miR156/SPL9 Regulates Reactive Oxygen Species Accumulation and Immune Response in *Arabidopsis thaliana*

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ABSTRACT

The functions of microRNA156 (miR156) and its targeted *SQUAMOSA PROMOTER BINDING PROTEIN-LIKE* (*SPL*) transcription factor genes in plant development have been widely investigated. However, the role of the miR156/*SPLs* regulatory network in plant immune systems remains obscure. Here, we found that the accumulation of reactive oxygen species (ROS) and the transcripts of basal salicylic acid (SA) signaling pathway genes were lower in *Arabidopsis Pro35S:MIR156* seedlings (miR156 overexpression mutants) but higher in *Pro35S:MIM156* (miR156 repression mutants) and *ProSPL9:rSPL9* (*SPL9* overexpression mutants) seedlings compared with wild-type Col-0 plants (WT). As a result, *Pro35S:MIR156* mutants induced greater susceptibility to *Pseudomonas*

syringae pv. *tomato* DC3000 following syringe infiltration than WT, while *Pro35S:MIM156* and *ProSPL9:rSPL9* mutants showed enhanced resistance. In addition, foliar H₂O₂ application resulted in activation of SA-mediated defense response and ablation of miR156-induced susceptibility to *P. syringae* pv. *tomato* DC3000 infection. Collectively, our results provide new insights into the function of the miR156/*SPL* network in *Arabidopsis* immune response by regulating ROS accumulation and activating the SA signaling pathway.

Keywords: *Arabidopsis thaliana*, immune response, miR156, *P. syringae* pv. *tomato* DC3000, reactive oxygen species, SA signaling pathway, *SPL9*.

MicroRNAs (miRNAs) are small, endogenous RNAs that posttranscriptionally repress gene expression by cleaving target mRNAs or repressing their translation (Baulcombe 2004; Nozawa et al. 2012). MicroRNA156 (miR156) is a highly conserved miRNA in plants. It targets and suppresses the expression of the *SQUAMOSA PROMOTER BINDING PROTEIN-LIKE* (*SPL*) transcription factor gene family, which plays a pivotal role in the regulation of a variety of biological processes in plants (Axtell and Bowman 2008; Wang 2016; Wang and Wang 2015). Overexpression of miR156 leads to prolonged expression of juvenile phase, delayed flowering (Chuck et al. 2007; Wang et al. 2009; Xie et al. 2006), shortened plastochrons, and overproduction of leaves (Schwarz et al. 2008; Wang and Weigel 2008). miR156 positively regulates branching and leaf angle in maize and rice (Bai et al. 2012; Chuck et al. 2007; Ishii et al. 2013; Jiao et al. 2010), whereas it negatively regulates tuberization in potato and nodulation in *Lotus japonicus* (Bhogale et al. 2014; Wang et al. 2015). Furthermore, *OsSPL14* and *OsSPL16* enhance grain yield in rice but *LeSPL-CNR* (an *Arabidopsis AtSPL3* ortholog) reduces tuber yield in tomato (Manning et al. 2006; Miura et al. 2010; Wang et al. 2012). The miR156/*SPLs* network also functions in the regulation of secondary

metabolism and abiotic stress responses. miR156-targeted *AtSPL9* negatively regulate the biosynthesis of anthocyanins and sesquiterpenes but positively modulates the accumulation of glucosinolates (Gou et al. 2011; Mao et al. 2017; Yu et al. 2015). The miR156/*SPL* module controls copper homeostasis (*Arabidopsis AtSPL7*, *Physcomitrella patens PpSBP2*, and *Chlamydomonas reinhardtii CRR1*) and responses to drought (*Medicago sativa SPL13*), heat, and cold stress (Arshad et al. 2017; Stief et al. 2014; Yamasaki et al. 2007, 2009; Yang et al. 2017).

miRNAs are critical in plant innate immunity to fungal, bacterial, viral, and nematode infections (Katiyar-Agarwal and Jin 2010; Li et al. 2012). Pathogenic attack always induces or represses the expression of miRNAs, which regulate the expression of key defense genes (Islam et al. 2018; Navarro and Jones 2006). The expression of miR156 has been reported to be repressed in loblolly pine infected by *Cronartium quercuum* (Lu et al. 2007). *SPL6* (*AtSPL6* and *NbSPL6*) has been found to positively regulate innate immunity in *Arabidopsis* and *Nicotiana benthamiana*, whereas the *Arabidopsis AtSPL14* induces fungal toxin *fumonisin B1* susceptibility and *AtSPL9* negatively regulates insect resistance (Mao et al. 2017; Padmanabhan et al. 2013; Stone et al. 2005). These findings imply that miR156 and *SPLs* might play a role in plant innate immunity, yet our knowledge of the immunity mediated by the miR156/*SPL* network is incomplete and requires extensive investigation.

Reactive oxygen species (ROS) are continually generated as a consequence of the normal metabolism of aerobic organisms (Gechev et al. 2006). Regulation of the accumulation and release of ROS is important, because high levels of ROS cause cell death and low levels regulate stress responses in plants (Camejo et al. 2016; Miller et al. 2010). ROS plays critical roles as signal molecules during plant–pathogen interactions (Camejo et al. 2016). miRNAs control responses to environmental stresses and viral resistance by

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regulating cellular ROS levels (Lu et al. 2011; Wu et al. 2017). miRNAs also regulate the production and signaling pathways of various hormones, directly or indirectly. In turn, these hormones modulate plant immunity and defense responses to invading pathogens by activating the expression of various defense-responsive genes (Islam et al. 2018; Navarro and Jones 2006; Vidhyasekaran 2015; Zhang et al. 2011). A recent study reported that *AtSPL9* negatively regulates jasmonic acid (JA) response (Mao et al. 2017). Based on these findings, we hypothesized that ROS and hormone pathways play a role in the immunity mediated by miR156 and its *SPL* target.

