

Expression of the Floral Repressor miRNA156 is Positively Regulated by the AGAMOUS-like Proteins AGL15 and AGL18

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The regulation of flowering time has crucial implications for plant fitness. MicroRNA156 (miR156) represses the floral transition in *Arabidopsis thaliana*, but the mechanisms regulating its transcription remain unclear. Here, we show that two AGAMOUS-like proteins, AGL15 and AGL18, act as positive regulators of the expression of *MIR156*. Small RNA northern blot analysis revealed a significant decrease in the levels of mature miR156 in *agl15 agl18* double mutants, but not in the single mutants, suggesting that AGL15 and AGL18 co-regulate miR156 expression. Histochemical analysis further indicated that the double mutants showed a reduction in *MIR156* promoter strength. The double mutants also showed reduced abundance of pri-miR156a and pri-miR156c, two of the primary transcripts from *MIR156* genes. Electrophoretic mobility shift assays demonstrated that AGL15 directly associated with the CARG motifs in the *MIR156a/c* promoters. AGL18 did not show binding affinity to the CARG motifs, but pull-down and yeast two-hybrid assays showed that AGL18 forms a heterodimer with AGL15. GFP reporter assays and bimolecular fluorescence complementation (BiFC) showed that AGL15 and AGL18 co-localize in the nucleus and confirmed their *in vivo* interaction. Overexpression of miR156 did not affect the levels of *AGL15* and *AGL18* transcripts. Taking these data together, we present a model for the transcriptional regulation of *MIR156*. In this model, AGL15 and AGL18 may form a complex along with other proteins, and bind to the CARG motifs of the promoters of *MIR156* to activate the *MIR156* expression.

INTRODUCTION

In many plant species, flowering marks the transition from ve-

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Received 12 November, 2014; revised 10 December, 2014; accepted 11 December, 2014; published online 30 January, 2015

Keywords: AGAMOUS-like 15, AGAMOUS-like 18, CARG motifs, floral transition, miRNA156

getative to reproductive growth; induction of flowering at the proper time increases the plant's reproductive success. In agriculture, the flowering transition crucially determines crop yield, as flowering is mandatory for the production of seeds and fruits in higher plants. By contrast, prolonged vegetative growth and a late floral transition can improve yields in vegetative crops. In *Arabidopsis* (*A. thaliana*), extensive studies on the mechanisms that determine the floral transition showed that environmental factors (i.e., temperature and day-length) and intrinsic conditions (i.e., age and gibberellin accumulation) regulate flowering time (Fornara et al., 2010). A better understanding of the factors that govern the floral transition will help in the manipulation of flowering time and the improvement of agricultural practice to increase crop yields (Chung et al., 2014).

MicroRNAs (miRNAs), short non-coding RNAs of 20-24 nucleotides, negatively regulate expression of their target genes (Carrington and Ambros, 2003). Several miRNAs affect flower development and the floral transition in *Arabidopsis*. For example, transgenic plants overexpressing miR172 flower early and have floral organ defects (Chen, 2004). Also, miR159 and miR319 regulate floral development via interaction with MYB and TCP transcription factors (Rubio-Somoza and Weigel, 2013). In association with phosphate starvation, miR399 has been suggested to regulate flowering time in response to ambient temperature (Lee et al., 2010). The SQUAMOSA PROMOTER BINDING PROTEIN-LIKE 3 (SPL3) transcription factor promotes the floral transition by activating the expression of *FRUITFULL* (*FUL*), *LEAFY* (*LFY*), and *APETALA1* (*AP1*) (Yamaguchi et al., 2009). MiR156 negatively regulates *SPL3* expression via cleavage of the *SPL3* transcripts (Wu and Poethig, 2006); thus overexpression of miR156 prolongs the vegetative phase and delays the floral transition. Lee et al. (2012a) also suggested that, in response to ambient temperature, the miR156-SPL3 module regulates *FLOWERING LOCUS T* (*FT*) expression via the direct binding of SPL3 to the GTAC motifs within the *FT* promoter. Although much is known about miRNA-mediated target gene regulation, the upstream transcriptional regulation of the miRNAs themselves remains relatively unexplored.

MCM1-AGAMOUS-DEFICIENS-SRF (MADS) domain proteins are a family of DNA-binding transcription factors that contain the conserved DNA-binding domains MADS box (56 amino acids) at the N-terminal and a C-terminal extension of approximately 30 amino acids (Shore and Sharrocks, 1995). The SRF

core DNA-binding domain selectively binds to a consensus DNA sequence, the C-A/T rich-G (CArG) motif (West et al., 1997) to either activate or repress the expression of target genes. MADS-domain proteins play key roles in regulating developmental processes in eukaryotes (West et al., 1997). Interestingly, plants have more MADS-box gene families, compared to other kingdoms (Jack, 2001). This greater diversity suggests greater specificity of the downstream regulation by selective binding to the target genes (Tang and Perry, 2003). Although different MADS-domain proteins may have a similar binding site, they often show preferential binding (Shore and Sharrocks, 1995). In Arabidopsis, MADS-domain proteins affect several developmental processes, including root growth (Zhang and Forde, 1998), ovary, fruit, and seed coat development (Ferrandiz et al., 2000; and Nesi et al., 2002; Pinyopich et al., 2003), floral organ identity (Jack, 2001), and flowering time determination (Borner et al., 2000; Yoo et al., 2011).

