

miR156 modulates rhizosphere acidification in response to phosphate limitation in *Arabidopsis*

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Abstract Rhizosphere acidification is a general response to Pi deficiency, especially in dicotyledonous plants. However, the signaling pathway underlying this process is still unclear. Here, we demonstrate that miR156 is induced in the shoots and roots of wild type *Arabidopsis* plants during Pi starvation. The rhizosphere acidification capacity was increased in *35S:MIR156* (miR156 overexpression) plants, but was completely inhibited in *35S:MIM156* (target mimicry) plants. Both *35S:MIR156* and *35S:MIM156* plants showed altered proton efflux and H⁺-ATPase activity. In addition, significant up-regulation of H⁺-ATPase activity in *35S:MIR156* roots coupled with increased citric acid and malic acid exudates was observed. qRT-PCR results showed that most H⁺-ATPase and PPCK gene transcript levels were decreased in *35S:MIM156* plants, which may account for the decreased H⁺-ATPase activity in *35S:MIM156* plants. MiR156 also affect the root architecture system. Collectively, our results suggest that miR156 regulates the process of rhizosphere acidification in plants.

Keywords *Arabidopsis thaliana* · miR156 · Rhizosphere acidification · Pi deficiency

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Introduction

Phosphorus (P) is a macronutrient that plays a vital role in many physiological processes, such as energy transfer, signal transduction, the synthesis of biological molecules, photosynthesis, and respiration. Plant roots acquire P in the form of phosphate (Pi) from the soil (Vance et al. 2003). Although Pi is abundant in the soil, most of it is in the form of precipitates with cations and is immobile, limiting Pi uptake (Raghothama 1999). The excretion of organic acids and protons from the roots is one of the strategies plants use to cope with Pi deficiency. Root exudation acidifies the soil to release insoluble P, which helps plants to take up and use P (Vance et al. 2003; Zhang et al. 2004). The induction of rhizosphere acidification by Pi deficiency has been reported in many species. White lupin (*Lupinus albus* L.) develops so-called proteoid roots to acidify the rhizosphere soil under Pi deficient conditions (Li et al. 1997). During this process, the release of organic acids (mainly citric acid) is coupled to proton efflux (Neumann and Römheld 1999; Yan et al. 2002). Furthermore, it was found that there is a close link between the burst of citrate exudation and plasma H⁺-ATPase-catalyzed proton efflux (Tomasi et al. 2009; Zhu et al. 2005). Salicylic acid and citramalic acid were exuded by sugar beet roots to solubilize P in response to Pi deficiency (Khorassani et al. 2011). The roots of rape (*Brassica napus* L.) plants excreted malic and citric acids under Pi deficiency, resulting in rhizosphere acidification and solubilization of rock phosphate (Hoffland et al. 1989). Acid soluble Ca phosphates were used by chickpea and tomato in response to Pi deficiency (Dinkelaker 1990; Hinsinger and Gilkes 1997).

The rhizosphere acidification phenomenon was explored very early, but studies on the molecular mechanism of

acidification are still at the initial stage. A phosphoenol pyruvate carboxylase gene (*LaPEPC3*) was reported to be related to citrate exudation in the cluster roots of white lupin (Cheng et al. 2014; Penaloza et al. 2005). In *Arabidopsis*, phosphorylation of AtPPC1 by AtPPCK1 and AtPPCK1 contributed to the metabolic adaptations in response to Pi starvation (Gregory et al. 2009; Plaxton and Tran 2011). The root plasma membrane H⁺-ATPase was involved in the modulation of Pi uptake in soybean under Pi starvation (Shen et al. 2006). The plasma membrane H⁺-ATPase gene *OsA8* influenced the uptake and translocation of Pi in rice (Chang et al. 2009). H⁺-pyrophosphatase AVP1 (AVP, *Arabidopsis* vacuolar pyrophosphatase) mediated rhizosphere acidification and root proliferation in *Arabidopsis* (Yang et al. 2007).

In recent years, the role of plant microRNAs in responses to environmental stress has generated widespread interest. A number of significant achievements have already been made in determining the function of the key molecules miR399 and miR827 in the regulation of phosphorus homeostasis (Bari et al. 2006; Fujii et al. 2005; Huang et al. 2013; Lin et al. 2008). miR399 is upregulated under Pi deficiency and directs the cleavage of *PHOSPHATE2* (*PHO2*) (Aung et al. 2006; Bari et al. 2006; Chiou et al. 2006; Fujii et al. 2005). miR827 has been functionally characterized in both *Arabidopsis* and rice with regard to its roles in Pi starvation signaling (Lin et al. 2010; Wang et al. 2012). The target of miR827 in *Arabidopsis*, *NITROGEN LIMITATION ADAPTATION* (*NLA*) and *PHO2* direct the degradation of plasma membrane-localized PHT1s in different subcellular compartments (Lin et al. 2013; Liu et al. 2014). In addition, deep sequencing of *Arabidopsis* small RNAs was conducted to reveal miRNAs that were differentially expressed in response to phosphate (Pi) deficiency, such as miR156, miR169, miR395 and miR2111 (Hsieh et al. 2009). However, the molecular basis of these Pi responsive miRNAs remains to be explored.

miR156 and its targets the SQUAMOSA PROMOTER BINDING PROTEIN-LIKE (SPL) family play critical roles in diverse developmental processes such as phase transition, flowering time, leaf development and anthocyanin biosynthesis (Bergonzi et al. 2013; Gou et al. 2011; Usami et al. 2009; Wang et al. 2008, 2009; Wu et al. 2009; Zhou et al. 2013). Recently, miR156 has also been reported to participate in multiple stress responses such as recurring heat stress and salt stress (Cui et al. 2014; Stief et al. 2014). However, whether miR156 is involved in rhizosphere acidification under Pi deficiency was still unknown. Therefore, the objective of the present study was to investigate the influence of miR156 on the process of root acidification. Our results may serve as an important basis for detailed studies of miR156 that regulate rhizosphere acidification in plants.