To test our hypothesis, three *Arabidopsis* transgenic plants—*Pro35S:MIR156* (miR156 overexpression mutant), *Pro35S:MIM156* (miR156 repression mutant), and *ProSPL9:rSPL9* (*SPL9* [one of the *SPLs*] overexpression mutant) (Wang and Weigel 2008)—were used to determine whether the miR156/*SPL9* module affects ROS accumulation, the expression of salicylic acid (SA) response genes, and responses to bacterial challenge. The analysis indicated that the miR156/*SPL9* module controls innate immunity by regulating ROS accumulation and activating of the SA signaling pathway in *Arabidopsis*.

MATERIALS AND METHODS

Plant materials and growth conditions. Three mutants—*Pro35S:MIR156* (Wang and Weigel 2008), *Pro35S:MIM156* (Franco-Zorrilla et al. 2007; Wang and Weigel 2008), and *Pro35S:rSPL9* (Wang and Weigel 2008)—along with wild-type *Arabidopsis thaliana* ecotype Columbia (Col-0) were used in this study.

Surface-sterilized seed were germinated in 1/2 Murashige and Skoog medium (pH 5.7) supplemented with 4% sucrose, 1% agar, and 0.05% morpholineethanesulfonic acid. Plated seedlings were grown in a growth chamber at 22°C with short days (photoperiod of 8 h of light and 16 h of darkness), 60% relative humidity, and light intensity of 600 mmol photon m⁻²s⁻¹. The 1-week-old seedlings were sown in plastic pots (9 seedlings/pot) with commercial nutrient soil. These pots were kept in an incubator under the conditions described above. The 5-week-old plants grown with short days (8 h of light and 16 h of darkness) were considered juvenile (Supplementary Fig. S1) (Telfer et al. 1997).

Histochemical staining of O₂ and hydrogen peroxide. To evaluate the in situ accumulation of O₂ and hydrogen peroxide (H₂O₂), histochemical staining was performed using nitroblue tetrazolium (NBT) and 3,3'-diaminobenzidine (DAB), as previously described (Jabs et al. 1996; Torres et al. 2002), with some modifications. Briefly, 1-week-old seedlings were vacuum infiltrated with 1 mM NBT (Sigma-Aldrich) in 0.01 M phosphate buffer solution (PBS, pH 7.0) or DAB (Sigma-Aldrich) at 1 mg/ml with sodium citrate (pH 3.8), then incubated at room temperature in the dark for 2 or 24 h, respectively. Seedlings were washed with 75% (vol/vol) ethanol to bleach out chlorophyll for 10 min at 70°C. The washed seedlings were mounted in 20% glycerol and photographed using a stereoscope.

Detection of H₂O₂ and NADPH oxidase activity in seedling extracts. H₂O₂ content and NADPH oxidase activity in 1-week-old seedling and 5-week-old plant leaves extracts were determined. First, 200 mg of fresh seedlings and total juvenile plant leaves were ground with liquid nitrogen and extracted with 5 ml of ice-cold PBS (pH 7.5) containing 2% polyvinyl pyrrolidone. After incubation on ice for half an hour, the extractions were centrifuged at 12,500 × g at 4°C for 20 min and supernatants were collected for use. To determine the H₂O₂ content, an Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit (Invitrogen) was used according to the manufacturer's protocol. The absorbance was measured at 560 nm using a microplate reader (SpectraMax M5) and H₂O₂ was quantified according to the standard curve prepared with known concentrations of H₂O₂. To determine NADPH oxidase activity, an aliquot of the isolated supernatant was added to a reaction mixture (NADPH at 0.1 mmol/liter, NBT at 0.1 mmol/liter, sucrose at 250 mmol/liter, and Tris-HCl at 50 mmol/liter, pH 7.4) according to methods described by Gestelen (1977). The activity was calculated as the rate of decrease in the absorbance of NADPH at 530 nm with an absorption extinction coefficient of 6.22 mM⁻¹ cm⁻¹.

Total RNA extraction and quantitative real-time polymerase chain reaction. To analyze changes in mRNA levels, quantitative real-time polymerase chain reaction (RT-qPCR) was performed according to a previous report (He et al. 2017). Total RNA from seedlings and the total juvenile plant leaves were extracted using the TRIzol protocol (Invitrogen) in accordance with the manufacturer's instructions. Total RNA (1 µg) was synthesized to first-strand cDNA using a HiScript II Q RT for qPCR (+gDNA viper) kit (vazyme). RT-qPCR was performed on a QuantStudio 6 Flex Real-Time PCR System (Applied Biosystems) using a ChamQ SYBR qPCR Master Mix kit (vazyme), following the supplier's protocol. The RT-qPCR conditions were 95°C for 3 min followed by 40 cycles of 95°C for 15 s, 60°C for 15 s, and 72°C for 20 s. The mRNA expression levels were normalized against the expression of housekeeping gene *ACTIN2* (AT3G18780), and the fold change was calculated by the comparative cycle threshold method (2^{-ΔΔC_t} method) (Livak and Schmittgen 2001). At least three biological replicate samples were used. Differences were considered significant at *P* ≤ 0.05. The primers used in this study are listed in Table 1.