AGAMOUS-like 15 (AGL15), a MADS-domain protein, is expressed during embryo and seed development in both monocot and dicot plants (Perry et al., 1996). AGL15 may function in the regulation of the Arabidopsis MYB transcription factor *MYB17* during early development of the inflorescence as well as in seed germination (Zhang et al., 2009). Like many other MADS-domain proteins, AGL15 forms a heterodimer with other proteins (Hill et al., 2008). DNA-protein interactions have also been observed, as AGL15 preferentially binds to CArG motifs *in vitro* (Tang and Perry, 2003; Wang et al., 2002). AGAMOUS-like 18 (AGL18) functions redundantly with AGL15 in the regulation of flowering time in Arabidopsis. Overexpression of *AGL15* and *AGL18* in Arabidopsis produced similar phenotypes, including morphological alterations and late flowering time. Moreover, *agl15 agl18* double mutants flower early, but the *agl15* or *agl18* single mutants do not flower early (Adamczyk et al., 2007). As genes in the same family often have overlapping functions, AGL15 and AGL18 likely have functional redundancy and act as co-repressors of floral transition via regulation of multiple flowering time genes (Gu et al., 2013; Fernandez et al., 2014).

MADS-domain proteins also regulate the expression of some miRNAs; for example, the MADS-domain protein SHORT VEGETATIVE PHASE (SVP) binds to a CArG motif in the promoter of *MIR172* and negatively regulates its expression (Cho et al., 2012), and the SVP function delays flowering transition (Lee et al., 2010). The *AGAMOUS-like 16* (*AGL16*) transcript is subject to sequence-specific degradation by miR824 in the regulation of stomatal development (Kutter et al., 2007). Although these findings collectively suggest an interaction between MADS-domain proteins and miRNAs, the molecular interactions between MADS-domain proteins and miRNAs, such as between AGL15/AGL18 and miR156 remain unknown. In this study, we demonstrate that AGL15 and AGL18 function as co-regulators of *MIR156* expression, as *agl15 agl18* double mutants show a significant decrease in miR156 accumulation and weaker expression from the *MIR156* promoter. AGL15 bound to the putative CArG motifs present in the *MIR156* promoters. AGL15 and AGL18 proteins co-localized in the nucleus and interacted *in vitro* and *in vivo*. Collectively, these data suggest that AGL15 and AGL18 may function as direct co-activators of the transcription of *MIR156* in the determination of flowering time in Arabidopsis.

MATERIALS AND METHODS

Plant materials

Wild-type Arabidopsis (Col-0), *agl15-3*, *agl15-4*, *agl18-1*, *agl15-*

3 agl18-1, *agl15-4 agl18-1* (Adamczyk et al., 2007), and *35S::MIR156* (Kim et al., 2012) plants were grown on Murashige and Skoog (MS) medium in a growth room under long-day (LD) conditions (16 h light/ 8 dark), at 23°C, with light intensity of 120 $\mu\text{mol m}^{-2}\text{s}^{-1}$. 9-day-old seedlings were sampled, immediately frozen in liquid nitrogen, and stored at -80°C prior to RNA extraction.

Gene expression analysis

Total RNA was isolated from 9-day-old seedlings using Plant RNA Purification Reagent (Invitrogen, USA). The total extracted RNA was pretreated with DNase I (NEB, USA) to eliminate possible DNA contamination, and subsequently subjected to complementary DNA synthesis using the First Strand cDNA Synthesis Kit (Roche Applied Science, USA). To measure the transcript levels of flowering time genes, quantitative real-time RT-PCR (qPCR) was carried out using the Green I Master Mix (Roche Applied Science, USA) with gene-specific primers (Supplementary Table S1). Two reference genes (AT1G13320/AT2G28390) that are stably expressed (Hong et al., 2010) were used for quantification. All reactions were performed with two biological replicates and three technical replicates. Determination of mature miR156 levels was done by miRNA northern hybridization analysis, as described previously (Lee et al., 2010). For histochemical GUS analysis, transgenic plants expressing *MIR156::GUS* were generated. The promoter region (approximately 2 kb) of *MIR156b* was amplified by PCR (Supplementary Table S1), and then cloned into the pBI101 vector harboring a GUS reporter gene. The resulting *MIR156::GUS* construct was introduced into wild-type and double mutant (*agl15-3 agl18-1* and *agl15-4 agl18-1*) plants using a floral dip method (Clough and Bent, 1998).

Recombinant protein production and purification

For production of His-fused proteins, the open reading frame (ORF) of Arabidopsis *AGL15* or *AGL18* was cloned into the pET21a vector (EMD Biosciences, USA). The resulting construct was introduced into *E. coli* BL21. Cell pellets were harvested from an overnight culture, resuspended in 1X His-binding buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl pH 7.9), sonicated, and then centrifuged. The supernatant was collected, and protein purification was performed using the His-Bind Kit (Merck, Germany) according to the manufacturer's instructions. For GST-fused proteins, the same ORF was cloned into the pGEX-5T vector (GE Healthcare, USA) and introduced into *E. coli* cells. The subsequent protein production and purification were conducted as previously described (Cho et al., 2012).

Electrophoretic mobility shift assay

Prediction of putative CArG motifs in *MIR156a* and *MIR156c* was done using AthaMap (<http://www.athamap.de>) and PLACE (<http://www.dna.affrc.go.jp/PLACE/>). The DNA sequences of the predicted CArG motifs were synthesized, with HPLC purification (Cosmogen, Korea). Electrophoretic mobility shift assays (EMSA) were performed using the LightShift Chemiluminescent EMSA Kit (Thermo Scientific, USA) according to the manufacturer's instructions. Double-stranded 3' biotin-labeled oligonucleotides were used as DNA probes (Supplementary Table S1). Purified AGL15-GST or AGL18-GST protein (2 μg) was incubated with the biotin-labeled oligonucleotides at room temperature for 30 min. The reaction samples were electrophoresed in an 8% native polyacrylamide gel, and then transferred onto a nylon membrane. Visualization was done as described in the manufacturer's instructions (Thermo Scientific, USA).

