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Molecular characterization and expression analysis of the critical floral gene *MdAGL24-like* in red-fleshed apple

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ABSTRACT

The transition from vegetative to reproductive growth is the most dramatic phase change in plants. To better understand the molecular regulation of floral transition and flower development in red-fleshed apple (*Malus sieversii f. niedzwetzkyana*), we isolated and characterized a floral MADS-box gene, MdAGL24-like, which shares sequence similarity with AGAMOUS-LIKE 24 (AGL24) from other species. Spatial expression analysis showed that *MdAGL24-like* dynamically expressed in flowers, followed by roots and fruits. Subcellular localization analysis indicated that, like other transcript factors, MdAGL24-like was localized in the nucleus. Protein interaction analysis showed that MdAGL24-like could interact with MdSOC1 and MdAP1 in vivo and in vitro. MdAGL24-like and MdSOC1 could increase each other's expression by binding the CArG motifs in their promoters. Unlike MdSOC1, MdAGL24-like might indirectly promote the expression of MdLFY by upregulating the expression of MdSOC1. Ectopic expression of *MdAGL24-like* in wild-type *Arabidopsis* induced early flowering like the phenotypes induced by other *AGL24* genes. Similar to *AGL24* in *Arabidopsis, MdAGL24-like* could rescue the late-flowering phenotype of the *agl24* mutant to some extent. These results help clarify the molecular mechanism underlying flowering and provide a means of shortening the juvenile period in red-fleshed apples and other fruit trees.

1. Introduction

Floral induction of fruit trees is an important link in the plant life cycle and an essential step for the continuation of plant life [1]. Most perennial woody fruit trees have a long juvenile stage before flowering and fruiting [2,3]. Different varieties of fruit trees have juvenile phases of different lengths.

Apple (*Malus* × *domestica*) belongs to the Rosaceae subfamily Maloideae and is one of the most important fruit tree crops worldwild. China is currently the largest producer of apples in the world in terms of area and yield [4]. The development of the apple industry in China is associated with many problems such as the long juvenile stage (typically 4–8 years or more), major differences in fruit bearing in alternating years, and the long initial delay in fruiting of young trees. In fruit production, growth regulators, pruning, and shaping are used to regulate flowering. The long juvenile phase means that traditional breeding methods are too inefficient in terms of time to meet increasing market demands. Therefore, the elucidation of the molecular mechanism for flowering in fruit trees is important to accelerate the floral transition by genetic engineering.

In recent years, studies on floral induction of apple trees have mainly focused on verifying the function of key floral genes such as *FLOWERING LOCUS T* (*FT*), *FLOWERING LOCUS C* (*FLC*), and *LEAFY* (*LFY*). For instance, *MdFT1* and *MdFT2* both promote the early flowering of plants and shorten the juvenile phase in apple [5]. Another study identified two homologs of *TFL*, *MdTFL1* and *MdTFL2*, and showed that silencing *MdTFL1* resulted in transgenic apple lines with early flowering [6]. Overexpressing *MdAFL2* in *Arabidopsis thaliana* produced an early flowering phenotype [7]. The transition from vegetative to reproductive development is mediated by multiple genetic pathways in response to developmental cues and environmental signals [8–13]. The interaction between these signal pathways regulates a

Abbreviations: wt/WT, wild-tipe; d, day; LD, long days; -T/-H, -Trp /-His; -T/-H/-L, -Trp/-His/-Leu; -T/-H/-L/-A, -Trp/-His/-Leu/-Ade; AGL24, agamous-like 24; SVP, short vegetative phase; AP1, apetela1; SEP3, sepallata3; TFL, terminal flower; AFL, apple floricaula/leafy; TF, transcription factor; GFP, green fluorescent protein; GST, glutathione S-transferase; HIS, histidine; Anti, antibody; MS, murashige and skoog

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common set of goal genes, including *FLOWERING LOCUS T (FT)*, *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1 (SOC1)* and *LEAFY (LFY)* [14,15]. The flowering pathways involve many transcription factors, MADS-box TFs are a major transcription factor family. These share a highly conserved MADS box domain that recognizes and binds to the $CC(A/T)_6GG$ (CArG) box of target genes [16,17]. At present, studies on floral formation are relatively thorough in model plant *Arabidopsis thaliana*. In *Arabidopsis thaliana*, MADS-box TFs play a key role in plant flowering and gene regulatory networks [3]. For example, *AGL24* and *SOC1* in *Arabidopsis* are floral promotion factors [18–20], while *SVP* and *FLC* are inhibitors of flowering [21,22]. Interestingly, AGL24 and SVP are closely related genes but have opposite functions in various stages of reproductive development [12,13]. AGL24 is reported that it could directly activate the transcription of SOC1 and together with SOC1 regulates LFY [20,23], thereby linking floral induction with floral development. AGL24 also participates in the higher order MADS-box complex with APETELA1 (AP1) and SEPALLATA3 (SEP3) to regulate flower development [24–26]. Considerable efforts are currently being made to identify and characterize *AGL24*-like genes, such as *AtAGL24* [20], *RcMADS1* [27], *WAS206* (MPF2-like MADS-box gene related to *AGL24*) [28], and *PtAGL24* [29], in many annual and perennial plants.

However, MADS-box TFs that regulate flowering, particularly, floral induction and floral development, have not been functionally characterized in red-fleshed apples. Therefore, in this study, we isolated and characterized a MADS-box gene, *MdAGL24-like*, from red-fleshed apple. The expression patterns and protein interactions of MdAGL24-like were examined and its role in floral induction was identified by ectopic expression in *Arabidopsis*. Based on our findings, the potential role of *MdAGL24-like* was postulated. We attempted to elucidate the expression patterns of MADS-box TFs that regulate flowering in red-fleshed apples.

