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Function of Soybean miR159 Family Members in Plant Responses to Low Phosphorus, High Salinity, and Abscisic Acid Treatment

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Abstract: MicroRNAs (miRNAs) regulate plant growth and development and plant responses to biotic and abiotic stresses. Although extensive studies show that miR159 family members regulate leaf and flower development in *Arabidopsis thaliana*, the roles of miRNAs in soybean (*Glycine max*) are poorly understood. Here, we identified six *MIR159* genes in soybean, *MIR159a–MIR159f*, and investigate their expression patterns in plants under low-phosphorus (low-P), NaCl, or abscisic acid (ABA) treatments. In soybean leaves, *MIR159e* and *MIR159f* expression was induced by low-P treatment, while in roots, *MIR159b*, *MIR159c*, *MIR159e*, and *MIR159f* expression was upregulated. In flowers, low-P led to upregulation of *MIR159a*, *MIR159b*, *MIR159c*, and *MIR159f* but downregulation of *MIR159d* and *MIR159e*. In soybean nodules, *MIR159b* was upregulated but *MIR159a*, *MIR159c*, and *MIR159d* was downregulated under P deficiency. NaCl treatment induced *MIR159a*, *MIR159b*, *MIR159c*, and *MIR159e* expression in leaves and *MIR159a–MIR159f* expression in roots. ABA treatment upregulated *MIR159a*, *MIR159b*, and *MIR159c* but downregulated *MIR159d*, *MIR159e*, and *MIR159f* in leaves. These results suggest that miR159 family members function in plant abiotic stress responses. Moreover, total P content in leaves was significantly lower in plants overexpressing *MIR159e* than in the wild type, suggesting that miR159e may regulate P absorption and transport in soybean plants.

Keywords: ABA; gene function; miR159; NaCl; phosphorus; soybean

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1. Introduction

MicroRNAs (miRNAs) are non-coding small (20~24 nucleotide) RNAs that are widespread in plants and animals as well as in single-celled algae [1]. MiRNAs inhibit gene expression and translation after transcription [2]. Several conserved miRNA families have been identified. In plants, for example, miR156, miR159, miR164, miR168, miR169, miR171, miR319, miR390, miR393, miR399, and miR827 play crucial roles in growth and development as well as in response to biotic and abiotic stress.

As one of conserved miRNA family, miR159s have been found in Arabidopsis [3], rice [4], soybean [5], cotton [6], tomato [7], potato [8], strawberry [9], rapeseed [10], wheat [11], apple [12], lily [13], and tobacco [14], etc. As has been well documented, the miR159 family plays important roles in plant growth and development [15]. The levels of Arabidopsis (*Arabidopsis thaliana*) miR159 are regulated by gibberellic acid (GA), and overexpressing miR159 reduces the transcripts of *LEAFY*, inhibits flowering in short-day conditions, and perturbs anther development [3]. Arabidopsis *MYB101* and *MYB33* are targets of miR159; these genes encode MYB transcription factors that function in seed

germination [16]. Arabidopsis *MiR159* is present in pollen where it has a crucial role in fertility [17,18]. In contrast to Arabidopsis wild type, the double mutant *miR159a miR159b* has a larger meristem; accordingly, the transcript levels of *MYB33*, *MYB65*, and *MYB101* in *miR159a miR159b* are increased [19]. Rice miR159 positively regulates grain filling via downregulating the expression of miR167 that targets *OsARF12* [4]. As previously documented, tomato miR159 (Sly-miR159) control fruit growth and development via promoting GA biosynthesis. The target gene of Sly-miR159 *SlGAMYB2* directly inhibits the activities of SlGA3ox2 that catalyze the degradation of GA [7]. Overexpressing *Brassica napus* miR159 (bna-miR159) in Arabidopsis results in lower seed setting and shorter siliques [10].

miR159s are involved in the responses of plants to biotic stress. Soybean miR159-3p is down-regulated during cyst nematode infection; consequently, overexpressing miR159 in soybean hairy roots increases the resistance to *Heterodera glycines* [5]. Lily miR159a play positive roles to cope with grey mold via down-regulating its target gene *GAMYB* [13]. Tobacco miR159 inhibits constitutive pathogen defense [14].

miR159s play crucial roles in land plants to cope with abiotic stresses. For example, wheat miR159 is induced by osmotic stress [20]. miR159s, in particular, regulate plant responses to nutrient stress, such as low phosphate (low-P) stress. *MIR159a*, *MIR319a*, *MIR396a*, *MIR389b*, and *MIR1507a* are upregulated under low-P stress [21]. Nitrogen is a very important macronutrient for plant growth and development. Previous studies have revealed some low nitrate-responsive miRNAs such as miR169 and miR393 [22,23]. Moreover, ABA has been proved to regulate plants' responses to low nitrogen [24]. On the other hand, interactions between phosphate and nitrogen have been reported [25]. However, the roles of soybean miR159s in phosphorus and nitrogen nutrition remain elusive. Eight miRNAs associated with salt tolerance have been identified in peanut (*Arachis hypogea*): miR159-1, miR159-2, miR159-3, miR164-2, miR167-3, miR319-1, miR319-2, and miR211-1 [26]. A study of the responses of salt tolerance related miRNAs to high-salt habitats in the mangrove companion plant *Sesuvium portulacastrum* showed that miR159 is involved in the responses of different tissues to high-salt stress [27], whereas the responses of soybean (*Glycine max*) *MIR159* genes to abiotic stress and their roles in abiotic stress responses are unclear. It is well known that soil salinity and drought are common threats to crops. As global warming becomes more and more severe, the salinity and drought stress will be more common, which could lead to decreased crop yields worldwide. Moreover, the involvement of stress hormone ABA in salinity and drought has been well documented. Hence, in this study, we focus on the responses of soybean miR159 family members to low phosphate, salinity, and ABA.

The Arabidopsis *hyponastic leaves 1* (*hyl1*) mutant, which fails to process primary miRNA transcripts (pri-miRNAs), is sensitive to abscisic acid (ABA) during germination [28], suggesting that the ABA signaling pathway might be regulated by miRNAs. Indeed, miR159 responds to ABA and drought treatment [16,29]. Analysis of the upstream regions of three *MIR159* genes revealed the presence of ABA-responsive elements (ABREs) and associated stress factors such as AtMYC2 binding sites [30]. In line with this finding, ABRE-like elements were identified in the upstream region of the *MIR159a* promoter [16].

Here, we identified six *MIR159* genes in soybean: *MIR159a–MIR159f*. We then explored the responses of the soybean *MIR159* gene family to low-P, NaCl, and ABA treatment. Finally, we overexpressed *MIR159e* in soybean. Under LP conditions, the total P content of *MIR159e*-overexpressing transgenic plants was significantly lower than the wild type in leaves but not roots. These results suggest that *miR159e* modulates the absorption and transport of P in soybean.

2. Materials and Methods

2.1. Plant Materials and Growth Conditions

Seeds of soybean (*Glycine max*) genotype YC03-3 were sterilized with 10% NaClO, germinated in sand, and transferred to nutrient solution when the cotyledons were open and the apical bud had developed. To explore the responses of soybean miR159 family

members to low-P stress, NaCl stress, and ABA treatment, seedlings with the first developed trifoliate leaf were transferred to high phosphate (Pi), high N (HP, 250 μM KH_2PO_4; HN, 5.3 mM nitrogen), N-deficient (HP, 250 μM KH_2PO_4; LN, 530 μM nitrogen), or Pi-deficient (LP, 5 μM KH_2PO_4; HN, 5.3 mM nitrogen; LN, 530 μM nitrogen) medium for 0 days (0 D), 7 days (7 D), and 40 days (40 D), respectively.

