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Detection of Candidate Loci and Genes Related to Phosphorus Efficiency at Maturity through a Genome-Wide Association Study in Soybean

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Abstract: Phosphorus (P) deficiency is one of the major factors limiting soybean production, and approximately 90% of P absorbed by plants occurs during the reproductive stage. Thus, it is important to understand the genetic mechanism underlying soybean low-P tolerance, especially in the mature period. Here, we evaluated six P-efficiency-related traits at maturity of 219 soybean accessions, namely, plant height (PH), node number of the main shoot (NN), branch number of the main shoot (BN), pod number per plant (PN), 100-seed weight (100SW), and seed yield per plant (SY), under normal-phosphorus (NP) and low-phosphorus (LP) conditions across two environments. Then, a genome-wide association study (GWAS) in conjunction with a high-density NJAU 355 K SoySNP array was performed. As a result, 27 P-efficiency-related single nucleotide polymorphisms (SNPs) were identified. Furthermore, two repeated SNPs, AX-93897192 and AX-93897200, located on chromosome 19 that were associated with both PH and NN were considered as stable SNPs associated with P deficiency, and the candidate gene *GmABCG39* was identified. This work will be helpful in breeding high-P-efficiency soybean varieties.

Keywords: soybean; P efficiency; traits at maturity; GWAS; *GmABCG39*

Citation: Wang, Q.; Ning, L.; Yu, W.; Zhao, W.; Huang, F.; Yu, D.; Wang, H.; Cheng, H. Detection of Candidate Loci and Genes Related to Phosphorus Efficiency at Maturity through a Genome-Wide Association Study in Soybean. *Agronomy* **2022**, *12*, 2031. https://doi.org/10.3390/agronomy12092031

Academic Editors: Gianni Barcaccia, Alessandro Vannozzi and Fabio Palumbo

Received: 24 July 2022
Accepted: 23 August 2022
Published: 26 August 2022

1. Introduction

Phosphorus (P) is one of the essential mineral elements for plant growth and development. It not only is an important component of ATP, nucleic acids, and phospholipids but also plays important roles in plant signal transmission, energy transfer, respiration, and photosynthesis [1]. However, phosphate (Pi), the only form that can be absorbed and utilized by soybean plants, is relatively low in abundance in cultivated soils, and low P has become an important factor limiting soybean yield. Although the application of Pi fertilizer could solve this problem, the large use of Pi fertilizer causes a series of environmental problems [2,3]. Importantly, Pi fertilizer mainly comes from the mining of Pi rock resources, which are nonrenewable resources, and the world's Pi rock resources will be exhausted in the next 50–200 years [4]. Therefore, analysing the genetic mechanism of low-P tolerance and exploring P-efficiency-related genes in soybean could be effective in preventing P pollution, as well as improving soybean yield.

Compared with traditional quantitative trait locus (QTL) mapping approach, the genome-wide association study (GWAS) is based on linkage disequilibrium (LD) and can

be used for fine mapping at the genome-wide level [5]. At present, the GWAS has been successfully applied to clone genes regulating complex quantitative traits in *Oryza sativa* [6,7], *Zea mays* [8,9], and *Triticum aestivum* [10].

Soybean P efficiency is a quantitative trait controlled by multiple genes, and the GWAS can serve as a powerful tool to analyse the genetic mechanism of soybean low-P tolerance. To date, several single-nucleotide polymorphisms (SNPs) significantly related to soybean P efficiency have been identified by GWAS [11–15], and P-efficiency-related genes around these SNPs have been cloned. For example, the acid phosphatase genes *GmACP1* [15] and *GmACP2* [16], which were located near P-efficiency-related SNPs, were found to significantly improve P efficiency in soybean hairy roots. In addition, SNP AX-93932874, which was significantly associated with P efficiency, was found to be located in the 5′ untranslated region of *GmSPX-RING1*, which was found to negatively regulate P concentration in soybean hairy roots [12].

