RESEARCH PAPER

Epigenetic regulation of miR396 expression by SWR1-C and the effect of miR396 on leaf growth and developmental phase transition in Arabidopsis

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Abstract

In this study, we investigated the regulatory function of miR396 in the phase transition in Arabidopsis thaliana. Using AtMIR396a/b knockout mutants generated through clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9 (CRISPR/Cas9)-directed genome editing, we showed that miR396 negatively regulates the leaf size and vegetative phase transition, and the first leaf with abaxial trichomes appeared earlier in the mir396ab double mutant than in the wild type (WT) and was significantly delayed in miR396 overexpression lines. Moreover, mir396ab exhibited early flowering, whereas 35S:MIR396a/b and cib4-1 delayed flowering, and the flowering time was negatively correlated with FT gene expression. Furthermore, in arp6 and pie1 mutants, which are deficient in the ATP-dependent chromatin remodeling complex (SWR1-C), miR396 expression was significantly repressed. Compared with the WT, reduced H2A.Z deposit and stronger relative nucleosome occupancy in the promoter region of MIR396a was found in the arp6 mutant, indicating that SWR1-C contributes to the transcriptional activation of MIR396a via nucleosome dynamics. In addition, miR396 displayed specific spatio-temporal expression patterns in the leaf, which was altered in arp6 and pie1, and therefore affected the transcript levels of CIB4 and FT in these mutants. We propose that miR396 is not only a marker of cell differentiation, but also an age signal for leaf development and phase change. Meanwhile, SWR1-C-mediated epigenetic regulation contributes to the age-dependent enhancement of miR396 expression and differential miR396 accumulation among leaves.

Keywords: Arabidopsis, ATP-dependent chromatin remodeling complex, CRY-interacting bHLH4, flowering, GROWTH-REGULATING FACTOR, microRNA396, vegetative phase transition.

Introduction

After seed germination, the plant life cycle advances from the juvenile to the adult stage of vegetative growth, and later enters the reproductive, flowering phase. Specific morphological traits of the lateral organs can be observed in each of these stages (Telfer et al., 1997). Therefore, the mechanism of vegetative phase change and flowering initiation is of great importance for our understanding of plant ontogeny.

Abbreviations: bHLH, basic helix–loop–helix; ChIP, chromatin immunoprecipitation; CIB, CRY-interacting bHLH; FT, FLOWERING LOCUS T; GRF, GROWTH-REGULATING FACTOR; H3K27ac, histone H3 lysine 27 acetylation; H3K27me3, histone H3 lysine 27 trimethylation; SAM, shoot apical meristem; SPL, SQUAMOSA PROMOTER BINDING PROTEIN-LIKE; SWR1-C, ATP-dependent SWR1 chromatin remodeling complex; WT, wild type.

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MiRNAs are a class of regulatory RNAs of ~21 nucleotides that post-transcriptionally regulate gene expression by directing miRNA cleavage or translational inhibition in plant (Axtell, 2013). In Arabidopsis and many other plants, vegetative phase change is regulated by miR156, whose targets encode a class of SQUAMOSA PROMOTER BINDING PROTEIN-LIKE (SPL) genes (Chuck et al., 2007; Wang et al., 2009; Wu et al., 2009). The miR156 expression level exhibits a downward trend during shoot development, while its targets, such as SPL3 and SPL9, are expressed in an opposing gradient pattern. SPLs regulate transition to the reproductive phase by directly binding to the promoter of the MIR172b gene, which in turn suppresses AP2-like transcription factors to control floral organ identity and flowering (Wang et al., 2009; Wu et al., 2009; Jung et al., 2016). Additionally, miR159 contributes to the correct timing of vegetative development in Arabidopsis. The repression of miR156 by miR159 is predominantly mediated by MYB33, an R2R3 MYB domain transcription factor targeted by miR159, and functions as a modifier of vegetative phase change (Guo et al., 2017).

The regulatory role of miRNAs depends on their specific spatio-temporal expression pattern during plant development. Recent studies demonstrated that, besides transcription factors, epigenetic regulation is closely associated with the transcription of genes encoding miRNAs. Notably, the temporal decline in miR156 during vegetative phase change is mediated by a decrease in histone H3 lysine 27 acetylation (H3K27ac), and an increase in histone H3 lysine 27 trimethylation (H3K27me3) at MIR156A and MIR156C. The latter occurs along with an increase in the binding of polycomb repressive complexes2 (PRC2) to these genes. The CHD3 chromatin remodeler PICKLE (PKL) promotes vegetative phase change by stabilizing the nucleosome at the +1 position and by promoting low levels of H3K27ac and high levels of H3K27me3 at MIR156A and MIR156C (M. Xu et al., 2016). Additionally, the SWI2/SNF2 chromatin remodeling ATPase BRAHMA (BRM) and the ATP-dependent SWR1 chromatin remodeling complex (SWR1-C) have been reported to promote the transcription of MIR156A through directly binding to the MIR156A promoter (Y. Xu et al., 2016). SWR1-C catalyzes the replacement of H2A–H2B dimers with H2A.Z–H2B dimers in nucleosome structures, thus producing variant nucleosomes with dynamic properties. Mutations in components of the SWR1-C (ARP6 and SEF) and in genes encoding H2A.Z (HTA9 and HTA11) reduce the expression of MIR156A and MIR156C, and accelerate vegetative phase change, indicating that H2A.Z promotes juvenile vegetative identity (Xu et al., 2018).