2. Materials and methods

2.1. Plant materials and growth conditions

F2 hybrid populations of *Malus sieversii f. neidzwetzkyana* × *M. domestica cv.* Fuji.were grown in the Guanxian (N 36°29′, E115°27′) fruit tree breeding base of Shandong Agricultural University. The roots, leaves, stems tips, flowers, and fruits were taken from F2 hybrid populations, and frozen in liquid nitrogen, then stored at −80 °C for use.

The 'Orin' callus used for genetic transformation was cultured on MS medium containing 1 mg/l 2, 4-dichlorophenoxyacetic acid (2,4-D), 0.5 mg/l 6-benzyl- aminopurine (6-BA), 30 g/L sugar and 7 g/L Agar at 24℃ in the dark. Then WT callus was subcultured every 15 days.

2.2. RNA extraction and real-time PCR

Total RNA was isolated using an RNAprep pure Plant Kit (TianGen, http://www.tiangen.com/en/), following the manufacturer's instructions. RNA was then used to synthesize first-strand cDNA using a RevertAid™First Strand cDNA Synthesis Kit (TransGen, http://www.transbionovo.com/). Quantitative real-time (qRT) PCR primers were designed using Beacon DESIGNER7 software and synthesized (Table S2). qRT-PCR analysis were performed using a Bio-Rad CFX96 (Bio-Rad, http://www.bio-rad.com/) real-time quantitative PCR instrument. Three technical replicates were included for each sample. Using the apple actin gene *MdActin* [30] and *Arabidopsis thaliana* actin gene *TUB2* [20] as internal controls, respectively. mRNA levels were analyzed using the $2^{-\Delta\Delta Ct}$ method [31].

2.3. Cloning and bioinformatic analysis of MdAGL24-like

The *MdAGL24-like* gene coding sequence (CDS) was amplified from the flowers of red-fleshed apple lines using PCR. The product was separated by 1% agarose gel electrophoresis, and target band was recovered, then ligated to a PLB zero background vector (VT205) for sequencing.

The physiochemical properties of the MdAGL24-like protein were determined using the Expasy website (http://web.expasy.org/protparam/). The NCBI software tool BLAST was used to compare the amino acid sequence of MdAGL24-like with that AGL24 from other species. MEGA5.1 software was used to construct the phylogenetic tree based on the neighbor-joining approach.

2.4. Apple callus transformation of MdAGL24-like

The CDS of *MdAGL24-like* was recombined into the pRI101-AN vector containing a GFP tag sequence and then transformed into A. tumefaciens LBA4404. 6–10 d old apple callus was infected by the *Agrobacterium* containing *MdAGL24-like*-GFP and empty GFP, respectively. Then the callus was transferred to screening medium with 50 mg/l kanamycin (Solarbio, Beijing, China) and 250 mg/l carbenicillin (Solarbio). Transgenic callus was subcultured every 15 days.

2.5. Extraction of protoplasts and subcellular localization analysis

The callus (2–3 g) containing OE-MdAGL24-like fused to a GFP tag was added to 10 ml cell wall lysis solution and vacuumed for 30 min. It was then incubated in the dark at 24 °C for 12 h. The mixture was gently shaken to release protoplasts, then 10 ml W5 solution was added. The solution was filtered through nylon fabric (75 μ m pore size) and centrifuged with 400 rpm for 2 min. The supernatant was discarded and precipitate containing protoplasts was suspended with10 ml W5 solution. After 30 min, the supernatant was discarded and protoplasts was suspended with 2 ml MMG solution. The GFP fluorescence in protoplasts was detected using an epifluorescence microscope (Olympus BX53 F, Tokyo, Japan). The nuclei were stained using 4′,6-diamidino-2-phenylindole (DAPI).

2.6. Yeast two-hybrid assays (Y2H)

The CDS of *MdAGL24-like* was recombined into the pGBKT7 vector (Clontech, http://www.clontech.com) and CDSs of *MdSOC1* and *MdAP1* were recombined into the pGADT7 vector (Clontech). The recombinant plasmids were co-transformed into the yeast strain Y2H Gold and cultured on selective medium -Trp/- Leu (-T/-L) at 30 °C for 2-3d. To screen for interactions, the yeast cells were transferred to medium lacking Trp, Leu, His, and Ade (−T/−L/−H/−A) with or without X-α-gal. Interactions were identified using X-α-gal selective medium. Empty pGADT7 vector was used as a control.

2.7. Bimolecular fluorescence complementation assays (BiFC)

The CDS of *MdAGL24-like* was recombined into the 35S-pSPYNE-YFP vector and CDSs of *MdSOC1* and *MdAP1* were recombined into the 35S-pSPYCE-YFP vectors. The recombinant plasmids were transformed into A. *tumefaciens* LBA4404. *Agrobacterium* containing NYFP-*MdAGL24-like* and CYFP-*MdSOC1*, CYFP-*MdAP1* were cultured to an OD600 of approximately 0.8–1.0 before being mixed together in equal volumes (15 ml). Onion epidermal cells were then infected with the mixed agrobacteria for 25–30 min before being cultured on new MS solid media plates at 28 °C in the dark for 1–2 d. A confocal laser scanning microscope was used to detect YFP fluorescence (Carl Zeiss, http://www.zeiss.com/) with an excitation wavelength of 488 nm.

2.8. Pull-down assays

MdAGL24-like-PLB, *MdAP1*-PLB and PET32 A(+) (His-Tag), pGEX-4T-1 (GST-Tag) were doubly digested with BamHI and SalI, then *MdSOC1*-PLB and PET32 A(+) (His-Tag) were doubly digested with BamHI and XhoI. These fragments were then used to construct