Yeast 2-hybrid, *in vitro* GST pull-down, and bimolecular fluorescence complementation (BiFC) analyses

PCR-amplified *AGL15* and *AGL18* ORFs were cloned into pGAD7 or pGBK7 (Clontech, USA) for the yeast two-hybrid experiment. The yeast 2-hybrid analysis was carried out as described previously (Lee et al., 2012b). The *in vitro* GST pull-down assay was performed as described by Jang et al. (2009) with minor modifications; purified GST-*AGL18* or GST protein was immobilized with glutathione sepharose beads (GE Healthcare, USA), and then incubated with the purified *AGL15*-His protein. The bead-retained proteins were subsequently eluted, analyzed by 12% SDS-PAGE, and then visualized by Western blot analysis or autoradiography.

For bimolecular fluorescence complementation (BiFC) analysis, the *AGL15* and *AGL18* ORFs were cloned into pUC-SPYNE/pSPYNE-35S or pUC-SPYCE/pSPYCE-35S (Walter et al., 2004) to generate *AGL15*-YFP^C and *AGL18*-YFP^N constructs. BiFC assays were done in both tobacco (*N. benthamiana*) epidermal cells and Arabidopsis protoplasts. For transient expression in tobacco plants, *Agrobacterium* harboring the *AGL15*-YFP^C and *AGL18*-YFP^N constructs was infiltrated into tobacco leaves (Voinnet et al., 2003). Epidermal cells of the transformed tobacco leaves were examined using a Zeiss LSM 510 META confocal microscope (Carl Zeiss, Germany). For transient expression in Arabidopsis mesophyll protoplasts, Arabidopsis protoplasts were prepared from young leaves by macerozyme treatment and transfected with the *AGL15*-YFP^C and *AGL18*-YFP^N constructs as previously described (Yoo et al., 2007). Cellular localization of the fluorescent proteins was examined with a Zeiss LSM 510 META confocal microscope (Carl Zeiss, Germany). Co-expression of the YFP-fused bZIP63 proteins (i.e., bZIP-YFP^C/bZIP-YFP^N) was used as a positive control (Walter et al., 2004).

Subcellular localization assay

Analysis of subcellular localization was performed using a green fluorescent protein (GFP) reporter assay. The full-length *AGL15* or *AGL18* ORF was cloned into a Gateway vector harboring the 35S promoter and GFP reporter gene, resulting in *35S::AGL15::GFP* and *35S::AGL18::GFP* constructs. The *35S::AGL15::GFP* and *35S::AGL18::GFP* fusion constructs were introduced into Arabidopsis mesophyll protoplasts using a polyethylene glycol (PEG)-calcium method (Yoo et al., 2007). 4', 6-diamidino-2-phenylindole (DAPI) staining identified the nucleus. Expression of the GFP fusion constructs was monitored by using a Zeiss Leica LSM 510 META confocal microscope (Zeiss, Germany). Tobacco leaves were infiltrated with the constructs and visualized as described above. A *35S::GFP* construct was used as a positive control.

RESULTS

Downregulation of miR156 in *agl15 agl18* double mutants

To investigate how miR156 expression is regulated by transcription factors, we analyzed the abundance of mature miR156 in *agl15* and *agl18* single mutants and in *agl15 agl18* double mutants, using northern blot analysis (Fig. 1A). Under inductive long day (LD) conditions, neither *agl15* nor *agl18* single mutants showed a significant reduction of mature miR156 levels, as compared to the wild type plants (Fig. 1A). However, we observed downregulation of miR156 levels in both *agl15-3 agl18-1* and *agl15-4 agl18-1* double mutants, suggesting that *AGL15* and *AGL18* act together to positively regulate *MIR156* expression.

We next analyzed the spatial expression patterns of *AGL15*, *AGL18*, and miR156. Small RNA northern blots showed that the levels of mature miR156 were high in the cotyledon of mature seedlings (Fig. 1B). Consistent with this observation, reverse transcription quantitative PCR (RT-qPCR) showed that *AGL15* and *AGL18* transcript levels were also high in the cotyledon. The levels of mature miR156 were low in the rosette leaves, cauline leaves, and stem. *AGL15* transcript levels in the same tissues were also lower than in the cotyledon, although *AGL18* showed slightly higher transcript levels in the rosette leaves. This result showed that transcript levels of *AGL15* and *AGL18* were largely correlated with the levels of mature miR156 levels. We then tested *pMIR156::GUS* reporter activity in *agl15 agl18* double mutants. GUS activity was detected in the vascular bundle of the cotyledon, true leaf, and shoot apical region in 6- and 8-day-old wild-type plants (Fig. 1C). Similar GUS patterns were observed in the *agl15 agl18* double mutants, but the GUS activity appeared to be lower. This finding further supported the notion that *AGL15* and *AGL18* regulate *MIR156* expression. To further investigate the genetic relationship, we measured the expression levels of *AGL15* and *AGL18* in *35S::MIR156* transgenic plants. The data showed that expression of *AGL15* and *AGL18* was not significantly altered in the transgenic lines (Fig. 1D), suggesting that *AGL15* and *AGL18* likely do not act downstream of miR156 in Arabidopsis. Because *agl15 agl18* double mutants showed a decreased level of *MIR156*, we performed qPCR to measure transcript levels of flowering time genes. We found that *FT*, *SPL3*, *SEP3*, and *FUL* expression levels significantly increased in *agl15-3 agl18-1* and *agl15-4 agl18-1* double mutants, compared to their expression in the wild type plants (Fig. 1E). Both double mutant lines showed similar increases in the expression of *FT*, *SPL3*, and *FUL*, whereas in the case of *SEP3*, the *agl15-3 agl18-1* mutants showed higher levels than *agl15-4 agl18-1* mutants. By contrast, *agl15* mutants did not show significant changes. Taken together, our results suggested that *AGL15* and *AGL18* positively regulate *MIR156* expression to regulate flowering time in Arabidopsis.