Antioxidant enzyme extraction and activity assay. The seedling and total juvenile plant leaf extracts for determination of antioxidant enzyme activity were prepared as described above. Superoxide dismutase (SOD) activity was assayed by measuring the photochemical reduction rate of NBT at an absorbance of 560 nm (Stewart and Bewley 1980). Catalase (CAT) activity was tested by detecting the degradation rate of H₂O₂ at an absorbance of 240 nm (Patra et al. 1978). Glutathione reductase (GR) activity was determined by measuring the dehydrogenation rate of NADPH at an absorbance of 340 nm (Foyer and Halliwell 1976). Monodehydroascorbate reductase (MDAR) activity was determined by measuring a decrease in absorbance at 340 nm due to NADH oxidation (Hossain et al. 1984). dehydrosacorbate reductase (DHAR) activities were qualified by the reduction rate of

TABLE 1. Primers used in this article

Gene ID	Primer name	Forward primer (5' to 3')	Reverse primer (5' to 3')
AT3G54660	<i>GR2</i>	CAAAGACTCCTCTGAGGGAAAG	GGGCTCCAATGCCGTAAATA
AT3G09940	<i>MDAR3</i>	TCAAGAGGAACACAAGGCTATT	CAAGACCCAATGTCTCAAAG
AT1G63940	<i>MDAR6</i>	GTTCCAGCTTCTACCAAGCTA	TCCTTCTCTAACTCTGTAGAGC
AT4G35090	<i>CAT</i>	TGTCATTGTCCGGTTCTCCA	GGTGGTGGGAGAAGAAGTCA
AT2G14610	<i>PR1</i>	TGCTCTTGTCTTCCTCGA	CTAACCACATGTTACCGGC
AT3G57260	<i>PR2</i>	AGAGATGGTGTGAGATTCC	GTGGTGTGAGTGGCTATA
At3G04720	<i>PR4</i>	TCATACACAGTGGCTACG	GTTGGTCGGAGAACAAGTA
AT1G75040	<i>PR5</i>	TCTCTTCTCGTGTTCATC	AAGCACCTGGAGTCAATT
AT1G64280	<i>NPR1</i>	CCGATGTCACCATAGGA	CATTCACCGCCATAGTG
AT5G45110	<i>NPR3</i>	AAGTTGTTGCCGAGATTC	AATGAAGAACCGTGTAACC
AT4G19660	<i>NPR4</i>	CTAACTCCACCTCCATCAA	TATCAGAACCTCATAACAAG
AT3G18780	<i>ACTIN2</i>	TGTTCCAGCCCTCGTTTGTGGG	TGCTGCTTGGTGCAAGTGCTGT

dehydroascorbate (DHA) at an absorbance of 265 nm (Nakano and Asada 1981). Peroxidase (POD) activity was determined by the absorbance of H_2O_2 oxidation at 470 nm (Chance and Maehly 1955). All optical density detections were performed on a SpectraMax M5 microplate reader.

SA measurements. The total leaves of 5-week-old plants were collected, ground into a powder in liquid nitrogen, and then used for hormone extraction and analysis as described previously (Fu et al. 2012), with some modifications. The internal standard $^2\text{H}_4$ -SA (140 pmol) was added to 200 mg of ground powder. The powder was

extracted with 2 ml of methanol and further purified by an Oasis mode anion exchange solid-phase extraction column (Waters). The eluate was analyzed by ultraperformance liquid chromatography–electrospray ionization tandem mass spectrometry (ACQUITY UPLC H-Class and Xevo TQ-S; Waters). Three biological replicates were used, each of which consisted of at least five pooled plants.

Bacterial growth assay. Using the *Pseudomonas syringae* pv. *tomato* DC3000 strain, bacterial challenge was performed as described previously (Whalen et al. 1991). Briefly, overnight cultured bacterial cells were collected, washed, resuspended, and

Fig. 1. Reactive oxygen species accumulation in the miR156/SPL9 module. **A**, In situ detection of leaf O_2 and H_2O_2 . Nitroblue tetrazolium (NBT) and 3,3'-diaminobenzidine (DAB) stains were used to detect the presence of O_2 and H_2O_2 in *Pro35S:MIR156*, *Pro35S:MIM156*, and *ProSPL9:rSPL9* mutants and wild-type Col-0 seedlings. The 1-week-old seedlings were collected and stained. The horizontal bar represents 2 mm. **B** and **C**, Quantitative measurements of H_2O_2 level and NADPH oxidase activity in Col-0 and mutant seedlings. Data represent means $\pm$ standard deviation from three biological replicates. Different letters at the top of each column indicate significant differences at $P \leq 0.05$ by Fisher's least significant difference tests. FW = fresh weight.