Although some progress has been made on soybean P efficiency via the GWAS, only a few functional genes have been cloned. Three possible reasons are as follows: (1) it is difficult to accurately identify P-efficiency-related specific SNPs caused by a lack of unified evaluation indexes for P efficiency; (2) it is difficult to select stable SNPs because soybean P efficiency is a complex quantitative trait controlled by a few select major genes, as well as additional genes with small effects; (3) it is difficult to identify the exact functional genes because the genotype data used for GWAS has low coverage in the whole-soybean genome, and the LD region is large. Researchers have always evaluated P-efficiency-related traits at the seedling stage, such as root architecture, plant dry weight, and P concentration, as evaluation indexes for soybean P efficiency [11,12]. However, 90% of the P absorbed by plants occurs during the reproductive stage during the whole soybean growth period [17]. In addition, SNPs that are significantly associated with multiple P-efficiency-related traits could be used as stable SNPs to search for nearby P-efficiency-related genes [15,18]. In summary, performing a GWAS on soybean P-efficiency-related traits at maturity with high-density genotype data and choosing SNPs that are associated with multiple P-efficiency-related traits could greatly improve the efficiency of identifying P-efficiency-related genes in soybean.

In this study, we evaluated six soybean P-efficiency-related traits at maturity, namely, plant height (PH), node number of the main shoot (NN), branch number of the main shoot (BN), pod number per plant (PN), 100-seed weight (100SW), and seed yield per plant (SY), under normal-phosphorus (NP) and low-phosphorus (LP) conditions across two environments. Then, a GWAS in conjunction with a high-density NJAU 355 K SoySNP array was conducted to identify P-efficiency-related SNPs and candidate genes. These findings will provide new loci involving soybean low-P tolerance and will be helpful for the improvement of high-P-efficiency soybean varieties.

2. Materials and Methods

2.1. Plant Materials and Growth Conditions

A natural soybean population was used in this study. The population consisted of 219 soybean accessions with different geographical origins, which were collected from 26 provinces in China and from America, Japan, and Brazil [19]. The natural population with abundant genetic variations has been successfully applied for the genetic analysis of complex quantitative traits in soybean [14,20,21]. All soybean materials were provided by the National Center for Soybean Improvement of China.

For the natural population, the 219 soybean accessions were sown in 15 L plastic pots at Jiangpu Experimental Station of Nanjing Agricultural University in 2012 (designated as E1) and 2013 (designated as E2). Before sowing, the concentrations of nitrogen (N), phosphorus (P), and potassium (K) in dry soil at the Jiangpu Experimental Station were measured to ensure that the P concentration was below 10 mg/kg. Then, the dry soil was designated as low-P soil, and monopotassium phosphate (KH_2PO_4) was added into low-P soil to bring the P concentration to 20 mg/kg, which was designated as normal-P soil. To

satisfy the demands of plants growth, urea (H_2NCONH_2) was added to both low-P soil and normal-P soil to bring the N concentration to 60 mg/kg, and potassium chloride (KCl) was added to low-P soil so that the K concentration was consistent with that of the normal-P soil. In general, the 219 soybean accessions were sown in 15 L plastic pots containing 10 kg of normal-P dry soil or low-P dry soil, with four or six seeds per pot, in accordance with a completely randomized block design with three replications. Then, the soybean seedlings were thinned to two in each plot approximately two weeks after germination. The normal-P dry soil was considered the NP condition, and the low-P dry soil was considered the LP condition. That is, each accession was subjected to NP and LP conditions, with six seedlings in three plastic pots.

To test the relative expression levels of *GmABCG39*, a candidate gene identified in this study, when the plants were exposed to low-P stress, seedlings of the low-P tolerant soybean accession "Kefeng No.1" and the low-P sensitive soybean accession "Nannong 1138-2" [12] were germinated in plastic pots in the greenhouse of Nanjing Agricultural University according to a previous study [22]. The treatment involving 1/2 Hoagland solution with 0.5 mM KH_2PO_4 was designated as the +P condition, and the treatment involving 1/2 Hoagland solution with 0.5 mM KCl was designated as the -P condition.