Pri-miR156a and pri-miR156c are affected in the *agl15 agl18* mutants

The mature miR156 is produced from primary transcripts of miR156 (pri-miR156) from eight different loci (*MIR156a-h*); therefore, we investigated which locus is mainly affected in the *agl15 agl18* double mutants. Absolute quantification of the levels of the primary transcripts revealed that pri-miR156a, pri-miR156c, and pri-miR156d are the main loci that produce miR156 in wild-type plants (data not shown). Thus we measured their transcript levels in wild-type and *agl15 agl18* double mutant plants. The expression of pri-miR156a and pri-miR156c were reduced in the double mutants, compared to the wild-type plants (Fig. 2), whereas pri-miR156d levels showed no significant changes in either of the double mutant lines (Fig. 2). This result suggested that *AGL15* and *AGL18* mainly regulate the transcription of *MIR156a* and *MIR156c*. Therefore, we focused on miR156a and miR156c for the subsequent studies.

AGL15 proteins bind to the CArG motifs in the promoters of *MIR156a* and *MIR156c*

Based on the gene expression data (Figs. 1 and 2), we hypothesized that the accumulation of mature miR156 might be caused by the direct interactions of *AGL15* and *AGL18* with the promoter sequences of *MIR156*. Using publicly available bioinformatics tools (AthaMap and PLACE) (Hehl and Bülow, 2014;

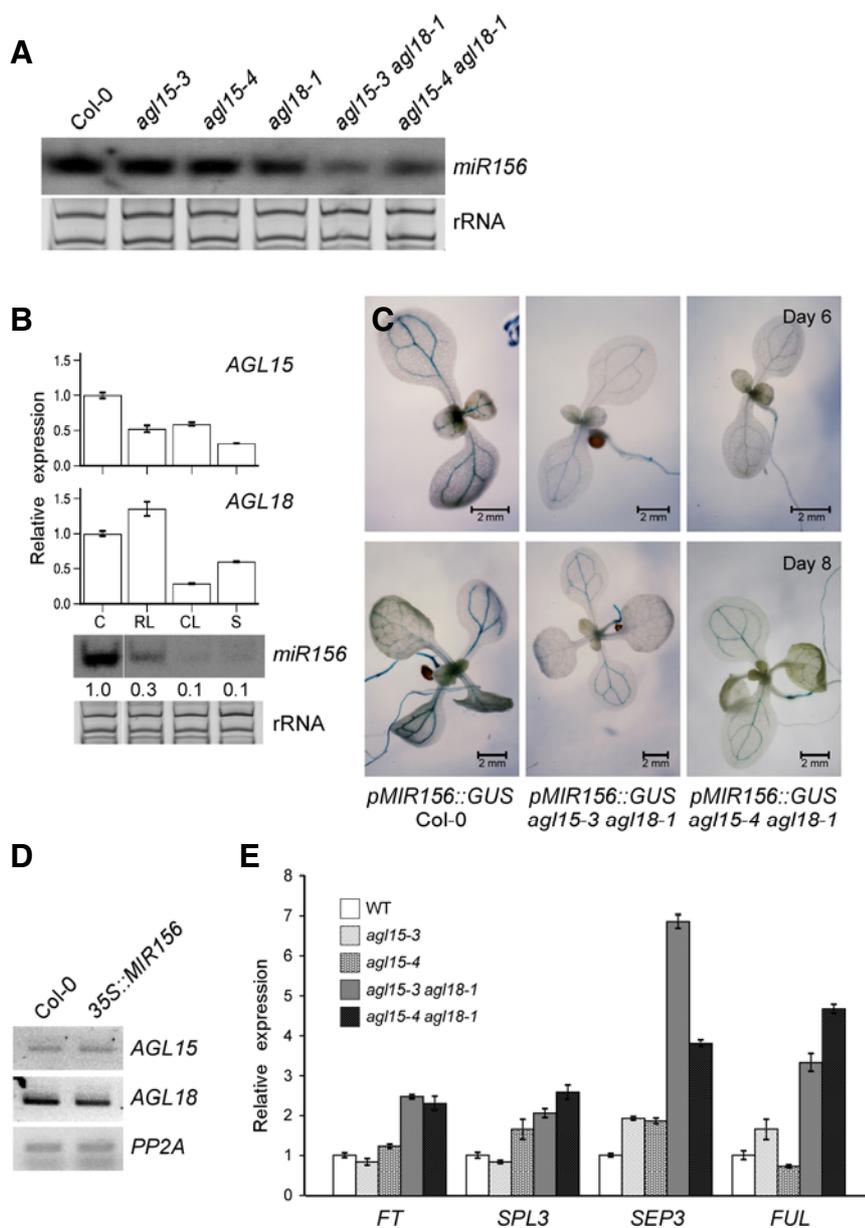


Fig. 1. Expression of mature miR156, AGL15, and AGL18. (A) Northern blot analysis of mature miR156 in Col-0, *agl15*, *agl18*, and *agl15 agl18* double mutants. Ethidium bromide (EtBr)-stained rRNA served as the loading control. (B) Spatial expression patterns of AGL15 and AGL18 compared with those of mature miR156. Y-axis indicates the relative transcript level of AGL15 or AGL18 in the cotyledon (C), rosette leaf (RL), cauline leaf (CL), and stem (S) of mature seedlings. The transcript levels of AGL15 and AGL18 in the cotyledon (C) were set to one. Ethidium bromide (EtBr)-stained rRNA served as the loading control. (C) Histochemical analysis of 6-day-old and 8-day-old seedlings of *pMIR156::GUS agl15 agl18* plants. Note that vascular bundle-specific GUS expression was reduced in the *agl15 agl18* double mutants. (D) Transcript levels of AGL15 and AGL18 in Col-0 and 35S::MIR156 plants determined by RT-PCR. PP2A was used as an internal control. (E) Transcript levels of FT, SPL3, SEP3, and FUL in Col-0, *agl15*, and *agl15 agl18* double mutants.

