The role of miR156/SPLs modules in Arabidopsis lateral root development

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SUMMARY

miR156 is an evolutionarily highly conserved miRNA in plants that defines an age-dependent flowering pathway. The investigations thus far have largely, if not exclusively, confined to plant aerial organs. Root branching architecture is a major determinant of water and nutrients uptake for plants. We show here that MIR156 genes are differentially expressed in specific cells/tissues of lateral roots. Plants overexpressing miR156 produce more lateral roots whereas reducing miR156 levels leads to fewer lateral roots. We demonstrate that at least one representative from the three groups of miR156 targets SQUAMOSA PROMOTER BINDING PROTEIN-LIKE (SPL) genes: SPL3, SPL9 and SPL10 are involved in the repression of lateral root growth, with SPL10 playing a dominant role. In addition, both MIR156 and SPLs are responsive to auxin signaling suggesting that miR156/SPL modules might be involved in the proper timing of the lateral root developmental progression. Collectively, these results unravel a role for miR156/SPL modules in lateral root development in Arabidopsis.

Keywords: miR156/SPL, lateral root, auxin signaling, homeostasis, feedback loops, Arabidopsis thaliana.

INTRODUCTION

miR156 is one of the most abundant and evolutionarily conserved miRNAs in plants. This miRNA accumulates in seedlings and its levels decrease with age (Wu and Poethig, 2006; Wang et al., 2009; Wu et al., 2009). Overexpression of miR156 produces a larger number of juvenile leaves and delays flowering. However, overexpression of a target mimic of miR156, MIM156, promotes flowering after plants have produced only a few adult leaves (Todesco et al., 2010). In Arabidopsis thaliana, 11 out of the 17 SQUAMOSA PROMOTER BINDING PROTEIN-LIKE (SPL) genes are targeted by miR156. SPL3, SPL4, and SPL5 encode small proteins comprising mostly of the DNA binding domain, the squamosa promoter binding domain (SBP) box. In SPL3 the miR156 complementary site is located in the 3'UTR of mRNA and miR156 regulates SPL3 expression through transcript cleavage as well as translational inhibition (Gandikota et al., 2007). SPL3, SPL4, and SPL5 function mostly in the control of flowering time and phase change, and SPL3 is a direct upstream activator of LFY, FUL and AP1 (Yamaguchi et al., 2009). SPL9 and SPL15 belong to the second group of SPL genes. Expression of a miR156-insensitive form of SPL (rSPL9) renders plants to flower extremely early and virtually skip the juvenile phase (Wang et al., 2009; Wu et al., 2009). In addition to having an effect on leaf initiation rate, SPL9 positively regulates genes involved in trichome development and negatively regulates anthocyanin accumulation (Wang et al., 2008; Yu et al., 2010; Gou et al., 2011). The third group of SPL genes includes SPL10, SPL11 and SPL2, which regulate morphological changes of lateral organs such as lamina shape and trichome distribution with shoot maturation in the reproductive phase (Shikata et al., 2009); however, the specific function of this group has not yet been addressed. Both SPL9 and SPL10 can promote miR172 expression by direct binding to the MIR172B promoter (Wu et al., 2009; Zhu and Helliwell, 2010). Moreover,
miR156 and miR172 are positively regulated by the transcription factors they target and these negative feedback loops contribute to the stability of the juvenile and adult phases (Wu et al., 2009).

Recently, Arabidopsis miR156 has been reported to mediate responses to recurring heat stress through SPL transcription factors (Stief et al., 2014). It has been found that the developmental decline in miR156 levels is partially mediated by sugars at both transcriptional and post-transcriptional levels and this effect is primarily attributable to a change in MIR156A and MIR156C expression (Yang et al., 2011, 2013; Yu et al., 2013). The function of other MIR156 genes such as MIR156B and MIR156D are still largely undefined. Furthermore, investigations on different miR156/SPL modules until now have been focused almost exclusively on their roles in shoot/leaf development, flowering time control and stress responses. It is not known whether any of the miR156/SPLs modules are involved in underground tissue development and hormone signaling.

Root system architecture is fundamentally important for plant growth and survival because of its role in water and nutrient acquisition and anchorage by plants. Dicotyledonous plants, such as Arabidopsis, are characterized by a primary root that produces lateral roots and root hairs, all of which can be modulated elaborately to adapt to the changing environment (Osmond et al., 2007). In Arabidopsis, lateral roots originate exclusively from pericycle founder cells located opposite xylem poles, which undergo several rounds of divisions to produce lateral root primordia that eventually emerge from the primary root (Malamy and Benfey, 1997; Péret et al., 2009). Auxin is important for all these stages of lateral root development. The precise priming of pericycle cells to become founder cells is a consequence of auxin signaling in the basal meristem and the establishment of auxin gradient during primordia formation is mediated by auxin transporters (Bennett et al., 1996; Benková et al., 2003; De Smet et al., 2007; Swarup et al., 2008).