An evolutionarily conserved miRNA, miR396, plays diverse regulatory roles in leaf, root, and floral development (Liu et al., 2009; Wang et al., 2011; Debernardi et al., 2012; Bazin et al., 2013; Bao et al., 2014; Debernardi et al., 2014; Liang et al., 2014; Schommer et al., 2014; Das Gupta and Nath, 2015; Duan et al., 2015; Gao et al., 2015; Rodriguez et al., 2015; Yang et al., 2015; Li et al., 2016; Tang et al., 2018). In Arabidopsis, the miR396 family targets seven genes belonging to the GROWTH-REGULATING FACTOR (GRF) family of transcription factors (Jones-Rhoades and Bartel, 2004; Debernardi et al., 2012). miR396 regulates cell proliferation and meristem size, and controls the final number of cells in leaves; therefore, it affects leaf size and longevity (Rodriguez et al., 2010; Debernardi et al., 2014). More recently, miR396 has been shown to control carpel number and pistil development, and to specify meristematic cells of gynoecia and anthers via regulation of the GRF–GIF complex in Arabidopsis (Lee et al., 2018). The miR396-targeted SHORT VEGETATIVE PHASE (SVP) is required to repress flowering and is related to the development of abnormal flower symptoms by the Phyllody Symptoms1 Effector (Yang et al., 2015). In rice (Oryza sativa), OsmiR396d and its OsGRF targets, together with OsGIF1, are involved in the regulation of floral organ development via OsJM706 and OsGR4 (Liu et al., 2014). Blocking the effect of miR396 greatly increases grain yield by modulating the development of auxiliary branches and spikelets through direct induction of OsGRF6, suggesting an miRNA-mediated regulation module for controlling spikelet development in rice (Gao et al., 2015). Another study, on tomato, showed that the flowers, sepals, and fruits all became noticeably larger when STTM396a/396a-88 was overexpressed (Cao et al., 2016). Besides GRF genes, bHLH74, which encodes a basic helix–loop–helix (bHLH) transcription factor, has also been identified as a target for miR396 in Arabidopsis. The repression of bHLH74 by miR396 is required for margin and vein pattern formation in leaves (Debernardi et al., 2012). Arabidopsis bHLH74, also known as CIB4 (cryptochrome-interacting bHLH4), was reported to promote flowering through interacting with CRY2 (cryptochrome 2) and activating the expression of the FLOWERING LOCUS T (FT) gene (Liu et al., 2013). These results suggest a possible role for miR396 in phase transition and flower initiation. However, further experiments are still needed to confirm the possibility and elucidate the underlying mechanism.

In this study, the regulatory role of miR396 in leaf development and phase transition was studied using AtMIR396a/b knockout mutants obtained through clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9 (CRISPR/Cas9)-directed genome editing. Moreover, mutants deficient in SWR1-C were used to investigate the epigenetic control of miR396 expression. We showed that miR396-mediated GRF/CIB4 repression affects juvenile to adult transition and flowering, and the spatio-temporal distribution of miR396 during leaf development is regulated by SWR1-C through changes in chromatin structure.

**Materials and methods**

**Plant materials and growth conditions**

All genetic stocks used in this study were in the Columbia (Col-0) ecotype. Plants used for gene expression assays and transformation were grown in soil in a growth chamber at 23°C and either long days (LDs; 16 h/8 h, light/dark) or short days (SDs; 12 h/12 h, light/dark). For leaf shape analysis, fully expanded leaves were removed, attached to cardboard with double-sided tape, flattened with transparent tape, and then scanned in a digital scanner. The length, width, and lamina area of successive rosette leaves were measured using the ImageJ software. Abaxial trichomes were scored 2–3 weeks after planting with a stereomicroscope. Flowering time was measured by counting the total number of rosette leaves and the number of days to flower when the floral buds are visible. Approximately 12–20 plants were counted and averaged for each measurement.
Vector construction and transformation

In order to knock out the MIR396a and MIR396b genes in Arabidopsis through the CRISPR/Cas9 system, a total of six guide RNAs (gRNAs) were designed to target MIR396a (gRNA1, gRNA2, gRNA3, and gRNA4) and MIR396b (gRNA5 and gRNA6), respectively (see Supplementary Fig. S1A at JXB online). Each U6p–gRNA–scaffold cassette was amplified through PCR and then, after BsaI digestion and T4 ligase ligation, two or four U6p–gRNA–scaffold cassettes were assembled. The resulting product was cloned into the plasmid vector pDe-CAS9 (TAIR Accession: Vector: 6531115762) (Supplementary Figs S1B, C, S2). All primers used in this work are listed in Supplementary Table S1. The clones used for vector construction were verified by sequencing.