adjusted to a final concentration of 1×10^8 CFU ml⁻¹ in sterilized MgCl₂ (10 mmol/liter). Leaves 1 and 2 of 5-week-old *Arabidopsis* were syringe infiltrated with the bacteria. At least five of each mutant plant were used, and 10 mM MgCl₂ was used as the mock control. The inoculated plants were kept at 100% humidity overnight, then raised in a growth chamber under the conditions described above for 3 more days. Disease symptoms in *Arabidopsis* leaves were observed and photographed. Using the methods described by Katagiri et al. (2002), the number of bacterial populations in four different plants of each genotype was determined. Leaf disks with disease symptoms were ground and suspended in 1 ml of sterilized MgCl₂ (10 mM). Leaf extracts were 10-fold serially diluted and plated onto King's B medium supplemented with rifampicin at 50 mg/liter. After incubation at 28°C for 2 days, we counted the CFU grown on the plates. To further investigate the role of ROS in miR156/SPL9 module-mediated responses to bacteria, wild-type Col-0 (WT) and *Pro35S:MIR156* leaves were sprayed with 10 mM H₂O₂ (Sigma) in 0.1% Triton X-100 12 h before being syringe infiltrated with *P. syringae* pv. *tomato* DC3000; 0.1% Triton X-100 was used as the mock control.

Statistical analysis. Differences were analyzed using a one-way analysis of variance with Fisher's least significant difference tests. A *P* value ≤ 0.05 was considered statistically significant. All analyses were performed using ORIGIN 8.

RESULTS

Changes in H₂O₂ levels caused by the miR156/SPL9 module. ROS act as a second messenger in pathogen interactions and miRNA responses (Camejo et al. 2016; Islam et al. 2018). To determine whether the miR156/SPL9 module affects H₂O₂ levels in *Arabidopsis*, we attempted to detect in situ accumulation of O₂ and H₂O₂ using NBT and DAB staining methods, respectively. Both methods produced lower levels of staining in *Pro35S:MIR156* seedlings compared with that in WT seedlings (Fig. 1A). To exclude the possibility that this was an indirect effect of other miRNAs, we utilized *Pro35S:MIM156* plants in which miR156 activity is specifically reduced by constitutive expression of a target mimic (Franco-Zorrilla et al. 2007; Wang and Weigel 2008). Higher levels of staining were observed in *Pro35S:MIM156* seedlings relative to that in WT (Fig. 1A). *ProSPL9:rSPL9*, which is an *SPL9* overexpression mutant but insensitive to miR156 action (Wang and Weigel 2008), displayed similar results to *Pro35S:MIM156* (Fig. 1A). Similar effects of miR156 and its *SPL9* target on H₂O₂

content in seedlings were also observed (Fig. 1B). To confirm whether H₂O₂ accumulation is affected by increased activity of NADPH oxidase, we measured the activity of NADPH oxidase in extracts of WT and mutant seedlings. Compared with WT plants, NADPH oxidase activity was significantly lower in *Pro35S:MIR156* plants but higher in *Pro35S:MIM156* and *ProSPL9:rSPL9* plants (Fig. 1C). These results indicate that the miR156/SPL9 module plays a role in modulating ROS accumulation.

Involvement of H₂O₂ in the miR156/SPL9 module. To explore the underlying molecular mechanisms of the miR156/SPL9 module in H₂O₂ accumulation, we firstly examined the expression of genes involved in antioxidant activity using RT-qPCR. Three antioxidant genes (*GR*, *CAT*, and *MDAR6*) were downregulated in *Pro35S:MIR156* seedlings but upregulated in *Pro35S:MIM156* and *ProSPL9:rSPL9* seedlings compared with that in WT (Fig. 2). An upregulation of *MADR3* was observed only in *Pro35S:MIM156* seedlings, whereas minimal changes in expression was detected in *Pro35S:MIR156* and *ProSPL9:rSPL9* seedlings (Fig. 2).

We also examined the activity of antioxidant enzymes. The activities of SOD, CAT, MDAR, and GR were significantly lower in *Pro35S:MIR156* plants but much higher in *Pro35S:MIM156* and *ProSPL9:rSPL9* plants than that in WT (Fig. 3). POD activity was suppressed in *Pro35S:MIR156* seedlings but enhanced in *Pro35S:MIM156* plants, while DHAR activity was elevated in *Pro35S:MIM156* and *ProSPL9:rSPL9* plants. These results further support the role of the miR156/SPL9 module in ROS accumulation.

Involvement of the SA-signaling pathway in the miR156/SPL9 module. Various hormone pathways have been reported to be involved in miRNA responses (Islam et al. 2018; Vidhyasekaran 2015). H₂O₂ and O₂ accumulation is always associated with the induced expression of SA-induced pathogenesis-related (PR) proteins in plants (Klessig and Malamy 1994). Based on these reports and the level changes in ROS accumulation detected above, we investigated the expression of SA-responsive genes by RT-qPCR. The expression levels of *PR1* and *PR2* were significantly downregulated in *Pro35S:MIR156* seedlings but upregulated in *Pro35S:MIM156* and *ProSPL9:rSPL9* seedlings compared with that in WT (Fig. 4A). Significant downregulation and upregulation of *PR5* were observed in *Pro35S:MIR156* and *Pro35S:MIM156* seedlings, respectively, whereas upregulation of *PR4* was only observed in *Pro35S:MIM156* seedlings (Fig. 4A).