2.2. Evaluation of Soybean P-Efficiency-Related Traits at Maturity

When the soybean plants matured, three plants with similar growth conditions were harvested for each accession under NP and LP conditions. Then, the PH (from the cotyledonary node to the top of the main stem), NN, BN, and PN were evaluated. The soybean seeds of each plant were dried at 35 °C in an oven to a constant weight, and then the total weight of the seeds was measured, which represented the SY. At the same time, 100 seeds were taken from those harvested from each plant randomly and weighed. This step was repeated three times, and the average weight was designated as the 100SW.

2.3. Statistical Analysis of Phenotypic Data

The descriptive statistical analysis and correlation analysis were conducted using SAS 9.2 (SAS Institute Inc., Cary, NC, USA). Origin 8.5 software (OriginLab, Northampton, MA, USA) was used to construct histograms, and Manhattan and quantile–quantile (QQ) plots were generated using R software (https://www.r-project.org/, accessed on 10 May 2022).

2.4. GWAS of Soybean P-Efficiency-Related Traits

A GWAS with the high-density NJAU 355 K SoySNP array [19] was conducted on P-efficiency-related traits, including PH, NN, BN, PN, 100SW, and SY, under NP and LP conditions across environments E1 and E2. The multiple mixed linear model (MLMM) in the GAPIT package was used, and the threshold was set to 1/n (n is the number of SNP markers, $p \leq 4.82 \times 10^{-6}$ or $-\log_{10}(p) \geq 5.32$) to identify significant SNPs [19].

2.5. Expression Analysis of Candidate Genes

SoyBase (https://www.soybase.org/, accessed on 10 May 2022) was used to determine the expression levels of candidate genes in different tissues based on RNA sequencing (RNA-seq) data, and the low-P induced transcriptome analysis of candidate genes were searched in NCBI database (https://www.ncbi.nlm.nih.gov, accessed on 10 May 2022).

For induced expression analysis of *GmABCG39*, total RNA was isolated from the roots of "Kefeng No.1" and "Nannong 1138-2" grown under +P and −P conditions for 15 days by the use of a kit according to the instructions (Tiangen, Beijing, China). After the RNA was reverse-transcribed into cDNA (Takara, Kyoto, Japan), Real Universal SYBR Green (Tiangen, Beijing, China) was used to generate a 20-µL reaction system to perform quantitative real-time PCR on a Bio-Rad CFX96 Real-Time System (Bio-Rad, CA, USA). The primers used for *GmABCG39* were 5′-TCATCAACCAAGCATAGACA-3′and 5′-CCTCAAGATTAGCCTCCATT-3′, and *Gmtubulin* (GenBank accession number: AY907703)

was used as a reference gene [22]. The expression level of *GmABCG39* was calculated by the $2^{-\Delta\Delta CT}$ method [23].

2.6. Bioinformatic Analysis of Candidate Genes

The 2-kb genomic sequence located upstream of the start codon of *GmABCG39* was identified as the promoter region, and prediction of cis-acting elements was conducted online via the New PLACE database (https://www.dna.affrc.go.jp/PLACE/?action=newplace, accessed on 10 January 2022). The protein sequence of GmABCG39 was used as a query sequence to predict the likely interacting proteins by the STRING database (https://cn.string-db.org, accessed on 30 June 2022).