Higo et al., 1998), we identified eight putative CARG motifs in the upstream regions of *MIR156a* and *MIR156c* (Figs. 3A and 3B). The sequence of the identified putative CARG motifs, as well as their locations relative to the major transcription start sites (TSSs) (Xie et al., 2005), are shown in Fig. 3C.

To determine whether AGL15 and AGL18 bind to the *cis*-acting element(s) in the promoters of *MIR156a* and *MIR156c*, we first produced GST-tagged, full-length, soluble AGL15-GST (approximately 56 kDa) and AGL18-GST (approximately 55 kDa) (asterisks in Supplementary Fig. S1A). We then purified AGL15-GST and AGL18-GST on glutathione affinity columns. For both recombinant proteins, a single major band eluted from the column at the expected molecular weight (lanes 2 and 4 in Supplementary Fig. S1B).

We used an electrophoretic mobility shift assay (EMSA) to test the possibility that the positive regulation of miR156 ex-

pression by AGL15 and AGL18 occurs via direct binding to the *cis*-acting elements of *MIR156a* and *MIR156c*. Purified AGL15-GST and AGL18-GST recombinant proteins and synthesized double-stranded biotin-labeled oligonucleotides of putative CARG motifs were allowed to interact before electrophoresis. AGL15-GST proteins bound to a subset of the CARG motifs in the *MIR156a* and *MIR156c* sequences. A shifted band was observed for CARG IV and V of *MIR156a* and CARG III of *MIR156c* (Fig. 4A). In contrast, putative CARG motifs I, II, III, and negative control (NC) of *MIR156a*, and CARG motifs I, II, and NC of *MIR156c* did not show detectable shifted bands. This suggested that the binding of AGL15-GST protein to the CARG motifs was specific. AGL18-GST produced no apparent band-shifts (Fig. 4B). We further tested the binding of AGL15-GST to the CARG motifs by using unlabeled competitor probes of identical sequence. Our competition assays showed that the

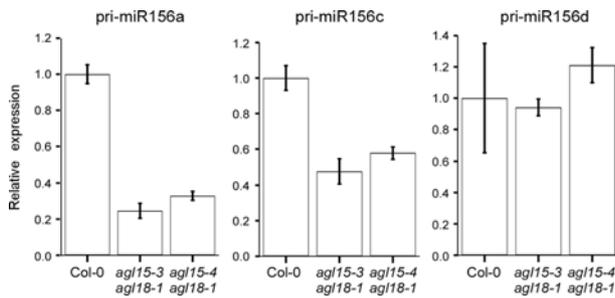


Fig. 2. Relative levels of primary transcripts of miR156a, c, and d by qPCR. The levels of each primary transcript in wild-type Col-0 plants were set to one.

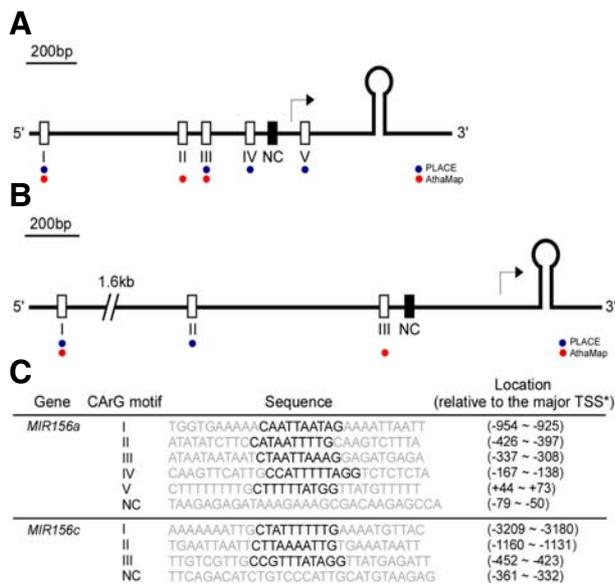


Fig. 3. Schematic representation of the locations of CARG motifs within the upstream region of the fold-back structure of miR156a (A) and miR156c (B). The putative CARG motifs predicted by PLACE and AthaMap (Hehl and Bülow, 2014; Higo et al., 1998) are indicated by blue and red dots, respectively. Open and closed boxes indicate putative CARG motifs and negative control (NC), respectively. The major transcription start site (TSS) previously reported (Xie et al., 2005) is denoted with an arrow. (C) Sequence information for the CARG motifs used for probes. Core CARG motifs are marked in bold. TSSs of *MIR156a* and *MIR156c* were previously reported (Xie et al., 2005).

addition of unlabeled identical probes greatly reduced the intensity of the shifted bands from CARG motifs IV and V of *MIR156a* and from CARG motif III of *MIR156c* (Fig. 4C). Taken together, our results suggest that AGL15 binds to the CARG motifs IV and V of *MIR156a* and to the CARG motif III of *MIR156c* to directly regulate the transcription of pri-miR156a and pri-miR156c.

