Interaction between Two Timing MicroRNAs Controls Trichome Distribution in Arabidopsis

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Abstract

The miR156-targeted SQUARE PROMOTER BINDING PROTEIN LIKE (SPL) transcription factors function as an endogenous age cue in regulating plant phase transition and phase-dependent morphogenesis, but the control of SPL output remains poorly understood. In Arabidopsis thaliana the spatial pattern of trichome is a hallmark of phase transition and governed by SPLs. Here, by dissecting the regulatory network controlling trichome formation on stem, we show that the miR171-targeted LOST MERISTEMS 1 (LOM1), LOM2 and LOM3, encoding GRAS family members previously known to maintain meristem cell polarity, are involved in regulating the SPL activity. Reduced LOM abundance by overexpression of miR171 led to decreased trichome density on stems and floral organs, and conversely, constitutive expression of the miR171-resistant LOM (rLOM) genes promoted trichome production, indicating that LOMs enhance trichome initiation at reproductive stage. Genetic analysis demonstrated LOMs shaping trichome distribution is dependent on SPLs, which positively regulate trichome repressor genes TRICHOMELESS 1 (TCL1) and TRIPTYCHON (TRY). Physical interaction between the N-terminus of LOMs and SPLs underpins the repression of SPL activity. Importantly, other growth and developmental events, such as flowering, are also modulated by LOM-SPL interaction, indicating a broad effect of the LOM-SPL interplay. Furthermore, we provide evidence that MIR171 gene expression is regulated by its targeted LOMs, forming a homeostatic feedback loop. Our data uncover an antagonistic interplay between the two timing miRNAs in controlling plant growth, phase transition and morphogenesis through direct interaction of their targets.

Introduction

MicroRNA (miRNA) was first identified as the regulator of the juvenile-to-adult transition in Caenorhabditis elegans [1], and a similar function was later assigned to plant miRNA: miR156 and its target SQUARE PROMOTER BINDING PROTEIN LIKE (SPL) genes define an endogenous aging and flowering pathway [2,3]. The miR156 overexpression delays phase transition and startup of flowering. Similarly, accumulation of SPLs accelerates aging [2–4]. In Arabidopsis there are 17 SPL genes, 11 of which contain a miR156 target site. The miR156-targeted SPLs can be divided into four clades based on their protein structures, and the clades composed of SPL5/4/5 and SPL9/15 both promote phase transition [2–4]. Acting downstream of miR156-targeted SPLs, miR172 also plays roles in developmental timing of Arabidopsis [3]. In addition, there are other transcription regulators that affect SPL activities, such as DELLAs, the negative regulator of gibberellin signaling pathway, interact with SPLs to control flowering [5].

Trichomes are specialized epidermal cells, acting as barriers to protect plants from herbivores, UV irradiation, and excessive transpiration. In Arabidopsis, trichome distribution is spatially and temporally regulated, and the distribution pattern serves as a trait to distinguish between juvenile and adult leaves [6,7]. During the early vegetative phase, trichomes are evenly distributed on the adaxial side of rosette leaves. Plants start transition from juvenile to adult phase when trichomes initiate on leaf abaxial side [7]. After entering into the reproductive stage, the number of trichomes is gradually reduced along the inflorescence stems. Floral organs are nearly glabrous except for a few trichomes on the abaxial surface of sepals. Genetic screens of Arabidopsis mutants have identified sets of regulators governing trichome formation. In brief, the trichome initiation complex of Arabidopsis comprises a WD40 protein TRANSPARENT TESTA GLABRA1 (TTG1), an R2R3 MYB protein GLABRA1 (GL1), and a basic helix-loop-helix transcription factor GL3 or its homolog, ENHANCER of GL3 (EGL3) [8–10]. This ternary complex initiates trichome cell development by activating GL2, which encodes a homeodomain leucine zipper transcription factor [11,12]. A group of single-repeat R3 MYB factors, including TRIPTYCHON (TRY) [13], CAPRICE (CPC) [14,15], ENHANCER OF TRY AND CPC1 (ETC1) [16], ETC2 [17], ETC3 [18], TRICHOMELESS1 (TCL1) [19] and TCL2 (20,21), redundantly suppress trichome initiation by competing with GL1 for the binding site of GL3 to prevent the active complex formation [12]. In addition to these specific negative

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HAIRY MERISTEM (HAM) from the first functionally characterized member of this subclade family. There are three LOM genes in transcription factors belonging to the GRAS-domain containing pathways. Marketing the regulatory network controlling trichome formation on stem, we show that a group of GRAS family members, LOST MERISTEMS 1 (LOM1), LOM2 and LOM3, targeted by timing miR171, function in modulating the SPL activity through direct protein-protein interaction. LOMs promote trichome formation through attenuating the SPL (such as SPL9) activity of trichome repression. The LOM-SPL interaction affects many aspects of plant growth and development, including flowering, aging and chlorophyll biosynthesis. Interestingly, miR171A gene expression is regulated by its own targets (LOMs), forming a feedback loop to program plant life. Our study establishes an age-dependent regulatory network composed of two timing miRNAs which act oppositely through direct interaction of their target proteins.

Author Summary

MicroRNAs are important aging regulators in many organisms. In Arabidopsis the miR156-targeted SQUAMOSA PROMOTER BINDING PROTEIN LIKE (SPL) transcription factors play important roles as an endogenous age cue in programming phase transition and phase-modulated morphogenesis, including trichome patterning. However, how the timely increasing SPL output is modulated remains elusive. By dissecting the regulatory network controlling trichome formation on stem, we show that a group of GRAS family members, LOST MERISTEMS 1 (LOM1), LOM2 and LOM3, targeted by timing miR171, function in modulating the SPL activity through direct protein-protein interaction. LOMs promote trichome formation through attenuating the SPL (such as SPL9) activity of trichome repression. The LOM-SPL interaction affects many aspects of plant growth and development, including flowering, aging and chlorophyll biosynthesis. Interestingly, miR171A gene expression is regulated by its own targets (LOMs), forming a feedback loop to program plant life. Our study establishes an age-dependent regulatory network composed of two timing miRNAs which act oppositely through direct interaction of their target proteins.