Materials and methods

Plant growth and stress treatments

Dry seeds of *Arabidopsis thaliana* were surface sterilized using 0.1 % HgCl₂ for 5 min, washed 5–7 times using sterilized water and placed on MS medium plates (containing MS salts, 3 % sucrose and 0.6 % agar, pH 5.8) at 4 °C for 3 day. The plates were placed in growth chambers (day/night cycle of 16/8 h at 22 °C; light intensity of 90–120 mmol m⁻² s⁻¹; relative humidity of 80 %) for 1–2 weeks. Low Pi stress treatment of plants was made by modification of MS medium such that the Pi concentration in LP medium was 12.5 μM. The agar of LP medium was replaced by agarose (Promega, Madison, WI, USA) to avoid contamination with phosphorous. For nutrition solution treatment, the LP solution was made without Pi.

Quantitative RT-PCR

Total RNA was isolated with TRIzol reagent (Invitrogen, Carlsbad, CA, USA). After DNase I treatment, cDNA was synthesized from 0.5 μg total RNA using Moloney murine leukemia virus reverse transcriptase (Invitrogen) with the oligo(dT) primer. Mature miR156 expression levels were determined following the methods of Xing et al. (2010) and Yang et al. (2009). Total RNA was extracted using a miRNA extraction kit (Kang Wei, Beijing, China). After DNase I treatment, 200 ng RNA was used for first-strand cDNA synthesis. SYBR Green was used in subsequent qRT-PCR. All qRT-PCR experiments were performed with two or three biological replicates. All specific primers used are listed in Table S2. Relative expression levels were normalized to *ACTIN2* or *18S rRNA*.

Histochemical analysis of GUS activity

Plants expressing the *PromiR156a:GUS* and *PromiR156d:GUS* transgenes in the wild-type background were used for histological analysis. Seedlings of transgenic plants grown in MS or low Pi (LP, 12.5 μM) medium were incubated at 37 °C for at least 4 h in staining buffer (100 mM phosphate buffer pH 7.0, 0.5 mM K₃Fe(CN)₆, 0.5 mM K₄Fe(CN)₆, 0.1 % Triton X-100, 0.1 % X-gluc) in the dark. Chlorophyll was removed with an ethanol series consisting of 50, 70 and 100 % ethanol washes at room temperature for 30 min each. The GUS activity was determined according to Jefferson et al. (1987).

Detection of rhizosphere acidification

After cultivation for 9 day on MS medium, seedlings were placed on MS medium or LP medium that contained

0.75 % (w/v) agar (or agarose) and 0.006 % (w/v) bromocresol purple (pH 5.8). Rhizosphere acidification was visualized after incubation for 8 h. The pH calibration was performed according to the color of medium containing bromocresol purple with different pH (from 5.2 to 6.8).

Quantitative measurements were performed as previously described (Santi and Schmidt 2009) with some modifications. Three hundred microliters of MS or low Pi (LP) solution (0.5 % sucrose, 0.006 % bromocresol purple, pH 5.8) was added into 96-well plates. The 9-day-old seedlings were transferred to the plates and three seedlings were pooled as one sample; three samples were set per treatment. The plates were placed in growth chambers for 2–3 h. A 100 μ L solution of each sample was removed to another 96-well plate. Rhizosphere acidification capacity was accessed by reading the adsorption at 590 nm (A_{590}). To determine the pH values of the rhizosphere, the adsorption was converted to pH according to the pH calibration. The pH calibration was performed according to the color of MS solution containing bromocresol purple with different pH (from 5.3 to 6.0).

Assay of cytoplasmic pH in root epidermal cells

The assay was performed as described previously (Gao et al. 2004; Moseyko and Feldman 2001) with some modifications. *35S:MIR156* and *35S:MIM156* plants containing pH sensitive GFP were created by crossing with N9561 lines obtained from the Nottingham Arabidopsis Stock Centre (<http://arabidopsis.info/StockInfo?NASCid=9561>). Nine-day-old seedlings were transferred from 0.6 MS medium to MS solution for 1 day, and then transferred to MS solution with or without Pi for 2 h. The root hairs of seedlings were used to monitor intracellular pH. GFP fluorescence was monitored using a FV 1000 confocal microscope (exciters, 405 and 488 nm; emitter, 525 nm). Fluorescence images were taken every 3 s. The F405 to F488 ratio was used as a measure of pH. pH levels were pseudo-color-coded according to the calibrated 410/470 nm ratio image of the same root hair.

Measurement of H⁺-ATPase activity

Fourteen-day-old *Arabidopsis* seedlings were transferred to MS or LP solution (0.5 % sucrose, pH 5.8) for 1 day. After the seedling roots were isolated, the plasma membranes were prepared as described previously (Larsson et al. 1987) and the protein was quantified according to Bradford (1976). The H⁺-ATPase activity was determined as described previously (Ames 1966; Yan et al. 2002).

Collection of root exudates and analysis of carboxylates

Three hundred microliters of MS or LP solution (0.5 % sucrose, pH 5.8) were added into 96-well plates.

Ten-day-old seedlings were transferred to the plates and three seedlings were pooled as one sample. The plates were placed in growth chambers for 1 day and the solution was collected. The roots were carefully rinsed twice with deionized water, dried and weighed. The root exudate solution was filtered through a 0.2 μ m filter and analyzed by HPLC with a 300 \times 7.8 mm column (Aminex HPX-87H); the mobile phase was 10 mmol/L H₂SO₄ with a flow rate of 1 mL min⁻¹ at 55 °C.

