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Ectopic expression of *CsMADS24*, an *AGAMOUS* ortholog from cucumber, causes homeotic conversion of sepals into carpels in transgenic *Arabidopsis* plants

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Abstract: The floral homeotic C-function MADS gene *AGAMOUS* (*AG*) in *Arabidopsis* plays crucial roles in specifying stamen and carpel identities as well as determining floral meristem. However, there have been only a few studies of floral homeotic C-function genes in cucumber thus far. In the present study, *CsMADS24*, a putative *AG* ortholog from cucumber, was isolated and characterized. Sequence analysis and protein sequence alignment revealed that the deduced CsMADS24 protein contained the typical MIKC structure and the N-terminal extension, as well as two highly conserved AG motifs (I and II). Phylogenetic analysis showed that CsMADS24 fell into the clade of core eudicots, while being distant from the AG orthologs of basal eudicots, monocots and gymnosperms. Expression analysis by RT-PCR showed that *CsMADS24* was exclusively expressed in female flower buds. *In situ* hybridization revealed that *CsMADS24* expression was only detected in the carpels. Functional analyses indicated that the sepals were partly converted into carpelloid-like structures in 35S::35S::*CsMADS24* transgenic plants. In addition, earlier flowering and delayed floral organ abscission during the development of siliques were also observed in transgenic *Arabidopsis*. Our findings demonstrate that the *AG* ortholog plays an exclusive role in carpel specification of cucumber, providing a basis for revealing the mechanisms of reproductive development in cucumber.

Keywords: cucumber; MADS-box; gene expression; *AGAMOUS*; phylogenetic analysis; transgenic *Arabidopsis*

INTRODUCTION

Normal development of floral organs is the basis for plant breeding, as well as for key physiological processes for improving the yield of agricultural products. Research on floral organ development can be of great importance for plant improvement. The analysis of the homeotic floral mutants of *Arabidopsis thaliana* and *Antirrhinum majus* resulted in the formulation of a genetic model named as the ABCDE model, which explains how the functions of five classes of genes (A, B, C, D and E) are combined to specify four different floral organs [1-4]. Nearly all of these genes encode MIKC-type MADS-box transcription factors, which contain a 60-amino acid MADS-box domain in the N-terminal region, a less conserved intervening region contributing to the DNA binding specificity and dimerization, a moderately conserved keratin-like domain involved in protein-protein interaction, and a highly variable C-terminal region [5-7].

In *Arabidopsis*, the C-class gene *AGAMOUS* (*AG*) plays a critical role in specifying stamen and carpel identities as well as determining floral meristem [8,9]. The *Arabidopsis ag* mutant has normal sepals and petals, but there was a homeotic conversion of stamens into petals, and the carpals were replaced by another flower [9,10]. Overexpression of *AG* induces a conversion of sepals into carpels and petals into stamens in the flower [9]. To date, the *AG* orthologs have been identified and characterized from a diverse number of plant species, such as *Lilium longiflorum* [11], *Fraxinus pennsylvanica* [12], *Hosta plantaginea* [13], *Magnolia wufengensis* [14], *Prunus lannesiana* [15], *Magnolia stellata* [16], *Carya*

illinoinensis [17] and *Fraxinus nigra* [18]. *Arabidopsis* plants with ectopic expression of *AG* orthologs from these plants also displayed homeotic changes. Similar results were obtained for the overexpression of *AG* orthologs using transgenic approaches in other plant species as well, such as tobacco [19-22], tomato [23] and petunia [24]. In addition, the endogenous *AG* gene function of specifying stamen and carpel identities in the *Arabidopsis ag* mutant could be partially or fully compensated by ectopic expression of *AG* orthologs from other plant species, such as *MastAG* [16], *FaesAG* [25] and *TcAG* [26]. These findings indicate that *AG* orthologs can also be responsible for the formation of reproductive organs.

Cucumber (*Cucumis sativus* L.) is an economically- and nutritionally-important vegetable crop cultivated worldwide and it is also a model system for flower development studies [27,28]. Our previous study revealed that there is only one *AG* subfamily member (*CsMADS24*) in cucumber [5], but its roles in determining reproductive floral organs have not yet been elucidated. In this study, *CsMADS24* was isolated and its sequence, expression pattern and the effect of overexpression in *Arabidopsis* were analyzed. The results showed that *CsMADS24* may be a functional *AG* ortholog in cucumber.

