Regulation of MicroRNA-Mediated Developmental Changes by the SWR1 Chromatin Remodeling Complex[1]

Kyuha Choi, Juhyun Kim, Sebastian Y. Müller, Mijin Oh, Charles Underwood, Ian Henderson, and Ilha Lee

Laboratory of Plant Developmental Genetics, School of Biological Sciences, Seoul National University, Seoul, 151-742, Korea (K.C., J.K., M.O., I.L.); and Department of Plant Sciences, University of Cambridge, Downing Street, Cambridge, CB2 3EA, United Kingdom (K.C., S.Y.M., C.U., I.H.)

ORCID IDs: 0000-0002-4072-3807 (K.C.); 0000-0001-5462-3391 (J.K.); 0000-0002-0148-7020 (S.Y.M.); 0000-0001-5730-6279 (C.U.); 0000-0002-3516-4326 (I.L.).

The ATP-dependent SWR1 chromatin remodeling complex (SWR1-C) exchanges the histone H2A-H2B dimer with the H2A.Z-H2B dimer, producing variant nucleosomes. Arabidopsis thaliana SWR1-C contributes to the active transcription of many genes, but also to the repression of genes that respond to environmental and developmental stimuli. Unlike other higher eukaryotic H2A.Z deposition mutants (which are embryonically lethal), Arabidopsis SWR1-C component mutants, including *arp6*, survive and display a pleiotropic developmental phenotype. However, the molecular mechanisms of early flowering, leaf serration, and the production of extra petals in *arp6* have not been completely elucidated. We report here that SWR1-C is required for miRNA-mediated developmental control via transcriptional regulation. In the mutants of the components of SWR1-C such as *arp6*, *sef*, and *pie1*, miR156 and miR164 levels are reduced at the transcriptional level, which results in the accumulation of target mRNAs and associated morphological changes. Sequencing of small RNA libraries confirmed that many miRNAs including miR156 decreased in *arp6*, though some miRNAs increased. The *arp6* mutation suppresses the accumulation of not only unprocessed primary miRNAs, but also miRNA-regulated mRNAs in miRNA processing mutants, *hyl1* and *serrate*, which suggests that *arp6* has a transcriptional effect on both miRNAs and their targets. We consistently detected that the *arp6* mutant exhibits increased nucleosome occupancy at the tested *MIR* gene promoters, indicating that SWR1-C contributes to transcriptional activation via nucleosome dynamics. Our findings suggest that SWR1-C contributes to the fine control of plant development by generating a balance between miRNAs and target mRNAs at the transcriptional level.

Chromatin structure is closely associated with the regulation of transcription. ATP-dependent chromatin remodeling complexes contribute to precise spatiotemporal transcription through distinct combinations of regulatory DNA sequences, DNA-binding transcription regulators, and chromatin-modifying enzymes (Cosma, 2002; Li et al., 2007). The ATP-dependent SWR1 chromatin remodeling complex (SWR1-C) catalyzes the replacement of H2A-H2B dimers with H2A.Z-H2B dimers in nucleosome structures, thus producing variant nucleosomes with dynamic properties (Mizuguchi et al., 2004; Luk et al., 2010). The H2A.Z-containing nucleosomes preferentially localize around transcription start sites and in the vicinity of the genes where SWR1-C is recruited, through the direct DNA binding of the SWR1 and SWC2, components of the complex (Raisner et al., 2005; Deal et al., 2007; Zilberman et al., 2008; Jin et al., 2009; Ranjan et al., 2013; Yen et al., 2013).

This SWR1-C-mediated histone exchange can have both positive and negative effects on transcription (Noh and Amasino, 2003; Choi et al., 2005; Deal et al., 2005; Choi et al., 2007; Deal et al., 2007; March-Diaz et al., 2008; Kumar and Wigge, 2010; Smith et al., 2011; Coleman-Derr and Zilberman, 2012; Jarillo and Pineiro, 2015). For example, a mutation in a component of SWR1-C reduces the transcription rate of floral repressor genes *FLC*, *MAF4*, and *MAF5*, thus contributing to early flowering. This shows the positive role played by H2A.Z deposition in transcription (Noh and Amasino, 2003; Choi et al., 2005; Deal et al., 2005; Martin-Trillo et al., 2006; Choi et al., 2007; Deal et al., 2007). In contrast, negative effects of SWR1-C on transcription have been reported in genes involved in systemic-acquired

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Address correspondence to ilhalee@snu.ac.kr; kc404@cam.ac.uk.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: Ilha Lee (ilhalee@snu.ac).

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resistance (SAR), jasmonate (JA)-mediated immunity, the P-starvation response (PSR), and genes that respond to high temperatures (March-Diaz et al., 2008; Kumar and Wigge, 2010; Smith et al., 2011; Coleman-Derr and Zilberman, 2012). Such genes are de-repressed in the *arp6* mutant under non-inductive conditions. Thus, the increased expression of *SAR* and *PSR* genes leads to a respective increase in pathogen resistance and root hair development in *arp6* (March-Diaz et al., 2008; Smith et al., 2011). The dual transcriptional roles of SWR1-C may be a result of its cooperative activities with different transcription regulators such as chromatin modifiers, post-translational modifications of H2A.Z and other histone variants, and DNA methylation, which generates broad and distinct ranges of nucleosome stability (Santisteban et al., 2000; Millar et al., 2006; Albert et al., 2007; Jin and Felsenfeld, 2007; Sarcinella et al., 2007; Venkatasubrahmanyam et al., 2007; Zilberman et al., 2008; Hardy et al., 2009; Jin et al., 2009; Marques et al., 2010; Choi et al., 2011; Coleman-Derr and Zilberman, 2012; Stroud et al., 2012; Wollmann et al., 2012).

It has been reported that H2A.Z is required for embryonic stem cell differentiation and gene activation through nucleosome depletion in mice (Li et al., 2012). However, how SWR1-C in plants influences transcriptional regulation and development remains to be established. H2A.Z deposition mutants were embryonically lethal in tested metazoans (Vandaal and Elgin, 1992; Iouzalen et al., 1996; Faast et al., 2001; Whittle et al., 2008), while Arabidopsis SWR1-C mutants such as *arp6* and *sef/atswc6* survive embryogenesis, but develop pleiotropic developmental phenotypes such as early flowering, serrated leaf shape, reduced fertility, decreased organ size, spontaneous necrosis, longer petioles, increased root hairs, and extra floral organs (Noh and Amasino, 2003; Choi et al., 2005; Deal et al., 2005; Martin-Trillo et al., 2006; Choi et al., 2007; Deal et al., 2007; March-Diaz et al., 2008; Kumar and Wigge, 2010; Smith et al., 2011). The developmental defects seen in SWR1-C mutants are largely mirrored in Arabidopsis *h2a.z* triple mutants and knock-down plants, suggesting that SWR1-C is required for H2A.Z deposition (Choi et al., 2007; March-Diaz et al., 2008; Kumar and Wigge, 2010; Coleman-Derr and Zilberman, 2012). Thus, understanding the molecular basis of the developmental phenotypes in SWR1-C mutants may provide insight into how H2A.Z influences chromatin structure and plant development. However, the developmental phenotypes of Arabidopsis SWR1-C mutants have not been fully investigated. For example, the *arp6 flc*, *arp6 ft*, and *sef ft* double mutants undergo earlier flowering than *flc* and *ft*, respectively. This indicates that *arp6* and *sef* influence *FLC*- and *FT*-independent flowering pathways (Choi et al., 2005; Choi et al., 2007). The molecular mechanisms behind the FLC/FT-independent effect on flowering and other developmental defects, including leaf serration and extra petal formation, remain unknown in *arp6*.

MicroRNAs (miRs) repress protein production post-transcriptionally (Chen, 2009). In flowering plants, miRNAs control diverse developmental processes including phase transitions, leaf shape, and floral organ identity. Plant miRNAs are spatiotemporally transcribed by RNA polymerase II, similar to miRNA-regulated target genes (Chen, 2009). Modules of miRNAs and their target transcription factors, such as the miR156-SPLs/miR172-AP2 LIKEs and miR319-TCPs/miR164-CUCs modules, regulate each other via feedback loops in Arabidopsis (Baker et al., 2005; Nikovics et al., 2006; Sieber et al., 2007; Chen, 2009; Wang et al., 2009; Wu et al., 2009; Koyama et al., 2010; Hasson et al., 2011; Huijser and Schmid, 2011). The miR156-*SPLs*/miR172-*AP2 LIKEs* module regulates age-dependent floral transitions: the miR156 level decreases gradually with age, and the transcript levels of the target genes, the *SPLs*, are subsequently increased. Sequentially, SPL genes increase the transcript level of miR172, which suppresses the flowering repressors, *AP2 LIKEs*, post-transcriptionally. SPLs also induce the transcription of *SOC1*, *FUL*, *AP1*, and *LFY* for floral induction (Wu and Poethig, 2006; Wang et al., 2009; Wu et al., 2009; Yamaguchi et al., 2009; Huijser and Schmid, 2011). The development of leaf margin serration is regulated by miR319-TCPs/miR164-CUCs modules, in which a transcription factor, TCP3, regulated by miR319, induces the transcription of *MIR164A* (Koyama et al., 2010). Subsequently, mature miR164a suppresses the expression of *CUC2*, a gene required to prevent the development of leaf serration (Nikovics et al., 2006). In a similar manner, floral organ number, and lateral root formation are regulated by the miR164-CUC/NAC1 module (Guo et al., 2005; Sieber et al., 2007).

Although DNA-specific transcription factors and the subunits of an Arabidopsis mediator complex are known to regulate the transcription of *MIR* genes (Chen, 2009; Wang et al., 2009; Kim et al., 2011), the correlation between chromatin structure and *MIR* gene transcription has not been fully explored. Here, we demonstrate that Arabidopsis SWR1-C is required to maintain the active transcription of several *MIR* genes, including *MIR156* and *MIR164*, in addition to heat shock or JA-responsive genes. We also demonstrate that the transcriptional misregulation of miR156 and miR164 in the mutations of the components of SWR1-C such as *arp6*, *sef*, and *pie1* may contribute to early flowering, leaf serration, and the formation of extra petals. Genome-wide small RNAs analysis revealed that many miRNAs including miR156 are decreased in *arp6*, although we found that some miRNAs, miR397, miR398, and miR408 were increased in *arp6*. In addition, we observed that the transcription of many other *MIR* genes is decreased by *arp6*, and that nucleosome occupancy is higher at the promoters of the *MIR* genes in *arp6* mutants than in the wild type, which suggests that SWR1-C directly regulates them at transcriptional level. Finally, we show that the transcription of both *MIRs* and their target genes is attenuated in *arp6* mutants, such that the transcription level of both primary transcripts (pri-miRNA) and the miRNA-target genes failed to be elevated in *hyl1* and *serrate* miRNA

processing mutants. We propose that such fine-tuning allows SWR1-C mutants in Arabidopsis to survive, while exhibiting a distinct developmental phenotype.