MicroRNAs (miRNAs) are post-transcriptional regulators of growth and development in both plants and animals. Several miRNAs involved in auxin signaling, nutritional homeostasis and stress responses have essential roles in root development. NAC1 which transduces auxin signal for lateral root emergence is targeted by miR164. The auxin-induced expression of miR164 and cleavage of NAC1 transcript provide a homeostatic mechanism to downregulate auxin signals for lateral root development (Xie et al., 2002; Guo et al., 2005). miR160 is a major regulator of root growth and gravitropism. This conserved miRNA negatively regulates ARF10, 16 and 17 transcripts and plants overexpressing miR160c have shorter and agranivorous roots with an enlarged tumour-like apex (Wang et al., 2005). miR167 can target mRNA encoding IAR3, which hydrolyzes an inactive form of auxin to release bioactive auxin. Overexpression of the cleavage-resistant form of IAR3 mRNA increases lateral root development (Kinoshita et al., 2012). miR166/165 degrade mRNAs encoding class III homeodomain zipper transcription factors in the endodermis and stele periphery and determine xylem cell types in a dosage-dependent manner (Carlsbecker et al., 2010). Other than the above miRNAs it is not known whether miR156 also shape the architecture of Arabidopsis root system.

Here, we demonstrated a role of miR156/SPL module in root development in Arabidopsis. We found that overexpression of five copies of the primary MIR156A transcript caused increased primary root growth and promoted lateral root development whereas overexpression of five copies of miR156 mimic (MIM156) retarded lateral root growth. Plants expressing cleavage-resistant forms of SPL3, SPL9 and SPL10 all exhibited decreased lateral root development with SPL10 having the most dominant effect.

RESULTS

Differential expression patterns of MIR156 genes in Arabidopsis

There are 8 MIR156 genes in the Arabidopsis genome: MIR156A, MIR156B, MIR156C, MIR156D, MIR156E, MIR156F, MIR156G and MIR156H. As the first step toward elucidating the role of different MIR156 genes in plant development we generated transgenic plants expressing promoter–GUS (uidA) fusion of different MIR156 genes. Each promoter contained ~2 kb upstream sequence of the respective MIR156 genes. After having screened 32 independent T1 lines for each construct, we focused on five MIR156 genes (A, B, D, E and F) with promoter–GUS lines that showed consistency and reproducible expression among different T1 lines. We further examined the promoter–GUS lines of these MIR156 genes for their expression at different developmental stages and at different tissues. GUS staining of transgenic plants revealed that MIR156A was highly expressed in the first two pairs of leaves of seedlings and its expression then decreased precipitously in adult leaves (Figure 1a); this result is consistent with its major function in regulating juvenile-adult phase transition (Yang et al., 2013). MIR156A was also highly expressed in the shoot apical meristem (Figure 1b), the pedicel of floral bud (Figure 1c) and the internode connecting the siliques (Figure 1d). By contrast, MIR156B was specifically expressed in the central cylinder of the primary root (Figure 1e) and at the bases and flanks of lateral root primordia (Figure 1f), and the shoot apical meristem (Figure 1g). MIR156D was highly expressed in the seedlings (Figure 1h). It was especially active in primary and lateral root tip (Figure 1i), particularly, in the root apical meristem (Figure S1). Previously, Huijser and colleagues has reported that PMIR156D–GUS was expressed in filaments, sepals and style but not in anthers (Xing et al., 2010). These results were also confirmed in our findings (Figure 1j,k).
miR156/SPLs involved in lateral root development

![](image)

**Figure 1.** Expression patterns of different MiR156 genes. (a–d) PMiR156A-GUS expression pattern in different tissues. (a) Thirty-day-old seedling. Arrows indicate strong expression in juvenile leaves. (b) Shoot apical meristem. Arrow indicates expression in shoot apical meristem. (c) Pedicel of floral bud. Arrow indicates expression in the pedicel. (d) Silique. (e–g) PMiR156B-GUS expression pattern in different tissues. (e) Ten-day-old seedling. (f) Lateral root primordium. (g) Shoot apical meristem. (h–k) PMiR156D-GUS expression pattern in different tissues. (h) Twenty-day-old seedling. (i) Roots. (j) Flower. (k) Silique. For each genotype, at least three independent transgenic lines were analyzed and all lines showed similar expression patterns. Images shown are representative individuals of at least 15 GUS-stained seedlings. Bars ~ 2 mm in (a, e, h, i); 50 μm in (b, f, g, j); and 250 μm in (c, d, k).

(l) Relative expression of primary MiR156 transcripts in roots of both 10- and 15-day-old seedlings. The transcript levels were normalized to that of ACTIN2 gene. The expression level of MiR156A in 10-day-old roots was set as 1.0. Error bars represent standard errors (n = 3). The experiment was repeated three times with similar results.

GUS analysis of plants carrying PMiR156E-GUS transgene showed that it was expressed in the cotyledons (Figure S2a), stomata (Figure S2b), the internode connecting silique (Figure S2c) and the internode connecting the flower (Figure S2d). MiR156F was highly expressed in the seedlings (Figure S2e), leaf veins (Figure S2f), stamen filaments (Figure S2g) and the pedicel of flower (Figure S2h).

To further understand the expression of these MiR156 genes in roots, we performed expression analysis using quantitative PCR. The expression level of MiR156B was the highest in roots of both 10- and 15-day-old seedlings whereas MiR156E and MiR156F were expressed significantly lower than the other MiR156 genes (Figure 1l). In addition, expression levels of MiR156A, MiR156B and MiR156D decreased in roots from 10- to 15-day-old seedlings (Figure 1l). Yu et al. (2013) and Yang et al. (2013) have reported that MiR156A and MiR156C mainly function in regulating phase transition in Arabidopsis. Our promoter analysis has shown that other MiR156 genes, particularly MiR156B and MiR156D, were expressed in different root cell types and tissues, implying a possible role for these two MiR156 genes in the development of root architecture.

