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The miR172 target TOE3 represses *AGAMOUS* expression during *Arabidopsis* floral patterning

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ABSTRACT

microRNA172 (miR172) regulates phase transition and floral patterning in *Arabidopsis* by repressing targets that encode the APETALA2 (AP2) and AP2-like transcription factors. The miR172-mediated repression of the *AP2* gene restricts *AGAMOUS* (*AG*) expression. In addition, most miR172 targets, including AP2, redundantly act as floral repressors, and the overexpression of the target genes causes delayed flowering. However, how miR172 targets other than AP2 regulate both of the developmental processes remains unclear. Here, we demonstrate that miR172-mediated repression of the *TARGET OF EAT 3* (*TOE3*) gene is critical for floral patterning in *Arabidopsis*. Transgenic plants that overexpress a miR172-resistant *TOE3* gene (*rTOE3*-ox) exhibit indeterminate flowers with numerous stamens and carpelloid organs, which is consistent with previous observations in transgenic plants that overexpress a miR172-resistant *AP2* gene. TOE3 binds to the second intron of the *AG* gene. Accordingly, *AG* expression is significantly reduced in *rTOE3*-ox plants. TOE3 also interacts with AP2 in the nucleus. Given the major role of AP2 in floral patterning, miR172 likely regulates *TOE3* in floral patterning, at least in part *via* AP2. In addition, a miR156 target SQUAMOSA PROMOTER BINDING PROTEIN-LIKE 3 directly activates *TOE3* expression, revealing a novel signaling interaction between miR156 and miR172 in floral patterning.

1. Introduction

Flower formation is a key developmental process that ensures the reproductive success of angiosperms. Angiosperm flowers are typically comprised of four different organs that are arranged in concentric whorls: sepals and petals, which are perianth organs that constitute the outer two whorls, and stamens and carpels, which are reproductive organs that constitute the inner two whorls.

The identities of the four floral organs are specified by the combinatorial interactions of three major classes of homeotic genes, which are designated as classes A, B and C [1,2]. In the first whorl of *Arabidopsis* flowers, A-class genes, such as *APETALA1* (*AP1*) and *AP2*, establish the sepal identity. In the second whorl, overlapping activities of B-class genes, including *PISTILLATA* (*PI*) and *AP3*, and A-class genes specify the petal identity. Similarly, the C-class gene *AGAMOUS* (*AG*), in conjunction with B-class genes, determines the stamen identity. The carpel identity is determined by *AG* in the fourth whorl. Furthermore, four redundant E-class genes, *SEPALLATA1* (*SEP1*), *SEP2*, *SEP3*, and *SEP4*, are required for the specification of all four floral organ identities [3,4].

The roles of AP2 and AG have been well established in floral organ development. AP2 restricts *AG* expression to the inner two whorls during the early stages of flower development [1,5]. In *ap2* mutants, the *AG* expression domain expands into the outer two whorls, which induces the formation of carpel-like organs in the first whorl and petal loss in the second whorl [5–7]. *AP2* transcripts have been demonstrated to accumulate predominantly in the outer two whorls, which further supports the hypothesis that AP2 restricts *AG* expression [8]. The second intron of the *AG* gene harbors at least two sequence elements that respond to AP2-mediated regulation [6,7]. AP2 has been demonstrated to directly bind to the non-canonical AT-rich target sequences in the second intron of *AG* and repress its expression by recruiting a transcriptional corepressor TOPLESS (TPL) and a histone deacetylase HDA19 [9,10].

The *AP2* gene is regulated post-transcriptionally by microRNA172 (miR172) [11–14]. Accordingly, the overproduction of miR172 disrupts the floral structure, which is similar to the phenotype observed in strong *ap2* mutants [12,14,15].

Abbreviations: AG, AGAMOUS; AP2, APETALA2; CaMV, cauliflower mosaic virus; ChIP, chromatin immunoprecipitation; FT, FLOWERING LOCUS T; GUSβ, -glucuronidase; miR172, microRNA172; MS, Murashige & Skoog; qRT-PCR, quantitative real-time RT-PCR; SOC1, SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1; SPL3, SQUAMOSA PROMOTER BINDING PROTEIN-LIKE 3; TOE3, TARGET OF EAT 3.

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Additionally, transgenic plants that express a miR172-resistant *AP2* gene under the control of its native promoter exhibit severe floral defects, such as indeterminate flowers with numerous reproductive organs and enlarged floral meristems [16]. These phenotypes suggest that the miR172-mediated repression of *AP2* would be crucial for defining the boundary between the perianth and reproductive organs and for maintaining floral determinacy [8,16].

Recent studies have shown that the miR172-mediated repression of *AP2* is more complex than initially proposed. The expression domain of miR172 is largely complementary to the *AP2* expression domain. The *AP2* gene is expressed primarily in the outer two whorls, whereas miR172 expression is restricted to the inner whorls during flower development [8]. During the early stages of flower development, the domains governing miR172 accumulation and *AP2* expression transiently overlap in the stamen primordia, where the *AG* gene is also expressed [8]. Therefore, the miR172-mediated repression of *AP2* likely occurs in a cadastral manner in the third whorl, which contributes to AG function in normal stamen formation.miR172 also regulates the phase transition between vegetative and reproductive phases, which is referred to floral transition, by repressing target genes that encode AP2 and AP2-like transcription factors, such as TARGET OF EAT 1 (TOE1), TOE2, TOE3, SCHLAFMÜTZE (SMZ), and SCHNARCHZAPFEN (SNZ). miR172 targets, except for TOE3, redundantly act as floral repressors, and their overexpression results in delayed flowering [12,15,17,18]. Therefore, a hexuple mutant lacking all of the known miR172 targets recapitulates the early flowering phenotype of miR172-overproducing plants [15].