RESULTS

ARP6 Is Required for Transcriptional Activation After Environmental Induction

There are many reports showing that SWR1-C-mediated histone exchange can have both positive and negative effects on transcription (Choi et al., 2005; Deal et al., 2005; March-Diaz et al., 2008; Kumar and Wigge, 2010; Smith et al., 2011; Coleman-Derr and Zilberman, 2012; Jarillo and Pineiro, 2015). To investigate the molecular mechanism behind the mediation of transcriptional activation by Arabidopsis SWR1-C, we checked whether *arp6* mutation affects the binding of transcription factors to the promoter. To achieve this, we exploited the FRIGIDA transcriptional activation complex's (FRI-C) accessibility to the *FLC* promoter, because the transcriptional activation of *FLC* by FRI-C is well established (Choi et al., 2011). We performed a chromatin immunoprecipitation (ChIP) assay to detect the presence of FRI binding on the *FLC* promoter using a *FRI-myc* transgenic line, which possesses a transgene containing the myc-tagged *FRI* genomic sequence under the control of an endogenous promoter in a Columbia background. The enrichment of FRI-myc was reduced by the *arp6* mutation (Fig. 1A). This indicates that *ARP6* is required for the recruitment of FRI-C, a transcriptional activator, on the *FLC* promoter, and is consistent with the previous reports showing that *arp6* exhibits reduced *FLC* transcription and decreased enrichment of RNA polymerase II (Choi et al., 2011).

Since we observed that *ARP6* promotes the recruitment of the transcription factor complex, FRI-C, and RNA polymerase II to the *FLC* promoter for strong activation, we checked whether *ARP6* is also required for strong induction of the environmentally inductive heat shock protein gene, *HSP70*. In the previous report, it was shown that at room temperature, *HSP70* is de-repressed by the *arp6* mutation, and thus the function of SWR1-C was proposed to negatively impact the transcription of heat shock factor genes (Kumar and Wigge, 2010). We confirmed that *HSP70* transcripts were de-repressed by the *arp6* mutation at 20°C, as previously reported (Fig. 1B). However, after heat shock treatment at 40°C, *HSP70* transcripts increased approximately 5-fold in the wild type, but barely increased in *arp6*. Thus, the wild type exhibited a much higher transcript level than *arp6*. Such reductions in the *HSP70* transcript level in *arp6* compared to that of the wild type were observed consistently at 1.5 h and 2 h after heat shock and even after conditions were returned to room temperature for 30 min (Fig. 1B). Interestingly, the *HSP70* transcript level is reduced in *arp6* at low temperature (0°C on ice), which also induces *HSP70* expression. Such results clearly show that *ARP6* has a positive effect on the transcription of *HSP70* under inductive conditions, but has a negative effect on basal expression. To determine whether *ARP6* has the opposite effect on the transcription of environmentally inductive genes for induction and basal expression, we analyzed the effect of *arp6* on the expression of the genes induced by JA. Similar to *HSP70*, under the

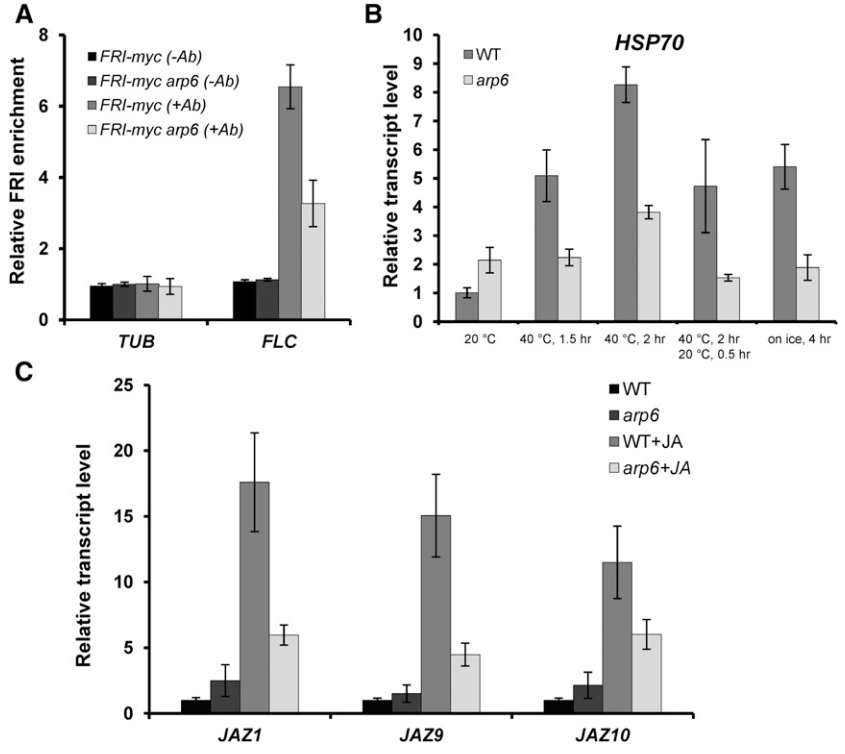

Figure 1. Positive transcriptional role of *ARP6* for *FLC*-, *HSP70*-, and JA-responsive genes. A, Reduced binding of FRI to the *FLC* promoter in *arp6*. The 10-d-old whole plants expressing *FRI-myc* in wild type and *arp6* were used for the ChIP-qPCR assay. (−Ab) Control experiments of ChIP without antibody; (+Ab) with antibody. *Tubulin 2* (*TUB*; +1556 approximately +2038 bp from the TSS) was used as a negative control for FRI binding while *FLC* promoter (−366 to approximately −492 for TSS) was used as a positive control. ChIP-qPCRs for (+Ab) were normalized by that of (−Ab) at *TUB* and *FLC*. B, Relative transcript level of *HSP70* in *arp6* under inductive conditions. For RT-qPCR, the 10-d-old plants were treated with inductive conditions consisting of high temperature (40°C for 90, 120, or 120 min followed by 20°C for 15 min) or on ice for 4 h. C, Transcript level of *JAZ1*, *JAZ9*, and *JAZ10* in *arp6* treated with methyl JA. Adult leaves of 30-d-old wild type and *arp6* were sampled at 1.5 h after methyl JA treatment (300 μM) for RT-qPCR. UBQ was used as a reference gene for RT-qPCR. Error bars show SD of the three biological replicates of the ChIP and RT-qPCR analyses. WT, wild type.

non-inductive conditions, without JA treatment, three JA-responsive genes, *JAZ1*, *JAZ9*, and *JAZ10*, exhibited slightly stronger transcript levels in *arp6* than in the wild type. This suggests that *ARP6* has a negative effect on the transcription of these genes (Fig. 1C). However, when JA is treated, *arp6* exhibited lower transcript levels of *JAZ1*, *JAZ9*, and *JAZ10* than the wild type. This result also indicates that SWR1-C has a positive effect on the transcription of inductive genes. Thus, it seems that SWR1-C has a negative effect on the basal-level transcription, but a positive effect on the inductive transcription.

Regulation of Age-Dependent Flowering by *ARP6* Mediating Transcriptional Activation

To search for additional evidence of SWR1-C-mediating gene activation in Arabidopsis, we explored the molecular links between *arp6* phenotypes and gene expressions. We previously speculated on the presence of another down-regulated floral repressor gene(s) in *arp6*, in addition to *FLC*, *MAF4*, and *MAF5*, which contributes to early flowering in a photoperiod-, *FLC*-, and *FT*-independent manner (Choi et al., 2005; Deal et al., 2005; Martin-Trillo et al., 2006; Choi et al., 2007; Deal et al., 2007). We tested whether miR156-*SPLs* and miR172-*AP2 LIKEs* pathways were affected in *arp6* (Fig. 2), because these miRNAs pathways regulate age-dependent flowering (Wang et al., 2009; Wu et al., 2009; Yamaguchi et al., 2009; Huijser and Schmid, 2011). We observed via small RNA blot analysis that levels of mature miR156 were reduced in *arp6* (Fig. 2B). miR156 is an upstream regulator of sequential phase transition pathways, and acts as a floral repressor, inhibiting SPL-family floral activators (Wang et al., 2009; Huijser and Schmid, 2011). Therefore, we investigated whether *MIR156* might be another floral repressor gene positively regulated by *ARP6* at the transcription level. The pri-miRNAs of *MIR156A*, *B*, and *MIR157C* genes, which are all processed to mature miR156, were reduced in *arp6*, similar to *FLC* (Fig. 2C), indicating that H2A.Z deposition is necessary for the transcriptional activation of these *MIR156/7* genes. As a result of the reduced miR156, the levels of the mRNAs of miR156-regulated genes *SPL3*, *-4*, and *-9* (but not *SPL11*) were found to be higher in *arp6* than in wild-type plants (Fig. 2D). The similar transcriptional changes to miR156 and its targets were observed in *arp6* grown under short-day conditions, wherein the age-dependent pathway mainly contributes to flowering induction (Supplemental Fig. S1). This may contribute to the early flowering of *arp6*, *arp6 ft*, and *sef ft* via the age-dependent pathway (Fig. 2, A, D, and E), because SPL3, -4 and -9 proteins directly increase the transcription of *SOC1*, *FUL*, *AP1*, and *LFY* (Wang et al., 2009; Yamaguchi et al., 2009; Huijser and Schmid, 2011).

We also observed that mature miR172 and pri-miR172b levels were moderately decreased, while the level of pri-miR172a was increased, in *arp6* (Fig. 2, B and C). This is unexpected because the miR172 level is inversely correlated with the miR156 level, and miR156-regulated SPL9 directly induces the transcription of *MIR172B*, which mainly contributes to the levels of mature miR172 in Arabidopsis (Fig. 2A; Wang et al., 2009). Therefore, the accumulation of *SPL9* transcripts in *arp6* due to lower miR156 levels may not be sufficient to increase the transcription of *MIR172B* under long-day conditions. This suggests that not only SPL9 protein, but also SWR1-C, may be required for *MIR172B* transcription—a finding that is consistent with previous observations of H2A.Z, which found that H2A.Z regulates transcription via other transcription factors and chromatin factors (Santisteban et al., 2000; Choi et al., 2011; Li et al., 2012). However, the *arp6* mutant exhibits a higher *SPL9* transcript level under short-day conditions than under long-day conditions, which may contribute to the increase in *MIR172B* transcription under short-day conditions (Supplemental Fig. S1). Because SPL9 directly activates *SOC1*, *AP1*, and *LFY* transcription (Wang et al., 2009; Yamaguchi et al., 2009; Huijser and Schmid, 2011), highly expressed *SPL9* may offset the effect of floral repressors, *AP2-Like*, thus causing early flowering in *arp6* in long days. In addition, in short days, both higher increase of *SPL9* and increased level of miR172 seem to cause much earlier flowering in short days (approximately 20 leaves in *arp6* versus 60 leaves in wild type) than long days (approximately 6 versus approximately 10 leaves).