Interaction of AGL15 and AGL18

To test the protein-protein interactions between AGL15 and AGL18, we first performed a yeast two-hybrid experiment using

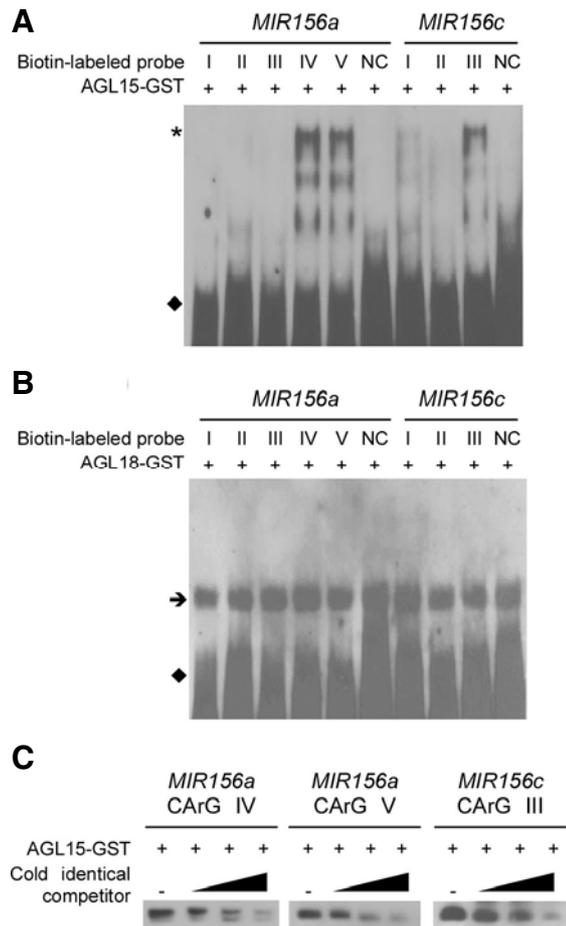


Fig. 4. Electrophoretic mobility shift assay (EMSA) with AGL15-GST protein (A) and AGL18-GST protein (B). Shifted bands are indicated with an asterisk. An arrow and closed diamonds indicate non-specific bands and free probes, respectively. (C) Competitor assay of AGL15-GST proteins binding to CARG IV and CARG V motifs (*MIR156a*) and a CARG III motif (*MIR156c*). Unlabeled probes of identical sequence were used as competitors at 100X, 500X, and 2500X molar excess. Only shifted bands are shown for simplicity.

pGADT7 and pGBKT7 as bait and prey, respectively. Yeast cells in the two hybrid assay were grown in selective medium lacking leucine and tryptophan (-Leucine -Tryptophan) and assayed for β -galactosidase (β -gal) reporter activity. The result showed that the yeast colonies expressing AGL15 as the prey (i.e., pGAD:AGL15/pGBK:AGL15 and pGAD:AGL15/pGBK18) turned blue in the presence of X-Gal (Fig. 5A; lower panel). When AGL18 was used as the prey (i.e., pGAD:AGL15/pGBK:AGL18 and pGAD:AGL18/pGBK18), the color development was also observed, but to a lesser extent.

To validate the interaction between AGL15 and AGL18, we also conducted *in vitro* glutathione S-transferase (GST) pull-down assays with recombinant AGL15 and AGL18. We constructed GST-fused AGL18 and His-fused AGL15, expressed these proteins in *E. coli*, and purified them using glutathione sepharose beads. GST-fused AGL18 and His-fused AGL15 were incubated together, pulled down with glutathione sepha-

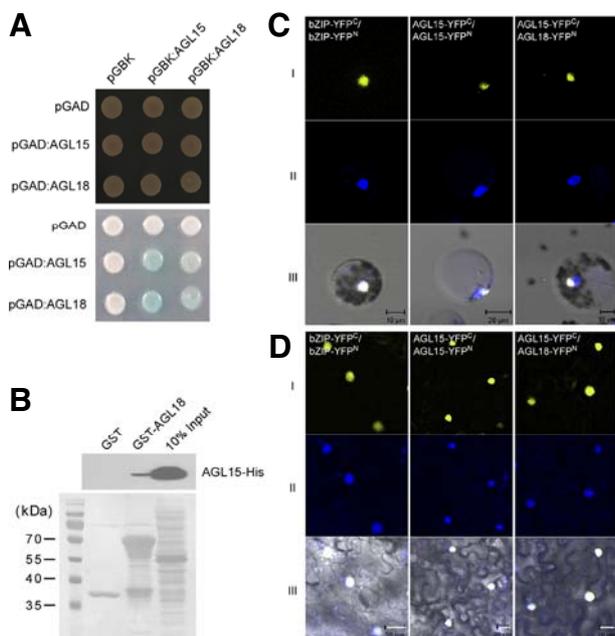


Fig. 5. Protein–protein interactions of AGL15 and AGL18 in yeast, *in vitro*, and *in vivo*. (A) Protein interactions between AGL15 and AGL18 in yeast two-hybrid analysis. Transformed yeast cells were grown on selective SD/-Leu/-Trp (SD-LT) medium (upper panel) and β-galactosidase assay was performed on SD-LT medium (lower panel). (B) Pull-down assay between AGL15-His and GST-AGL18 proteins. The signals were detected using an anti-His antibody. Proteins stained with Ponceau S are shown below. (C and D) Bimolecular fluorescence complementation using AGL18-YFP^N and AGL15-YFP^C (right column) (YFP^N, N-terminal YFP fragment; YFP^C, C-terminal YFP fragment) in Arabidopsis mesophyll protoplasts (C), and tobacco leaves (D). Rows I and II indicate YFP fluorescence and the nucleus stained by 4', 6-Diamidino-2-phenylindole (DAPI), respectively. Row III indicates the merged image of YFP, DAPI signals, and bright field images. bZIP-YFP^C and bZIP-YFP^N were used for the positive control (Walter et al., 2004).

rose, and detected by Western blotting. The band signals were detected using anti-His antibody (Fig. 5B). The pull-down assay demonstrated that *in vitro* synthesized AGL15 interacts with AGL18 (Fig. 5B), but not with GST alone. This result confirmed the heterodimer formation between AGL15 and AGL18 proteins detected in the yeast two-hybrid assay.

