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RESEARCH PAPERS

The Arabidopsis SPL9 Transcription Factor Regulates Phosphate Acquisition and *miR399f* Expression under Phosphate Deprivation

K. Lei[a, b], J. Ren[a], M. Niu[a], D. Gu[a], and G. An[a, *]

[a] *Henan Key Laboratory of Plant Stress Biology, Department of Biology, Henan University, Kaifeng, 475004 China*

[b] *Pharmacy Institute of Henan University, Kaifeng, 475004 China*

**e-mail: angy@henu.edu.cn*

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Abstract—Transcription factors play an important role in the signaling of Pi deficit. We show that Arabidopsis SPL9 regulates the phosphate (Pi) deficit response. We report here that the SPL9 of *Arabidopsis* modulates the phosphate (Pi) deficiency response. The Pi absorption and Pi content, rhizosphere acidification capability, and anthocyanin content in *35S:rSPL9* plants (the *miR156*-resistant version of SPL9) were all measured. To study the interaction of SPL9 protein with *miR399f* promoters, yeast single hybrid assays, transient transfection and ChIP assays were used. The phenotypes of *35S:SPL3 35S:SPL9* lines were also studied in the absence of Pi. The results show that Pi uptake and content increased in *35S:rSPL9* plants compared to wild-type plants. Under Pi-deficient conditions, *35S:rSPL9* transgenic plants showed lower rhizosphere acidification capacity and reduced rhizosphere acidification phenotypes. In response to low Pi stress, *35S:rSPL9* plants reduced their anthocyanin accumulation. The SPL9 protein interacts directly to the *miR399f* promoters at their GTAC sites. SPL3 and SPL9 were also found to have comparable or redundant activities during Pi deficiency responses. These findings indicate that SPL9 in Arabidopsis is involved in the Pi deficit response and regulates *miR399f* expression.

Keywords: *Arabidopsis thaliana*, Pi deficiency, SPL9, *miR399f*

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INTRODUCTION

Plant metabolism, structure, and growth all require phosphorus, an essential macronutrient. Nonetheless, phosphate (Pi) scarcity persists in over 70% of the world's agricultural land. [1]. To compensate for the lack of Pi, plants have redesigned their root system architecture (RSA) to improve Pi uptake [2–4] by releasing and activating of organic acids and acid phosphatases [5], or via altering the pattern of Pi-related gene expression [3, 6].

MicroRNAs (miRNAs) play critical roles in Pi deficient signaling. *MiR399*, which is produced by low Pi levels and controls the cleavage of PHOSPHATE2 (PHO2) transcript cleavage, is the most thoroughly researched miRNA that is impacted by stress caused by low Pi levels [7–9]. PHO2, a *miR399*-targeted gene, mediates the degradation of post-ER compartment-localized PHT1s as well as PHOSPHATE1 (PHO1) degradation in the pericycle cells' internal membrane [10, 11]. Furthermore, miR156 and its target gene SQUAMOSA PROMOTER BINDING PROTEIN-LIKE 3 (SPL3) play an important role in Pi deficiency-induced plant responses [12].

SPL proteins are transcription factors that are exclusive to plants and have highly conserved SBP domains [13]. The SBP domain is made up of 79 amino acids and comprises two zinc finger motifs that are required for the SPL protein to bind to the core binding cis-element (GTAC) [13, 14]. SPL9 participates in a variety of metabolic, developmental, and stress-mediated activities, including the arrival of flowering [15], vegetative phase change [16, 17], shoot maturation [18], trichome distribution [19], biosynthesis of anthocyanin [20], phytohormone responses [21–23] and abiotic stress tolerance [24].

The role of SPL9 under Pi deficit was studied in the current study, and it was identified as a Pi stress response regulator. SPL9 was found to be involved in rhizosphere acidity and anthocyanin accumulation. Furthermore, SPL9 overexpression increased Pi absorption and regulated *miR399f* expression in *Arabidopsis* through binding to the GTAC boxes in the *miR399f* promoter.

Abbreviations: Pi—phosphate; RSA—root system architecture; *35S:rSPL9*—the *miR156*-resistant version of SPL9; PHO2—PHOSPHATE2; PHO1—PHOSPHATE1; SPL—SQUAMOSA PROMOTER BINDING PROTEIN-LIKE; WT—wild type; DFR—*DIHYDROFLAVONOL-4-REDUCTASE*.

MATERIALS AND METHODS

Plant materials and growth conditions. In this study, the seeds of *Arabidopsis thaliana* (Columbia-0 ecotype; Arabidopsis) were used. Previously characterized lines *35S:rSPL9* [15], *35S:rSPL3* [25], *PromiR399f:GUS* [26], *ProSPL9:GUS* [27], *SPL9pro:GFP-rSPL9* [22] were used in this work. All seeds were surface sterilized and then allowed to sprout on Murashige and Skoog (MS) medium made up of 1% agar, 3% sucrose, and 1.25 mM KH_2PO_4. The seedlings were moved to *35S:rSPL9* supplemented normal (MS, 1.25 mM KH_2PO_4) or low Pi (LP, 2.5 μM KH_2PO_4) media when they were 7-days-old. For hydroponic culture, MS medium without any additional sucrose was used.