MicroRNA172 suppresses five members of the AP2 family: AP2, TOE1, TOE2, SMZ, and SNZ, all of which act as floral repressors in a redundant manner (Huijser and Schmid, 2011). Despite the lower miR172 level, we observed that among the mRNAs of AP2 family genes, *TOE1* and *TOE2* levels were significantly lower in *arp6*, suggesting that SWR1-C may be required for the transcription of both *MIR* genes and miRNA-regulated genes. However, because the miR172-TOE1/2 module involves feedback-loop regulation, wherein TOE1 and TOE2 increase *MIR172* transcription while miR172 inhibits *TOE1/2* post-transcriptionally (Wu et al., 2009), we cannot dismiss the possibility that the reduced transcription of *TOE1* and *TOE2* in *arp6* mutants may lead to decreased *MIR172* transcription and miR172 levels. Thus, we show that three *MIR156* genes and two *AP2-LIKE* genes involved in floral repression are down-regulated at the transcriptional level in *arp6*, leading to early flowering. This indicates that *ARP6* has a positive effect on the transcription of the genes involved in the miR156/172-mediated flowering pathways (Fig. 2).

Control of miR164-Mediated Developments by *ARP6*

In addition to the early flowering phenotype, *arp6* mutants show leaf serration and extra petals (Figs. 2 and 3, A and E; Supplemental Fig. S2; Choi et al., 2005; Deal et al., 2005; Martin-Trillo et al., 2006; Choi et al., 2007). We investigated whether these phenotypes are associated with the misregulation of the miR164 family, because miR164a controls leaf margin formation

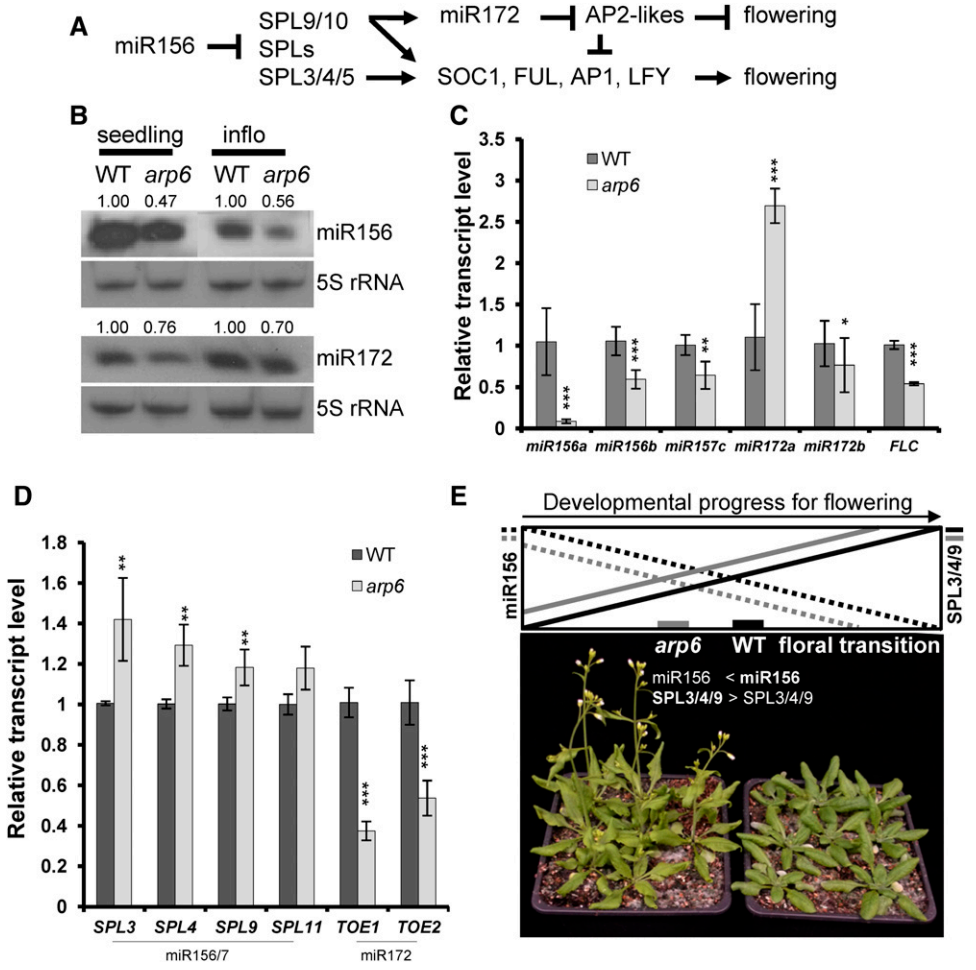

Figure 2. miR-mediated age-dependent flowering in *arp6*. A, Genetic hierarchy among modules of miR156-SPLs, miR172-AP2 LIKEs, and floral activators for flowering-time control. Arrows indicate a positive effect, and T bars indicate a negative effect. B, Small RNA blots of miR156 and miR172. The 20-d-old whole seedlings and inflorescences of wild type and *arp6* were used for the small RNA blot. C, Transcript levels of primary miRNAs, *pri-miR156*, *pri-miR172*, and *FLC* in wild type and *arp6*. D, Transcript level of miR156- and miR172-regulated genes in *arp6*. E, Early flowering in *arp6* caused by a lower level of miR156 and higher SPL activity. In the top diagram, black continuous and dotted lines represent the wild type, and gray lines represent the *arp6*. The dotted lines show the miR156 level, and continuous lines represent SPL3, 4, and 9 levels. Photos show an *arp6* mutant (left) and wild type (right) grown at the same time under long-day conditions. Total RNAs from plants grown for 20 d under long-day conditions were extracted for RT-qPCR analysis. *TUB* was used as a reference gene for RT-qPCR. Error bars indicate SD of three biological replicates, with two technical replicates per sample. Three asterisks indicate a *p*-value of Student's *t*-test lower than 0.01 among the means of three biological replicates in *arp6*, compared to wild type; two asterisks indicate the value is lower than 0.05; one asterisk indicates that the value is lower than 0.1. WT, wild type; inflo, influorescence.

(Nikovics et al., 2006), and miR164c from *MIR164C*, also known as *EARLY EXTRA PETAL1*, prevents the production of extra petals by repressing several NAC-like family transcription factors, including CUP-SHAPED COTYLEDON1 (CUC1) and CUC2 (Fig. 3A; Baker et al., 2005; Sieber et al., 2007). In the miR164a-*CUC2* module, we observed that both mature miR164a and pri-miR164a levels were decreased in *arp6* (Fig. 3, B and C). Consequently, the mRNA levels of miR164a-target genes *CUC1* and *CUC2* (but not *NAC1*) were increased in 20-d-old whole plants (Fig. 3D), which may cause leaf serration in *arp6* (Fig. 3E; Nikovics et al., 2006). Similarly, both mature miR164c and pri-miR164c levels, which are high in wild-type inflorescences but not in seedlings, were reduced in *arp6* inflorescences. Correspondingly, the transcription levels of the miR164c targets, *CUC1* and *CUC2*, were significantly increased, which may lead to the development of extra petals in *arp6* (Fig. 3, B–E; Baker et al., 2005; Sieber et al., 2007). Our results suggest that the *arp6* mutation may result in both the development of leaf serration and extra petals, due to the transcriptional attenuation of *MIR164* genes and the subsequent accumulation of *CUC* transcripts.

It was revealed that in the determination of leaf margin shape, miR319-regulated-TCP3 acts as an activator of *MIR164A* (Koyama et al., 2010). We measured

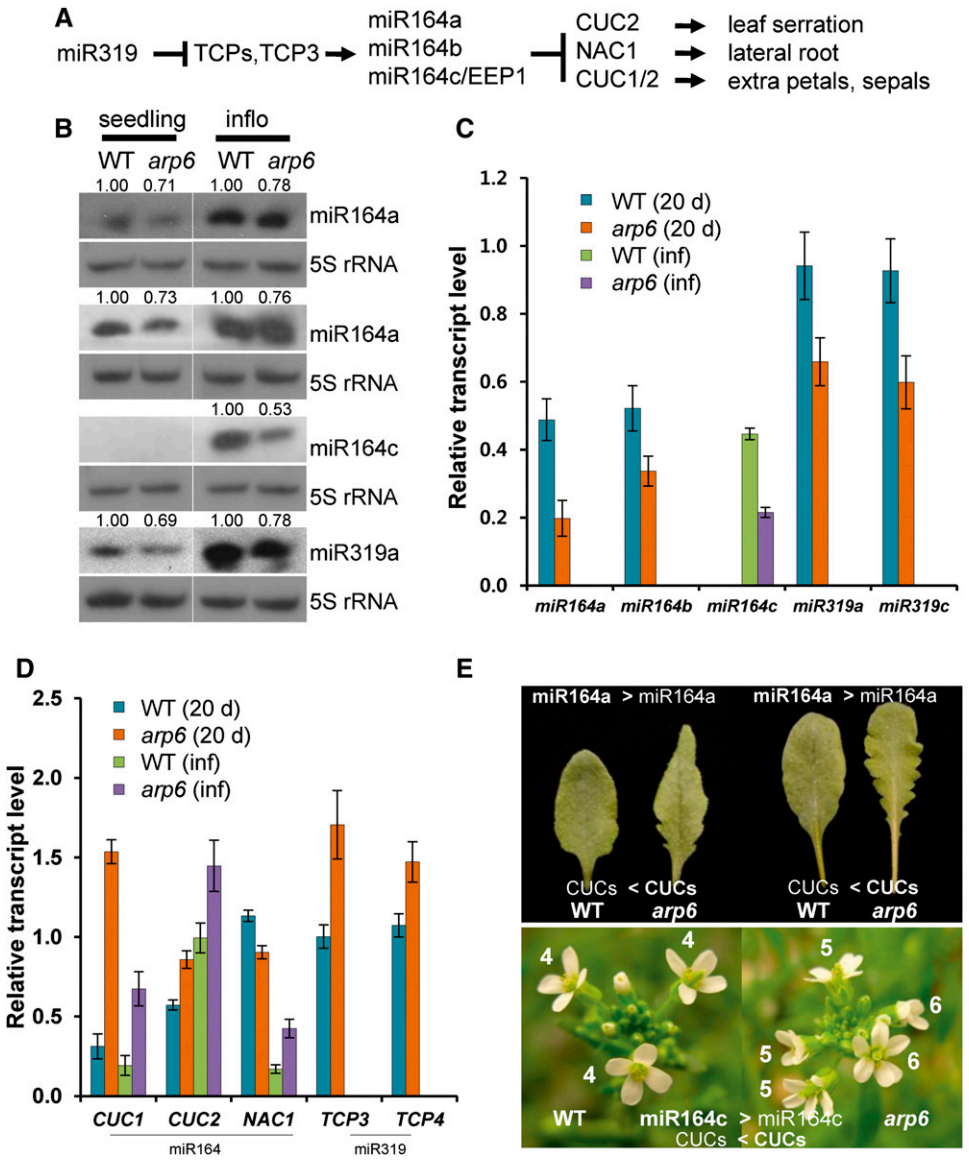

Figure 3. Effects of *arp6* on the miR-mediated organ boundary formation. A, Diagram of genetic hierarchy between miR319-TCPs and miR164-CUCs modules. B, Small RNA blots of miR164a, miR164c, and miR319 from seedlings and inflorescences. C, Transcript levels of the primary miRNAs *pri-miR164* and *pri-miR319*. D, Transcript levels of miR164- and miR319-regulated genes. E, Photos of serrated leaves and extra petals in *arp6*. Leaves of wild type and *arp6* grown at long days (left) and short days (right) are displayed. Numbers of petals from early arising flowers in wild type (right) and *arp6* (left) grown at short days were denoted (bottom panels). Error bars indicate SD of three technical replicates. The same RNAs from seedlings and inflorescences in Fig. 1 were used for RT-qPCR. WT, wild type; inflo, influorescence.