In vivo protein-protein interaction

Bimolecular fluorescence complementation (BiFC) assays were conducted to confirm the *in vivo* AGL15 and AGL18 interaction in Arabidopsis protoplasts. We co-expressed fusion pairs of the C-terminal half of yellow fluorescent protein (YFP) fused to AGL15 (AGL15-YFP^C). AGL15 and AGL18 sequences were also fused to the YFP N-terminal half (i.e., AGL15-YFP^N and AGL18-YFP^N). Co-expression of AGL15-YFP^C and AGL18-YFP^N resulted in yellow fluorescent signals in Arabidopsis protoplasts (Fig. 5C). The strong YFP fluorescence signals were only observed in the nucleus of the transfected cells, as confirmed by DAPI staining of the nuclei. The BiFC assays also detected self-interaction of AGL15, as the AGL15-YFP^C/AGL15-YFP^N transformed cells generated signals. The BiFC experi-

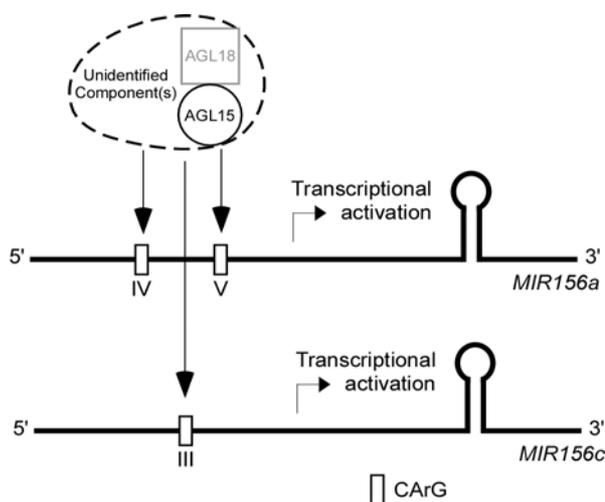


Fig. 6. A proposed model for the function of AGL15 and AGL18 in regulating the expression of miR156a and miR156c. AGL15 and AGL18 form a complex, possibly with other unidentified component(s); this complex then binds to the CArG motifs of the *MIR156a* and *MIR156c* promoters to activate gene expression.

ment was also conducted in tobacco leaves and nucleus-specific signals were observed (Fig. 5D). As a positive control, co-expression of bZIP-YFP^C and bZIP-YFP^N also produced nuclear signals. These data suggest that the major interaction of AGL15 and AGL18 likely occurs in the nucleus, as expected for transcription factors.

DISCUSSION

The AGAMOUS-like genes AGL15 and AGL18 act together to activate miR156 expression

As AGL15 and AGL18 act redundantly to suppress the floral transition (Adamczyk et al., 2007), we hypothesized that this suppression occurred via activation of miR156, a known floral suppressor (Wu and Poethig, 2006). The down-regulation of miR156 observed only in the double mutants (Fig. 1) indicates that, in addition to their functional redundancy, AGL15 and AGL18 positively co-regulate the expression of miR156. The spatial expression analysis further revealed that the similar expression patterns of AGL15, AGL18, and miR156 are consistent with their possible genetic interactions. The high transcript levels of the three genes in the cotyledon suggest that the cotyledon may be the possible site of their interaction. In the rosette leaf, high expression levels of AGL18 may partially compensate for the low AGL15 expression levels, which result in a decrease in miR156 accumulation. Reduced accumulation of miR156 in cauline leaf and stem are consistent with the reduced AGL15 and AGL18 expression.

The upregulation of floral activator genes (i.e., *SPL3*, *FUL*, *SEP3*, and *FT*) observed only in the double mutant lines (Fig. 1E) further supports the role of AGL15 and AGL18 as co-repressors of the floral transition. MiR156 directly targets *SPL3*, which is a direct activator of *FUL* (Yamaguchi et al., 2009), *FT*, and *SEP3* (Lee et al., 2012a); the accumulation of *SPL3* transcripts is inversely proportional to the levels of miR156 (Wu and Poethig, 2006). In line with previous reports, our results suggested that upregulation of *SPL3* and with its downstream genes (i.e.,

FUL, *FT*, and *SEP3*) is the consequence of the downregulation of miR156 in the double mutant lines. Downregulation of *SPL3*, *FUL*, *SEP3*, and *FT* can potentially explain the late-flowering phenotype of *AGL15* and *AGL18* gain-of-function lines (Adamczyk et al., 2007). Histochemical assays showed a reduction of GUS reporter expression in two independent double mutant lines (i.e. *agl15-3 agl18-1* and *agl15-4 agl18-1*). This indicates reduced *MIR156* promoter strength in the mutants, implying that *AGL15* and *AGL18* both affect miR156 expression at the transcriptional level. However, the fact that *GUS* expression was not completely abolished in the double mutant lines suggested that miR156 expression was partially independent of *AGL15* and *AGL18* and that other components could affect miR156 expression. The expression levels of both *AGL15* and *AGL18* were not affected by the overexpression of miR156, suggesting that miR156 does not regulate their expression, and therefore acts downstream of *AGL15* and *AGL18*. The drastic reduction of expression levels of miR156a and miR156c in the double mutant backgrounds implies that their transcriptional regulation directly involves *AGL15* and *AGL18*. The unaltered expression levels of pri-miR156d in *agl15 agl18* double mutants is consistent with our observation that pri-miR156d levels were lower than pri-miR156a and pri-miR156c, the two loci that mainly contribute to the production of mature miR156.

