

Isolation and Functional Characterization of an *AGAMOUS-LIKE 18* (*AGL18*) MADS-box Gene from Cucumber (*Cucumis sativus* L.)

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Abstract

MADS-box proteins play vital roles in plant growth and development. However, few studies have addressed the biological functions of MADS-box genes in cucumber. In this study, a MADS-box gene, *CsMADS25*, was cloned from cucumber (*Cucumis sativus* L.). The open reading frame (ORF) of *CsMADS25* was 810 bp in length and encoded a deduced protein consisting of 269 amino acids with a calculated MW of 30.53 kDa and a theoretical pI of 5.38. Sequence alignment showed that *CsMADS25* shared the highest amino acid identity with *CmMADS09* from *Cucumis melo*. Phylogenetic tree analysis indicated that *CsMADS25* was clustered with *AGL18* proteins with high bootstrap values. qRT-PCR analysis showed that the expression of *CsMADS25* was observably regulated by various abiotic stresses and GA treatments. Overexpression of *CsMADS25* resulted in dwarf and small-leaf phenotypes in transgenic *Arabidopsis* plants, and the leaf index value (leaf length/width ratio) of transgenic plants was dramatically increased compared with that of wild-type (WT) plants. These findings suggest that *CsMADS25* might play important roles in various developmental processes and in response to abiotic stress of cucumber.

Keywords: abiotic stress; *AGL15*; dwarf; small leaves; transgenic *Arabidopsis*

Introduction

MADS-box gene family members are transcription factors proven to play prominent roles in various developmental processes of plants. Based on their protein domain structure and evolutionary lineage, the MADS-box genes can be categorized into two main types (type I and II), which were originated from a gene duplication event (Smaczniak *et al.*, 2012; Yin *et al.*, 2017). The type II MADS, which was also designated as MIKC and known to harbor four basic components: a highly conserved N-terminal MADS-box (M), a weakly conserved Intervening region (I), the second most-conserved Keratin domain (K), and a highly divergent C-terminal region (C), can be further divided into MIKC^C and MIKC* types based on their domain structures (Kaufmann *et al.*, 2005; Smaczniak *et al.*, 2012; Grimplet *et al.*, 2016).

AGAMOUS-LIKE15 (*AGL15*) and its closest member *AGAMOUS-Like18* (*AGL18*) constitute the *AGL15* clade, which is a subfamily of MIKC^C type MADS named after the first identified member (*AGL15*, At5g13790) in *Arabidopsis* (Heck *et al.*, 1995), and functions in controlling of diverse developmental processes such as floral transition and floral organs abscission. For example, overexpression of *AGL15* in *Arabidopsis* could promote the longevity of floral organs including sepals and petals, alter leaf morphology, and delay flowering, fruit maturation and perianth abscission (Fernandez *et al.*, 2000; Fang and Fernandez, 2002). *SlMBP11* is an *AGL15*-like gene in tomato, which is mainly expressed in the reproductive stage and displays much lower transcription in the vegetative stage, and its overexpression in tomato also resulted in a delayed perianth senescence phenotype (Guo *et al.*, 2017). Similarly, *Arabidopsis* plants overexpressing *AGL18* also displayed a prolonged lifecycle with inhibited perianth abscission

(Adamczyk *et al.*, 2007). In addition, the *agl15* mutants did not exhibit any obvious phenotype, while the *agl15agl18* double mutants displayed an early flowering phenotype with rapid increases in the expression of *SUPPRESSOR OF OVEREXPRESSION OF CO1 (SOC1)* and *FLOWERING LOCUS T (FT)* (Adamczyk *et al.*, 2007; Fernandez *et al.*, 2014). AGL15 can directly interact with a large number of proteins including some other MADS-domain proteins such as AGL18, AGL24 and SHORT VEGETATIVE PHASE (SVP) (de Folter *et al.*, 2005; Zheng *et al.*, 2009; Serivichyaswat *et al.*, 2015), as well as bind to the promoters of *SOC1* and other floral regulators [such as *SVP* and *FLOWERING LOCUS C (FLC)*] containing CARG boxes in *Arabidopsis* (Zheng *et al.*, 2009; Immink *et al.*, 2012; Fernandez *et al.*, 2014). Additionally, AGL18 can directly interact with histone deacetylase complex proteins and plays a role in photoperiodic flowering-time regulation by periodic histone deacetylation of *FT* (Gu *et al.*, 2013). These findings suggest that AGL15 and AGL18 have co-operative and redundant roles in controlling the floral transition of *Arabidopsis* through regulating *SOC1*, *FT*, and other floral regulators. A recent study also revealed that a *Brassica juncea* AGL18 ortholog (BjAGL18-1) can interact with the promoters of *SOC1* and *AGL24* to regulate the flowering time of *B. juncea* (Li *et al.*, 2018). Moreover, the AGL15 subfamily members were also reported to be involved in somatic embryo development (Thakare *et al.*, 2008; Zheng *et al.*, 2013, 2016; Yang *et al.*, 2014; Zheng and Perry, 2014), lignin biosynthesis (Cosio *et al.*, 2017), seed development (Chen *et al.*, 2018), and stress tolerance (Guo *et al.*, 2016), indicating their multiple roles in the development of plants.

