MicroRNA-Mediated Regulation of Stomatal Development in *Arabidopsis*  

Claudia Kutter, Hanspeter Schöb,¹ Michael Stadler, Frederick Meins, Jr.,² and Azeddine Si-Ammour³  

Friedrich Miescher Institute for Biomedical Research, CH-4058 Basel, Switzerland  

The proper number and distribution of stomata are essential for the efficient exchange of gases between the atmosphere and the aerial parts of plants. We show that the density and development of stomatal complexes on the epidermis of *Arabidopsis thaliana* leaves depend, in part, on the microRNA-mediated regulation of *Agamous-like16* (*AGL16*), which is a member of the MADS box protein family. *AGL16* mRNA is targeted for sequence-specific degradation by miR824, a recently evolved microRNA conserved in the Brassicaceae and encoded at a single genetic locus. Primary stomatal complexes can give rise to higher-order complexes derived from satellite meristemoids. Expression of a miR824-resistant *AGL16* mRNA, but not the wild-type *AGL16* mRNA, in transgenic plants increased the incidence of stomata in higher-order complexes. By contrast, reduced expression of *AGL16* mRNA in the *agl16-1* deficiency mutant and in transgenic lines overexpressing miR824 decreased the incidence of stomata in higher-order complexes. These findings and the nonoverlapping patterns of *AGL16* mRNA and miR824 localization led us to propose that the miR824/AGL16 pathway functions in the satellite meristemoid lineage of stomatal development.

INTRODUCTION

MicroRNAs (miRNAs) are short, ~21-nucleotide-long, noncoding RNAs with essential functions in regulating gene expression in multicellular plants and animals. Animal miRNAs pair imperfectly to the 3′ untranslated region of mRNAs and act preferentially by repressing productive translation. By contrast, plant miRNAs usually pair perfectly in coding regions of target mRNAs and act primarily by cleavage (Millar and Waterhouse, 2005). Most miRNA families are conserved in more than one distantly related plant species and are believed to have ancient functions in regulating organogenesis (Floyd and Bowman, 2004; Zhang et al., 2006). Several families of transcription factors have developmental functions that depend on these conserved miRNAs (Jones-Rhoades et al., 2006). Examples include TCP regulation of leaf shape by miR159 (Palatnik et al., 2003), NAC determination of organ boundaries by miR164 (Laufs et al., 2004), APETALA2-like regulation of flowering time and specification of floral whorl identity by miR172 (Aukerman and Sakai, 2003; Chen, 2003), and HD-ZIPIII regulation of organ polarity and the arrangement of vascular tissues by miR166 (Williams et al., 2005). By contrast, none of the many developmental functions of the large plant-specific MADS box protein family (Theissen and Saedler, 1995; Parenicova et al., 2003) are known to depend on miRNAs.

Here, we show that mRNA encoding the MIK-type MADS box protein AGAMOUS-LIKE16 (*AGL16*) is the target of a recently identified *Arabidopsis thaliana* miRNA, miR824 (Rajagopalan et al., 2006; Fahlgren et al., 2007), that is conserved in the Brassicaceae but not in distantly related species. Although no function for AGL16 is known, the *Arabidopsis* MIK-type MADS box transcription factors *APETALA1*, *APETALA3*, *PISTILLATA*, and *AGAMOUS* are required for proper flower formation. Other members of this family have crucial roles in establishing meristem identity, timing of flowering, fruit development, and root growth (Becker and Theissen, 2003).

The finding that *AGL16* mRNA accumulates in stomatal guard cells (Alvarez-Buylla et al., 2000) suggested that *AGL16* has stomata-related functions. Brassicaceae species including *Arabidopsis* form anisocytic stomatal complexes consisting of a central stoma and guard cells surrounded by three neighboring cells in which one cell (E3) is usually smaller than the other two cells (E1 and E2) (Pant and Kidwai, 1967). Primary stomatal complexes are usually of monoclonal origin (von Groll and Altmann, 2001). They arise by asymmetric division of a meristemoid mother cell that generates a meristemoid with a triangular shape and a larger sister cell. This meristemoid undergoes additional asymmetric divisions to produce neighboring epidermal cells and then converts to a guard mother cell that divides symmetrically to produce two guard cells. Primary stomatal complexes can also give rise to higher-order secondary and even tertiary complexes derived from satellite meristemoids. Stomata are almost invariably separated from each other by at least one epidermal cell. This minimum spacing is maintained by asymmetric division of satellite meristemoids to give an epidermal cell and a guard mother cell distal to existing stomata (Nadeau and Sack, 2002a; Bergmann and Sack, 2007).
We found that expression of a miR824-resistant AGL16 mRNA, but not the wild-type AGL16 mRNA, in transgenic plants increased the incidence of stomata in higher-order complexes. By contrast, reduced expression of AGL16 mRNA in the agl16-1 deficiency mutant and in transgenic plants overexpressing miR824 decreased the incidence of stomata in higher-order complexes. These findings and the nonoverlapping patterns of AGL16 mRNA and miR824 localization led us to propose that the miR824/AGL16 pathway functions in the satellite meristemoid lineage of stomatal development.

