MADS-box family genes in sheepgrass and their involvement in abiotic stress responses

Junting Jia 1,2†, Pincang Zhao 1,3†, Liqin Cheng 1, Guangxiao Yuan 1, Weiguang Yang 1,2,4, Shu Liu 1,2, Shuangyan Chen 1, Dongmei Qi 1, Gongshe Liu 1* and Xiaoxia Li 1*

Abstract

Background: MADS-box genes are categorized into A, B, C, D and E classes and are involved in floral organ identity and flowering. Sheepgrass (Leymus chinensis (Trin.) Tzvel) is an important perennial forage grass and adapts well to many adverse environments. However, there are few studies on the molecular mechanisms of flower development in sheepgrass, especially studies on MADS-domain proteins.

Results: In this study, we cloned 11 MADS-box genes from sheepgrass (Leymus chinensis (Trin.) Tzvel), and phylogenetic analysis of the 11 genes with their homologs revealed that they are divided into nine subclades. Tissue-specific expression profile analysis showed that most of these MADS-box genes were highly expressed in floral organs. LcMADS1 and LcMADS3 showed higher expression in the stamen than in the other tissues, and LcMADS7 showed high expression in the stamen, glume, lemma and palea, while expression of LcMADS2, LcMADS9 and LcMADS11 was higher in vegetative organs than floral organs. Furthermore, yeast two-hybrid analyses showed that LcMADS2 interacted with LcMADS7 and LcMADS9. LcMADS3 interacted with LcMADS4, LcMADS7 and LcMADS10, while LcMADS1 could interact with only LcMADS7. Interestingly, the expression of LcMADS1 and LcMADS2 were significantly induced by cold, and LcMADS9 was significantly up-regulated by NaCl.

Conclusion: Hence, we proposed that LcMADS1, LcMADS2, LcMADS3, LcMADS7 and LcMADS9 play a pivotal role in sheepgrass sexual reproduction and may be involved in abiotic stress responses, and our findings provide useful information for further exploration of the functions of this gene family in rice, wheat and other graminaceous cereals.

Keywords: MADS-box genes, Sheepgrass, Abiotic stress, Gene expression, Sexual reproduction, Yeast two-hybrid assay

Background

In plants, MADS-box genes play important roles in many aspects of developmental processes, especially in floral induction and flower development [1]. According to their roles in flower development, MADS-box genes are classified into A, B, C, D and E classes [2, 3]. Many MADS-box genes have also been identified in rice, and MADS-box proteins determine the identity of flower organs by forming higher order complexes [4, 5]. Previous studies indicated that the A-class gene OsMADS15 plays a role in palea development [6]. However, plants with a triple mutant of OsMADS14, OsMADS15, and OsMADS18 have no obvious phenotype in flower development [7]. There are two B-class PI orthologous genes in rice, OsMADS2 and OsMADS4, and when OsMADS2 is silenced, transgenic plants display differences in lodicules but no changes in stamens. The OsMADS4 gene mutant plants display no alteration in lodicule or stamen phenotypes. However, when OsMADS2 and OsMADS4 are silenced together, transgenic plants display palea-like structures in place of lodicules and carpel-like organs instead of stamens [8, 9]. Two B-class PI-like genes (TaPI-1 and TaPI-2/TaAGL26) have been identified in wheat, and they are closely related to the rice PI-like genes OsMADS4 and OsMADS2, respectively [10]. The C-class gene OsMADS3 plays a role in stamen

* Correspondence: liugs@ibcas.ac.cn; lixx2013@ibcas.ac.cn
†Equal contributors
1Key Laboratory of Plant Resources, Institute of Botany, The Chinese Academy of Sciences, Beijing, China
Full list of author information is available at the end of the article

© The Author(s). 2018 Open Access This article is distributed under the terms of the Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The Creative Commons Public Domain Dedication waiver (http://creativecommons.org/publicdomain/zero/1.0/) applies to the data made available in this article, unless otherwise stated.
and ovule identity and is involved in lower meristem activity in early flower development and late flower development [11], while the D-class OsMADS13 is essential for *O. sativa* ovule development [12, 13]. Furthermore, expression pattern analysis demonstrates that the E-class genes specify all four whorls of floral organs and flower meristem determinacy [2, 13]. Previous studies indicated that some plant MADS-box genes are involved in abiotic stress responses. For example, *OsMADS26*, *OsMADS22* and *OsMADS55* were found to be involved in stress tolerance [14–16]. MADS-box genes have also been shown to be affected by low temperature stress in the tomato [17]. All of these findings reveal that some MADS-box genes may be involved in abiotic stress-related processes. However, until now, there have not been many reports about MADS-box gene involvement in abiotic stress responses.

Sheepgrass is a perennial forage grass with high protein content, vegetative productivity and palatability [18]. It can adapt well to many adverse environmental conditions, including drought, high salinity and alkalinity, and cold [19]. In sheepgrass, the basic unit of the inflorescence is the spikelet, and a spikelet contains two glumes and a number of flowers, which is similar to barley or wheat [20, 21]. The MADS-domain proteins are involved in many developmental processes in plants (e.g., floral organ identity and flowering). However, there are few studies on MADS-domain proteins in sheepgrass.

