Identification and Characterization of a SEPALLATA-like MADS-box Gene from Cucumber (Cucumis sativus L.)

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Abstract

SEPALLATA (SEP) genes encode the E class MADS-box transcription factors that play vital roles in various aspects of plant growth and development. In this study, we isolated a SEP-like gene from cucumber (Cucumis sativus L.), which was previously named as CsMADS01. CsMADS01 had a coding sequence (CDS) of 741 bp, and coded a deduced protein of 246 amino acid residues that was predicted to be located in the nucleus. The putative CsMADS01 protein was typically characterized by the MIKC-type MADS (including MADS, I, K, and C domains) and shared high homology with other SEP-like proteins. Phylogenetic analysis of CsMADS01 and SEP proteins from other plants revealed that CsMADS01 was a member of the SEP1/2 clade of SEP proteins and was the most closely related to ZjMADS48 from Chinese jujube. Promoter analysis showed that several cis-elements related to stress response and hormones were present in the promoter region of CsMADS01. Expression analysis indicated that CsMADS01 was highly expressed during the development of male and female flowers, and the expression increased gradually along with fruit development. Ectopic expression of CsMADS01 in Arabidopsis resulted in a phyllody-like phenotype and the transgenic plants never flowered. These results suggest that CsMADS01 plays an important role in the growth and development of cucumber.

Keywords: cucumber; gene expression; MADS-box; SEPALLATA (SEP); transgenic Arabidopsis

Introduction

MADS-box family genes encode transcription factors characterized by the presence of 58-60 highly conserved N-terminal DNA binding domain (termed as the MADS domain), which is conserved across a wide range of organisms including plants, fungi, and mammals (Riechmann and Meyerowitz, 1997; Xu et al., 2014; Nardelli et al., 2018). According to the phylogenetic analysis, the MADS-box genes can be divided into two functional types named as type I and type II (Alvarez-Buylla et al., 2000; Masiere et al., 2011), and the type II members are also termed as MIKC-type MADS for the presence of three additional domains from N-terminal to C-terminal: intervening (I) domain, keratin (K) domain, and C-terminus (C) domain, when compared with the type I members (Kaufmann et al., 2005; Ren et al., 2017; Zhou et al., 2019b). The MIKC-type MADS genes are well-known for their roles in floral organ development. Except for APETALA2 (AP2), all A, B, C, D, and E class genes belong to the MIKC-type MADS-box genes according to the hypothesis of floral organ ABCDE model, and these genes specify the formation of floral organs in a combinatorial way (Weigel and Meyerowitz, 1994; Gutierrez-Cortines and Davies, 2000; Zahn et al., 2006; Theissen et al., 2016).

SEPALLATA (SEP) genes are the E class genes that are preferentially expressed in flowers and fruits for plant floral organ development (Honma and Goto, 2001; Theissen and Saedler, 2001). The SEP genes were first described in tomato and petunia, and inhibition of their expression would result in highly aberrant flowers (Angenent et al., 1994; Pnueli et al., 1994). Arabidopsis contains four SEP

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genes (SEP1-4), which are expressed in all four whorls of floral organs and play redundant roles in flower meristem identity and organ identity. In sep1 sep2 sep3 triple-mutants, flowers possessed only sepal, while all floral organs were replaced by leaf-like organs in sep1 sep2 sep3 sep4 quadruple mutants (Pelaz et al., 2000; Ditta et al., 2004; Malcomber and Kellogg, 2005). In addition, the sep1-sep2-sep4 triple mutants displayed a phenotype similar to that of wild-type (WT) plants in floral organ development, revealing that SEP3 is much more critical for flower development than other SEP genes in Arabidopsis (Ditta et al., 2004). Rice contains five floral homeotic genes (OsMADS1, 5, 7, 8 and 34), all of which display both functional conservation (E function) and diversification (Cui et al., 2010; Gao et al., 2010; Kobayashi et al., 2012; Khanday et al., 2013; Lin et al., 2014; Meng et al., 2017; Wu et al., 2018). In addition, the SEP proteins can form MADS-box protein complexes with proteins of other classes for floral organ development. For example, OsMADSS5 and OsMADSS4 can physically interact with candidate class A, B, C, D, E, and AGL6-like floral homeotic proteins to control inflorescence and spikelet morphogenesis (Kobayashi et al., 2012; Hu et al., 2015; Meng et al., 2017; Wu et al., 2018). Besides floral organ development, SEP genes are also involved in many other aspects of growth and development in plants, such as fruit development (Ito et al., 2017; Li et al., 2017), stress response (Chen et al., 2019), bud growth and dormancy (Zhang et al., 2017b). These findings reveal that the E class SEP genes play essential roles in many aspects of plant growth and development with both redundancy and specificity (Soza et al., 2016; Zhang et al., 2017b).

