Article

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

Bodi Li [1,#], Ping Tao [1,3,#], Feng Xu [1], Pingan He [1] and Jinxiang Wang [1,2,*]

[1] College of Natural Resources and Environment & Root Biology Center, South China Agricultural University, Guangzhou 510642, China
[2] Key Laboratory of Agricultural and Rural pollution Control and Environmental Safety in Guangdong Province, Guangzhou 510642, China
[3] Institute of Biological and Medical Engineering Guangdong Academy of Sciences, Guangzhou 510316, China
* Correspondence:
# Cofirst author

Abstract: MicroRNAs (miRNAs) regulate plant growth and development and their 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.

Keywords: MiR159; soybean; phosphorus; NaCl; ABA; gene function

1. Introduction

MicroRNAs (miRNAs) are non-coding small (20~24 nucleotide) RNAs that are widespread in plants and animals, as well as 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 responses to biotic and abiotic stress. MiR159, in particular, regulates plant responses to nutrient stress, such as low-phosphate (low-P) stress. *MIR159a*, *MIR319a*, *MIR396a*, *MIR389b*, and *MIR1507a* are upregulated under low-P stress [3].

The miR159 family plays important roles in plant growth and development and in plant adaptation to abiotic stress [4]. Eight miRNAs associated with salt tolerance in have been identified in peanut (*Arachis hypogea*): miR159-1, miR159-2, miR159-3, miR164-2, miR167-3, miR319-1, miR319-2, and miR211-1 [5]. 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 [6]. However, the roles of miR159 family members in soybean (*Glycine max*) remain poorly understood.

 doi:10.20944/preprints202306.0772.v1

The Arabidopsis (*Arabidopsis thaliana*) *hyponastic leaves 1* (*hyl1*) mutant, which fails to process primary miRNA transcripts (pri-miRNAs), is sensitive to abscisic acid (ABA) during germination [7], suggesting that ABA signaling pathway might be regulated by miRNAs. Indeed, miR159 responds to ABA and drought treatment [8,9]. 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 [10]. In line with this finding, ABRE-like elements were identified in the upstream region of the *MIR159a* promoter. MYB101 and MYB33 are targets of miR159; these genes encode MYB transcription factors that function in seed germination [11]. However, the responses of soybean *MIR159* genes to abiotic stress and their roles in abiotic stress responses are unclear.

In this study, 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 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 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 [12].

2.3. qRT-PCR analysis

To quantify transcript levels in soybean genotype YC03-3 under different P conditions, qRT-PCR was used. 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 above. 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. YC03-3 plants grown in soil were sampled and subjected to total RNA extraction using the TRIzol method. cDNAs were obtained using reverse transcriptase. The transcript abundance of the above-mentioned genes was determined via qRT-PCR, and *AtEF1a* was used as a reference gene to normalize the qRT-PCR results.

 doi:10.20944/preprints202306.0772.v1

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 [13].

2.5. Data analysis

MEGA 6.0 software [14] was used to construct the phylogenetic tree using the neighbor-joining method. The promoters were analyzed using the TSSP program of Phytozome (http://phytozome.org) and softberry (http://softberry.org); target genes were predicted using psRNA Target (http://psrna target.org) [15].

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 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). 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: 40266722-40266935 +
MIR159b	GAGUUCCCUGCACUCCAAGUC	Gm07: 5424789-5424974 –
MIR159c	AUUGGAGUGAAGGGAGCUCCG	Gm16: 2830034-2830218 –
MIR159d	AGCUGCUUAGCUAUGGAUCCC	Gm09:40267077-40267097+
MIR159e	GAGCUCCUUGAAGUCCAAUU	Gm07: 9561934-9562144 –
MIR159f	GAGUUCCCUGCACUCCAAGUC	Gm16: 2819636-2819815 –

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.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 12 June 2023 doi:10.20944/preprints202306.0772.v1