In this study, 11 MADS-box genes were cloned and further studied. A phylogenetic tree combining MADS proteins from sheepgrass and other species was constructed to examine their evolutionary relationships. MADS-box genes of sheepgrass were differentially expressed in vegetative and reproductive organs, and the interaction relationships of the MADS-box genes were validated by yeast two-hybrid assays. At the same time, the abiotic stress-induced expression patterns of these genes were also analyzed by qRT-PCR. Our work suggested that the MADS-box genes in sheepgrass are involved in sexual reproduction and abiotic stress and will also complement previous reports on the functions of this gene family in Triticeae.

### Methods

#### Plant materials, growth conditions and stress treatments

Two-year-old sheepgrass of the variety Zhongke 1 was used for the experiments. Sheepgrass was planted under natural conditions in a field at the Beijing Botanical Garden, Chinese Academy of Sciences, Beijing, China (40° 07′ N, 116° 11′ E). The leaves, stems, roots and flower tissues including glumes, lemmas, paleas, stamens and carpels were collected at the time of flowering. All samples were immediately frozen in liquid nitrogen and stored at −80 °C for tissue-specific expression analysis. For stress treatments, seeds were grown in a soil mixture containing 2:1 peat moss and vermiculite (v/v) in a greenhouse at 22–27 °C under 16-h light/8-h dark conditions. The seedlings of four-week-old sheepgrass were used for different stress treatments. For cold treatment, seedlings were placed in a chamber at 4 °C. For the ABA, drought and salt treatments, seedlings were irrigated with 100 μM ABA, 300 mM mannitol and 200 mM NaCl, respectively. Plants were sampled at the 0, 4th, 8th and 12th hour after abiotic stress treatments and immediately frozen in liquid nitrogen for further analysis.

#### Cloning of the 11 MADS-box genes from sheepgrass

To clone the full-length sequence of the MADS-box genes, total RNA was isolated from 100 mg of frozen inflorescences (Fig. 1a) using a TRIzol kit (TaKaRa, Dalian, China) according to the manufacturer’s instructions. Gene-specific primers were designed and used to amplify the full-length coding sequences (CDS) (Additional files 1: Table S1). The amplification conditions were as follows: 95 °C for 5 min, followed by 38 cycles of 95 °C for 30 s, 56 °C for 30 s, 72 °C for 1 min, and a final extension at 72 °C for 7 min. All the PCR products were purified, ligated into a pMD18-T vector (TaKaRa, Dalian, China) and sequenced.

#### Phylogenetic analysis

Eleven *L. chinensis* MADS-box predicted amino acid sequences and 35 related MADS-box genes in other plant species (five *Arabidopsis* MADS-box genes, twelve *O. sativa* MADS-box genes, four *H. vulgare* MADS-box genes, three *Aegilops tauscbii* subsp. *tauscbii* MADS-box genes, eleven *T. aestivum* MADS-box genes, one *B. distachyon* MADS-box gene) were selected to infer their evolutionary relationships using the maximum likelihood method in MEGA 6.0 [22, 23]. The other confirmed MADS-box protein sequences were obtained by a BLAST search from the National Center for Biotechnology Information (NCBI). The software DNAMAN was used to perform a multiple protein sequence alignment of 11 LcMADSs and 10 rice MADS-box factors.

#### RNA extraction and cDNA synthesis

Total RNA from different tissues, including glume, lemma, palea, stamen, carpel root, stem, and leaf, was isolated using an RNAiso Plus kit (Takara, Japan), according to the manufacturer’s instructions, and treated with DNase I (Takara, Japan). The quality of RNA was determined using 1% agarose gel electrophoresis. Then, the acceptable RNA was treated with DNase I (Takara, Japan) at 37 °C for 20–30 min. The cDNA was synthesized using the PrimeScript® RT Reagent Kit (TaKaRa, Dalian, China).
Real-time RT-PCR analysis
To analyze the expression profiles of these 11 MADS-box genes in different organs and under different abiotic stress conditions, quantitative real-time polymerase chain reaction (qRT-PCR) was performed using a LightCycler 480 System (Roche, Germany). For the qRT-PCR, the cDNA was diluted 1:50 with EASY Dilution (Takara, Japan). Each reaction contained 20 μl (10 μl SYBR Premix Ex Taq II (2×), 2.0 μl cDNA, 0.8 μM of each primer and 6.4 μl ddH₂O). The following program was performed: 95 °C for 30 s, 40 cycles of 95 °C for 5 s and 68 °C for 20 s. Three biological and three technical replicates were executed with \(LcACTIN\) as the internal control. The results of the qRT-PCR were analyzed with the \(2^{-\Delta\Delta Ct}\) method, and all primers used in this study are listed in Additional files 1: Table S1.

Yeast 2-hybrid analysis
Yeast two-hybrid (Y2H) analysis was performed to investigate the interactions among the 11 MADS-box proteins. Yeast two-hybrid analysis was performed using the GAL4 system (Clontech, Mountain View, CA, USA). The ORFs of the 11 MADS-box genes were inserted into the pGADT7 and the pGBK7 vectors and co-transformed into \(Saccharomyces\) \(cerevisiae\) strain AH109 to express the fusion proteins. The positive transformants were selected on SD/−Trp-Leu medium and confirmed by PCR. The empty vectors were used as a negative control. The interactions among these 11 MADS-box proteins were assayed on SD/−Trp-Leu-His-Ade medium supplied with 3 mM 3-AT at 30 °C for 4–6 d. For the autoactivation test, the single transformants with pGBK7 were tested by incubation on SD select medium (SD-His-Trp + 0, 1, 2, 3, 4, 5 or 10 mM 3-AT).