Our previous report has indicated that there are four SEP genes (CsMADS01–CsMADS04) in cucumber genome (Hu and Liu, 2012), while only CsMADS02 was cloned and functionally characterized (Zhou et al., 2019a). In the present study, another SEP gene (CsMADS01) was isolated from cucumber and its expression profile during flower and fruit development was examined. In addition, a phyllody-like phenotype was observed in the CsMADS01-overexpressing Arabidopsis plants. These findings suggest that CsMADS01 plays an important role in the growth and development of cucumber.

Materials and Methods

Plant materials and growth conditions

Cucumber (Cucumis sativus var. sativus line 9930) and Arabidopsis thaliana ecotypes Columbia-0 (Col-0) were used in this study. Cucumber plants were planted in the field of Jiangxi Agricultural University, Nanchang, China. Different developmental stages of female flowers (MF) and male flowers (FF) were collected for expression profile analysis according to our previous study (Zhou et al., 2019b). The wild-type (WT) and transgenic Arabidopsis seeds were germinated on one-half Murashige and Skoog medium (1/2 MS) and the seedlings were grown on a plant growth chamber in a long-day photoperiod (16-h light/8-h dark cycle) at 22-24 °C.

RNA extraction and cloning of the CsMADS01 gene

Total RNA was extracted from cucumber female flowers with the TransZol Reagent kit (TransGen, China) following the manufacturer’s protocol. After checking the integrity with a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, USA), the first-strand cDNA was synthesized with Transcript All-in-One First-Strand cDNA Synthesis SuperMix for qPCR (One-Step gDNA Removal) (TransGen, China). The semi-quantitative reverse transcription PCR (RT-PCR) was carried out to amplify the coding sequence (CDS) of the CsMADS01 gene by using the specific primers CsMADS01-1F (5'-aaaaCCATGGATGGAAGAGGAAGATC-3') and CsMADS01-1R (5'-aaaaAGATCTTCACAGCTTCAAGCACCAGGGAG-3') based on the sequence of CsMADS01 (Gene ID: Cs0004117) (Hu and Liu, 2012), with the procedure described in our previous study (Zhou et al., 2019b). PCR products were cloned into the pMD18-T vector (TaKaRa, Japan) and sequenced (Shanghai Sangon, China).

Bioinformatics analysis

The putative CsMADS01 protein sequence was uploaded to ProtParam (http:// web.expasy.org/protParam/) to calculate the theoretical molecular weight (MW), isoelectric point (pI), and grand average of hydropathy index (GRAVY). The SOPMA tool (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_sopma.html) was used to analyze the secondary structure of CsMADS01. The subcellular localization of CsMADS01 was predicted using the online servers ProtComp 9.0 (http://linux1.softberry.com/berry.phtml), and WoLF PSORT (https://www.genscript.com/tools/wolf-psort.html). The amino acid sequences of CsMADS01 and other selected MADS-box proteins from various plant species were aligned by Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/), and the alignments were used to create a neighbor-joining (NJ) phylogenetic tree with the MEGA 7.0 software. The bootstrap was set as 1000 replicates. To predict cis-elements in promoter of CsMADS01, the 1,500 bp regions upstream of the initiation codon (ATG) of CsMADS01 was examined by the PlantCARE database (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/).