3. Results

3.1. Soybean P-Efficiency-Related Traits at Maturity Exhibited Wide Genetic Variation

To evaluate the genetic variation of P-efficiency-related traits, six P-efficiency-related traits at maturity, including PH, NN, BN, PN, 100SW, and SY, of a natural soybean population were measured. In general, PH, NN, BN, PN, 100SW, and SY all showed wide genetic variation under NP and LP conditions. The values of PH, NN, BN, PN, SW, and SY ranged from 25–201 cm, 8–34, 0–11, 15–156, 3.27–34.95 g, and 2.20–42.17 g, respectively, under NP condition, and the corresponding values ranged from 20–170 cm, 7–29, 0–13, 13–128, 2.71–32.10 g, and 1.51–37.78 g under LP condition (Table 1). The mean values of the six P-efficiency-related traits under NP condition were higher than those under LP condition, and the results of variance analysis indicated that P levels significantly affected PH, NN, BN, PN, 100SW, and SY in E1 and E2 (Table 1). In addition, with values ranging from 92.97–96.25%, PH and 100SW showed higher broad-sense heritability (h^2) than the other five traits did (Table 1).

Table 1. Descriptive statistical analysis of the six soybean P-efficiency-related traits at maturity under NP and LP conditions in E1 and E2.

Traits	Environments	Treatments	Mean Values	CV [a]	Range	P [b]	G [c]	P × G [d]	h^2 [e]
PH (cm)	E1	NP	74.24 ± 32.30	43.51%	25–201	***	***	ns	NP 93.45% LP 92.97%
	E1	LP	69.14 ± 29.48	42.64%	20–170				
	E2	NP	81.77 ± 30.42	37.21%	33–186	***	***	ns	
	E2	LP	72.28 ± 26.85	37.15%	29–168				
NN	E1	NP	17.79 ± 4.72	26.53%	8–34	***	***	***	NP 85.81% LP 85.80%
	E1	LP	16.95 ± 4.36	25.71%	7–29				
	E2	NP	16.91 ± 3.87	22.88%	9–30	***	***	ns	
	E2	LP	15.32 ± 3.56	23.26%	8–26				
BN	E1	NP	3.58 ± 1.58	44.22%	0–9	***	ns	***	NP 52.00% LP 60.34%
	E1	LP	3.46 ± 1.77	51.01%	0–13				
	E2	NP	3.82 ± 1.25	32.78%	1–11	***	ns	ns	
	E2	LP	3.78 ± 1.23	32.57%	1–10				
PN	E1	NP	60.96 ± 20.85	34.20%	15–132	***	***	ns	NP 68.53% LP 72.01%
	E1	LP	52.17 ± 18.31	35.10%	13–121				
	E2	NP	59.77 ± 21.27	35.58%	24–156	***	***	ns	
	E2	LP	51.17 ± 18.72	36.59%	13–128				
100SW (g)	E1	NP	13.24 ± 5.28	39.92%	4.59–34.95	***	***	***	NP 96.25% LP 96.08%
	E1	LP	12.34 ± 4.86	39.40%	4.33–32.10				
	E2	NP	12.46 ± 5.01	40.20%	3.27–34.64	***	***	ns	
	E2	LP	11.43 ± 4.72	41.27%	2.71–31.3				
SY(g)	E1	NP	15.20 ± 6.87	45.19%	2.20–42.17	***	***	***	NP 65.38% LP 66.86%
	E1	LP	10.89 ± 5.11	46.91%	1.51–35.41				
	E2	NP	13.08 ± 5.46	41.77%	2.29–37.95	***	***	***	
	E2	LP	9.41 ± 4.21	44.73%	2.56–37.78				

PH: Plant height; NN: Node number of the main shoot; BN: Branch number of the main shoot; PN: Pod number per plant; 100SW: 100-seed weight; SY: Seed yield per plant; [a]: Coefficient of variation; [b]: Phosphorus level; [c]: Genotype; [d]: Phosphorus level × genotype; [e]: Broad-sense heritability; NP (normal phosphorus): Soil available P = 20 mg/kg; LP (low phosphorus): Soil available P < 10 mg/kg; ***: Significance at the 0.001 probability level; ns: Not significant (Student's *t*-test).