Determination of phosphate content. 7-days-old seedlings were cultivated on MS or LP conditions for 14 days before their Pi contents were determined using the Chiou et al. [9] procedure.

Measurement of Pi uptake. On MS media, seedlings of wild-type (WT) and transgenic lines were cultivated for 11 days. Each biological sample was made up of groups of 10 seedlings. After weighing the shoot and root samples, they were lysed in 500 μL of H_2O_2 (30%) and 200 μL $HClO_4$ for 3 h. Following that, a scintillation cocktail (3 mL) was added into each vial, and the radioactivity was determined using a scintillation counter.

The rhizosphere acidification detection. After nine days of growth on MS medium, seedlings were transferred to MS or LP (2.5 μM) media containing 0.006% (w/v) bromocresol purple (pH 5.8) and 0.75% (w/v) agar. The phenotype of rhizosphere acidity was observed after 8 h of incubation. The methodology used by Santi et al. [28] was modified slightly to determine net proton flux. 300 μL of either MS or LP solution (0.006% bromocresol purple, 0.5% sucrose, pH 5.8) was applied to 96-well plates. Transferring 9-day-old seedlings to the plates; each sample comprised three seedlings. After 3 h in the growth chamber, 100 μL of each sample solution was transferred to a new 96-well plate. The rhizosphere acidification capacity was determined by measuring absorbance at 590 nm.

Determination of anthocyanin. The method of Kim et al. [29] was slightly modified and used for anthocyanin estimation. 10 mg of fresh seedlings were extracted overnight at 4°C in a 1% HCl-methanol solution. 200 μL of methenyl trichloride and deionized water were added to the samples, and they were then centrifuged for 10 min at 14000 *g*. The top phase was carefully removed, and the amount of anthocyanin was determined spectrophotometrically and calculated as follows:

$$\text{Anthocyanin content}\left(\text{mg}/\text{mL}\right) = A_{530}\left(0.33 \times A_{657}\right).$$

GUS activity analysis. For histological tests, the transgenic *PromiR156d:GUS* and *PromiR156a:GUS* in the wild-type background were analyzed. Transgenic *PromiR156d:GUS* and *PromiR156a:GUS* in the wild-type context were investigated for histological testing. Transgenic plants were cultivated in MS or LP (12.5 μM) media, and their seedlings were treated in a staining buffer comprising 0.1% Triton X-100, 100 mM phosphate buffer (pH 7.0), 0.5 mM $K_4Fe(CN)_6$, 0.5 mM $K_3Fe(CN)_6$, and 0.1% X-gluc for at least 4 h at 37°C. Chlorophyll was removed at room temperature using a series of 50, 70, and 100% ethanol washes, with each wash lasting 30 min. Finally, the GUS activity was investigated using the procedure published by Lei et al. [30].

Yeast one-hybrid assay. Yeast one-hybrid experiments were used to investigate SPL9 binding to *miR399f*. To create pJG-SPL9, a full-length SPL9 encoding cDNA was introduced into pJG. The effectors contained the GAL4-activation domain. As a positive control, the *ProDFR:LacZ* reporter plasmid was created. There were also *PromiR399f-1:LacZ*, *PromiR399f-2:LacZ*, and *PromiR399f-3:LacZ* reporter plasmids created. The *Saccharomyces cerevisiae* strain EGY48 was then converted and chosen on synthetic complete medium lacking Leu and Ura [31].

Transient transfection assay. According to the Liu et al. [32] procedure, the transcriptional activity of the *miR399f* promoter was examined in Arabidopsis protoplasts. *PromiR399f:LUC* was the component that was employed as the reporter. To create the effector construct under the control of the 35S promoter, the SPL9 coding sequence was introduced and cloned into the pBI221 vector. Yoo et al. [33] provided the procedure for *35S:GUS* synthesis and subsequent transfection. The LUC activity was normalized relative to the GUS activity.

Chromatin immunoprecipitation-qPCR assay. *GFP-rSPL9* and WT *Arabidopsis* seedlings grown on MS media for 7 days were treated with low Pi for additional 7 days. The seedlings were then collected for chromatin immunoprecipitation (ChIP) tests, as described previously [12]. In brief, two grams of seedlings were cross-linked in 1% formaldehyde solution for 6 minutes under vacuum. To immunoprecipitate genomic DNA, anti-GFP antibodies (GeneTex, USA) were utilized. Finally, using the primers specified in Supplementary Table S1, qPCR was used to examine the GFP-specific enrichment of fragments from the *miR399f* promoter.

