Isolation and Functional Analysis of CmMAX1 from Chrysanthemum

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ABSTRACT. More axillary buds 1 (MAX1), initially identified in arabidopsis (Arabidopsis thaliana), is a key regulatory gene in strigolactone synthesis. CmMAX1, an ortholog of MAX1 was cloned from chrysanthemum (Chrysanthemum morifolium cv. Jinba). It had an open reading frame of 1611 bp and encoded 536 amino acid of P450 protein, with a conserved heme-binding motif of PFG × GPR × C × G, as well as PERF and KExxR motifs. The predicted amino acid sequence of CmMAX1 was most closely related to the MAX1 ortholog identified in lotus (Nelumbo nucifera), NnMAX1, with 55.33% amino acid sequence similarity. Expression analysis revealed there was no significant difference of CmMAX1 expression among various tissues. Phosphorus (P) deficiency significantly improved the expression levels of CmMAX1. Strigolactone, auxin, and cytokinin negatively regulated the expression of CmMAX1. Overexpression of CmMAX1 reduced the branch numbers of arabidopsis max1-1. These results suggest that CmMAX1 may be a candidate gene for reducing the shoot branching of chrysanthemum.

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Fig. 1. Sequence alignments and phylogenetic analysis of CmMAX1 in *Chrysanthemum morifolium*. (A) Alignments of the predicted amino acid sequences of CmMAX1 compared with *Arabidopsis thaliana* (MAX1), *Medicago truncatula* (MtMAX1), and *Petunia hybrida* (PhMAX1). Three conserved functional domains—P (proline) F (phenylalanine) G (glycine) · GPR (arginine) · C (cysteine) · G, PE (glutamic acid) RF, and K (lysine) ExxR—are marked by a black frame. (B) Phylogenetic analysis. The phylogenetic tree was constructed using the maximum likelihood method. The accession numbers of selected MAX1s are as follows: *Manihot esculenta* MeMAX1 (XP_021598065.1), *Hevea brasiliensis* HbMAX1 (XP_021681567.1), *Jatropha curcas* JcMAX1 (XP_012077261.1), *Populus euphratica* PeMAX1 (XP_011019422.1), *Gossypium hirsutum* GhMAX1 (XP_016689695.1), *Durio zibethinus* DzMAX1 (XP_022731995.1), *Theobroma cacao* TcMAX1 (XP_007012311.1), *Gossypium arboreum* GaMAX1 (XP_017619614.1), *Ziziphus jujuba* ZjMAX1 (XP_015884519), *Prunus avium* PaMAX1 (XP_021823617.1), *Rosa chinensis* RcMAX1 (XP_024165263.1), *Juglans regia* JrMAX1 (XP_018844671.1), *A. thaliana* MAX1 (NP_565617.2), *Citrus sinensis* CsMAX1 (XP_006475901.1), *Ipomoea nil* InMAX1 (XP_019190529.1), *Sesamum indicum* SiMAX1 (XP_020550592.1), *Erythranthe guttatus* EgMAX1 (XP_012857912.1), *Capsicum annum* CaMAX1 (XP_016537773.1), *Nicotiana attenuata* NaMAX1 (XP_019250188.1), *Vitis vinifera* VvMAX1 (XP_002279086.3), *Nelumbo nucifera* NnMAX1 (XP_010262061.1), *Punica granatum* PgMAX1 (PKI70646.1), *Salvia miltiorrhiza* SmMAX1 (AJD25243.1), and *M. truncatula* MtMAX1 (AGI65360.1).
reverse primer (5’-AAGTCCATCTTVAGTTGHGTTGT-3’), the 3’ and 5’ ends of CmMAX1 were amplified using the 3’ RACE System for Rapid Amplification of cDNA Ends (Invitrogen) and the 5’-full RACE core set (Takara), respectively. The CmMAX1 full-length cDNA was isolated by combining the three parts of the fragments. The products amplified using Primestar HS DNA Polymerase (Takara) were cloned into the PEASY-Blunt Simple Cloning Vector (Trans, Beijing, China) to determine the sequence of CmMAX1. The protein molecular formula was determined using the Protparam analysis tool on an ExPASy server (Gasteiger et al., 2005). Alignment of multiple sequences was carried out using DNAMAN software (version 5.2.2; Lynnon Biosoft, San Ramon, CA). Phylogenetic analysis was conducted using the MEGA 5.05 program (Tamura et al., 2011) by the maximum likelihood method.