The constructs described were electroporated into Agrobacterium tumefaciens GV3101, and used to transform Arabidopsis with the floral dip method (Clough and Bent, 1998). Transformants were selected on 6 μg ml−1 Basta (Sigma-Aldrich, Darmstadt, Germany). Lines containing a mutation were selected based on PCR analysis and sequencing. The backbone of T-DNA including Cas9 has been segregated through PCR-based screening of T2 and T3 generations. Cas9-free homozygous stocks of T3 seeds were used for further study.

Other transgenic lines, including proMIR396a:GUS, proMIR396b:GUS, 35S:MIR396a#3, 35S:MIR396b#10, proCIB4:CIB4-GUS, and proCIB4:mCIB4-GUS, were generated in our previous study (Bao et al., 2014).

GUS staining

To visualize the expression of reporter, transgenic plants were subjected to β-glucuronidase (GUS) staining by incubating seedlings in a solution of 1 mg ml−1 5-bromo-4-chloro-3-indolyl-β-d-glucuronic acid, 1 mM potassium ferricyanide, 0.1% Triton X-100, 0.1 M sodium phosphate buffer (pH 7.0), and 10 mM EDTA overnight at 37 °C, followed by clearing in 70% ethanol. Photos were taken by a Canon SLR camera.

Small RNA analysis

Total RNA was extracted using TRIzol reagent (Invitrogen, USA). For RNA gel blot, 20–30 μg of total RNA was separated on a 17% polyacrylamide gel containing 7 M urea, blotted to HyBond-N+ membranes (Roche, Mannheim, Germany) and fixed by UV cross-link. Blots were hybridized using a digoxigenin end-labeled locked nucleic acid (LNA) oligonucleotide probe designed against miR396 (Roche DIG Oligonucleotide Tailing Kit, 2nd Generation). The miRNA level was standardized with that of 5S rRNA. Alternatively, quantification of mature miR396 was performed with the FastStart Essential DNA Green Master (Roche). The miRNA level was standardized with U6. Sequences of oligonucleotide probe and primers are listed in Supplementary Table S1.

Quantitative RT-PCR

Total RNA was extracted using TRIzol reagent (Invitrogen), and 1 μg was used for first-strand cDNA synthesis using oligo(dT) primers and the ReverTra Ace® qPCR RT Master Mix with gDNA Remover (2× SYBR) (ToYoBo, Osaka, Japan). Real-time PCR was performed on the Mastercycler ep realplex® system (Eppendorf, Hamburg, Germany) with the FastStart Essential DNA Green Master (Roche). The amplification program was as follows: 15 s at 95 °C, and then 15 s at 95 °C, 20 s at 58 °C, and 20 s at 72 °C for 40 cycles, followed by a thermal denaturing step. Relative transcript levels were calculated with the ΔΔCt method using the UBQ1 or ACTIN2 gene as a reference. Sequences of primers are listed in Supplementary Table S1.

Nucleosome occupancy assay

Nuclei were isolated from 1 g of 12-day-old wild-type (WT), arp6, and pic1-1 plants, as described previously (Choi et al., 2016). The isolated chromatin was digested in the buffer [0.05 U of micrococcal nuclease (MNase; Sigma-Aldrich), 4 mM CaCl₂, 10 mM Tris–HCl (pH 8.0), 10 mM NaCl, and 1 mM EDTA] at 28 °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 precipitated with 2.5 vol of anhydrous ethanol and 1/10 vol of sodium acetate (3 M, pH 5.2) 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. The ACT2–73 loci served as the control, and relative nucleosome occupancies were calculated using the 2ΔΔCt method. The sequences of the primer pairs are provided in Supplementary Table S1.

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) was performed according to Hu and Xu (2016). First, 15–day-old seedlings (1 g) of 35S:ARP6-myc and Col-0 were harvested and cross-linked in 1% formaldehyde for the analysis of ARPs abundance at MIR396a. The anti-c-Myc Agarose Affinity Gel antibody (A7470, Sigma-Aldrich) was used for immunoprecipitation. Chromatin was also extracted from leaves (1, 2, 3, and 4) of 21-day-old seedlings of arp6 and Col-0 for the analysis of H2A.Z level at MIR396a, and the sonicated chromatin was then immunoprecipitated with 5 μl of anti-H2A.Z antibody (active motif). The ChIP results were normalized against the input control. The information on the primer pairs for ChIP-qPCR detection is presented in Supplementary Table S1.