The frequency distribution analysis revealed that PH, NN, BN, PN, 100SW, and SY in E1 and E2 appeared to exhibit normal or approximately normal distributions (Figure 1), which indicated that P-efficiency-related traits were quantitative characteristics that were controlled by multiple genes.

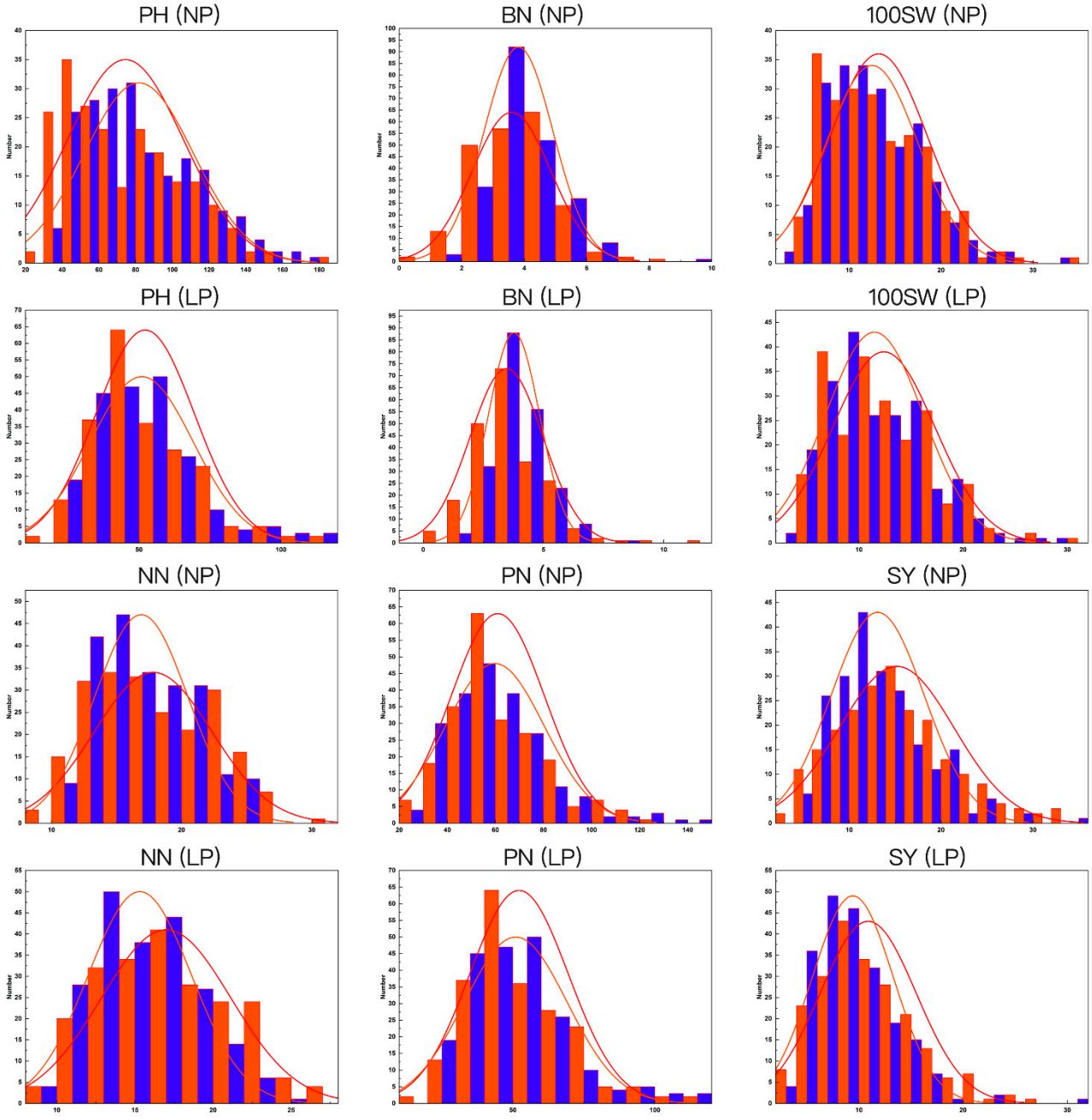

Figure 1. Frequency distribution of phenotypic values of the six soybean P-efficiency-related traits at maturity under NP and LP conditions in E1 and E2. PH (cm): Plant height; NN: Node number of the main shoot; BN: Branch number of the main shoot; PN: Pod number per plant; 100SW (g): 100-seed weight; SY (g): Seed yield per plant; NP (normal phosphorus): Soil available P = 20 mg/kg; LP (low phosphorus): Soil available P < 10 mg/kg; the colours red and purple represent values in E1 and E2, respectively.

3.2. Significant Correlations Were Detected among the Six P-Efficiency-Related Traits

Correlation analysis was conducted to further determine whether significant correlations occurred among the six P-efficiency-related traits in soybean. As shown in Table 2,

PH, NN, BN, PN, and SY all exhibited significant positive correlations with each other under both NP and LP conditions. In addition, PN and 100SW showed significant negative correlations under NP and LP conditions (Table 2). Taken together, these results demonstrated that significant correlations occurred among soybean P-efficiency-related traits at maturity.