Factors, hormone signaling components also affect the trichome regulatory complex [22].

We previously reported that the miR156-targeted SPLa temporally repress trichome distribution on stem and inflorescence through activating TCL1 and TRY [23]. Plants overexpressing miR156 developed ectopic trichomes on the stem and floral organs, whereas plants with elevated SPLa produced fewer trichomes after bolting [23]. Since miR156-SPLa define a major endogenous age cue, this provides a straightforward mechanism that connects plant phase transition with trichome development. Due to the easiness of observation, trichome formation on stem is an ideal system to investigate the interaction of miR156-SPL module with other developmental or environmental signaling pathways.

LOST MERISTEMS (LOMs), also known as AtHAMS because the first functionally characterized member of this subclade is HAIRY MERISTEM (HAM) from Petunia hybrid [24], are transcription factors belonging to the GRAS-domain containing family. There are three LOM genes in Arabidopsis, LOM1/2/3 (also known as AtHAM1/2/3 [25] or SCL6-2/3/4 [26]), which are targets of miR171 [27,28]. Like other GRAS members, LOMs contain a highly conserved GRAS domain on C-terminus and a variable N-terminus. LOM1 and LOM2 are ≈65% identical at amino acid sequence level, and LOM3 has a shorter N-terminal domain. Previous studies revealed that LOMs function in diverse processes such as meristem maintenance, shoot branching, chlorophyll biosynthesis and root growth [25-27,29]. Here, we report that miR171-targeted LOMs functionally interfere with selected SPLs through protein-protein interaction.

Results

miR171-LOM regulates trichome distribution

In Arabidopsis genome, there are four miR171 coding genes, MIR171A, B, C and MIR170. We found that overexpression (OE) of MIR171A/B/C under the 35S promoter reduced trichomes on stem very significantly (Figure 1A and 1B), which was not reported previously. To see if the reduction of trichomes was caused by decreased level of LOMs due to miR171 accumulation, we first checked the abundance of mature miR171 and the transcript levels of LOMs in these transgenic plants. Indeed, miR171 was over-accumulated (Figure 1C) and LOM expression declined drastically (Figure 1D). In addition, miR171 overexpression resulted in phenotypic changes similar to those of lom1 lom2 lom3 triple mutant (termed lom1 hereinafter) as reported [26]: the narrowed rosette leaves and the higher chlorophyll content (Figure 1E-1H). We then analyzed the effects of LOM over-accumulation by examining the 35S:LUC::LOM1 plants, in which a miRNA-resistant LOM1 (rLOM1) was fused to firefly luciferase (LUC) gene and expressed constitutively. In addition to yellow-green leaves (Figure 1I) as reported [26], the 35S:LUC::LOM1 plants produced supernumerary trichomes on stems, inflorescences and pedicels compared to the wild-type and lom1 mutant plants (Figure 1K-1N), indicating that LOM1 accumulation induces ectopic trichomes after bolting.

To further examine the role of LOMs in trichome production, we analyzed transgenic Arabidopsis expressing Myc-tagged rLOM1, 2 and 3, under the 35S or their native promoters, respectively. LOM1::Myc::LOM1 and LOM2::Myc::LOM2 plants produced supernumerary or ectopic trichomes on stems, inflorescences, and even on pedicels that are glabrous in wild-type plants (Figure 2A-2C); and 35S::Myc::LOM1/2/3 plants exhibited a similar phenotype as that of 35S::LUC::LOM1 with higher but varied degrees of trichome enrichment (Figure 2D-2F). To exclude the effect of Myc tag on LOM activities, we generated 35S::LOM1 transgenic Arabidopsis, which exhibited the same phenotypic change as 35S::Myc::LOM1 and 35S::LUC::LOM1. Clearly, the three miR171-regulated LOMs have the ability to promote trichome initiation at flowering stage.

Stem trichome repressors act at downstream of miR171-LOMs

Based on the Arabidopsis trichome model [12], ectopic trichomes could be triggered by either the increase of trichome promoting factors or the reduction of single-repeat R3 MYB repressors. For example, gibberellin and cytokinin promote trichome formation by activating the expression of C2H2 transcription factor genes GL1, GIS2 and ZFP8, which successively promote the transcription of GL1 and GL3 [30–32]. However, analysis of the lom1 and the 35S:LUC::LOM1 plants by quantitative RT-PCR (qRT-PCR) did not show an evident change of transcript levels of GIS, GIS2, ZFP8 and GL3 (Figure S1A). We then examined the expression of the R3 MYB repressor genes and found that transcript changes of CPC, ETC1 and TCL2 were uncoupled from trichome production in the lom1 and the 35S:LUC::LOM1 plants (Figure S1B and S1C), whereas the mRNA levels of TRY and TCL1 matched the phenotypes (Figures 2G and 2H). Indeed, among these single MYB-domain factors, TRY and TCL1 are the major negative regulators of stem and inflorescence trichomes [13,19,23]. TRY expression in the main stems was up-regulated in lom1 and repressed in 35S:LUC::LOM1 plants (Figure 2G). And similarly, TCL1 expression was also down-regulated in 35S:LUC::LOM1 inflorescences (Figure 2H), consistent with the ectopic trichomes on inflorescence stems and pedicels. These data indicate that transcriptional repression of TRY and TCL1 occurred in LOM-overexpressors that produced ectopic trichomes.