According to our previous study, *CsMADS25* belongs to the AGL15 subfamily (Hu and Liu, 2012), but little is known about its biological functions. In this study, we examined the tissue expression profiles of *CsMADS25* in cucumber, and investigated its possible role in plant growth and development by constitutive expression in *Arabidopsis*.

Materials and Methods

Plant materials and growth conditions

Cucumis sativus var. *sativus* line 9930 was grown in an artificial chamber based on the recommended protocols, and cold, salt, and drought treatments were conducted in our previous research (Zhou *et al.*, 2018). The leaf samples were harvested at 0, 3, 6 and 12 h, rapidly frozen with liquid nitrogen, and stored at -80°C until use.

Transgenic and wild-type (WT, ecotype Col-0) *Arabidopsis* seeds were sown on the plates of 1/2 MS solid medium. After placing at 4°C for 2 days, the plates were transferred to an artificial chamber at 22°C under 16 h day/8 h night photoperiod.

RNA extraction and cDNA synthesis

The total RNA from all samples was isolated using the TRIzol reagent (TaKaRa, Dalian, China) according to the manufacturer's protocol. Nanodrop 2000 was employed to examine the RNA quality and concentration by measuring

the A230, A260, and A280. Genomic DNA (gDNA) elimination and first-strand cDNA reverse transcription were conducted by using $3\ \mu\text{g}$ of total RNA with the TransScript One-Step gDNA Removal and cDNA Synthesis SuperMix kit (TransGen, Beijing, China).

Cloning of CsMADS25 and bioinformatics analysis

Using the cucumber leaf cDNA as the template, the open reading frame (ORF) of *CsMADS25* was amplified by reverse transcription PCR (RT-PCR) with a pair of primers (5'-ATGGGTAGAGGCAGAGTTGAGAT-3' and 5'-TTACATACCAAATGACGAGAGAGT-3') based on its locus ID (Csa021473) in cucumber genome database (Huang *et al.*, 2009). The amplified products were attached to the pMD18-T vector (TaKaRa, Dalian, China) and sequenced at Tsingke Biological Technology Company (Beijing, China).

The online server ProtParam ([http:// web.expasy.org/protparam/](http://web.expasy.org/protparam/)) and SOPMA (Geourjon and Deleage, 1995) were used to predict the primary and secondary structures of *CsMADS25* protein. The subcellular localization of *CsMADS25* protein was predicted by ProtComp 9.0 ([http:// linux1.softberry.com/berry.phtml](http://linux1.softberry.com/berry.phtml)) and Plant-mPLoc ([http:// www.csbio.sjtu.edu.cn/bioinf/plant-multi/](http://www.csbio.sjtu.edu.cn/bioinf/plant-multi/)). Multiple sequence alignment of *CsMADS25* and AGL15 subfamily members from other plant species was performed using the Clustal Omega online tool (<https://www.ebi.ac.uk/Tools/msa/clustalo/>) by aligning their amino acid sequences. Subsequently, the alignment was used to generate a phylogenetic tree using the MEGA 5.0 software by the neighbor-joining (NJ) method with 1000 replicates for the bootstrap values.