MATERIALS AND METHODS

Plant material and growth conditions

Cucumis sativus var. *sativus* line 9930 and *Arabidopsis thaliana* Col-0 were used in this study. Cucumber seeds were germinated and grown in trays containing a soil mixture (peat:sand:pumice, 1:1:1, v/v/v). The seedlings were adequately watered and grown at day/night temperatures of 24/18°C, respectively, with a 16 h photoperiod. Wild-type (WT) and transgenic *Arabidopsis* seeds were placed in Petri plates containing solid 1/2 Murashige and Skoog (MS) medium. After being kept at 4°C in darkness for 2 days, the plates were transferred in a growth chamber to the greenhouse under long-day conditions (16 h light/8 h dark) at 22°C for 10 days; the seedlings were then transplanted into pots with soil for further study.

Cloning of the *CsMADS24* gene

Total RNA was extracted using Plant RNA Purity Reagent (Invitrogen, USA) from the cucumber inflorescences. Single-stranded cDNA was synthesized by priming with the oligo(dT) using M-MLV reverse transcriptase (TaKaRa, Japan). The ORF sequence of *CsMADS24* was amplified by semi-quantitative reverse transcription PCR (RT-PCR) with the *CsMADS24* specific primers CsMADS24-1F (5′-ATGAGTTGTTATGAGGAAG-3′) and CsMADS24-1R (5′-TTACACAAGTTGAAGAGAG-3′) using the following procedure: 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 1 min, and then a final extension at 72°C for 10 min. The PCR product was cloned into the pMD18-T vector (TaKaRa, Japan) and sequenced.

Bioinformatics analysis

The exon-intron structure of *CsMADS24* was analyzed by comparing the open reading frame (ORF) sequence and genomic DNA with Gene Structure Display Server (GSDS, http://gsds.cbi.pku.edu.cn/). The online servers ProtParam (http://web.expasy.org/protparam/) and SOPMA (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_sopma.html) were employed to examine the physicochemical characteristics and secondary structure of the CsMADS24 protein, respectively. The subcellular localization of the CsMADS24 protein was analyzed using ProtComp Version 9.0 (http://linux1.softberry.com/berry.phtml) and CELLO v.2.5 (http://cello.life.nctu.edu.tw/).

Multiple sequence alignments were performed on the protein sequences using Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) with the default parameters and alignments adjusted manually by GeneDoc. A neighbor-joining (NJ) phylogenetic tree was constructed on the basis of a comparative analysis of amino acid sequences of CsMADS24 and the AG members from different plant species using the MEGA 5.0 software, which was supported by bootstrapping based on 1000 replicates. The accession numbers of these proteins are listed in Supplementary Table S1.

Expression pattern analysis of *CsMADS24* in cucumber

To determine the expression of *CsMADS24* during flower development, five floral developmental stages of female flowers (FF) and male flowers (MF) were sorted according to their corolla length, which ranged from FF1/MF1=(2.5±1) mm to FF5/MF5=(20±2) mm [29]. RT-PCR was carried out as described above with the following primers: CsMADS24-2F (5′-CCGAATTCGATCCAGAAAGA-3′) and CsMADS24-2R (5′-CCCATTGATCCTTCCTCTCC-3′), and amplification for *CsMADS24* and the cucumber internal control *Actin* gene (*CsAct3*, GenBank accession number: DQ115883) was performed for 30 and 25 cycles, respectively.

In situ hybridization

The cucumber floral development could be divided into 12 stages, from meristem initiation (stage 1) to anthesis (stage 12); the male and female flower buds became morphologically distinguishable from each other at stage 6 [27]. Flower buds collected from cucumbers grown in the greenhouse at stage 6 were fixed, embedded, sectioned, and hybridized with digoxigenin-labeled probes as described previously [28]. Sense and antisense RNA probes were amplified by PCR using the Digoxigenin RNA labeling kit (Roche) with the following primers: CsMADS24-3F (5′-GTGAAATTGGAGAAAGGAAT-3′) and CsMADS24-3R (5′-GATAGGGTGATTATGATGGG-3′).