RESULTS

The Arabidopsis miRNA miR824 Shows Limited Evolutionary Conservation in the Brassicaceae

We identified miR824 in a screen of ~350 unique small RNA (smRNA) clones from Arabidopsis. It has a unique genomic location on chromosome IV (NC_003075.3, positions 12,625,136 to 12,625,157). Recently, high-throughput pyrophosphate sequencing has shown that miR824 is present in the left arm of a 689-nucleotide stem-loop hairpin (Figure 1A) (Rajagopalan et al., 2006; Fahlgren et al., 2007). RNA gel blot hybridization confirmed a 689-nucleotide stem-loop hairpin (Figure 1A) (Rajagopalan et al., 2006; Fahlgren et al., 2007). RNA gel blot hybridization confirmed that miR824 is encoded in the Arabidopsis genome and shows a ubiquitous pattern of expression in stems, leaves, inflorescences, and roots (see Supplemental Figure 1A online). In Arabidopsis, the miRNA pathway depends on HYAPONASTIC LEAVES1 (HYL1), HUA ENHANCER1 (HEN1), and DICER-LIKE1 (DCL1) but not on DCL2, DCL3, and DCL4 or RNA-DEPENDENT RNA POLYMERASE-ASE6 (RDR6) (Meins et al., 2005; Vazquez, 2006). Expression of miR824 was impaired in the homoyzgous deficiency mutants hyl1-1, henn1-1, and dcl1-8 but not in dcl2-5, dcl3-1, dcl4-2, or rdr6-15 (see Supplemental Figures 1B and 1C online), confirming that miR824 is a bona fide miRNA produced exclusively by the known miRNA pathway, as recently reported by Rajagopalan et al. (2006). Moreover, miR824 is associated with ARGONAUT1 protein, indicating that miR824 is efficiently incorporated into the RNA-induced silencing complex, as described for other miRNAs (Qi et al., 2006).

A BLAST search of all available plant genome databases detected the 21-nucleotide miR824 sequence only in other Brassicaceae species, specifically, Brassica rapa, B. napus, and B. oleracea. In each case, this sequence was present on the 5’ arm of the predicted hairpin structure, which was identical in the subspecies B. napus and B. rapa (Figure 1A). RNA blot hybridization showed that miR824 is expressed in the three Brassica species tested but not in rice (Oryza sativa) or tobacco (Nicotiana tabacum) (Figure 1B). To identify miR824 orthologs that might have escaped detection in sequence similarity searches or smRNA gel blots, we compared Arabidopsis miR824 and its neighboring genomic sequences with a putative syntenic region in the rice, poplar (Populus spp), and maize (Zea mays) genomes. As reported previously (Rajagopalan et al., 2006), no homologous loci were detected in those species even using very low-stringency parameters (data not shown). Taken together, these results suggest that miR824 is a member of the class of recently evolved Arabidopsis miRNA genes (Allen et al., 2004) that have been conserved in the same eudicot family but not in more distantly related eudicots and monocots.

miR824 Is Derived from a Spliced, Capped, and Polyadenylated Primary miRNA Encoded at a Single Locus

The stem-loop hairpin containing miR824 is encoded by a transcribed gene, designated MIR824, corresponding to the gene At4g24415.1. Seven ESTs representing this gene were identified, including three full-length cDNAs in the RIKEN EST collection of capped, polyadenylated mRNAs (Figure 2A; see Supplemental Figure 2A online). The capped, polyadenylated 2897-nucleotide transcript, named pri-miR824, is noncoding since no translation start or stop codon could be predicted using the Augustus algorithm (Stanke and Morgenstern, 2005), pri-miR824, like Arabidopsis pri-miR163 (Kurihara and Watanabe, 2004), is spliced. RT-PCR using primers annealing at the ends of pri-miR824 confirmed the expression of two splice variants (data not shown): the 1640-nucleotide variant pri-miR824.1 represented by ESTs R12637 and RAFL04-20-O22, and the 1497-nucleotide variant pri-miR824.2 represented by ESTs RAFL07-10-K03 and GSLTPGH76ZA12 (Figure 2A; see Supplemental Figure 2A online).

The region upstream of the transcription start of pri-miR824 contains a TATA box with the conserved TATAAA motif at position ~30 as well as several distal cis elements implicated in transcriptional regulation (see Supplemental Figure 2B online). To identify the promoter region of MIR824, we bombarded Arabidopsis leaves with 2954 bp of genomic DNA upstream of the pri-miR824 transcription start fused to a luciferase (Luc) reporter gene. Leaves exhibited strong, comparable luciferase activity 48 h after bombardment with ProMIR824:Luc and a Luc gene driven by the strong, double-enhancer cauliflower mosaic virus 35S RNA promoter (Pro2x35S). No activity was detected after bombardment with the promoterless Luc gene (Figures 2B to 2D). These results show that the region 2954 upstream of pri-miR824 is a functional promoter.

We identified an insertion mutant, m3, carrying a T-DNA insertion mapped to a position 169 bp upstream of the transcription start of MIR824 (see Supplemental Figure 2B online) that showed enhanced miR824 expression (Figure 2E). Because the T-DNA promoter is in the same orientation as pri-miR824 transcription (data not shown), enhanced expression probably results from read-through transcription as reported by others using the same T-DNA vector (Ren et al., 2004). Relative to ecotype Columbia (Col-0), homozygous m3 plants accumulated 24-fold higher levels of miR824, 5-fold higher levels of polyadenylated pri-miR824, and 21-fold higher levels of the spliced version pri-miR824.1 (Figure 2E). By contrast, no changes in the accumulation of miR171 or its target, SCARECROW-LIKE6-III (SCL6-III) (Llave et al., 2002), were detected, indicating that the T-DNA insertion in m3 does not have a general effect on miRNA biogenesis and function. These findings confirm that miR824 is encoded at the MIR824 locus as a polyadenylated pri-mRNA that is spliced and then processed.

AGL16 Is the Unique Target of miR824

BLAST searches of Arabidopsis coding sequences allowing up to three mismatches, deletions, or substitutions identified a unique locus complementary to miR824 within the last exon of
Figure 1. Fold Back Structure, Expression, and Conservation of miR824 in the Brassicaceae.

(A) 5’ to 3’ stem-loop hairpin structures of the pre-miR824 predicted for Arabidopsis and three closely related Brassica species. The mature miR824 sequence is indicated in red, and the miR824* sequence is indicated in blue. G:U wobble pairing is shown by a circle. Note that the miR824/miR824* sequences are identical in Arabidopsis and the Brassica species.