Results

miR156 is induced during Pi starvation

Eight loci (*MIR156A* to *H*) that encode pri-miR156 have been reported in *A. thaliana* and miR156 may be involved in Pi deficiency (Hsieh et al. 2009; Xing et al. 2010; Yang et al. 2013). To investigate the function of miR156 in Pi deficiency, the expression of mature miR156 in *Arabidopsis* was analyzed. The seeds of wild-type (WT) seedlings were sowed on MS medium for 7 day and then transferred to Pi sufficient or LP medium for 5 day. Quantitative real time (qRT) PCR results indicated that miR156 was induced significantly in both roots and shoots during Pi starvation (Fig. 1a). To further study the expression pattern of miR156, we observed the GUS staining of *PromiR156a:GUS* and *PromiR156d:GUS* plants. As shown in Fig. 1b, when grown in low Pi, the *PromiR156a:GUS* seedlings displayed stronger GUS activity in their shoots. Additionally, marginally stronger GUS activity was detected in the root tips of *PromiR156d:GUS* seedlings. Consistent with the above qRT-PCR results, GUS activity in *PromiR156a:GUS* and *PromiR156d:GUS* plants also increased in response to Pi deficiency (Fig. 1c). However, the induction level of *PromiR156a:GUS* and *PromiR156d:GUS* plants were much lower compared with qRT-PCR results. Thus, the expression of *MIR156A-H* were examined (Fig. S1a). The results show that *MIR156B* and *MIR156E* play dominant roles within the *miR156* gene family in response to Pi starvation. Although *MIR156A* and *D* were also induced, their expression levels were lower than those of *MIR156B* and *MIR156E*.

The only target family of miR156 is SQUAMOSA PROMOTER BINDING LIKE (SPL) transcription factors (Rhoades et al. 2002). To confirm whether SPLs was repressed in Pi starvation, the expression of SPLs transcript in wild type seedlings after transferring from MS to LP medium were also examined by qRT-PCR. As shown in Fig. S2, the expression of SPL genes was generally lower at low Pi. In addition, *SPL3* mRNA level is the most significant repression of expression at low Pi conditions.

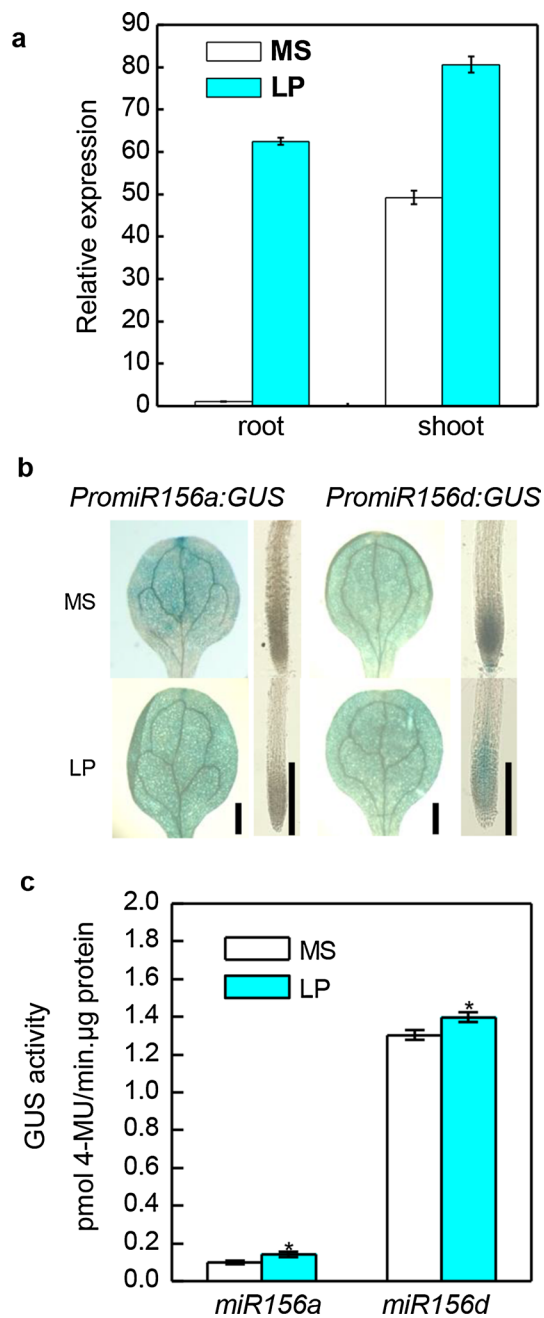


Fig. 1 miR156 is induced in response to phosphate starvation. **a** qRT-PCR analysis of miR156 expression. Wild-type seedlings were grown on MS medium for 7 day and transferred to low Pi (LP) medium for 6 h. **b** GUS staining of *PromiR156a:GUS* and *PromiR156d:GUS* transgenic lines. Tissues were stained 7 day after transfer to MS or LP medium. Bar 1 mm. **c** The assay of GUS activity in *PromiR156a:GUS* and *PromiR156d:GUS* plants in response to normal states and Pi deficiency. Error bars indicate the \pm SD of three biological replicates

The rhizosphere acidification capacity was changed in miR156 transgenic plants under Pi deficiency

Rhizosphere acidification is one of the strategies by which

plants promote the release of undissolved Pi from soil minerals. We tested whether miR156 was involved in the process of rhizosphere acidification. The transgenic plants *35S:MIR156* (miR156 overexpression; Schwab et al. 2005) and *35S:MIM156* (Franco-Zorrilla et al. 2007) were analyzed. It is noteworthy that *35S:MIM156* is an artificial target mimics which contains a motif with sequence complementarity to miR156, but the pairing is interrupted by a mismatched loop. It sequesters miR156, thereby resulting in increased SPL mRNA accumulation and activity. The expression of *MIR156B* was induced dramatically in *35S:MIR156* plants (Fig. S1b), which is consistent with the previous report (Schwab et al. 2005). The expression of *MIR156B* was also repressed significantly in *35S:MIM156* plants.