The expression of nonexpresser of *PR* (*NPR*) genes, which have been reported to be required for SA-induced *PR* gene expression (Cao et al. 1997; Vernooij et al. 1994), was also detected. As

Fig. 2. Expression of antioxidant genes in the miR156/SPL9 module. The 1-week-old seedlings were collected and used to examine the mRNA expression levels of antioxidant genes by quantitative real-time polymerase chain reaction. *ACTIN2* was used as the internal reference gene. Data represent means $\pm$ standard deviation from three biological replicates; an asterisk (*) indicates significant differences between wild-type and mutant plants at *P* ≤ 0.05 by Fisher's least significant difference tests. GR = glutathione reductase, CAT = catalase, and MDAR = monodehydroascorbate reductase.

expected, the expression levels of *NPR1* and *NPR3* were lower in *Pro35S:MIR156* seedlings but higher in *Pro35S:MIM156* and *ProSPL9:rSPL9* seedlings relative to that in WT (Fig. 4). Significant downregulation and upregulation of *NPR5* were observed in *Pro35S:MIR156* and *Pro35S:MIM156* mutants, respectively, whereas only small differences in expression were observed in *ProSPL9:rSPL9* seedlings (Fig. 4B). In addition, we observed that the same expression patterns of SA-responsive genes were present in juvenile leaves of the indicated mutants plants (Fig. 4C and D). Taken together, our results suggest that the miR156/SPL9 module controls the activation or inactivation of the SA signaling pathway.

Effect of the miR156/SPL9 module in disease response.

The elevated production of ROS and activation of the SA signaling pathway implicates an immune function of the miR156/SPL9 module. To test this, a virulent *P. syringae* pv. *tomato* DC3000 strain (Whalen et al. 1991) was chosen to determine whether the miR156/SPL9 module affects plant responses to bacterial infection. We inoculated WT and transgenic plants with *P. syringae* pv. *tomato* DC3000 by

syringe infiltration, and the progress of infection was observed over 4 days postinoculation (dpi). *Pro35S:MIR156* leaves displayed a broader area with disease symptoms compared with WT (Fig. 5A). In contrast, *Pro35S:MIM156* and *ProSPL9:rSPL9* leaves displayed milder symptoms compared with WT. To confirm whether bacterial proliferation inside the plants was in accordance with disease symptoms, bacterial growth was measured in WT and mutant plants at 4 dpi. Consistent with the visible symptoms, *Pro35S:MIR156* plants exhibited significantly higher bacterial population than WT at 4 dpi, whereas *Pro35S:MIM156* and *ProSPL9:rSPL9* plants exhibited significantly lower bacterial proliferation (Fig. 5B). These results indicate that *Pro35S:MIR156* is more susceptible to *P. syringae* pv. *tomato* DC3000, while *Pro35S:MIM156* and *ProSPL9:rSPL9* are more resistant to *P. syringae* pv. *tomato* DC3000 than WT.

To confirm that the SA signaling pathway mediated by the miR156/SPL9 module was involved in the immune system responses, we evaluated the levels of *PR1* and *NPR1* transcripts. After bacterial challenge, the *PR1* and *NPR1* transcript levels were

Fig. 3. Activity of antioxidant enzymes in miR156/SPL9 module. The 1-week-old seedlings were collected and used to determine the activities of antioxidant enzymes. Data represent means $\pm$ standard deviation from three biological replicates. SOD = superoxide dismutase, CAT = catalase, GR = glutathione reductase, MDAR = monodehydroascorbate reductase, POD = peroxidase, and DHAR = dehydroascorbate reductase. Different letters at the top of each column indicate significant differences at $P \leq 0.05$ by Fisher's least significant difference tests. FW = fresh weight.

increased 750- and 32-fold, respectively, in WT (Fig. 5C and D). The levels of these genes in *Pro35S:MIR156* (410- and 17-fold, respectively) were much lower whereas, in *Pro35S:MIM156* (1,368- and 103-fold, respectively) and *ProSPL9:rSPL9* (1,208- and 81-fold, respectively), these levels were dramatically increased compared with that in WT (Fig. 5C and D). Consequently, the shift in resistance ability of the indicated mutants to bacterial challenge is likely due to suppression or activation of the SA signaling pathway.

Ablation of miR156-mediated bacterial susceptibility by H₂O₂. To further investigate the role of ROS in the miR156/SPL9 module-mediated immunity to bacteria, we treated WT and *Pro35S:MIR156* leaves with 10 mM H₂O₂; 0.1% Triton X-100 was used as a mock control and, 12 h after treatment, leaves were syringe infiltrated with *P. syringae* pv. *tomato* DC3000. Four days later, we observed milder leaf symptoms and detected lower levels of bacterial population in WT and *Pro35S:MIR156* plants treated with H₂O₂ compared with the mock control (Fig. 6). However, *Pro35S:MIR156* plants still exhibited significantly higher levels of bacterial growth compared with WT (Fig. 6B). These results suggest that H₂O₂ enhanced resistance in *Arabidopsis* and masked the effect of miR156 on susceptibility to *P. syringae* pv. *tomato* DC3000 infection.

To determine whether the effect of ROS in responses to bacterial infection is related to the SA signaling pathway, we treated WT and *Pro35S:MIR156* plants with H₂O₂ for 12 h. The expression of SA signaling genes was significantly increased in the treated plants compared with the mock controls (Fig. 7). In *Pro35S:MIR156* plants, the transcript levels of SA-responsive genes were less affected than that in WT after H₂O₂ treatment (Fig. 7). These results suggest that the ablation of miR156-induced susceptibility to *P. syringae* pv. *tomato* DC3000 infection by H₂O₂ was due to activation of the SA signaling pathway.