To dissect genetic interaction between LOMs and the trichome repressor genes, we crossed 35S:LUC::LOM1 to 35S::TRY and 35S::TCL1 plants, respectively. Overexpression of LOM1 failed to induce trichome production as both hybrid progeny exhibited the glabrous phenotype like 35S::TRY or 35S::TCL1 (Figure 2I). On the other hand, down-regulation of LOMs in try tcl1 double mutant by MIR171B overexpression did not reduce trichome production.
Figure 1. MiR171-LOMs regulate trichome initiation. (A) Trichome distribution on main stem of wild-type (Col-0) and MiR171OE (35S::MiR171A/B/C) plants; MiR171 overexpression decreased trichome density. Scale bar represents 5 mm. (B) Overexpression of MiR171 reduced the total number of trichomes on stems. Trichome numbers on main stems (0 to 5 cm from bottom) were counted and data were given as mean ± s.d. (n = 18) and analyzed by t test. **P < 0.01. (C) RNA blot showing high accumulation of mature miR171 in 10-day-old MiR171OE plants. U6 was used as an internal reference. (D) qRT-PCR showing repressed expression of LOM1, LOM2 and LOM3 in MiR171OE plants. Expression level in the wild-type was set to 1. Errors bars indicate s.d. (n = 3). Three biological replicates were analyzed, with consistent results. (E–J) View of 15-day-old plants of indicated genotypes. Overexpression of MiR171 resulted in dark-green and narrower leaves. MiR171OE transgenic plants showed similar phenotypes as lomt (lom1 lom2 lom3) mutant (I). On the contrary, 35S::LUC-LOM1 transgenic plants (J) showed yellow-green leaves. Scale bar in (E) represents 1 cm for (E–J). (K–M) Spatial distribution of trichomes on main stem of wild-type, lomt and 35S::LUC-LOM1 plants. Arrows in (M) indicate the ectopic trichomes on the main inflorescence stems and pedicels. Scale bar in (K) represents 1 cm for (K–M). (N) Trichome density on main stem of wild-type, lomt and 35S::LUC-LOM1 plants. The Int.1 to Int.3 represents the main stem internodes from bottom to top. The y axis indicates trichome number per centimeter of each internode. Data are given as mean ± s.d. (n > 16) and analyzed by t test. **P < 0.01.

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as it worked in wild-type background (Figure 2J). These data indicate that miR171-LOMs act upstream of TRY and TCL1 in promoting trichome formation. We also introduced 35S::LUC-rLOM1 into gl1 and gl3 egl3 backgrounds respectively, and the resultant plants barely developed trichomes (Figure 2K), demonstrating that, whether promoted by LOMs or not, trichome initiation in Arabidopsis requires the GL1-GL3/EGL3-TTG1 ternary complex.

LOMs attenuate SPL activities of trichome repression

Our previous report showed that SPLs repress stem and inflorescence trichome production through activating TCL1 and TRY gene expression [23]. Because up-regulation of LOMs (35S::LUC-rLOM1) and down-regulation of SPLs (35S::MIR156F) both reduced TCL1 and TRY expression and triggered ectopic trichomes, we wondered if miR171-LOMs induced trichome formation through affecting the miR156-targeted SPLs. To test this hypothesis, we crossed 35S::MIR171B to 35S::MIR156F plants. Although 35S::MIR171B repressed trichome formation on stem in wild-type plants, it did not change the ectopic trichome distribution induced by 35S::MIR156F (Figure 3A–3D), suggesting a requirement of miR156-targeted SPLs in miR171-mediated trichome suppression. Correspondently, mutation of LOM genes caused a further trichome reduction in 35S::MIR156 plants (Figure 3E and 3F), suggesting an enhancement of SPL activity in the absence of LOMs. High level of LOM1 (LOM1::Myc-rLOM1) stimulated while high level of SPL9 (SPL9::GFP-rSPL9) repressed stem trichome formation (Figure 3G and 3H). The plants expressing both (LOM1::Myc-rLOM1 × SPL9::GFP-rSPL9) showed an intermediate phenotype in terms of stem trichomes, though less

Figure 2. Elevated LOMs trigger ectopic trichomes and down-regulate TRY and TCL1. (A–F) Trichomes on main stems and inflorescences of different plants as indicated. The insertion panel in the top right corner in (A–C) represents magnified image of the area marked by square; note that ectopic trichomes appeared on inflorescence stems and pedicels in (B) and (C). Scale bar shared in (A–F), 1 cm. (G) Relative expression level of the trichome repressor gene TRY in main stem of wild-type, lomt and LUC-rLOM1OE plants. The Int.1 to Int.3 represent the stem internodes from bottom to top. Expression level in Int.1 of wild-type was set to 1. Error bars indicate s.d. (n = 3). (H) Relative expression level of TCL1 in inflorescences of wild-type, lomt and LUC-rLOM1OE plants. Errors bars indicate s.d. (n = 3). For (G) and (H) three biological replicates were analyzed with similar results. (I-K) Trichome distribution on main stems (Int.1) of different genetic background plants as indicated. Note that TCL1 and TRY are epistatic to LOM1, as LUC-rLOM1OE did not induce trichome production in TCL1OE and TRYOE backgrounds (I). MIR171OE did not reduce trichome density in try tcl1 double mutant (J). 35S::LUC-rLOM1 failed to rescue the glabrous phenotype of gl1 and gl3 egl3 mutants (K). Scale bars in (I–K) represent 1 cm.

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than the wild-type (Figure 3G and 3H). Together, these data suggest an antagonistic effect of LOMs on the SPLs. Although the intermediate phenotype mentioned above, at this stage it does not rule out the possibility of independent effects of these proteins on common downstream targets, such as TRY and TCL1.