(B) RNA blot hybridization of miR824 in leaves of Brassica species, Arabidopsis Col-0, O. sativa, and N. benthamiana. The 5S rRNA and tRNA loading standards are stained with ethidium bromide. The size of the RNA is indicated at left in nucleotides (nt).

(C) RNA ligase–mediated rapid amplification of cDNA ends (RLM-RACE) mapping of AGL16 mRNA cleavage sites in Col-0. The arrow indicates the site of cleavage in 10 of 10 sequenced clones. Perfect base pairing between miR824 and AGL16 mRNA is –38.6 kcal/mol. Conserved miRNA binding sites (red), conserved nucleotides (black), and nonconserved nucleotides (gray highlight) in Arabidopsis and Brassica species are indicated.

(D) to (F) Sequence complementarity between transcripts encoded by miRNA genes and their targets. The red line in (D) indicates complementarity between MIR824 and AGL16 in Arabidopsis. The orange line in (E) indicates complementarity between the MIR824 ortholog in B. oleracea (MIRBr824) and its target sequence AGL16. The blue lines in (F) indicate complementarity between MIR171 and its two targets genes, SCL6-III and SCL6-IV, in Arabidopsis. The gray line in each panel indicates the expected background complementarity at each position in the duplex (see Supplemental Methods online). The percentage of paired bases in a window of 20 nucleotides is shown for a potential duplex structure between miRNA precursor and target sequences. The duplex was anchored at the miRNA complementary site, indicated by vertical dashed lines. Values for each window are plotted aligned to its center position. Sequences of miRNA gene transcripts and their target RNAs, 88 nucleotides in length and centered on the miRNA complementary site, are shown below each plot with the miRNAs highlighted in red.

the gene At3g57230 (AGL16). The base pairing at this site is almost perfect, except for one G:U wobble pairing at the 3’ end of miR824, and has the predicted free energy of pairing –38.6 kcal/mol (Reeder et al., 2006). This highly conserved miR824 pairing region was also identified in B. rapa (AC189325.1), B. napus (CX281097), and B. oleracea (EH417933) ESTs encoding AGL16 orthologs, suggesting that miR824-mediated regulation of AGL16 has been conserved in the evolution of the Brassicaceae (Figure 1C). The presence of AGL16 transcripts in these Brassica species was also verified by RNA gel blot hybridization (see Supplemental Figure 1E online). RLM-RACE showed for all 10 clones sequenced that cleavage of AGL16 transcripts, like targets of many other miRNAs, occurs between nucleotides 10 and 11 of the miR824 pairing region (Figure 1C). Relative to the wild type, AGL16 mRNA
consistently accumulated at higher concentrations in the miRNA defective mutants dcl1-8, hyl1-1, and hen1-1 (see Supplemental Figure 1D online). Taken together, these results indicate that miR824 has a unique target gene, AGL16, and that miR824-mediated negative regulation of AGL16 is conserved in the Brassicaceae.

miR824 Originated in Brassicaceae Genomes by Duplication of AGL16

When MIR824 was used in a BLAST search for complementary Arabidopsis transcripts, the only significant result was AGL16, which showed 64% complementarity over a region of 189 nucleotides (E-value = 3.2e-5) that extends well beyond the 21-nucleotide-long miR824 pairing site (Figure 1D). Similar extended complementarity over a region of 105 nucleotides (E-value = 2e-10) was found for MIR824 and its AGL16 homolog in B. oleracea (Figure 1E). By contrast, the highly conserved Arabidopsis MIR171 gene only shows high complementarity for the pairing sites in its two miR171 target genes, SCL6-III and SCL6-IV (Figure 1F). Extended complementarity with target sequences is a feature of recently evolved Arabidopsis MIR genes, such as those encoding miR161 and miR163 (Allen et al., 2004), miR841 (Rajagopalan et al., 2006), or miR778, miR780, and miR856 (Fahlgren et al., 2007), which originated by duplication of their target genes. Therefore, we propose that MIR824 was generated by a duplication of AGL16 and that this duplication event occurred before the divergence of Brassica lineages.

Characterization of Transgenic Lines Altered in AGL16 mRNA Expression

The report that AGL16 mRNA accumulates in guard cells of leaves (Alvarez-Buylla et al., 2000) suggested to us that miR824-mediated...
regulation of AGL16 might have a role in stomatal development. To test this hypothesis, we generated transgenic plants showing decreased AGL16 mRNA expression, increased ectopic expression of AGL16 mRNA, and ectopic expression of a mutant form of AGL16 mRNA resistant to interaction with miR824. The homozygous, monogenic mutant agl16-1 carries a T-DNA insertion in the last exon of AGL16 (see Supplemental Figure 3A online) and shows an ~14-fold reduction in AGL16 mRNA relative to the wild type (see Supplemental Figure 3B online). AGL16m transformants carry a miR824-resistant form of AGL16 generated by introducing seven silent mutations to block cleavage at the miR824 pairing site controlled by the strong Pro2x35S promoter (Figure 3A). AGL16 transformants carry wild-type AGL16 cDNA controlled by the same promoter as a control for nonspecific effects of ectopic AGL16 overexpression.