RESULTS

SPL9 Modulates the Uptake of Pi in Response to Pi Deficiency

Pi deficiency symptoms were tested in transgenic plants bearing the *35S: rSPL9* (the *miR156*-resistant variant of SPL9) construct to see if SPL9 has a role in controlling the plant's response to Pi deficiency. Plants grown on MS and LP (Pi-deficient) plants were evaluated in terms of their Pi concentrations. Figure 1a

demonstrates that, as compared to wild-type plants, branches of *35S:rSPL9* plants contained more Pi, demonstrating that Pi accumulation is aided by SPL9 overexpression in Arabidopsis. The seedlings were then kept in a ^{33}P-supplemented Pi uptake solution for 2 hours and the Pi was measured. As demonstrated in Fig. 1b, the roots of *35S:rSPL9* plants assimilate ^{33}P at a significantly higher rate than wild-type plants in Pi deficit. Thus, enhanced Pi uptake resulted in an increase in Pi content in the *35S:rSPL9* plant shoots. Furthermore, the Pi contents of *spl9* mutant were previously examined, and there is no difference between wild-type plants and *spl9-4* mutant [34], indicating that SPLs play a redundant role in regulating Pi absorption.

The Rhizosphere Acidification Capacity and Accumulation of Anthocyanin Changed in SPL9 Transgenic Plants in Pi-deficient Condition

To better understand the role of SPL9 during Pi deprivation, pH fluctuations in the rhizosphere were measured using a pH indicator bromocresol purple added MS agar media [5]. The *35S:rSPL9* transgenic lines showed a reduction in low Pi-induced rhizosphere acidification as compared to wild-type plants. There observed a considerable decrease in net proton flux in *35S:rSPL9* plants as compared to wild-type plants (Figs. 2a, 2b), similar to the acidification phenotype. Anthocyanin accumulation, which is another hallmark plant response to Pi deprivation, was also measured. The wild-type seedlings turned light purple after 3 weeks of growth on low Pi hydroponic media, but the complete shoots of *35S:rSPL9* plants remained significantly lighter in color (Fig. 2c). The quantitative analysis revealed a significant decrease in anthocyanin content in the *35S:rSPL9* seedlings (Fig. 2d). This revealed that under low Pi stress, SPL9 plays a key role in rhizosphere acidification and anthocyanin accumulation.

The modification of RSA in Arabidopsis has been used to demonstrate the adaptive response to Pi deprivation [35]. So, it was investigated how SPL9 overexpression affected the plant RSA. On vertically positioned agar plates, we tracked the RSA of wild-type (7-day-old) and *35S:SPL9* plants for 7 days. The plants were grown on MS and LP media. In contrast to the wild-type plants, the *35S:SPL9* plants showed a noticeable reduction in the length and number of lateral roots under both MS and LP conditions (Fig. 3), showing that SPL9 regulates lateral root development irrespective of the Pi status of the plant.

miR399f and SPL9 are Expressed in Opposite Patterns

Pi deficiency dramatically promotes miR399 expression [7]. We examined GUS expression in transgenic *PromiR399f:GUS* and *ProSPL9:GUS* tissues from plants grown on MS and LP medium to see if SPL9 exhibited a similar pattern of expression. The