Results
Cloning and sequence analysis of 11 MADS-box genes in sheepgrass
As shown in Fig. 1a, the fertile floret of sheepgrass consists of two bract-like structures (a lemma and a palea), two lodicules, three stamens, and one pistil (stigma and ovary) from the outside to the inside. In our previous study, transcriptome sequencing techniques were used to study the self-incompatibility mechanisms of sheepgrass. Compared with mature stigmas, ovaries and leaves, we identified 1025 specifically or preferentially expressed genes of mature stigmas [24]. Based on the transcriptome data, we found that the transcript profiles of 21 putative MADS-box gene sequences were significantly different in the three tissues of sheepgrass (Fig. 1b, Additional files 2: Table S2), and among them, 7 MADS-box gene sequences were only highly expressed in ovaries, 4 were highly expressed in both stigmas and ovaries, and 3 were highly expressed in ovaries and leaves. However, 7 MADS-box gene sequences were obviously highly expressed in leaves. Furthermore, the full-length coding sequences (CDS) of 11 MADS-box genes were cloned and named \(LcMADS1, LcMADS2, LcMADS3, LcMADS4, LcMADS5, LcMADS6, LcMADS7, LcMADS8, LcMADS9, LcMADS10\) and \(LcMADS11\). The CDS and predicted amino acid sequences of these 11 \(LcMADS\) genes are listed in Additional files 3: Table S3, and all the sequences were submitted to NCBI (GenBank No. 1963149).

Multiple sequence alignment indicated that the 11 sheepgrass MADS-box genes had the typical MADS
domain structure in their coding proteins, and the MADS domain that localized in the N-terminus of each protein was highly conserved (Fig. 2). In addition, these sheepgrass MADS-box genes showed high consistency with their orthologs in *Oryza sativa* (Fig. 2).

**Phylogenetic analysis**

To determine the evolutionary relationship between the 11 sheepgrass MADS-box genes and those of other species, a phylogenetic tree was created according to the maximum likelihood method. Our results showed that these 11 MADS-box proteins in sheepgrass belonged to nine clades and shared high similarity with the *T. aestivum* or *H. vulgare* MADS-box sequences. The phylogenetic analysis clearly confirmed that *LcMADS1* and *LcMADS2* were closely related to *OsMADS14* and *OsMADS18*, respectively, and each of these genes belonged to the AP1-like subfamily (A-class), which is involved in specifying sepals and petals in *Arabidopsis* [25]. *LcMADS3*, *LcMADS5* and *LcMADS4*, together with their homologs *OsMADS2*, *OsMADS4* and *OsMADS16*, were assigned into the PI and AP3 clades (B-class), indicating that these three sheepgrass MADS-box genes function in the development of lodicules and stamens.

*LcMADS7*, *LcMADS8*, *LcMADS9* were divided into the SEP-like, MIKC*-type, and SVP-like clades, respectively (Fig. 3).

**Tissue-specific expression analysis of 11 sheepgrass MADS-box genes**

Based on the differential expression results of these MADS-box genes in the heat map analysis, we found that the expression of MADS-box genes was significantly different in three tissues of sheepgrass (Fig. 1b). To further investigate the expression patterns of the 11 MADS-box genes in different tissues and organs, we harvested the root, stem, leaf, and floral organs, which included glumes, lemmas, paleae, stamens and carpels for qRT-PCR. The results showed that most of these MADS-box genes in sheepgrass were highly expressed in the floral organ (Fig. 4a). PI-like genes *LcMADS3* and *LcMADS5* were highly expressed in stamens and carpels, but weak expression was detected in vegetative organs. The SEP-like gene *LcMADS7* showed high expression in all floral organs, such as stamens, glumes, lemmas and paleas. Interestingly, the expression of the *LcMADS2*, *LcMADS9* and *LcMADS11* genes was higher in vegetative organs (stem, leaf and root) than in floral organs.
(Fig. 4b), while the transcript abundance of LcMADS8 and LcMADS10 was higher in carpels than other tissues or organs (Fig. 4c).

**MADS-box genes from sheepgrass involved in abiotic stress**

Previous studies showed that some MADS-box genes are involved in stress tolerance [2], and our study investigated the responses of 11 MADS-box genes to abiotic stresses in sheepgrass. In this study, seedlings were exposed to 100 μM ABA, 300 mM mannitol, 200 mM NaCl, or a low-temperature treatment (4 °C).

As a whole, LcMADS1, LcMADS2, LcMADS3, and LcMADS9 were significantly induced by abiotic stresses (Fig. 5). LcMADS1 and LcMADS2 were strongly induced by cold (Fig. 5a, b). LcMADS3 was up-regulated when treated with ABA and mannitol, and its expression levels were higher at 12 h in the ABA treatment and at 4 h in the mannitol treatment (Fig. 5c). Furthermore, LcMADS9 was significantly induced by NaCl and its expression level reached its peak at 12 h (Fig. 5d). We also analyzed the stress-induced expression profile of the other LcMADS genes in sheepgrass, and our results indicated that LcMADS8 and LcMADS11 were up-regulated when treated with ABA, while LcMADS4 was down-regulated by ABA and mannitol treatment (Fig. 6a, b). However, the differences in the responses to different stresses were not significant for LcMADS5, LcMADS7, LcMADS10 or LcMADS11 (Fig. 6). Thus, we suggested that some MADS-box genes in sheepgrass may also be involved in the abiotic stress response as an escape strategy.