Nine-day-old *35S:MIR156*, *35S:MIM156* and WT seedlings were transferred to MS or low Pi (12.5 μ M) medium that contained 0.006 % (w/v) bromocresol purple. Bromocresol purple was chosen as the pH indicator; its color changed from yellow to purple when the pH increased. After 8 h, WT plants (the control) acidified the low Pi medium. At the same time, the rhizosphere acidification capacity was increased in *35S:MIR156* plants, but was completely inhibited in *35S:MIM156* plants (Fig. 2a). To detect whether plasma membrane H⁺ pumping was involved in the process of root acidification, the H⁺-ATPase inhibitor vanadate (1 mM) was added into the medium. After 8 h of cultivation, the color of the rhizosphere did not change for any of the P-deficient plants, indicating that root acidification was completely inhibited by 1 mM vanadate (Fig. 2b).

To further quantify the pH variation of plants in the rhizosphere, 9-day-old seedlings were transferred to 96-well plates containing MS or LP standard nutrient solution (0.5 % sucrose, 0.006 % bromocresol purple, pH 5.8). After 3 h incubation in the test solution, the absorption at 590 nm (A_{590}) was read, and then converted into pH according to a pH curve. The results showed that the rhizosphere pH in MS solution was 5.57 ± 0.01 for the WT, 5.46 ± 0.02 for the *35S:MIR156* plants and 5.69 ± 0.01 for the *35S:MIM156* plants. In the LP solution, the rhizosphere pH was 5.32 ± 0.03 for the WT, 5.2 ± 0.04 for the *35S:MIR156* plants and 5.43 ± 0.03 for the *35S:MIM156* plants (Fig. 2c).

The above results showed that rhizosphere acidification by the plants was enhanced under low Pi stress and the pH decreased obviously. Compared with the WT, the rhizosphere acidification capacity of the *35S:MIR156* plants was enhanced whereas that of the *35S:MIM156* plants was decreased.

miR156 modulates the cytosolic pH of root hair cells in response to Pi deficiency

Plant roots acidify the rhizosphere soil and increase proton export in response to P deficiency (Yan et al. 2002).

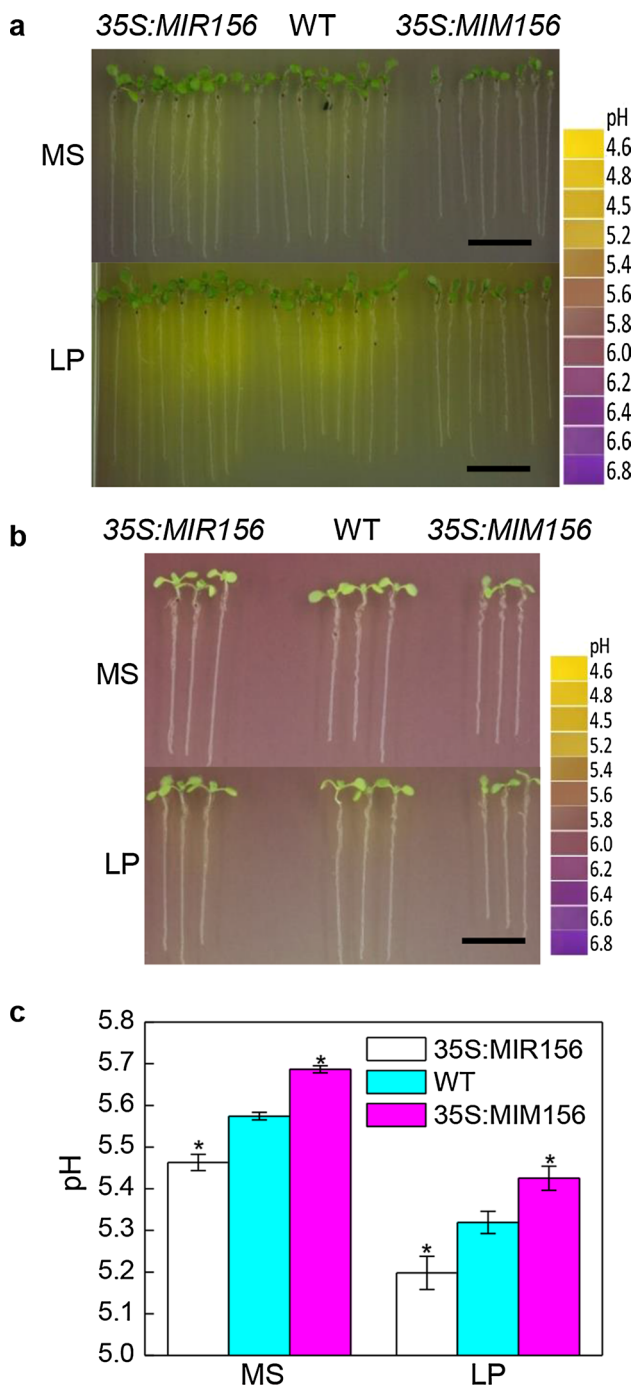


Fig. 2 Acidification capacity are enhanced or decreased in *35S:MIR156* and *35S:MIM156* transgenic plants. **a** Seedlings grown for 9 day were transferred to MS and low Pi (LP) medium with the addition of 0.006 % (w/v) bromocresol purple for 1 day. Four biological replicates were analyzed, with similar results. *Bar* 1 cm. **b** 1 mM vanadate was added in the corresponding medium for study of the inhibitory effect of vanadate. Four biological replicates were analyzed, with similar results. *Bar* 1 cm. **c** For the determination of pH values of the rhizosphere, 9-day-old plants were incubated in the test solution for 24 h. *Error bars* show standard deviation of 12 plants analysed in two independent experiments. *Asterisks* represent significant differences from the wild type ($P \leq 0.05$ from a Student's *t* test)