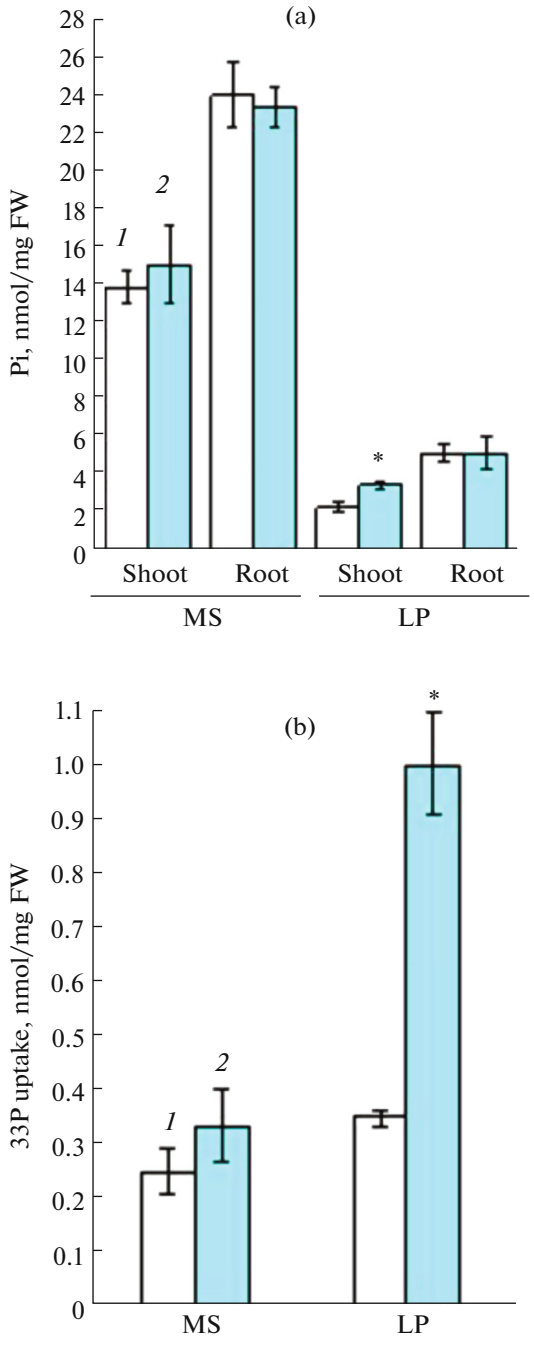

Fig. 1. Pi concentrations and Pi uptake in wild-type (*1*) and *35S:SPL9* (*2*) plants under Pi deficient conditions. (a) Pi levels in the roots and shoots of plants grown on MS or LP agar (or agarose) plates. The 7-day-old seedlings were kept on MS or Pi-deficient medium for 14 days before being collected to measure the Pi concentration. (b) The rate of ^{33}Pi uptake in roots. Seedlings were grown on MS medium for 11 days before being transferred for 2 h to solutions of MS or LP enriched with ^{33}Pi; ^{33}Pi absorption in the roots was then measured. The assay was repeated three times, each time with roughly 30 seedlings. Any significant difference in comparison to the wild-type ($P < 0.05$) is indicated by asterisks. The SD ($n = 3$) is represented by the error bars.