DISCUSSION

Recent studies have revealed a number of miRNAs involved in responses to pathogens, and artificial miRNAs have been designed as new strategies for disease control (Islam et al. 2018). Overexpression of miR160a and miR398b enhances resistance to blast

fungus *M. oryzae* in rice, and overexpression of miR393 increases resistance to *P. syringae* in *Arabidopsis* (Li et al. 2013; Navarro and Jones 2006). However, upregulation of miR773 and miR398b induce susceptibility to *P. syringae* pv. *tomato* DC3000 in *Arabidopsis*, and upregulation of miR528 also induces susceptibility to *Rice strip virus* in rice (Li et al. 2013; Wu et al. 2017). Furthermore, suppression of nbe-miR166h-p5 attenuated the leaf yellowing symptom of *Turnip mosaic virus* and *Potato virus X* in *N. benthamiana*, and decreased viral accumulation (Wang et al. 2018). These findings imply that miRNAs play a complex role in regulating immunity depending on the type of pathogen and plant species involved. Here, we found that overexpression of miR156 (*Pro35S:MIR156* plants) increased susceptibility to *P. syringae* pv. *tomato* DC3000 infection, while interference with miR156 activity by target mimics (*Pro35S:MIM156* plants) conferred considerable resistance to bacterial attack (Fig. 5A and B). Therefore, miR156 might play a negative role in regulating plant immunity. Furthermore, we demonstrated that upregulation of the miR156 target gene *SPL9* (*ProSPL9:rSPL9* plants) elevated resistance to *P. syringae* pv. *tomato* DC3000 infection (Fig. 5A and B), which is consistent with the previously reported positive immune function of *AtSPL6* and *NbSPL6* (Padmanabhan et al. 2013). However, their specific mechanisms may be different. Nucleotide-binding domain leucine-rich repeats interact with NbSPL6 only during an active defense response, whereas AtSPL6 acts only against the bacterial pathogen *P. syringae* expressing the AvrRps4 effector but not avrRpm1 and avrRpt2 effectors (Padmanabhan et al. 2013). Our data showed that *ProSPL9:rSPL9* plants exhibited constitutive systemic acquired resistance, including elevated ROS levels and increased SA signaling pathway transcripts, resulting in an enhanced resistance of *P. syringae* pv. *tomato* DC3000 infection (Figs. 1, 2, 3, 4, and 5; Supplementary Figs. S2, S3, and S4). In plants, age-related resistance is a common phenomenon, because old and mature plants may display enhanced resistance to attacks by pathogens and herbivores (Kus et al. 2002; Price 1991). *SPL* transcripts gradually increase during the transition to flowering due to the decline of miR156 levels along with plant growth (Wang et al. 2009; Wu and Poethig 2006; Wu et al. 2009). Cumulative accumulation of glucosinolates compensates for miR156-targeted

Fig. 4. Expression of salicylic acid (SA) signaling genes in miR156/SPL9 module. The 1-week-old seedlings and 5-week-old leaves were collected and used to examine the mRNA expression levels of SA-signaling genes by quantitative real-time polymerase chain reaction. *PR* = pathogenesis related and *NPR* = nonexpresser of *PR* genes. Data are shown as relative expression levels of indicated mutants in comparison with the wild-type Col-0 plants. *ACTIN2* was used as the internal reference gene. Data represent $\pm$ standard deviation from three biological replicates; an asterisk (*) indicates significant differences between wild-type and mutant plants at $P \leq 0.05$ by Fisher's least significant difference tests.

SPL9 regulated age-dependent decline of JA-induced insect resistance in adult plants (Mao et al. 2017). Therefore, the age-related miR156-targeted *SPL9* may also contribute to the immunosenescence in plants.

According to the previous studies, miRNAs are involved in regulating ROS release (Jia et al. 2017; Lu et al. 2011; Wu et al. 2017), and elevated ROS accumulation is observed following abiotic (drought, cold, heat, high-light, wound, and heavy-metal) and biotic (bacteria, fungi, and viruses) stress in plants (Camejo et al. 2016; Gechev et al. 2006; Yang et al. 2017). Under cold stress, *N. benthamiana* leaves transiently overexpressing miR156 exhibit lower H₂O₂ accumulation (Yang et al. 2017). In apple, the *MdMIR156a5* and *MdMIR156a12* transcriptional regulatory network controls the vegetative phase change in response to the plastid-nucleus redox signals such as glutathione (GSH). However, *MdMIR156a6*-overexpressing or *miR156*-mimetic transgenic *N. benthamiana* showed no regular differences in ROS and GSH

levels (Du et al. 2015; Jia et al. 2017). In this work, we found that overexpression of miR156 could reduce ROS accumulation, whereas specific repression of miR156 activity increased ROS levels (Fig. 1), indicating that miR156 may negatively regulate ROS levels in *Arabidopsis*. Similarly, previous reports have documented that miR398 and miR528 also negatively regulate ROS levels by targeting and repressing expression of their target genes (Leng et al. 2017; Lu et al. 2011; Wu et al. 2017). Cu or Zn superoxide dismutases (*CSD1* and *CSD2*), targets of miR398, catalyze the conversion of O₂⁻ to H₂O₂ and O₂, directly detoxifying ROS generated in the cytoplasm and chloroplasts (Lu et al. 2011; Perry et al. 2010). L-ascorbate oxidase, a target of miR528, plays a direct role in regulating basal ROS levels by oxidizing apoplastic L-ascorbic acid (Pignocchi et al. 2006; Wu et al. 2017). Our data showed that overexpression of *SPL9* increased levels of O₂⁻ and H₂O₂ observed by situ staining, higher H₂O₂ content, and elevated NADPH oxidase activity (Fig. 1), suggesting that *SPL9* may play a