However, how the intracellular pH changes is not clear. *35S:MIR156* and *35S:MIM156* plants containing ratio-metric pH-sensitive GFP were obtained by crossing with the Pt-GFP line N9561 (Moseyko and Feldman 2001). Nine-day-old seedlings were transferred to MS solution for 1 day. Then, the seedling roots were used to monitor cytosolic pH by incubating them in low Pi solution for 2 h. We monitored GFP fluorescence using a confocal microscope. The 405/488 nm ratio image was converted to intracellular pH according to a pH standard curve. As shown in Fig. 3b, the cytosolic pH of the WT was increased from 6.98 ± 0.17 to 7.31 ± 0.24 after low Pi treatment, while the pH of *35S:MIR156* plants changed from 6.82 ± 0.24 to 8.21 ± 0.18 , which was higher than the WT. The pH of *35S:MIM156* plants increased marginally from 7.01 ± 0.09 to 7.12 ± 0.17 , which was marginally lower than the WT after low Pi treatment (Fig. 3a).

Next, the root cells were treated with a H^+ -ATPase inhibitor (1 mM vanadate) for 15 min. We observed that the ratio image became extremely weak, indicating that the pH in root epidermal cells decreased significantly (Fig. 3b).

These results suggested that proton export by root cells was induced in response to Pi deficiency, which caused cytoplasmic alkalization. The intracellular pH of *35S:MIR156* plants was remarkably changed under Pi starvation. On the contrary, the pH of *35S:MIM156* plants did not change significantly. When the H^+ -ATPase activity was inhibited, the cytosolic pH of all of the transgenic plants and WT plants was decreased (Fig. 3b), which indicates that H^+ -ATPase is involved in the process of Pi deficiency response.

The H^+ -ATPase activity of *35S:MIR156* plants was enhanced during Pi starvation

To gain a deeper insight into the relationship between rhizosphere acidification and H^+ -ATPase, the plasma membrane ATPases of WT, *35S:MIR156* and *35S:MIM156* roots were assayed. Fourteen-day-old seedlings were treated with MS or LP solution for 1 day. The roots were collected, the total membrane protein was extracted and the H^+ -ATPase activity was determined. The plasma membrane H^+ -ATPase activity of WT and *35S:MIR156* plants was increased dramatically. Moreover, the activity of *35S:MIR156* plants was higher than the WT. By contrast, the H^+ -ATPase activity of *35S:MIM156* plants decreased significantly (Fig. 4). Consistent with a previous report (Yan et al. 2002), plasma membrane H^+ -ATPase was closely related with rhizosphere acidification. The increased H^+ -ATPase activity of *35S:MIR156* plants may be one of the reasons for their higher rhizosphere acidification capacity. Likewise, the decreased H^+ -ATPase activity of *35S:MIM156* plants after