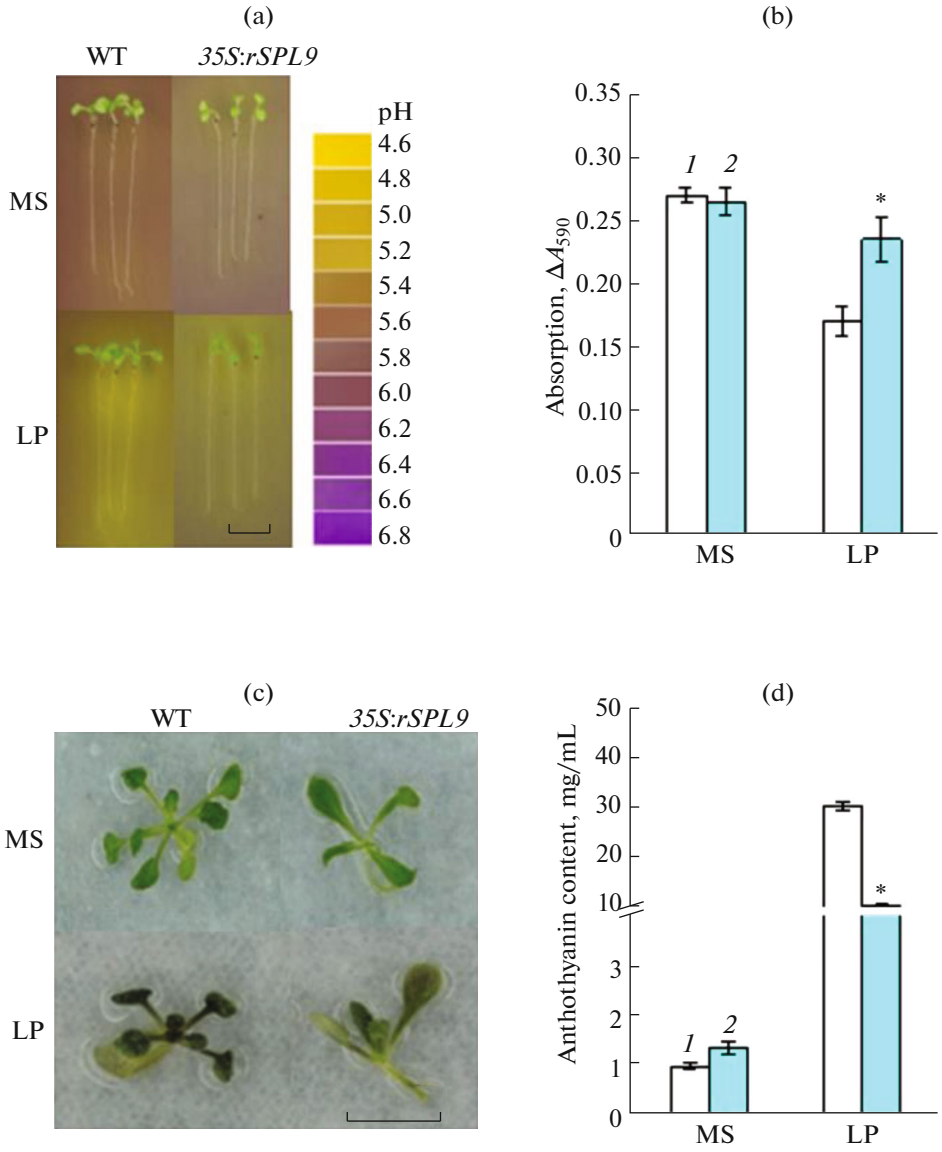

Fig. 2. Responses to rhizosphere acidity and anthocyanin accumulation in wild-type (*1*) and *35S:SPL9* transgenic (*2*) plants. (a) Acidification of seedling roots 9 days after switching from a medium containing sufficient Pi (1.25 mM) to a medium lacking in Pi (2.5 μM), as indicated by the staining of bromocresol purple dye, a pH indicator. Bar = 1 cm. (b) Estimation of acidification capacity. Nine-days-old plants were incubated for 3 h in MS or a Pi-deficient solution (0.006% bromocresol purple, 0.5% sucrose, pH 5.8). Two independent tests with 12 plants each were conducted, and SD are shown as error bars. (c) Anthocyanin accumulation in plants cultivated in MS (Pi-sufficient) or LP (Pi-deficient) hydroponic conditions at 21 days. Bar = 1 cm. (d). The SD (n = 3) is represented by error bars. Any significant difference in comparison to the respective control ($P < 0.05$, Student's t-test) is represented by an asterisk.

promoter *miR399f* showed weak activity on GUS staining in the cotyledon and leaf vascular tissues on MS media, as expected (Supplementary Figs. S1a–S1d). The *miR399f* promoter was strongly expressed in rosette leaves, cotyledons, and lateral and primary roots of seedlings grown on Pi-deficient medium (Supplementary Figs. S1e–S1h). GUS staining, on the other hand, was found in cotyledons and rosette leaves of *ProSPL9:GUS* seedlings grown on MS media (Supplementary Figs. S1i–S1l). Under Pi deficiency, cotyledons and rosette leaves of *ProSPL9:GUS* seedlings showed clear inhibition of SPL9 promoter activity (Supplementary Figs. S1m, S1n). In general, the expression patterns of GUS reporter in *PromiR399f:GUS* leaf tissues and ProSPL9::GUS seedlings were opposite, prompting us to investigate whether SPL9 might influence miR399 transcription.

The Binding of SPL9 to the miR399f Promoter

According to the promoter sequence analysis, there were three GTAC-boxes inside the 700 bp *miR399f*

promoter (Fig. 4a), raising the possibility that SPL9 regulates *miR399f* expression directly by binding to the GTAC box in the *miR399f* promoter. Yeast one-hybrid and transient transfection tests were used to examine this. SPL9 activity was investigated in yeast using *lacZ* reporter gene expression mediated by the *miR399b* and *miR399f* promoters. The GAL4-activation domain was present in the effectors. SPL9 binds to the DFR promoter (Fig. 4b), which was employed as a positive control. SPL9 protein production increased *lacZ* expression in yeast bearing four copies of the GTAC motif, which was present in the promoter of *miR399f*, as shown in Fig. 4. By contrast, yeast cells harboring the *miR399b* promoter as a reporter gene, did not become blue, indicating that the SPL9 protein was linked to the *miR399f* promoter.

A protoplast transient transfection assay and a ChIP-qPCR assay were used to determine whether *miR399f* genes are targets of SPL9. When Arabidopsis protoplasts were co-transformed with the *PromiR399f:LUC* reporter and pBI221-SPL9 or pBI221 (negative control) as an effector construct, SPL9 increased *miR399f* promoter activity compared to pBI221 (Fig. 4d). Furthermore, the ChIP-qPCR test from *GFP-rSPL9* seedlings revealed that the fragment area A and B of the *miR399f* promoter was substantially enriched under low Pi conditions (Fig. 4f). These findings suggest that SPL9 interacts to the *miR399f* gene promoter in vivo.

SPL9 and SPL3 are Functionally Similar or Redundant in Regulating Low Pi Responses

SPL3 has previously been shown to play an important role in Pi starvation-induced plant response and to regulate *miR399f* expression [12]. We created a *35S:SPL3/35S:SPL9* line by crossing *35S:SPL3* and *35S:SPL9* transgenic plants to examine the interaction between SPL3 and SPL9. When cultivated on LP media, the *35S:SPL3*, *35S:SPL9*, and *35S:SPL3 35S:SPL9* lines showed higher Pi content in the shoots, lower rhizosphere acidification capability in the roots, and lower anthocyanin levels in the shoots (Fig. 5). The low Pi-sensitive phenotypes of *35S:SPL9* plants were more similar to those of *35S:SPL3* plants than to those of *35S:SPL3 35S:SPL9* plants. These findings suggested that SPL9 and SPL3 may have comparable or redundant roles in controlling low Pi responses.