Table 2. Correlation coefficients between the six soybean P-efficiency-related traits at maturity under NP and LP conditions.

	PH (cm)	NN	BN	PN	100SW (g)	SY (g)
PH (cm)		0.89 ***	0.43 ***	0.46 ***	−0.02	0.29 ***
NN	0.87 ***		0.42 ***	0.52 ***	−0.1	0.26 ***
BN	0.45 ***	0.41 ***		0.49 ***	−0.04	0.23 ***
PN	0.43 ***	0.50 ***	0.41 ***		−0.40 ***	0.22 **
100SW (g)	0.01	−0.08	−0.15	−0.42 ***		0.74 ***
SY (g)	0.34 ***	0.33 ***	0.28 ***	0.26 ***	0.64 ***	

PH: Plant height; NN: Node number of the main shoot; BN: Branch number of the main shoot; PN: Pod number per plant; 100SW: 100-seed weight; SY: Seed yield per plant; upper right: Correlation coefficients under NP (normal phosphorus: soil available P = 20 mg/kg) condition; lower left: Correlation coefficients under LP (low phosphorus: soil available P < 10 mg/kg) Condition; **, and ***: Significance at the 0.01, and 0.001 probability levels, respectively (Student's *t*-test).

3.3. SNPs Related to P-Efficiency Were Identified by a GWAS

To understand the genetic mechanism underlying soybean P efficiency, a GWAS was performed for PH, NN, BN, PN, 100SW, and SY under NP and LP conditions across E1 and E2, and a total of 27 significant SNPs were identified (Table 3, Figures 2 and S1). Among these SNPs, eight, six, two, six, two, and three were found to be associated with PH, NN, BN, PN, 100SW, and SY, respectively, under both NP and LP conditions (Table 3 and Figure 2).

Table 3. SNPs significantly detected for the six soybean P-efficiency-related traits at maturity under NP and LP conditions in E1 and E2.