Two independent T2 lines overexpressing wild-type AGL16 (AGL16.1/2) and AGL16m (AGL16m1/2) were studied in detail. Quantitative PCR of AGL16 mRNA showed that, relative to wild-type Col-0, AGL16 transcripts were increased by 5- to 7-fold in AGL16.1/2 plants and by 16- to 19-fold in AGL16m1/2 plants, in which AGL16 is resistant to miR824-mediated cleavage (Figure 3B). The relative accumulation of unrelated SCL6-III mRNA, which is the target of miR171, was not affected in the AGL16 and AGL16m lines. These results and the finding that miR824 and miR171 levels were not appreciably affected in the AGL16.1/2 or AGL16m1/2 lines (Figure 3C) indicate that overexpression of AGL16 and AGL16m does not have a general effect on miRNA biogenesis and targeting. Because the primers used for quantitative PCR do not distinguish between AGL16 and AGL16m transcripts, we confirmed that AGL16m RNA is expressed in the AGL16m transformants by treating the RT-PCR products with HinCII restriction endonuclease, which only cuts the miRNA-resistant form (Figures 3A and 3D). Interestingly, the 163 bp fragment representing the endogenous AGL16 signal was reduced in AGL16m lines relative to controls. This might reflect a negative regulation of AGL16 RNA by AGL16m RNA, as reported

Figure 3. Expression of miR824 and AGL16 mRNA in AGL16.1/2 and AGL16m1/2 Leaves.

(A) Nucleotide sequence of AGL16 (AGL16m) with silent mutations in the miR824 recognition site. miR824 pairs to AGL16 RNA at the nucleotide segment corresponding to amino acids Ser-197 and Leu-203. Predicted free energies of pairing to miR824 are indicated at left. Mutated nucleotides are underlined. Vertical lines indicate perfect base pairing, circles indicate G:U wobble pairing, and gray shading indicates the HinCII restriction site (GTT/GAC) introduced into AGL16m.

(B) Fold expression relative to the wild type of uncleaved target AGL16 mRNA (black bars) and SCL6-III mRNA (gray bars), determined by RT-quantitative PCR using primers spanning the miR824 and the miR171 complementary sites, was normalized to TIP41-like (At4g34270) mRNA.

(C) RNA gel blot hybridization of low molecular weight RNA in leaves of Col-0, AGL16.1/2, and AGL16m1/2 using probes for miR824 and miR171. tRNA and 5S rRNA were stained with ethidium bromide. The sizes of the RNAs are indicated at left in nucleotides (nt).

(D) AGL16 and AGL16m transcripts were distinguished by HinCII digestion, which only digests AGL16m cDNA, after RT-PCR amplification of endogenous and AGL16m transcripts.
AGL16 Expression Is Important for the Development of Stomatal Complexes

To detect the effects of altered AGL16 regulation on stomatal development, we traced the cell lineage of at least 110 individual stomatal complexes of each line. Figure 4A shows the proportion of primary, secondary, and higher-order stomatal complexes estimated for comparable abaxial regions of the fifth fully expanded leaves. Similar results were obtained in three independent experiments. Under our culture conditions, the proportion of stomatal complex types of AGL16m1/2 did not differ significantly from that of Col-0 ($\chi^2, df = 2, P > 0.94$). These three lines developed predominantly primary stomatal complexes (69 to 71%), with considerably lower incidences of higher-order secondary (26 to 28%) and tertiary (3 to 4%) stomatal complexes and no quaternary stomatal complexes. By contrast, the distributions of the AGL16m1/2 plants differed significantly from that of Col-0 ($\chi^2, df = 2, P < 3.0 \times 10^{-5}$) and consistently showed a dramatic shift to higher-order stomatal complexes in which secondary and tertiary forms predominate. Moreover, 2 to 5% of the complexes in AGL16m1/2 plants were quaternary forms, which were never detected in Col-0 or AGL16.1/2 plants. The distributions obtained with agl16-1 and m3 plants deficient in AGL16 mRNA accumulation also differed significantly from that of Col-0 ($\chi^2, df = 1, P < 2.7 \times 10^{-5}$), but unlike AGL16m1/2 they showed a higher percentage of primary complexes relative to Col-0 (91 and 93%, respectively) and lacked tertiary complexes. Figures 4B and 4C show representative scanning electron microscopy images of individual primary and quaternary stomatal complexes. All lines exhibited the anisocytic structure and guard cell morphology of wild-type Col-0.

We followed the time course of development in individual stomatal lineages on the abaxial surface of the first true leaf. Images were collected daily for 4 d starting at 6 d after germination. Figure 5 shows that the first mature stomata with surrounding guard cells appeared at 8 d after germination in all of the lines tested. Wild-type Col-0 and AGL16.1 showed similar kinetics of stomatal development (Figures 5A and 5B). The first satellite meristemoids appeared at 9 d after germination, followed by the first mature secondary complexes at 10 d after germination. During this period, line agl16-1 only formed primary stomatal complexes in the particular sample shown (Figure 5C). By 6 d after germination, which was the earliest sampling time, line AGL16m1 had already formed a high incidence of satellite meristemoids before the appearance of the first mature stomatal complexes at 8 d after germination (Figure 5D). Moreover, line AGL16m1 continued to form higher-order satellite meristemoids at 10 d after germination, after the initiation of satellite meristemoids had ceased in the other lines. To summarize, reduced AGL16 expression significantly lowered the proportion of higher-order stomatal complexes but did not alter the kinetics of primary stomatal complex development. Expression of miR824-resistant AGL16m mRNA, on the other hand, increased the incidence of early meristemoid formation, prolonged the period of satellite meristemoid initiation, and significantly increased the proportion of higher-order stomatal complexes.

The Density of Higher-Order Stomatal Complexes Is Positively Correlated with AGL16 Expression

Mutants altered in stomatal development often exhibit changes in stomatal density—that is, the number of stomata per square millimeter of epidermis (Bergmann and Sack, 2007)—or in stomatal index, defined as the ratio of the number of stomata to the
number of epidermal cells plus stomata (Berger and Altmann, 2000). We compared the density of stomata on the abaxial surface of the fifth rosette leaves of plants grown under the same conditions in the same experiment. Similar results (data not shown) were obtained for the adaxial leaf surface. Figure 6A shows that the stomatal density of AGL16.1/2 plants did not differ significantly (t test, \( P = 0.11 \) and 0.25) from that of Col-0. Stomatal density was reduced significantly in agl16-1 (\( -1.2\)-fold; \( P < 0.025 \)) and in the m3 mutant (\( -1.2\)-fold; \( P < 0.05 \)) but was increased significantly in AGL16m2 (\( -2.2\)-fold; \( P < 0.05 \)) and in AGL16m1 (\( -2.9 \) fold; \( P < 0.01 \)). Interestingly, none of the lines altered in AGL16 expression showed a significant effect on stomatal index (Figure 6B).