Expression pattern of CsMADS25

The *CsMADS25*-specific primers (5'-CGAGGAGTTTCAGCACAGAA-3' and 5'-GGTCTCATTGGTAGAAGCAGTT-3') were employed to investigate the expression profile of *CsMADS25* under various stress conditions by quantitative real-time PCR (qRT-PCR). qRT-PCR was performed on CFX96 Real-Time PCR System (Bio-Rad, USA) by the SYBR PrimeScript PCR Kit (TaKaRa, Dalian, China) in a $20\ \mu\text{L}$ -volume reaction with a procedure as follows: 1 cycle of 95°C for 5 min, 40 cycles of 95°C for 5 s and 60°C for 40 s. Each reaction was run in three biological replicates to ensure the reliability of the results. The *CsAct3* gene was used as the internal control for qRT-PCR analysis (Wan *et al.*, 2010), and the primers were 5'-GGCAGTGGTGGTGAACAT-3' and 5'-GATTCTGGTGATGGTGTGAGTC-3'.

To investigate the expression of *CsMADS25* in response to gibberellins (GAs), the RPKM values (0, 6, and 12 h under GA_3 treatment) of *CsMADS25* were obtained based on a previous study (Zhang *et al.*, 2017).

Plasmid construction, plant transformation and phenotypic evaluation

To construct the overexpression vector of *CsMADS25*, the ORF of *CsMADS25* was amplified by PCR using the primers 5'-aaaa AAGCTTATGGGTAGAGGC

AGAGTTGAGAT-3' and 5'-aaa TCTAGATTA CATAACC AAAATGAC GAGAGAGT-3', and inserted into the pHB vector, which contained a double 35S cauliflower mosaic virus (CaMV) promoter. The recombinant vector was introduced into *Agrobacterium tumefaciens* strain *GV3101* by electroporation. The transformation of *Arabidopsis* ecotype Col-0 plants was performed by the floral-dip method (Clough and Bent, 1998). Seeds of different transgenic lines were screened on 1/2 MS medium containing 50 mg/L hygromycin. The hygromycin-resistant transgenic plants were further confirmed by examining the expression of *CsMADS25* by RT-PCR using *CsMADS25*-specific primers with the *AtTubulin4* gene (5'-GCGAACAGTTCACAGC TATGTTCA-3' and 5'-GAGGGAGCCATTGAC AACATCTT-3') as an internal control. Positive T₃ homozygous transgenic lines were tested for further study.

Statistical analysis

At least three biological replicates were set and the data were shown as mean \pm standard deviation (SD). Data were evaluated by SPSS software using student's *t*-test with two-way ANOVA. *P* value < 0.05 was considered as statistically significant.

Results

Isolation and sequence analysis of *CsMADS25*

A 810-bp PCR fragment was amplified using specific primers based on the ORF sequence of the *CsMADS25* gene (Gene ID: Csa021473) (Hu and Liu, 2012). The resulting ORF sequence encoded a 269 amino-acid residue protein with a MADS-box and a K-box (Fig. 1A, 1B). In addition, one putative phosphorylation site, many DNA binding sites and dimerization interfaces were present in the MADS-box of the *CsMADS25* protein (Fig. 1B). The results of physicochemical analysis by ProtParam server showed that *CsMADS25* has a theoretical MW of 30.53 kDa and a pI of 5.38 (Table 1). ProtComp 9.0 and Plant-mPloc analyses indicated that *CsMADS25* is a nucleus-localized protein. In addition, the secondary structure prediction by SOPMA showed that *CsMADS25* possesses 48.70% alpha helices, 13.01% extended strands, 2.97% beta turns, and 35.32% random coils, respectively (Fig. 1C; Table 1). A previous study proposed that *CsMADS25*

belongs to the AGL15 subfamily (Hu and Liu, 2012). Multiple sequence alignments of amino acid sequences showed that *CsMADS25* has the highest amino acid identity with *CmMADS09* from *Cucumis melo* (91.79%). Also, it shares high identities at the amino acid level with other AGL15 proteins, such as *GmAGL18* (56.02%), *OsMADS59* (55.84%), *ZjMADS42* (51.64%), *AGL18* (46.15%), *GhAGL15.2* (43.88%), *RcAGL15* (43.64%), *ZjMADS45* (43.04%), *CmMADS08* (42.92%), *GmAGL15* (42.40%), *SlMBP11* (40.00%), and *AGL15* (36.59%) (Fig. 2). In addition, the alignment results showed that all these proteins possess highly conserved regions, mostly in the MADS-box and K-box, while the I-region and C-region are more variable (Fig. 2).

