, *SitMADS63* and *SitMADS64*) were selected for further expression profiling after PEG, drought and ABA treatments. Overall, except *SitMADS9* up-regulated in 3 h, nine *SitMADS* genes (*SitMADS6*, *SitMADS23*, *SitMADS42*, *SitMADS46*,

SitMADS51, *SitMADS52*, *SitMADS58*, *SitMADS63* and *SitMADS64*) were significantly induced and up-regulated after 1 h, whereas down regulated after 3 h under PEG-6000 treatment (Fig. 5). To further confirm whether the expression of *SitMADS* genes was influenced by drought stresses, we treated the ten genes with real drought treatment. Ten *SitMADS* genes (*SitMADS6*, *SitMADS9*, *SitMADS23*, *SitMADS42*, *SitMADS46*, *SitMADS51*, *SitMADS52*, *SitMADS58*, *SitMADS63* and *SitMADS64*) significantly responded to drought stress. The results were consistent with the trend of PEG simulated drought experiment. In addition, the expression pattern of ten *SitMADS*s under 100 μ M ABA treatment were also analyzed. *SitMADS51*, *SitMADS63* and *SitMADS64* had an increase in transcript levels at 3 h by ABA treatment, while *SitMADS52* showed a trend of first decreased and then slightly increased (Fig. 5).

Discussion

The evolutionary process of *SitMADS* genes

MADS genes comprise a large family of transcription factors that play important roles in plant growth, development, and response to abiotic stress (Zhang et al. 2012). Currently, our understanding of the role of *MADS* family in the drought-resistant foxtail millet is limited. Here, we identified 70 *SitMADS*s from the genome, which were divided into two types (Type-I and Type-II) based on their evolutionary relationships (Fig. 1A, 1B). In recently published study, 72 *SitMADS* genes were identified (Zhao et al. 2021). We speculate that this may be caused by different screening conditions and verification methods. In our study, 512 genes, encoding MADS-box protein from nine representative species (*Volvox carteri*, *Micromonas pusilla* CCMP 1545, *Arabidopsis thaliana*, *Ananas comosus*, *Oryza sativa*, *Brachypodium distachyon*, *Sorghum bicolor*, *Zea mays* and *Setaria italica*), were combed (Online Resource F1 and S1). As we all know, *Micromonas pusilla* CCMP1545 belongs to the *Prasinophyceae* which are ancient members of the green lineage, the lineage giving rise to higher plants. However, only one type-II *MADS* genes were identified in *Micromonas pusilla* CCMP1545 and *Volvox carteri*. We suspect that the type-II appeared before the type-I genes during the process of evolution of *MADS* gene family. In *Sorghum bicolor*, *Oryza sativa*, *Zea mays*, *Brachypodium distachyon* and *Setaria italica*, we also identified 65, 72, 75, 77 and 70 *MADS* genes, respectively (Online Resource F1 and S1). This result showed that the number of members of *MADS* gene family was similar in Poaceae.

Difference in the acquisition tropism of introns between type-I and type-II *SitMADS* genes

The conserved motifs and exon-intron organization of all the identified *SitMADS* genes were examined to gain more insight into the evolution of the MADS-box family in *Setaria italica*. Genes belong to same subgroup shared similar motifs and exon-intron organization, and the number of introns of type-II *SitMADS* genes were conspicuous higher than type-I (Online Resource F2 and S6). Among 27 type-I *SitMADS* genes, except *SitMADS4*, *SitMADS12*, *SitMADS17*, *SitMADS22*, *SitMADS29*, *SitMADS34*, *SitMADS43*, *SitMADS65* and *SitMADS66*, all had no intron. On the contrary, there were seven introns in

most of type-II *SitMADS* genes (Online Resource F2). This phenomenon was also reflected in other species, such as *Arabidopsis thaliana*, Chinese cabbage, rice and cotton (Balanza et al. 2018; Bemmer et al. 2017; Diao et al. 2018; Parenicova et al. 2003). In recent years, researchers had found that introns in animal, plants and micro-organisms had the ability to enhance gene expression (Shaul 2017). And intron could not only regulated gene expression in normal cell development but also played an important role in stress response (Jacob and Smith 2017; Naro and Sette 2017). In the results of this paper, type-II genes had higher transcriptional activity under stress (Fig. 5), such as *SitMADS52*. This consequence could provide a reasonable explanation for the difference in the tendency of deletion or acquisition of introns between type-II and type-I *SitMADS* genes, and supplied a hypothesis that the type-II *MADS* gene has the function to resist drought stress.