We distinguished between the effects on primary and higher-order stomatal lineages by comparing the densities of the two types of stomata calculated from the proportion of higher-order stomata (Figure 4A) and the total stomatal density (Figure 6A). Figure 6C shows that the density of primary stomata of lines altered in AGL16 expression did not differ significantly from that of Col-0. In striking contrast, the density of higher-order stomata was greatly decreased by fourfold to fivefold in the AGL16 mRNA-deficient m3 (t test, \( P < 7.5 \times 10^{-4} \)) and agl16-1 (\( P < 2.3 \times 10^{-4} \)) mutants, while the density of higher-order stomata was greatly increased by fourfold to sevenfold in AGL16m1 (\( P < 5.8 \times 10^{-3} \)) and AGL16m2 (\( P < 2.4 \times 10^{-3} \)). These results indicate that the small but significant effects of altered AGL16 expression on total stomatal density reflect robust effects on the incidence and degree of higher-order stomatal complexes. This confirms the conclusion that AGL16 has a function in the development of higher-order stomata.

Aside from the stomatal phenotypes described above, the lines agl16-1 (see Supplemental Figure 3C online), AGL16.1/2, and m3 (see Supplemental Figure 4A online) did not show obvious developmental abnormalities. By contrast, the AGL16m1/2 lines exhibited a bushy growth habit, increased numbers of leaves that are reduced in size, and abnormalities in leaf morphology, including increased green pigmentation, twisting and elongation of the apical–basal axis, and upward curling of the leaf margin (see Supplemental Figure 4A online). In addition, trichomes on the leaves of the AGL16m1/2 lines sometimes formed two branches or, more frequently, four branches, rather than the three branches expected for wild-type Col-0 (see Supplemental Figures 4B to 4D online).

miR824 and AGL16 mRNA Are Localized in Different Cell Types of the Stomatal Complex

We were unable to detect either miR824 or AGL16 mRNA by in situ hybridization of whole mounts of Arabidopsis leaves. As an alternative, we chose young leaves of B. rapa, since highly conserved homologs of both the AGL16 target and miR824 are expressed in this species (Figures 1B and 1E). Sense miR824

---

**Figure 5.** Time Course of Stomatal Development on the Abaxial Surfaces of First Rosette Leaves.

Drawings of the relevant features of nail polish pictures taken from sequential dental resin impressions showing the development of representative stomatal complexes on the abaxial surfaces of first true leaves of Col-0 (A), AGL16.1 (B), agl16-1 (C), and AGL16m1 (D) plants monitored daily starting at 6 d after germination (dpg). Satellite meristemoids are colored in green, guard mother cells in yellow, kidney-shaped guard cells in gray, and jigsaw-shaped epidermal cells are not colored. Bar = 10 \( \mu m \).
was detected in satellite meristemoids (Figure 7A), which are easily identifiable by their triangular shape and position close to mature stomata (Pant and Kidwai, 1967), and guard mother cells (Figure 7B) but not in mature guard cells (Figures 7A and 7B). By contrast, AGL16 mRNA was detected only in mature guard cells and not in cells of stomatal complexes, where miR824 is localized (Figure 7D). No signals were obtained in controls using probes for antisense miR824 (Figure 7C) or AGL16 mRNA (Figure 7E). These results show that although both miR824 and its target are localized in stomatal complexes, they are never detected in the same cell type.

DISCUSSION

miR824-Mediated Regulation of AGL16 mRNA Is Required for Proper Stomatal Complex Development

Members of the MADS box protein family have many established functions in plant growth and development (Kaufmann et al., 2005). Here, we show that a MADS box protein also functions in stomatal development and is subject to miRNA regulation. miR824 is encoded at a single genetic locus and has as a unique target AGL16 mRNA, which is cleaved at the miRNA recognition site in a sequence-dependent manner, leading to decreased accumulation of the target RNA. Decreased accumulation of AGL16 mRNA, both in the agl16-1 deficiency mutant and in the miR824-overexpressing m3 mutant, resulted in a fourfold to fivefold decrease in the density of higher-order stomata. Overexpression of AGL16m mRNA resistant to miR824-mediated cleavage had the opposite effect: the density of higher-order stomata was increased by fourfold to sevenfold. The fact that this increase was not observed with the AGL16.1/2 lines indicates that this effect depends on miR824 resistance rather than on the ectopic expression of AGL16 mRNA. These results, together with the finding that both miR824 and AGL16 mRNA are localized in stomatal complexes, but never in the same cell type, strongly suggest that the normal development of stomatal complexes depends on the proper downregulation of AGL16 by miR824.