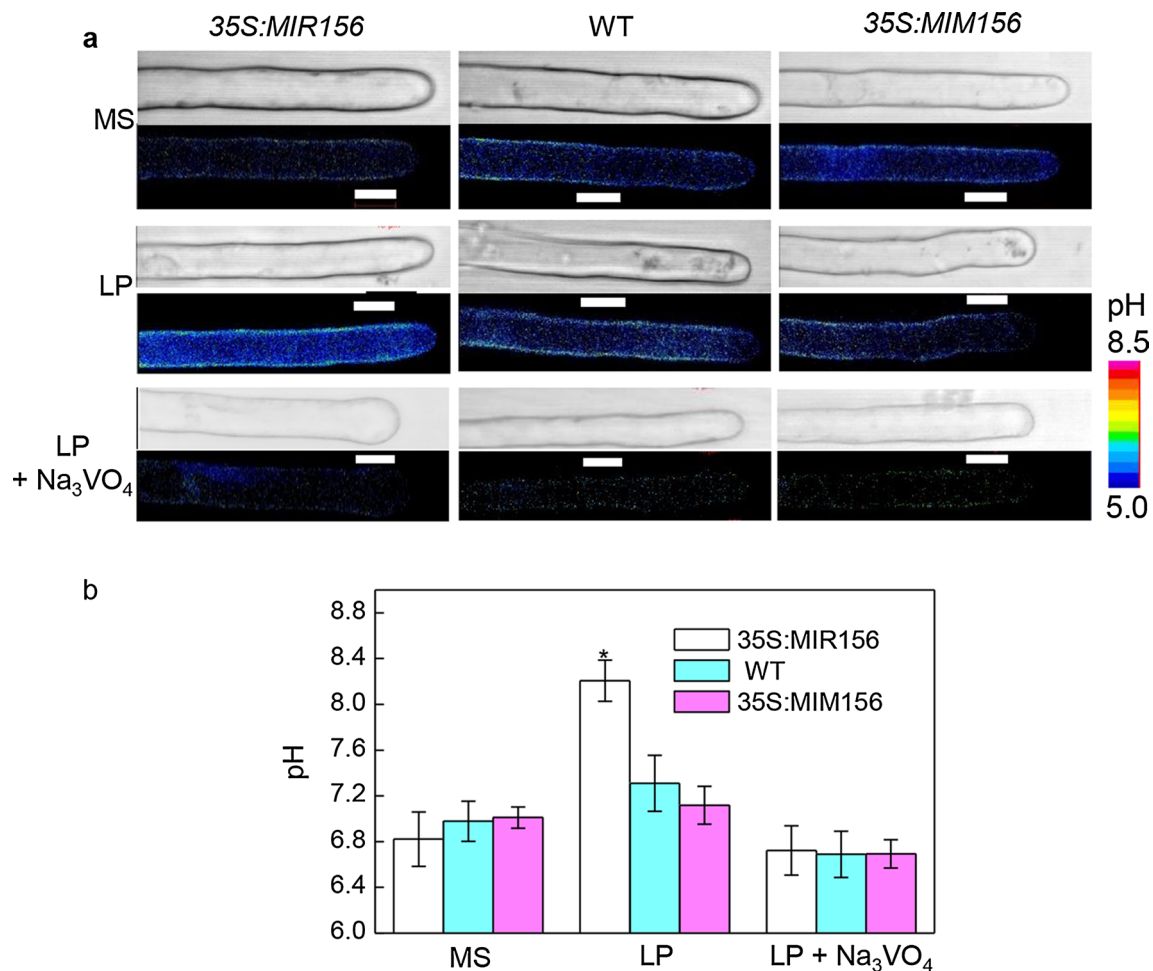


Fig. 3 Cytoplasmic pH in root epidermal cells of *35S:MIR156* and *35S:MIM156* transgenic plants. **a** 405/488 ratio fluorescence images of different processing in root epidermal cell of *35S:MIR156*, *35S:MIM156* transgenic plants and wild type plants. Bar 10 μm . **b** cytoplasmic pH in root epidermal cell of *35S:MIR156*, *35S:MIM156*

transgenic plants and wild type plants. Error bars represent mean \pm SD of three biological replicates with two technical replicates each. Asterisks represent significant differences from the wild type ($P \leq 0.05$ from a Student's t test)

LP treatment may inhibit their rhizosphere acidification capacity.

Root organic acid exudates of *35S:MIR156* plants were increased in response to Pi deficiency

It is generally accepted that enhanced exudation of organic anions from roots is connected with Pi deficiency (Ryan et al. 2001). To determine whether organic acid exudates were changed in *35S:MIR156* and *35S:MIM156* plants, the root exudates of WT, *35S:MIR156* and *35S:MIM156* plants were tested by HPLC. We mainly assayed citric acid, malic acid and succinic acid from the root exudates of the WT and transgenic plants. These acids were present in root exudates from both MS and LP treatments (Fig. 5). As expected, the concentrations of the three excreted compounds from the WT were all higher with LP than with MS

treatment. Under both treatments, the citric acid and malic acid exuded from *35S:MIR156* plants were significantly higher than the WT, whereas the exudates of *35S:MIM156* plants were not significantly different from the WT except that the concentration of citric acid was marginally higher. These results showed that the root organic acid (mainly citric acid and malic acid) exudates of *35S:MIR156* plants were increased in response to Pi deficiency. Maybe this is one of the reasons for the increased rhizosphere acidification of *35S:MIR156* plants.

Expression of acidification-related genes

To understand the molecular basis of changes in rhizosphere acidification, we monitored the expression of H⁺-ATPase and H⁺-pyrophosphatase genes, such as *AHA1*, *AHA2*, *AHA7* and *AVP1*. Seven-day-old seedlings were

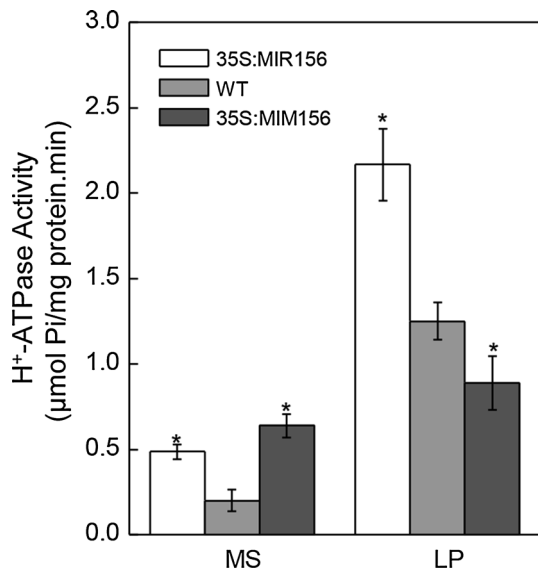


Fig. 4 Comparison of H⁺-ATPase activity of plasma membranes derived from different roots of *35S:MIR156*, *35S:MIM156* transgenic plants and wild type plants. Plants were grown on MS medium for 14 day and transferred to nutrition solution with or without Pi for 1 day. Bars represent mean ± SD of three biological replicates with two technical replicates each. Asterisks represent significant differences from the wild type ($P \leq 0.05$ from a Student's t test)