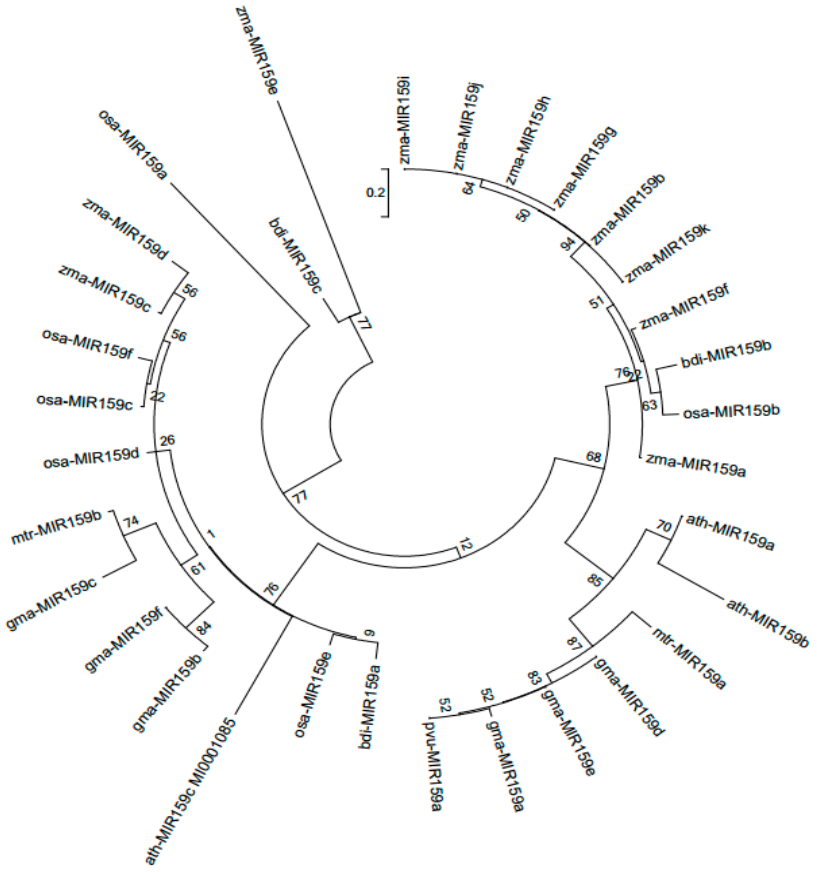

Figure 1. Phylogenetic analysis of the *MIR159* family in soybean and other plant species. Note: Abbreviations: gma, Glycine max; ath, Arabidopsis thaliana; osa, Oryza sativa; mtr, Medicago truncatula; zma, Zea mays; bdi, Brachypodium distachyon; pvu, Phaseolus vulgaris.

ABREs can be found in the promoter regions of *MIR159* genes in Arabidopsis [10], and *MIR159e* responds to low-P stress in soybean [16]. 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.

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	

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 12 June 2023 doi:10.20944/preprints202306.0772.v1

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 [3], 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, 2I) but were inhibited in roots (Figure 2B, 2J), whereas *MIR159b* expression was not significantly changed in leaves but was induced in roots (Figure 2D), *MIR159c* expression was inhibited in both leaves and roots (Figure 2E, 2F), 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, 2L). These results suggest that miR159 family members may regulate the responses of soybean to low-P stress in various ways.

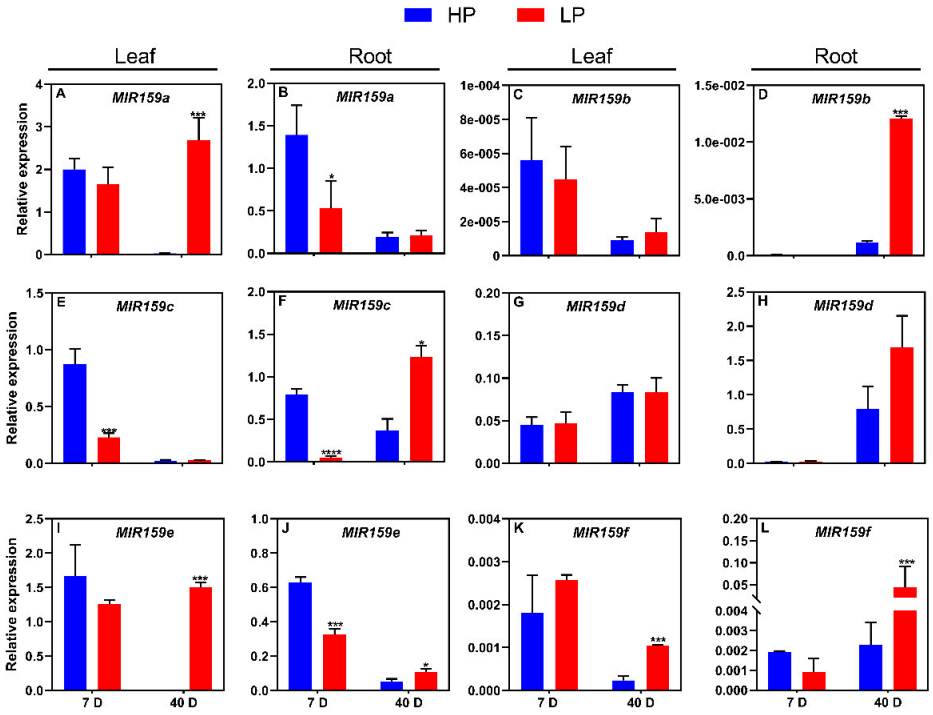

Figure 2. Responses of the soybean *MIR159* gene family to low-P stress in leaves and roots. *MIR159* transcript levels were quantified in samples from soybean plants of genotype YC03-3 that were transplanted when the first three-emerging compound leaf was fully unfolded and the second three-emerging 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). Results are means ± SE from 3 independent experiments. Student's *t*-test was used to determine the differences between control and nutrient-deficiency conditions (*, $P < 0.05$; ***, $P < 0.001$).