The plants were grown in growth chambers under a 16 hour (h) light/8 h dark cycle. The nutrient solutions were aerated for 15 min every 3 h and replaced with fresh nutrient solutions every 2 days. The roots, leaves, flowers and nodules were sampled, frozen in liquid nitrogen, and stored at −80 °C. After 0 or 6 h of ABA treatment, including −ABA (0 μM) or +ABA (300 μM), the treated seedlings were sampled, quickly frozen in liquid nitrogen, and stored at −80 °C. After 0 or 6 h of NaCl treatment, including −NaCl (0 mM) or +NaCl (200 mM), the seedlings were sampled, frozen in liquid nitrogen, and stored at −80 °C.

2.2. Extraction of Total RNA, Reverse-Transcription, and Extraction of Genomic DNA

Total RNA was extracted from soybean seedlings using the TRIzol method. The RNA was reverse transcribed to cDNA using M-MLV reverse transcriptase. DNA was extracted from the samples using the CTAB method. Other molecular experiments were performed using standard methods as described [31].

2.3. qRT-PCR Analysis

To quantify transcript levels in soybean genotype YC03-3 under different P conditions, quantitative real-time PCR (qRT-PCR) was performed. The roots and leaves of YC03-3 seedlings that were subjected to low-P stress were quickly frozen in liquid N and stored at −80 °C. Total RNA extraction and cDNA synthesis were performed using standard methods as described [31]. Reverse transcription reaction was performed using PrimeScriptTM RT regent Kit (Takara) in a final volume of 10 μL, including 2 μL of 5× PrimeScript™ Buffer, 0.5 μL of PrimeScript™ RT Enzyme Mix I, and 1 μM of Universal RT-primer, and it was incubated at 37 °C for 15 min followed by enzyme inactivation at 85 °C for 5 s. Soybean *EF1a* was used to normalize the PCR data. The expression levels of the six *MIR159* genes (*MIR159a*, *MIR159*b, *MIR159*c, *MIR159*d, *MIR159*e, *MIR159*f) were measured in leaves, roots, and nodules. Forward and reverse primers for miRNA qRT-PCR amplification were designed according to previous studies [12,32] (Supplemental Table S2).

2.4. Measurement of Fresh Weight, Soluble Phosphate (Pi), and Total P Contents in Soybean Seedlings

Samples of soybean plants that were subjected to low-P stress for 40 days, as described, were weighed to quantify the fresh weight, including the fresh root and leaf. The lengths of roots and shoots were measured, and these values were used to calculate the root-to-shoot ratio. Soybean seedlings were dried at 105 °C for 30 min, oven-dried at 75 °C and then weighed. Soluble Pi and total P concentrations were measured exactly as described [32,33].

2.5. Vector Construction and Soybean Transformation

In short, the overexpressing soybean miR159e vector construction and whole soybean plant transformation were made based on the methods described [34]. The sequence of miR159e precursor was PCR amplified from the cDNA of soybean YC03-3 genotype using specific primers (forward: AAcccgggTAGCAAGGGTTTAGGTGGTG, reverse primer: AAtctagaAGAGCAAGAACGAGATTATGG) that contain SmaI and XbaI site, respectively. Then, the PCR product was cloned into the binary vector pTF101.1 that harbor a Bar gene under the control of 2× 35S CaMV promoter.

The cotyledonary-node was transformed. Primary transformants were grown to maturity in the greenhouse. Overexpressing miR159e lines derived from double 35S promoters were determined by PCR and Bar resistance [34]. Single copy T-DNA insertion homozygous transformant lines were used in this study.

2.6. Phytogenetic Tree Reconstruction and Data Analysis

MEGA 6.0 software [35] was used to construct the phylogenetic tree using the neighbor-joining method. The promoters were analyzed using the TSSP program of Phytozome (https://phytozome-next.jgi.doe.gov/) (accessed on 4 July 2023) and softberry (http://www.softberry.com/) (accessed on 4 July 2023); target genes were predicted using psRNA Target (http://plantgrn.noble.org/psRNATarget/) (accessed on 4 July 2023) [36].

All data were analyzed with Excel 2010. Student's *t*-test was employed to identify significant differences between treatment groups. GraphPad Prism 8 was used to draw the figures.

3. Results

3.1. Identification of MIR159 Gene Family Members in Soybean

To explore the *MIR159* gene family, their target genes, and the evolution of this family in soybean, we obtained sequences of miR159 family members in soybean, rice (*Oryza sativa*), Arabidopsis, alfalfa (*Medicago truncatula*), maize (*Zea mays*), and *Brachypodium distachyon* from the miRBase website (http://www.mirbase.org) (accessed on 4 July 2023). We found that the soybean *MIR159* family consists of *MIR159a*, *MIR159b*, *MIR159c*, *MIR159d*, *MIR159e*, and *MIR159f*. *MIR159a* and *MIR159d* are located on chromosome 9, *MIR159b* and *MIR159e* are on chromosome 7, and *MIR159c* and *MIR159f* are on chromosome 16 (Table 1).

Table 1. The *MIR159* family in soybean.

Name	Mature miRNA Sequence	Position on Chromosome
MIR159a	GAGCUCCUUGAAGUCCAAUUG	Gm09: 40,266,722–40,266,935 +
MIR159b	GAGUUCCCUGCACUCCAAGUC	Gm07: 5,424,789–5,424,974 −
MIR159c	AUUGGAGUGAAGGGAGCUCCG	Gm16: 2,830,034–2,830,218 −
MIR159d	AGCUGCUUAGCUAUGGAUCCC	Gm09: 40,267,077–40,267,097 +
MIR159e	GAGCUCCUUGAAGUCCAAUU	Gm07: 9,561,934–9,562,144 −
MIR159f	GAGUUCCCUGCACUCCAAGUC	Gm16: 2,819,636–2,819,815 −

Note: Gm, *Glycine max*; +, sense strand; −, antisense strand.

To decipher the evolutionary history of *MIR159* in plants, we compared the *MIR159* sequences of soybean with those from the plant species listed above. After aligning them using the CLUSTALW program, we analyzed their sequences using MrBayes3.2, finding that plant *MIR159s* belong to three subgroups (Figure 1). The soybean *MIR159* genes fell into two subgroups: *MIR159a*, *MIR159d*, and *MIR159e* belong to subgroup I, and *MIR159b*, *MIR159c*, and *MIR159f* belong to subgroup II. Subgroup III contains only the *MIR159* gene from monocotyledons (Figure 1). These results indicate that *MIR159* genes are conserved across plant species, implying that they might play important roles in plant growth and development.

ABREs can be found in the promoter regions of *MIR159* genes in Arabidopsis [16], and *MIR159e* responds to low-P stress in soybean [32]. To identify the *cis*-elements in these gene promoters, we analyzed the 2000-nucleotide sequence upstream of the start codon (ATG) in all six *MIR159* genes (Table 2). As expected, we found phosphorus-responsive elements, NaCl-responsive elements, and ABREs in the promoters of all six soybean *MIR159* genes (Table 2). Among the phosphorus-responsive elements, we identified three or four TATA-box and W-box binding elements in each of the six *MIR159* gene promoters, but no TATA-box-like binding elements, and we found that only *MIR159c* and *MIR159f* contain P1BS binding elements. Among the NaCl-responsive elements, all six *MIR159* gene promoters contain one ABRE-like and one ACGT sequence binding element, but *MIR159b* and *MIR159c* lack RD22 binding elements and *MIR159f* lacks AtMYB2 binding elements. Finally, among ABREs, all six *MIR159* promoters contain a DPBF binding element, while *MIR159c* and *MIR159f* lack ABRE, and only *MIR159e* contains an RY binding element (Table 2). These results suggest that the transcription of *MIR159* genes in soybean seem to be regulated by PHR1, WRKY, and ABF transcriptional regulators.