the levels of miR319 and *TCP3/4* transcripts in *arp6*, which could influence the miR164-CUC2 module (Hasson et al., 2011). Interestingly, the amount of both miR319 and pri-miR319 was reduced in the *arp6* mutant (Fig. 3, B and C). As a consequence of lower miR319 levels, mRNAs of *TCP3* and *TCP4* accumulated; however, this was not sufficient to increase the transcription of *MIR164A* in *arp6*, as with *MIR172B* regulated by SPL9 and ARP6 (Figs. 2B and 3, B and C). It appears that SWR1-C is necessary for the transcription of both *MIR319* and *MIR164* (Fig. 3, B and C).

Despite the lower levels of miR164 observed in whole seedlings and reduced pri-miR164 levels in roots (Figs. 3D and 4A), the transcript level of *NAC1*, a miR164-target gene promoting root branching, was decreased. This caused *arp6* to display lateral root formation comparable to that of wild-type plants, although the root length was shorter (Fig. 4, B and C, Supplemental Tables S2 and S3; and Guo et al., 2005). Therefore, the loss of H2A.Z deposition has a differential effect on the expression of each miR164-regulated gene. Unlike miR156-SPLs and miR164-CUC modules in which target mRNAs were accumulated in *arp6*, the transcriptional attenuation of both miR164 and its target *NAC1* in *arp6* might lead to wild-type-level root branching (Figs. 3D and 4; Supplemental Table S2). The miR164-NAC1 module in *arp6* shows an example indicating why the reduced miRNA cannot produce all miRNA-associated phenotypes, because of the diverse effects of *arp6* on the transcription of each miRNA-regulated gene, as shown below (Figs. 5–9).

pie1 and *sef* Show Similar Transcriptional Changes in miR156/SPLs and miR164/CUCs Modules to *arp6*

Because *arp6* shows similar phenotypes to *pie1* and *sef/atswc6* (Noh and Amasino, 2003; Choi et al., 2005;

Deal et al., 2005; Martin-Trillo et al., 2006; Choi et al., 2007; March-Diaz et al., 2007), we investigated whether *pie1* and *sef* mutants also show similar transcriptional changes in the genes involved in miR156 and miR164 modules. As shown previously, *arp6*, *sef*, and *pie1* have reduced *FLC* expressions (Fig. 5A). We observed that like *arp6*, *sef* and *pie1* mutants show reduced transcription of *MIR156/7* and increased expression of *SPLs*, compared to wild type in fold-change analyses using RT-quantitative (q)PCRs (Figs. 2, C and D; 5, B and C). Expressional fold changes of *MIR172A/B* and *TOE1/2* in *sef* and *pie1* were similar to *arp6* (Figs. 2, C and D; 5, B and C). We also observed that transcript level of *MIR164A*, a major *MIR* gene related to leaf serration, was reduced, and subsequently *CUC1/2* expressions were increased in *sef* and *pie1*, similar to *arp6* (Figs. 3, C and D; 5, D and E). These similar developmental phenotypes and transcriptional misregulation of miR156 and miR164 modules in *arp6*, *sef*, and *pie1* suggest that Arabidopsis SWR1-C including ARP6, SEF, and PIE1 is required for the transcription of genes involved in the miR156 and miR164 modules.

Transcriptional Activation of Some *MIR* Genes and miRNA-Target Genes by *ARP6*

Since we observed that the *arp6* mutation led to the misregulation of miR156 and miR164 pathway genes, we explored whether *ARP6* could also be involved in the transcriptional regulation of other *MIR* genes (Fig. 6). Indeed, the levels of some miRNAs and their precursors decreased to approximately 30% to 80% of those observed in wild-type plants, but miR165 and miR159 levels either were not changed or were increased slightly, and the precursors of miR165 and miR159 were also moderately elevated (Fig. 6, A and B). This suggests that changes in the mature miRNAs may be determined mainly at the transcriptional level in *arp6*. Additionally, the transcription of several miRNA/transacting (ta) siRNA biogenesis genes was also affected (Fig. 6D). Therefore, the miRNA level seems to be regulated by both transcription and miRNA processing. The significant reduction of *RDR6* transcription may lead to lower levels of tasiRNAs in *arp6* (Fig. 6, A and D).

Next, we measured the transcript levels of miRNA-regulated genes in *arp6* (Fig. 6C). We observed that the transcripts of some miRNA-target genes, including *AGO2* and *GRF1*, were increased, similar to *SPL3/4/9* and *CUC1* and *2*, while the expression of most miRNA-target genes, including *MYB33*, *ARF4*, *AGO1*, and *AGL16*, was reduced, much like *NAC1*, *TOE1*, and *TOE2* (Fig. 6, A and C). The reduction in the expression of these miRNA-regulated genes occurs regardless of the decrease in the levels of miRNAs targeting them. These results imply that miRNA-regulated genes whose transcripts were reduced in *arp6* may require SWR1-C for active transcription, and thus the SWR1-C defect overwhelms the effect of the decrease in miRNA. It suggests that the influence of the *arp6* mutation on gene regulation may vary among miRNAs-regulated genes, as seen in miR156- or miR164-regulated genes (Figs. 2–4). This probably depends on the transcriptional potential of each gene promoter. To test whether SWR1-C regulates *MIR* genes directly, we performed a ChIP analysis using *35S-myc:ARP6* transgenic plants, which fully complements the phenotypes of *arp6* (Choi et al., 2007). We observed the enrichment of myc-tagged recombinant ARP6 around the promoters of the tested *MIR156A*, *MIR164A*, *MIR164C*, *MIR166A*, *MIR168A*, and *MIR172B*, as well as *TOE1* and *FLC*, compared to the gene body of tubulin (*TUB*; Fig. 6E).

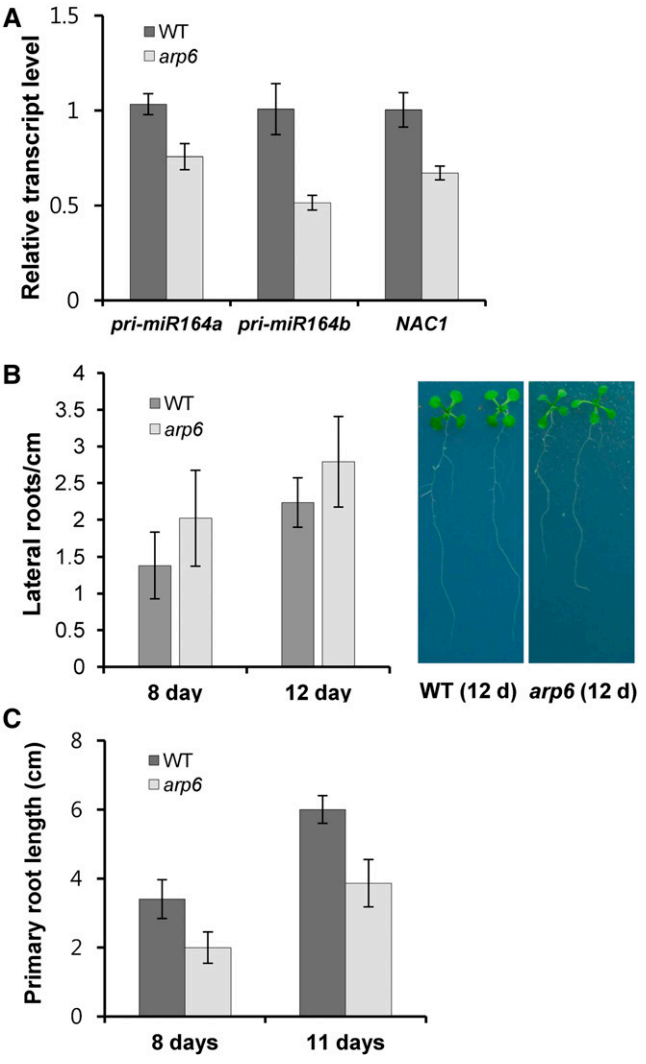

Figure 4. miR164-NAC1 module-mediated root branching in *arp6*. A, Relative transcript level of *pri-miR164a*, *pri-miR164b*, and *NAC1* in roots. RNAs were isolated from roots of plants grown in MS media for 30 d. B, Numbers of lateral roots. Plants were grown on plates of MS medium containing 1% Suc for 8 d and 12 d. The roots of 10 plants per genotype were harvested to count the lateral roots per centimeter (Supplemental Table S2). C, Primary root length in 8- and 11-d-old wild type and *arp6*. Nine to 15 plants were used (Supplemental Table S3). WT, wild type.