MiR159 family members regulate flower development in Arabidopsis [17], and delay flowering under low-P stress [18], but their responses to nutrient deficiency in soybean are unknown. 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*,

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 12 June 2023 doi:10.20944/preprints202306.0772.v1

MIR159c and *MIR159f* was significantly induced in flowers under LP stress, whereas *MIR159d* and *MIR159e* expression was inhibited by this treatment (Figure 3A).

Soybean is a legume crop capable of biological nitrogen fixation; some miR159 family members regulate nodule growth and development in legumes [19]. We therefore cultured soybean plants under high-phosphorus and low-nitrogen (HPLN, control) and low-phosphorus and low-nitrogen (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.

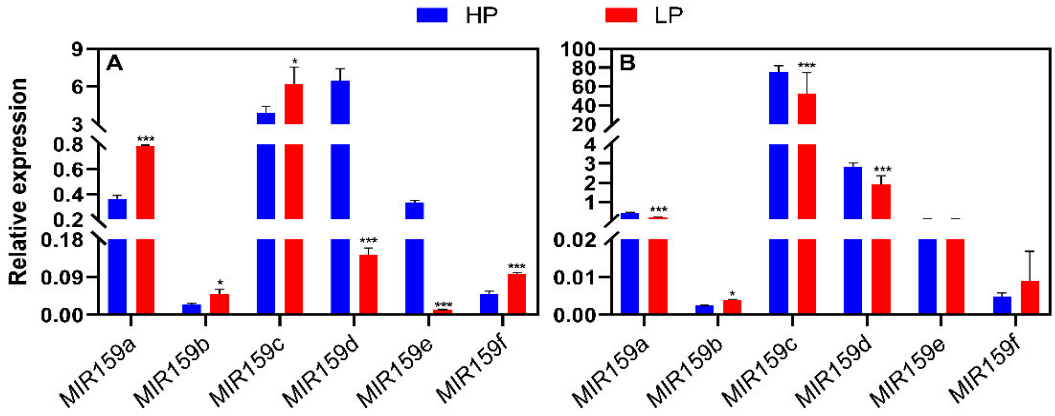

Figure 3. Responses of the soybean *MIR159* gene family to low-P stress in flowers and nodules. **(A)** *MIR159* gene expression in flowers; **(B)** *MIR159* gene expression in nodules. Soybean genotype YC03-3 was transplanted when the first three-emerging compound leaf was fully unfolded and the second three-emerging 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 (*, $P < 0.05$; ***, $P < 0.001$).

3.3. Responses of the soybean MIR159 gene family to salt stress

MiR159 responds to high-salt stress in *S. portulacastrum* [6]. 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, 4B), *MIR159b* (Figure 4C, 4D), *MIR159c* (Figure 4E, 4F), and *MIR159e* (Figure 4I, 4J) were significantly upregulated in leaves and roots under this treatment; *MIR159d* was upregulated only in roots (Figure 4G, 4H); and *MIR159f* was down-regulated in leaves but upregulated in roots (Figure 4K, 4L). These results suggest that the soybean *MIR159* gene family plays important roles in plant responses to salt stress.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 12 June 2023 doi:10.20944/preprints202306.0772.v1