Gene members had a similar conserved motif organization within the same subgroup

A total of 4 conserved motifs were predicted in 70 *SitMADS* proteins (Online Resource F2 and S7). Comparing with type-I *SitMADS* proteins, most type-II *SitMADS* proteins contained a specific conserved K domain, and related proteins of the phylogenetic tree had similar motif organization, such as *SitMADS6*, *SitMADS9*, *SitMADS38*, *SitMADS51* and *SitMADS58* (ANR1). The results proved that the distribution of protein motifs were clade-specifically that suggesting similarity of function.

The different replication events happened in *SitMADS* genes

Gene replication events, including segmental replication and tandem replication, were 7 important processes for the expansion of gene families in the process of evolution (Cannon et al. 2004; Freeling 2009). Among the *AtMADS* gene family, 53% of the MIKC group might come from segment replication (Parenicova et al. 2003). In the rice genome, most of repetitive genes belong to MIKC^C group in which 30 *OsMADS-box* genes were tandem replication and 16 *OsMADS* genes were segmental replication (Arora et al. 2007), the same phenomenon has been found in *Zea mays* (Zhao et al. 2010).

In *Setaria italica*, two tandem repeat regions (*SitMADS19*, *SitMADS20*, *SitMADS53*, *SitMADS54* and *SitMADS55*, MIKC^C subgroup) were detected (Fig. 2A), and segmental replication events were happened in 19 gene family members (27%) which had high homologous relationship in the *SitMADS* gene family (Fig. 2B). It is worth noticing that 37, 23 and 4 pairs of orthologous genes were discovered between *Oryza sativa*, *Zea mays*, *Arabidopsis thaliana* and *Setaria italica*, respectively. Some of them were presented in all of these species (Fig. 3, Online Resource S4), such as *SitMADS28* and *SitMADS41*, suggesting that these orthologous pairs may have existed prior to evolutionary divergence. The different replication events, which happened in *SitMADS* genes, led to an expansion of gene numbers and an evolution of function.

Gene expression pattern of *MADS* genes in *Setaria italica*

Gene expression pattern was a crucial aspect in the study of gene function, which could better understand the role of *SitMADS-box* genes in the growth and development in plants. The expression patterns of *MADS* genes in different tissues have been described in many species, such as *Arabidopsis thaliana*, *Oryza sativa*, *Zea mays*, *Sorghum bicolor* and *Lycopersicon esculentum* (Arora et al. 2007; Parenicova et al. 2003; Wang et al. 2019; Yin et al. 2017; Z. Zhang et al. 2012; Zhao et al. 2010). In previous studies, type-II genes had been studied more intensively, while less research had been reported on type-I genes (He et al. 2019; Liu et al. 2021; Schilling et al. 2020; Zobell et al. 2010). In this paper, RT-qPCR was performed to examine the expression pattern of 10 *SitMADS* genes (including eight type-I and two type-II genes). *SitMADS23* (Ma) and *SitMADS42* (Mb) were found to have the highest transcriptional activity in the root, the same trend was also detected in *CnMADS80* (Ma) and *JcMADS03* (Mb) (Tang et al. 2020; Won et al. 2021). This results suggested that the type-I genes without introns may increase more rapidly in transcript levels in roots under drought stress. In *Arabidopsis thaliana*, *AtAGL12* had been shown to be involved in the regulation of root meristem proliferation, and *AtANR1* was also shown to control lateral elongation in response to stress (Tapia-Lopez et al. 2008; Zhang and Forde 1998). Roots are the most sensitive part to feel drought stress, because drought stress results from an insufficient of water in the soil (Kaashyap et al. 2018; Kim et al. 2018; Kim et al. 2015; Koevoets et al. 2016; Zhu et al. 2019). *SitMADS6*, *SitMADS9*, *SitMADS51* and *SitMADS58*, belonged to ANR1, were also had the tissue expression specificity in root.

Gene expression analysis under drought/ABA treatment

The *cis*-acting element of promoters is essential for determining the initiation and efficiency of gene transcription and is involved in the regulation of gene expression under abiotic stress. Among the 10 candidate genes, *SitMADS6*, *SitMADS9*, *SitMADS46*, *SitMADS51* and *SitMADS63* contained *MBS* (drought stress response element) and the others contain at least one *cis*-acting element associated with abiotic stresses (Online Resource F3 and S5). In *Oryza sativa*, *OsMADS27* (belonged to ANR1) was up regulated expression more than 2 folds under abiotic stress (Arora et al. 2007). In our study, *SitMADS58*, homologous gene of *OsMADS27*, was significantly induced under PEG-6000 treatment (Fig.5), which may participate in abiotic stress-response. The expression of *SIMBP8* (type-II) was induced by dehydration, seedling growth of *SIMBP8*-RNAi plants became more tolerant to drought stress than WT (wild type) plants in soil (Yin et al. 2017). Similarly, *SitMADS6*, *SitMADS9*, *SitMADS46*, *SitMADS51*, *SitMADS52*, *SitMADS58*, *SitMADS63* and *SitMADS64* (type-II) were up-regulated under PEG-6000 and drought treatment, of which *SitMADS46*, *SitMADS51*, *SitMADS52*, *SitMADS58* and *SitMADS64* were up-regulated more than 10 folds compared to the control group (Fig. 5). This result suggested that type-II *MADS* genes may be involved in the resisting drought stress signaling pathways.