**miR156-targeted SPLs are differentially expressed in roots**

Transcripts of SPLs are well known targets of miR156. In Arabidopsis, there are 11 SPLs targeted by miR156. Most of these SPL genes can be further divided into three groups, represented by SPL3 (SPL3, SPL4, and SPL5), SPL9 (SPL9 and SPL15) and SPL10 (SPL2, SPL10, and SPL11) (Schwarz et al., 2008). To investigate which SPLs are possibly involved in root development, we first examined the expression patterns of SPL genes in different tissues. In roots of both 10- and 15-day-old wild-type seedlings, SPL3, 4 and 5 were expressed relatively lower than those of other SPLs (Figure 2a). When the expression of SPL4 in 10 days was set as 1.0, SPL3 in 15-day-old roots was expressed 15-fold higher compared with SPL4 level. Transcripts for the mid-size protein SPL9 were expressed eight-fold higher than the SPL4 transcripts in 15-day-old roots. Genes encoding SPL10, SPL11 and SPL2 were highly expressed to the level of 53- to 92-fold in roots compared with the other SPLs (Figure 2a). Nevertheless, the expression level of SPL3 was higher in flower, silique and leaf tissues compared with other SPL genes (Figure S3a–d). This was consistent with its important role in the regulation of flowering time.

Analysis of SPL10 promoter-GUS fusion revealed its high activity in the meristems, cortical cells and vascular tissues of primary roots (Figure 2b). It was also highly expressed at different stages of lateral root development (Figure 2c–e). Nevertheless, SPL9 promoter-GUS was only specifically expressed in the stele and the quiescent center of primary root (Figure 2f, see also the enlarged image in...
Figure S4), the primed pericycle cells (Figure 2g) and later in the two layers of pericycle cells (Figure 2h,i). These results together suggest that all these three groups of SPL transcripts targeted by miR156 may be involved in regulating root development, with SPL10 playing a major role.

**miR156/SPLs modules play a role in root system architecture**

To investigate if lateral root development is affected by miR156 and its SPL targets, we generated transgenic plants carrying a construct expressing five copies of the primary MIR156A (P35S:5MIR156) to ensure higher abundance of miR156 (Figure S5) and also plants expressing five copies of MIM156 (P35S:5MIM156) to effectively sponge and neutralize miR156 activity (Wang et al., 2008). We selected two strong expression lines derived from each construct to examine the effects of 5MIR156 and 5MIM156 overexpression on root architecture. Seedlings were grown on 0.2 x Murashige and Skoog (MS) medium for 5 days, then transferred onto medium placed vertically and root growth was followed for another 5 days under long-day conditions. We found that no lateral root had emerged from the primary roots of P35S:5MIM156 seedlings at this stage of seedling development (Figure 3a). By contrast, P35S:5MIR156 seedlings showed a nearly two-fold increase in lateral root number compared with wild-type (WT) seedlings (Figure 3a,c). In addition, P35S:5MIR156 seedlings had longer primary roots than WT whereas P35S:5MIM156 developed shorter primary roots (Figure 3b), suggesting a role for miR156 in primary root growth as well. As plants developed, P35S:5MIR156 seedlings consistently produced more lateral roots than WT whereas lateral roots in P35S:5MIM156 seedlings emerged only when the seedlings were 15-day-old and the number of lateral roots produced was much lower compared with WT, even when plants were 20-day-old (Figure 3c).

We also investigated root growth under short-day condition (8 h light/16 h dark). Under this condition lateral roots of seedlings developed much slowly compared with long-day condition (Figure S6a). Nevertheless, P35S:5MIR156
seedlings still produced nearly two-fold more lateral roots than WT whereas P35S:5MIR156 seedlings consistently developed fewer lateral roots (Figure S6a). The leaf number of P35S:5MIR156 and P35S:5MIM156 seedlings also showed a similar trend as lateral roots under both long-day and short-day conditions (Figure S6b,c). Taken together, these results indicate that miR156 might act as a positive regulator of lateral organ growth.

To further determine which SPL is involved in lateral root development, P35S:rSPL3, PSPL9:rSPL9 and PSPL10:rSPL10 seedlings were analyzed. Interestingly, these transgenic plants all showed defects in lateral root development (Figure 3a). Primary root length in the P35S:5MIR156 and P35S:5MIM156 seedlings was about the same as WT seedlings (Figure 3b). However, PSPL9:rSPL9 seedlings showed shorter primary root than WT (Figure 3b) suggesting partial regulation of primary root growth by SPL9. The P35S:rSPL3 and PSPL9:rSPL9 seedlings produced fewer lateral roots than WT in 10-day-old seedlings (Figure 3d). As plants grew, lateral root number began to increase but the number of lateral roots produced was still much fewer than that of WT. In PSPL10:rSPL10 seedlings there was nearly no emerged lateral root even after 15 days of growth (Figure 3d). Moreover, PSPL10:rSPL10 seedlings consistently produced fewer lateral roots even compared with P35S:rSPL3 and PSPL9:rSPL9 seedlings (Figure 3d). Similar results were obtained when seedlings were compared under short-day conditions (Figure S6d). The leaf number in rSPLs seedlings also exhibited the same trend as lateral roots under both long-day and short-day conditions (Figure S6e,f). Therefore, our results showed that these three representative SPLs: SPL3, SPL9 and SPL10 are all involved in lateral root development, with SPL10 playing a major role. Taken together, our data suggest that miR156/SPLs modules are involved in root system architecture development in Arabidopsis.