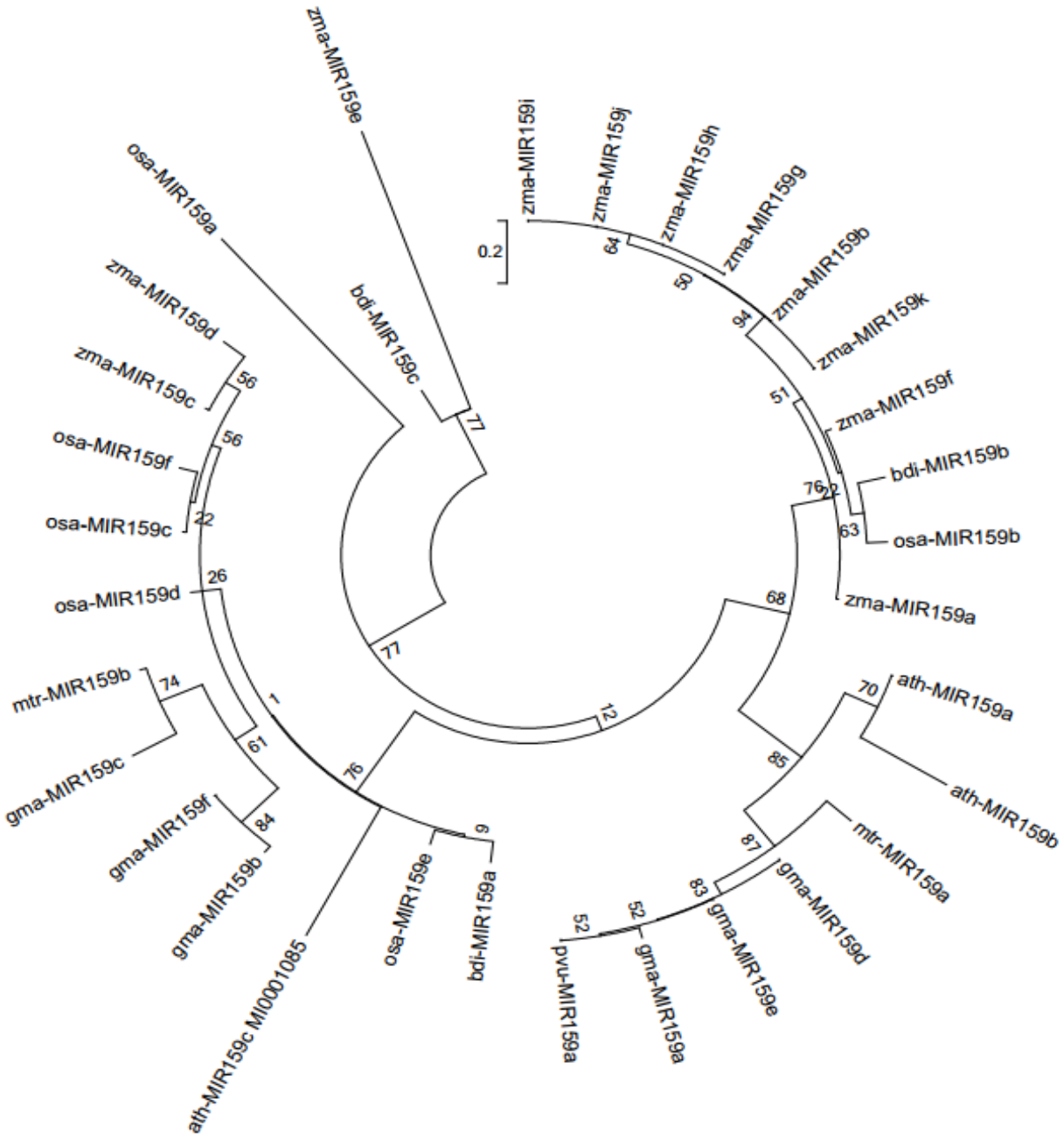

Figure 1. Phylogenetic analysis of the *MIR159* family in soybean and other plant species. Note: Abbreviations: gma, *Glycine max*; ath, *Arabidopsis thaliana*; osa, *Oryza sativa*; mtr, *Medicago truncatula*; zma, *Zea mays*; bdi, *Brachypodium distachyon*; pvu, *Phaseolus vulgaris*.

Table 2. Analysis of elements in the *MIR159* promoters in soybean.

	Low-P Responsive Elements			NaCl-Responsive Elements				ABA-Responsive Elements				
	TATA -Box	TATA -Box Like	W-box	PHR1 Element	ABRE-Like	ACGT Sequence	rd22	AtMYB2	MYC2	ABRE	DPBF	RY Elements
MIR159a	4	1	4		1	1	1	2	1	1	1	
MIR159b	4	1	3		1	1		1	1	1	1	
MIR159c	4	1	4	1	1	1		2	2		1	
MIR159d	4	1	4		1	1	1	3	1	1	1	
MIR159e	4		4		1	1	1	4	3	1	1	1
MIR159f	4	1	3	1	1	1	1		1		1	

3.2. Responses of the Soybean MIR159 Gene Family to Low-P Stress

Various miRNAs are involved in plant responses to low-P (LP) stress. Although *MIR159a* is upregulated in soybean under LP stress [21], the responses of other *MIR159* gene family members to LP stress were not known. To examine this issue, we analyzed the expression of *MIR159* genes in soybean roots, leaves, flowers, and nodules after 7 and 40 days of LP stress via quantitative reverse-transcription PCR (qRT-PCR). Compared to those in the high-phosphorus (HP) control, after 7 days of LP stress, *MIR159a* and *MIR159e* transcript levels were not significantly altered in leaves (Figure 2A,I) but were inhibited in roots (Figure 2B,J), whereas *MIR159b* expression was not significantly changed in leaves but was induced in roots (Figure 2C,D), *MIR159c* expression was inhibited in both leaves and roots (Figure 2E,F), and *MIR159d* and *MIR159f* expression was unaltered. After 40 days of LP stress, however, *MIR159a* expression was increased in leaves (Figure 2A), *MIR159c* expression was induced in roots (Figure 2F), and *MIR159e* and *MIR159f* expression was induced in both leaves and roots (Figure 2I,L). These results suggest that miR159 family members may regulate the responses of soybean to low-P stress in various ways.