Phylogenetic relationships of *CsMADS25* and AGL15 subfamily members from other plant species

To better characterize the phylogenetic relationships of *CsMADS25* and other AGL15 members, a phylogenetic tree was generated by NJ method according to the alignment of the protein sequences. As a result, *CsMADS25* along with *CmMADS09*, *AGL18*, *GmAGL18*, and *ZjMADS42* were clustered into AGL18 subgroup, while other proteins were clustered into the AGL15 subgroup (Fig. 3), suggesting that *CsMADS25* is an AGL18 protein.

Expression patterns of *CsMADS25* in response to various stresses and GA treatments

qRT-PCR analysis was conducted to investigate the transcription of *CsMADS25* in response to various stresses including cold, salt, and drought conditions. Under cold stress, the expression of *CsMADS25* showed no significant change at 3 h and 6 h, but exhibited an observable decline at 12 h (Fig. 4A). Under salt treatment, the transcript level of *CsMADS25* notably decreased at 6 h, and then increased and finally reached the maximum at 12 h (Fig. 4B). Under drought treatment, the transcription of *CsMADS25* significantly declined at 3 h, and stayed unchanged at 6 h and 12 h (Fig. 4C).

To determine the transcript level of *CsMADS25* in response to GA, the RPKM values of *CsMADS25* were obtained based on a previous study (Zhang *et al.*, 2017). As a result, the expression of *CsMADS25* was unchanged at 6 h under GA treatment, and down-regulated at 12 h (Fig. 4D).

Table 1. The prediction of primary and secondary structures of *CsMADS25* protein

| Structures | Results |
|-----------------------|---------|
| Primary structure | |
| Number of amino acids | 269 |
| MW (kDa) | 30.53 |
| pI | 5.38 |
| Instability index | 53.46 |
| GRAVY | -0.764 |
| Secondary structure | |
| Alpha helix | 48.70% |
| Extended strand | 13.01% |
| Beta turn | 2.97% |
| Random coil | 35.32% |