**Perturbation of SPL expression level correlates with lateral root development and affects miR156 expression in roots**

To investigate if lateral root development is correlated with the abundance of miR156 and its target SPL transcripts, we first confirmed that the abundance of miR156 was greatly...
increased in roots of P35S:5MIR156 and decreased in roots of P35S:5MIM156 seedlings (Figure 4a). SPL3, SPL9 and SPL10 transcripts levels were all decreased in roots of P35S:5MIM156 seedlings but increased in roots of P35S:5MIM156 seedlings (Figure 4b–d). In P35S:rSPL3, PSPL9:rSPL9 and PSPL10:rSPL10 seedlings, SPL3, SPL9 and SPL10 transcripts in roots were respectively increased and SPL10 transcripts were elevated to the highest levels compared with those of SPL3 and SPL9 (Figure 4b–d).

Next, we obtained spl mutants from the SALK collection (Figure S7a). qRT-PCR analysis showed the down-regulation of SPL3 and SPL10 transcripts in roots of 15-day-old spl3 and spl10 seedlings, respectively (Figure S7b,c). Double mutant spl9 spl15 (CS67865) was also previously identified (Schwarz et al., 2008; Wang et al., 2008). Seedlings of spl3, spl10 and spl9 spl15 all produced more lateral roots than WT (Figure 4e). The double mutant spl9 spl15 produced more lateral roots than other spl single mutants (Figure 4e). These results provide evidence that lateral root development can be modulated by changing SPL expression level through miR156/MIM156 overexpression or mutation in the SPL genes and that the expression levels of SPLs are quantitatively correlated with lateral root numbers.

Given that miR156 are positively regulated by its targets SPL9 and SPL10 in maintaining the stability of the vegetative phase change (Wu et al., 2009), we evaluated the activity of the PMIR156D-GUS at the tip of lateral roots in 15-day-old P35S:5MIR156, P35S:5MIM156 and PSPL10:rSPL10 seedlings (Figure 4f). Moreover, in both 10- and 15-day-old seedlings, the MIR156D expression level was increased in roots of both PSPL9:rSPL9 and PSPL10:rSPL10 seedlings but was unchanged in P35S: rSPL3 seedlings compared with WT (Figure 4g), suggesting that miR156 is modulated by its targets SPL9 and SPL10 but not by SPL3 in roots. These results further sug-

![Figure 4](image-url)

**Figure 4. Perturbation of SPL expression affects lateral root development and MIR156 accumulation.**

(a–d) (a) Quantitative RT-PCR of miR156 in roots of 10-day-old P35S:5MIR156 and P35S:5MIM156 seedlings. The expression level of mature miR156 in WT was set as 1.0. (b–d) Relative expression of SPL3 (b), SPL9 (c), and SPL10 (d) in roots of 15-day-old seedlings in the indicated genotypes. The transcript levels were normalized to that of ACTIN2 gene. The expression level of each gene in WT was set as 1.0. Error bars represent standard errors (n = 3).

(e) Quantification of lateral root numbers in 10-day-old seedlings of different spl mutants. Error bars represent standard errors (n = 50 seedlings). Asterisk indicates statistical differences, ***P < 0.001; **P < 0.01; *P < 0.05.

(f) PMIR156D-GUS activity in lateral roots of 10-day-old seedlings in the indicated genotypes. For each genotype, at least four independent lines were analyzed and all lines showed similar expression patterns. Images shown are representative individuals of at least 15 GUS-stained seedlings. Bar = 50 μm.

(g) Relative expression of MIR156D in roots of 10- and 15-day-old rSPLs seedlings. The transcript levels were normalized to that of ACTIN2 gene. The expression levels of MIR156D in 10- and 15-day-old WT were set as 1.0, respectively. The experiment was repeated twice with similar results.

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gest that miR156 and its targets SPL9 and SPL10 can form negative feedback loops to buffer against small changes in the expression of its targets to maintain the stability of the lateral root development.