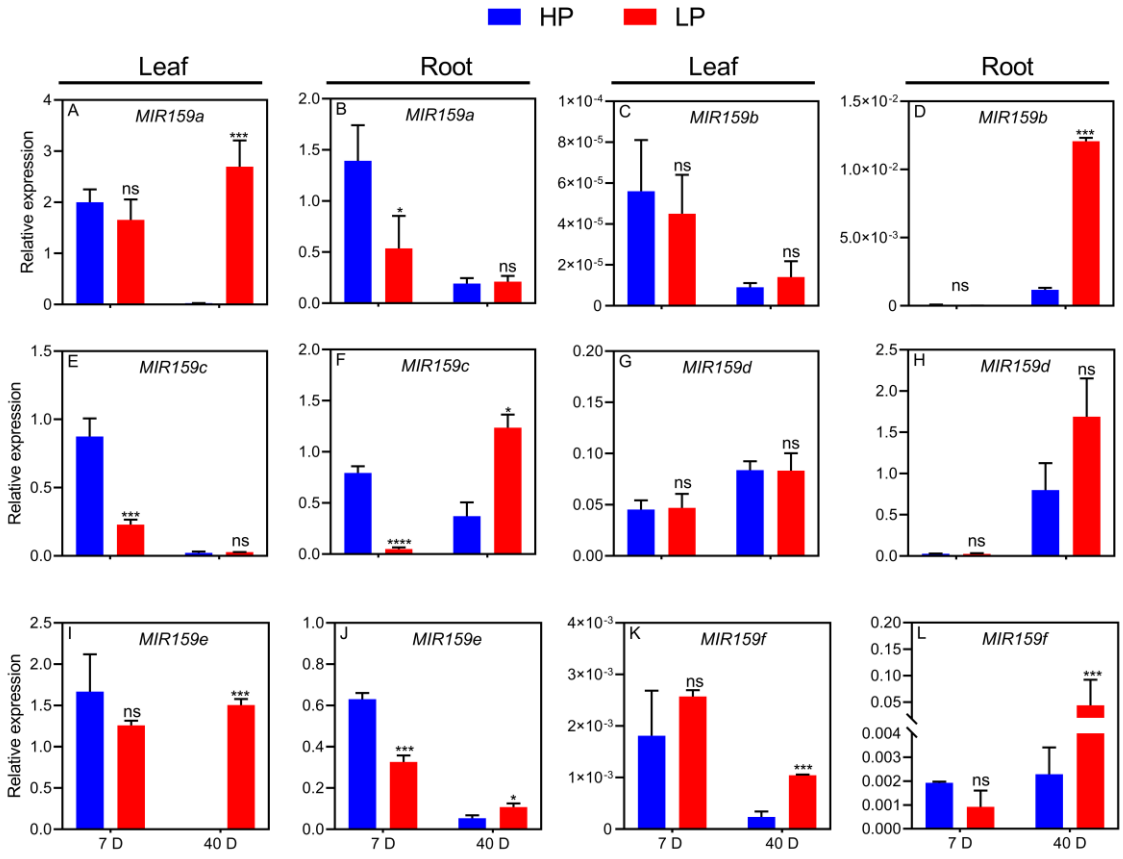

Figure 2. Responses of the soybean *MIR159* gene family to low-P stress in leaves and roots. *MIR159* transcript levels were quantified in samples from soybean plants of genotype YC03-3 that were transplanted when the first ternately compound leaf was fully unfolded and the second ternately compound leaf was not fully unfolded and treated with high and low P (HP, 250 μM KH_2PO_4; LP, 5 μM KH_2PO_4) for 7 days (7 D) or 40 days (40 D). (**A**) *MIR159a* expression in leaf, (**B**) *MIR159a* expression in root, (**C**) *MIR159b* expression in leaf, (**D**) *MIR159b* expression in root, (**E**) *MIR159c* expression in leaf, (**F**) *MIR159c* expression in root, (**G**) *MIR159d* expression in leaf, (**H**) *MIR159d* expression in root, (**I**) *MIR159e* expression in leaf, (**J**) *MIR159e* expression in root, (**K**) *MIR159f* expression in leaf, (**L**) *MIR159f* expression in root. Results are means ± SE from 3 independent experiments. Student's *t*-test was used to determine the differences between control and nutrient-deficiency conditions (ns, no significance; * $p < 0.05$; *** $p < 0.001$, **** $p < 0.0001$).

Given that MiR159 family members regulate flower development in Arabidopsis, and overexpressing *MIR159* delays flowering in short-day conditions [3]. Hence, in this study, we analyzed the expression levels of soybean *MIR159* gene family members in flowers after 25 days of LP stress. Compared with that under HP stress, the expression of *MIR159a*, *MIR159b*, *MIR159c* and *MIR159f* was significantly induced in flowers under LP stress, whereas *MIR159d* and *MIR159e* expression was inhibited by this treatment (Figure 3A).

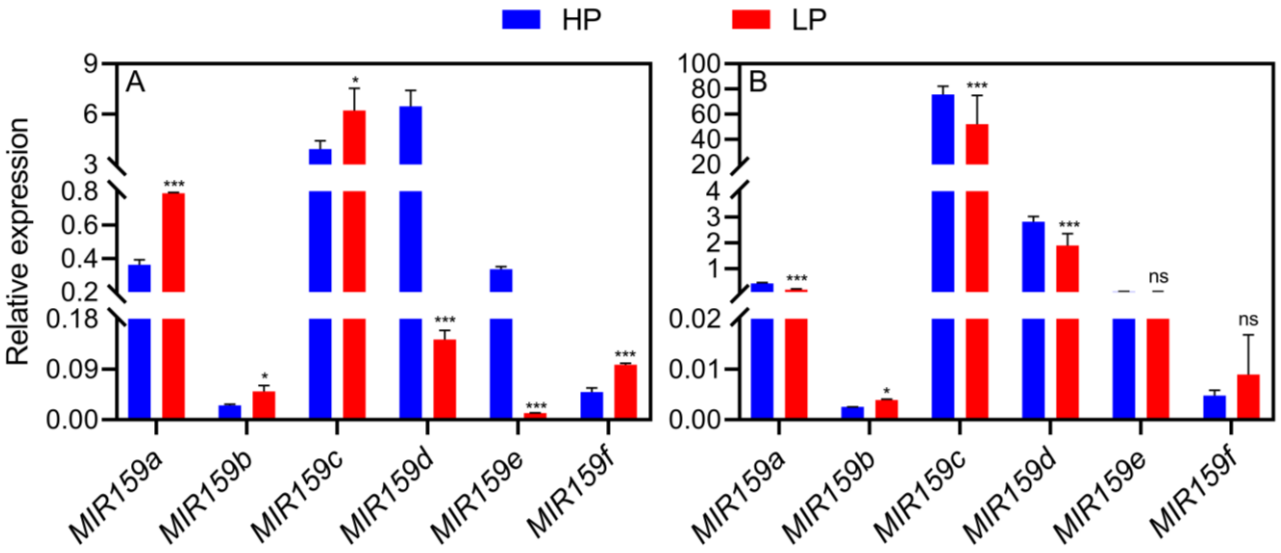

Figure 3. Responses of the soybean *MIR159* gene family to low-P stress in flowers and nodules. (**A**) *MIR159* gene expression in flowers; (**B**) *MIR159* gene expression in nodules. Soybean genotype YC03-3 was transplanted when the first ternately compound leaf leaf was fully unfolded and the second ternately compound leaf was not fully unfolded, and treated with high and low P (HP, 250 μM KH_2PO_4; LP, 5 μM KH_2PO_4) for 25 days. Results are means ± SE from 3 independent experiments. Student's *t*-test was used to determine the differences between control and nutrient deficiency conditions (ns, no significance; * $p < 0.05$; *** $p < 0.001$).

Soybean is a legume crop capable of biological nitrogen fixation; some miR159 family members appear to regulate nodule growth and development in legumes [37]. Given that low-nitrogen (N) promotes nodulation, we cultured soybean plants under high-phosphorus and low-N (HPLN, control) and low-phosphorus and low-N (LPLN) conditions for 7 days and examined the samples 33 days after inoculation with rhizobia. We then examined the expression of *MIR159* gene family members in nodules. Compared to HPLN, under LPLN conditions, the expression of *MIR159a*, *MIR159c*, and *MIR159d* in nodules was significantly inhibited, *MIR159b* expression was induced, and *MIR159e* and *MIR159f* expression did not significantly change (Figure 3B). These results indicate that *MIR159* genes respond to low P stress in nodules.

3.3. Responses of the Soybean MIR159 Gene Family to Salt Stress

MiR159 responds to high-salt stress in *S. portulacastrum* [6,27]. We therefore used qRT-PCR to measure the transcript levels of *MIR159* family genes in leaves and roots of soybean plants subjected to short-term (6 h) treatment with 200 mM NaCl. *MIR159a* (Figure 4A,B), *MIR159b* (Figure 4C,D), *MIR159c* (Figure 4E,F), and *MIR159e* (Figure 4I,J) were significantly upregulated in leaves and roots under this treatment; *MIR159d* was upregulated only in roots (Figure 4G,H); *MIR159f* was down-regulated in leaves but upregulated in roots (Figure 4K,L). These results suggest that the soybean *MIR159* gene family plays important roles in plant responses to salt stress.