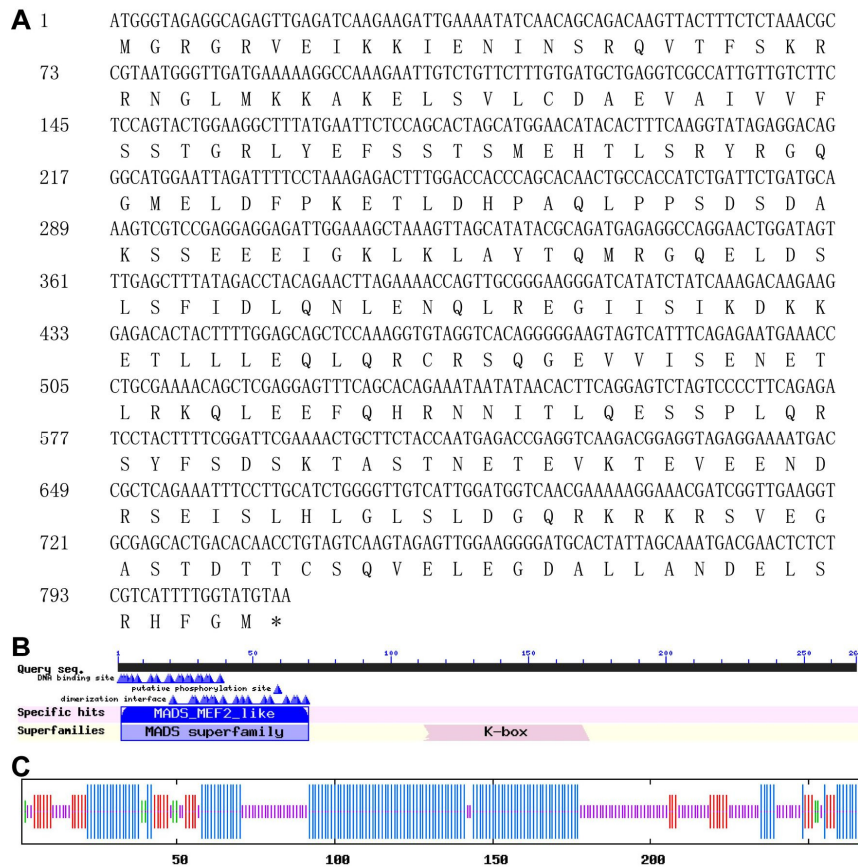


Fig. 1. Sequence analysis of *CsMADS25* and its deduced protein. (A) ORF and deduced amino acid sequences of *CsMADS25*. * represent stop codon. (B) Prediction of *CsMADS25* by NCBI Conserved Domain database (<https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>). (C) SOPMA analysis of the secondary structure of *CsMADS25* protein. The blue, red, green, and purple vertical lines represent alpha helix, extended strand, beta turn, and random coil, respectively

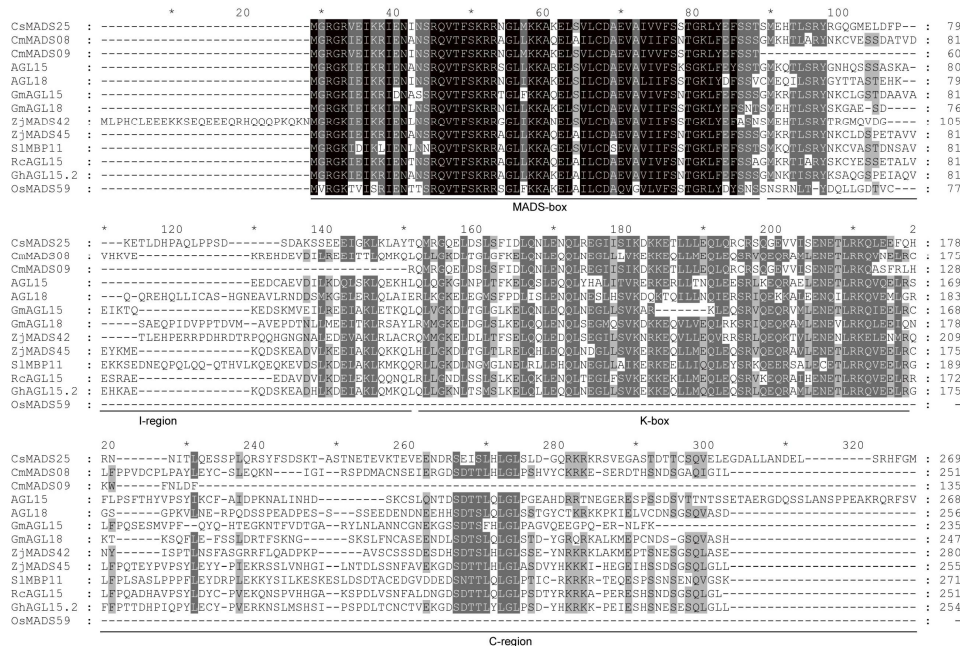


Fig. 2. Amino acid sequence alignment of *CsMADS25* and other members of AGL15 subfamily. Amino acid sequences of these proteins were aligned using Clustal Omega. The MADS-box, I-region, K-box, and C-region are underlined. Accession numbers of these proteins are shown in Table S1

Dwarf and small-leaf phenotypes of Arabidopsis caused by CsMADS25 overexpression

To discern the function of *CsMADS25* during plant growth and development, the ORF of *CsMADS25* was overexpressed in *Arabidopsis* under the control of the double 35S promoter (Fig. 5A). A total of 46 transgenic lines were obtained, and most of them exhibited a significant dwarf phenotype, suggesting that the dwarf phenotype may be attributed to the overexpression of *CsMADS25* in *Arabidopsis*. Two lines (OE1 and OE2) from T₃ generation were randomly selected to determine the expression of *CsMADS25* using RT-PCR (Fig. 5B).