**Quantitative real-time PCR (qRT-PCR) and semiquantitative reverse transcription analysis.** For the experiment of CmMAX1 expression in different tissues, roots, stems, leaves, axil, and apex were collected from 30-d-old chrysanthemum seedlings. The P deficiency experiment was conducted by treating the roots of the chrysanthemum seedlings with P-free nutrient solution for 12 h, then the roots were harvested for the further experiments. For the hormone treatment experiments, 5 μL of 2 μM GR24 (a synthetic analog of strigolactone), 50 μM 6-benzylamino adenine (6-BA), or 100 μM indole-3-acetic acid (IAA) solutions were applied to node 4 (the fourth bud from the top downward) of 30-d-old chrysanthemum seedlings. After 2 h, the buds treated with different hormones were collected. RNA extraction, qRT-PCR, semiquantitative reverse transcription,
and analysis were performed as described by Dong et al. (2013). To analyze the transcription from the transgenes in 35S::CmMAX1 and analysis were performed as described by Dong et al. (2013). CmMAX1 sequence was amplified with 5'-GAGTTGAAGTTGGAGGTTACG-3' (forward) and 5'-GACCAAGGCACATTGAGG-3' (reverse). Plasmid 35S::CmMAX1 was made by ligating the open reading frame (ORF) of CmMAX1 into the binary vector PBI121 at the XbaI and SacI sites. The construct was transformed into arabidopsis mutant max1-1 plants via Agrobacterium tumefaciens strain LBA4404 by the floral-dip method (Clough and Bent, 1998). Transformants were screened using a Murashige and Skoog medium containing 50 mg L⁻¹ kanamycin. Homozygous T₃ lines were identified and used in this study. The number of rosette branches was scored and the height of the main stem was measured for phenotype analysis.

STATISTICAL ANALYSIS. All data were repeated at least three times. Tukey’s test at P < 0.05 was used to evaluate the degree of rescue of the max1 phenotype by the CmMAX1 gene.

Results

CLONING OF CmMAX1 AND SEQUENCE ANALYSIS OF CmMAX1. CmMAX1 cDNA included a complete ORF of 1611 bp, which encoded proteins of 536 amino acids. The deduced amino acid sequence contained a heme-binding motif (PFQ×GPR×C×G), PERF, and a K-helix motif (KExxR), which were the most highly conserved regions of P450 (Poulos et al., 1987), with a calculated molecular mass of 61.29 kDa and an isoelectric point of 8.92. It was speculated that the molecular formula of the CmMAX1 protein was C₃₀₂₃H₄₁₇₈N₁₇₀O₇₇₁S₁₇₁.

PHYLOGENETIC ANALYSIS OF CmMAX1 HOMEODOMAIN PROTEINS. Sequence alignment of the deduced amino acid sequence of the chrysanthemum MAX1 ortholog with other known MAX1 proteins was performed (Fig. 1A). CmMAX1 shared 60.7%, 60.7%, and 57.9% similarity with PhMAX1, MtMAX1, and MAX1 proteins, respectively. To investigate the evolutionary relationships among the predicted MAX1 proteins, a phylogenetic tree was constructed by an alignment of MAX1 proteins from a taxonomically diverse set of species (Fig. 1B). The phylogenetic tree revealed that CmMAX1 was closest to NnMAX1.

Tissue Specificity of CmMAX1 Expression. To identify the tissues in which CmMAX1 is most highly expressed, the level of CmMAX1 transcripts in different tissues was examined. As shown in Fig. 2, the CmMAX1 transcript was detected in all tissues examined. The difference of CmMAX1 expression in roots, stems, leaves, and axil was not significant, and the expression level of CmMAX1 in apex was lowest.

P Deficiency Improved Expressions of CmMAX1. To study the relationship between CmMAX1 expression and P deficiency, we analyzed the expression of CmMAX1 in chrysanthemum seedlings treated with a P deficiency. Our results showed that the expression level of CmMAX1 in P-deficient plants increased about six times compared with the control (Fig. 3).

Expression of CmMAX1 was repressed by different phytohormones. To study the regulation of CmMAX1 by strigolactone, auxin, and cytokinin, we detected the expression of CmMAX1 after 2 h of treatment with GR24, IAA, or 6-BA. The results showed that all three hormones repressed the expression of CmMAX1, and the expression level of CmMAX1 decreased to about 0.21, 0.27, and 0.32 of the control, respectively (Fig. 4).