Results

Generation of transgenic Arabidopsis with targeted knockout of MIR396a and MIR396b genes using the CRISPR/Cas9 system

To investigate the biological function of miR396 in Arabidopsis development, we used four constructs to edit two members of miR396–encoding genes. Six gRNAs were designed; each gRNA lay at the stem of a MIR396 precursor, or was located up or downstream from the precursor (Supplementary Fig. S1A). The distance of two Cas9 cuts is expected to be 411, 160, and 300 bp for the gRNA1/gRNA2, gRNA3/gRNA4, and gRNA5/gRNA6 pairs, respectively. Two or four gRNAs were ligated in tandem and driven by one AtU6 promoter in the same construct (Supplementary Fig. S1B). After transformation, we selected transgenic plants with target mutations in MIR396a and MIR396b. The PCR amplification of MIR396a/b fragments across the corresponding target sites in transgenic Arabidopsis plants is shown in Supplementary Fig. S3A, and only Cas9-free and T3 homozygotes were used in molecular analysis. Representative sequences of chromosomal fragment deletions aligned with that of the WT are shown in Supplementary Fig. S3B. Most mutations derived by Cas9 in transgenic lines were 100–400 bp long deletions at the target sites; ab#4 also had a 61 bp addition. For ab#1, three deletions were detected, two in the MIR396a site and one in the MIR396b site. The double mutant ab#2 was generated through crossing at#4 with b#26.

Northern blot analysis showed that miR396 levels were decreased slightly in the single locus mutant lines we tested, while in double mutants miR396ab#1 and ab#2, no signal of mature miR396 could be detected (Fig. 1A). This indicates that both MIR396a and MIR396b contribute to the accumulation of miR396. Through qRT-PCR, we further measured the level of miR396a/b.
MIR396 primary transcripts (pri-MIR396a and pri-MIR396b). As expected, deletion in MIR396a only resulted in a decrease in pri-MIR396a, whereas mutation in MIR396b resulted in a decrease in pri-MIR396b. Additionally, both pri-MIR396a and pri-MIR396b were undetectable in the double mutant mir396ab#1 (Fig. 1B). We also detected the levels of target genes in these mutants. Compared with their corresponding controls in the WT, most target genes were up-regulated by mutations in MIR396a and/or MIR396b (Fig. 1C). Furthermore, the levels of target transcripts varied more significantly in double mutants mir396ab#1 and ab#2 than in single mutants a#16, a#42, b#6, and b#26.

miR396 affects the transition from juvenile vegetative phase to adult vegetative phase

It has been reported that miR396 can regulate leaf cell proliferation, and hence control leaf size (Rodriguez et al., 2010). We observed the leaf phenotype in these MIR396a/b knockout mutants. According to the statistics of length, width, and area of the lamina of successive rosette leaves of Col.0 and mutant plants, there were significant differences between leaf size in the WT and the four mutants we tested. In double mutants mir396ab#1 and ab#2, the sizes of L1, L3, L5, and L6 were greatly increased after growing in LD conditions for 4 weeks, which is due to the increase in both the length and the width of rosette leaves (Fig. 2A–D).

In Arabidopsis, the phenotype of leaves reflects vegetative phase change. Juvenile leaves are small and round, and lack trichomes on their abaxial surface, whereas adult leaves are elongated, have serrated margins, and produce abaxial trichomes (Poethig, 2013). We found that mir396a#42, b#26, ab#1, and ab#2 produced abaxial trichomes approximately one plastochron earlier than did the WT under LD conditions, whereas miR396 overexpression lines (OE396a#3 and b#10) exhibited a significant delay in abaxial trichome production (Fig. 3A, B); they entered precociously into the adult phase, as evidenced by the premature initiation of abaxial trichomes and changes in leaf shape. The phenotype of abaxial trichome production was normal in mir396ab#1 and ab#2 when plants were grown in SD conditions, whereas two lines of OE396 entered the adult phase significantly earlier than the WT (Supplementary Fig. S4). The expression of seven target genes was detected in samples of leaf 3 (22-day-old seedlings), and remarkable increases in transcript levels were found in six target genes, with the exception of GRF7 (Supplementary Fig. S5A), whereas in the shoot apex, GRF1 and GRF9 transcripts were significantly elevated compared with that in the WT (Supplementary Fig. S5B).

It has been reported that the initiation of vegetative phase transition in flowering plants is regulated by the miR156–SPL pathway (M. Xu et al., 2016). We tested whether the alteration of miR396 expression causes a change in SPL genes in rosette leaves. SPL9, SPL10, and SPL15 were significantly
The spatio-temporal distribution of miR396 is regulated by SWR1-C down-regulated by the deficiency of miR396, other SPL genes, including SPL4 and 5, were up-regulated by miR396 (Fig. 3C). Considering the importance of the shoot apical meristem (SAM) in lateral organ production and phase transition, the expression of SPL genes in the shoot apex was measured. A slight increase in SPL4 and SPL10 transcripts can be found in mir396ab#1, compared with those in their WT control (Fig. 3D). This indicated that the effect of miR396 on vegetative phase transition might be independent of the miR156–SPL pathway, or through the downstream genes targeted by SPL transcription factors.

miR396 affects flowering

Changes in the relative timing of juvenile to adult shoot development are expected to result in considerable effects on traits such as leaf morphology and the onset of flowering. We became interested in the role of miR396 in flowering because of the studies suggesting that one of the miR396 target genes, CIB4, together with CIB1, CIB2, and CIB5, acts redundantly to activate the transcription of FT, and are positive regulators of CRY2-mediated flowering (Liu et al., 2013). We subsequently checked whether miR396 is associated with flowering. As shown in Fig. 4A–C, mir396ab#1 began to flower with fewer leaves under LD conditions, while an overexpression of miR396 led to a dramatic late flowering phenotype, producing >13 leaves at the time of bolting, as observed in both OE396a#3 and OE396b#10. Under SD conditions, both mir396ab#1 and ab#2 produced fewer leaves at flowering, while OE396a#3 and OE396b#10 displayed the late flowering phenotype just as they did under LD conditions (Supplementary Fig. S6).