miR156/SPLs modules are involved in lateral root primordia progression

To gain further insight into the developmental basis for the lateral root defects in miR156/SPLs seedlings, we quantified the distribution of stages of lateral root primordial (LRP) in different genotypes (Malamy and Benfey, 1997). In 8-day-old seedlings, the numbers of stages I–IV LRP in P35S:5MIR156 seedlings were two-fold lower than that of WT (Figure 5a), whereas in P35S:rSPL3, PSPL9:rSPL9 and PSPL10:rSPL10 (rSPLs) roots the numbers were nearly two-fold higher. There was no detectable difference at later primordial stage from stage VI to stage VII among WT, P35S:5MIR156 and rSPLs seedlings. Consequently, rSPLs seedlings exhibited twice as many LRP than WT, whereas in P35S:5MIR156 seedlings the number was reduced by more than 50%. However, the number of emerged lateral roots in P35S:5MIR156 seedlings was two-fold higher than in WT (Figure 5a). By contrast, rSPLs roots showed significant decrease in the number of emerged lateral root (LR) compared with WT. The total numbers of emerged and non-emerged (stages I–VII) primordia did not differ across different genotypes examined (Figure 5a). In 10-day-old seedlings, the lateral root length of P35S:5MIR156 seedlings was longer than that of WT seedlings whereas emerged lateral root length in P35S:rSPL3 and PSPL9:rSPL9: rSPL9 seedlings was shorter than that of WT seedlings (Figure 5b). Note that the P35S:5MIM156 and PSPL10: rSPL10 seedlings did not show any lateral root emergence until 15 days. To further analyze this, we examined the meristem size of lateral roots in different genotypes and found no difference among P35S:5MIR156, rSPLs and WT seedlings (Figure S8), suggesting that miR156/SPLs modules do not regulate lateral root meristem size. Taken together, our results suggest that perturbation of miR156/SPLs mainly affect the rate of primordia progression through the postemergence developmental stages rather than the initiation process.

Figure 5. miR156/SPLs modules are involved in lateral root primordia progression.
Seeds were germinated and grown for 8 days on solidified 0.2× MS agar medium for the examination of root primordia development.
(a) Quantification of lateral root primordia from stages I to VII in seedlings of the indicated genotypes according to Malamy and Benfey (1997). Error bars represent standard errors (n = 30 seedlings).
(b) Quantification of lateral root length in roots of 10-day-old seedlings of the indicated genotypes. Note that there was no lateral root emergence in 10-day-old PSPL10:rSPL10 seedlings. Error bars represent standard errors (n = 50 seedlings). Asterisk indicates statistical differences, ***P < 0.001; **P < 0.01; *P < 0.05. The experiment was repeated twice with similar results.

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miR156 expression is responsive to auxin during lateral root development

Auxin plays an important role in inhibiting primary root elongation and promoting lateral root development. To determine whether auxin acts as a signal to promote lateral root development through miR156/SPLs modules, we examined the responses of promoters of different MiR156 genes to indole-3-acetic acid (IAA) treatment. Homozygous lines expressing PMiR156A-GUS, PMiR156B-GUS and PMiR156D-GUS were germinated and grown for 5 days on solidified 0.2× MS agar medium and then transferred to plates supplemented with 10 μM IAA for another 6 days. Samples were collected each day after IAA treatment and analyzed for GUS activity. The GUS activity of PMiR156A was undetectable in roots without auxin treatment; however, weak GUS activity appeared in the central cylinder of primary roots 6 days after auxin treatment (Figure 6a, upper panel). MiR156B was specifically expressed at the base of lateral root. Upon IAA treatment, the GUS activity of PMiR156B was highly induced in the entire primordia and the central cylinder of the lateral root (Figure 6a, middle panel), and after 6 days the GUS activity was detected in the newly emerged primordia of lateral roots (Figure 6a, middle panel). MiR156D was specifically expressed at the tip of primary root. After IAA treatment, the GUS activity of PMiR156D increased significantly in root tip (Figure 6a, lower panel), particularly in the quiescent center area. After 6 days of treatment, the GUS from PMiR156D was strongly expressed in the newly emerged lateral root tips (Figure 6a, lower panel). The increased GUS staining in different genotypes in response to auxin was also clearly seen in the entire roots (Figure S9). In the wild-type seedlings that were treated with IAA, the primary MiR156B transcript level in roots was increased from day 1 to 6 (Figure 6b), with the highest level at day 6. Primary MiR156D transcript level in roots showed significant increase from day 4 to 6, whereas primary MiR156A transcript levels showed relatively small change compared with primary MiR156B and MiR156D (Figure 6b). The level of mature miR156 was also increased after IAA treatment (Figure S10). Moreover, the entire transgenic seedlings carrying PMiR156A-GUS or PMiR156B-GUS also showed increased response to IAA treatment (Figure S11). These results suggest that auxin can induce expression of MiR156B and MiR156D in root.

SPLs expression is responsive to auxin during lateral roots growth

The above results suggest that auxin could act as a signal to stimulate lateral root formation through induction of MiR156 at the transcriptional level. To elucidate the responses of miR156 targets SPLs to auxin signaling, we evaluated the contribution of representative SPL3, SPL9 and SPL10 to the lateral root growth during auxin induction. We used the cleavage-resistant form of SPL mRNAs so as to avoid complication by miR156-mediated cleavage.
WT, P35S::rSPL3, PSPL9::rSPL9 and PSPL10::rSPL10 seedlings were grown in solidified 0.2× MS agar medium for 5 or 10 days and then immersed in liquid MS medium with or without 10 μM IAA for 24 h. After the treatment, SPL9 expression in roots was induced about six-fold in 5-day-old and 56-fold in 10-day-old PSPL9::rSPL9 seedlings when compared with the no-treatment control (Figure 7d). Similarly, SPL10 expression in roots was induced about three-fold in 5-day-old and 2.5-fold in 10-day-old PSPL10::rSPL10 seedlings when compared with the no-treatment control (Figure 7b). Moreover, GUS expression levels were also elevated in roots of PSPL9::GUS and PSPL10::GUS seedlings after incubation with IAA for 24 h (Figure 7c). We could not detect consistent change of SPL3 expression in roots of P35S::rSPL3 seedlings between 5- and 10-day-old seedlings after the treatment (Figure S12). This is not surprising since the rSPL3 mRNA was not expressed from the native promoter but from a 35S promoter. In another experiment, 5-day-old rSPLs seedlings were transferred to solidified 0.2× MS agar medium placed vertically with different concentrations of IAA for 24 h. We found that SPL9 expression level in roots of PSPL9::rSPL9 seedlings were elevated two-fold at 0.001 μM IAA and peaked up to 4.5-fold at 10 μM IAA compared with the no-treatment control (Figure 7d). Similarly, SPL10 expression level in roots of PSPL10::rSPL10 seedlings showed an increase at 0.1 μM IAA and peaked up to two-fold at 10 μM IAA compared with untreated control (Figure 7e). From these results, we conclude that SPL9 is more responsive to IAA than SPL10. Taken together, these results suggest that both miR156 and its targets SPL9 and SPL10 are responsive to auxin during progression of lateral root development.