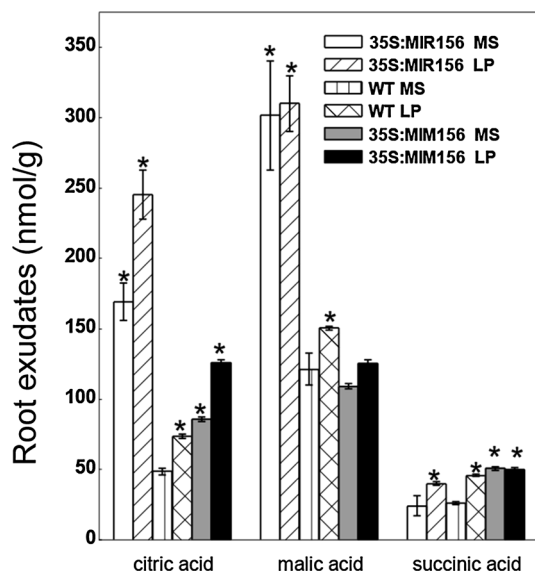


Fig. 5 The assay of organic acid contents in root and root exudates. *35S:MIR156*, *35S:MIM156* transgenic plants and wild type plants were grown on MS medium for 21 day and transferred to nutrition solution with or without Pi for 1 day. The root exudates were collected and assayed by HPLC. Bars represent mean ± SD of three biological replicates. Asterisks represent significant differences from the wild type with MS treatment ($P \leq 0.05$ from a Student's t test)

transferred to LP medium for 1 day, and were then harvested for RNA extraction. The qRT-PCR results showed that transcripts of all four genes were lower in *35S:MIM156* seedlings than in the WT under Pi deficiency (Fig. 6). The transcript level of *AHA7* in *35S:MIR156* seedlings was the same as in the WT in response to Pi deficiency, whereas transcripts of *AVP1* and the other two genes (*AHA2* and *AHA7*) were increased and decreased marginally respectively compared with the WT. In addition, the expression of *PPCK1* and *PPCK2*, encoding the PEPC protein kinase, was also evaluated. Compared with WT plants, the expression of *PPCK1* was decreased in *35S:MIM156* seedlings, while the expression of *PPCK2* was also decreased in *35S:MIR156* during Pi deficiency. These results indicated that the overexpression and suppression of miR156 had different effects on the genes involved in rhizosphere acidification.

As transcription factor family, the core binding motif of SPLs is GTAC consensus sequence (Birkenbihl et al. 2005). An analysis of the promoter regions of the above acidification related genes, indicated that all of them have one or more GTAC sequence on their promoters (Table S1).

MiR156 affect the root system architecture

It has been reported that the root system architecture (RSA) affect the process of rhizosphere acidification (Li et al. 1997; Yan et al. 2002). Therefore, it was logical to examine the RSA of *35S:MIR156*, *35S:MIM156* and wild-type seedlings under normal and low Pi states (Fig. S3a–c). *35S:MIM156* plants caused decreases in the number and length of lateral roots compared with wild type under normal and low Pi conditions. Besides, the number of lateral roots increased in *35S:MIR156* under LP condition. The number and length of root hairs were also detected under both conditions (Fig. S3d–f). It was found that the length of root hairs increased in *miR156* overexpression mutants under both states. Moreover, the number of root hairs of *35S:MIR156* increased only in normal condition. Together, these data mean that miR156 is involved in the lateral root and root hair development process. The involvement of miR156 in RSA development may also contribute to the phenotype of rhizosphere acidification.

Discussion

Rhizosphere acidification is a common response to Pi deficiency, especially in dicotyledonous plants. The roots of plants exude more H⁺ than HCO₃⁻ or OH⁻, resulting in