ABA (Absciscic Acid) had been studied primarily with respect to its critical roles in abiotic stress. Many drought stress-response genes were also described as ABA-induced genes (Nakashima and Yamaguchi-

Shinozaki 2013). Previous studies have shown that *SIMBP11* was induced by ABA, which played an important role through an ABA-independent signaling network(Guo et al. 2016). In *Medicago sativa*, the expression levels of *MsMADS001*, *MsMADS065*, *MsMADS075* and *MsMADS112* changed regularly with the time point of ABA treatment(Dong et al. 2021). In the *SitMADS*s promoter region, the *ABRE* (ABA-responsive *cis*-acting element) was detected. The expression of *SitMADS51*, *SitMADS63* and *SitMADS64* were induced under ABA treatment and some *SitMADS* genes were down-regulated (Fig. 5). Moreover the expression of the *SitMADS51*, *SitMADS63* and *SitMADS64* showed increasing under PEG and drought treatment compared with the control, suggesting that *SitMADS51*, *SitMADS63* and *SitMADS64* may play a certain role in drought stress through an ABA-independent signaling network. Overall, these above findings provide insight into the potential functional roles of *SitMADS* genes in stress tolerance, which needs to be further analyzed.

Conclusion

In this study, 70 *MADS* genes were first identified in the *Setaria italica*. The family was divided into two subgroups (type-I and type-II) based on the phylogenetic tree, exon-intron organization and distribution of conserved motifs. Chromosomal locations of the *SitMADS* gene family members were also determined. Finally, the expression pattern of *SitMADS*s was explored using qRT-PCR, revealing their expression specificity in different tissues and differential expression under osmotic stress, drought stress and ABA treatment. Ten candidate genes were highly expressed in roots and significantly induced by PEG-6000 treatment and drought treatment. The expression of *SitMADS51*, *SitMADS63* and *SitMADS64* was induced at 3 h of ABA treatment. These results suggested that *SitMADS*s play vital role in resistance to drought stress. Our work provides functional insight into the role of *SitMADS* genes in stress-resistance.

Methods

Identification of *MADS-box* genes

Setaria italica genome and annotation files were downloaded from the plant genomics resource (<https://phytozome.jgi.doe.gov/pz/portal.html>). The *MADS-box* genes in *Setaria italica* genome were identified by the HMMER-3.0 program. The hidden Markov model (HMM) profiles of the SRF domain (PF00319) were retrieved from the Pfam website(Finn et al. 2014) and were used to identify the putative *MADS-box* proteins with the best domain e-value cutoffs of $< 3e-18$. To validate the accuracy of these predicted genes, we used the online tool SMART (<http://smart.embl-heidelberg.de/>)(Schultz et al. 1998), pfam (<http://pfam.xfam.org/>)(Bateman et al. 2000) and NCBI-CDD (<https://www.ncbi.nlm.nih.gov/cdd>) to confirm that all candidate genes had *MADS-box* domains(Huang et al. 2015). The typical characters, including the theoretical isoelectric point (PI), the instability index, the aliphatic index, and the molecular weight theoretical isoelectric points (PI), were analyzed by the online tool ProtParam (<http://web.expasy.org/protparam/>).

Gene structure and conserved motifs

The Gene Structure Display Server (GSDS 2.0) (<http://gsds.gao-lab.org/>) was used to analyze the composition of exons and introns based on the foxtail millet genome annotation file and to display the structure of the *MADS-box* genes. MEME(Bailey et al. 2009) was applied to predict the conserved motifs of the *MADS-box* with the following parameter settings: number of repetitions -any, maximum number of motifs -4, minimum motif width -6 and maximum motif width -100. Graphic beautification by TBtools (<http://www.tbtools.com/>)(Chen et al. 2020).