Effects of IAA and NPA on root architecture upon overexpressing MIr156 and Spls

To determine how auxin and the polar auxin transport inhibitor NPA (1-Naphthylphthalamic acid) affect miR156/SPLs modules in the modulation of root system architecture, we examined root architecture responses of WT, P35S::5MIR156 and rSPLs seedlings grown in solidified

![Figure 7](https://example.com/figure7.png)

**Figure 7.** Spls expression is responsive to auxin during lateral root growth.

(a)–(c) Seedlings of different genotypes were mock- or IAA-treated (10 μM) in liquid 0.2× MS medium for 24 h. (a) Relative expression of SPL9 in roots of 5- and 10-day-old PSPL9::rSPL9 seedlings. (b) Relative expression of SPL10 in roots of 5- and 10-day-old PSPL10::rSPL10 seedlings. The transcript levels were normalized to that of ACTIN2 gene. The expression levels of SPL9 and SPL10 in 5- and 10-day-old roots without IAA treatment were set as 1.0, respectively. (c) Relative GUS expression in roots of 5-day-old PSPL9::GUS and PSPL10::GUS seedlings. The transcript levels were normalized to that of ACTIN2 gene. The expression levels of GUS in PSPL9::GUS and PSPL10::GUS roots without IAA treatment were set as 1.0, respectively.

(d, e) Seedlings of different genotypes were treated with increasing concentrations of IAA on solidified 0.2× MS agar medium placed vertically for 24 h. (d) Relative expression of SPL9 in roots of 5-day-old PSPL9::rSPL9 seedlings. (e) Relative expression of SPL10 in roots of 5-day-old PSPL10::rSPL10 seedlings. The transcript levels were normalized to that of ACTIN2 gene. The expression levels of SPL9 and SPL10 in PSPL9::rSPL9 and PSPL10::rSPL10 roots without IAA treatment were set as 1.0, respectively. Error bars represent standard errors (n = 3). The experiment was repeated twice with similar results.
0.2 × MS agar medium with or without different concentrations of IAA or NPA. The number of lateral roots was increased in WT, P3SS:5MIR156 and rSPLs seedlings with increasing concentrations of IAA from 0.0001 to 10 μM (Figure 8a). P3SS:5MIR156 seedlings showed a consistent two-fold increase in the number of lateral roots compared with WT in response to IAA treatments. By contrast, rSPLs seedlings still had lower numbers of lateral roots than WT in response to IAA treatments (Figure 8a), suggesting that IAA is necessary but not sufficient to rescue the lateral root defects in rSPLs seedlings. In agreement with this finding, we found that the number of lateral roots produced was reduced in all the examined genotypes upon treatment with the polar auxin transport inhibitor NPA (Figure 8b). However, P3SS:5MIR156 seedlings consistently showed a higher number of lateral roots than WT whereas rSPLs seedlings produced fewer or nearly no lateral roots (Figure 8b).

**DISCUSSION**

miR156 and its target genes SPLs have been shown to be important players in plant development but the investigations have largely, if not exclusively, confined to plant aerial organs. For example, miR156/SPLs are known to control plant phase transition, and related traits such as, cell size and number, trichome development, anthocyanin synthesis, leaf morphology and flowering time. Whereas the direct targets of both SPL3 and SPL9 have been well characterized (Schwarz et al., 2008; Wang et al., 2008; Usami et al., 2009; Wu et al., 2009; Yu et al., 2010; Gou et al., 2011), the specific function of SPL10 remain largely undefined. Expression of MIR156A and MIR156C is partially regulated by sugar for precise developmental timing in plants. Yet, the function of other MIR156 genes and whether other endogenous factors also play a role in this developmental transition are largely unexplored (Yang et al., 2013; Yu et al., 2013). In particular, no work has been done so far on the possible role of miR156/SPL modules in the development of underground tissue.

Here, we have analyzed transgenic plants expressing individual MIR156 promoter-GUS fusion genes and uncovered expression of specific MIR156 genes, MIR156B and MIR156D in roots, suggesting a role for these genes in root development. The following lines of evidence support the view that miR156/SPLs modules play a role in lateral root development (Figure 9):

**Figure 9.** A working model for the role of miR156/SPLs modules in lateral root development.

Different SPLs genes are targeted by miR156 to maintain the stability of lateral root development in response to hormone fluctuations, with SPL10 playing a dominant role. Positive feedback loops of SPL9 and SPL10 on miR156 reinforce the proper timing of lateral root development.