Transformation of *Arabidopsis* and analysis of transgenic plants

The *CsMADS24* ORF in pMD18-T vector was cleaved using *Pst* I and *Xba* I, and subcloned into binary vector pHB under the control of a double cauliflower mosaic virus 35S promoter in the sense orientation. The resulting 35S::35S::*CsMADS24* construct was induced into *Agrobacterium tumefaciens* strain *GV3101*. The transformed bacteria were used to obtain transgenic *Arabidopsis* plants by the floral dip procedure [30]. Transgenic lines were checked by germinating the seeds on 1/2 MS medium containing 50 mg/L hygromycin for 10 days at 22°C. Putative hygromycin-resistant transformants were further confirmed by RT-PCR analysis using CsMADS24-2F and CsMADS24-2R. *AtTubulin4* was used as an internal control with the following primers: AtTubulin4-F (5′-GCGAACAGTTCACAGCTATGTTCA-3′) and AtTubulin4-R (5′-GAGGGAGCCATTGACAACATCTT-3′). T_2 and T_3 homozygous transgenic plants were used for phenotypic evaluation.

RESULTS

Cloning and sequence analysis of the *CsMADS24* gene from cucumber

The *CsMADS24* gene was cloned from *C. sativus* by RT-PCR using primers designed on the basis of a previous report [5]. The ORF of *CsMADS24* is 765 bp in length and encodes a peptide of 254 amino acid residues with an estimated molecular weight of 28.98 kDa, a pI of 9.03 and an average hydropathy index (GRAVY) of -0.833. NCBI Conserved Domain Database (CDD) analysis demonstrated that the MADS and K domains were present in the CsMADS24 protein (Fig. 1A). A BLAST search of CsMADS24 against GeneBank showed that it was identical to CUS1 (100% amino acid identity) [31] and has only one non-conserved amino acid substitution of CAG2 [32]. GSDS analysis performed by comparing the sequences of ORF and genomic DNA suggested that *CsMADS24* harbored

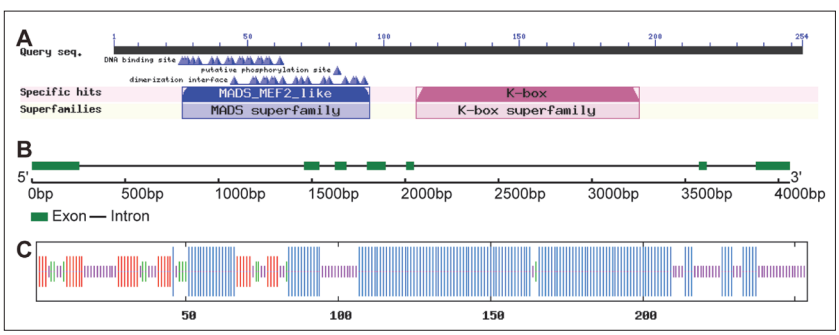

Fig. 1. Characterization of the *CsMADS24* gene and deduced CsMADS24 protein. **A** – Amino acid sequence-based CDD search of the CsMADS24 protein. **B** – Exon-intron structure of the *CsMADS24* gene by GSDS. Exons and introns are indicated by dark green boxes and black lines, respectively. **C** – Secondary structure of CsMADS24. The alpha helix, extended strand, beta turn, and random coil residues are represented in the order from the longest to the shortest.