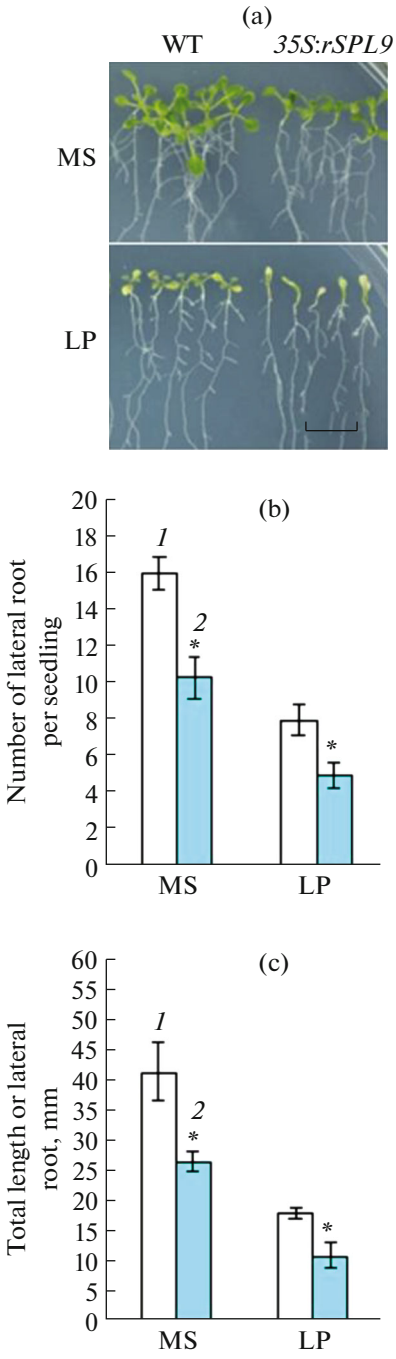

Fig. 3. Root architecture in wild type (*1*) and *35S:SPL9* plants (*2*). (a) Plant lateral roots. Plants were cultivated on 1/2MS agar medium for 5 d before switching to MS or LP (Pi-deficient) medium for 9 d. Bar = 1 cm. (b) Total number of lateral roots per plant. (c) The total length of all first-order lateral roots per plant. Any significant difference compared to the respective control ($P < 0.05$, Student's *t*-test) is presented as an asterisk.

DISCUSSION

SPL9 is an Important Regulator of Pi Deficiency Responses in Plants

Transcription factors have a significant impact on plants responses to Pi deficiency, according to prior studies. Some transcription factors, including PHR1, ZAT6, basic helix-loop-helix32, WRKY75, WRKY6, WRKY42 and WRKY45, have been identified to be involved in Pi deficiency-induced reactions [35]. Previous transcriptomic analyses conducted in SPL9 overexpression plants may help to shed light on the functions of SPL9 under low Pi stress [34]. This research also reveals that the transcription factor SPL9 in Arabidopsis modulates plant response to Pi defi-