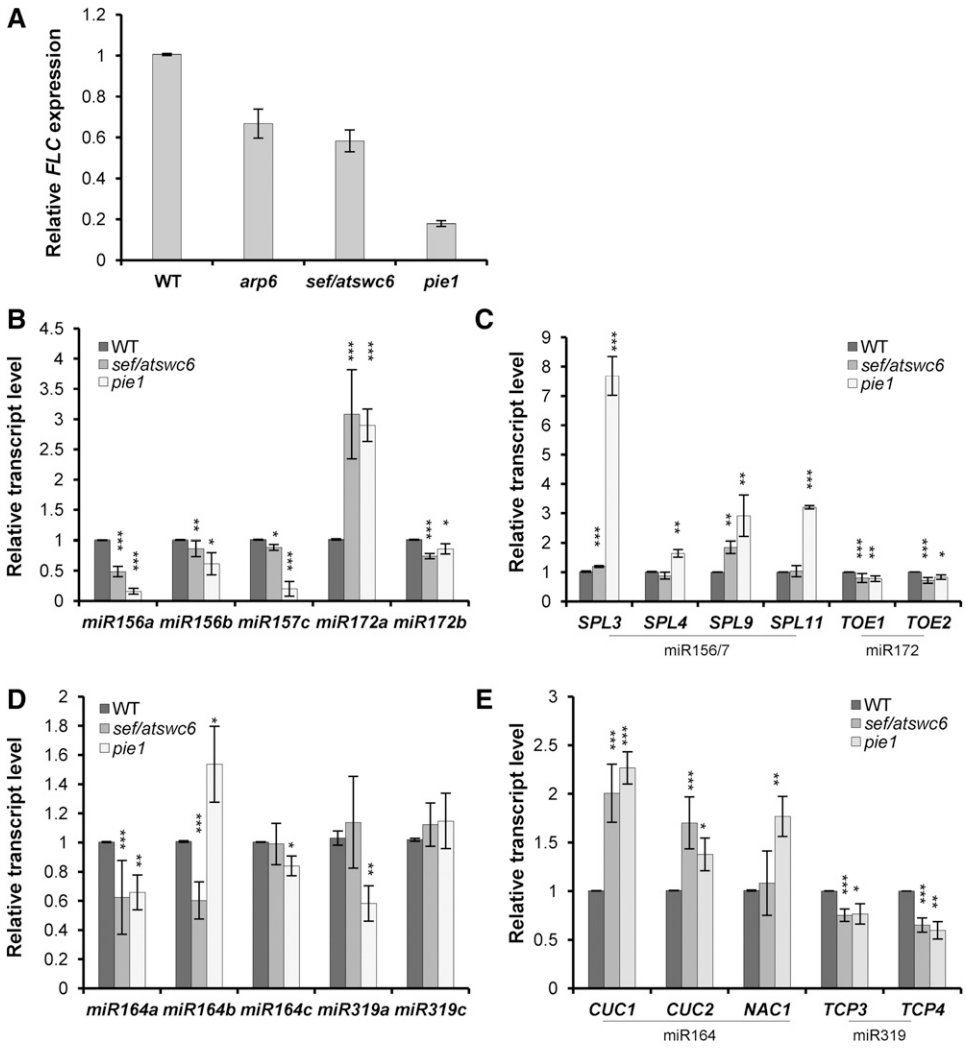

Figure 5. Effects of *pie1* and *sef/atswc6* on the transcription of miR156/SPLs and miR164/CUCs modules. A, Relative expression of *FLC* in wild type, *arp6*, *sef*, and *pie1*. B, Transcript levels of pri-miR156 and pri-miR172. C, Transcript levels of miR156- and miR172-regulated genes. D, Transcript levels of pri-miR164 and pri-miR319. E, Transcript levels of miR164 and miR319-regulated genes. *TUB* was used as a reference for normalization of qPCR. WT, wild type.

Genome-Wide Change in the Levels of miRNAs in *arp6*

To examine how *arp6* affects abundance of miRNAs at genome level, we generated and analyzed four biological replicates of small RNA libraries from 7-d whole seedlings. We found that *arp6* has fewer small RNAs with the sizes of 20 and 21 nt than wild type, indicating that *arp6* may reduce transcription or production of miRNAs that are mostly 20 to 21 nt. On the other hand, the levels of 23 to 24 nt small RNAs, which are mainly associated with repetitive sequences, were comparable in wild type and *arp6* (Fig. 7A). We confirmed that the abundance of miR156 (which is a 20-nt small RNA) in the library was significantly reduced in *arp6*, consistent with the results from small RNA-blot analysis (Figs. 2, B and C; 7, B and C). Some miRNAs including miR157, miR163, miR399, miR319, and miR403 were also reduced in *arp6*, supporting a positive transcriptional role of *ARP6* in the *MIR* genes (Fig. 7, B and C; Supplemental Table S4). Additionally, we observed that some miRNAs such as miR398 and miR408 were increased in *arp6* while some were not changed (Fig. 7, B and D; Supplemental Table S4). It is noteworthy that miR398 and miR408 are small RNAs that are induced by copper deficiency and heat stresses (Abdel-Ghany and Pilon, 2008; Guan et al., 2013). Therefore, it may indicate that *ARP6* is required for the basal repression of some miRNAs, which are environmentally inducible, similar to the heat inducible gene *HSP70*.

In summary, genome-wide miRNA analysis showed that approximately 37% of miRNAs were reduced but approximately 23% increased by *arp6* mutation, suggesting that *ARP6* is required for transcriptional regulation of approximately 60% of miRNA. In contrast, approximately 40% of miRNAs were not affected by *arp6* mutation.

arp6 Mutation Attenuates Both the pri-miRNAs and mRNAs of miRNA-Target Genes of miRNA Biogenesis Mutants

To check whether *ARP6* is required for the direct transcriptional regulation of *MIR* genes and miRNA-regulated genes (Figs. 2–6), we measured the levels of pri-miRNAs and miRNA-regulated genes present in

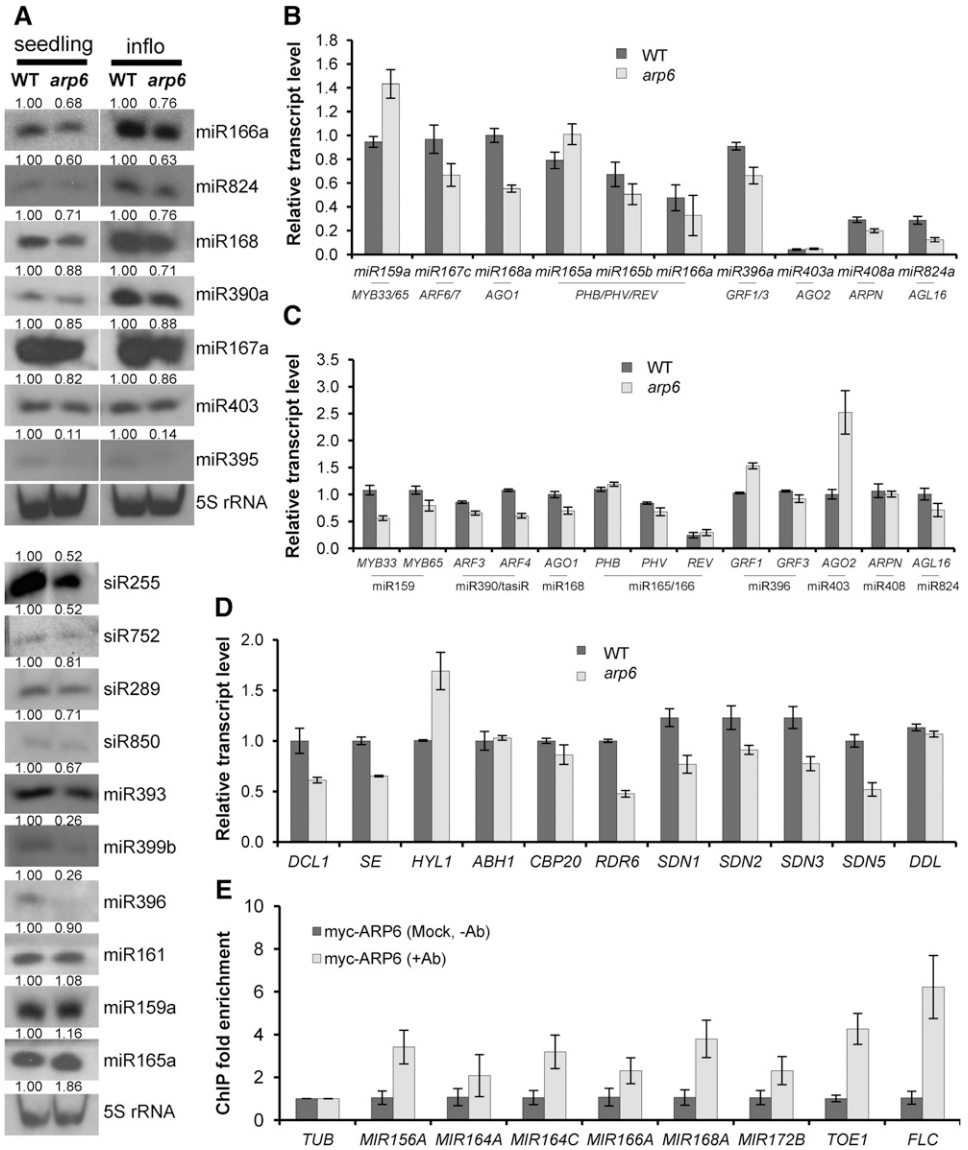

Figure 6. Effect of *arp6* on the transcript levels of genes involved in miRNA processing and miRNA target genes. A, Small RNA blots of miRNAs and siRNAs in *arp6* and wild type. B, Transcript levels of *pri-miRNAs* in *arp6* and wild type, detected by RT-qPCR. The miRNA-target genes are shown below the corresponding pri-miRNAs. C, Transcript levels of the genes regulated by miRNAs in *arp6* and wild type. The miRNAs are shown below their target genes. D, Transcript levels of miRNA-biogenesis genes in *arp6* and wild type. E, ChIP-qPCR analysis of myc-tagged ARP6 enrichment at the *MIR* genes miR172-target *TOE1* and *FLC*. Primer pairs were used for amplification in the promoter region (−560 to approximately −700) for *MIR156A*; (−738 to approximately −840) for *MIR164A*; (−594 to approximately −613) for *MIR164C*; (−447 to approximately −591) for *MIR166A*; (−847 to approximately −948) for *MIR168A*; (−649 to approximately −761) for *MIR172B*; (−452 to approximately −521) for *TOE1*; and (−366 to approximately −492) for *FLC*. Dark gray bars (−Ab) indicate control experiments performed under the same conditions as sample experiments (light gray bars, +Ab), with the exception of the addition of antibody. Primer pairs amplifying the *TUB* gene body (+1556 to approximately +2038) were used for normalization and fold enrichment. WT, wild type; Ab, antibody.

the double mutants of *arp6 hyl1-1* or *arp6 se-1* (Fig. 8). As expected, higher levels of pri-miRNAs were detected in *hyl1* and *serrate* mutants in which there is inefficient processing of pri-miRNAs into mature miRNAs, leading to fewer miRNAs and abundant mRNAs of miRNA-regulated genes (Fig. 8, A and B; Lu and Fedoroff, 2000; Lobbes et al., 2006). Strikingly, the *arp6* mutation significantly suppressed the accumulation of most unprocessed pri-miRNAs in *hyl1-1* and *se-1*, supporting the positive role of *ARP6* in the transcription of *MIR* genes (Fig. 8, A and B). In addition, we observed that the uncut and accumulated transcripts of many miRNA-target genes in *hyl1-1* were reduced by *arp6* mutation, indicating that SWR1-C positively regulates the transcription of miRNA-regulated genes (Fig. 6C). This data rules out the effect of *arp6* on the misregulation of *MIR* and miRNA processing genes that cause post-transcriptional changes in miRNA-regulated gene expression (Figs. 2–5, and 6, A–D), and strongly suggests that SWR1-C mediates gene activation in miRNA-mediated plant developmental pathways.