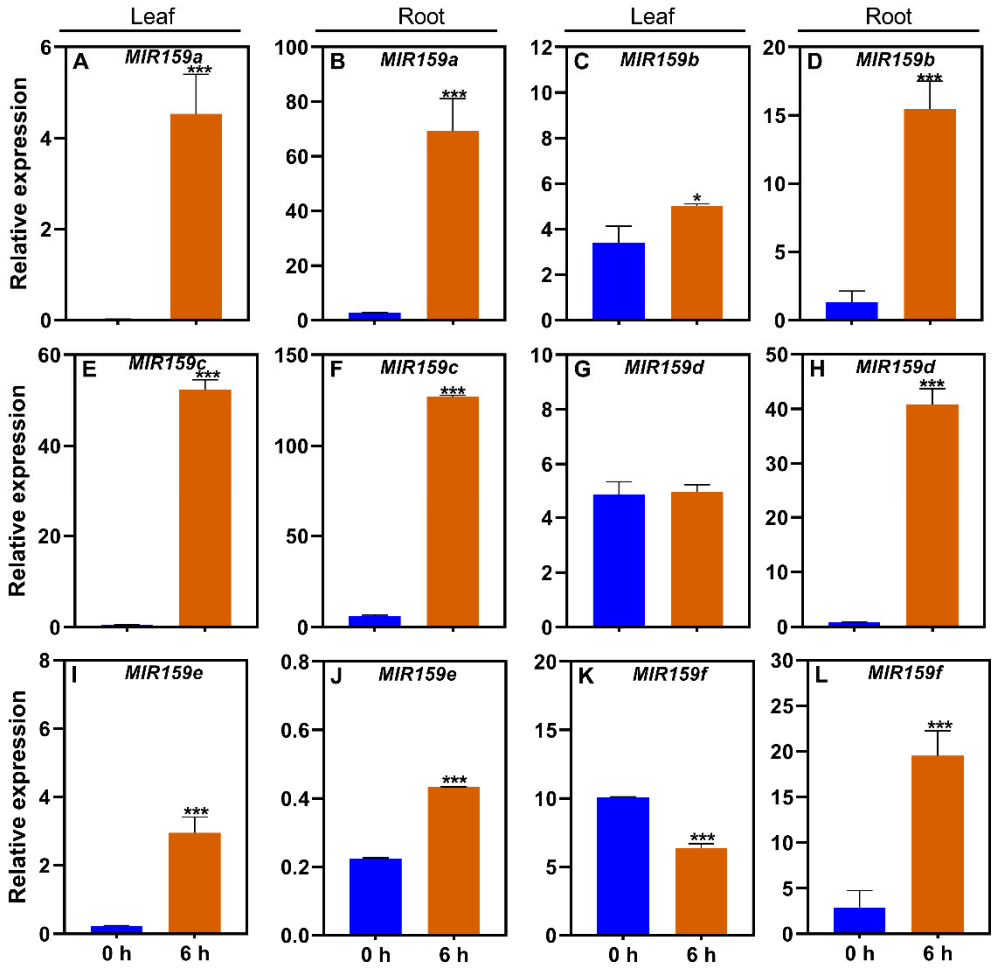

Figure 4. Responses of the soybean *MIR159* gene family to NaCl stress in leaves and roots. *MIR159* transcript levels were quantified in samples from soybean plants of genotype YC03-3 that were transplanted when the first three-emerging compound leaf was fully unfolded and the second three-emerging compound leaf was not fully unfolded, and treated NaCl with for 0 or 6 h. –NaCl, 0 mM; +NaCl, 200 mM; h, hours. Results are means ± SE from 3 independent experiments. Student's *t*-test was used to determine the differences between control and nutrient deficiency conditions (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

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 [20], 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 hours of ABA treatment (Figure 5B). In response to ABA treatment, *MIR159b* was upregulated in leaves and roots (Figure 5C, 5D), *MIR159*C was upregulated in leaves and downregulated in roots (Figure 5E, 5F), and *MIR159d* was downregulated in leaves (Figure 5G) and upregulated in roots (Figure 5H). *MIR159e* was down-regulated in leaves and roots (Figure 5I, 5J), and *MIR159f* was down-regulated in leaves and upregulated in roots (Figure 5K, 5L). These results suggest that *MIR159* family genes play important roles in regulating ABA responses in soybean.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 12 June 2023 doi:10.20944/preprints202306.0772.v1