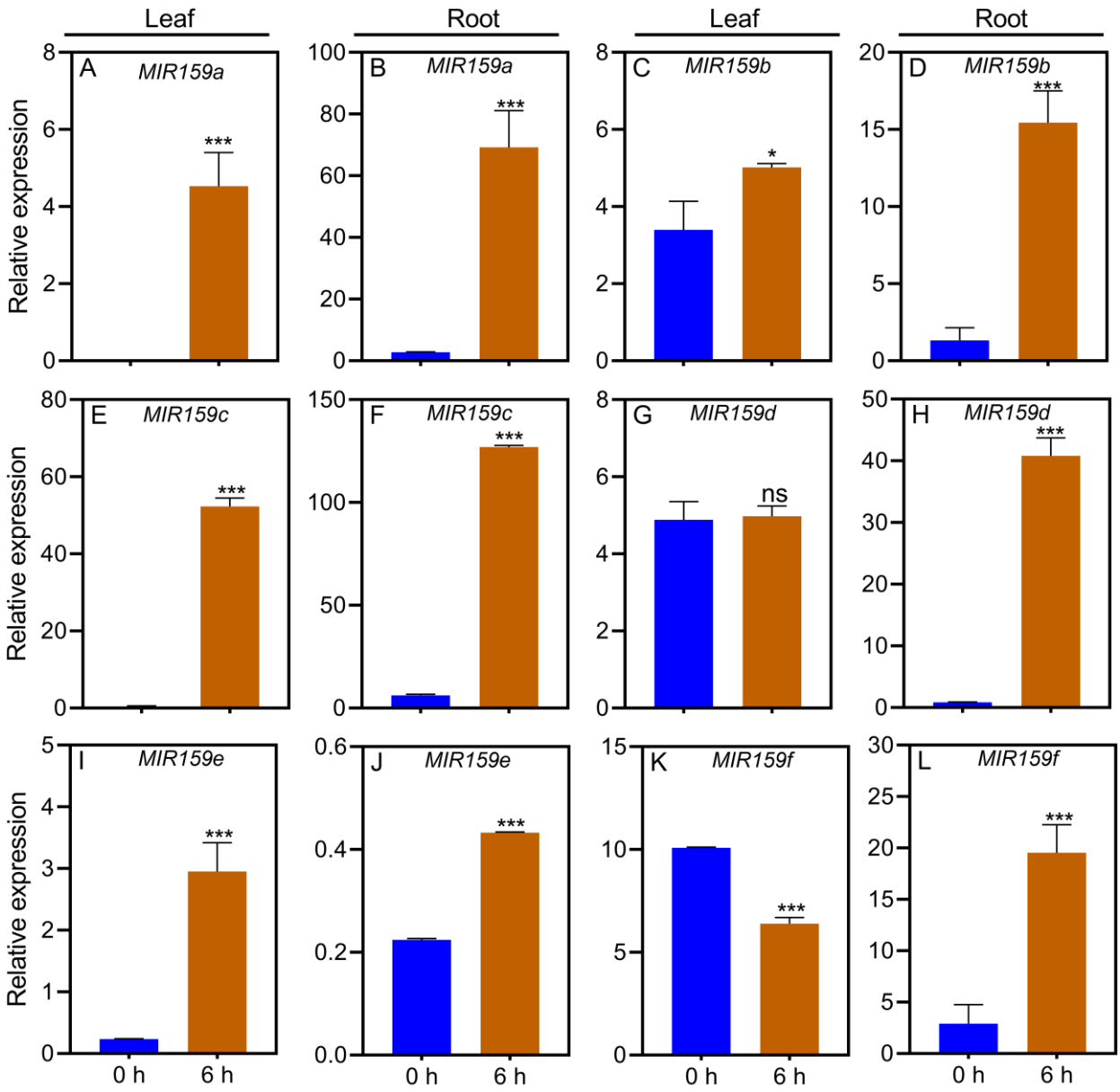

Figure 4. Responses of the soybean *MIR159* gene family to NaCl stress in leaves and roots. *MIR159* transcript levels were quantified in samples from soybean plants of genotype YC03-3 that were transplanted when the first ternately compound leaf compound leaf was fully unfolded and the second ternately compound leaf was not fully unfolded, and treated NaCl with for 0 or 6 h. −NaCl, 0 mM; +NaCl, 200 mM; h, hours. (**A**) *MIR159a* expression in leaf, (**B**) *MIR159a* expression in root, (**C**) *MIR159b* expression in leaf, (**D**) *MIR159b* expression in root, (**E**) *MIR159c* expression in leaf, (**F**) *MIR159c* expression in root, (**G**) *MIR159d* expression in leaf, (**H**) *MIR159d* expression in root, (**I**) *MIR159e* expression in leaf, (**J**) *MIR159e* expression in root, (**K**) *MIR159f* expression in leaf, (**L**) *MIR159f* expression in root. Results are means ± SE from 3 independent experiments. Student's *t*-test was used to determine the differences between control and nutrient deficiency conditions (ns, no significance; * $p < 0.05$; *** $p < 0.001$).

3.4. Responses of the Soybean MIR159 Gene Family to ABA

To further assess the effects of *MIR159* family genes in stress response, we investigated their expression upon exposure to the hormone ABA, a downstream factor in stress response pathways. *MIR159a* responds to ABA treatment in Arabidopsis [16], but the responses of other soybean *MIR159* family genes to ABA remained unclear. In this study, qRT-PCR

demonstrated that *MIR159* gene expression was induced or inhibited in response to ABA. Compared to the control (−ABA), *MIR159a* was upregulated in leaves (Figure 5A), but not in roots, after 6 h of ABA treatment (Figure 5B). In response to ABA treatment, *MIR159b* was upregulated in leaves and roots (Figure 5C,D), *MIR159*C was upregulated in leaves and downregulated in roots (Figure 5E,F), and *MIR159d* was downregulated in leaves (Figure 5G) and upregulated in roots (Figure 5H). *MIR159e* was down-regulated in leaves and roots (Figure 5I,J), and *MIR159f* was down-regulated in leaves and up-regulated in roots (Figure 5K,L). These results suggest that *MIR159* family genes play important roles in regulating ABA responses in soybean.