The miR824/AGL16 Pathway Functions in the Satellite Meristemoid Lineage

Stomatal development in Arabidopsis involves iterative cell specification and differentiation to generate mature guard cells, amplification of precursor cells by successive asymmetric divisions, and the proper spacing and orientation of precursor cells that will eventually form stomata (Bergmann and Sack, 2007). Altered expression of AGL16 mRNA did not affect the basic anisocytic structure of stomatal complexes or the morphology of guard cells. This is consistent with the finding that the stomatal index of AGL16 mutants ranged from 25 to 31%, as expected for a mixed population of primary and higher-order stomatal complexes, and did not differ significantly from that of the wild type. The proper spacing of stomata depends on an extracellular inhibitory signal that moves from meristemoids and guard mother cells to the neighboring cells, where it maintains neighboring cell identity and represses the satellite meristemoid lineage (Nadeau and Sack, 2002a; von Groll et al., 2002; Bergmann and Sack, 2007). Recent evidence suggests that this signal is a peptide encoded by EPIDERMAL PATTERNING FACTOR1 (EPF1) or STOMATAL DENSITY AND DISTRIBUTION1 (SDD1), both of which require TOO MANY MOUTHS (TMM) perception (Hara et al., 2007). The loss-of-function mutations sdd1 (Berger and Altmann, 2000), trmn (Yang and Sack, 1995), epf1 (Hare et al., 2007), and yoda (yda) (Bergmann et al., 2004) result in ectopic satellite meristemoids and the formation of characteristic clusters of stomata. We did not observe these clusters in any of our mutant lines (see Supplemental Figure 5 online). Thus, AGL16 expression is not a major factor in determining the plane of division and stomatal spacing.
The incidence of higher-order stomatal complexes depends on the number of entry divisions and subsequent asymmetric divisions that give rise to satellite meristemoids (von Groll and Altmann, 2001; Bergmann and Sack, 2007). Reduced AGL16 expression in deficient mutants markedly decreased the incidence of higher-order stomata but did not affect the incidence of primary stomata, which arise directly from the meristemoid mother cell lineage. Therefore, AGL16 is a positive regulator that functions downstream of the entry division in the satellite meristemoid lineage.

AGL16m1/2 lines, but not AGL16.1/2 lines, showed precocious, prolonged initiation of satellite meristemoids and an increased incidence of higher-order stomata, indicating that miR824, like AGL16, functions in the satellite meristemoid lineage, but as a negative regulator.

Our working hypothesis is that AGL16 acts, presumably as a transcription factor, to promote the expression of genes required for continued asymmetric divisions, satellite meristemoid identity, or both processes. miR824 localized in satellite meristemoids, and guard mother cells downregulate AGL16 mRNA to inhibit amplification divisions. An unresolved issue is the discrepancy between the localization of AGL16 mRNA in mature guard cells and its proposed function in the satellite meristemoid lineage. One possible explanation is that AGL16 mRNA produced by immature guard cells might move to the neighboring cells via plasmodesmata, as described for certain other plant mRNAs (Kim, 2005). Although mature guard cells are symptomatically isolated, histological evidence suggests that this occurs late during maturation of the guard cell wall (Wille and Lucas, 1984). Thus, high levels of AGL16 mRNA would be expected to accumulate in symplastically isolated, mature guard cells lacking miR824. According to this speculative hypothesis, in a newly forming stomatal cell lineage an extracellular signal dependent on the EPF1/TMM/YODA and/or the SDD1/TMM/YODA pathways determines the spacing and orientation of asymmetric divisions and an intercellular signal, AGL16 mRNA, acts in concert with miR824 to regulate entry and amplification. At present, we do not know how the two pathways interact functionally or where the MIR824 and AGL16 genes are transcribed. Reporter gene approaches in combination with studies of multiple mutants affecting the relevant developmental pathways are needed to test our hypothesis.

**Pleiotropic Abnormalities of AGL16m Mutants**

AGL16 mRNA (Gong et al., 2004) and miR824 are expressed in all aerial parts and roots of Arabidopsis, raising the possibility that the miR824/AGL16 pathway has pleiotropic functions in several different developmental programs. Deficiencies in AGL16 mRNA did not have detectable developmental effects other than on the satellite meristemoid lineage. This suggests that there is functional compensation for extrastomatal deficiencies, as has been reported for other members of the highly redundant MADS box
protein family (Ferrandiz et al., 2000; Liljegren et al., 2000; Pinyopich et al., 2003; Ditta et al., 2004; Gregis et al., 2006). Independent AGL16m lines consistently exhibited abnormalities in growth habit, leaf shape, and branching of trichomes, which have been reported to accumulate AGL16 mRNA (Alvarez-Buylla et al., 2000). Dramatic effects have also been reported for several other miRNA-resistant transformants obtained with Pro2x3SS-regulated constructs (reviewed in Jones-Rhoades et al., 2006). While we cannot rule out the possibility that our effects are due to the Pro2x3SS promoter, they are ultimately regulated by the miR824, since overexpression of wild-type AGL16 mRNA did not result in abnormalities. This supports the hypothesis that the miR824/AGL16 pathway has pleiotropic functions and that redundancy can compensate for AGL16 deficiencies but not for overexpression resulting from impaired, miR824-mediated regulation, which only targets AGL16 mRNA.

The miR824/AGL16 Pathway Is Conserved in the Brassicaceae

miR824 was recently described as a nonconserved miRNA originated by duplication of the founder gene AGL16 (Fahlgren et al., 2007). No miR824 orthologs could be identified in other plant species, even by lowering stringency in BLAST analysis (Rajagopalan et al., 2006). We demonstrate in our study that miR824 is conserved in the Brassicaceae but not in more distantly related species and that its only target, AGL16, is also highly conserved in the Brassicaceae. The MADS box genes Os MADS57 in rice and Pp PPM1, Pp MAD1, and Pp Phypa1_1 109598 in the moss Physcomitrella patens are targeted by miR444 (Sunkar et al., 2005) and miR538 (Axtell et al., 2007), respectively, but we were unable to detect MIR824 syntenic regions or similarity to the MiR444 and MiR538 loci in either species. MiR824, like MIR161 and MIR163 (Allen et al., 2004) and the recently described MIR778, MIR780, and MIR856 (Fahlgren et al., 2007), appears to be a rather recently evolved gene generated by the duplication of its unique AGL16 target that probably evolved ~12 to 20 million years ago, before the divergence of the Brassica and Arabidopsis lineages (Town et al., 2006). Thus, in contrast with the highly conserved miRNA families miR156/157, miR172, and miR170/171, with ancient functions in plant development (Axtell and Bartel, 2005), we believe that the miR824–AGL16 interaction is restricted to the Brassicaceae and might account for some Brassica-specific taxonomic features of stomatal organization.