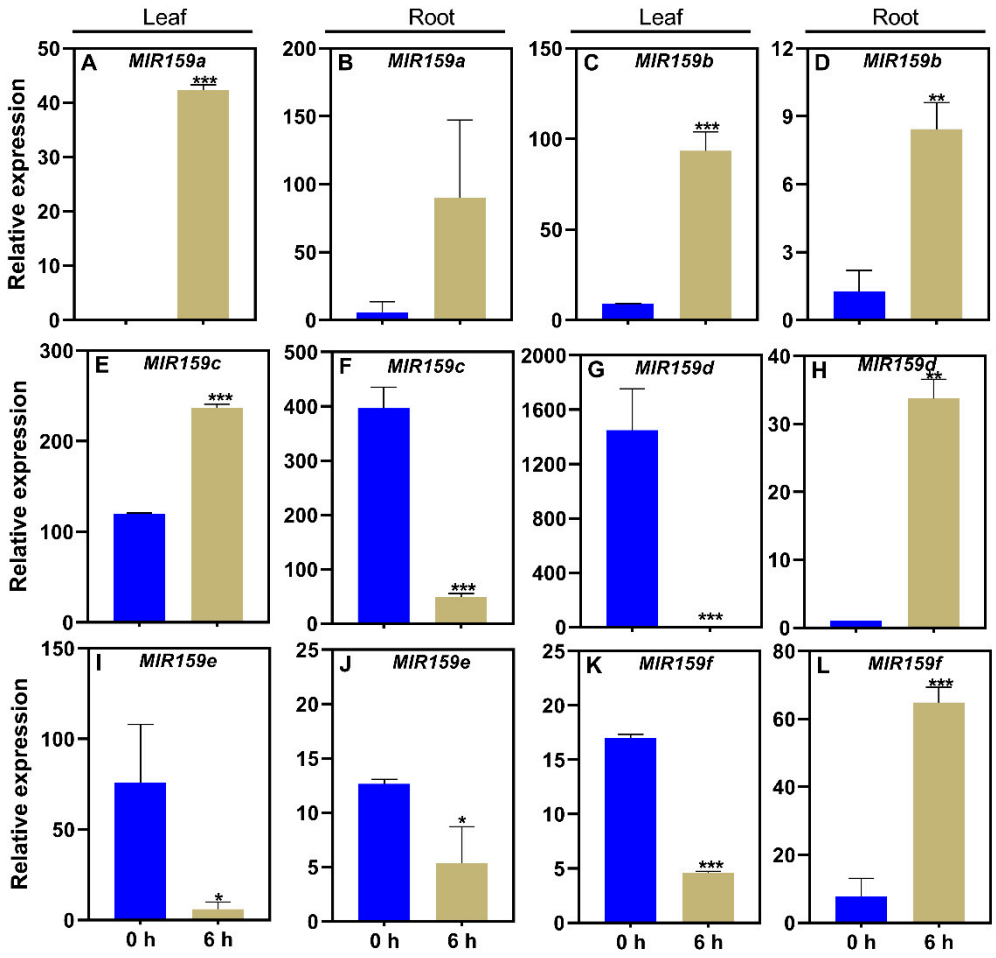

Figure 5. Responses of the soybean *MIR159* gene family to ABA in leaves and roots.

MiR159 transcript levels were quantified in samples from soybean plants of genotype YC03-3 that were transplanted when the first three-emerging compound leaf was fully unfolded and the second three-emerging compound leaf was not fully unfolded, and treated with ABA for 0 h or 6 h. –ABA, 0 μM; +ABA: 300 μM; h, hour. Results are means ± SE from 3 independent experiments. Student's *t*-test was used to determine the differences between control and nutrient-deficiency conditions (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

3.5. Overexpressing MIR159e decrease total phosphorus content in soybean leavea 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. 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 phosphoru contents in soybean.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 12 June 2023 doi:10.20944/preprints202306.0772.v1

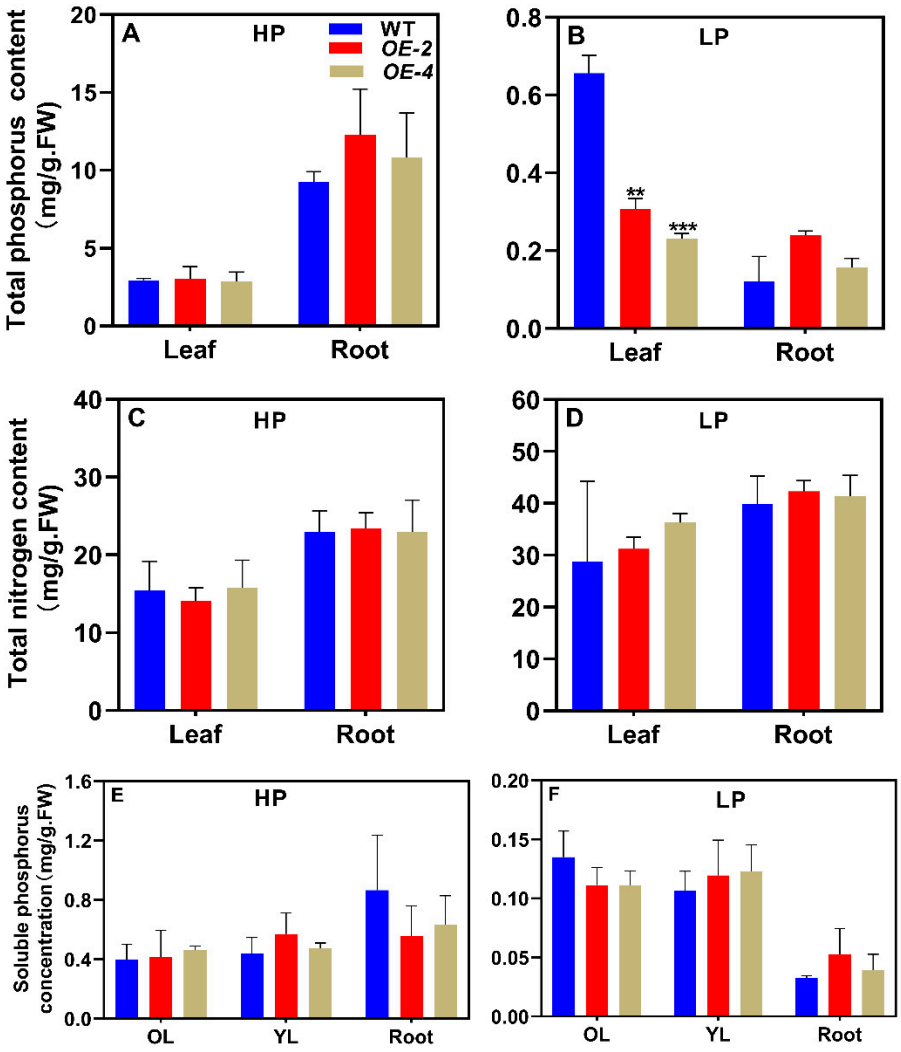

Figure 6. Overexpressing *MIR159e* affects total phosphorus, total nitrogen, and soluble phosphorus contents in soybean. **WT**, wild type (YC03-3); **OL**, old leaves; **YL**, young leaves. Soybean genotype YC03-3 was transplanted when the first emerging compound leaf was fully unfolded and the second emerging 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 (40D). Results are means ± SE from 3 independent experiments. Student's *t*-test was used to determine the differences between control and nutrient deficiency conditions (**, $P < 0.01$; ***, $P < 0.001$).