Phylogenetic analysis and classification

To understand the phylogenetic relationship and to classify the *MADS-box* genes, individual unrooted neighbor-joining (NJ) trees were constructed for type-I and type-II genes based on the full-length amino acid sequences of *MADS-box* genes from *Arabidopsis thaliana*, *Oryza sativa* and *Setaria italica* (<https://phytozome.jgi.doe.gov/pz/portal.html>). The *MADS-box* proteins were aligned to each reference by using Muscle(Edgar 2004). Phylogenetic trees were constructed by the MEGA7 software(Kumar et al. 2016). All the NJ tree nodes were evaluated by bootstrap analysis with 1000 replicates(Saitou and Nei 1987), model-selected P-distance, missing data treatment set Pairwise deletion.

Chromosomal locations and gene duplication

MADS-box genes were mapped to the nine chromosomes of foxtail millet according to GFF files (<https://phytozome.jgi.doe.gov/pz/portal.html>), and the results were visualized with a self-implemented Perl script. The gene duplication events were analyzed by Multiple Collinearity Scan toolkit (MCScanX), with the default parameters(Wang et al. 2012). The CDS region of the *MADS* genes were compared by using the KaKs_Calculator 2.0 software(Koch et al. 2000) for the development of segmental duplication genes. The divergence time was calculated with the formula $T = Ks/2r$, with *Ks* being the synonymous substitutions per site and *r* being the rate of divergence for nuclear genes from plants. The *r* was taken to be 5.3×10^{-8} synonymous substitutions per site per year for monocotyledon plants.

Cis-element analysis of promoters

Cis-acting regulatory elements as binding sites of transcription factors connect gene structure and function(Rani 2007) and play important roles in gene expression. The *cis*-elements in *SitMADS*s promoters were predicted using PlantCARE (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>).

Plant materials and drought/ABA treatment

Seeds of Yugu1(drought-resistant), an model cultivar of *Setaria italica* L., were cross-bred by Anyang Academy of Agricultural Sciences and provided by Xianmin Diao (Chinese Academy of Agricultural Science). Mature non-dormant seeds were disinfected in 70% alcohol for 30 seconds, then washed with sterile water and placed in a covered sterile petri dish containing MS solid medium. The plants were placed in the light incubator in the tissue culture room of the Analysis and testing Center of Hebei normal University of Science and Technology (Qinhuangdao). They grew at 28 °C day/20 °C night with 60% relative humidity for 4 weeks. The experiment began under 16 h light/8 h dark photoperiod prior. For the PEG-6000 simulated drought stress treatment, 40 uniformly developed seedlings were selected and divided into four groups. Control group, did not apply PEG stress treatment, while the simulated drought treatment group (PEG0.5 h, PEG1 h and PEG3 h) removed the seedlings from the petri dish and soaked them in 20% (v/v) PEG-6000 solution for 0.5 h, 1 h and 3 h, respectively. The seedlings were quickly frozen in liquid nitrogen and then stored in the refrigerator at -80 °C. For the ABA stress treatment, the treatment method was similar to that of PEG-6000 simulated drought stress treatment. The seedlings were immersed in ABA solution containing 100 µM and soaked for 0.5 h, 1 h and 3 h respectively under the condition of avoiding light. The seedlings were quickly frozen in liquid nitrogen and then stored in the refrigerator at -80°C. In the real drought stress treatment, the germinated seeds were transplanted into individual pots (15 cm diameter, 35 cm depth) containing nutrient soil and vermiculite (1:1, v/v) and placed in an intelligent artificial climate incubator. The control group was watered to field capacity every day, and the real drought group stopped watering at the 4-5 leaf stage, and when the soil moisture content was less than 20%, the materials were taken at the same time as the control group, and the liquid nitrogen was frozen and stored at -80 °C.

RNA extraction and gene expression analysis

Total RNA was extracted by RNA Extraction Kit (MiniBEST Plant RNA Extraction Kit, TaKaRa, Japan), all RNA was analyzed by agrose gel electrophoresis and then quantified with a Nanodrop ND-1000 spectrophotometer. DNA-free RNA were reverse transcribed to cDNA by UEIris RT-PCR System for First-Strand cDNA Synthesis (US EVERBRIGHT® INC, Suzhou, China). Primers for RT-qPCR were designed for the coding sequences of *SitMADS6*, *SitMADS9*, *SitMADS23*, *SitMADS42*, *SitMADS46*, *SitMADS51*, *SitMADS52*, *SitMADS58*, *SitMADS63* and *SitMADS64* of *SitMADS* gene family (Online Resource S8), and the production was limited to 80-200 bp. Real-time quantitative PCR was performed using a BIO-RAD CFX connect™ quantitative PCR instrument for amplification, using *SitEF-1a-2* as an internal reference. The amplification system was 10 µL, cDNA 0.5 µL, primers 0.3 µL each in forward and reverse directions, AugeGreen™ qPCR Master Mix(US EVERBRIGHT® INC, Suzhou, China) 5 µL, and ddH2O 3.9 µL. The reaction was carried out as follows: 95°C for 2 min, followed by 45 cycles of 95 °C, /5 s, 60 °C, /5 s and 72 °C, /25 s. The experiment was set up with 3 biological replicates. The expression data were analyzed by GraphPad Prism 8 software.