Seeds of WT and transgenic *Arabidopsis* lines (OE1 and OE2) were placed on solid 1/2 MS plates and kept at 4 °C

in darkness for 2 days. The plates were then transferred to a growth chamber at 22 °C for 10 days, and the seedlings were transplanted into the pots with soil and grown in a growth chamber under long-day conditions (16 h light/8 h dark) for another 10 days. Finally, the phenotypes of these plants were recorded. Compared with WT plants, the transgenic lines (OE1 and OE2) exhibited a significant dwarf phenotype with shorter internode lengths (Fig. 5C). In addition, the transgenic plants displayed smaller rosette and cauline leaves at the vegetative stage than WT plants, and a significantly reduced number of trichomes on the leaf surface (Fig. 5D). Although the leaf length and width of the transgenic plants were reduced, the leaf index value (leaf length/width ratio) of transgenic plants was much higher than that of WT plants (Fig. 5E).

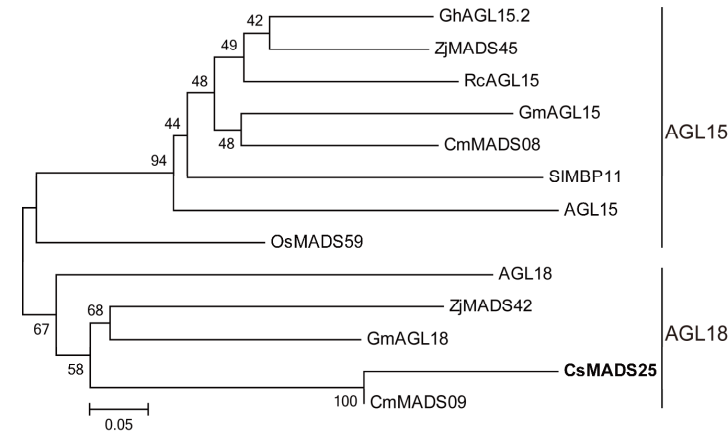


Fig. 3. Phylogenetic analysis of *CsMADS25* and other members of AGL15 subfamily. The phylogenetic tree was created by MEGA 5.0 with the neighbor-joining method by bootstrap analysis with 1000 replicates. *CsMADS25* is bolded. Accession numbers of these proteins are provided in Table S1

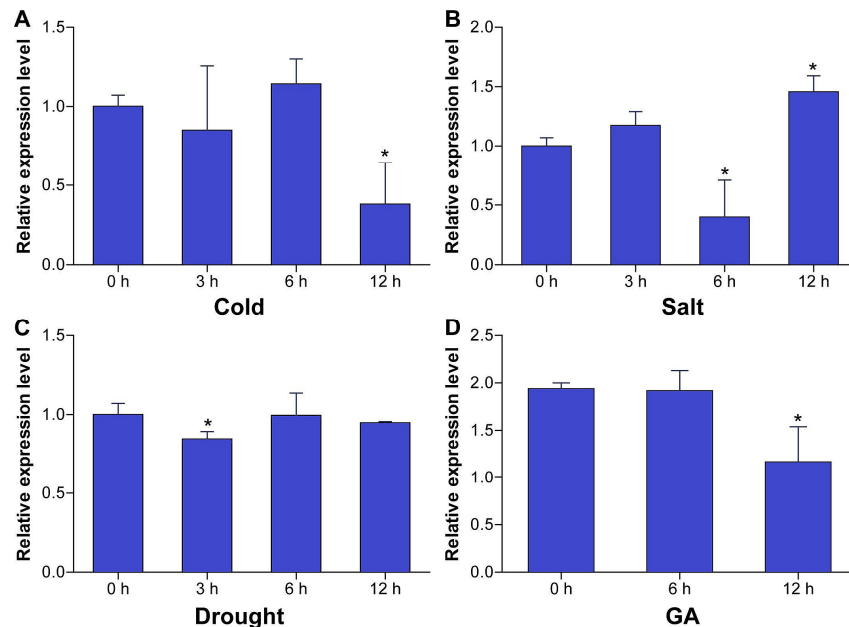


Fig. 4. Expression profiles of *CsMADS25* in response to various stresses and GA treatments. (A-C) qRT-PCR analysis of *CsMADS25* under various stresses including cold (A), salt (B), and drought (C). The relative expression of *CsMADS25* under various stresses at 0 h was normalized as 1.0. (D) The expression profile of *CsMADS25* in response to GA treatment. The shoot apices from cucumber plants (13-3B) were harvested at 0, 6 and 12 h under GA₃ treatment, and the RPKM values of *CsMADS25* were obtained based on the data described in a previous study (Zhang *et al.*, 2017)