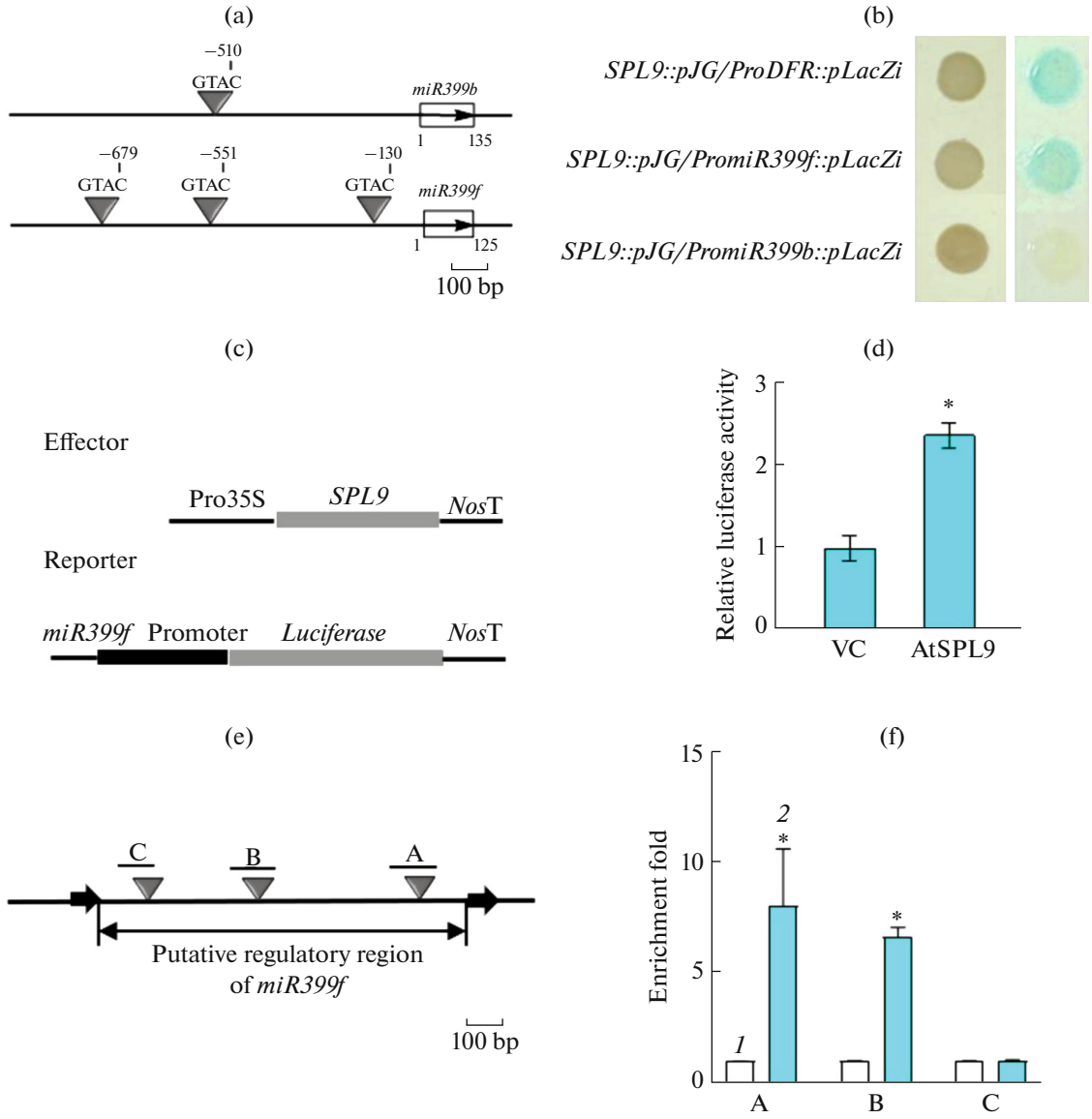

Fig. 4. Yeast one-hybrid and transient transfection analyses of the SPL9 protein. (a) The relative placements of GTAC boxes as shown in the diagram of the *miR399b* and *miR399f* promoters. The adenine residue of ATG, the initiation codon for translation, was assigned position +1. (b) A yeast one-hybrid assay demonstrating that SPL9 promotes the expression of the *ProDFR:LacZ* and *PromiR399f:LacZ* reporter genes. (c, d) In *Arabidopsis* protoplasts, transient transfection assays show that the SPL9 protein promotes production of the LUC reporter gene, which is controlled by the *miR399f* promoter. The luciferase activity was set to 1× in protoplasts transfected with the empty effector and reporter vectors. (e) The *miR399f* promoter regions are depicted schematically. Gray triangles represent the GTAC motifs. Fragments amplified in the ChIP-qPCR experiment are shown by black horizontal bars. ChIP-qPCR analysis of *miR399f* genomic fragments. The ratio of (2) *GFP-rSPL9* signal to (*1*) WT signal is represented as fold enrichment. The SD ($n = 3$) is represented by error bars. Any significant difference compared to the respective control ($P < 0.05$, Student's *t*-test) is presented as an asterisk.

ciency. SPL9 overexpression increased the Pi content of Arabidopsis shoots and the Pi uptake of Arabidopsis roots (Fig. 1). Furthermore, SPL9 overexpression reduced rhizosphere acidification and anthocyanin accumulation (Fig. 2). SPL9 regulates *miR399f* expression via binding to the *miR399f* promoter, according to biochemical investigations (Fig. 4). We found that SPL9, a plant response regulator to Pi deficiency, directly regulates *miR399f* expression to balance plant Pi uptake.

SPL3, SPL9 and SPL10 proteins are involved in the suppression of lateral root development [36]. Under low Pi stress, the *35S:rSPL9* plants also showed decreased lateral root lengths and numbers (Fig. 3). SPL9 and its target gene *DFR* (*DIHYDROFLAVONOL-4-REDUCTASE*) are also implicated in anthocyanin metabolism and abiotic stress tolerance [24, 25]. SPL9 could possibly have a role in Pi deficient reactions such anthocyanin accumulation.