We used the cib4-1 (bhlh74-1) mutant and CIB4 overexpression transgenic lines to determine the effect of CIB4 on flowering time regulation. cib4-1 showed a moderate late flowering phenotype (Fig. 5A–C), whereas 35S:mCIB4 began flowering earlier than the WT and produced fewer leaves (~7.79±0.59) at the time of bolting (Supplementary Fig. S7). Further detection of CIB4 and FT transcript levels showed that early flowering in mir396ab#1 was at least partly due to the elevated expression of FT (Fig. 5D). Other flowering-associated genes, such as SOC1 and AP1, did not seem to be responsible for the observed phenotype in mutants (Fig. 5D).

The expression of miR396 during developmental phase transition

The expression profile of miR396 was monitored during phase transition. Under LD conditions, the transcripts of pri-MIR396a and pri-MIR396b in the shoot apices of WT plants declined to 20% and 30% of the starting level during the first 3 weeks after germination, respectively (Fig. 6A). The expression of miR396 during developmental phase transition.

Fig. 2. Leaf phenotype of MIR396a/b knockout mutants. (A) Shape of successive rosette leaves of Col.0 wild-type (WT), mir396a#42, mir396b#26, mir396ab#1, and mir396ab#2 mutant plants grown in long-day (LD) conditions for 32 d (n>15). Scale bar=1 cm. (B–D) The statistics of length, width, and lamina area of successive rosette leaves of WT and mutant plants. Asterisks indicate significant differences from WT plants, as determined by Student’s t-test (*P<0.05; **P<0.01, compared with the WT).
of target genes, such as **GRF2** and **CIB4**, exhibited an upward trend (Fig. 6B). Promoter–reporter transgenic lines helped us to observe the spatial expression of **MIR396a** and **MIR396b**. GUS staining revealed that the promoters of **MIR396a** and **MIR396b** were strongly active in old leaves and shoot apices (Fig. 6C, D; Supplementary Fig. S8A, B). In flowering plants, the transcription of **MIR396a** and **MIR396b** was detected in the sepal and carpel (Fig. 6E, F). The sites of CIB4–GUS fusion protein accumulation overlapped the expression regions of **MIR396**. Moreover, in the **proCIB4-mCIB4** transgenic plants, which have a modified version of CIB4 (**mCIB4**) with mutations that impaired its interaction with miR396, the
The spatio-temporal distribution of miR396 is regulated by SWR1-C

GUS signal of CIB4 expression became stronger in the leaves and shoot apices (Supplementary Fig. S8C, D), indicating that CIB4 was post-transcriptionally regulated by miR396.

SWR1-C regulates the transcription of MIR396a

The next question we focused on was how the expression of miR396 itself was regulated. There is increasing evidence that epigenetic control of gene expression is a major regulatory mechanism in phase and flowering transitions in higher plants (Kohler et al., 2012; M. Xu et al., 2016; Y. Xu et al., 2016). The SWR1-C in Arabidopsis was reported to affect many MIRNA genes via exchanging the histone H2A–H2B dimer with the H2A.Z–H2B dimer (Choi et al., 2016). We detected miR396 levels in two mutants of SWR1-C (arp6 and pie1). Compared with the WT, the level of miR396 in arp6 and pie1 was significantly reduced, which was mainly due to the decrease of pri-MIR396a (Fig. 7A, B). ChIP analysis of H2A.Z levels at MIR396a was carried out to reveal the effect of ARP6 on the expression of MIR396a. Chromatin was isolated from 15-day-old seedlings under LD conditions and was immunoprecipitated with the antibody to H2A.Z. The qPCR amplification of nine regions around the MIR396a transcription start site (TSS) showed that the relative level of H2A.Z was more decreased in the arp6 mutant than in the WT (Fig. 7C), suggesting that the conversion of histone H2A to H2A.Z by ARP6 might be responsible for the activation of MIR396a transcription. MNase-qPCR analysis showed that the relative occupancy in the promoter region of MIR396a was stronger than that in the WT, indicating that SWR1-C promoted the expression of MIR396a at the transcriptional level through the alteration of chromatin structures at these loci (Fig. 7D). We then performed a ChIP analysis to test whether SWR1-C regulates MIR396a directly using 35S:myc-ARP6 transgenic plants. The enrichment of Myc-tagged ARP6 was observed at two tested sites around the MIR396a promoter, but was unchanged at the ACT2 promoter (Supplementary Fig. S9). Overall, these data indicate that ARP6 is required for the regulation of transcription around the transcription start site of MIR396a.