ARP6 Is Required for Distinct Nucleosome Occupancy Around the Transcription Start Sites of *MIR156*, *MIR164*, and *FLC*

As it has been revealed that transcription is controlled by the alteration of nucleosomal occupancy around promoters, transcription start sites (TSSs), and the genes downstream of TSSs (Workman and Kingston, 1998; Albert et al., 2007; Li et al., 2007; Hu et al., 2013; Soboleva et al., 2014), we examined whether *arp6* mutation could affect nucleosomal occupancy in the promoter regions and TSSs of *MIR156A*, *MIR164A*, and *FLC* as effective sites of SWR1-C (Jin et al., 2009; Hu

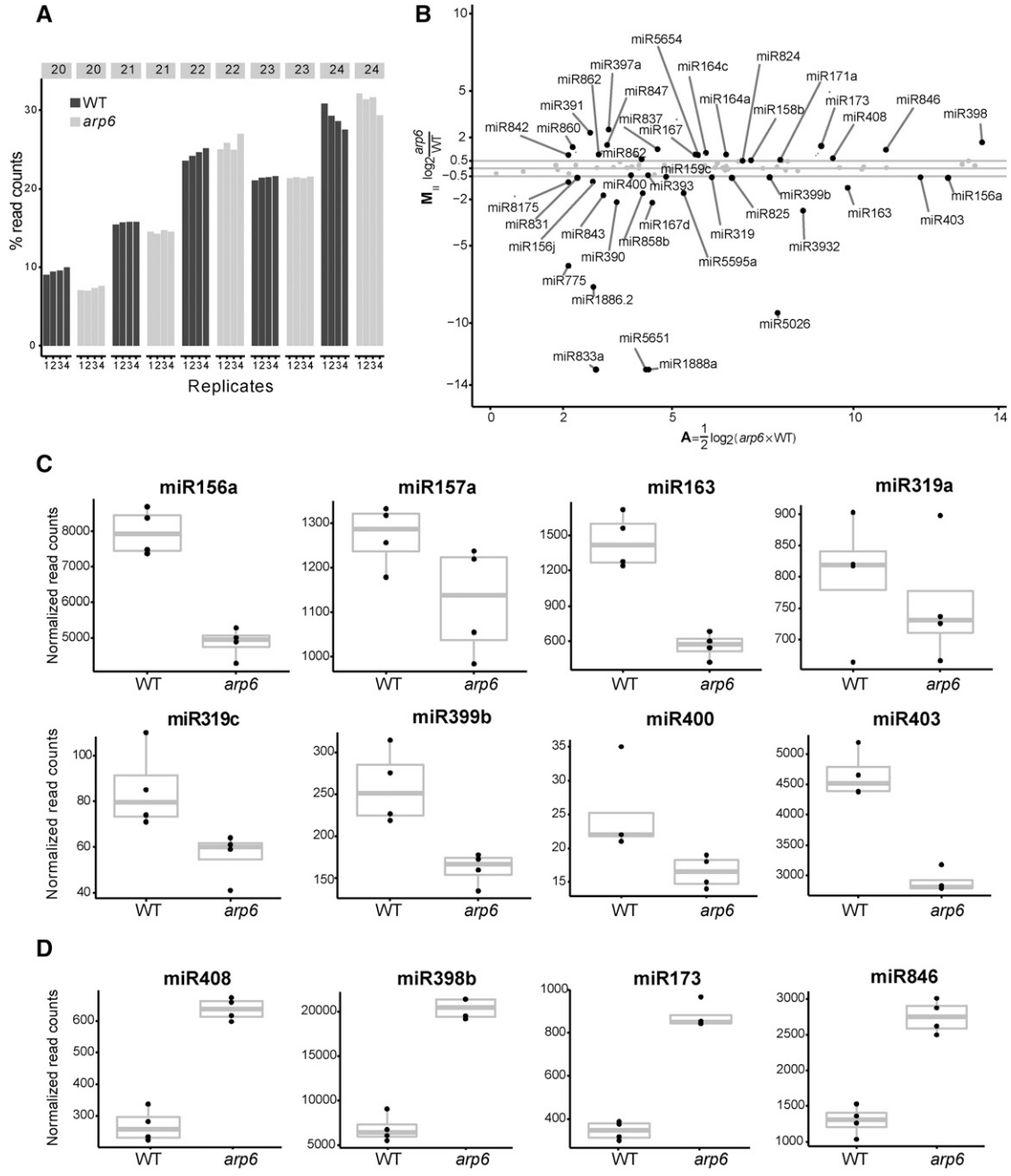

Figure 7. Genome-wide analysis of miRNAs in wild type and *arp6*. A, Proportion of small RNAs from 20 to 24 nt size in wild type and *arp6*. Bars indicate proportion of small RNAs in size at each library. Replicates (1–4) represent four biological replicates of small RNA libraries per genotype. B, MA plot showing fold changes of miRNAs in *arp6*. The *y* axis (M) represents fold change. The *x* axis (A) indicates mean miRNA read counts of wild type and *arp6*. Black dots indicate miRNAs with greater than ±0.5 of fold changes; gray represent unchanged miRNAs. C, Boxplots of decreased miRNAs in *arp6*. D, Boxplots of increased miRNAs in *arp6*. (C and D) The *y* axis ticks indicate normalized miRNA read counts (see Materials and Methods). Black dots show miRNA reads from wild-type and *arp6* small RNA libraries. The bold vertical gray lines indicate the median of four libraries. WT, wild type.

et al., 2012; Li et al., 2012; Ranjan et al., 2013; Yen et al., 2013; Fig. 9A). We performed a micrococcal nuclease (MNase)-qPCR assay during which the greater enrichment of nucleosomal DNAs reflects more nucleosome occupancy and less DNA accessibility (Supplemental Fig. S3). The *arp6* mutants exhibited higher nucleosome occupancy than did wild-type plants at the *MIR156*, *MIR164*, and *FLC* promoters, but it was unchanged at the *ACTIN2* (*ACT2*) promoter and the gene bodies of *ACT2*, *FWA*, and *EVADE* (*EVD*). The control, *ACT2*, is

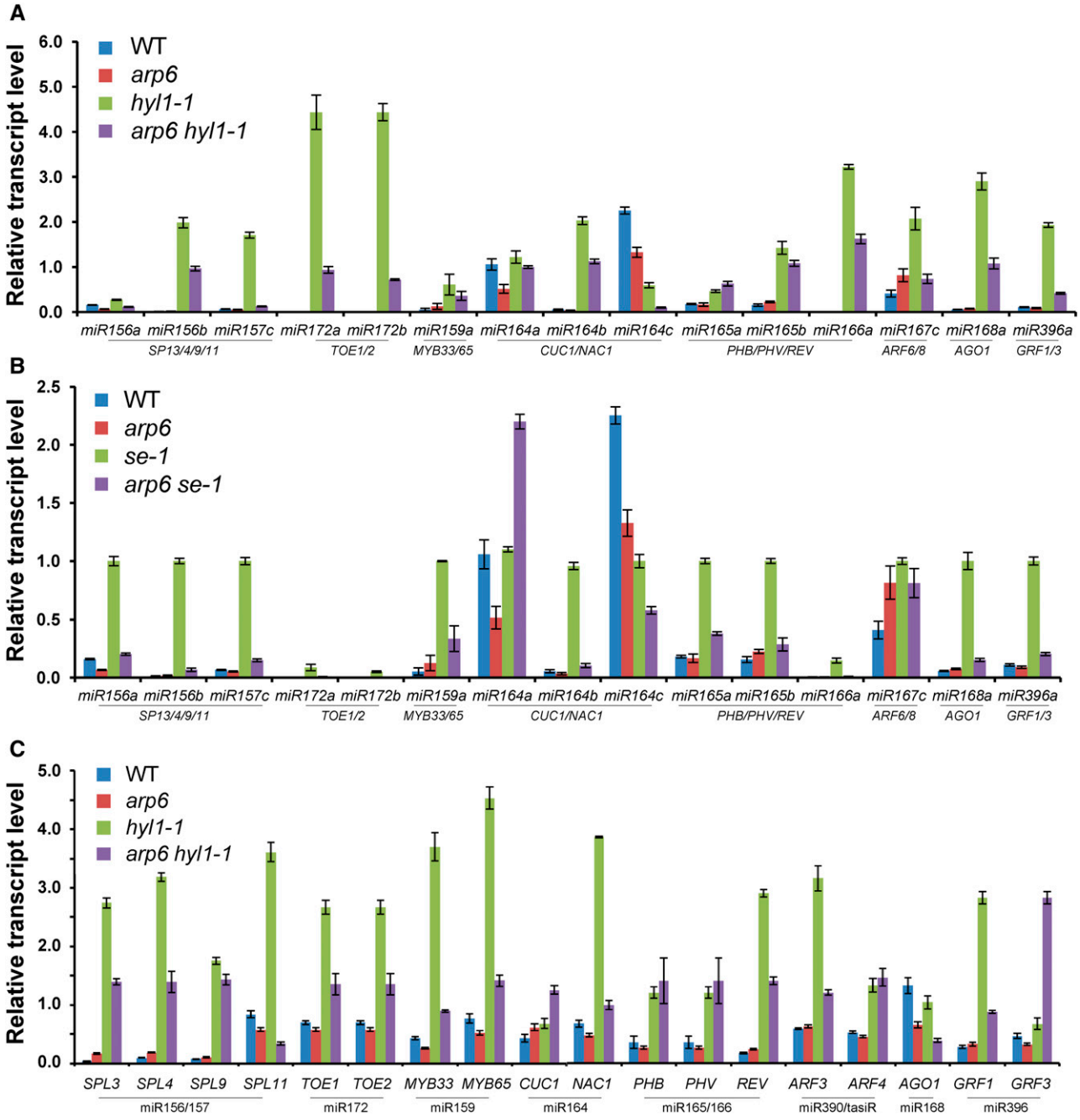

Figure 8. Effects of *arp6* mutation on the transcript levels of *pri-miRNAs* and miRNA-target genes in miRNA biogenesis mutants. A, Transcript levels of *pri-miRNAs* in wild type, *arp6*, *hyl1-1*, and *arp6 hyl1-1*. Total RNAs from whole seedlings grown under long-day conditions for 20 d were used for RT-qPCR. B, Quantification of *pri-miRNAs* in wild type, *arp6*, *se-1*, and *arp6 se-1* RT-qPCR. C, Transcript levels of miRNA-regulated genes in wild type, *arp6*, *hyl1*, and *arp6 hyl1*. The relationships between miRNAs and their regulated genes are shown between underlines. *TUB* was used as a reference for normalization of qPCR. WT, wild type.

expressed similarly in wild type and *arp6*, while *FWA* and *EVD* are the DNA-hypermethylated gene and transposon, respectively, with low transcription and H2A.Z deposition (Zilberman et al., 2008). Nucleosome occupancy in *arp6* was slightly reduced at the 5′ end region of the +1 nucleosome (+1 nuc) position but highly increased at the promoters of *MIR156*, *MIR164*, and *FLC* genes (Fig. 9A). This suggests that the *arp6* mutation, which is defective in H2A removal and H2A.Z deposition, may affect the dynamics of nucleosomes in the promoters of the *MIR* genes and *FLC*—potentially leading to the decreased accessibility of positive

transcription regulators or transcriptional machineries, as shown in the promoter of *FLC* (Fig. 1A).