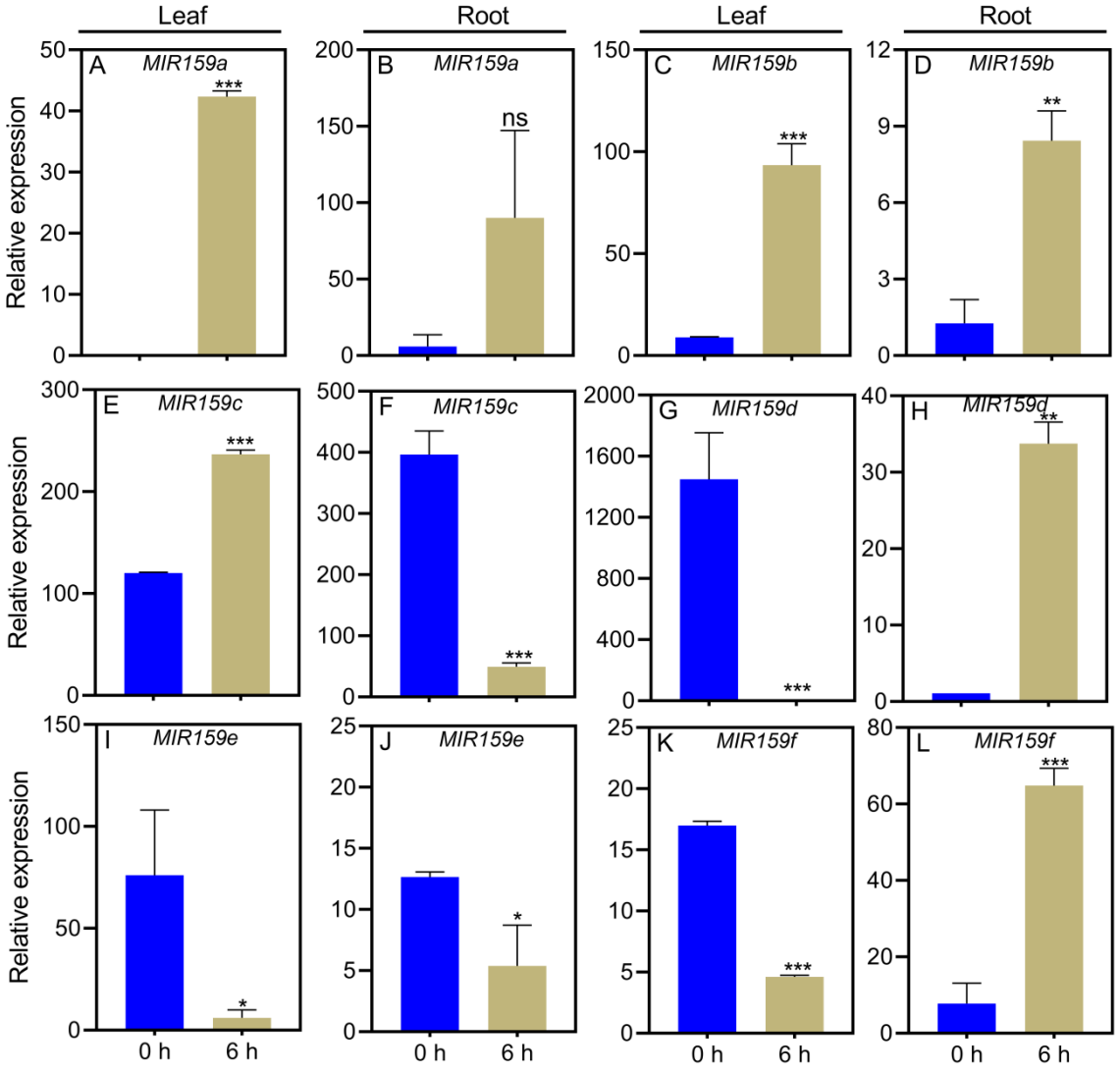

Figure 5. Responses of the soybean *MIR159* gene family to ABA in leaves and roots. *MiR159* transcript levels were quantified in samples from soybean plants of genotype YC03-3 that were transplanted when the first ternately compound leaf was fully unfolded and the ternately compound leaf was not fully unfolded and treated with ABA for 0 h or 6 h. −ABA, 0 μM; +ABA: 300 μM; h, hour. (**A**) *MIR159a* expression in leaf, (**B**) *MIR159a* expression in root, (**C**) *MIR159b* expression in leaf, (**D**) *MIR159b* expression in root, (**E**) *MIR159c* expression in leaf, (**F**) *MIR159c* expression in root, (**G**) *MIR159d* expression in leaf, (**H**) *MIR159d* expression in root, (**I**) *MIR159e* expression in leaf, (**J**) *MIR159e* expression in root, (**K**) *MIR159f* expression in leaf, (**L**) *MIR159f* expression in rootResults are means ± SE from 3 independent experiments. Student's *t*-test was used to determine the differences between control and nutrient-deficiency conditions (ns, no significance; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

3.5. Overexpressing MIR159e Decrease Total Phosphorus Content in Soybean Leaves under LP Conditions

To examine its functions, we overexpressed *MIR159e* driven by the constitutive CaMV 35S promoter in soybean genotype YC03-3. By screening for Basta resistance conferred by the *Bar* gene, we identified dozens of transgenic lines [34]. Following confirmation using a chi-squared test, we selected two single-copy T-DNA insertion lines, #2 and #4, for subsequent experiments. After 40 days of growth under HP (250 μM KH_2PO_4) or LP (5 μM KH_2PO_4) conditions, the growth performance of lines #2 and #4 was not significantly different from that of wild-type YC03-3 (Supplemental Figure S1). Under HP conditions, the P (Figure 6A) and total nitrogen contents (Figure 6C) in #2 and #4 plants also were not significantly different from YC03-3 in leaves or roots. However, under LP conditions, the total P contents were significantly reduced in leaves of #2 and #4 plants compared to YC03-3 (Figure 6B), although the total nitrogen contents were not significantly different from YC03-3 (Figure 6D). Finally, under both HP and LP conditions, the soluble P concentrations of #2 and #4 plants in old leaves, new leaves, and roots were not significantly different from those of wild-type plants (Figure 6F). These results indicate that overexpressing *MIR159e* alters total phosphorus contents in soybean.