LOMs interact with SPLs

In order to further elucidate the correlation between LOMs and SPLs in trichome regulation, we analyzed TCL1 promoter activities in plants of different backgrounds. GUS activity conferred by TCL1::GUS was decreased in 35S::LUC-rLOM1 and increased in 35S::MIR156F plants (Figure 4A–4C), consistent with qRT-PCR results (Figure 2H). The increase of GUS activity in 35S::MIR156F was abolished when the mutant promoters of TCL1mut3::GUS and TCL1mut4::GUS (see [23]) were used, in which the SPL binding sites in TCL1 promoter were disrupted (Figures 4D–4G). Clearly, the SPL binding motifs were involved in regulation of TCL1 by LOMs, further supporting that LOMs modulate trichome formation via SPLs. 35S::LUC-rLOM1 and 35S::MIR156F plants exhibited other phenotypic similarities in addition to ectopic trichome production, such as short plastochron, delayed flowering time, yellow-green leaves, enhanced shoot branching and limp stem (see [26] and Figure S2), suggesting that the antagonistic effects between LOMs and SPLs could be general rather than limited to trichome regulation. It was reported that DELLAs physically interact with SPLs and gibberellin promotes flowering partially through releasing this interaction [5]. Since both LOM and DELLA proteins belong to two close subclades of the GRAS family [33], we wondered if LOMs also interacted with SPLs. We first examined the subcellular

Figure 3. LOMs and SPLs regulate trichome formation antagonistically. (A–C) Trichome distribution on stems and inflorescences of 35S::MIR171B (A), 35S::MIR156F (B) and the progeny of 35S::MIR171B × 35S::MIR156F plants (C). Bars = 5 mm in (A–C). (D) Number of main inflorescence stem internodes with trichomes, counting from the first pedicel. Data are given as mean s.d. (n = 14). (E) Trichomes on stems (Int.1) of the indicated genotypes; note that trichome production was further reduced in 35S::MIM156 lomt plants. (F) Trichome numbers of plants in (E). The y axis indicates trichome number on main stem (0 to 5 cm from the bottom). Data are given as mean s.d. (n = 20); α represents P values (t-test) <0.01 relative to wild-type, and β represents P values (t-test) <0.01 relative to lomt and MIM156, respectively. Note that the trichome number of MIM156 lomt was very significantly less than that in MIM156 or lomt plants. (G) Trichomes on the stems (Int.1) of the indicated genotypes. SPL9::GFP-rSPL9 transgenic plants were almost glabrous, and the LOM1::Myc-rLOM1 plants produced more trichomes than the wild-type. (H) Trichome numbers (0 to 5 cm from the basal of main stems) of plants in (G). Data are given as mean s.d. (n = 20). α represents P values (t-test) <0.01 relative to wild-type, and β represents P values (t-test) <0.01 relative to Myc-rLOM1 and GFP-rSPL9, respectively. doi:10.1371/journal.pgen.1004266.g003
localization of SPL9 and LOM1. **35S::GFP-rSPL9** and **35S::mCherry-rLOM1** were transiently expressed in *Nicotiana benthamiana* leaves, and the fusion proteins of GFP-SPL9 and mCherry-LOM1 were found co-localized in speckles in the nucleus (Figure 4H). We then performed a biomolecular fluorescence complementation (BiFC) assay by fusing SPL9 to the C-terminal half of LUC (cLUC-rSPL9) and LOMs to the N-terminal half (LOMs-nLUC). A strong LUC activity was detected in leaves co-infiltrated with the respective chimerical constructs (Figure 4I). The interaction between SPL9 and LOMs was further confirmed in a yeast two-hybrid assay (Figure 4J). Domain deletion revealed that the N-terminal domain of LOM1 was responsible for its interaction with SPL9 (Figure 4K). In addition, LOMs were able to bind to SPL2 (Figure S3A), which is remarkably also involved in trichome development [34]. Taken together, these results demonstrate that the miR171-targeted LOMs physically interact with the miR156-targeted SPL9 and SPL2 and may result in attenuation of the SPL function such as regulating trichome patterning. Based on the facts that miR156 and miR171 are conserved in plant kingdom and excessive miRNAs cause opposite phenotypes, the interaction between the two miRNA targets coordinate many developmental and morphogenesis events beyond trichome formation.

**LOMs repress SPL activities of gene regulation**

To substantiate the potentially wide implication of LOM-SPL interaction, we examined the effect of LOMs on the flowering pathway. In long-day conditions, LOM1 overexpression delayed flowering time (Figure 5A and Figure S3B–S3E) and consistently down-regulated the expression of the MADS-box gene, **SUPPRESSOR OF OVEREXPRESSATION OF CONSTANS 1 (SOC1)**, which was under the direct control of miR156-targeted SPLs [2]; by contrast, **SOC1** was up-regulated in *lomt* mutant along with earlier flowering (Figure 5A and 5B). As the N-terminal domain of LOM1 (nLOM1) was responsible for interaction with SPL9 (Figure 4K), we overexpressed nLOM1 fused to the nuclear localization signal (NLS). The **35S::nLOM1-NLS-YFP** plants showed normal development (Figures 5C and 5D) except for late flowering and more trichomes on stems (Figures 5E–5G). A drastic drop of the **SOC1** transcript levels (Figure 5F) was accompanied by severe delay of flowering (Figure 5E) in **35S::nLOM1-NLS-YFP** plants. It is the same to **rLOM1OE** plants that nLOM1-NLS-YFP triggered more stem