Gene expression analysis

RT-PCR was performed to examine the expression of CsMADS01 in different developmental stages of MF and FF using the reaction procedure previously described (Zhou et al., 2019a). The primer sequences are listed in Table S1. For expression analysis of CsMADS01 during fruit development, the RPMK values of CsMADS01 were obtained from the fruitENCODE database (http://www.epigenome.cuhk.edu.hk/ZhongWeb/cucumb er/cucumber_index.jsp) as described previously (Lü et al., 2018).

Vector construction, Arabidopsis transformation and phenotypic analysis

The CDS of CsMADS01 in pMD18-T vector was
digested with the restriction sites NeI and BglII, and ligated into the pCAMBIA1301 vector to generate the overexpression construct pCAMBIA1301-CsMADS01. Then, the resulting plasmid was introduced into Agrobacterium tumefaciens GV3101, and GV3101 containing pCAMBIA1301-CsMADS01 was transformed into Col-0 via the floral dip method (Clough and Bent, 1998). The transgenic Arabidopsis seeds were screened by growing on 1/2 MS medium supplemented with 50 mg/L hygromycin, and transferred to soil. The transcription levels of CsMADS01 were examined in the transgenic plants by RT-PCR, and the AtTubulin4 gene was used as the internal control, with the procedure described previously (Zhou et al., 2018). The primer sequences are listed in Table S1. T3 homozygous offspring were employed for phenotypic analysis.

Results and Discussion

Cloning and sequence analysis of the CsMADS01 gene

RT-PCR was used to amplify the ORF of CsMADS01 gene using specific primers based on the sequence of the CsMADS01 gene (Gene ID: Csa004117) in our previous study (Hu and Liu, 2012). The gene contained the sequence of the complete CDS (741 bp), which encoded a putative protein of 246 amino acids (Fig. 1A). The SMART analysis showed that CsMADS01 possessed the representative MADS and K-box domains, which were located in the positions of 1-60 and 80-172 amino acids, respectively (Fig. 1A). The secondary structure analysis by SOPMA revealed that CsMADS01 had a theoretical MW of 28.22 kDa, a pI of 9.20, and a GRAVY value of -0.628, suggesting that it is a hydrophilic and basic protein. Finally, ProtComp and WoLF PSORT results revealed that CsMADS01 was localized to the nucleus. These results indicated that CsMADS01 encodes a MADS-box transcription factor.

Characterization of CsMADS01

BLAST search showed that CsMADS01 had the highest homology to Csa4G126990 in cucumber. It should be noted that the amino acid sequence of Csa4G126990 was identical to the positions of 63-246 amino acids of CsMADS01, and the positions of 1-60 of CsMADS01 was the MADS-box domain (Figs. 1 and 2), which is an indispensable domain for SEP members. Coincidentally, the amino acid sequence of CsMADS01 was identical to the corrected sequence of Csa4G126990, which was formally named as CsSEP2 (Wang et al., 2016). In addition, the CDS and gDNA sequences of CsMADS01 were also identical to the full sequences of CsSEP2 (Table S2) (Wang et al., 2016).
et al., 2016). These results demonstrated that CsMADS01 is identical to CsSEP2, which may be a functional SEP in cucumber.

Our previous study has shown that CsMADS01 is a member of SEP subfamily (Hu and Liu, 2012). We then compared the amino acid sequence of CsMADS01 with that of other SEP proteins in literature references by using Clustal Omega. The amino acid sequence of CsMADS01 showed 53.68-85.19% identity with these SEP proteins, such as OsMADS34 in rice (53.68% identity) (Gao et al., 2010; Kobayashi et al., 2010), GhSEP in Ginkgo biloba (55.00% identity) (Cheng et al., 2016), CaMADS in cucumber (60.68% identity) (Chen et al., 2019), CsMADS02 in cucumber (69.70% identity) (Zhou et al., 2019a), AtSEP2 in Arabidopsis (74.07% identity) (Pelaz et al., 2000), BoMADS5 in Brassica oleracea (76.33% identity) (Sheng et al., 2019), AtSEP1 in Arabidopsis (76.45% identity) (Pelaz et al., 2001), FaMADS9 in strawberry (80.74% identity) (Seymour et al., 2011), MdMADS8 in apple (81.48% identity) (Ireland et al., 2013), ZjMADS48 in Chinese jujube (82.32% identity) (Zhang et al., 2017a), and PrupSEP1/PrpMADS7 in peach (85.19% identity) (Xu et al., 2008; Li et al., 2017). In addition, all the SEP proteins contained a highly conserved MADS domain, followed by less conserved I and K domains, and a highly variable C domain (Fig. 2), which is the characteristic of the MIKC+ group of Type II MADS-box proteins (Ren et al., 2017; Zhou et al., 2017). Moreover, two additional conserved motifs, SEP I and SEP II, were present in the C-terminus of these proteins (Fig. 2), which is typical for SEPs and was also found in other SEP proteins, such as RanSEP3 (Soza et al., 2016), and HrSEP-2 (Mitoma and Kanno, 2018).