FUNCTIONAL ANALYSIS OF CmMAX1. The CmMAX1 ORF driven by cauliflower mosaic virus 35S promoter (35S::CmMAX1) was expressed in the arabidopsis max1-1 mutant background. Four independent lines—line 1, line 3, line 6, and line 7—were selected for further analysis. As expected, all four lines displayed high expression levels of the CmMAX1 gene. The number of rosette branches with a length of at least 5 mm was scored to quantify the degree of rescue of the max1-1 mutant phenotype by the CmMAX1 gene overexpression. CmMAX1 expression reduced the mean number of branches from 8.2 in max1-1 to 3.25, 3.05, 4.2, and 3.5, respectively (Tukey’s test at P < 0.05; Fig. 5A and B), improving the height of plants from 202 mm in max1 to 266.8, 270.3, 255.5, and 263 mm (Tukey’s test at P < 0.05; Fig. 5A and C).
MAX1 encodes a cytochrome P450 family member that acts downstream of D27 to produce a carotenoid-derived branch-inhibiting hormone (Waters et al., 2012). Mutation of MAX1 led to the bushy phenotype in arabiopsis (Stirnberg et al., 2002). However, MAX1 orthologs were absent from Physcomitrella, present in monocots and eudicots as multiple copies and as a single copy, respectively, which indicated that MAX1 had a different pattern compared with the other genes. Thus, studies on MAX1 have important implications for understanding the evolution of the MAX pathway. In addition, expression and functional analysis of MAX1 orthologs will provide valuable information for molecular breeding.

In this study, another ortholog of MAX1 was cloned from chrysanthemum cultivar Jinba, then submitted to Nelson’s P450 databases, and named CYP711A34 (Nelson et al., 1993). We found only one copy existed in chrysanthemum, which was consistent with the hypothesis that MAX1 is generally present as a single copy in eudicots (Hepworth, 2012). Sequence analysis revealed that CmMAX1 had deduced an amino acid sequence that contains heme-binding motif PFG × GPR × C × G, the PERF motif, and a K-helix motif KEtxxR. All three motifs were known to be involved in heme-binding or thought to stabilize the heme-binding pocket (Paquette et al., 2009). A phylogenetic tree based on the amino acid sequences was constructed. It showed that all the CYP711 members originated from the same ancestral origin, but diverged subsequently at different phases of evolution. CmMAX1 was closely related to NnMAX1 from lotus. It shared 55.33% amino acid similarity with NnMAX1, demonstrating CmMAX1 was closely related to NnMAX1 from lotus. It shared 55.33% amino acid similarity with NnMAX1, demonstrating CmMAX1 was a member of CYP711 according to the theory that P450 proteins with more than 55% similarity were grouped into the same subfamily (Mizutani and Ohta, 2010).

During the transition phase from vegetative growth to reproductive growth in plants, the hormone levels will be altered, which might lead to changes in gene expression. Given this, we tested the expression of CmMAX1 during the vegetative growth stage. Expression analysis showed that CmMAX1 was detected in tissues varied little. This result was different from the previous report in petunia, in which PhMAX1 was expressed highest in the low-stem samples (Drummond et al., 2012).

Studies in multiple species have shown that the biosynthesis of strigolactones is increased during P deficiency (Wang et al., 2015; Xi et al., 2015; Yoneyama et al., 2007). P starvation upregulated the expression level of DgCCD7 and DgCCD8 in chrysanthemum (Xi et al., 2015). In our study, the expression levels of CmMAX1 increased significantly compared with normal condition, indicating P deficiency can induce the expression of CmMAX1.

Strigolactone, auxins, and cytokinin are the most important hormones regulating shoot branching. Strigolactone and cytokinin were thought to be the second messengers of auxin to inhibit or promote directly the growth of axillary buds (Domagalska and Leyser, 2011). CmMAX1 decreased significantly after 2 h of GR24 treatment, which indicated there was a feedback regulation in the synthesis pathway of strigolactone. The application of 6-BA caused a decrease in the expression of CmMAX1, suggesting that cytokinins may regulate the synthesis of strigolactone by inhibiting the expression of CmMAX1. Studies in arabiopsis showed that MAX3 and MAX4 transcripts are regulated positively by auxin (Hayward et al., 2009). However, IAA treatment led to the significant decrease of CmMAX1 expression (Fig. 4), suggesting the complex regulation of the strigolactone synthesis pathway by auxin.

The overexpression of CmMAX1 in arabiopsis not only reduced max1-1 branching, but also increased plant height (Fig. 3C). This result demonstrates that CmMAX1 is a functional gene. Overexpression of PgMAX1 (Picea glauca) in arabiopsis was found to be capable of complete rescue of max1-1, and the overexpression of SmMAX1 (Selaginella moellendorffii) obviously reduced branching, although SmMAX1 only shares 38.7% amino acid similarity with MAX1 (Hepworth, 2012). These results indicated the partial conservation of MAX1 function across a wide evolutionary range.

We obtained the full length of CmMAX1 cDNA from chrysanthemum cultivar Jinba, and detected the expression of tissues and different treatments. Moreover, we confirmed the function of the CmMAX1 gene in vivo by the complementation experiment. Our results presented here reveal that the MAX1 ortholog was present and active in chrysanthemum, and also that CmMAX1 might be a candidate gene for the regulation of shoot branching.

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