SWR1-C affects the spatio-temporal expression pattern of miR396/CIB4 module

To investigate the biological importance of SWR1-C-mediated regulation, the expression of MIR396, CIB4, and FT was monitored in the mutants of SWR1-C, such as arp6 and pie1. We detected the expression of miR396 in leaves 1 + 2 at two different time points (12 d and 22 d). In the WT, the transcription of MIR396a was more enhanced in 22-day-old leaves than in 12-day-old leaves, and the rapid growth of MIR396a expression was slowed in arp6 and pie1 (Fig. 8A). As regards pri-MIR396b, it displayed a slight increase with development in the WT, while it remained unchanged or decreased in arp6
and pie1 (Fig. 8B). The variation in CIB4 transcription was negatively related to that of pri-MIR396a, with a significant fall at 22 d compared with at 12 d, and was relatively smoothly in arp6 and pie1 mutants (Fig. 8C). The expression of FT was hardly detectable at 12 d, and increased dramatically at 22 d, in both the WT and mutants, suggesting a complex regulatory mechanism for its expression (Fig. 8D).

Next, we examined the levels of pri-MIR396, CIB4, and FT transcripts in different leaves on the 22-day-old seedlings. We showed that the highest expression of MIR396a was located in old leaves (leaves 1 + 2), then leaves 3 + 4, and the lowest expression was in young developing leaves (leaf 5+apex) (Fig. 9A). A similar pattern was observed in MIR396b expression (Fig. 9B). In arp6 and pie1, both MIR396a and MIR396b transcription was decreased, and the difference among different leaves was not as significant as that in the WT. There was a progressive increase in CIB4 expression from leaves 1 + 2, leaves 3 + 4, to leaf 5+apex in the WT, and even more so in the two mutants (Fig. 9C). The expression of FT was higher in arp6 and pie1 compared with their corresponding controls (leaves located in the same sites) in the WT (Fig. 9D). Collectively, these results implied that SWR1-mediated epigenetic regulation is responsible for the spatio-temporal expression pattern of the miR396/CIB4 module in leaves.

**Discussion**

The miR396/target module has been recognized as a master regulator for cell proliferation during leaf development. In this study, we provided evidence that miR396 might be involved in vegetative phase change and flower initiation. In contrast to previous studies, which usually used miRNA overexpression or target mimicry transgenic lines, we generated miR396 knockout mutants based on CRISPR/Cas9 genome editing to dissect the function of miR396. Through northern blot and qRT-PCR analysis, no signal of mature miR396 or MIR396 precursors could be detected in double mutants mir396ab#1 and mir396ab#2 (Fig. 1A, B). Therefore, CRISPR/Cas9-guided
The spatio-temporal distribution of miR396 is regulated by SWR1-C. The expression level of miR396 in the WT was set at 1.0. Error bars represent the SD from three technical replicates. Similar results were obtained in two independent experiments. Locations of the amplified PCR products are based on TSSs as follows: A1 (–738 to approximately –846), A2 (–631 to approximately –756), A3 (–556 to approximately –654), A5 (–239 to approximately –403), A6 (+5 to approximately +195), A7 (+19 to approximately +217), A8 (+206 to approximately +405), A9 (+384 to approximately +580). (D) MNase-qPCR assay to analyze relative nucleosome occupancy at the promoter of MIR396a in the WT, arp6, and pie1. Locations of the amplified PCR products are based on the TSSs as follows: A (–179 to approximately –118), B (–259 to approximately –159), and C (–653 to approximately –564). The input chromatin without MNase treatment was used for normalization and fold enrichment at each site. Values are relative to the WT and represent the mean ±SD from three technical replicates. Similar results were obtained in three independent experiments. Asterisks indicate significant differences from WT plants, as determined by Student’s t-test (*P<0.05; **P<0.01, compared with the WT). (This figure is available in color at JXB online.)

The miRNA miR396 controls leaf cell proliferation and modifies vegetative phase transition

The mir396ab double mutant displayed a significantly increased leaf size (Fig. 2). This result confirms the function of miR396 in leaf cell proliferation, and is consistent with previous reports based on the miR396 overexpression line and plants expressing a miR396-insensitive version of GRF2 (mGRF2) (Rodriguez et al., 2010).

The spatial expression pattern of miR396 throws light on its function in leaf growth control. After the establishment of leaf polarity, the leaf blade grows to reach its final size and shape through cell proliferation. Cell proliferation first occurs throughout the leaf primordium, and then becomes restricted to the leaf base, while the cells located in the distal part of young developing leaves leave the mitotic cell cycle and begin expansion without division (Rodriguez et al., 2014). Studies using promoter–reporter fusion lines have shown that miR396 is expressed in a gradient along the longitudinal axis of the leaf, with higher expression at the distal part. On the other hand, target genes, such as GRF2, have been shown to exhibit an opposite pattern to that of miR396, with higher levels in the proximal part of the leaf. This demonstrates that miR396 restricts the expression of GRF genes to the cells undergoing mitosis, where they promote proliferation. Thus, we propose that miR396 might control the size of the proliferation zone or the progression of the cell cycle arrest front, which is crucial for determining the final size and the shape of the leaf.