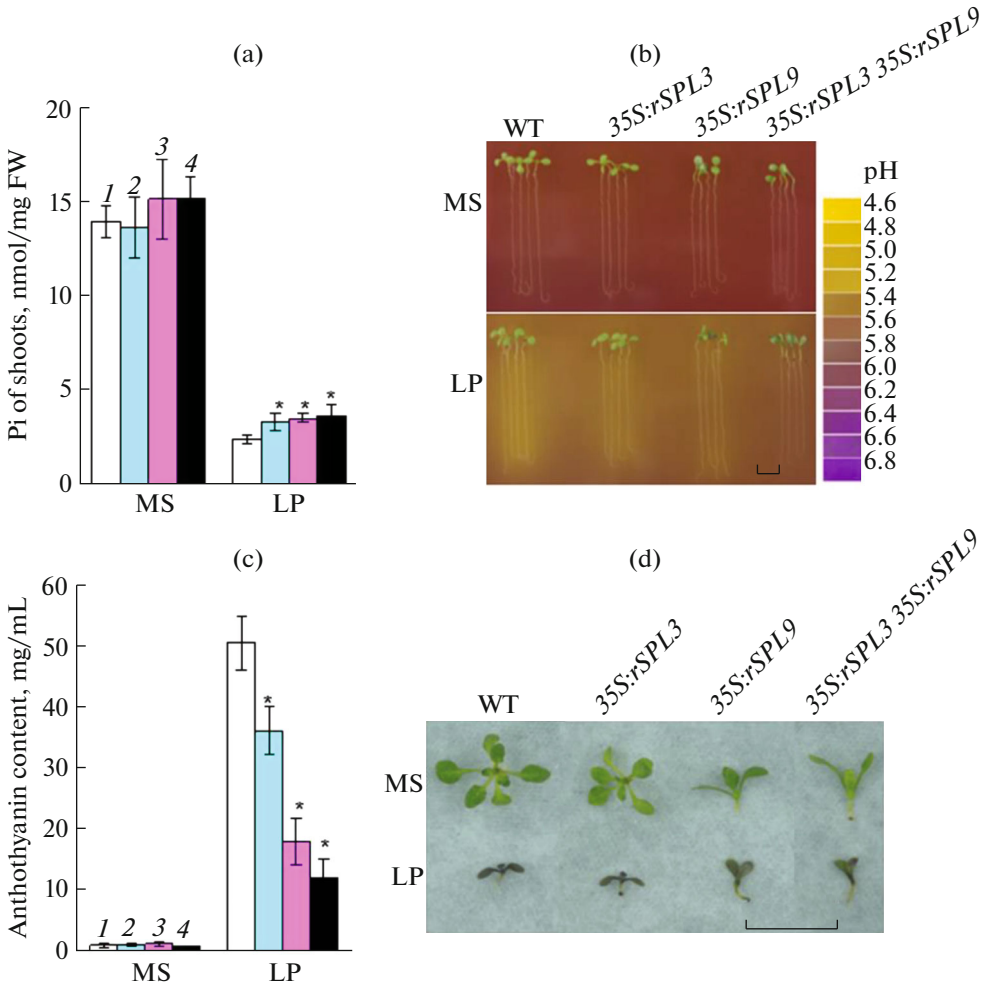

Fig. 5. The wild-type (*1*), *35S:rSPL3* (*2*), *35S:rSPL* (*3*) and *35S:rSPL3/35S:rSPL9* (*4*) transgenic lines responses to modulation of Pi-deficiency. (a) Pi concentration in the shoots of plants grown on MS (Pi-sufficient) or LP (Pi-deficient) agar plates. Seedlings were grown on MS or LP media for 14 d before being harvested to determine the Pi content. (b) The acidification of seedling roots after 9 days of being switched from Pi-sufficient (1.25 mM Pi) media to Pi-deficient (2.5 μM) medium, as shown by the staining with bromocresol purple, a pH indicator dye. Bar = 1 cm. (c) Anthocyanin levels in plants cultivated on MS or LP media for three weeks. Any significant difference from the respective control (Student's *t*-test; $P < 0.05$) is indicated with an asterisk. (d) Anthocyanin accumulation in 14-day-old seedlings grown in hydroponic media which contained sufficient Pi (MS) or was deficient in Pi (LP). Bar = 1 cm.

SPL9 and SPL3 Have Redundant Functions during Pi Deficiency Responses

SPL proteins are plant-specific transcription factors, and contains 17 family members in *Arabidopsis*. Previous research has demonstrated that SPL transcription factors function redundantly; for example, overexpression of either SPL3 or SPL9 accelerates blooming [36], In vegetative development, *SPL3*, *SPL9* and *SPL10/SPL11* have overlapping functions [37, 38]. SPL3 has previously been shown to directly regulate *miR399f* expression by binding to regions I and II within the *miR399f* promoter [12]. SPL9 was also shown to regulate *miR399f* expression in this study. Furthermore, both SPL3 and SPL9 are implicated in rhizosphere acidification, anthocyanin accumulation, and Pi uptake. Thus, the transcription factors SPL3 and SPL9 may play comparable or redundant functions in low Pi responses. However, SPL9 may have a dominant role because its overexpression lines exhibited phenotypes more comparable to *35S:SPL3/35S:SPL9* plants.

Finally, our biochemical and physiological investigations suggest that SPL9 plays an important role in the low Pi response. To respond to changes in the environment caused by Pi accessibility, the SPL9 transcription factor regulates the expression of *miR399f*.

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COMPLIANCE WITH ETHICAL STANDARDS

The authors declare that they have no conflict of interest. This article does not contain any studies involving animals or human participants as objects of research.

SUPPLEMENTARY INFORMATION

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