**Figure 4. LOMs repress SPL function through direct protein-protein interaction.** (A–C) **TCL1::GUS** reporter in indicated backgrounds. GUS activity was increased in **MIR171BOE** (B) and decreased in **LUC-rLOM1OE** (C) compared to wild-type (A) inflorescences. (D–G) The increase of GUS activity in **MIR171BOE** plants was abolished when the SPL binding sites in **TCL1** promoter was mutated (see [23]). **MIR171BOE** did not increase the activity of **TCL1mu3::GUS** (E) and **TCL1mu4::GUS** (G), compared to that shown in (D) and (F), respectively. Scale bar in (A) represents 1 cm for (A–G). (H) **35S::GFP-rSPL9** and **35S::mCherry-rLOM1** were transiently expressed in *N. benthamiana* leaves and the fluorescence merged in nucleus. Scale bar, 10 μm. (I) BiFC assay of the interaction between LOMs and SPL9. **rLOMs-nLUC** or **nLUC** was transiently co-expressed with **cLUC-rSPL9** or **cLUC** in *N. benthamiana* leaves; only the combination of **rLOMs-nLUC** and **cLUC-rSPL9** triggered the luciferase activity when luciferin was infiltrated. Scale bar, 1 cm. (J) Yeast two-hybrid assay of protein-protein interaction. **SPL9-AD** combined with **LOM1/2/3-AD** conferred yeast growth in -Leu-Trp-His medium. (K) **SPL9-AD** interacts with the N-terminal (1–250) of LOM1 (nLOM1) in yeast two-hybrid system.

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trichomes (Figure 5G). However, remarkable flowering delay and ectopic trichomes were not observed in 35S::nLOM1-YFP plants, in which the NLS was removed and the nLOM1-YFP signal was diffused in cytoplasm (Figure 5H). Because nLOM1 does not contain the DNA binding domain, these data further support that it is the LOM-SPL interaction that caused, or at least contributed to, the flowering delay and trichome increase.

Figure 5. Overexpression of N-terminal of LOM1 in nucleus triggers a flowering delay. (A) The number of leaves at flowering of wild-type, lomt mutant and LUC::LOM1OE plants under long-day condition. Data are given as mean s.d. (n=61), and analyzed by t test. **P<0.01. (B) qRT-PCR analysis of expression of SOC1 in 7-day-old long-day-grown seedlings. SOC1 was repressed in 35S::MIR156F and increased in 35S::MIM156 as reported [2]. (C and D) View of 10-day-old wild-type (C) and 35S::nLOM1-NLS-YFP (D) plants. 35S::nLOM1-NLS-YFP plants look normal. Scale bars, 1 cm. (E) 35S::nLOM1-NLS-YFP delayed flowering very significantly under long-day condition. The number of days to flowering was counted when the floral buds were visible. Data are given as mean s.d. (n=18) and analyzed by t test, **P<0.01. (F) Expression of SOC1. SOC1 was down-regulated in 35S::nLOM1-NLS-YFP plants, consistent with delayed flowering. (G) Trichome numbers of wild-type and 35S::nLOM1-NLS-YFP plants. The y axis indicates the total trichome number on main stems. Data are given as mean s.d. (n=32) and analyzed by t test, **P<0.01. (H) Subcellular localization of nLOM1-YFP and nLOM1-NLS-YFP in leaf trichome (left panel) and leaf vein (right panel). Scale bars, 100 μm.

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We noted that, among the three LOMs, LOM3 has a shorter and more diversified N-terminal region (Figure S4). Interestingly, in comparison with 35S::Myc::LOM1 and 35S::Myc::LOM2 plants, overexpression of nLOM3 only led to a mild increase of trichome production (Figure 2F) and less serious developmental abnormalities. This result again points to the importance of the N-terminal domains of LOM proteins.
LOMs induce miR171 accumulation

To see whether LOMs affected the SPL output through mediating miR156 accumulation, we compared the miR156 amount in wild-type, lomt and 35S::LUC-rLOM1 plants by RNA blots. Under long-day conditions, the miR156 accumulation was similar in 10-day-old seedlings (Figure 6A). Although slightly higher in 20-day-old 35S::LUC-rLOM1 plants, the miR156 level was not altered in the stem of lomt or LUC-rLOM1OE plants (Figure 6B), neither was SPL9 expression (Figure S5). Thus it is unlikely that LOMs have a significant effect on miR156 abundance.

Interestingly, the miR171 level was clearly increased in 35S::LUC-rLOM1 but decreased in lomt mutant plants (Figure 6A), and overexpression of LOM2 or LOM3 also promoted miR171 accumulation (Figure 6C). qRT-PCR showed that the MIR171A transcript was much higher (up to 3-fold) in 35S::LUC-rLOM1 but lower in lomt mutant than in wild-type plants, whereas the expression of MIR171B and C showed a less degree response to LOM1 (Figure 6D). LOM2 and LOM3 expression was slightly reduced in 35S::LUC-rLOM1 plants (Figure 6E), possibly due to the elevated miR171 levels. We also generated the MIR171A/B/C::GUS reporter lines and crossed them to 35S::LUC-rLOM1 and 35S::MIR171B plants, respectively. Compared to wild-type, GUS activities of MIR171A::GUS and MIR171B::GUS were enhanced in 35S::LUC-rLOM1 and weakened in 35S::MIR171B plants, respectively (Figure 6F).

To investigate if MIR171 genes were directly regulated by their targets, we used an inducible system to test the activity of LOM1 in activating MIR171 expression. The 10-day-old 35S::rLOM1-GR lomt seedlings were sprayed with 10 mM dexamethasone (DEX) to allow the translocation of LOM1-GR fusion protein into the nucleus. An obvious transcript increase of MIR171A and, to a less extent, of MIR171B was observed after 4 hours, whereas MIR171C was not induced during this period (Figure 7A). Furthermore, in transient assays using N. benthamiana leaves, the level of luciferase activity controlled by the MIR171A promoter was elevated significantly when LOM1 was co-expressed, while the MIR171B and MIR171C promoters exhibited a weak or marginal response (Figure 7B). To identify the LOM1 binding regions, we dissected the truncated promoters of the three MIR171 genes (Figure 7C). Successive deletions from the 5'-end revealed a 143-bp promoter fragment of MIR171A (~201 to