4. Discussion

Increasing evidence indicates that miRNAs play crucial roles in plant adaptation to nutrient stress. 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 [21]. Nutrient deficiency in soils inhibits the growth and development of legumes, thereby reducing yields. MiR159 is a highly conserved miRNA family whose members are important in regulating vegetative growth, flowering, anther development, and

 doi:10.20944/preprints202306.0772.v1

seed germination in various plants [20,22,23]. However, the roles of the miR159 family in soybean are poorly understood.

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 (Table 2). We also examined the expression patterns of *MIR159* genes in response to P (Figure 2, Figure 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. Evolutionary analysis suggested that miR159 shares a common ancestor with miR319, another highly conserved miRNA family [24]. Soybean *MIR159* genes are arranged in clusters (Table 1), similar to *MIR399* genes [16]. There are 3, 6, 11, 3, 3, and 1 *MIR159* genes in alfalfa, maize, rice, Arabidopsis, Brachypodium distachyon, and common bean, respectively. Arabidopsis contains three *MIR159* genes, 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 [25].

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. However, *MIR159e* lacks TATA-box elements, and only *MIR159c* and *MIR159f* contain P1BS binding elements (Table 2). *MIR159c* and *MIR159f* transcription might be regulated by PHR-type transcriptional regulators in soybean [26]. *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 [3]. *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, 2J), which is consistent with previous reports [16]. *MIR159* was upregulated in leaves and roots after 40 days of low-P stress (Figure 2K, 2L). 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). Unlike some other stresses, low -P stress delays flowering [18].

Overexpressing *MIR159a* in gloxinia (*Sinningia speciosa)* delays flowering under short-day conditions and downregulates the expression of *LEAFY* (*LFY*), *AGAMOUS*, *APETALA1* (*AP1*) and *AP3* genes in flower buds [27]. *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 [28]. The Arabidopsis *mir159a mir159b* double mutant shows plant dwarfing, reduced apical dominance, reduced fertility, and an irregular seed shape [17]. 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. 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 [29,30]. *MIR159* is expressed at a relatively high level in alfalfa nodules [31]. Here, we showed that *MIR159a*, *MIR159c*, and *MIR159d* were down-regulated, *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.

Compared to wild-type soybean (YC03-3), transgenic plants overexpressing *MIR159* induced drought resistance, suggesting that miR159 functions in drought resistance and perhaps plant responses to high-salt stress [32]. *MIR159* expression is induced in peanut by salt stress, and miR159

 doi:10.20944/preprints202306.0772.v1

regulates the expression of its target genes [33]. In addition, miR159 in different tissues of *S. portulacastrum* is involved in plant responses to high-salt stress [6]. 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 2, 1, 2, 3, and 4 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.

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 [20]. 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 [34]. Previous studies demonstrated that the Arabidopsis *hyl1* mutant is sensitive to ABA during seed germination [7], suggesting that the ABA signaling pathway might be regulated by miRNAs. By isolating and cloning Arabidopsis miRNAs during early germination, Abe et al. (2003) determined that miR159 responds to ABA and drought treatment. Analysis of the upstream regions of the three Arabidopsis *MIR159* genes revealed ABREs and binding sites for stress-related transcription factors such as AtMYC2 [10]. The expression of *MIR159a* and *MIR159b* is induced by ABA in Arabidopsis at the seed germination stage, but not at the seedling stage [20]. 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.

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 [35,36]. 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 [37]. 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-nitrogen stress. *MIR159e* might be involved in nitrogen and P, but the physiological and molecular mechanisms are unclear.

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-nitrogen conditions, the total nitrogen content was higher in nodules than in leaves and roots, indicating that nodules play a role in nitrogen accumulation and provide nitrogen for plant growth (Supplemental Figure S2). These results are consistent with previous findings [36]. 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 nutrient balance in plants. Overexpressing *MIR159* delayed flowering in ornamental gloxinia (*Sinningia speciosa*) [27]. However, in the current study, overexpressing *MIR159e* in soybean did not delay 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.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/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.

Author Contributions: J.W., B.Li. and P.T. conceived and designed the experiments. B. Li., P.T., F.X., and P.H. performed the experiments. B. Li. analyzed the date. J.W. and B. Li wrote the original manuscript. J.W. revised and approved the final version of the paper. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by Natural Science Foundation of China (Grant number: 31572184).