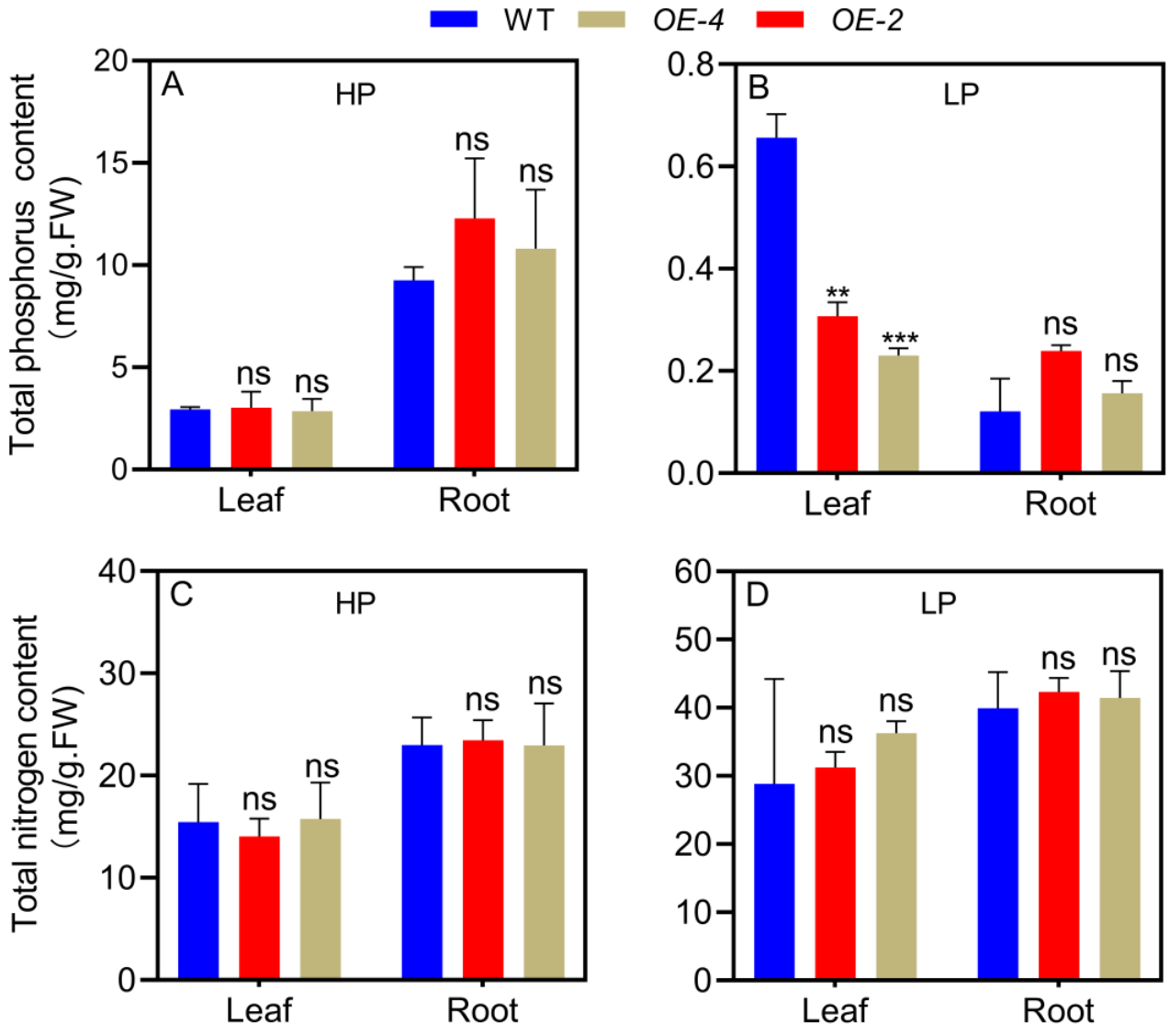

Figure 6. *Cont.*

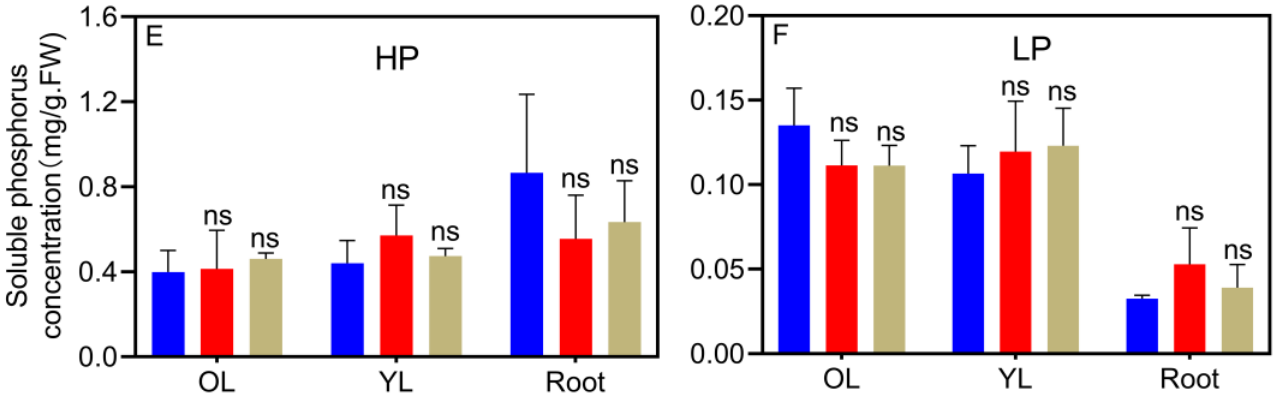

Figure 6. Overexpressing *MIR159e* affects total phosphorus, total nitrogen, and soluble phosphorus contents in soybean. WT, wild type (YC03-3); OL, old leaves; YL, young leaves. Soybean genotype YC03-3 was transplanted when the first ternately compound leaf was fully unfolded and the second ternately compound leaf was not fully unfolded, and treated with high and low P (HP, 250 μM KH_2PO_4; LP, 5 μM KH_2PO_4) for 40 days (40 D). (**A**) Total phosphorus contents in HP conditions, (**B**) Total phosphorus contents in LP conditions, (**C**) total nitrogen content in HP conditions, (**D**) total nitrogen content in LP conditions, (**E**) soluble phosphorus concentration in HP conditions, (**F**) soluble phosphorus concentration in LP conditions. Results are means ± SE from 3 independent experiments. Student's *t*-test was used to determine the differences between control and nutrient deficiency conditions (ns, no significance; ** $p < 0.01$; *** $p < 0.001$).

4. Discussion

Increasing evidence indicates that miRNAs play crucial roles in plant adaptation to nutrient stress [22,23]. In the past decades, most studies on the roles of miRNAs in nutrient stress have been performed in model plants, such as Arabidopsis and rice, but little is known about their roles in legumes. Soybean is an important leguminous crop with great ability to fix nitrogen from the atmosphere, providing protein and edible oil for human consumption. MiRNAs are a class of non-coding small RNAs that function in the post-transcriptional regulation of their target genes by forming RNA-induced silencing complexes to shear the transcripts of their target genes or to inhibit the translation of these transcripts [38]. MiR159 is a highly conserved miRNA family whose members are important in regulating vegetative growth, flowering, anther development, and seed germination in various plants [3,15]. Of note, a recent study verified that rice miR159 regulates grain filling via negatively affecting miR167, and the transcription factor *OsARF12* is the target of miR167, thus overexpressing *OsARF12* enhance field yield [4]. In future, it is necessary to explore whether soybean miR159s modulate flowering development, seed set or grain filling.

Here, we identified six *MIR159* genes in the soybean genome (Table 1), predicted *cis*-acting elements in their promoters, and identified candidate miR159 target genes (Supplementary Table S1). We also examined the expression patterns of *MIR159* genes in response to P (Figures 2 and 3), NaCl (Figure 4), and ABA (Figure 5) in soybean roots, leaves, flowers, and nodules. The highly conserved miRNA159 family is found in vascular plants and bryophytes [15]. Evolutionary analysis suggested that miR159 shares a common ancestor with miR319, another highly conserved miRNA family [39]. Soybean *MIR159* genes are arranged in clusters (Table 1), similar to soybean *MIR399* genes [32]. There are 3, 6, 11, 3, 3, and 1 *MIR159* genes in alfalfa, maize, rice, Arabidopsis, *Brachypodium distachyon*, and common bean, respectively (https://www.mirbase.org/) (accessed on 4 July 2023). Arabidopsis contains three *MIR159* genes [3,39], but the soybean genome contains six, suggesting that the roles of soybean miR159 family members are more complex. In addition, the larger size of the soybean *MIR159* gene family might be related to the two genome duplication events that occurred during the evolution of the soybean genome [40].

Like structural genes, *MIRNA* genes are also regulated at the transcriptional level [2]. In this study, three or four TATA-box and W-box binding elements were found in the promoters of all six soybean *MIR159* genes. Thus, it is interesting to screen WRKYs to regulate the transcription of soybean MIR159 in the near future. However, *MIR159e* lacks TATA-box elements, and only *MIR159c* and *MIR159f* contain P1BS binding elements (Table 2). Hence, *MIR159c* and *MIR159f* transcription might be regulated by PHR-type transcriptional regulators in soybean [41]. *MIR159a* was upregulated in leaves in response to 40 days of low-P stress but was downregulated in roots after 7 days of this treatment (Figure 2A), which is consistent with the results of previous studies [21]. *MIR159c* expression was inhibited in leaves after 7 days of low-P stress (Figure 2E) but induced in roots after 40 days (Figure 2F). *MIR159e* was upregulated in both leaves and roots under low-P stress (Figure 2I,J), which is consistent with our previous reports [32]. *MIR159* was upregulated in leaves and roots after 40 days of low-P stress (Figure 2K,L). In addition, in flowers, *MIR159a*, *MIR159b*, *MIR159c*, and *MIR159f* expression was induced, while *MIR159d* and *MIR159e* expression was inhibited after 25 days of low-P stress (Figure 3A). Therefore, the transcription of these *MIR159* genes is regulated by low-P stress and may be related to the presence of low-P-responsive elements (Table 2).

Overexpressing *MIR159a* in gloxinia (*Sinningia speciosa)* delays flowering and downregulates the expression of *LEAFY* (*LFY*), *AGAMOUS*, *APETALA1* (*AP1*), and *AP3* genes in flower buds [42]. Overexpressing Arabidopsis *MIR159* delays flowering and anther development [3]. *MIR159* and *MYB33* are co-transcribed in the aleurone layer and embryo in germinating Arabidopsis seeds, and the two genes show the same spatiotemporal expression pattern [18]. The Arabidopsis *mir159a mir159b* double mutant shows plant dwarfing, reduced apical dominance, reduced fertility, and an irregular seed shape [18]. However, whether soybean miR159 is functionally conserved with Arabidopsis miR159 requires further study. Soybean *MIR159* is expressed in floral organs and is induced by low-P stress (Figure 3A), but the role of soybean miR159 in regulating flower development remains unclear. Unlike some other stresses, low-P stress delays flowering [43]. In this study, we determined that the expression of *MIR159a*, *MIR159b*, *MIR159c*, and *MIR159f* increases under low-P conditions. This likely leads to the inhibition of flowering-related genes, such as *LFY* and *AP*, thereby delaying flowering under low-P conditions. Of course, further research is needed to confirm or disprove this theory.