DISCUSSION

Transcriptional Regulation by SWR1-C in Arabidopsis

In Arabidopsis, the negative effect of SWR1-C on the transcription of responsive genes is well understood (March-Diaz et al., 2008; Kumar and Wigge, 2010; Smith et al., 2011; Jarillo and Pineiro, 2015). In addition, we demonstrate here that SWR1-C is required to maintain the active transcription of many *MIR* genes, miRNA-regulated genes, and heat shock- and JA-responsive genes. How Arabidopsis SWR1-C maintains both the repressive and activating transcription states of inductive genes such like *HSP70* and *JAZs* remains unclear, although the dual effects of H2A.Z on transcription have been demonstrated in different species (Thambirajah et al., 2009; Marques et al., 2010; Talbert and Henikoff, 2010; Hu et al., 2012; Li et al., 2012; Soboleva et al., 2014; Jarillo and Pineiro, 2015). One possibility is that H2A.Z triggers the production of more or less stable nucleosomes with other histones, depending on the transcription state (Jin and Felsenfeld, 2007; Henikoff, 2009; Jin et al., 2009; Thambirajah et al., 2009). Different transcription states may lead to specific combinations of active or repressive histone modifications such as H2A.Z and H3 acetylation, thereby affecting SWR1-C activity and specificity for diverse nucleosome stability (Keogh et al., 2006; Millar et al., 2006; Thambirajah et al., 2009; Altaf et al., 2010; Draker et al., 2012; Watanabe et al., 2013).

In repressive transcription states, Arabidopsis H2A.Z deposition may result in more stable nucleosomes that act as barriers to the access of RNA polymerase II. Indeed, Arabidopsis H2A.Z is highly detected in the environment-responsive genes under non-inductive conditions, suggesting that H2A.Z plays a direct negative transcriptional role of them (Kumar and Wigge, 2010; Coleman-Derr and Zilberman, 2012). However, in highly active transcription states, our results suggest that Arabidopsis SWR1-C makes nucleosomes less stable, thus promoting transcription (Fig. 1, B and C). Consequently, H2A.Z is rapidly evicted from the nucleosome during transcription, so that the detected H2A.Z level displays an anticorrelation with the transcript level (Deal et al., 2007; Kumar and Wigge, 2010). It is also possible that SWR1-C acts in concert with other active chromatin modifiers such as EFS and COMPASS to promote active transcription as seen in *FLC* gene (Kim et al., 2005; Choi et al., 2011; Jiang et al., 2011). Another possibility is that Arabidopsis SWR1-C may help promote or maintain the accessibility of active or repressive transcription regulators to the chromatin (Fig. 9B). The results of our ChIP assay using FRI support this hypothesis, as FRI, a specific activator, is less accessible to the *FLC* promoter in *arp6* than in wild type (Fig. 1A). Additionally, our MNase-qPCR analysis showed that *arp6* has higher nucleosome occupancy at the promoter of *FLC*, *MIR156A*, and *MIR164A*, indicating reduced chromatin accessibility (Fig. 9A). This is similar to the proposed function of mouse H2A.Z that mediates nucleosome depletion to promote the accessibility of active or repressive regulators during embryonic stem cell differentiation (Hu et al., 2012; Li et al., 2012).

Although Arabidopsis SWR1-C is required to maintain the active transcription states of developmentally regulated genes, such as *FLC* and *MIR* genes, it is also required for the repressive transcription states especially of environmentally responsive genes (Figs. 2–7). Not only the protein-coding genes, such as *HSP70* and *JAZs*, but also some *MIR* genes, such as miR398 and miR408, may require SWR1-C for their basal repression prior to environmental induction (Fig. 7, B and D; Kumar and Wigge, 2010; Coleman-Derr and Zilberman, 2012; Qin et al., 2014). Thus, SWR1-C may contribute to maintain the repressive transcription states for the environmentally induced genes. It is likely that Arabidopsis SWR1-C contributes to an expansion of the range of transcription plasticity by modulating nucleosome stability and/or the accessibility of transcription regulators to chromatin (Fig. 9B).

However, not all the genes show changes in the expression in *arp6*, *sef*, and *pie1* mutants compared to wild type, although H2A.Z localizes at almost all the genes in Arabidopsis (Noh and Amasino, 2003; Choi et al., 2005; Deal et al., 2005; Martin-Trillo et al., 2006; Choi et al., 2007; Deal et al., 2007; March-Diaz et al., 2007; Jarillo and Pineiro, 2015). Approximately 5% to 10% protein coding genes and 40% *MIR* genes show differential expression in *arp6*. It indicates that *arp6* does not significantly effect on the accessibility of transcription regulators or nucleosome stability/accessibility in most of the genes (Figs 6 and 7). Thus, the transcription of many genes and *MIR* genes seems not to be changed in mutants of the components of SWR1-C. However, *FLC*, and some *MIR* genes and the environmentally responsive genes affected by *arp6* are regulated by SWR1-C, probably in concert with other transcription factors and chromatin-modifying factors. It would be intriguing to explore further how Arabidopsis SWR1-C affects the accessibility of transcription regulators or nucleosome stability in responsive and developmental genes under different conditions.

How Does Arabidopsis SWR1-C Contribute to miRNA-Mediated Development?

Our results suggest that SWR1-C contributes to the transcription of some miRNA-regulated genes as well as *MIR* genes, thereby affecting plant development (Figs. 2–9). Here, we propose that SWR1-C is required to fine-tune quantitative gradients between miRNAs and their target mRNAs through transcriptional activation during plant development. In our model (Fig. 9C), the wild-type plant exhibits proper spatiotemporal transcription of *MIR* genes and miRNA-regulated genes by SWR1-C (Fig. 9C[a]), while the *arp6* mutant

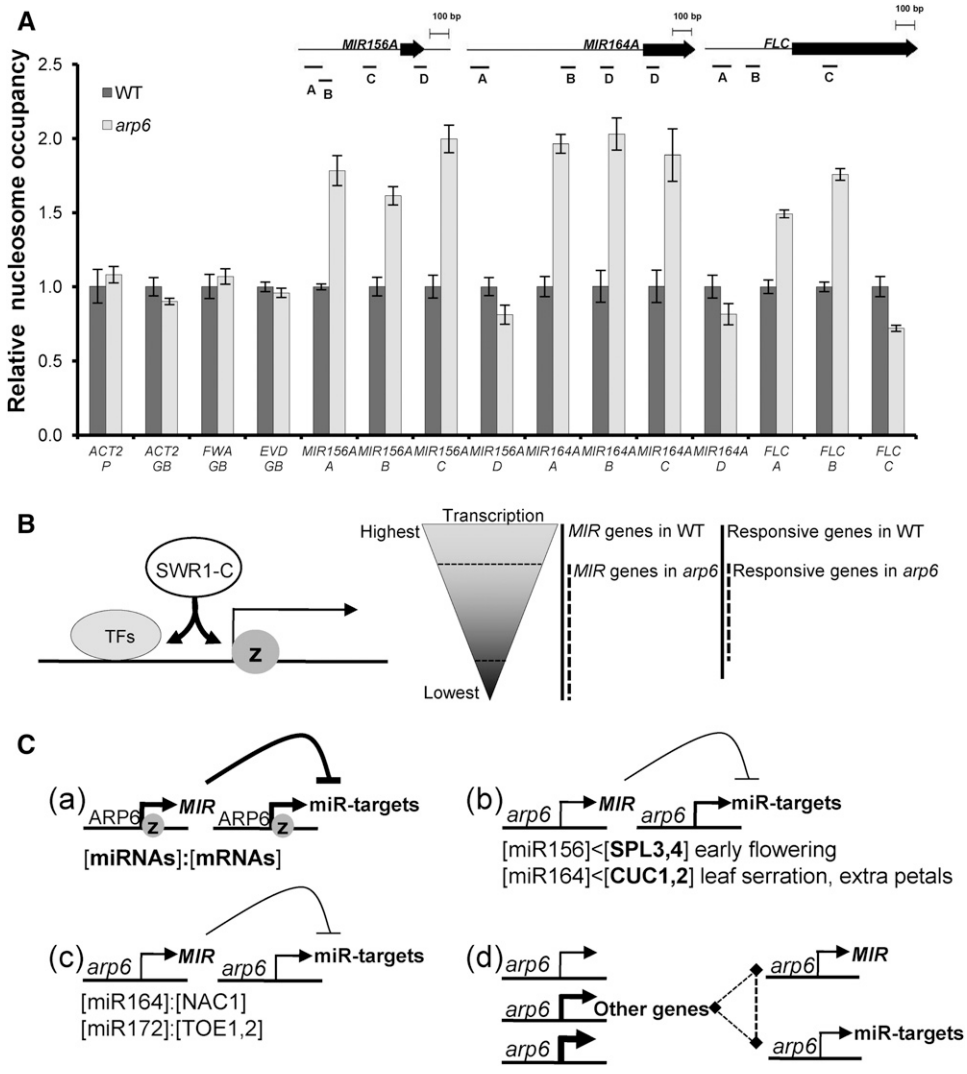

Figure 9. Altered nucleosome occupancy around the promoters of *MIR156*, *MIR164*, and *FLC* by *arp6*. A, MNase-qPCR assay to analyze relative nucleosomal occupancy at the promoter and 5′ end of *MIR156*, *MIR164*, and *FLC* in wild type and *arp6*. Locations of the amplified PCR products are based on TSS as below: *MIR156A* A (−496 to approximately −402), B (−421 to approximately −351), C (−192 to approximately −123), and D (+69 to approximately +134, +1 nuc); *MIR164A* A (−899 to approximately −800), B (−430 to approximately −352), C (−22 to approximately −154), and D (+17 to approximately +85, +1 nuc); and *FLC* A (−317 to approximately −414), B (−168 to approximately −239), and C (+157 to approximately +234, +1 nuc). The input chromatin without MNase treatment was used for normalization and fold enrichment at each site. Error bars indicate SD of three biological replicates. B, Model of the role of Arabidopsis SWR1-C in transcription. SWR1-C may maintain both the transcription state and the accessibility of transcription regulators to the promoter. C, Control of miRNA-mediated plant developments by SWR1-C. The proper quantitative balance between transcript levels of miRNAs and their target mRNAs is regulated by SWR1-C in wild type (a) but that is disturbed by the *arp6* mutation (b, c, d). The stronger effect of *arp6* on the transcription of *MIR* genes than that of target genes causes the increased level of target mRNAs, and thus the phenotypes of early flowering, serrated leaves, and extra floral organs are prominent (b). Similar effect of *arp6* on the transcription of both miRNA and target mRNA may not lead to the development of specific phenotypes (c). Multiple influences of *arp6* on the expression of miRNA pathway genes, other developmental genes, and environmentally responsive genes may cause other developmental defects such as longer hypocotyls and increased root hair density (d). WT, wild type.

exhibits the miR156 and miR164-associated phenotype, with reduced miRNA levels and increased miRNA-target mRNAs (Figs. 2, 3, and 9C[b]). However, in most cases, the *arp6* mutant has a phenotype similar to that of the wild-type plants due to the effect of *arp6* on the transcription of both miRNA and miRNA target genes. For example, root branching in *arp6* is almost normal, because *arp6* effects on the transcription of both *MIR164* and the miR164-regulated *NAC1* gene (Figs. 4, 6, and 9C [c]). It is also important to note that SWR1-C regulates the transcription of many genes affecting miRNA pathways both positively and negatively (Fig. 9C[d]).