**Interaction analysis of 11 sheepgrass MADS-box proteins revealed by the yeast two-hybrid assay**

Protein interactions are essential not only for the normal roles that proteins play but also for expanding the functional diversities of proteins [26]. The yeast two-hybrid
assay is an effective method to discover interaction relationships in vitro and to understand molecular networks. We performed the yeast two-hybrid assays to investigate the protein-protein interaction relationships among the 11 MADS-box genes (two A-class genes, three B-class genes, one C-class gene, one SEP gene, one AGL12-like gene, one OsMADS32-like gene, one SVP-like gene, and one MIKC*-type gene) in sheepgrass. No self-activation was observed for any of the single constructs with pGBKT7 on SD selective medium (SD-His-Trp + 3 mM 3-AT). Of all the combinations of the 11 MADS-box proteins, only 13 combinations showed positive results on SD/−Trp-Leu-His-Ade medium (Fig. 7). The results showed that the A-class proteins LcMAD1 and LcMAD2 could both interact with the E-class protein LcMAD7, and LcMAD2 could also interact with the SVP-like LcMAD9 protein (Fig. 7a). The interaction of proteins of three B-class proteins, LcMADS3, LcMADS4, and LcMADS5, are shown in Fig. 7b. The results indicated that LcMADS3 interacted with LcMADS4 and could also interact with LcMADS10 and LcMADS7. LcMADS4 could interact with another B-class protein, LcMADS5. However, LcMADS5 interacted with the C-class protein LcMADS6 (Fig. 7b). In addition, our results showed that LcMADS7 could interact with four LcMADSs, but the interaction between LcMADS2 and LcMADS7 was very weak. LcMADS10, LcMADS9 and LcMADS7 could form homodimers, and the MIKC*-type gene LcMADS8 could not interact with any other LcMADSs (Fig. 7c).

**Discussion**

In the plant ABC (DE) model, MADS-box genes are critical transcription factors that are involved in floral organ identity specification [3, 27, 28]. A-, B-, C-, D-,
Fig. 5 Differential expression levels of four MADS-box genes in response to cold, salt, drought and ABA stress. Relative expression levels of (a) LcMADS1, (b) LcMADS2, (c) LcMADS3, and (d) LcMADS9 are shown. The transcript levels of the LcMADS genes at 0 h were used as controls.

Fig. 6 Differential expression of seven MADS-box genes in response to ABA (a) drought (b), cold (c) and salt (d) stress. The transcript levels of LcMADS genes at 0 h were used as controls.
and E-class genes have been confirmed in grass species, and there are four A-class genes, three B-class genes, two C-class genes, two D-class genes, and seven E-class genes (homologs of SEP and AGL6 genes in eudicots) in the *O. sativa* genome [11, 29–31]. Sheepgrass is a member of the tribe Triticeae and is one of the important perennial forage grasses with high quality and stress resistance in China [19, 32, 33]. In our previous studies, a large set of 1025 genes specifically expressed in the stigma was identified [24], and 21 putative MADS-box gene sequences were found in sheepgrass (Fig. 1b, Additional files 2: Table S2). Based on the transcriptome sequencing data, we identified 11 sheepgrass MADS-box genes (Fig. 2, Additional files 3: Table S3). These genes encoding proteins were divided into nine clades based on their evolutionary relationships, and all of them have close orthologs in *H. vulgare*, *T. aestivum* and *O. sativa*, indicating the large extent of conservation among the sheepgrass and other grass MADS-box gene families. Furthermore, two genes belong to the A class, and three genes belong to the B class (Fig. 3). This finding will provide valuable information for further studies of the functions of different MADS-box genes classes in sheepgrass.

Previous studies suggested that the A-class (AP1 and AP2) MADS-box genes are specific to the outermost sepals and that carpels are controlled by the C (AG)-class genes, while the combination of A-, B- (AP3 and P1) and E-class genes controls petal identity [34–36]. A large number of SEP-like genes have also been identified in monocots [37, 38]. In maize, there are at least eight SEP-like genes with distinguishable expression patterns that most likely reflect diverse functions [39]. In this study, the tissue expression patterns of 11 MADS-box genes revealed that most of these MADS-box genes in sheepgrass were highly expressed in floral organs, similar to the expression profiles of their homologous genes in *T. aestivum* and *O. sativa*. The tissue-specific expression analysis indicated that the *LcMADS1* gene was expressed higher in stamens, carpels, and glumes (Fig. 4a), while the functions of their homologs in rice *OsMADS14* were not only involved in specifying meristem identity but also palea and lodicule identities [5]. One AP1-like gene, *LcMADS2*, was expressed in reproductive organs similar to the expression of its homolog in *T. aestivum* and was also expressed in the leaf and root (Fig. 4b), consistent with the expression of its homolog *OsMADS18* [29]. Consistent with the expression patterns of PI in *Arabidopsis*, homologues in sheepgrass *LcMADS3* and *O. sativa OsMADS2* were also clearly expressed in the stamen and the carpel (Fig. 4a) [9, 40]. The *LcMADS9* gene had high homology with rice *OsMADS22* and *OsMADS55*, and overexpression of *OsMADS22* and *OsMADS55* led to abnormal floral morphologies including leaf-like sepals [41]. However, *OsMADS22* is expressed in non-vegetative tissues, and its ectopic expression induces spikelet meristem indeterminacy [42], while *LcMADS9* was not only expressed higher in flowers but also expressed relatively highly in vegetative organs, such as the stem and leaf (Fig. 4b). In addition, the expression pattern of the SEP-like gene *LcMADS7* was strongly expressed in the glume, lemma, palea and stamen (Fig. 4c).