Fig. 5. Effect of miR156/SPL9 module in response to bacteria infection. **A**, Disease symptoms on *Arabidopsis* leaves caused by *Pseudomonas syringae* pv. *tomato* DC3000 infection. Leaves were 5 weeks old and syringe infiltrated with a bacterial suspension (1×10^8 CFU in sterilized 10 mM MgCl₂) of *P. syringae* pv. *tomato* DC3000. MgCl₂ (10 mM) without bacteria was syringe infiltrated as mock control. Photos of the disease symptoms were taken after 4 days postinoculation (dpi). The white bar represents 1 centimorgan. **B**, Number of CFU after 4 dpi. Different letters at the top of each column indicate significant differences at $P \leq 0.05$ by Fisher's least significant difference tests. **C**, *PR1* and **D**, *NPR1* expression in the Col-0 and mutants after infection. Error bars represent the standard error of three biological repeats.

positive role in regulating ROS accumulation. In addition, we also observed the opposite effects of miR156 and its *SPL9* target on the expression and activity of antioxidant enzymes (Figs. 2 and 3). Plants have different ROS level at different ages (Camejo et al. 2016; Gechev et al. 2006). Consistent with previous studies (Du et al. 2015; Jia et al. 2017), we found that the levels of ROS in juvenile leaves were higher than those in young seedlings (Figs. 1, 2, and 3). Furthermore, our data showed that the levels of ROS accumulation in juvenile WT plants and indicated mutants displayed the same change patterns as those in young seedlings (Figs. 1, 2, and 3). Thus, the negative regulation of miR156 on ROS accumulation may depend on the transcription function of *SPL9* indirectly but not directly on miR156 itself.

ROS has been demonstrated to be involved in responses to plant pathogen invasion (Camejo et al. 2016; Klessig and Malamy 1994). By inducing defense-related gene expression, increased H_2O_2 accumulation causes enhanced resistance to *Erwinia carotovora* subsp. *carotovora*, *Phytophthora infestans* race 0, *P. capsici*, and

Pseudomonas syringae pv. *tomato* DC3000 infection (Wang et al. 2013; Wu et al. 1995). ROS accumulation has also been shown to be directly modulated by miRNAs and to contribute to defenses against pathogen infection (Pignocchi et al. 2006; Wu et al. 2017). Here, we demonstrated that levels of ROS accumulation are positively correlated with susceptibility to *P. syringae* pv. *tomato* DC3000 (Figs. 1, 2, 3, and 5). We also proved that exogenous application of H_2O_2 attenuated leaf symptoms and reduced bacterial proliferation during *P. syringae* pv. *tomato* DC3000 infection (Fig. 6). In addition, we confirmed that miR156 suppressed the ability of H_2O_2 to enhance resistance to bacterial infection (Fig. 6). Although the details of *SPL9* on regulating ROS accumulation require further investigation, our data suggest that ROS-mediated defense may, at least partially, contribute to the miR156/*SPL9* module-regulated immune system.

miRNAs have also been reported to target genes involved in hormone pathways such as JA and auxin pathways (Mao et al. 2017; Navarro and Jones 2006; Zhang et al. 2011, Zhang et al. 2016).

Fig. 6. Effect of foliar H_2O_2 application on *Pseudomonas syringae* pv. *tomato* DC3000 infection in Col-0 and *Pro35S:MIR156* plants. **A**, Disease symptoms in *Arabidopsis* leaves caused by *P. syringae* pv. *tomato* DC3000 infection. The 5-week-old leaves were sprayed with 10 mM H_2O_2 or 0.1% Triton X-100 (mock control) for 12 h, then syringe infiltrated with a bacterial suspension (1×10^8 CFU in sterilized 10 mM $MgCl_2$) of *P. syringae* pv. *tomato* DC3000. Photos of the disease symptoms were taken after 4 days postinoculation (dpi). The white bar represented 1 centimorgan. **B**, Number of CFU after 4 dpi. Different letters at the top of each column indicate significant differences at $P \leq 0.05$ by Fisher's least significant difference tests.

Fig. 7. Effect of foliar H_2O_2 treatment on salicylic acid-dependent gene expressions in Col-0 and *Pro35S:MIR156* plants. The 5-week-old leaves were sprayed with 10 mM H_2O_2 or 0.1% Triton X-100 (mock control). After 12 h, treated leaves were collected and used for RNA extraction. *PR* = pathogenesis related and *NPR* = nonexpresser of *PR* genes. Data are shown as relative expression levels of H_2O_2 -treated plants in comparison with the mock control plants. Actin was used as the internal reference gene. Data represent $\pm$ standard deviation from three biological replicates; an asterisk (*) indicates significant differences between wild-type and mutant plants at $P \leq 0.05$ by Fisher's least significant difference tests.