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**Figure 8.** Effects of IAA and NPA on root architecture of P3SS:5MIR156 and rSPLs seedlings.

Seeds were germinated and grown for 5 days on solidified 0.2 × MS agar medium, and then transferred to solidified agar plates with or without treatment for further 5 days. (a) Effects of IAA on root system architecture of seedlings in the indicated genotypes. (b) Effects of NPA on root system architecture of seedlings in the indicated genotypes. Error bars represent standard errors (n = 50 seedlings). Asterisk indicates statistical differences, ***P < 0.001; **P < 0.01; *P < 0.05. The experiment was repeated three times with similar results.
Overexpression of miR156 increases and MIM156 decreases lateral root number, respectively, indicating that miR156 plays a positive role in the lateral root developmental program.

Single (spl3 and spl10) and double mutants (spl9 spl15) with deficiencies in specific SPLs produce more lateral roots whereas plants expressing the cleavage-resistant forms of SPL3, SPL9 and SPL10 produce fewer lateral roots. These results from mutants and overexpression plants are consistent with previous finding that SPL transcripts are targeted by miR156.

SPL9 and SPL10 positively modulate miR156 expression in roots. This feedback loop has been proposed to maintain the stability of phase transition (Wu et al., 2009).

Although acting antagonistically, transcription of MIR156B and MIR156D and of SPL9 and 10 are activated by auxin suggesting an interesting link between miR156/SPL modules and auxin signaling in lateral root development.

Spatial and temporal regulation of plant development by miR156/SPLs modules

As miR156 is encoded by eight genomic loci and 11 out of 17 SPLs are targeted by miR156, to elucidate how MIR156 regulates each SPL or several SPLs in a tissue specific manner it is necessary first to examine the specific expression pattern of each gene member. Our MIR156 promoter–GUS analysis provided direct evidence of differential function of miR156 genes both spatially and temporally during plant development. First, MIR156A is highly expressed in the first two pairs of true leaves and decreased precipitously in subsequent leaf pairs, which is consistent with its fundamental role in the regulation of plant phase transition (Wu et al., 2009). Second, MIR156B is only expressed in the bases and flanks of lateral roots whereas MIR156D expression is specifically localized in the meristem area (Figure 1). Conversely, SPL10 is also highly expressed in the initiation sites of lateral roots while SPL9 is specifically expressed in the meristem zone (Figure 2). Note that both MIR156B and SPL10 are expressed at the highest level compared with other members of its respective group. Collectively, these results suggest that MIR156B may be primarily responsible for targeting SPL10 and MIR156D for SPL9 at their respective specific tissues/cell types, and the two miR156s cooperate to finally ensure a rapid regulation of their targets. On the other hand, SPL10 is also expressed in other parts of roots, where it may also be targeted by other MIR156 genes. This issue remains to be resolved by expression of miR156 or MIM156 under specific MIR156 promoters to examine which SPLs are affected.

Xing et al. (2010) reported that miR156-targeted SPLs are required for securing male fertility and examined PMIR156A–GUS, PMIR156D–GUS and PMIR156H–GUS expression patterns in flower tissues in search of the responsible miR156 genes. They found that PMIR156D–GUS is expressed in flowers, an observation confirmed by our results here. Their central focus was on PMIR156H–GUS which is strongly expressed in anthers and ovules but shows decreased expression during anther development; this temporal expression pattern was paralleled by that of the primary MIR156H transcripts. In our work here, we analyzed promoter expression pattern and function of a number of different MIR156 genes and its SPL targets. We showed that expression of each MIR156/SPL module is restricted to specific tissues/cell types for defining accurate developmental timing of lateral root growth. Previous work showed that miR156-targeted SPL genes function in the development of lateral organs in the aerial parts of the plant by regulating leaf initiation rate and leaves emerged from the shoot apical meristem. Our results here extend the role of miR156/SPLs modules to the development of lateral roots as well.

SPL10 and its role in lateral root development

It has been reported that SPL3 can directly bind to LFY, FUL and AP1 to promote flowering whereas SPL9 can positively regulate TCL1 and TRY expression to affect trichome distribution and interact with PAP1 to negatively regulate anthocyanin biosynthesis (Schwarz et al., 2008; Wang et al., 2008; Usami et al., 2009; Wu et al., 2009; Yu et al., 2010; Gou et al., 2011). However, the specific developmental role of SPL10 was hitherto largely undefined. Here, we found that although SPL3, SPL9 and SPL10 were all involved in repressing lateral root development, SPL10 seemed to play a larger role. Several lines of evidence support this view:

(i) The expression level of the SPL10 group was the highest in roots and the promoter activity of SPL10 was quite strong in whole roots (Figure 2).

(ii) PSPL10:rSPL10 seedlings produced the least number of lateral roots even compared with other rSPLs seedlings as plants grow (Figure 3d).