Data Availability Statement: All data are provided as figures and table which are included in this paper.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Jonesrhoades, M.W., Bartel, D.P., Bartel, B. MicroRNAs and their Regulatory roles in Plants. Annu. Rev. Plant Biol, **2006**, 57(1): 19–53. [CrossRef] [PubMed].
2. Rogers, K., Chen, X. Biogenesis, turnover, and mode of action of plant microRNAs. Plant Cell, **2013**, 25(7): 2383–2399. [CrossRef] [PubMed].
3. Zeng, H.Q., Zhu, Yi.Y., Huang, Si.Q., Yang, Z.M. Analysis of phosphorus-deficient responsive miRNAs and cis-elements from soybean (*Glycine max L.*). J Plant Physiol, **2010**, 167(15): 1289–1297. [PubMed].
4. Mallory, A. C., Vaucheret, H. Functions of microRNAs and related small RNAs in plants. Nat Genet, **2006**, 38: S31–S36. [CrossRef] [PubMed].
5. Zhang, S., Xie, M., Ren, G., Yu, B. CDC5, a DNA binding protein, positively regulates posttranscriptional processing and/or transcription of primary microRNA transcripts. PNAS, **2013**, 110(43): 17588–17593. [CrossRef] [PubMed].
6. Tang, M.N., Liu, Q., Zhang, Y. Response of salt-tolerant mirnas to high-salt habitats in mangrove companion plant hippocampal teeth. MPB, **2017**,15(3):1137–1142. [CrossRef] [PubMed].
7. Lu, C., Fedoroff, N. A Mutation in the Arabidopsis HYL1 Gene Encoding a dsRNA Binding Protein Affects Responses to Abscisic Acid, Auxin, and Cytokinin. Plant Cell, **2000**, 12(12): 2351–2366. [PubMed].
8. Lopez-Molina, L., Mongrand, S., Mclachlin, D.T., Mclachlin, B.T., Chait, Nam-Hai.C. ABI5 acts downstream of ABI3 to execute an ABA-dependent growth arrest during germination. Plant J, **2002**, 32(3): 317–328. [CrossRef] [PubMed].
9. Zhang, B.H., Pan, X.P., Wang, Q.L., Cobb, G.P., Anderson, T.A. Identification and characterization of new plant microRNAs using EST analysis. Cell Res, **2005**, 15(5): 336–360. [PubMed].
10. Abe, H., Urao, T., Ito, T., Seki, M., Shinozaki, Kazuo., Yamaguchi-Shinozaki, Kazuko. Arabidopsis AtMYC2 (bHLH) and AtMYB2 (MYB) Function as Transcriptional Activators in Abscisic Acid Signaling. Plant Cell, **2003**, 15(1): 63–78. [PubMed].
11. Fujii, H., Chiou, T.J., Lin, S.I., Aung, K. A miRNA Involved in Phosphate-Starvation Response in Arabidopsis. Current Biology CB, **2005**, 15(22): 2038–2043. [CrossRef] [PubMed].