Unlike other miR172 targets, the mechanism by which TOE3 affects the floral transition remains elusive, since its overexpression does not cause delayed flowering [17]. The expression pattern of the *TOE3* gene differs from other miR172 targets [15,17]. Putative binding sites for SMZ and AP2 have been identified in the *TOE3* gene promoter [15,18], which suggests that TOE3 functions downstream of SMZ or AP2. In addition, the ectopic expression of a miR172-resistant version of *TOE3* (*rTOE3*) causes floral defects [19], which suggests that the miR172-mediated regulation of the *TOE3* gene is important for flower development.

Recent studies have indicated that the miR172-mediated signaling cascades that regulate the phase transition and flower development are not simple. miR172 abundance is temporally regulated by miR156 and its targets, which encode a subset of the SQUAMOSA PROMOTER BINDING PROTEIN-LIKE (SPL) transcription factors [20,21]. SPL9 and SPL10 directly activate the *MIR172b* gene [20], signifying that a reduction in miR156 levels correlates with an elevation in miR172 levels during plant development. Interestingly, miR172 is also regulated by its target, AP2, which directly binds to the genomic region of the *MIR172b* gene [15], further extending the signaling complexity of miR172 and its targets.

In this work, we demonstrate that the miR172-mediated regulation of the *TOE3* gene is critical for both floral transition and floral patterning in *Arabidopsis*. The ectopic expression of the *rTOE3* gene causes late flowering and indeterminate flowers with numerous reproductive organs. We also demonstrate that TOE3 binds to the second intron of the *AG* gene and interacts with AP2 in the nucleus, supporting the *AP2*-dependent function of *TOE3* in floral patterning. In addition, the *TOE3* gene is activated by SPL3, which reveals a novel signaling interaction between miR172 and miR156.

2. Materials and methods

2.1. Plant materials and growth conditions

All of the *Arabidopsis thaliana* lines used were in the Columbia (Col-0) background. *Arabidopsis* plants were grown in soil or on 1/2× Murashige & Skoog (MS)-agar plates under long days (LDs, 16 h light and 8 h dark). *Arabidopsis* seeds were cold-imbibed for 3 days at 4 °C in complete darkness and allowed to germinate at 23 °C. Cool-white light illumination (120 μmol of photons m^{-2} s^{-1}) was provided by fluorescent FLR40D/A tubes (Osram, Seoul, Korea).

An *Arabidopsis* loss-of-function mutant of *TOE3* gene (*toe3-2*, SAIL-74-B10) was isolated from a pool of T-DNA insertion lines deposited in the *Arabidopsis* Biological Resource Center (ABRC, The Ohio State University, Columbus, OH, USA). A single T-DNA element insertion in the *toe3-2* mutant was verified by genomic PCR using the primer pair LP (5′-CTTGGATAGGGGCATTTTAGG-3′) and RP (5′-ACTGAGAGCTACGTGAGCGAG-3′).

2.2. Generation of transgenic plants

To generate transgenic plants overexpressing *rAP2*, *rTOE1*, *rTOE3*, and *TOE3* genes (*rAP2*-ox, *rTOE1*-ox, *rTOE3*-ox, and *TOE3*-ox, respectively), six copies of the MYC (6xMYC) epitope tag-coding sequence were fused in-frame to the 3′ end of each gene sequence and overexpressed under the control of the cauliflower mosaic virus (CaMV) 35S promoter in Col-0 plants. We also generate transgenic plants overexpressing a miR156-resistant *SPL3* gene (*rSPL3*-ox), in which six copies of the MYC-coding sequence were fused in-frame to the 3′ end of the *SPL3*-coding sequence that does not contain the miR156-binding site. The p*TOE3-rTOE3* transgenic plants were also generated by expressing the *rTOE3* gene driven by the *TOE3* gene promoter that spans approximately a 4 kb sequence region upstream of the transcription start site. *Agrobacterium*-mediated *Arabidopsis* transformations were performed according to a modified floral dip method [22]. Because most of the transgenic plants, except for *TOE3*-ox, exhibited female sterility, they were maintained as F1 seeds by crossing to Col-0 plants. Note that the TOE3 proteins expressed in the p*TOE3-rTOE3* transgenic plants did not contain MYC tags.

2.3. Analysis of gene transcript levels

Quantitative real-time RT-PCR (qRT-PCR) was employed to measure gene transcript levels. Total RNA samples were extracted from appropriate plant materials using the RNeasy Plant Total RNA Isolation Kit (Qiagen, Valencia, CA, USA). Total RNA samples were pretreated with an RNase-free DNase I (Roche, Indianapolis, IN, USA) to eliminate genomic DNA before use.qRT-PCR was performed in 96-well blocks with the Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using the SYBR Green I master mix in a reaction volume of 20 μl. All qRT-PCR assays were performed in biological triplicates using total RNA samples extracted from three independent plant materials treated under identical conditions with gene-specific primer pairs listed in Supplementary Table S1. The RNA preparation, data processing and determination of reaction specificities were performed as described previously [23].

2.4. Histochemical analysis

The promoter sequence of the *TOE3* gene spanning approximately a 4 kb sequence region upstream of the transcription start site was amplified by genomic PCR using the primer pair 5′-AAAAAGCAGGCTTGAAAGTTTCCTTCACCATG-3′ and 5′-AGAAAGCTGGGTCTCCTGAGTATTGACATCACAT-3′. The PCR product was cloned into the pDONR211 vector (Invitrogen, Carlsbad, CA, USA) and subcloned into the binary pKGWFS7 vector (Invitrogen) using the Gateway technology. The fusion construct was transformed into Col-0 plants.

For histochemical analysis of β-glucuronidase (GUS) activity, plant materials were soaked in 90% acetone for 20 min on