All together, this evidence strongly suggests that *AGL15* and *AGL18* act upstream of miR156 to positively co-regulate its transcription. Despite the ambient-temperature responsive nature of miR156 (Lee et al., 2010), neither *AGL15* nor *AGL18* expression patterns showed a strong correlation with temperature (data not shown). Lee et al. (2007) reported higher expression of *SVP*, the key factor of the ambient temperature flowering pathway (Lee et al., 2008), in the leaf, whereas *AGL15* is expressed more strongly in the cotyledon. This suggests that *AGL15* and *AGL18* act independently of the ambient temperature pathway, but may be involved in the endogenous aging pathway (Wang et al., 2009).

AGL15 binds to the promoters of *MIR156a* and *MIR156c*

Although the members of the MADS-domain protein family interact with similar or identical DNA sequences (Tang and Perry, 2003), our findings showed that *AGL15* and *AGL18* proteins did not bind to the same CARG motifs. DNA-binding site recognition does not determine the specific physiological role of MADS-domain proteins, as several proteins of the MADS-domain family can bind the same CARG motifs, yet have different developmental roles (Riechmann et al. 1996). Tang and Perry (2003) also reported that *AGL15* preferentially binds CARG motifs with a longer A/T-rich core, but we observed that the length of A/T-core of the *AGL15*-bound motifs was not necessarily longer than those of their counterparts. This implies that factors other than the length of the A/T-rich core (e.g. the sequences flanking the motif) might also contribute to the determination of the preferential binding site(s) for *AGL15*. Interestingly, in our assays, *AGL18* did not seem to directly interact with the promoters of *MIR156*, as observed in the EMSA experiment (Fig. 4). However, considering that the levels of mature miR156 were downregulated only in *agl15 agl18* double mutants, it is possible that *AGL18* may have a supporting role in regulation of *MIR156*. Nevertheless, we still cannot exclude the possibility that *AGL18* binds to another factor, which allows it to bind to the *MIR156* promoter.

AGL15 forms a heterodimer with AGL18

Our yeast 2-hybrid and *in vitro* pull-down results indicated that

AGL18 interacts with *AGL15*. In addition, *AGL15* also interacted with itself, forming a homodimer (Fig. 5). Co-localization of *AGL15* and *AGL18* in the nucleus, as observed in the GFP reporter assay (Supplementary Fig. S2), implies that the two proteins likely function at the same site and therefore is consistent with their interaction with each other. The localization of *AGL15* in the nucleus is also consistent with a previous report that it is a nuclear protein (Perry et al., 1996). However, we cannot exclude the possibility that *AGL15* and *AGL18* may be preferentially localized to the nucleolus, within the nucleus. The *in vivo* study of the protein-protein interaction further confirmed heterodimer formation between the two proteins. As expected, *AGL15* also formed a homodimer *in vivo*, consistent with a previous study in yeast (Hill et al., 2008). The similar results obtained from tobacco leaves infiltrated with the same constructs provide additional support for the protein-protein interaction. Collectively, our evidence suggests that, although *AGL18* does not directly bind to the CARG motifs on *MIR156* promoters, it interacts with *AGL15*, possibly forming a complex that activates *MIR156* expression.

Increasing evidence indicates a role for the network of MADS-domain protein members in flowering time control, with individuals contributing more or less to the transition (Yoo et al., 2011). With the fact that neither the phenotype previously reported (Adamczyk et al., 2007) nor the abundance of miR156 of each single mutant in this study resembles those of the double mutants, we suggest that *AGL15* and *AGL18* function as a complex along with other additional components, which exhibit redundant or additional DNA-binding properties. Therefore, the absence of either *AGL15* or *AGL18* in the individual single mutant might not be sufficient to cause a major conformational change, and hence the complex's function as a transcriptional activator is maintained. Further studies are required to identify the other components of the complex.

Based on our findings, we propose a working model (Fig. 6) demonstrating the potential upstream regulation of *MIR156* genes by *AGL15* and *AGL18*. These MADS box proteins form a complex, which may include other additional components that directly bind to CARG motifs in the promoters of the *MIR156* gene to activate its expression, which subsequently prevents premature floral transition. In binding, *AGL15* may act as a molecular surface for the interaction between the complex and *MIR156* sequences. Fernandez et al. (2014) reported the direct binding of *AGL15* to the *FT* locus, which contributes to delaying the reproductive phase transition; our evidence additionally suggests that *AGL15* and *AGL18* delay the floral transition by regulating *MIR156*, which eventually regulates the expression of multiple flowering genes. Further analysis of the molecular mechanism of the *AGL15/18* interaction with the *MIR156* sequence will give more information on how flowering time is fine-tuned in *Arabidopsis*.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

ACKNOWLEDGMENTS

We would like to express our appreciation to Dr. J.H. Lee and Dr. S.Y. Kim for their valuable intellectual and technical support. This work was supported by a National Research Foundation of Korea grant funded by the Korea government (Ministry of Science, ICT, and Future Planning) (2008-0061988) to J.H.A. P.S. was supported by BK21+ program. H.S.R. and W.K. were supported by Korea University.

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