METHODS

The sequences of all primers and smRNA markers are listed in Supplemental Table 1 online.

Plant Materials and Growth Conditions

Unless indicated otherwise, all Arabidopsis thaliana mutants and transgenic lines are in the Col-0 background. Seeds of wild-type ecotypes and the T-DNA insertion mutants agf16-1 (SALK_104701) and m3 (SALK_042802) were obtained from the Nottingham Arabidopsis Biological Stock Centre. Seeds of the T-DNA insertion mutant rdr6-15 (Garlic_617_H07), described by Allen et al. (2004), were obtained from the Syngenta Arabidopsis Insertion Library collection. Arabidopsis plants grown in GS90 soil and Brassica rapa subsp pekinensis, Brassica napus subsp oleifera, and Brassica oleracea var abelagabra plants grown axenically in liquid half-strength Murashige and Skoog (MS) medium were raised at 21°C (16 h of 100 μE·m⁻²·s⁻¹ light, 8 h of dark). Orzya sativa var japonica and Nicotiana benthamiana plants grown in GS90 soil were raised at 26°C (16 h of 300 μE·m⁻²·s⁻¹ light, 8 h of dark). Homozygous SALK lines were selected by growing plants axenically on half-strength MS agar containing 25 mg/L kanamycin and genotyped using the allele-specific primers F-AGL16geno, R-AGL16geno, F-M3geno, R-M3geno, and the pROK2 left border primer. The rdr6-15 mutant was genotyped using the primers F-RDR6geno, R-RDR6geno, and the left border primer L71. Transformants were produced by the floral dip method using Agrobacterium tumefaciens strain GV3101 (Clough and Bent, 1998) carrying the T-DNA binary vectors pAG16 and pAGL16m constructed as summarized in the Supplemental Methods online. pAG16 expresses the wild-type AGL16 coding sequence, and pAGL16m expresses the mutated AGL16 coding sequence shown in Figure 3A regulated by the strong, double-enhancer cauliflower mosaic virus 35S RNA promoter. Primary transformants were selected on half-strength MS agar containing 25 mg/L hygromycin, 50 mg/L kanamycin, and 100 mg/L lentinin and scored for the segregation of hygromycin resistance in the second selfed generation.

High Molecular Weight RNA Analysis

Total RNA was extracted from tissues frozen in liquid N₂ of 4-week-old plants using Trizol reagent (Invitrogen), and the poly(A)⁺ RNA fraction was purified using the PolyATtract mRNA isolation system (Promega). For RNA gel blot hybridization, gels were loaded with 20 μg of total RNA or 2 to 3 μg of poly(A)⁺ RNA. The hybridization probes were cDNAs from total RNA of Col-0 leaves amplified by RT-PCR with gene-specific primers for AGL16 (At3g57230), SCL6-III (At3g60630), TUBULIN (At5g23860), and pri-miR824.1 randomly labeled with [g-³²P]dCTP using the RadPrime labeling kit (Invitrogen). For RT-PCR, a 163-bp fragment spanning the AGL16:mirR824 pairing region was amplified with the primers F-AGL16 and R-qPCRAGL16, and the product was digested with HincII (New England Biolabs). Real-time PCR was performed on an optical 96-well plate with the ABI PRISM 7000 sequence detection system (Applied Biosystems) using SYBR Green (Applied Biosystems) to monitor double-stranded DNA synthesis. Triplicate reactions for each sample contained 12.5 μL of SYBR Green Master Mix reagent (Applied Biosystems), 10 μL of cDNA (0.25 μg/μL), and 2.5 μL of each gene-specific primer (2.5 μM) in a final volume of 25 μL. The standard thermal profile used for all PCRs was 50°C for 2 min, 95°C for 10 min, and 50 cycles of 95°C for 15 s and 60°C for 40 s. Data were analyzed using SDS 1.1 software (Applied Biosystems). AGL16 levels were normalized by amplification of the Tip41-like gene (Czechowski et al., 2005) using the comparative C₇ method (Ramakers et al., 2003). Reactions without cDNA or without reverse transcription were included as controls.

Low Molecular Weight RNA Analysis

smRNAs were cloned from the aerial parts of bolting Arabidopsis Col-0 plants by the method of Elbashir et al. (2001), except that BanII rather than EcoRI was used for concatemerization. The genomic location of smRNAs was determined by BLAST screening of the National Center for Biotechnology Information gene database. Fractionation of smRNAs and RNA gel blot hybridization were performed as described by Akbergenov et al. (2006) using γ-³²P-labeled oligonucleotide DNA probes. Unless indicated otherwise, gels were loaded with 10 μg of low molecular weight RNA. The size markers were 21+ and 24-nucleotide RNA oligonucleotides (Microsynth).
Mapping the AGL16 Cleavage Site

RLM-RACE of total RNA isolated from 4-week-old Col-0 leaves was performed using the FirstChoice RLM-RACE (Ambion) protocol. cDNA synthesis with SuperScript SS-III reverse transcriptase (Invitrogen) was primed using RLMA GL16, which is specific for the 3’ untranslated region of AGL16. The first round of PCR amplification performed with the 5’ RACE outer primer and RLMA GL16 was followed by nested PCR with the 5’ RACE inner primer and the AGL16-specific primer AGL16GS. The amplification product was gel-purified and cloned in the pCRII-TOPO vector (Invitrogen). Ten independent clones were sequenced.