**Figure 6.** LOMs regulate MIR171 expression. (A) RNA blot analysis of mature miR171 and miR156 in wild-type, lomt and LUC-rLOM1OE plants. Seedlings (10-day-old) and rosette leaves (20-day-old plants) were harvested for total RNA isolation. (B) RNA blot analysis of mature miR171 and miR156 in stem (Int.2 from the bottom) of 35-day-old plants. (C) RNA blots of miR171 in 10-day-old LOM overexpression plants; 35S::Myc-rLOM1/2/3 promoted miR171 accumulation. (D) qRT-PCR analysis of the pri-miRNA transcripts of MIR171A, B and C in 10-day-old plants of indicated genotypes. All three pri-miRNA transcripts were up-regulated in LUC-rLOM1OE and reduced in lomt mutant plants. Error bars indicate s.d. (n = 3). Three biological replicates were analyzed, with similar results. (E) Expression of three LOM genes in 10-day-old plants of indicated genotypes. LOM2 and LOM3 were slightly reduced in LUC-rLOM1OE plants probably due to elevated miR171 accumulation. Error bars indicate s.d. (n = 3). Three biological replicates were analyzed, with similar results. (F) GUS staining of MIR171A/B/C::GUS in different backgrounds. The GUS activity was stronger in LUC-rLOM1OE and weaker in MIR171BOE plants than that in wild-type.

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which conferred the LOM1 induction (Figure 7D and Figure S6). However, the response of MIR171B and MIR171C promoters to LOM1 was negligible in N. benthamiana leaves (Figure 7E, 7F and Figure S6). Finally, we performed a chromatin immunoprecipitation (ChIP) assay using the 10-day-old 3SS:Myc-rLOM1 plants, which showed LOM1 bound strongly to A4 region (Figure 7G), corresponding to the fragments identified in promoter deletion assays (Figure 7D and Figure S6). LOM1 weakly bound to promoters of MIR171B and MIR171C directly (Figure 7H and 7I). Together, these data reveal a regulatory feedback loop between LOMs and MIR171 genes, particularly MIR171A (Figure 8), which explains the seemingly contradictory phenomenon that miR171 and its target LOMs show similar expression patterns and both are mounting to a high level in inflorescence (Figure S7).

Discussion

By dissecting molecular mechanisms that control Arabidopsis trichome distribution, we found that the miR171-targeted LOMs directly interact with the miR156-targeted SPL9 and SPL2, leading to inhibition of the SPL activities. Remarkably, miR156 and miR171 function antagonistically in regulating many aspects of plant growth and development, including but far beyond the age-dependent trichome formation. Because both miR156 and miR171 are timing regulators, the interaction between their targets shed a new light on the endogenous network of plant aging. A similar but mechanistically distinct scenario has been elucidated in C. elegans, where lin-4 and miR-239 oppositely regulate lifespan by common downstream genes [35,36]. Except promoting the expression of miR172, SPLs positively regulate miR156 expression as well [3]. However, the miR171-LOM module is different from the miR156-SPL. The miR156 and its targets, such as SPL9, show reverse expression patterns [2–4], whereas miR171 and LOMs have a congruous temporal expression pattern (Figure S7). The level of LOMs elevates with age, leading to progressively activation of MIR171 genes, which in turn keep the LOM transcripts under the fine control. This regulatory feedback loop ensures the homeostasis of miR171 and miR156.

Figure 7. LOM1 directly binds MIR171A promoter. (A) Expression of MIR171A, B and C in rosette leaves of 10-day-old 3SS:rLOM1-GR lomt seedlings after a 4-h DEX or a mock treatment. Error bars indicate s.d. of three technical replicates, and the results were consistent in three biological replicates. (B) Relative reporter activities showing the induction of MIR171 transcription by LOM1. N. benthamiana leaves were used for transient expression of the reporter (MIR171A/B/C::LUC) in combination with the effector (empty vector or 3SS::rLOM1). The relative LUC activities normalized to the REN activity are shown (LUC/REN, n = 3). (C) Schematic diagram of MIR171A (top), MIR171B (middle) and MIR171C (bottom) upstream genomic regions. Black box represents stem-loop of the pri-miR171; P1–P4 represent the start sites of truncated promoters used in dual-LUC assays in (D–F); A1–A4, B1–B3 and C1–C3 represent the DNA amplicons for ChIP assays in (G–I). (D–F) Relative activities of truncated promoters of MIR171A (D), MIR171B (E) and MIR171C (F) in response to LOM1 induction. (G–I) ChIP enrichment of MIR171 promoter regions bound by Myc-LOM1. DNA fragments, isolated from rosette leaves of 10-day-old 3SS:Myc-LOM1 and wild-type plants, were quantitatively analyzed by PCR, and β-TUBULIN-2 promoter was used as a reference. Error bars indicate s.d. of three quantitative PCR replicates. doi:10.1371/journal.pgen.1004266.g007
its targets. Furthermore, since the LOMs themselves are transcription factors, the effect of LOM-SPL interaction can be bilateral. Taken chlorophyll content as an example, miR156 overexpression suppresses chlorophyll biosynthesis and this suppression is LOM-dependent (Figure S8A and S8B). Several transgenic lines of 35S::rLOM1-YFP and 35S::Myc-rLOM2 produced yellowish leaves during early vegetative stage due to decreased chlorophyll content as reported [26]. But this phenotype became less evident with plant aging (Figure S8C–S8E) when the SPL level was increasing, suggesting a possibility that the miR156-targeted SPLs enhance chlorophyll biosynthesis at least partially through negatively regulating the LOM activity. Finally, although overexpression of LOM1 and SPL9 simultaneously resulted in an intermediate phenotype, the two transcription factors may act independently on downstream genes. Identification of the targets of LOM factors will further our understanding of the biological significance of LOM-SPL interaction.