Secondly, we found that miR396 affects the correct timing of vegetative phase change. The first leaf with abaxial
Fig. 8. SWR1-C mediates the expression of genes in the miR396–CIB4 pathway during development. The transcriptional levels of pri-MIR396a (A), pri-MIR396b (B), CIB4 (C), and FT (D) in leaves 1 + 2 of the wild type (WT), arp6, and pie1 in long-day conditions. Plants were grown for 12 d or 22 d. RNA was collected from leaves 1 + 2. Values are relative to the WT and represent the mean ±SD from three technical replicates. Similar results were obtained in three independent experiments. Means with different letters are significantly different (P<0.05, by Duncan’s multiple range test).

Fig. 9. SWR1-C modulates pri-MIR396a and pri-MIR396b expression patterns in different leaves. The expression levels of pri-MIR396a (A), pri-MIR396b (B), CIB4 (C), and FT (D) in different leaves in the wild type (WT), arp6, and pie1. Total RNA was isolated from leaf 1 + 2, leaf 3 + 4, and leaf 5+apex samples in 22-day-old seedlings grown under LDs. Values are relative to the WT and represent the mean ±SD from three technical replicates. Similar results were obtained in three independent experiments. Means with different letters are significantly different (P<0.05 by Duncan’s multiple range test).
trichomes appeared earlier in the mir396ab double mutant than in the WT, and was significantly delayed in 35S:MIR396a/b (Fig. 3). Also, the phenotype of mir396ab could be rescued when we crossed 35S:MIR396a/b to mir396ab#1 or ab#2 (Supplementary Fig. S10A). This is in accordance with the temporal expression pattern of miR396 during leaf development. The miRNA miR396 is expressed at a low level in leaf primordia, steadily increases its level with leaf growth, and expands to the whole organ at the late development stages (Rodriguez et al., 2010). This demonstrates that miR396 is not only a marker of cell differentiation, but also an age signal for leaf development.

How does this leaf-derived age signal integrate with other factors to affect the onset and progression of vegetative phase transition? One possible explanation is that miR396 regulates vegetative phase change in the SAM, which can continuously produce lateral organs (including leaves) during the life cycle of a plant. According to a root and leaf ablation experiment, Yang et al. (2011) proved that vegetative phase change is initiated by a signal(s) produced by the root primordia, which acts by repressing the transcription of specific members of the miR156 gene family. Although the expression of miR396 seemed to be weak in the SAM and leaf primordia, previous data did suggest a role for miR396 in SAM development and maintenance through the regulation of cell proliferation. The overexpression of miR396 led to fewer proliferating cells in the SAM, along with a reduction in the SAM size (Rodriguez et al., 2010). This hypothesis was in accordance with the results of the quantitative detection of miR396 and its targets in the shoot apices (Fig. 6A, C). The temporally regulated decrease in miR396 expression was observed in the shoot apices, together with a gradual increase in its target genes during the first 3 weeks after germination. Consequently, this hypothesis implies that miR396 probably modifies vegetative phase change through its coordination role between cell proliferation and cell differentiation in the SAM.

**CIB4 and FT contribute to miR396-mediated flowering time regulation**

The reproductive phase appears successively after the end of the vegetative phase, and both phases are regulated by closely linked pathways. For example, the age-dependent decline in miR156 results in an increase in SPL genes that promote flowering through activating FT, MADS-box genes, and LFY (Wang et al., 2009; Yamaguchi et al., 2009). Interestingly, SPL is able to activate another miRNA, miR172, which targets APETALA2 (AP2), a group of transcriptional repressors of FT (Mathieu et al., 2009; Wu et al., 2009). This feed-forward loop ensures an irreversible vegetative to reproductive transition. In this study, we showed that plants deficient in miR396 resulted in accelerated flowering partly due to an elevated CIB4 level (Figs 4, 5), whereas the overexpression of miR396 delayed the time of flowering, with a similar phenotype to cib4-1 (Figs 4, 5A–C). When we crossed 35S:mCIB4 to OE396, it was found that they exhibited accelerated phase transition due to accumulation of miR396-resistant CIB4 (Supplementary Fig. S11), suggesting that miR396-mediated CIB4 regulation affects flowering. Furthermore, there was a positive correlation between the level of CIB4 and FT transcript levels (Fig. 5D), which supports the finding that CIB4 activates the transcription of FT by binding to its promoter and served as a positive regulator in CRY2-mediated flowering (Liu et al., 2013).

However, CIB4 alone seems not to be sufficient to affect flowering. Although 35S:mTIR1 flowered significantly earlier than the WT in LD conditions, proCIB4:mCIB4-GUS exhibited a normal flowering phenotype (Supplementary Fig S7), suggesting a redundant role for CIB4 and other CIB members in flowering. Therefore, we propose that the phenotype of mir396ab double mutants might be explained by a cumulative effect of both GRFs and CIB4. Further studies regarding the effects of GRFs on flowering are needed.