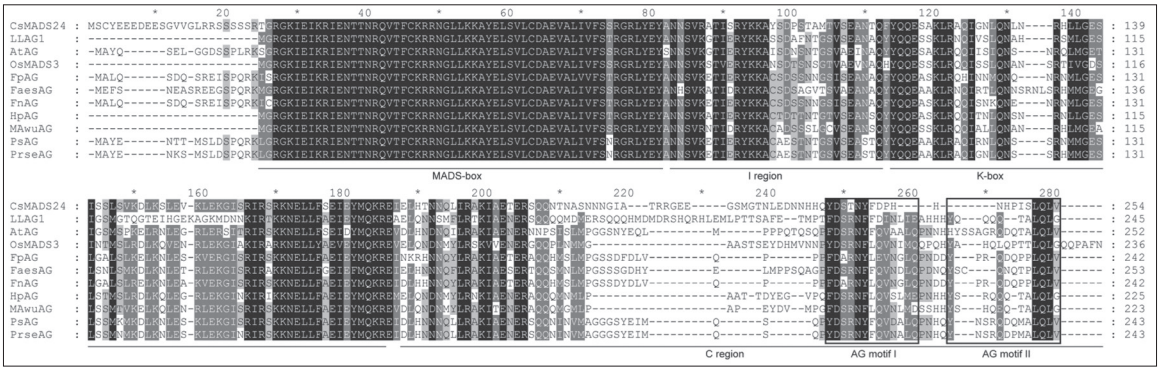

Fig. 2. Sequence alignment of CsMADS24 protein with other AG orthologs from different plant species. The sequence of CsMADS24 was aligned with those of AG orthologs from *Lilium longiflorum* (LLAG1, AAR98731), *Arabidopsis thaliana* (AtAG, P17839), *Oryza sativa* (OsMADS3, AAA99964), *Fraxinus pennsylvanica* (FpAG, AFP99884), *Fagopyrum esculentum* (FaesAG, AFO83615), *Fraxinus nigra* (FnAG, APJ35634), *Hosta plantaginea* (HpAG, ACB70410), *Magnolia wufengensis* (MAwuAG, AEO52692), *Prunus serotina* (PsAG, ACH72974), and *Prunus serrulata* (PrseAG, ADK95058). The MADS domain, I region, K domain, and C region are underlined. Two highly conserved AG motifs (I and II) are indicated in boxes.

7 exons and 6 introns (Fig. 1B). Sequence analysis via SOPMA showed that the secondary structure of CsMADS24 includes 56.30% alpha helix, 11.81% extended strand, 4.72% beta turn and 27.17% random coil sequences (Fig. 1C). In addition, ProtComp and CELLO analyses suggested that CsMADS24 was localized to the nucleus.

Multiple sequence alignment and phylogenetic analysis of CsMADS24

An alignment of the deduced CsMADS24 protein sequence with that of other AG orthologs from different plant species in literature references was performed by Clustal Omega. The results showed that CsMADS24 was well aligned with the sequences and shared 60.18, 61.54, 63.01, 63.60, 63.98, 64.91, 68.84, 69.01, 70.61, and 71.49% identities with the AG orthologs LLAG1 [11], AtAG [9], OsMADS3 [33], FpAG [12], FaesAG [25], FnAG [18], HpAG [13], MAwuAG [14], PsAG [34], and PrseAG [15], respectively (Fig. 2). Like other AG orthologs, CsMADS24 possesses a highly conserved MADS domain, a short I region, a weakly conserved K domain and a highly variable C-terminal (C) domain. In addition, the N-terminal extensions, which usually appear in AG members from eudicots, were also found at the N-terminal region of CsMADS24 (Fig. 2). Furthermore, two highly conserved AG motifs (I and II) were present in CsMADS24 and other aligned proteins, further indicating that CsMADS24 is an AG ortholog (Fig. 2).

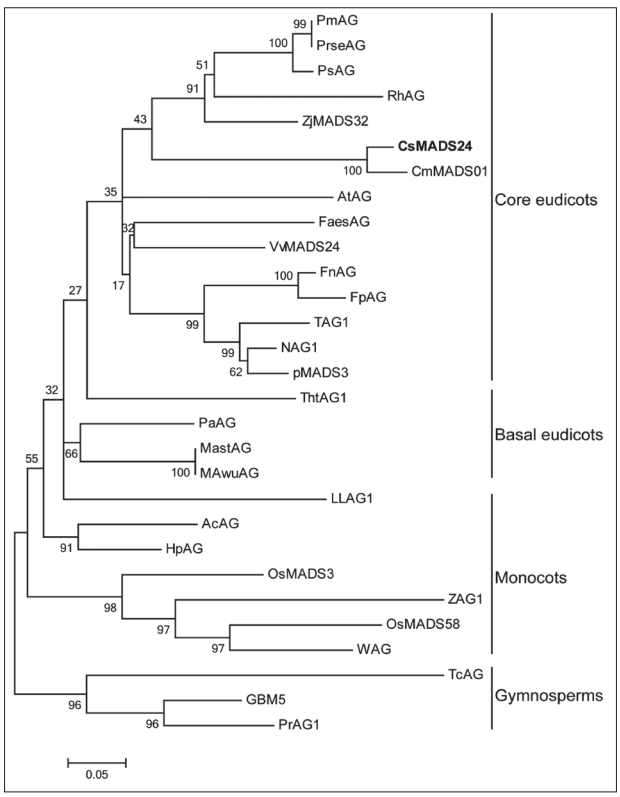

Fig. 3. Phylogenetic analysis of AG orthologs from different plant species. The accession numbers of AG proteins used for phylogenetic tree analysis are shown in Table S1. CsMADS24 from *C. sativus* is bold.