12. Huo, W.G., Li, B.D., Kuang, J.B., Functional Characterization of the Steroid Reductase Genes GmDET2 and GmDET2b from Glycine max." Int J Mol Sci, **2018**, 19(3):726. [PubMed].
13. Zhou, J., Jiao, F. C., Wu, Z. C., Li, Y. Y., Wang, X. M., He, X. W.; et al. (2008). OsPHR2 is involved in phosphate-starvation signaling and excessive phosphate accumulation in shoots of plants. Plant Physiol, **2008**, *146*, 1673–1686. [PubMed].
14. Kumar, S., Stecher, G., Peterson, D., Tamura. MEGA-CC: Computing core of molecular evolutionary genetics analysis program for automated and iterative data analysis. Bioinformatics, **2012**, 28(20): 2685–2686. [CrossRef] [PubMed].
15. Dai, X., Zhao, P. X. psRNATarget: A plant small RNA target analysis server. Nucleic Acids Research, **2011**, 39(Web Server issue): W155–W159. [CrossRef] [PubMed].
16. Xu, F., Liu, Q., Chen, L., Kuang J.B., Liao H. Genome-wide identification of soybean microRNAs and their targets reveals their organ-specificity and responses to phosphate starvation. BMC Genomics, **2013**, 14(1): 66. [PubMed].
17. Allen, R.S., Li, J., Alonsoperal, M. M., MicroR159 regulation of most conserved targets in Arabidopsis has negligible phenotypic effects. Silence, **2010**, 1(1): 1–18. [PubMed].
18. Nord, E.A., Lynch, J.P. Delayed reproduction in Arabidopsis thaliana improves fitness in soil with suboptimal phosphorus availability. Plant Cell & Environment, **2008**, 31(10): 1432–1441. [CrossRef] [PubMed].
19. Xu, F., Wang, J.X. Research progress of MicroRNA regulating nutrient stress response of leguminous crops. Plant Nutrition and Fertilizer Journal, **2016**，22(1):236–244. [CrossRef] [PubMed].
20. Reyes, J.L., Chua, N. ABA induction of miR159 controls transcript levels of two MYB factors during Arabidopsis seed germination. Plant Journal for Cell & Molecular Biology, **2007**, 49(4): 592–606. [CrossRef] [PubMed].
21. Khraiwesh, B., Zhu, J.K., Zhu, J. Role of miRNAs and siRNAs in biotic and abiotic stress responses of plants. Biochimica et Biophysica Acta, **2012**, 1819(2): 137–148. [PubMed].
22. Millar, A.A., Gubler, F. The Arabidopsis GAMYB-Like Genes, MYB33 and MYB65, Are MicroRNA-Regulated Genes That Redundantly Facilitate Anther Development. Plant Cell, **2005**, 17(3): 705–721. [PubMed].
23. Allen, R.S., Li, J., Stahle, M.I., Genetic analysis reveals functional redundancy and the major target genes of the Arabidopsis miR159 family. PNAS, **2007**, 104(41): 16371–16376. [PubMed].
24. Li, Y., Jin, Y.X. Evolution of MIR159/319 MicroRNA Genes and Their Post-transcriptional Regulatory Link to siRNA Pathways. BMC Evol Biol, **2011**, 11(1): 122. [CrossRef] [PubMed].
25. Schmutz, J., Cannon, S.B., Schlueter, J., Ma, J.X., Mitros, T., Nelson, W., Hyten, D. Genome sequence of the paleopolyploid soybean (Glycine max (L.) Merr.). Nature, **2010**, 463(7278): 178–183. [PubMed].
26. Bustos, R., Castrillo, G., Linhares, F., Puga, M.I., Rubio, V., P'erez-Perez, J., Solano, R., Leyva, A., Paz-Ares, J. A central regulatory system largely controls transcriptional activation and repression responses to phosphate starvation in Arabidopsis. PLOS Genet, **2010**, 6(9): e1001102. [PubMed].
27. Bian, X.Y., Song, D.F., Han, S.Y., Wang, J.H., Zhu, M.Y. Flowering time control in ornamental gloxinia (Sinningia speciosa) by manipulation of miR159 expression. Annals of Botany, **2013**, 111(5): 791–799. [PubMed].
28. Csukasi, F., Donaire, L., Casañal, A., Martínez-Priego, L., Botella, M.A., Medina-Escobar, N., Llave, C., Valpuesta, V. Two strawberry miR159 family members display developmental-specific expression patterns in the fruit receptacle and cooperatively regulate Fa-GAMYB. New Phytol, **2012**, 195(1): 47–57. [PubMed].
29. Olivera, M., Tejera, N., Lribarne, C., Ocaña, A., Lluch, C. Growth, nitrogen fixation and ammonium assimilation in common bean (Phaseolus vulgaris): Effect of phosphorus. Physiol Plantarum, **2004**, 121(3): 498–505. [CrossRef] [PubMed].
30. Schulze, J., Temple, G., Temple, S.J., Beschow, H., Vance, C.P. Nitrogen fixation by white lupin under phosphorus deficiency. Annals of Botany, **2006**, 98(4): 731–740. [PubMed].
31. Zucchero, J.C., Caspi, M., Dunn, K. ngl9: A third MADS box gene expressed in alfalfa root nodules. Mol Plant Microbe Interact, **2001**, 14(12):1463–1467.
32. Li Y.J., Alonso-Peral, M., Wong, G., Wang, M. B., Millar, A. A. Ubiquitous miR159 repression of MYB33/65 in Arabidopsis rosettes is robust and is not perturbed by a wide range of stresses. Bmc Plant Biology, **2016**, 16(1):179.
33. Zhang, T.T., Yan, C. X., Zhao, X.B., Li, C.J., Shi, C.R., Yu, J.J., Chan, S.H., Li, R.G. Identification and functional characterization of salt tolerance related microRNAs in roots of peanut (*Arachis hypogaea L.*). Journal of Peanut Science, **2016**，45(4):36–42. [CrossRef] [PubMed].
34. Panda, S.K., Sunkar, R. Nutrient- and other stress-responsive microRNAs in plants: Role for thiol-based redox signaling. Plant Signaling & Behavior, **2015**, 10(4): e1010916.
35. Raghothama, K.G. Phosphate Acquisition. Annu Rev Plant Phys, **1999**, 274(1–2): 665-693. [CrossRef] [PubMed].

36. Reinhart, B. J., Weinstein, E.G., Rhoades, M.W., Bartel, B., Bartel, D. MicroRNAs in plants. Genes & Development, **2002**, 16(13): 1616–1626. [CrossRef] [PubMed].
37. Wang, X.R., Yan, X.L., Liao, H. Genetic improvement for phosphorus efficiency in soybean: A radical approach. Ann Bot, **2010**, 106(1): 215–222. [CrossRef] [PubMed].
38. Tao, X., Liu, Z., Dai, X., Xiang, F. Primary root growth in Arabidopsis thaliana is inhibited by the miR159 mediated repression of MYB33, MYB65 and MYB101. Plant Sci, **2017**, 262: 182–189.