In previous reports, some plant MADS transcription factors acted as crucial regulators in response to abiotic stresses [43]. For example, MADS-box genes have been shown to be affected by low temperature, photoperiod, and plant hormones such as cytokinins, gibberellins and ethylene [17, 44–46]. In this study, we found that the A-class *LcMADS1* and *LcMADS2* genes were both significantly up-regulated under cold stress (Fig. 5a, b), while the B-class *LcMADS3* gene was found to exhibit high expression in response to mannitol (4 h) and ABA (12 h) (Fig. 5c). These results suggested the two classes of genes function in different stress responses. We found that *LcMADS9* was induced by salt (Fig. 5d), while its

---

**Fig. 7** Yeast two-hybrid assays of 11 MADS-box proteins of sheepgrass. Protein-protein interactions of (a) two A-class MADS-box proteins, (b) three B-class MADS-box proteins, and (c) three other classes of MADS-box proteins. Serial dilutions (10⁻⁵–10⁻¹) of AH109 cells containing different plasmid combinations were grown on the selective medium plates SD/-Trp/-His/-Ade/3 mM 3-AT.
homolog OsMADS22 exhibited a different expression pattern; it was up-regulated by more than two-fold in response to cold and dehydration treatments [2]. In O. sativa, OsMADS26, an AGL12-class gene, has also been reported to be involved in drought tolerance [15], and its ortholog LcMADS11 was up-regulated by ABA (Fig. 6a). Taken together, our results revealed novel roles of LcMADS genes in response to abiotic stresses and may provide useful clues for future research on grass MADS family gene responses to abiotic stress signaling processes.

Interactions between MADS-box proteins are central to the ABCDE model of flower formation and development [26, 47]. In Arabidopsis, SEP proteins can directly interact with ABC MADS-domain proteins and act as bridges for higher-order complexes [4, 48]. Here, we used yeast two-hybrid systems to investigate the protein-protein interactions among 11 sheepgrass MADS box proteins (Fig. 7), and a composite figure was used to understand their molecular networks and provide a framework for the interaction capacity for these MADS-box proteins in sheepgrass and rice (Fig. 8). The direct interaction of B-class proteins with the SEP subfamily proteins has been demonstrated in the chrysanthemum and the tomato [47, 49, 50], and our study reconfirmed that B-class proteins could interact with SEP proteins. Interestingly, orthologues of LcMADS3 were able to interact with LcMADS4 and LcMADS10 in both sheepgrass and rice (Fig. 8). Phylogenetic analysis showed that LcMADS3 and OsMADS2 belonged to PI-class genes (Fig. 3). OsMADS2 was able to interact with the AP3 protein OsMADS16 (which had a close relationship with LcMADS4) and OsMADS32 (homologous to LcMADS10) [51, 52], and the LcMADS3 protein displayed the same interaction patterns in sheepgrass (Fig. 8, dotted line). Furthermore, the SEP-like protein LcMADS7 had an extensive interaction network that included PI-like (LcMADS3), AP1-like (LcMADS1 and LcMADS2), and AGL12-like proteins (LcMADS11), and it also formed a homodimer (LcMADS7). However, the SEP-like OsMADS5 protein cannot homodimerize or heterodimerize with other SEP proteins [53]. Two proteins, LcMADS1 and LcMADS2, belonged to the AP1 clade, and both of these proteins interacted with LcMADS7, while LcMADS2 could also interact with LcMADS9 (Fig. 7). LcMADS1 and LcMADS2 were recently identified and exhibited homology to OsMADS14 and OsMADS18, respectively (Fig. 8). Whether their paralogs in O. sativa has the same protein interaction patterns requires further verification. Hence, these SEP-like proteins of sheepgrass and O. sativa had different interaction partners, indicating that SEP-like proteins might have potentially novel functions in sheepgrass.

**Conclusion**

We first cloned 11 MADS-box genes in sheepgrass, which play important roles in flower development, and taken together, our results showed the expression patterns of LcMADS genes in various tissues and under different abiotic stress conditions. Our results indicated that the MADS-box genes LcMADS1 and LcMADS3 were highly expressed in sheepgrass stamens. The expression levels of LcMADS2 and LcMADS9 were high in