In addition to LOMs, DELLAs could bind to SPLs as well. DELLAs are degraded in response to gibberellin [37–39], resulting in release of the factors they bind, and the SPL-DELLA interaction integrates the hormone signals to the miR156-SPL pathway in regulating plant flowering [5]. MiR171 is not only regulated by endogenous cues, but also responds to environmental stress, such as cold, high salt and hydration [40–43]. Thus the LOM-SPL interaction may transduce environmental stimuli into endogenous signaling to adjust plant phase transition and development. Because both LOMs and SPLs mount with plant age, and their over-accumulation have adverse effects on plant growth and development [2,3,26,44], we propose that plants have employed LOMs as a damper to measure the increasingly higher SPL output at protein level in aged plants when the miR156 level is low, and likewise SPLs may temporally restrict the activity of LOMs. Since the miR156-targeted SPLs function as a key endogenous age cue and SPL9 is one of the highly active SPL members, the LOM-SPL interaction has a profound contribution to programming plant life.

Mining of genome data revealed that both miR156 and miR171 are highly conserved in land plants from moss (Physcomitrella patens) to flowering plants of both monocots and dicots [43], and in crop plants they control important agronomic traits [46,47]. In the diploid cotton species of Gossypium raimondii there are 15 MIR171 and seven putative LOM genes, of which five LOMs contain the miR171 recognition sites (see [48] and Figure S9). It would be interesting to examine if LOMs are involved in regulating cotton fiber (seed trichome) development. Notably miR171a* is also functional in gene silencing and the miR171a*-SU(VAR)3-9 HOMOLOG8 pair was proposed to have evolved very recently in the Arabidopsis lineage [44]. A recent report showed that overexpression of miR171 (Hwa-pri-miR171a) in barley up-regulated miR156 and repressed vegetative phase transitions [49], which is in contrast with the opposite effects of the two miRNAs in Arabidopsis described herein and reported by others [26,29,50]. Whether the interplay between the two conserved aging miRNAs varies with plant taxa is a subject of further study.

Materials and Methods

Plant materials and constructs

Plants of Arabidopsis thaliana, ecotype Columbia (Col-0), and Nicotiana benthamiana were grown at 22°C in long days (16 h light/8 h dark). The lom triple mutant [26], 35S::LUC-LOM1 [26], 35S::MIR156F [23], 35S::MIM156 [23], SPL9::GFP-SPL9 [23], TCL1::GUS [23], TCL1mu3/4::GUS [23] have been described previously. The gl1 (SALK_039478), try (SALK_029760), tcl1 (SALK_055460) and gl3 gl3 (CS66490) mutants were obtained from Arabidopsis Biological Resource Center (ABRC).

For MIR171A/B/C::GUS constructs, the promoters of MIR171A/B/C (~2 kb) were PCR amplified using PrimeSTAR HS DNA polymerase (TaKaRa) and individually fused to the GUS coding region. For LOM constructs, the miRNA-resistant versions were created by two-round PCR. The resultant fragment was inserted into a vector which harbors a 35S::6×Myc cassette to generate 35S::Myc::LOM1/2/3. Then the 35S promoter was replaced by a native promoter to generate LOM1::Myc::LOM1 and LOM12::Myc::LOM2. At least 30 T1 seedlings were analyzed for each construct. For BiFC constructs, coding regions of the miRNA-resistant LOMs and SPL9 were PCR amplified and cloned into JW771 and JW772 [51], respectively. Primers are listed in Supplementary Table S1.

Trichome number and flowering time measurement

The trichome numbers were counted on each internode of stem, and the number was divided by the length of each internode to calculate the trichome density (number per centimeter). Flowering time was measured by counting the total number of leaves (rosette and cauline leaves) and the number of days to flowering under long-day condition. The data were given as mean s.d. and analyzed by t-test.

Figure 8. A model for miR171-LOM and miR156-SPL interaction in regulating trichome formation and other biological events. Trichomes are distributed on stem acropetally, with a high density at the basal part and sparse to nearly glabrous at apical part and inflorescence. The miR156-targeted SPLs up-regulate trichome negative factors TRY and TCL1 to repress trichome production on stem and inflorescence, whereas miR171-targeted LOMs counteract the SPLs through protein-protein interaction. LOMs positively regulate MIR171, forming a feedback loop to maintain their homeostasis, which influences the SPL output. Arrows indicate positive regulation, blunt ends indicate negative regulation, and the dashed line indicates the unidentified pathway.
Gene expression analyses

RNAs were extracted with Trizol reagent (Invitrogen) following the manufacturer’s instructions. Total RNAs of 1 μg were used for reverse transcription in a 20 μL reaction system with M-MLV Reverse Transcriptase kit (Invitrogen). The fragments of interest were amplified by RT-PCR using sequence-specific primers (see Supplementary Table S1). Real-time PCR was performed with SYBR Premix Ex Taq II (Takara), and amplification was monitored on the Mastercycler ep RealPlex2 (Eppendorf). The gene expression level was normalized to reference gene β-

Dual-LUC assays

A dual-luc method using N. benthamiana plants was used [55]. Briefly, the effector plasmid is 35S::LOM1 or empty vector, and the reporter plasmid, pGreen-0800-LUC, harbors two luciferases: the firefly luciferase (LUC) controlled by the MIR171 promoter, and the Renilla (REN) luciferase controlled by the constitutive 35S promoter. The Agrobacterium strain containing the reporter was mixed with the effector strain (at the reporter:effector ratio of 1:3). The mixture was infiltrated into leaves of N. benthamiana. Three days later, leaf samples were collected for the dual-luc assay using commercial Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer’s instruction. The LUC activity was normalized to REN. Three biological repeats were measured for each sample.