Phylogenetic analysis of CsMADS01 and other SEP subfamily members

Several gene duplications might have occurred in the most recent common ancestor of angiosperms and gymnosperms, resulting in the division of the SEP-like genes of angiosperms into two major clades, the LIOSEP clade (also called SEP1/2/4 clade or AGL12/3/4 clade) and the SEPS clade, within the SEP phylogeny (Malcomber and Kellogg, 2005; Zahn et al., 2005). To study the phylogenetic relationships of CsMADS01 and other SEP subfamily members, a phylogenetic tree was created based on the amino acid sequences of CsMADS01, CsMADS02 (Zhou et al., 2019a), and SEP proteins from Arabidopsis (Pelaz et al., 2000), rice (Arora et al., 2007), Chinese jujube (Zhang et al., 2017a), and Brassica oleracea (Sheng et al., 2019). These SEP proteins can be clustered into three clades, namely SEP1/2, SEP3, and SEP4 (Fig. 3), which is consistent with the evolution lines reported in previous studies (Xu et al., 2008; Ireland et al., 2013; Zhou et al., 2017; Zhou et al., 2019a). CsMADS01 shared high genetic homology with ZjMADS48, AtSEP1, AtSEP2, and BoMADS5 in the SEP1/2 clade (Fig. 3), suggesting that they may have similar functions.

Cis-element analysis of the CsMADS01 promoter

To investigate the possible regulatory functions of the CsMADS01 gene, the 1,500 bp region upstream of the translational initiation codon of CsMADS01 was analyzed. As a result, three kinds of hormone-related cis-elements, such as CGTCA-motif, ABRE, and TCA-element, which are involved in response to methyl jasmonate (MeJA), abscisic acid (ABA), and salicylic acid (SA), respectively, were identified in the CsMADS01 promoter. In addition, four kinds of stress-related cis-elements were present in the CsMADS01 promoter, such as W-box response to fungal elicitor, MBS response to drought and salt stress, ARE response to anaerobic induction. These results indicated that CsMADS01 plays possible roles in hormone and stress responses. Recently, a SEP gene in pepper, CaMADS, was found to act as a positive stress-responsive regulator in the cold, salt, and osmotic stress signaling pathways (Chen et al., 2019).

Expression analysis of CsMADS01 during flower and fruit development

Previous results have shown that CsMADS01 is primarily expressed in flowers (Hu and Liu, 2012; Wang et al., 2016). To investigate the possible function of CsMADS01 in flower development, RT-PCR was used to examine the expression of CsMADS01 in five different developmental stages of MF and FF as described in a previous study (Zhou et al., 2019b). As shown in Fig. 5A and Fig. 5B, CsMADS01 gene was continuously and highly expressed during the development of male and female

![Fig. 2. Multiple sequence alignment of CsMADS01 with SEP proteins from other plant species. The MADS, I, K, and C domains are underlined. Two conserved motifs, SEP I and SEP II, are boxed.](image-url)
Fig. 3. Phylogenetic analysis of CsMADS01 and other SEP subfamily members from different plant species. Different SEP proteins from cucumber (red round, Cs), *Arabidopsis* (brown square, At), rice (purple rhombus, Os), and *Brassica oleracea* (green square, Bol) were aligned with Clustal Omega and the phylogenetic tree was created using the NJ method in MEGA 7.0 by bootstrap analysis with 1000 replicates. CsMADS01 is bolded.