Traits	Marker	Chromosome	Position	E1		E2	
				p-Value [a]	R^2 (%) [b]	*p*-Value [a]	R^2 (%) [b]
PH-NP	AX-94111943	13	30,318,194	1.80×10^{-6}	12.20	ns	ns
	AX-93815278	13	30,596,851	3.77×10^{-6}	16.99	ns	ns
	AX-93897192	19	44,936,236	3.12×10^{-8}	16.03	ns	ns
	AX-93897200	19	44,950,887	ns	ns	3.43×10^{-10}	14.25
PH-LP	AX-94111943	13	30,318,194	3.33×10^{-6}	12.68	ns	ns
	AX-93815278	13	30,596,851	2.70×10^{-6}	17.46	ns	ns
	AX-93897192	19	44,936,236	6.15×10^{-8}	16.51	ns	ns
	AX-93897200	19	44,950,887	ns	ns	1.38×10^{-9}	13.97
NN-NP	AX-94139280	15	36,288,379	ns	ns	3.05×10^{-7}	31.16
	AX-93897200	19	44,950,887	ns	ns	1.89×10^{-8}	14.25
	AX-93659142	19	45,673,217	5.37×10^{-8}	34.69	ns	ns
NN-LP	AX-94139280	15	36,288,379	ns	ns	1.26×10^{-7}	31.13
	AX-93897192	19	44,936,236	ns	ns	3.05×10^{-8}	15.93
	AX-93659142	19	45,673,217	4.13×10^{-7}	35.17	ns	ns
BN-LP	AX-93900689	20	4,595,321	ns	ns	3.90×10^{-6}	16.67
	AX-93900715	20	4,650,611	ns	ns	4.92×10^{-6}	15.69
PN-NP	AX-94207363	20	40,532,229	ns	ns	4.11×10^{-6}	7.49

Table 3. *Cont.*

Traits	Marker	Chromosome	Position	E1		E2	
				p-Value [a]	R^2 (%) [b]	*p*-Value [a]	R^2 (%) [b]
PN-LP	AX-93822132	14	6,139,377	ns	ns	4.20×10^{-6}	4.90
	AX-94169276	18	11,868,836	4.01×10^{-6}	17.70	ns	ns
	AX-93871886	18	11,875,808	4.01×10^{-6}	17.70	ns	ns
	AX-94291170	19	32,625,440	3.50×10^{-6}	5.50	ns	ns
	AX-94207363	20	40,532,229	ns	ns	3.12×10^{-6}	7.35
100SW-NP	AX-93699741	3	40,213,701	ns	ns	1.88×10^{-6}	29.95
100SW-LP	AX-93699741	3	40,213,701	ns	ns	2.35×10^{-6}	29.90
SY-NP	AX-94287407	13	37,024,315	ns	ns	3.77×10^{-6}	48.31
	AX-93952890	18	60,070,870	ns	ns	1.06×10^{-6}	31.16
SY-LP	AX-94287407	13	37,024,315	ns	ns	3.17×10^{-6}	48.53

PH (cm): Plant height; NN: Node number of the main shoot; BN: Branch number of the main shoot; PN: Pod number per plant; 100SW (g): 100-seed weight; SY (g): Seed yield per plant; NP (normal phosphorus): Soil available P = 20 mg/kg; LP (low phosphorus): Soil available P < 10 mg/kg; [a]: Significance at $p \leq 4.82 \times 10^{-6}$; [b]: Percentage of phenotypic variation explained by the SNP; ns: Not significant (Student's *t*-test).