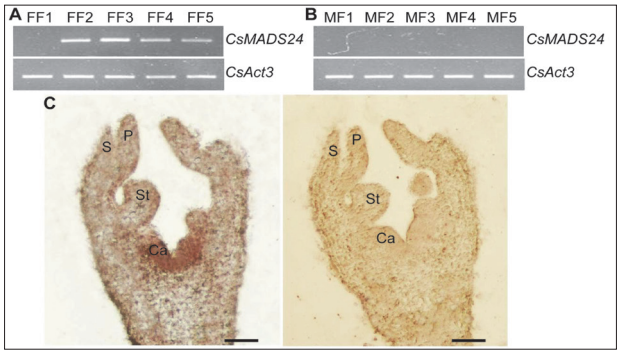

Fig. 4. Expression patterns of *CsMADS24* during different flower developmental stages in cucumber. **A**, **B** – Expression analyses of *CsMADS24* by RT-PCR using *CsAct3* as an internal control in female (**A**) and male (**B**) flowers at different developmental stages. **C** – mRNA *in situ* hybridization of *CsMADS24* in flower buds at stage 6. **D** – Negative control using the sense probe in flower buds at stage 6. S, sepal; P, petal; St, stamen; Ca, carpel. Scale bars = 100 μm.

To determine the evolutionary relationship between CsMADS24 and other AG orthologs, we constructed a phylogenetic tree based on their amino acid sequences. As shown in Fig. 3, CsMADS24 fell into the clade of core dicots, and was the most closely related to CmMADS01 from *Cucumis melo* [35].

Expression pattern of *CsMADS24* during flower development

Our previous results also showed that *CsMADS24* was expressed in flowers while no transcript was detected in roots, shoots and leaves [5]. To further examine the spatial expression pattern of *CsMADS24* during flower development, we investigated its expression at five different developmental stages of male and female flowers. These floral developmental stages were sorted according to their corolla length, which ranged from 2.5±1 mm in FF1/MF1 to 20±2 mm in FF5/MF5 [29]. In female flowers, the transcripts of *CsMADS24* were first detected at the FF2 stage, increasing at the FF3 stage and finally decreasing at stages FF4 and FF5 (Fig. 4A). However, in male flower buds, no transcript was detected in any of the five developmental stages (Fig. 4B).

To dissect the detailed expression patterns of *CsMADS24* during cucumber flower development, *in situ* hybridization was performed. As shown in Fig. 4C, *CsMADS24* RNA was detected in flower buds at stage 6 exclusively in the carpel.

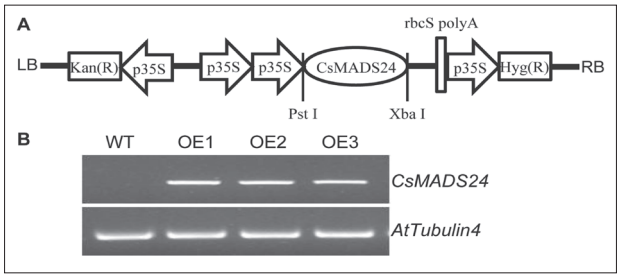

Fig. 5. Schematic diagram of 35S::35S::*CsMADS24* overexpression construct and RT-PCR detection of transgenic *Arabidopsis*. **A** – Schematic diagram of 35S::35S::*CsMADS24* overexpression construct. **B** – Transcript abundance of *CsMADS24* in transgenic lines (OE1, OE2 and OE3) and WT plants. WT, wild-type *Arabidopsis*. OE1-OE3, 35S::35S::*CsMADS24* transgenic *Arabidopsis* lines. The *AtTubulin4* gene was used as an internal control. RNA was isolated from leaves of transgenic and WT plants.