**SWR1-C promotes the transcription of MIR396a during leaf development**

SWR1-C regulates gene transcription through catalyzing the replacement of the histone H2A–H2B dimer with the H2A.Z–H2B dimer in nucleosome structure. Arabidopsis SWR1-C component mutants, including arp6, display a pleiotropic developmental phenotype, including early flowering, leaf serration, and the production of extra petals. Sequencing of small RNA libraries showed that many miRNAs, including miR156, decreased in arp6, though some miRNAs increased, and arp6 had a transcriptional effect on both miRNAs and their targets (Choi et al., 2016). Here, we showed that the levels of miR396 were significantly reduced in arp6 and pic1 mutants, which was mainly due to the decrease in pri-MIR396a transcription (Fig. 7A, B). A similar phenotype, such as the production of fewer juvenile leaves and early flowering in LDs, has been observed in the mir396ab double mutant, suggesting the contribution of MIR396 deficiency to flowering control. Similar results have been observed in studies on miR156, which showed that mutations in components of the SWR1 complex (ARP6 and SEF), and in genes encoding H2A.Z (HTA9 and HTA11) reduce the expression of MIR156A and MIR156C, and accelerate vegetative phase change (Xu et al., 2018). Overall, these results indicate that H2A.Z promotes juvenile vegetative identity, and SWR1-C is required for miRNA-mediated development control via transcriptional regulation.

The mechanism by which H2A.Z affects transcription of MIR396 is still unknown. One possibility is that H2A.Z decreases nucleosome occupancy. Our data support this hypothesis; arp6 displayed lower H2AZ deposits (Fig. 7C) and a stronger relative nucleosome occupancy (Fig. 7D) at MIR396a in the promoter region, indicating that SWR1-C contributes to the transcriptional activation of MIR396a via nucleosome dynamics. Another possibility is that the presence of H2A.Z influences the modification of other histone proteins. There is evidence that H2A.Z co-localizes with H3K4me3 near the TSS of many genes (Choi et al., 2016), and the abundance of H3K4me3 at FLC is reduced in arp6 mutants (Martin-Trillo et al., 2006). The arp6 gene reduces H3K4me3 at both MIR156A and MIR156C, but only increases H3K27me3 at
MIR156A (Xu et al., 2018). However, we did not find a difference in H3K4me3 abundance near the TSS of MIR396a locus between the WT and arp6 (data not shown).

The effect of SWR1-C on the expression pattern of MIR396 during development has been investigated through qRT-PCR. We showed that the transcription of MIR396a increased with leaf growth, and SWRC1-C is required for the enhancement of miR396 expression in old leaves (Fig. 8A, B). Furthermore, miR396 was preferentially expressed in old leaves, rather than in young developing leaves or leaf apices, and SWRC1-C is responsible for differential expression of miR396 among different leaves (Fig. 9A, B). These data indicate that SWR1-C-mediated epigenetic regulation contributes to the spatio-temporal expression pattern of miR396 during development.

Further analysis of the miR396 target gene revealed that the variation in CIB4 transcription was negatively related to the levels of pri-MIR396a (Figs 8C, 9C) and miR396 (Rodriguez et al., 2010), suggesting a dominant role for miR396 in the CIB4 transcript level. The effect on FT transcripts seemed to be complicated. The expression of FT increased as the plant grew, which is not affected by SWR1-C mutation (Fig. 8D), demonstrating that SWR1-C-mediated regulation is not responsible for the age-dependent increase in FT. Although a previous study concluded that ARP6 can promote the deposition of H3K4me3 at both FT and FLC (Xu et al., 2018), considering that FLC is a transcriptional repressor for FT expression (Li et al., 2008), it is difficult for us to explain the observations at present.

Based on the above data, we proposed a possible model to explain how miR396 participates in the regulation of leaf development and phase transition (Fig. 10). The knockout of both miR396a and miR396b increases leaf size, accelerates juvenile to adult transition, and modifies flowering. SWR1-C contributes to transcriptional activation of MIR396a via nucleosome dynamics, and is responsible for spatio-temporal miR396 expression in individual leaves and among different leaves.

**Supplementary data**

Supplementary data are available at JXB online.

Fig. S1. Targeted knockout of MIR396a and MIR396b genes in Arabidopsis using the CRISPR/Cas9 system.

Fig. S2. The schematic diagram of vector construction using the Golden Gate Shuffling strategy.

Fig. S3. Identification of mutants.

Fig. S4. The first leaf with abaxial trichomes in short-day conditions.

Fig. S5. The transcriptional levels of GRF transcription factors in the leaves and shoot apices of the WT and mir396ab#1.

Fig. S6. Flowering phenotype in short-day conditions.

Fig. S7. The effect of CIB4 on flowering in long-day conditions.

Fig. S8. GUS staining of 12-day-old seedlings expressing proMIR396a:GUS, proMIR396b:GUS, proCIB4:CIB4-GUS, and proCIB4:mCIB4-GUS.

Fig. S9. Chromatin immunoprecipitation (ChIP)-qPCR analysis of Myc-tagged ARP6 enrichment at the MIR396a gene.

Fig. S10. The phenotype of mir396ab×35S:MIR396 in long-day conditions.

Fig. S11. Flowering phenotype of 35S:MIR396×35S:mCIB4 in long-day conditions.

Table S1. List of primers used in this study.

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