Although single Arabidopsis H2A.Z deposition mutants such as *pie1*, *arp6*, and *sef* are viable and exhibit phenotype similar to *h2a.z* double (*hta9 hta11*) and triple (*hta8/9/11*) mutants, the *pie1;hta8/9/11* quadruple mutation resulted in a lethal phenotype at the early seedling stage. This suggests that Arabidopsis H2A.Z may still be incorporated in the nucleosome by other chromatin remodelers in the absence of SWR1-C, or that *PIE1* may have a H2A.Z-independent function (Coleman-Derr and Zilberman, 2012). It could be a reason why the Arabidopsis SWR1-C mutants are viable, even with the moderate transcriptional changes observed in this study and in the previous reports (Noh and Amasino, 2003; Choi et al., 2005; Deal et al., 2005; Martin-Trillo et al., 2006; Choi et al., 2007; Deal et al., 2007; March-Diaz et al., 2007; Coleman-Derr and Zilberman, 2012).

Here our findings show that *MIR* and miRNA-target genes that are positively regulated by SWR1-C provide a platform to elucidate the transcription mechanism via which SWR1-C modulates chromatin features by itself (cis) or cross talks with other factors (trans) to regulate plant development and responses to environmental changes.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Arabidopsis Columbia (Col-0) plants were used as the wild type. The *arp6/suf3-1* mutant allele in a Col-0 background was used as the experimental plant and all plants were grown under long-day (16 h of light/8 h of darkness at 22°C) or short-day conditions (8 h of light/16 h of darkness at 22°C) as previously described in Choi et al. (2005, 2007). The *hy11-1* and *se-1* alleles in the Col-0 background were crossed with *arp6* to obtain *arp6 hyl1-1* and *arp6 se-1* double mutants (Lu and Fedoroff, 2000; Lobbes et al., 2006). The T-DNA insertion *sef/atswc6* (SAIL_1142_C03) and *pie1* (SALK_003776) lines in Col-0 background from ABRC were used for gene expression analyses (Noh and Amasino, 2003; Choi et al., 2007).

Small RNA Blot

All small RNA blot procedures followed the method including chemical cross linking as described in Pall and Hamilton (2008). Ten micrograms of total RNAs were loaded per lane, after which the RNAs were transferred to the nylon membrane. The 5S rRNA probe was used to check that equal amounts of each sample were loaded, and for normalization. The intensity of the small RNA signals was analyzed using the Image J program (National Institutes of Health, Bethesda, MD). Information on the oligonucleotides used for probes of small RNA blots is provided in Supplemental Table S1.

Small RNA Library Construction

Total RNA was extracted from 7-d-old whole seedlings of Arabidopsis Col-0 and *arp6*. Ten μg of total RNA was used to construct a single small RNA library. Total RNA was mixed with the same volume of 2× RNA loading buffer (deionized formamide, 0.5 mM EDTA, pH 8.0, 0.1% w/v bromophenol blue, 0.1% w/v xylene cyanol), incubated at 65°C for 10 min and cooled on ice. Preloading-treated RNA and RNA ladder were loaded to 15% TBE-urea gel. Gel was run in a 1× TBE running buffer at 150 V until the bromophenol blue reached the bottom of the gel. After SYBR Gold (Life Technologies, Carlsbad, CA) staining for 3 min, gel containing the small RNA band was excised and placed into a shredder (a 0.5-mL microcentrifuge tube with three 21-G needle holes punctured into the bottom within a 2-mL nuclease-free microcentrifuge tube) and spun at 10,000*g*. Three volumes of 0.3 M NaCl (pH 7.0) were added and rotated at 4°C overnight. The solution and gel pieces were transferred into a Costar SpinX column (Dow Corning, Midland, MI) and spun at 17,000*g* for 5 min at 4°C. Flow-through was transferred to a new tube and ethanol-precipitated. Small RNA was dissolved in nuclease free water. Four biological replicate libraries per genotype were constructed according to the manual of NEBNext small RNA library prep set (cat. no. E7300S; New England BioLabs, Ipswich, MA) and then pooled, sequenced in NextSeq500 instrument (Illumina, San Diego, CA).

Computational Analyses of Small RNA Libraries

Small-RNA reads samples were pooled since all samples were split up into four lanes on the NextSeq 500 desktop sequencer (Illumina), trimmed using Trim Galore! 0.4.1 (Babraham Bioinformatics, http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/) and aligned against the Arabidopsis genome (TAIR10; https://www.arabidopsis.org/download/index-auto.jsp?dir=%2Fdownload_files%2FGenes%2FTAIR10_genome_release) using Bowtie v. 1.1.1 (http://bowtie-bio.sourceforge.net/index.shtml) requiring perfect matches. Non-mapping reads were discarded and individual small-RNA species were subsequently counted and imported into R, ver. 3.2 (https://cran.r-project.org/bin/windows/base/old/3.2.0/) for downstream analysis. Small RNA species were normalized using the TMM method implemented in edgeR (Bioconductor; https://bioconductor.org/packages/release/bioc/html/edgeR.html) using default parameters; however, since a shift of 21/22 nt small-RNAs could not be excluded in *arp6*, only 23/24-nt small-RNAs were used to determine the TMM library sizes for normalization. MicroRNAs were identified by matching small-RNA species against mature miRNAs derived from miRBase rel. 21 (http://www.mirbase.org/). Boxplots of microRNA quantity were created using ggplot2 (http://ggplot2.org/) based on normalized read count.

MA Plots

Normalized reads from *arp6* and wild-type libraries were plotted as an MA-plot using ggplot2; *y* axis (M) shows log2 fold change [log2(*arp6*/wild type)] and *x* axis (A) shows average abundance [0.5*log2 (*arp6**wild type); bioinformatic scripts are available on request from S.Y.M.]. Libraries are available for download at ArrayExpress E-MTAB-4498 (ArrayExpress, EMBL-EBI; https://www.ebi.ac.uk/arrayexpress/).

Differential Expression

The Bioconductor package baySeq v. 1.17.3 (https://github.com/Bioconductor-mirror/segmentSeq/commits/master) was used to test for differential expression between wild-type and *arp6* libraries for each miRNA species, based on counts of individual miRNA species in the respective libraries.

Reverse Transcription-Quantitative PCR

For reverse transcription-quantitative PCR (RT-qPCR), the total RNA was isolated using an RNeasy Plant Mini Kit (cat. no. 74904; Sigma-Aldrich). For the RT-qPCR of *JAZ* genes, total RNA was isolated from the adult leaves of 30-d-old plants. The leaves were treated with 300 μM of methyl jasmonate (cat. no. 392707; Sigma-Aldrich) for 1.5 h. The cDNA was generated using 5 μg of total RNA, reverse transcriptase (no. EP0441; Fermentas/Thermo Fisher Scientific, Guilford, CT), and oligo dT. qPCR was performed as described previously in Choi et al. (2007). The relative transcript levels were calculated using the $2^{\Delta\Delta}$Ct method. Sequences of oligonucleotides for the RT-qPCR used in this study are provided in the Supplemental Table S1.

Chromatin Immunoprecipitation Analysis

All chromatin immunoprecipitation (ChIP) analysis procedures were followed as reported previously in Choi et al. (2007). Two grams of 10-day-old *35S-myc:ARP6* or *FRIp::myc:FRI* grown under long-day conditions were used. To quantify the enrichment of 6 myc-tagged ARP6 or FRI in chromatin, monoclonal antic-myc (cat. no. sc-40; Santa Cruz Biotechnology, Santa Cruz, CA) was used for immunoprecipitation, and a negative control experiment was performed that was identical to the sample experiment, with the exception of the addition of antibody. The information on the primer pairs for ChIP-qPCR is presented in Supplemental Table S1.

MNase-qPCR Assay

Nuclei were isolated from 2 g of 10-day-old wild-type and *arp6* plants, as described previously in Kumar and Wigge (2010). The isolated chromatin was

digested in the buffer (0.05 units micrococcal nuclease (New England BioLabs), 4 mM $CaCl_2$, 10 mM Tris-HCl, pH 8.0, 10 mM NaCl, and 1 mM EDTA) at 37°C for 10 min followed by vortexing at 1000*g*. The digested mononucleosomal DNA of the supernatant was collected by centrifuging at 14,000*g* for 5 min, and genomic DNAs were purified using a PCR purification kit (Qiagen, Hilden, Germany) after digestion with protease K and a phenol/chloroform extraction. Undigested genomic DNA was prepared and used as the input control and for normalization of the qPCR. Relative nucleosome occupancies were calculated using the $2^{\Delta\Delta}$Ct method. The procedure and information on the primer pairs for MNase-qPCR are provided in Supplemental Fig. S3 and Supplemental Table S1, respectively.

Accession Numbers

Sequence data from this article can be found in the EMBL data libraries (ArrayExpress E-MTAB-4498, EMBL-EBI; https://www.ebi.ac.uk/arrayexpress/).

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Transcription levels of *pri-miR156* and *pri-miR172* in *arp6* and wild type grown for 2 d under short day conditions (A) and transcription levels of miR156- and miR172-regulated genes in *arp6* and wild type under short-day conditions (B).

Supplemental Figure S2. Comparison of leaf shape in *arp6* and wild-type.

Supplemental Figure S3. Effects of *sef* and *pie1* on transcription of miRNA gene and miRNA-regulated genes.

Supplemental Figure S4. MNase-qPCR assay in *arp6*.

Supplemental Table S1. Information about oligonucleotides used for the small RNA blot and qPCR.

Supplemental Table S2. Numbers of lateral root in wild type and *arp6*.

Supplemental Table S3. Root lengths of wild type and *arp6*.

Supplemental Table S4. Comparison of individual miRNA abundance in small RNA libraries between wild type and *arp6*.

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