Declarations

Author Declaration

Not Applicable

Ethics approval and consent to participate

Not Applicable

Consent for publication

Not Applicable

Availability of Data and materials

All data generated or analyzed during this study are included in this published article [and its online resources].

Competing interests

The authors declare that they have no conflict of interest.

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Authors contributions

XM was responsible for the main part of data analysis, experimental design and manuscript writing. NX, LD and LB performed the RT-qPCR. LD participated the RNA-Seq data analysis. PG and HZ performed the primer design and plant materials preparation. ZG performed the foxtail millet planting and samples collection. XL and HG were responsible for the programs and manuscript revision. All the authors have commented, read and approved the final manuscript.

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Figures

different bootstrap values. Different species were represented by different markers—including star (*Setaria italica*), triangle (*Oryza sativa*) and tick (*Arabidopsis thaliana*).

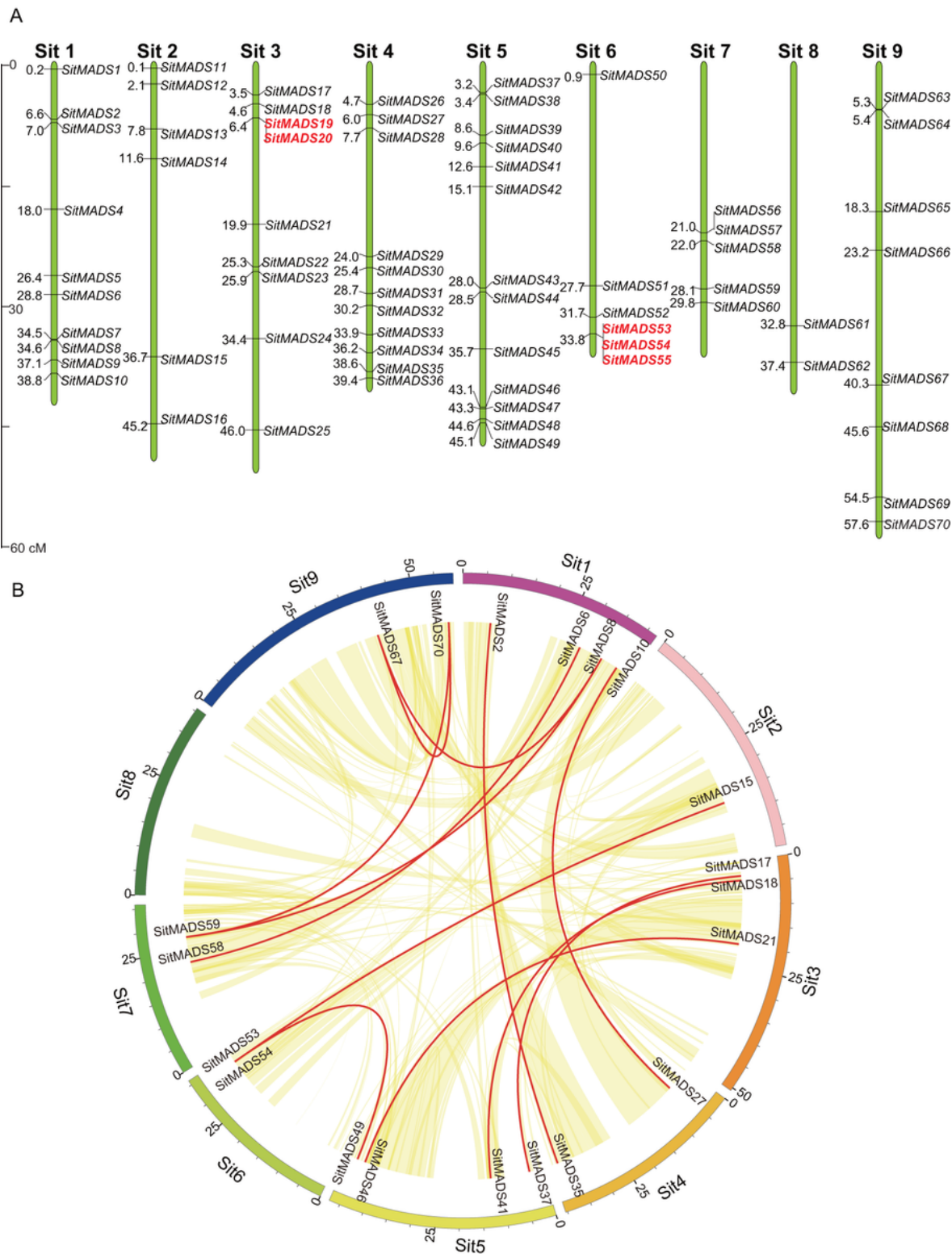


Figure 2

Schematic representations of the chromosomal distribution and interchromosomal relationships of the *MADS-box* genes in *Setaria italica*. **a** Distribution of the *MADS-box* gene on nine chromosomes in *Setaria*