Fig. 4. Putative cis-element analysis of the CsMADS01 promoter.
flowers, suggesting that it may play an essential role in flower development of cucumber. Similarly, all three SEP subfamily genes in Chinese jujube (ZjMADS30, ZjMADS47 and ZjMADS48) were highly expressed in the sepal, petal and pistil (Zhang et al., 2017a). In mulberry (Morus notabilis), all four SEP subfamily genes also displayed floral organ specific expression (Luo et al., 2018). Two SEP subfamily genes in B. olaracea, BolMADS5 and BolMADS6, also exhibited relatively high expression in four whorls of the flower including the sepal, petal, stamen, and pistil, while another SEP subfamily gene (BolMADS9) was rarely expressed in these tissues (Sheng et al., 2019). Our previous study also revealed that CsMADS02, another SEP gene in cucumber, also displayed the highest expression in flowers, especially female flowers (Zhou et al., 2019a). However, the expression pattern of CsMADS02 during female and male flower development was different from that of CsMADS01 (Figs. 5A and 5B). These results indicated that CsMADS01 and CsMADS02 may have different functions during flower development in cucumber. This phenomenon may due to the different evolution lines of the two SEP genes: CsMADS01 and CsMADS02 are members of the SEP1/2 and SEP3 evolution lines, respectively (Fig. 3). We also examined the expression of CsMADS01 during fruit development using the transcriptome data from the fruitENCODE database. As shown in Fig. 5C, the expression of CsMADS01 increased gradually from 10 DPA to 40 DPA during fruit development, while no expression was detected in the leaves. An exon skipping in the CsMADS01/CsSEP2 gene resulted in defective fruits and enlarged sepal in both female and male flowers (Wang et al., 2016). These results indicated that CsMADS01 may also function in fruit ripening. Similarly, two apple SEP1/2 evolution line genes MdMADS8 and MdMADS9 exhibited higher expression during fruit maturation, and play important roles in both fleshy fruit development and fruit ripening (Ireland et al., 2013). PrunSEP1 was also found to be involved in the regulation of ripening and softening of melting flesh peach (Li et al., 2017). A tomato SEP gene, MADS-RIN (or RIN), plays vital roles in regulating various aspects of fruit development and ripening through controlling many important ripening genes (Ito et al., 2017; Li et al., 2018; Li et al., 2019).

Overexpression of CsMADS01 caused a phyllody-like phenotype of transgenic Arabidopsis

To elucidate the potential functions of CsMADS01, the gene was overexpressed in Arabidopsis under the control of the cauliflower mosaic virus (CaMV) 35S promoter. After transformation of Arabidopsis, most transgenic plants displayed significantly altered phenotypes, and two independent transgenic lines with higher expression of CsMADS01, named as OE1 and OE2 (Fig. 6A), were used for phenotypic analysis. The transgenic plants displayed increased numbers of leaves coupled with increased formation of branches, leading to a leafy phenotype (Fig. 6B and 6C). Besides, the phenotype of high-expression transgenic line (OE2) was more severe than that of low-expression transgenic line (OE1), suggesting that the leafy phenotype was due to the ectopic expression of CsMADS01 in Arabidopsis. Similarly, altered leaf phenotypes were also reported for the ectopic expression of SEP genes in Arabidopsis, such as LMADS3 (Tzeng et al., 2003), TaMADS1 (Zhao et al., 2006), and CsMADS02 (Zhou et al., 2019a). In addition, the transgenic plants never flowered, while the WT plants could flower normally (Fig. 6D), indicating the flower organs were replaced by leaves in the transgenic plants, resulted in a phyllody-like phenotype. In Arabidopsis, all the four SEP genes play vital roles in specifying the identities of four whorls of the flower and determine floral meristem identity (Pelaz et al., 2000; Ditta et al., 2004; Malcomber and Kellogg, 2005). Single SEP gene mutations resulted in only subtle or no changes in phenotypes when compared with the WT, while floral organs were replaced by leaf-like organs in the sep1 sep2 sep3 sep4 quadruple mutant (Ditta et al., 2004). A phytoplasmal secreted protein PHYL1 interacts with and degrades the floral homeotic MADS domain proteins APETALA1 (AP1) and SEP1-4 proteins, resulting in the transformation of flower organs into leaf-like structures (Maejima et al., 2014; Maejima et al., 2015). In rice, OsMADS34 is also involved in the transition from vegetative to reproductive growth via the specification of inflorescence meristem identity (Gao et al., 2010), and can act as a transcriptional activator, promoting the formation of male and female flowers in rice (Sun et al., 2016). Therefore, it is possible that CsMADS01 could play a similar role in cucumber, especially in the development of female flowers, as it is highly expressed in both female and male flowers (Zhou et al., 2019a). Furthermore, the expression pattern of CsMADS01 in cucumber was similar to the expression pattern of OsMADS34 in rice (Gao et al., 2010), suggesting that CsMADS01 may also function in flower development in cucumber. (A and B) RT-PCR analysis of CsMADS01 in five different developmental stages (MF1 to MF5, and FF1 to FF5) in cucumber. (C) In silico expression analysis of CsMADS01 during fruit development in cucumber. The expression of CsMADS01 is presented as RPKM values.