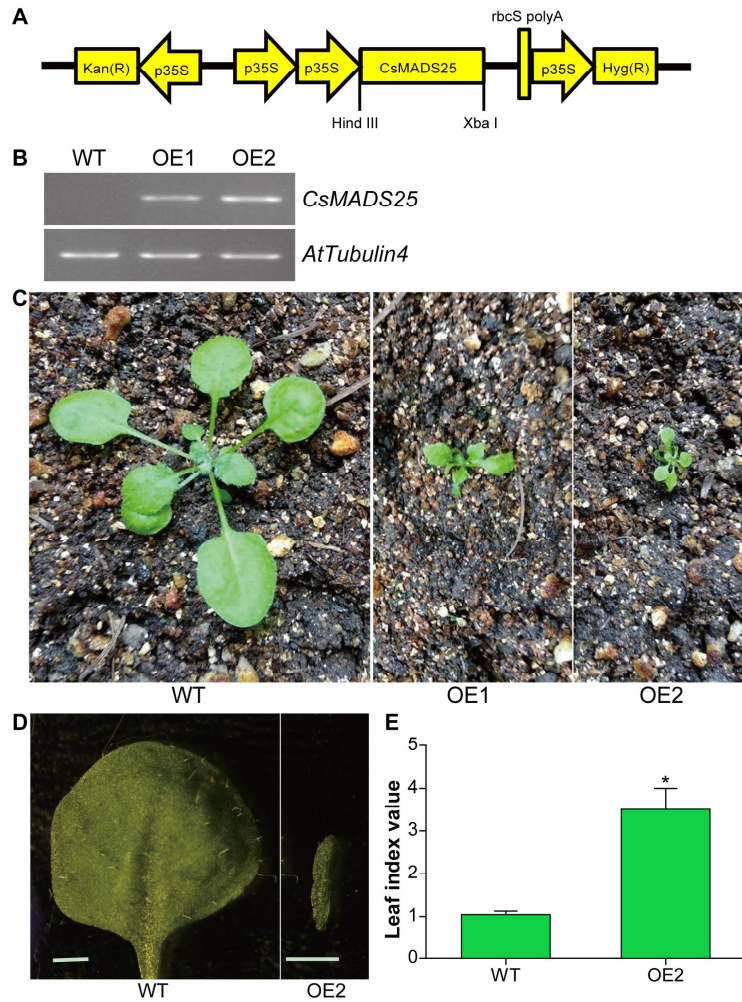


Fig. 5. Phenotypes of *CsMADS25*-overexpressing transgenic plants. (A) Schematic diagram of the 35S::35S::*CsMADS25* construct. (B) RT-PCR analysis of *CsMADS25* transcripts in WT and two 35S::35S::*CsMADS25* transgenic *Arabidopsis* lines (OE1 and OE2). The *AtTubulin4* gene was used as the internal control. (C) Morphology of *CsMADS25*-overexpressing transgenic lines (OE1 and OE2) and WT plants. (D) The 6th rosette leaf morphology of OE2 and WT plants. Bar = 0.1 cm. (E) Leaf index values of the 6th rosette leaves of OE2 and WT plants. Error bars represent the SD. *, $P < 0.05$.

Discussion

In the present study, we cloned the *CsMADS25* gene from cucumber, and predicted that *CsMADS25* is located in the nucleus, and contains the typical MADS-box and K-box as well as many DNA binding sites (Fig. 1), suggesting that *CsMADS25* is a MADS-box transcription factor gene in cucumber. Our previous study has shown that *CsMADS25* is a member of AGL15 subfamily in cucumber (Hu and Liu, 2012). Based on the alignment results, *CsMADS25* has a closer relationship with AGL18 proteins than with AGL15 proteins (Figs. 2, 3), suggesting that *CsMADS25* is an AGL18 protein.