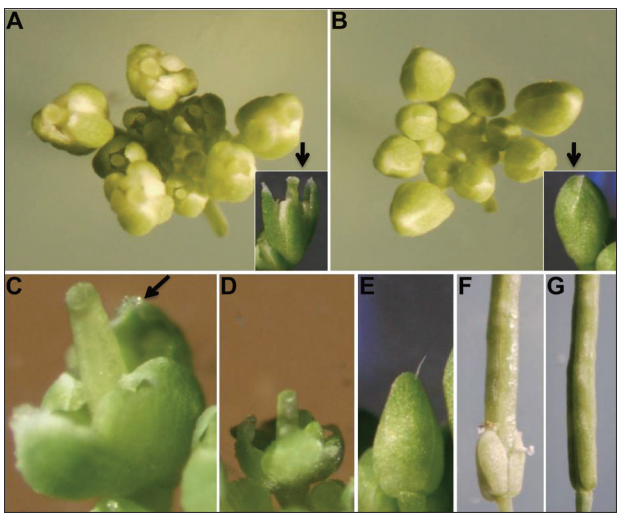

Fig. 6. Comparison of the phenotypes of 35S::35S::*CsMADS24* transgenic and WT *Arabidopsis* plants. **A**, **B** – Inflorescence with prematurely open flower buds of transgenic plants (**A**) in comparison with those of WT plants (**B**). **C** – Sepals are converted homeotically into carpel-like structures with stigmatic papillae (arrow). **D**, **E** – Floral development in 35S::35S::*CsMADS24* transgenic (**D**) and WT plants (**E**) at stage 10. **F** – The 35S::35S::*CsMADS24* fruits. **G** – WT fruits.

Phenotypes of *CsMADS24* overexpression in *Arabidopsis*

To gain further insight into the function of *CsMADS24*, we transformed the model species *Arabidopsis* with *CsMADS24*, which is under the control of two cauliflower mosaic virus 35S promoters (Fig. 5A). Among 64 35S::35S::*CsMADS24* independent transgenic plants, 23 exhibited significantly earlier

flowering phenotypes. The significantly earlier flowering transgenic *Arabidopsis* plants were identified by RT-PCR analysis, and three transgenic lines (OE1, OE2 and OE3) were selected for further analysis (Fig. 5B).

The phenotypes of 35S::35S::*CsMADS24 Arabidopsis* transgenic plants at different flower developmental stages were investigated using the method of a previous study [36]. The results showed that the transgenic plants flowered significantly earlier than WT plants (Fig. 6A, B). Usually, the flower buds of WT plants were open after stage 12; however, those of transgenic plants were prematurely open, sometimes before stage 10 (Fig. 6A, D). The sepals of transgenic plants were homeotically transformed into carpelloid-like structures with stigmatic papillae (Fig. 6C). At stage 10, the stigmatic papillae showed a relatively earlier and more rapid extension out of the sepals in transgenic plants than in WT plants (Fig. 6D). In WT plants, stigmatic papillae did not appear until stage 11, and at this time the flower buds were still enclosed by sepals (Fig. 6E). In addition, delayed floral organ abscission during the development of siliques was observed in transgenic plants (Fig. 6F), while in WT plants it was normal (Fig. 6G).

DISCUSSION

Cucumber is one of the economically-important vegetable crops cultivated worldwide, and ABCDE model genes may play very important roles in flower development. However, only a number of floral homeotic genes from cucumber have been investigated in detail [28,31,37,38]. In this study, an *AG* homologous gene, *CsMADS24*, was isolated from cucumber developing flowers.

The predicted protein encoded by *CsMADS24* has a typical MIKC-type domain, suggesting that it is a MADS-box gene, and CsMADS24 was found to have high identities in deduced amino acid sequences with the AG orthologs from core eudicot species, particularly with CmMADS01 from *C. melo*. In addition, CsMADS24 contains an N-terminal extension ahead of the MADS-box, which is usually present in the AG ortholog proteins from core eudicot species but absent in those from basal eudicot species and monocot species [25,39]. The N-terminal extension peptide seems to have no specific function since some AG orthologs lacking this extension are also functionally active *in vitro*, such as LLAG1 [11], OsMADS3 [33], HpAG [13], MAwuAG [14], PrAG1 [21], AcAG [40], and CiAG [17]. Moreover, CsMADS24 also harbors two highly conserved AG motifs (I and II), which are specific to C-class proteins [39]. These results indicate that *CsMADS24* is an *AG* homolog and may function in regulating flower organ development like other *AG*-like genes.

Our previous study revealed that *CsMADS24* is only expressed in flowers [5]. In the present study, RT-PCR results showed that *CsMADS24* is specifically expressed in female flowers and that the expression is much higher at certain specific stages of female flower development. The spatial expression pattern of *CsMADS24* is in line with that of *CUS1* and *CAG2* [31,32]. Compared with *AG* from *A. thaliana* and *AG* orthologs from other plant species, *CsMADS24* showed a higher specificity of expression. *In situ* hybridization revealed that *CsMADS24* RNA exclusively accumulated in the carpels, while no signal was detected in the stamens, which is different from the case of *AG* orthologs that have been identified so far, implying that *CsMADS24* may only be required for specifying carpel identity.