![Fig. 8 Interaction maps of putative orthologous MADS-domain proteins from sheepgrass and rice. The putative orthologs were the closest homologs derived from phylogenetic analysis. Each node represents putative orthologous proteins from two species. The nodes were named using sheepgrass MADS-domain proteins. Thick solid line, dashed dot line, and dotted line represent putative orthologous proteins were interacted in sheepgrass, rice, and both species, respectively.](image-url)
vegetative tissues. Meanwhile, \textit{LcMADS1}, \textit{LcMADS2}, \textit{LcMADS3} and \textit{LcMADS9} were significantly induced by abiotic stresses. In addition, we first demonstrated the interaction relationship between 11 sheepgrass MADS-box proteins, and our results indicated that \textit{LcMADS2} interacted with \textit{LcMADS7} and \textit{LcMADS9}. \textit{LcMADS3} interacted with \textit{LcMADS4}, \textit{LcMADS7} and \textit{LcMADS10}, while \textit{LcMADS1} could interact with only \textit{LcMADS7}. \textit{LcMADS7} could interact with four \textit{LcMADSs}. Hence, we proposed that \textit{LcMADS1}, \textit{LcMADS2}, \textit{LcMADS3}, \textit{LcMADS7} and \textit{LcMADS9} play a pivotal role in sheepgrass sexual reproduction and may be involved in abiotic stress responses. Our findings provide useful information for further exploration of the functions of this gene family in rice, wheat and other graminaceous cereals.

**Additional files**

- Additional file 1: Table S1. All primers used in this study. (XLSX 11 kb)
- Additional file 2: Table S2. The expression information of 21 putative MADS-box gene in stigma, leaf, ovary of sheepgrass based on the transcriptome data. (XLSX 11 kb)
- Additional file 3: Table S3. The sequences of 11 Sheepgrass MADS-box genes. (XLSX 15 kb)
- Additional file 4: Table S4. The GeneBank accession numbers of genes used in multiple sequence alignment. (XLSX 10 kb)

**Abbreviations**

- ABA: Abscisic acid; BLAST: Basic local alignment search tool;
- cDNA: Complementary DNA; CDS: Coding sequences; LcMADS: \textit{ Leymus chinensis} MADS-box genes; NaCl: Sodium chloride; NCBI: The national center for biotechnology information; qRT-PCR: Quantitative real-time polymerase chain reaction; SEP: SEPALATA

**Acknowledgements**

We thank Peiqiang Feng, Jinjue Liu and Tian Jiang (Chinese Academy of Sciences) for kindly providing the plasmids and for their excellent technical assistance in the yeast two-/three-hybrid experiments. We also thank Dr. Xiaobing Dong supplied much contribution in materials collection and management.

**Funding**

This work was supported by the National Basic Research Program of China (‘973’, 2014CB138704), the Project of Science and Technology Service Network Initiative of the Chinese Academy of Sciences (STS, KFJ-EW-STS-119). The funders had no role in the design of the study and collection, analysis, and interpretation of data and in writing the manuscript, but just provide the financial support.

**Availability of data and materials**

All relevant supplementary data is provided within this manuscript as Additional files 1, 2, 3 and 4. We have uploaded the sequence data and phylogeny data to the Treebase repository with the following Study Accession URL: http://purl.org/phylo/treebase/phylows/study/TB2/522364.

**Authors’ contributions**

GL, XL and LC conceived and designed the experiments. JJ performed most of the experiments. JJ, XL and PZ made substantial contributions to the data analysis and the manuscript writing. SC revised and edited the manuscript. GL gave the final approval the manuscript. GY, WY, SL and DQ were involved in performing the experiments; All authors read and approved the final manuscript.

**Ethics approval and consent to participate**

Not applicable.

**Consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

**Publisher’s Note**

Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

**Author details**

1. Key Laboratory of Plant Resources, Institute of Botany, The Chinese Academy of Sciences, Beijing, China. 2. University of Chinese Academy of Sciences, Beijing, China. 3. College of Biological and Food Engineering, Huaihua University, Huaihua, Hunan 418000, People’s Republic of China. 4. Institute of Animal Science of Heilongjiang Province, Qiqihar, Heilongjiang, China.