Increasing evidence shows that MADS-box genes play crucial roles in different aspects of vegetative organ development including leaf morphogenesis (Burko *et al.*, 2013; Pabon-Mora *et al.*, 2013). AGL15 subfamily members were also suggested to be involved in leaf development process. For example, the *agl15 agl18 agl24*

svp quadruple mutants displayed small and curled leaves compared with WT plants (Fernandez *et al.*, 2014), suggesting that AGL15 and AGL18 participate in leaf morphogenesis through interacting with other MADS-box proteins. In this study, the leaf morphology was obviously changed with reduced length and width in transgenic plants compared with WT (Fig. 5C, 5D). Similar results were also observed in tomato plants overexpressing *SIMBP11*, an AGL15 member from tomato (Guo *et al.*, 2017). However, the leaf index value (leaf length/width ratio) of transgenic plants was much higher than that of WT plants (Fig. 5E), while no difference was found in the leaf index value between WT and transgenic tomato plants overexpressing *SIMBP11*. These results suggest that *CsMADS25* may affect leaf morphogenesis in some way different from that of *SIMBP11*.

GA plays a vital role in stem elongation, and dwarfism is usually connected with deficiency in GA content or signal transduction (Olszewski *et al.*, 2002; Xiao *et al.*, 2016).

Increasing studies have shown that some MADS-box genes play essential roles in GA signal transduction pathway. For example, *OsMADS1*-overexpressing transgenic plants displayed a dwarf phenotype and shorter panicles due to the inactivation of GA in the panicle (Wang *et al.*, 2017). Suppression of *SLMBP15* in tomato could also lead to reduced plant height with decreases in both GA content and the expression of GA biosynthesis genes (Yin *et al.*, 2018). In this study, besides the changes in leaf shape, the size of transgenic plants was much smaller than WT (Fig. 5). Similarly, overexpression of *SLMBP11* also caused a reduction in GA biosynthesis, leading to a GA-deficiency dwarf phenotype (Guo *et al.*, 2017). Considering that the expression of *CsMADS25* was inhibited by GA treatment (Fig. 4D), and *CsMADS25* and *SLMBP11* shared a 40.00% identity at the amino acids level (Fig. 2), the dwarf phenotype of the transgenic plants may be due to the decrease in GA content. *AtGA2ox6* is a direct downstream target of *AGL15*, and a significant decrease in biologically active GA was observed in *AGL15*-overexpressing transgenic *Arabidopsis* plants (Wang *et al.*, 2004). Similarly, *GmAGL15* can manipulate GA metabolism in somatic embryogenesis by directly interacting with *GmGA2ox6* in soybean (Zheng *et al.*, 2016). Moreover, in transgenic *Arabidopsis* plants, overexpression of many genes including *JcGA2ox6* (Hu *et al.*, 2017) and *PpGA2ox* (Tan *et al.*, 2018) could result in dwarf phenotype with a reduction in endogenous GA content. The amino acid sequence of *CsMADS25* showed a 43.64% identity with that of *RcAGL15* (Fig. 2), and GA content was also reduced in *RcAGL15*-overexpressing transgenic *Arabidopsis* plants compared with in WT (Xu *et al.*, 2018). Therefore, overexpression of *CsMADS25* in *Arabidopsis* may affect GA metabolism, and then decrease the GA content to modulate the GA signal transduction.

Increasing evidence has demonstrated that some MADS-box genes can be regulated by various environmental stresses, and may be involved in stress-related developmental processes. For example, the transcription of *OsMADS26* was increased by osmotic treatment, and suppression of *OsMADS26* enhanced the drought tolerance of rice (Khong *et al.*, 2015). *SLMBP11* expression was induced by wounding, salt, and dehydration, and *SLMBP11* could act as an upstream regulator to modulate salt stress response in tomato (Guo *et al.*, 2016). The expression of *SLMBP8* was also induced by various abiotic stresses such as wounding, salt and dehydration, and *SLMBP8*-RNAi tomato plants showed enhanced tolerance to drought and salt stress (Yin *et al.*, 2017). In the present study, *CsMADS25* was also regulated by cold, salt and drought stresses (Fig. 4A-C), implying that *CsMADS25* may participate in the regulation of stress response in cucumber.

Conclusions

In summary, we isolated and characterized a MADS-box gene named as *CsMADS25*, which is an *AGL18* gene belonging to *AGL15* subfamily in cucumber. The expression of *CsMADS25* is regulated under various stresses and GA treatments. In addition, *CsMADS25*-

overexpressing transgenic *Arabidopsis* plants displayed dwarf height, smaller leaves and higher leaf index value compared with WT plants. Further research is needed to reveal the biological function of *CsMADS25* in the growth and development as well as stress response of cucumber.

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