italica. The chromosome number was written as Sit1-Sit9 above each chromosome. The tandem repeats genes were represented by red fonts. On the left, the size of each chromosome was estimated in cM. **b** Segmental duplications analysis of 19 *SitMADS* genes on nine chromosomes. Red lines represented duplicated MADS gene pairs and the yellow line indicated all synteny blocks in *Setaria italica*.

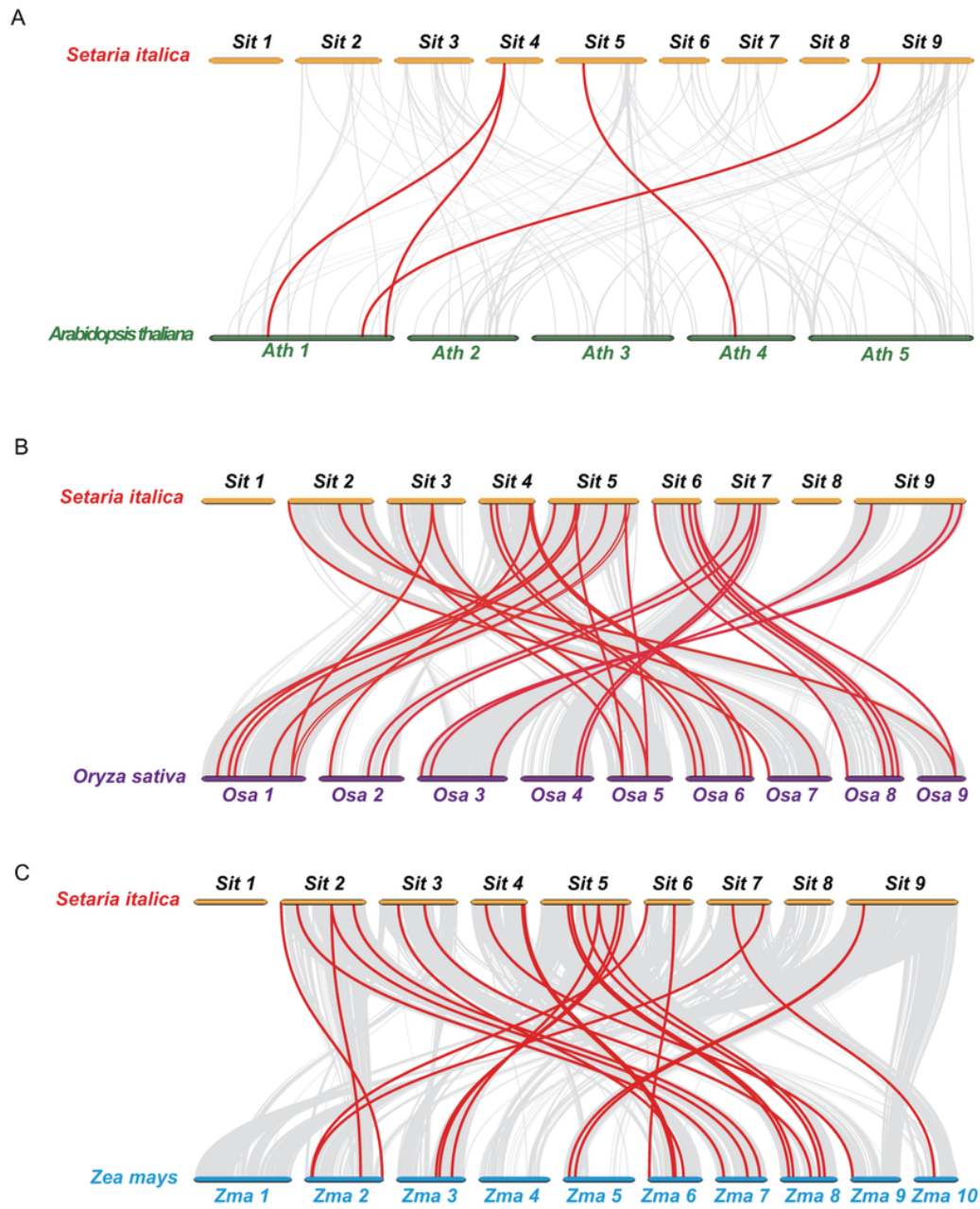


Figure 3

Synteny analysis of *SitMADS* genes in *Setaria italica* and other plant species. **a** Synteny analysis of the MADS genes in *Setaria italica* and *Arabidopsis thaliana*. **b** Synteny analysis of the *MADS* genes in *Setaria*

italica and *Oryza sativa*. **c** Synteny analysis of the *MADS* genes in *Setaria italica* and *Zea mays*. Gray lines in the background indicated the collinear blocks in *Setaria italica* and other plant genomes, while the red lines highlight the syntenic *MADS* gene pairs.