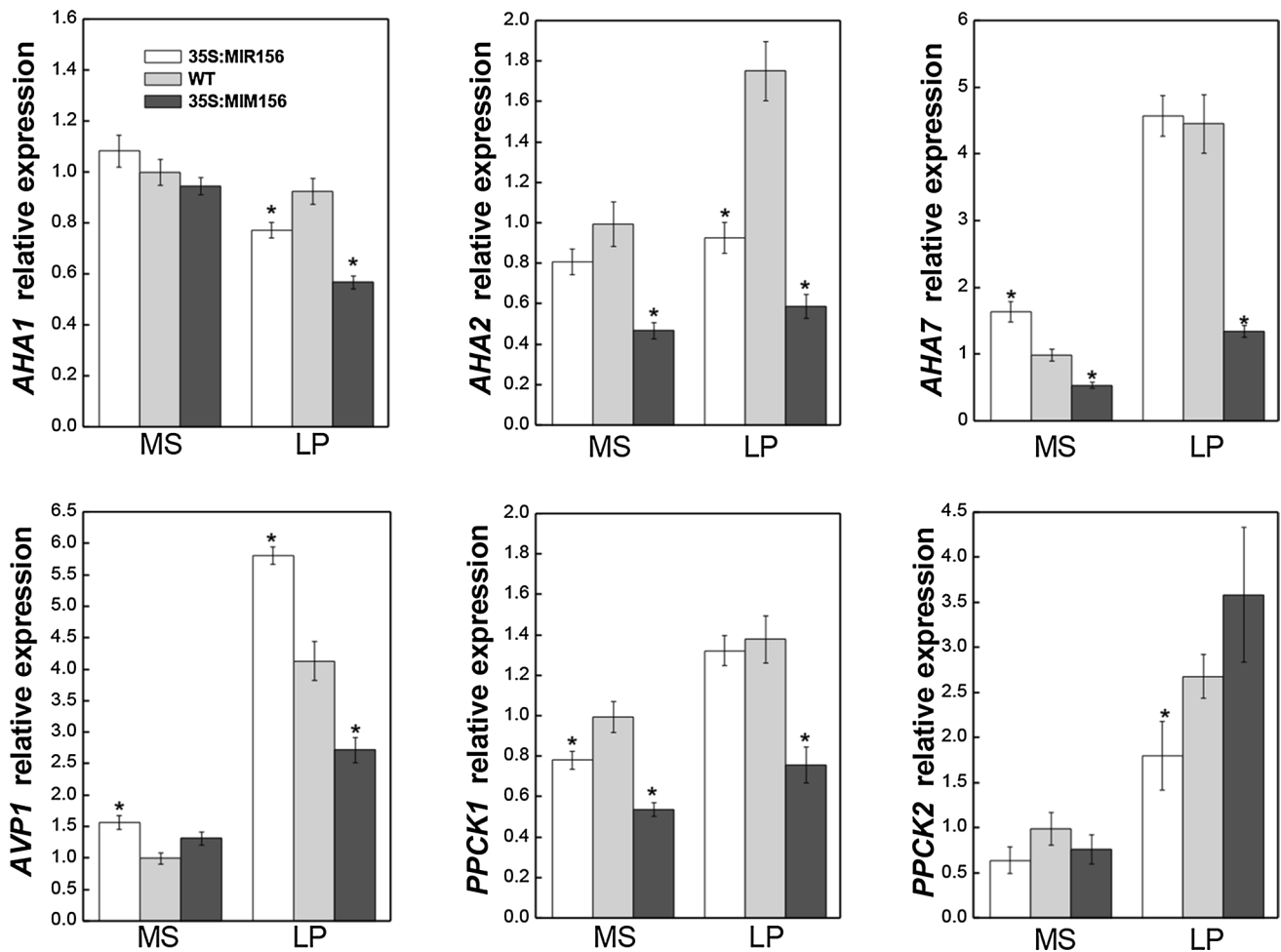


Fig. 6 Expression patterns of root acidification related genes in mutants and wild type. Seeds of the *35S:MIR156*, *35S:MIM156* transgenic plants and wild type plants were grown on MS agar medium for 7 day and then transferred to the MS or LP medium for 1 day. The

ACTIN2 transcript level was used for normalization. Bars represent mean \pm SD of three biological replicates with two technical replicates each. Asterisks represent significant differences from the wild type ($P \leq 0.05$ from a Student's *t* test)

acidification of the rhizosphere soil (Neumann and Römheld 1999; Raghothama 1999). A previous study screened dozens of rhizosphere acidification deficiency mutants, one of which was the *spl1* mutant (Lei et al. 2015). Our research showed that the upstream regulator of SPL genes miR156 also take part in the process of rhizosphere acidification. In *Arabidopsis*, the process of rhizosphere acidification occurred in a short time. There were significant color changes around the roots of seedlings grown on LP medium containing 0.006 % (w/v) bromocresol purple for 8 h. We observed that the acidification ability of *35S:MIR156* and *35S:MIM156* seedlings increased and decreased, respectively (Fig. 2a). In addition, the H^+ -ATPase inhibitor 1 mM vanadate blocked the acidification process completely (Fig. 2b). Yan et al. (2002) reported that plasma membrane H^+ -ATPase is essential for enhanced rhizosphere acidification by active proteoid roots in white lupin. Combined with the H^+ -ATPase activity assay results (Fig. 4), we confirmed

that plasma membrane H^+ -ATPase plays a vital role in the process of rhizosphere acidification in *Arabidopsis*.

To clarify the cytosolic pH changes of root hair cells in response to Pi deficiency, the cytosolic pH was assayed using pH-sensitive GFP fluorescence. The cytosolic pH of *35S:MIR156* plants increased compared with the WT under Pi deficiency (Fig. 3). This phenomenon was in accordance with the increased H^+ -ATPase activity of *35S:MIR156* plants. The increased H^+ -ATPase activity may promotes proton efflux, resulting in an increase of cytosolic pH in the root hair cells of *35S:MIR156* plants.

It was reported that citric acid release was closely correlated with H^+ release in white lupin (Zhu et al. 2005). In this study, we found significant up-regulation of H^+ -ATPase activity in *35S:MIR156* roots coupled with high citric acid and malic acid exudation (Fig. 5). These results demonstrate that H^+ release and organic acid exudates from roots contribute to rhizosphere acidification in *Arabidopsis*.

In *Arabidopsis*, 11 out of 17 SPL genes are targeted by miR156 (Rhoades et al. 2002). Thus, we tested the molecular mechanism by which miR156 is involved in rhizosphere acidification under Pi deficiency. MiR156 was induced and targeted SPL family was repressed in response to Pi starvation (Fig. 1, Figs. S1 and S2). Table S1 also showed that the promoter of these acidification related genes all have GTAC motifs. In addition, we found that *AHA1/2/7* and *PPCK* genes transcript levels were decreased in *35S:MIM156* plants, which may account for the decreased H⁺-ATPase activity in *35S:MIM156* plants (Fig. 6). Furthermore, the expression levels of these genes in *35S:MIR156* plants were the same as or lower than in WT plants. Only the expression of *AVP1* gene in *35S:MIR156* plants increased compared with WT. The *AVP1* gene might be the target of SPL gene, which needs further research.

On the other hand, the lateral roots and root hairs development of two transgenic plants of miR156 are different from wild type (Fig. S3), which suggest that miR156 is involved in regulation of lateral root and root hair development. The increase of root surface area may be attributed to the increased rhizosphere acidification capacity. Thus, further investigation is required to elucidate the mechanisms of interaction between the root development and the process of rhizosphere acidification.

Cui et al. (2014) reported that miR156 integrates environmental signals (such as salt stress) to ensure timely flowering. In this study, miR156 is also involved in the rhizosphere acidification process in response to Pi deficiency. MiR156 may be a key regulator which integrate the environmental signals into developmental process. Taken together, our results demonstrate that miR156 is involved in the rhizosphere acidification process in terms of proton release and carboxylate exudation. miR156 positively regulates the rhizosphere acidification process and contributes to coping with insoluble phosphorus stress. This research opens a whole new research direction in the search for novel miRNA signaling machinery related to rhizosphere acidification and is potentially a substantial advance in understanding the molecular mechanisms of rhizosphere acidification.

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