Small RNA blot

Total RNAs were extracted using Trizol reagent (Invitrogen), and 5–20 μg of the total RNA were resolved on 17% polyacrylamide gels under denaturing conditions (7 M urea). RNAs were then cross-linked in formaldehyde solution (1%) under a vacuum. The cross-linked material was washed and ground in liquid nitrogen, the resultant powder was re-suspended in extraction buffer (0.4 M sucrose, 10 mM Tris·HCl, pH 8.0, 10 mM MgCl2, 5 mM mercaptoethanol, 0.1 mM PMSF, and 1× protease inhibitor [Roche]) and lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% deoxycholate sodium, and 0.1% SDS), successively, followed by sonification (output 3, 6×10 s). An anti-Myc antibody (Abmart) was added for precipitation. After several washes, DNA samples were reversely cross-linked and then purified using a PCR purification kit (Qiagen). The relative amounts of the DNA amplicons were analyzed by quantitative PCR using the β-TUBULIN2 gene promoter as a reference. Relative enrichment was calculated by normalizing the value in 35S::Myc-LOM1 against the value in wild-type.

Supporting Information

Figure S1 Expression of trichome regulator genes. (A) qRT-PCR analysis of expression of trichome promoting genes in wild-type, lomt mutant and LUC::LOM1OE plants. Expression of GIS, GIS2, ZFP8 and GL3 did not change evidently. (B and C) Expression of trichome repressor genes in first internode (B) and inflorescence (C). Expression of CPC, ETC1 and TPL2 did not change in lomt and LUC::LOM1OE plants in first internode; CPC was higher in LUC::LOM1OE inflorescence. (TIF)

Figure S2 LOM1OE and MIR156FOE plants show similar phenotypes. View of 49-day-old plants of the indicated genotypes. Plants were grown under short-day condition (8 h light/16 h dark). Note that LOM1OE (35S::LUC::LOM1) and MIR156FOE (35S::MIR156F) plants were both yellow-green; on the contrary, lomt mutant and SPL accumulation plants (35S::MIM156 and SPL::GFP::SPL9) were dark-green. 35S::LUC::LOM1 and 35S::MIR156F plants produced more rosette leaves than wild-type. Bar = 2 cm. (TIF)

Figure S3 LOMs accumulation delays flowering. (A) SPL2 interacts with three LOMs in yeast. (B–D) Flowering time of
wild-type (B), lont (C) and 35S::LUC-rLOM1 (D) plants under long-day conditions. The x axis indicates the number of days after germination and the y axis indicates percentage of plants that flowered on a given day. (E) Flowering time of the plants indicated in (B–D). The number of days to flowering was counted when the first floral bud opens. Data are given as mean s.d. and analyzed by t-test. **P<0.01, relative to the wild-type; n, number of plants analyzed. (TIF)

Figure S4 Alignment of N-terminus of three LOMs of Arabidopsis thaliana. LOM1N and LOM2N are more similar in length and amino acid sequence identity. (TIF)

Figure S5 Expression of SPL9 in seedling and inflorescence. Analysis by qRT-PCR shows that expression of SPL9 did not change evidently in lont and 35S::LUC-rLOM1 plants in either seedling or inflorescence. (TIF)

Figure S6 Identification of promoter regions of MIR171 genes responsible for LOM1 induction. (A) LUC activities driven by different upstream fragments of the MIR171A gene. Nucleotides are numbered from the stem-loop of the pri-miRNA. The Agrobacterium strain containing the MIR1711::LUC reporter combined with the effector strain containing the 35S::LOM1 or the empty vector were infiltrated into N. benthamiana leaves. After 3 days, luciferin was infiltrated into the same region and the LUC activities were monitored. Note that LUC activities were increased when MIR1711::LUC was combined with 35S::MIR171C, in comparison with the empty vector control. Because A-P3 fragment still responded to LOM1 while A-P4 did not, the LOM1-binding motifs are located in the region between −343 and −201. (B) and (C) LUC activities driven by truncated promoters of MIR171B (B) and MIR171C (C) in response to LOM1. (TIF)

Figure S7 SPL9, LOMs and MIR171 show similar temporal expression pattern. (A) GUS staining of 35S::LOM1-GUS plants. GUS activity in newly emerged leaves was stronger than in elder ones. (B) Relative expression level of SPL9 and three LOMs in stem and inflorescence. Based on qRT-PCR, expression of SPL9 and three LOMs increased gradually along the stem from bottom to top (Int.1 to Int.5) and reached the highest level in inflorescence (Inf.). The expression level of each gene in Int.1 was set to 1. (C) MIR171 expression in main stem and inflorescence. Transcript abundance of pri-MIR171A, B and C increased gradually along the main stem from basal to apex and reached the highest levels in inflorescence. The expression level of each gene in Int.1 was set to 1. (D) The mature miR171 level was increasing with age, and the miR156 showed an opposite accumulation pattern. U6 was used as an internal reference. (TIF)

Figure S8 LOM-SPL module plays a role in chlorophyll synthesis. (A) View of wild-type, 35S::MIR156F, 35S::MIR171B and 35S::MIR156F×35S::MIR171B plants. Plants were grown in long-day condition for 20 days. Bar = 1 cm. (B) Chlorophyll content of the genotypes shown in (A). FW, fresh weight. **P<0.01 compared with wild-type. Chlorophyll was measured as described previously [56]. (C–E) View 35S::Myc-rLOM2 transgenic Arabidopsis at indicated age under long day condition. This transgenic line showed severe development defects, including up-curly yellowish leaves, soft stem, late flowering and infertility. Arrows in (D) indicate one leaf exhibiting different colors. After the transition shown in (D), the newly emerged leaves showed a wild-type shape and color, suggesting normal chlorophyll content (E). Stars in (E) indicate the elder up-curly yellow-green rosette leaves. Scale bars, 1 cm. (TIF)

Table S1 Oligonucleotide primers used in the study. (DOCX)

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Author Contributions
Conceived and designed the experiments: XYX XYC. Performed the experiments: XYX XYC. Wrote the paper: XYX XYC. Helpful and critical discussion: YBM LJW.

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Table S1 Oligonucleotide primers used in the study. (DOCX)
An Interplay between Two miRNAs

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