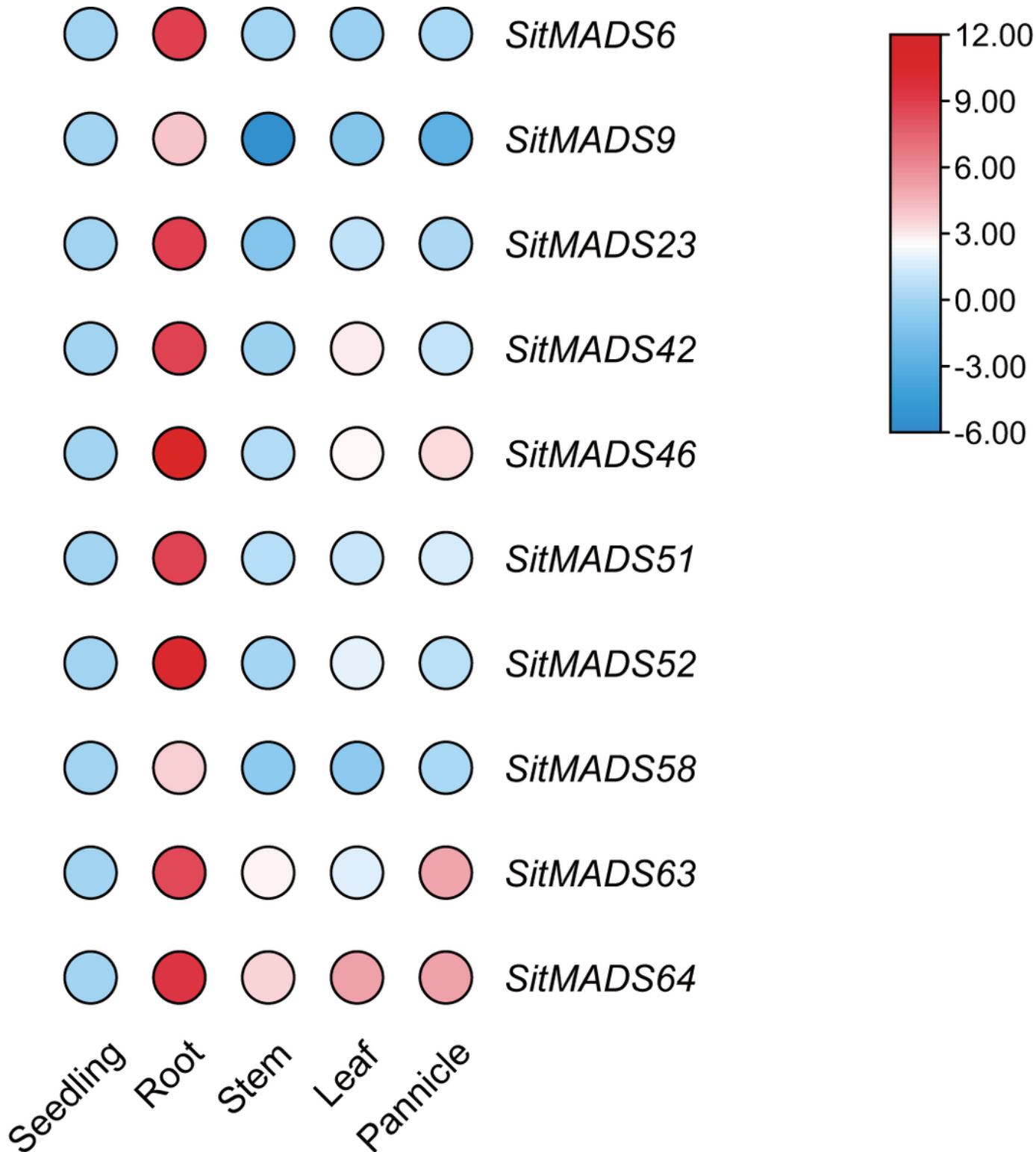


Figure 4

Expression profiles of 10 selected *SitMADS* genes in 5 samples including different tissues in *Setaria italica*. The expression patterns of *SitMADS* genes in root, leaf, stem, pannicle and seedling tissues by RT-qPCR. There were three biological and three technical replicates performed for each sample. The relative expression levels were evaluated by $2^{-\Delta\Delta CT}$. Color bar at the base represents $\log_2$ expression values, thereby blue color representing low level expression, pink shows medium level expression and red signifies high level expression.