The function of *CsMADS24* was further investigated by its ectopic expression in *Arabidopsis* to examine whether *CsMADS24* plays roles in specifying stamen and carpel identities. As a result, ectopic expression of *CsMADS24* significantly promoted early flowering in transgenic plants. A similar early flowering phenotype was observed in transgenic *Arabidopsis* with ectopic expression of *AG* orthologs from different plant species, such as *MAwuAG* [14], *PrseAG* [15], *FnAG* [18], and *FaesAG* [25]. In addition, the 35S::35S::*CsMADS24* transgenic *Arabidopsis* plants displayed a homeotic transition of sepals into carpelloid-like structures with earlier appearance of stigmatic papillae, suggesting that ectopic expression of *CsMADS24* is sufficient to convert sepals into carpels, but insufficient to convert petals into stamens. These results indicate that 35S::35S::*CsMADS24* transgenic *Arabidopsis* plants only had partially similar phenotypes compared to the *Arabidopsis* plants with *AG* ectopic expression [41]. Similar phenotypes were also reported previously for the ectopic expression of *AG* orthologs from several plant species. For example, *MastAG* from the ancestral angiosperm *Magnolia stellata* also plays a major role in carpel identity, and it can substitute for the endogenous *AG* gene of *A. thaliana* that specifies carpel identity, but fails to rescue stamen

development [16]. Ectopic expression of *pMADS3* was sufficient to convert petals into anthers, but failed to convert sepals into carpels [4]. In 35S::*FAR Arabidopsis* plants, the second whorl organs were stamenoid-like structures, but the first whorl organs showed almost no obvious phenotypic changes [42]. The ectopic expression of *MAwuAG* can convert the petals into stamenoid structures in *Arabidopsis*, but is insufficient to convert sepals in the first whorls into carpels [14]. These findings indicate that the establishment of complete C-function may require the joint effects of *AG* and other genes to specify the stamen and carpel identity in these plants. For example, *OsMADS3* plays a more crucial role in regulating stamen identity, while *OsMADS58* is required for carpel morphogenesis and meristem termination [33]. As *AG* orthologs from *Cyclamen persicum*, *CpAG1* plays a more predominant role in stamen formation and *CpAG2* mainly functions in carpel formation and termination of meristematic activity [43]. In addition, the transgenic *Arabidopsis* displayed delayed floral organ abscission during the development of siliques. Premature fruit shattering along the dehiscence zone was also observed in transgenic *Arabidopsis* plants with ectopic expression of *TrAG* and *TrSHP* [44] and *MAwuAG* [14]. These results indicate that *CsMADS24*, which might also have undergone neofunctionalization like *MAwuAG* [14], may have a function in regulating the development of floral reproductive organs in cucumber in a unique manner. We speculate that the differences in the functions of AG orthologs between cucumber and *Arabidopsis* may be due to the fact that *Arabidopsis* is androgynous, while cucumber is a monoecious plant with distinct male and female flowers on a single plant.

CONCLUSIONS

An *AG* ortholog named as *CsMADS24* was isolated from *C. sativus* and its function in flower development was analyzed. RT-PCR, *in situ* hybridization and transgenic analysis in *Arabidopsis* were performed to investigate the function of *CsMADS24*. The 35S::35S::*CsMADS24* transgenic *Arabidopsis* plants exhibited a homeotic transition of sepals into carpelloid-like structures, but the petals were not converted into stamenoid structures. These results indicate that *CsMADS24* plays an important role in regulating the reproductive organ identity of carpels in cucumber.

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Author contributions: SL conceived and designed the study. LG, GL and PH performed the experiments. YZ, LH, LJ and SL analyzed the data. YZ and SL wrote the paper. YZ, LH and SL revised the paper. All authors read and approved the manuscript.

Conflict of interest disclosure: The authors declare that they have no conflict of interest.

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Supplementary Data

Supplementary Table S1. AGAMOUS proteins used for phylogenetic tree analysis.
Available at: http://serbiosoc.org.rs/NewUploads/Uploads/Zhou%20et%20al_Supplementary%20Table%20S1.pdf