Received: 1 November 2017 Accepted: 1 March 2018

**References**

1. Causier B, Kieffer M, Davies B. Plant biology. MADS-box genes reach maturity. Science. 2002;296(5566):275–6.
2. Arora R, Agarwal P, Ray S, Singh AK, Singh VP, Tuagi AK, et al. MADS-box gene family in rice genome-wide identification, organization and expression profiling during reproductive development and stress. BMC Genomics. 2007;8:242.
3. Theissen G. Development of floral organ identity: stories from the MADS house. Curr Opin Plant Biol. 2001;4(1):75–85.
4. Horina T, Goto K. Complexes of MADS-box proteins are sufficient to convert leaves into floral organs. Nature. 2001;409(6819):525–9.
5. Wu F, Shi X, Lin X, Liu Y, Chong K, Theissen G, et al. The ABCs of flower development: mutational analysis of AP1/FUL-like genes in rice provides evidence for a homeotic (a)-function in grasses. Plant J. 2017;91(2):310–24.
6. Wang K, Tang D, Hong L, Xu W, Huang J, Li M, et al. DEP and AFO regulate reproductive habit in rice. PLoS Genet. 2010;6(11):1000818.
7. Kobayashi K, Yasuno N, Sato Y, Yoda M, Yamazaki R, Kimizu M, et al. Inference of flower meristem identity in rice is specified by overlapping functions of three AP1/FUL-like MADS box genes and PAP2, a SEPALATA MADS box gene. Plant Cell. 2012;24(5):1848–59.
8. Yadav SR, Prasad K, Vijayaghavan U. Divergent regulatory OsMADS2 functions control size, shape and differentiation of the highly derived rice floret second- and third-order organ. Genetics. 2007;176(1):283–94.
9. Yao SC, Dhmori S, Kimizu M, Yoshida H. Unequal genetic redundancy of rice PISTILLATA orthologs, OsMADS2 and OsMADS4, in lodicule and stamen development. Plant Cell Physiol. 2008;49(5):853–7.
10. Hama E, Takumi S, Ogihara Y, Murali K. Pistillody is caused by alterations to the class-B MADS-box gene expression pattern in alphawhisps. Planta. 2004;220(5):712–20.
11. Yasui Y, Tanaka W, Sakamoto T, Kurata T, Hirano HY. Genetic enhancer analysis reveals that FLORAL ORGAN NUMBER2 and OsMADS3 co-operatively regulate maintenance and determinacy of the flower meristem in Rice. Plant Cell Physiol. 2017;58(5):893–903.
12. Dreni L, Pilatore A, Yun DP, Ereni S, Pajoro A, Caporali E, et al. Functional analysis of all AGAMOUS subfamily members in Rice reveals their roles in reproductive organ identity determination and meristem maintenance. Plant Cell. 2011;23(8):2849–60.
13. Yamada K, Satake T, Shitsukawa N, Hirabayashi C, Takumi S, Murai K. Class D (sister) MADS-box genes are associated with ectopic ovule formation in the pistil-like stamens of alloplasmic wheat (Triticum aestivum L.). Planta. 2004;218(5):712–20.
14. Quan K, Li L, Hu P, Xu SP, Xu ZH, Xue HW. A brassinolide-suppressed rice MADS-box transcription factor, OsMDD1, has a negative regulatory role in BR signaling. Plant J. 2006;47(5):319–31.
15. Khong GN, Pati PK, Richaud F, Parizot B, Bidzinski P, Mai CD, et al. OsMADS26 negatively regulates resistance to pathogens and drought tolerance in Rice. Plant Physiol. 2015;169(4):2395–49.
16. Lee S, Choi SC, An G. Rice SVP-group MADS-box proteins, OsMADS22 and OsMADS55, are negative regulators of brassinosteroid responses. Plant J. 2008;54(1):93–105.

17. Lozano R, Angosto T, Gomez P, Payan C, Capel J, Huijser P, et al. Tomato flower abnormalities induced by low temperatures are associated with changes of expression of MADS-box genes. Plant Physiol. 1998;117(1):99–100.

18. Liu GS, Liu JS, Qi DM, Chu CC, Li HJ. Factors affecting plant regeneration from tissue cultures of Chinese leymus (Leymus chinensis). Plant Cell Tissue Org Cult. 2004;76(2):175–83.

19. Chen S, Huang X, Yan X, Liang Y, Wang Y, Li X, et al. Transcriptome analysis in sheepgrass (Leymus chinensis): a dominant perennial grass of the Eurasian steppe. PLOS One. 2013;8(7):e67974.

20. Bommert P, Satoh-Nagasawa N, Jackson D, Hirano HY. Genetics and evolution of inflorescence and flower development in grasses. Plant Cell Physiol. 2005;46(1):69–78.

21. Schmidt RJ, Ambrose BA. The blooming of grass flower development. Curr Opin Plant Biol. 1998;1(1):66–7.

22. Jones DT, Taylor WR, Thornton JM. The rapid generation of mutation data matrices from protein sequences. Comput Appl Biosci. 1992(3):275–82.

23. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: molecular evolutionary genetics analysis version 6.0. Mol Biol Evol. 2013;30(12):2725–9.

24. Zhou Q, Jia J, Huang X, Yan X, Wang Y, Li X, et al. Functional and evolutionary genetics analysis version 6.0. Mol Biol Evol. 2013;30(12):2725–9.

25. Kempin SA, Savidge B, Yanofsky MF. Molecular basis of the cauliflower phenotype in Arabidopsis. Science. 1995;267(5200):197–522.

26. de Folter S, Immink RG, Kieffer M, Parenicova L, Henz SR, Weigel D, et al. Comprehensive interaction map of the Arabidopsis MADS box transcription factors. Plant Cell. 2005;17(7):1424–33.

27. Smaczniak C, Immink RG, Angenent GC, Kaufmann K. Developmental and evolutionary diversity of plant MADS domain factors: insights from recent studies. Development. 2012;139(17):3081–98.

28. Zhou Y, Xu Z, Yong X, Ahmad S, Yang W, Cheng T, et al. SEP-class genes in Arabidopsis thaliana functions in floral organ and meristem identity. Curr Opin Plant Biol. 2004;7(17):1935–64.

29. Liu GS, Liu JS, Qi DM, Chu CC, Li HJ. Factors affecting plant regeneration from tissue cultures of Chinese leymus (Leymus chinensis). Plant Cell Tissue Org Cult. 2004;76(2):175–83.

30. Zhang L, Mao D, Xing F, Bai X, Zhao H, Yao W, et al. Loss of function of OsMADS32 interaction of the AP1/SEP/AGL6 superclade of MADS-box genes in the basal eudicot Epimedium sagittatum. Ann Bot. 2014;113(4):653–78.

31. Yamaguchi T, Lee DY, Miyao A, Hirochika H, An G, Hirano HY. Functional characterization of OsMADS18, a member of the AP1/SQUA subfamily of MADS-box genes. Plant Physiol. 2004;135(6):2207–19.

32. Zhang L, Mao D, Xing F, Bai X, Zhao H, Yao W, et al. Loss of function of OsMADS3 via the insertion of a novel retrotransposon leads to recessive male sterility in rice (Oryza sativa). Plant Sci. 2015;238:188–97.