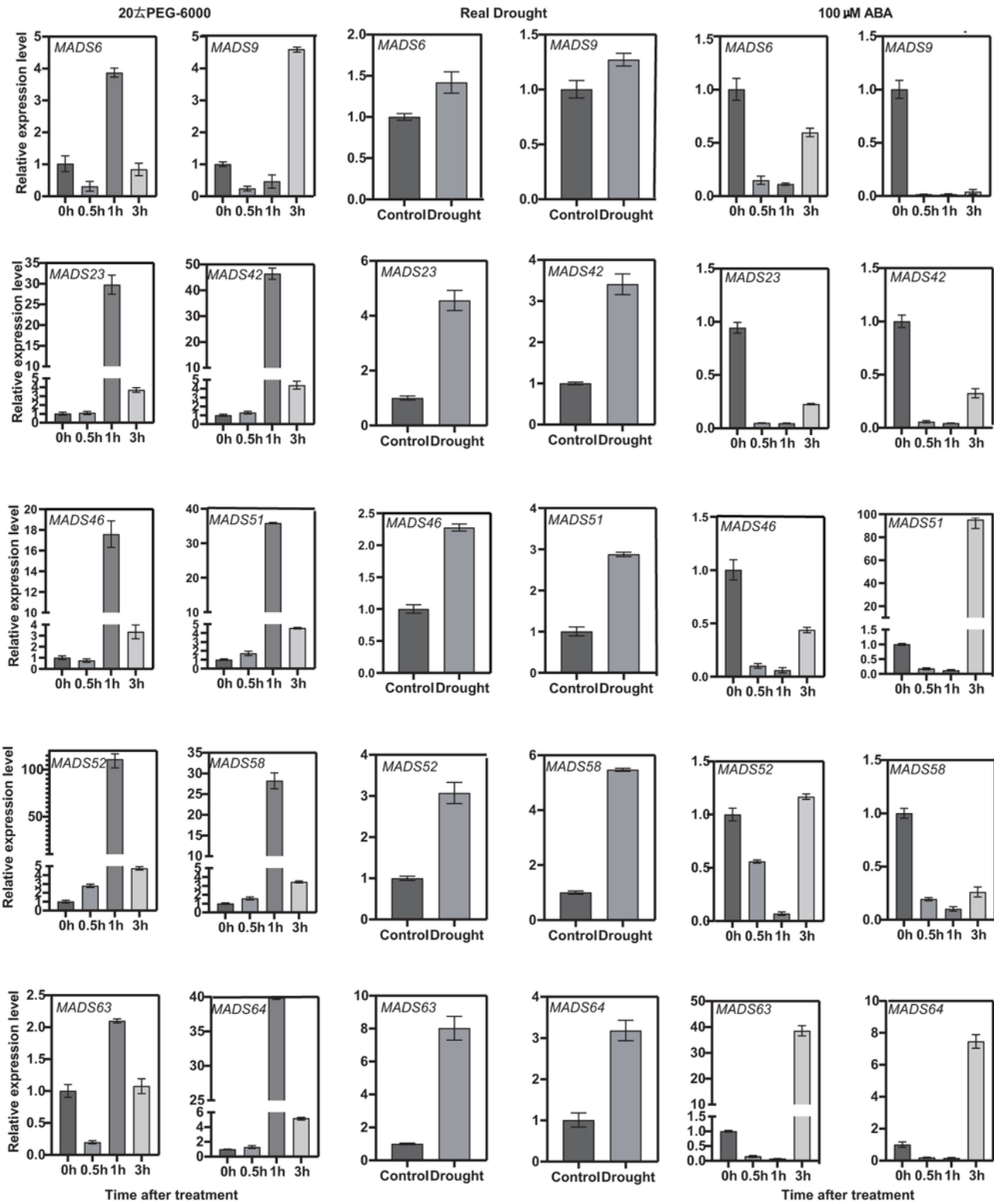


Figure 5

Expression profiles of 10 selected *SitMADS-box* genes in response to different treatments. Analysis of the *SitMADS-box* gene expression patterns under 20% PEG-6000, real drought and 100 μ M ABA treatment by RT-qPCR. Data were normalized to *SitEF-1a-2* gene and error bars were obtained from three biological replicates and three technical replicates in each sample

Supplementary Files

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