miR319 decreases endogenous JA levels and downregulates expression of JA biosynthesis- and signaling-related genes in rice (Zhang et al. 2016) by targeting *TEOSINTE BRANCHED/CYCLOIDEA/PCsF* genes. miR156-targeted *SPL9* negatively regulates JA response through interaction with JAZ proteins in *Arabidopsis* (Mao et al. 2017). It has been well known that cross-talk between SA and JA pathways displays antagonistic effects on pathogen attack (Kim et al. 2013; Petersen et al. 2000; Spoel et al. 2003). *Arabidopsis NahG* plants, which are defective in accumulating SA, produce higher JA levels and expression of JA-responsive genes upon *P. syringae* pv. *tomato* DC3000 infection (Spoel et al. 2003). In contrast, the *mpk4* mutant, exhibiting elevated SA levels and constitutive *PR* gene expression but decreased JA-responsive gene expression, increases resistance to *P. syringae* pv. *tomato* DC3000 infection (Petersen et al. 2000). Here, we found suppression of *PR* gene transcripts in the *Pro35S:MIR156* mutants but significant increases in their expression in the *Pro35S:MIM156* and *ProSPL9:rSPL9* plants (Fig. 4). The expression of *NPR* genes, master regulators of SA-induced *PR* gene expression (Ding et al. 2018), displayed the same changes in expression as the *PR* genes in indicated plants (Fig. 4). Unexpectedly, we observed no significant effect of miR156/SPL9 module on SA accumulation (Supplementary Fig. S5). Nevertheless, we found that suppression SA signaling transcripts in *Pro35S:MIR156* plants caused susceptibility to the *P. syringae* pv. *tomato* DC300 pathogen, whereas activation of the SA pathway in *Pro35S:MIM156* and *ProSPL9:rSPL9* plants enhanced pathogen resistance (Figs. 4 and 5A and B). In addition, we also demonstrated that the resistance ability of the indicated mutants was accompanied by different transcript levels of *PR1* and *NPR1* following bacterial challenge (Fig. 5C and D). These results are similar to those of previous studies, which found that elevated expression of SA marker genes such *PR1* and *NPR1* is often associated with enhanced disease resistance in *Arabidopsis* (Chen et al. 1993; Hondo et al. 2007; Rogers and Ausubel 1997; Wu et al. 2012). Taken together, the results suggest that activation of SA signaling and suppression of JA signaling play an important role in the miR156/SPL9 module-mediated immune defense to pathogen infection, which also provides new evidence that plants infected by SA-inducing biotrophic pathogens often suppress JA-dependent defense responses (Spoel et al. 2007; Vidhyasekaran 2015).

A recent study reported crosstalk between ROS- and SA-dependent pathways, leading to pathogen resistance (Karpiński et al. 2013). Similarly, our data analysis revealed that the levels of ROS accumulation were in keeping with the basal expression of SA signaling pathway genes (Figs. 1, 2, 3, and 4), indicating a tight interaction between these two independent pathways. To further investigate the role of ROS and SA signaling pathways in miR156/SPL9 module-mediated defense responses to bacterial infection, we treated WT and *Pro35S:MIR156* plants with exogenous H₂O₂. H₂O₂ treatment ablated miR156-induced *P. syringae* pv. *tomato* DC3000 susceptibility (Fig. 6). Exogenous H₂O₂ treatment efficiently induced the expression of SA signaling pathway genes (Fig. 7). In addition, the induced levels of SA-dependent gene transcripts evoked by exogenous H₂O₂ are positively correlated with plant defense to *P. syringae* pv. *tomato* DC3000 infection (Figs. 6 and 7). These data indicate that H₂O₂-mediated defense can ablate miR156-induced susceptibility to *P. syringae* pv. *tomato* DC3000 infection by activating the SA signaling pathway. This follows the pattern seen in recent studies that ROS acts synergistically with the SA pathway during pathogen infection (Camejo et al. 2016; Gechev et al. 2006; Vidhyasekaran 2015). SA inhibits CAT activity and thereby causes an increase in H₂O₂ levels, triggering the expression of defense-related genes during pathogen invasion (Klessig and Malamy 1994). In tobacco, SA enhances ROS levels by binding to an SA-binding protein, SABP3, to mediate a hypersensitive response to disease (Slaymaker et al. 2002). Enhanced ROS levels have also been reported to induce SA-induced *PR* gene expression and trigger resistance to pathogen invasion (Chaouch et al. 2010;

Rojas et al. 2012). In *cat2* mutants overproducing H₂O₂, SA accumulation is induced and signaling pathways are activated in response to *P. syringae* pv. *tomato* DC3000 infection (Chaouch et al. 2010). In *N. benthamiana* and *Arabidopsis*, H₂O₂ generated by glycolate oxidase activates the SA signal pathway, leading to increased resistance to nonhost pathogens (Rojas et al. 2012). Consistent with the concept of mutual synergistic effects between ROS and SA, our findings enhance the case for crosstalk between ROS and SA pathways in response to bacterial infection in the miR156/SPL9 module.

In conclusion, we have demonstrated that ROS accumulation and SA signaling gene expression were suppressed in miR156 overexpression mutants but elevated in miR156 suppression mutants and *SPL9* overexpression mutants. As a result, miR156 induced susceptibility to *P. syringae* pv. *tomato* DC3000 infection, while *SPL9* played an active role in antibacterial immunity in *Arabidopsis*. Furthermore, foliar H₂O₂ treatment triggered activation of the SA signaling pathway, leading to masking of the miR156-mediated *P. syringae* pv. *tomato* DC3000 susceptibility. Taken together, our results provide new insights into the immune functions of the miR156/SPL9 network, which appear to involve regulation of ROS accumulation and activation of the SA signaling pathway.

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