33. Yamaguchi T, Lee DY, Miyao A, Hirochika H, An G, Hirano HY. Functional diversification of the two C-class MADS box genes OS/MADS and OS/MADS55 in Oryza sativa. Plant Cell. 2005;17(1):10.

34. Fornara F, Parenicova L, Falasca G, Pelucchi N, Masiero S, Ciannammea S, et al. Functional characterization of OsMADS18, a member of the AP1/SQUA subfamily of MADS-box genes. Plant Physiol. 2004;135(4):2207–19.

35. de Folter S, Immink RG, Kieffer M, Parenicova L, Henz SR, Weigel D, et al. Comprehensive interaction map of the Arabidopsis MADS box transcription factors. Plant Cell. 2005;17(7):1424–33.

36. Sun W, Huang W, Li Z, Song C, Liu D, Liu Y, et al. Molecular basis of the cauliflower phenotype in Arabidopsis. Science. 1995;267(5200):197–522.

37. Becker A, Theissen G. The major clades of MADS-box genes and their role in the development and evolution of flowering plants. Mol Phylogenet Evol. 2001;16(3):89–105.

38. Liu GS, Liu JS, Qi DM, Chu CC, Li HJ. Factors affecting plant regeneration from tissue cultures of Chinese leymus (Leymus chinensis). Plant Cell Tissue Org Cult. 2004;76(2):175–83.

39. Chen S, Huang X, Yan X, Liang Y, Wang Y, Li X, et al. Transcriptome analysis in sheepgrass (Leymus chinensis): a dominant perennial grass of the Eurasian steppe. PLOS One. 2013;8(7):e67974.

40. Prasad K, Vijayraghavan U. Double-stranded RNA interference of a rice PV Glycoalkaloid, OsMADS2, uncovers its second-whorl-specific function in floral organ patterning. Genetics. 2003;165(4):2301–5.

41. Lee JH, Park SH, Ahn JH. Functional conservation and diversification between rice OsMADS22/OsMADS55 and Arabidopsis SVP proteins. Plant Sci. 2012;185:189–97.

42. Sentoku N, Kato H, Kitano H, Imai R. OsMADS22, an STMSADS11-like MADS-box gene of rice, is expressed in non-vegetative tissues and its ectopic expression induces spikelet meristem indeterminacy. Mol Gen Genomics. 2005;273(1):1–9.

43. Gupta SK, Rai AK, Kanwar SS, Chand D, Singh NK, Sharma TR. The single functional blast resistance gene Pi54 activates a complex defence mechanism in rice. J Exp Bot. 2012;63(2):757–72.

44. Ando S, Sato Y, Kamachi S, Sakai S. Isolation of a MADS-box gene (ERAF17) and correlation of its expression with the induction of female flowers by ethylene in cucumber plants (Cucumis sativus L). Planta. 2001;213(6):943–52.

45. Bonhomme F, Kurz B, Melzer S, Bernier G, Jacquard A, Cytokinin and gibberellin activate SAMSAd a, a gene apparently involved in regulation of the floral transition in Sisiphus alba. Plant J. 2000;24(1):103–11.

46. Lee JH, Park SH, Ahn JH. Functional conservation and diversification between rice OsMADS22/OsMADS55 and Arabidopsis SVP proteins. Plant Sci. 2015;238:188–97.

47. Shchennikova AV, Shulga OA, Immink R, Skyanbin KG, Angenent GC. Identification and characterization of four chrysanthemum MADS-box genes, belonging to the APETALA1/FRUITFULL and SEPALLATA subfamilies. Plant Physiol. 2004;134(6):1632–41.

48. Yang Y, Jack T. Defining subdomains of the K domain important for protein–protein interactions of plant MADS-box proteins. Mol Plant. 2006;235(5):504–13.

49. Sentoku N, Kato H, Kitano H, Imai R. OsMADS22, an STMADS11-like MADS-box gene from sheepgrass, confers salt stress tolerance in transgenic Arabidopsis. Mol Genet Genomics. 2005;273(1):1–9.

50. Bonhomme F, Kurz B, Melzer S, Bernier G, Jacquard A, Cytokinin and gibberellin activate SAMSAd a, a gene apparently involved in regulation of the floral transition in Sisiphus alba. Plant J. 2000;24(1):103–11.

51. Lee JH, Park SH, Ahn JH. Functional conservation and diversification between rice OsMADS22/OsMADS55 and Arabidopsis SVP proteins. Plant Sci. 2015;238:188–97.

52. Seok HY, Park HY, Park JI, Lee YM, Lee SY, An G, et al. Rice ternary MADS protein complexes containing class B MADS heterodimer. Biochem Biophys Res Commun. 2010;401(4):598–604.

53. Cu K, Han J, Zhao S, Su K, Wu D, Xu X, et al. Functional conservation and diversification of class E floral homeotic genes in rice (Oryza sativa). Plant J. 2010;61(5):767–81.

Submit your next manuscript to BioMed Central and we will help you at every step:

• We accept pre-submission inquiries
• Our selector tool helps you to find the most relevant journal
• We provide round the clock customer support
• Convenient online submission
• Inclusion in PubMed and all major indexing services
• Maximum visibility for your research

Submit your manuscript at www.biomedcentral.com/submit