(iii) SPL10 exerted more influence on the expression of MIR156D in roots than SPL9 (Figure 4g).

miR156/SPLs modules and auxin signaling

Auxin is involved in all stages of lateral root development including initiation, patterning and emergence process (De Smet et al., 2007; Péret et al., 2009; Tian et al., 2014). In our work, both SPL9 and SPL10 can increase the expression of MIR156D, suggesting that miR156 and its targets can form negative feedback loops to buffer against small changes of its target and maintain the stability of lateral root growth. Moreover, both MIR156 promoter–GUS activity and primary MIR156 transcript levels showed increased expression in response to auxin and the resistant form rSPLs also showed the same responses. It is interesting to note that auxin induced both miR156 and its target genes.
SPLs in root. Overexpression of SPLs prevented lateral root development (Figure 3). Auxin-mediated induction of miR156 caused a decrease in SPLs transcripts. As unstable mRNAs are less likely to encode stable proteins (Guo et al., 2005), we hypothesize that the concerted decrease in both mRNA and protein levels of SPLs would foster a permissive environment for lateral root growth. The induction of SPLs by auxin would serve as a mechanism to terminate the signaling and reset the regulatory circuit. Collectively, these findings suggest a link between auxin and miR156/SPLs modules in Arabidopsis lateral root development.

Previous studies have shown that SPLs mainly function as activators, for instance, SPL3 activates AP1, FUL1 and LEAFY transcription; SPL9 and SPL10 promote MIR172B expression and SPL9 activates TCL1 and TRY expression (Wu et al., 2009; Yamaguchi et al., 2009; Yu et al., 2010). Conversely, SPL9 has also been reported to act as a negative regulator in anthocyanin biosynthesis (Gou et al., 2011).

It will also be interesting to further identify downstream targets regulated by SPL10 to understand the molecular link between the miR156/SPLs and lateral root development.

EXPERIMENTAL PROCEDURES

Plant materials and growth conditions

Arabidopsis thaliana ecotype Col-0 was used as the wild-type (WT) control for all experiments. Transgenic P35S:rSPL3, PSPL9: rSPL9 and PSPL10:rSPL10 plants were kindly provided by Dr Jia-Wei Wang (Wang et al., 2008). All plants were grown in a growth chamber at 22°C under long-day conditions (16 h light/8 h dark) unless specified as short-day conditions (8 h light/16 h dark).

Preparation of constructs

The Arabidopsis MIR156A was amplified by PCR, cloned into pENTR vector (Invitrogen, http://www.invitrogen.com) and sequenced. Construct carrying a five-copy primary MIR156A gene was generated as described (Niu et al., 2006). Subsequently the five-copy primary MIR156A gene was cloned into the same pENTR construct and then recombined into pBA-DC using the LR Clonase II enzyme (Invitrogen). P35S:MIM156 was generated as described (Franco-Zorrilla et al., 2007). P35S: MIR156 construct was generated in the same way as P35S:SMIM156.

The MIR156 promoter fragments were amplified by PCR using primers covering 2 kbp upstream of the transcription start site. The fragments were cloned into pENTR vector, and then recombined into pKGWFS7 destination vector. The primer sequences are given in Table S1.

Expression analyses

Total RNA was extracted from seedlings or roots with the plant RNaseasy Mini kit (Qiagen, https://www.qiagen.com/). Total RNA was treated with DNase I and reverse-transcribed with oligo(dT) primers and Superscript reverse transcriptase (Invitrogen). Quantitative RT-PCR was performed with SYBR-Green PCR Mastermix (TaKaRa, http://www.takara-bio.com/). ACTIN2 transcripts were used as internal control. The qRT-PCR primers are shown in Table S1. The detection and quantification of mature miR156 using qRT-PCR was performed according to a published protocol (Varkonyi-Gasic et al., 2007). RNA gel blots were performed essentially according to Reyes and Chua (2007).

Root measurements

Seeds were surface sterilized with 50% (v/v) bleach with 0.02% (v/v) Triton X-100 for 10 min, and then washed three times with distilled water. Seeds were germinated and grown on agar plates containing 0.2× MS medium with 1% sugar. For root analysis, 5-day-old seedlings were transferred onto plates placed vertically for observation. All lateral roots were analyzed with a stereo microscope (Olympus DP25, http://www.olympus-global.com/en/). The root length was analyzed by ImageJ software (National Institutes of Health, http://rsb.info.nih.gov/ij/). Lateral root primordia were quantified 8 days after germination according to Malamy and Benfey (1997).

Histochemical analysis

We followed essentially the protocol for GUS staining (Senecoff et al., 1996). For each line and treatment, at least 20 seedlings were analyzed.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article. Figure S1. Enlarged root image of PMIR156D-GUS with staining in root apical meristem. Figure S2. Expression patterns of MIR156E and MIR156F. Figure S3. Relative expression of SPL3, SPL9 and SPL10 in different tissues. Figure S4. Enlarged root image of PSPL9-GUS with staining in stele and quiescent center. Figure S5. Detection of the mature miR156 expression by small RNA blot in roots of 10-d-old seedlings. Figure S6. Quantification of lateral root and leaf numbers in different genotypes. Figure S7. T-DNA insertion mutants. Figure S8. Quantification of lateral root (LR) meristem size in seedlings from different genotypes. Figure S9. Effect of IAA treatments on different PMIR156-GUS lines in roots. Figure S10. The detection of mature miR156 by qRT-PCR after IAA treatment. Figure S11. Effect of IAA treatments on seedling expression of different PMIR156-GUS lines. Figure S12. Relative expression of SPL3 in roots of 5- and 10-d-old P35S:rSPL3 seedlings after IAA treatment. Tables S1. Primers used in this study.
miR156/3-SPLs involved in lateral root development

REFERENCES