Nodules are the sites of symbiosis between leguminous crops and rhizobia. Since nitrogen fixation in nodules requires ATP, maintaining the balance between P and other nutrients is crucial [44,45]. *MIR159* is expressed at a relatively high level in alfalfa nodules [46]. Here, we showed that *MIR159a*, *MIR159c*, and *MIR159d* were downregulated, *MIR159b* was upregulated, and *MIR159e* and *MIR159f* expression was unchanged under low-P stress (Figure 3B). We also noted that *MIR159* genes in soybean showed different response patterns to low-P stress in roots (Figure 2) and nodules (Figure 3B), possibly due to different nutritional conditions.

MIR159 expression is induced in peanut by salt stress, and miR159 regulates the expression of its target genes [26]. In addition, miR159 in different tissues of *S. portulacastrum* is involved in plant responses to high-salt stress [27]. In the current study, qRT-PCR results showed that *MIR159* responds to high-NaCl concentrations in soybean (Figure 4). One ABRE-like and one ACGT sequence binding element were identified in each of the six *MIR159* gene promoters in soybean (Table 2). However, *MIR159b* and *MIR159c* lack RD22 binding elements, which are found in the other four soybean *MIR159* genes. *MIR159a*−*MIR159e* contain two, one, two, three, and four AtMYB2 binding elements, respectively, whereas *MIR159f* did not lack AtMYB2 binding elements (Table 2). Therefore, to understand the functions of soybean miR159s, it will be important to identify the transcriptional regulators of the response of this plant to salt stress and the binding of these regulators to the *MIR159* promoters. Consistently, miR159 in potato (*Solanum tuberosum* subsp. andigena) is induced by salinity [8]. We noticed that the transcripts of wheat miR159 and its target gene TaGRAS178 are increased by osmotic stress [20], and

tomato miR159 is repressed by drought, thus its target gene is induced [29]. Taken together, these studies indicates that plant miR159 play important roles in responses to drought, salinity, and osmotic stress.

Analysis of ABREs showed that all six *MIR159* promoters in soybean contain a DPBF-binding element, whereas *MIR159c* and *MIR159f* lack ABREs and only *MIR159e* contains RY-binding elements (Table 2), suggesting that soybean miR159s may respond to ABA signals. In line with this notion, both *MIR159a* and *MIR159b* were upregulated by ABA treatment in Arabidopsis [16]. ABA plays an important role in regulating root development, seed maturation and germination, and drought and salt stress tolerance. Several ABA-responsive miRNAs have been identified, such as miR399f in Arabidopsis [47]. Previous studies demonstrated that the Arabidopsis *hyl1* mutant is sensitive to ABA during seed germination [28], suggesting that the ABA signaling pathway might be regulated by miRNAs.

ABA signaling directly regulates stomatal opening and transpiration and is the prime candidate responsible for deciding the cellular fate under stress conditions. Moreover, ABA sensitivity is tightly associated with plant drought tolerance and seed dormancy [48,49]. Analysis of the upstream regions of the three Arabidopsis *MIR159* genes revealed ABREs and binding sites for stress-related transcription factors such as AtMYC2 [30]. Jiang et al. (2022) established a signaling cascade involving miR159, MYB33, and ABA-responsive basic leucine zipper transcription factor ABI5 regulating Arabidopsis seed germination under drought stress [50]. A loss of *MIR159* increases Arabidopsis drought tolerance and ABA sensitivity [51]. Sly-miR159 as an important regulator of fruit morphology in tomato, a model species of fleshy-fruit development [7]. The expression of *MIR159a* and *MIR159b* is induced by ABA in Arabidopsis at the seed germination stage, but not at the seedling stage [16]. In the current study, the results in Figure 5 suggest that miR159 family members play important roles in regulating plant responses to ABA in soybean. All six *MIR159* gene promoters contain DPNF binding elements, whereas *MIR159e* and *MIR159f* lack ABREs, and only *MIR159e* contains RY elements (Table 2), which might help explain the different responses of individual soybean *MIIR159* family members to exogenous ABA. On the other hand, strawberry *MIR159a* is induced by exogenous GA, but not miR159b [9], GA application enhanced the abundance of miR159, lowered the expression of its target gene *GmMYB33*, and delayed the development of *Heterodera glycines* [5]. Thus, further studies are needed to reveal the responses of soybean miR159s to other phytohormones such as GA, brassnosteroids, auxin, ethylene, and cytokinins.

In transgenic soybean plants overexpressing *MIR159e* and wild-type soybean plants under different P treatments for 40 days, the fresh weights of both transgenic and wild-type plants were lower under low-P vs. high-P treatment (Supplemental Figure S1A), which is consistent with previous findings [32,43]. Under HP and LP conditions, there was no significant difference in fresh weight, primary root length, root area, total P, soluble P, or total nitrogen contents between *MIR159e*-overexpressing transgenic and wild-type YC03-3 plants (Supplemental Figure S1). However, the primary roots of the Arabidopsis *miR159ab* double mutant are longer than those of the wild type, and its meristem is enlarged [19]. Under HPLN conditions, *MIR159e*-overexpressing soybean plants showed reduced total P contents in leaves and roots (Supplemental Figure S2A) and reduced soluble P concentrations in old leaves and nodules compared to the wild type (Supplemental Figure S2C). These results indicate that overexpressing *MIR159e* affects the uptake and reuse of P in soybean roots under low-N stress. *MIR159e* might be involved in N and P nutrition, but the physiological and molecular mechanisms are unclear. Interestingly, overexpressing *TamiR159* delays rice heading, decreases male sterility, and increases the sensitivity to heat [11].

Whether overexpressing *MIR159e* affected the expression of a phosphate transporter gene and the abundance of its encoded protein in soybean; this should be further studied under low P or low-nitrogen conditions. Under low-N conditions, the total nitrogen content was higher in nodules than in leaves and roots (Supplemental Figure S2B), indicating

that nodules play a role in nitrogen accumulation and provide nitrogen for plant growth. Overexpressing *MIR159e* did not affect the total nitrogen content in leaves, roots, or nodules under HPHN, LPHN, or HPLN conditions (Figure 6, Supplemental Figure S2). These results suggest that miR159e might not be involved in regulating nitrogen balance in soybean. Overexpressing *MIR159* delayed flowering in ornamental gloxinia (*Sinningia speciosa*) [42]. However, in this study, overexpressing *MIR159e* in soybean did not affect flowering. It will be important to design experiments to determine whether overexpressing *MIR159e* alters the sensitivity of soybean to ABA and NaCl. In the future, CRISPR-Cas9 or miRNA-STTM technology is needed to knock out or knock down MIR159 expression to further analyze their functions.

5. Conclusions

In this study, we demonstrated that the soybean genome contains six *MIR159* family members, which are divided into two subgroups. The promoter regions of soybean *MIR159* genes contain phosphorus signal response and NaCl and ABA response elements, and the transcription of various soybean *MIR159* genes is regulated by low-P stress, NaCl, and ABA, suggesting that the miR159 family plays important roles in abiotic stress in soybean. Overexpressing soybean *MIR159e* alters P nutrition in soybean roots under low-N conditions.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy13071798/s1, Supplemental Table S1. Predicted miR159 target genes in soybean; Supplemental Figure S1. Effects of *MIR159e* over expression on growth and development. WT, wild type (YC03-3); *OE-2*, *miR159eOE-2*; *OE-4*, *miR159eOE-4*; HP, plants treated with a high phosphorus concentration of 250 μM; LP, plants treated with a low-P concentration of 5 μM; treatment time was 40 days. Results are means ± SE from three independent experiments. Student's *t*-test was used to compare the differences between Col-0 and transgenic *MIR159eOE* plants (* $p < 0.05$); Supplemental Figure S2. Total phosphorus, nitrogen, and soluble phosphorus contents in soybean overexpressing *MIR159e* under low-nitrogen conditions. WT, wild type (YC03-3); HPLN, high phosphorus and low nitrogen; treatment was performed for 40 days. Results are means ± SE from three independent experiments. Student's *t*-test was used to compare the differences between Col-0 and the transgenic line *MIR159eOE* in the same plant part sampled at the same time point (* $p < 0.05$); Supplemental Table S2. List of primer pairs used in this study.

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