Fig. 5. Expression analysis of CsMADS01 during flower and fruit development in cucumber. (A and B) RT-PCR analysis of CsMADS01 in five different developmental stages (MF1 to MF5, and FF1 to FF5) in cucumber. (C) In silico expression analysis of CsMADS01 during fruit development in cucumber. The expression of CsMADS01 is presented as RPKM values.
repressor to influence grain yield by suppressing the transcription of related genes (Ren et al., 2016). The sterile lemmas, lemmas, paleas, and the inner floral organs were converted into leaf-like structures in the osmads1-2 osmads5-3 osmads34-1 triple mutant (Wu et al., 2018). A recent study showed that compared with Rosa chinensis cv. Old Blush, R. chinensis cv. Viridiflora displayed a phyllody phenotype, in which flower organs were converted into leaf-like organs, and this phenomenon is associated with the modified expression of the genes related to flowering and flower development, including RcSEP1 and RcSEP3 (Yan et al., 2016). Therefore, it can be speculated that the phyllody-like phenotype of CsMADS01-overexpressing transgenic Arabidopsis plants may be due to the misexpression of Arabidopsis endogenic SEPs and other flower organ identity genes. Similarly, ectopic expression of a wheat SEP-like gene, TaMADS1, caused early flowering and abnormal development of all floral organs in Arabidopsis, including the conversion of sepals into leaf-like structures, which may be due to the regulation of some flower meristem identity genes (Zhao et al., 2006).

Conclusions

In summary, we performed the isolation and functional characterization of CsMADS01 from cucumber. This gene was previously named as CsSEP2 by a previous study (Wang et al., 2016), and is involved in floral organ and fruit development. The deduced CsMADS01 protein contains the typical MADS, I, K, and C domains, as well as two conserved motifs (SEP I and SEP II) specific for SEP proteins. Overexpression of CsMADS01 in Arabidopsis led to a phyllody-like phenotype and the transgenic plants never flowered. These results lay a foundation for further revealing the regulatory mechanisms of flower and fruit development in cucumber.
Acknowledgements

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Conflict of Interest

The authors declare that there are no conflicts of interest related to this article.

References


Table S1. RT-PCR primers used for gene expression analysis in this study

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<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
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<tr>
<td>CsMADS01</td>
<td>AGAAACCTTCTTGGGAGGA</td>
<td>GAAGCCATTGACTTGTGGG</td>
</tr>
<tr>
<td>CsAct3</td>
<td>GACATTCAATGTGCTTGCTATG</td>
<td>CATACCGATGAGATGGCTG</td>
</tr>
<tr>
<td>AtTubulin4</td>
<td>GCGAACAGTTCAAGCTATGCTCA</td>
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