Transcript Expression

The plasmids used for transient expression were pGNN35Sluc+, carrying a luciferase gene regulated by the Pro2x3SS promoter (Molinier et al., 2004), pProMIR824:Luc, carrying 2954 bp of genomic region upstream of the pri-miR824 start of transcription fused to the luciferase gene of pGNN35Sluc+, and pGNLUC, carrying a promoterless luciferase/35S terminator cassette. Their relevant features and construction are described in the Supplemental Methods online. The plasmids were delivered biologically into leaves of plants at 3 weeks after germination as described by Klehr and Meins (2004). Images were made at 48 h after bombardment using a N2-cooled CCD camera (Gloor Instruments) as described by Fritsch et al. (2004).

In Situ Hybridization

For in situ hybridization of AGL16 mRNA, whole mounts of the third true leaf of B. rapa at 1 week after germination were prepared and hybridized with digoxigenin-labeled probes as described by Friml et al. (2003). The probes used were sense and antisense RNAs complementary to the region between nucleotides 469 and 753 of the AGL16 transcript. For miR824 and miR824*, the hybridization procedure of Bhattacharyya et al. (2006) with digoxigenin-labeled miR CURY LNA probes (Exiqon) was used. Hybridization signals were detected using the nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate staining procedure (Roche Biochemicals).

Microscopy

Samples were prepared for scanning electron microscopy using a Balzers SCU 020 cryopreparation unit. Images were collected with a JEOL JSM 6300 scanning electron microscope at 15 kV. Stomatal development was monitored using the dental resin impression method described by Berger and Altman (2000) and Geisler et al. (2000). Images of nail polish copies of dental resin imprints were collected using a Kappa CF 8/5 camera and a Zeiss Axioplan 2 microscope. The time course of stomatal development was determined from tracings made of images of the same stomatal complexes obtained with the first true leaves of 6- to 10-d-old seedlings grown axenically on half-strength MS agar. Stomatal density, stomatal index, and the proportion of primary and higher-order stomatal complexes were quantified from images of the surfaces of the fifth rosette leaves of plants at 4 weeks after germination. Counts were made of five different 1-mm² regions of three to five biological replicates. Comparable regions of epidermis were scored for each type of plant in the same experiment.

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: AGL16 (At4g24415), MIR824 (At4g24415), pri-miR824,1 (AF389288), pri-miR824,2 (AK226543), SCL6-III, (At3g60630), S-TUBULIN, (At4g23860), and TIP41-like (At4g34270).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. Expression of miR824 and AGL16 mRNA in Arabidopsis miRNA Mutants and Brassica Species.

Supplemental Figure 2. Genomic Locations of MIR824 ESTs and the T-DNA Insertion in line m3.

Supplemental Figure 3. Partial Characterization of the Arabidopsis AGL16 Insertion Mutant agl16-1.

Supplemental Figure 4. Phenotype of Transgenic Arabidopsis Plants Altered in AGL16 mRNA Expression.

Supplemental Figure 5. Positioning of Stomatal Complexes on the Abaxial Epidermis of Transgenic Arabidopsis Plants Altered in AGL16 mRNA Expression.

Supplemental Table 1. Sequences of Oligonucleotides Used in This Study.


ACKNOWLEDGMENTS

We thank Stephen Schauer for dcl1-8 (sin1-2), Xuemei Chen for hen1-1, Nina Fedoroff for hyf1-1, Thomas Hohn for dcl4-2, and Syngenta for rice and Brassica seeds. We thank Daniel Mathys at the Basel University Microscopy Center for expert scanning electron microscopy. We are grateful to Thomas Tuschi and Jutta Meyer for instruction in smRNA cloning, to Andreas Gisela for help with in silico analysis of the clones, and to Olivier Fritsch for assistance with luciferase assays. We also thank our colleagues Thomas Boller, Helge Grosshans, and Franck Vazquez for their critical comments and the Novartis Research Foundation for financial support.

Received January 12, 2007; revised July 26, 2007; accepted July 27, 2007; published August 17, 2007.

REFERENCES


Correction


In Figure 1A, two nucleotides in the stem loop hairpin of B. rapa/B. napus were inadvertently mislabeled. Position 22 is the first nucleotide (U) of miR824, indicated in red, and position 588 is the first nucleotide (C) of miR824*, indicated in blue.

On page 2424, line 6, paragraph 2, of the section “The miR824/AGL16 Pathway Functions in the Satellite Meristemoid Lineage” should read as follows: Recent evidence suggests that this signal, which requires TOO MANY MOUTHS (TMM) for its perception, is a peptide encoded by EPIDERMAL PATTERNING FACTOR1 (EPF1) or is processed by STOMATAL DENSITY AND DISTRIBUTION1 (SDD1) (Hara et al., 2007).
MicroRNA-Mediated Regulation of Stomatal Development in Arabidopsis
Claudia Kutter, Hanspeter Schöb, Michael Stadler, Frederick Meins, Jr. and Azeddine Si-Ammour
Plant Cell 2007;19;2417-2429; originally published online August 17, 2007;
DOI 10.1105/tpc.107.050377

This information is current as of July 8, 2017

Supplemental Data /content/suppl/2007/08/17/tpc.107.050377.DC1.html
References This article cites 58 articles, 29 of which can be accessed free at:
/content/19/8/2417.full.html#ref-list-1
eTOCs Sign up for eTOCs at:
http://www.plantcell.org/cgi/alerts/ctmain
CiteTrack Alerts Sign up for CiteTrack Alerts at:
http://www.plantcell.org/cgi/alerts/ctmain
Subscription Information Subscription Information for The Plant Cell and Plant Physiology is available at:
http://www.aspb.org/publications/subscriptions.cfm
Errata An erratum has been published regarding this article. It is appended to this PDF and can also be accessed at:
/content/19/11/3831.full.pdf

© American Society of Plant Biologists
ADVANCING THE SCIENCE OF PLANT BIOLOGY