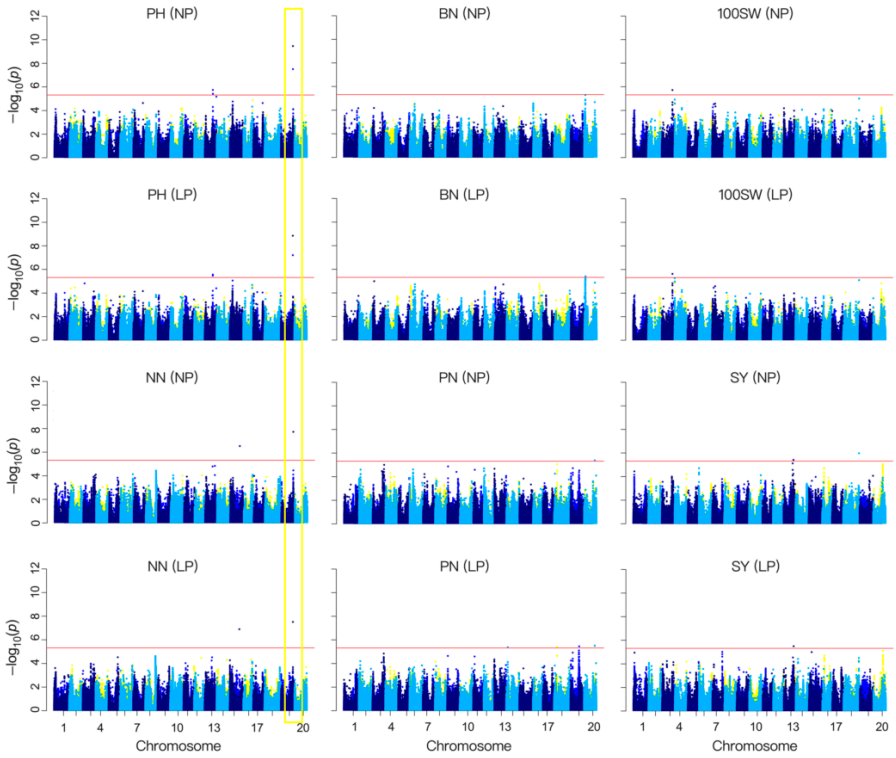

Figure 2. Manhattan plots of the GWAS results of phenotypic values of the six soybean P-efficiency-related traits at maturity under NP and LP conditions in E1 and E2. PH (cm): Plant height; NN: Node number of the main shoot; BN: Branch number of the main shoot; PN: Pod number per plant; 100SW (g): 100-seed weight; SY (g): Seed yield per plant; NP (normal phosphorus): Soil available P = 20 mg/kg; LP (low phosphorus): Soil available P < 10 mg/kg; the red lines indicate the significant threshold ($-\log_{10}(p) = 5.32$); the colours purple and yellow indicate E1; the colours dark purple and blue indicate E2; the yellow box indicates SNPs AX-93897192 and AX-93897200 located on chromosome 19.

Among 27 significant SNPs, both AX-94111943 and AX-93815278 were associated with PH under NP and LP conditions in E1; AX-93897192 was associated both with PH under NP and LP conditions in E1 and with NN under LP condition in E2; AX-93897200 was

associated both with PH under NP and LP conditions and with NN under NP condition in E2; AX-94139280 was associated with NN under NP and LP conditions in E2; AX-93659142 was associated with NN under NP and LP conditions in E1; AX-94207363 was associated with PN under NP and LP conditions in E2; AX-93699741 was associated with 100SW under NP and LP conditions in E2; and AX-94287407 was associated with SY under NP and LP conditions in E2 (Table 3).

3.4. Prediction of Candidate Genes Associated with Soybean P-Efficiency

Among the repeated SNPs, both AX-93897192 and AX-93897200, which were associated with PH and NN, were located on chromosome 19, and the two SNPs were only approximately 14 kb apart (Table 3). Then, the two SNPs were identified as stable SNPs related to soybean P efficiency, and candidate genes around them were searched. As shown in Table 4, a total of 32 candidate genes were identified. Among them, the homologous gene of *Glyma.19g192700* in *Arabidopsis* was *At3g52910*, which belongs to the growth regulating factor (GRF) family. A previous study demonstrated that overexpression of *Arabidopsis GRF9* in tomato plants regulated resistance to P deficiency [24]. *Glyma.19g192900* was named *GmABCG39* in soybean [25], and members of ABCGs (subfamily G of the ATP-binding cassette family) were found to play important roles in defences against various biotic and abiotic stresses [26,27]. Homology of *Glyma.19g193400* was *At1g19490* (*AtbZIP62*), which regulated responses to drought stress [28], and both *Glyma.19g193900* and *Glyma.19g194000* encoded purple acid phosphatases. *Glyma.19g194500* and *Glyma.19g195200* were defined as "protein abscisic acid-insensitive 5" and "auxin responsive protein", respectively, in the Phytozome database, and abscisic acid [29] and auxin [30] were shown to be involved in P signalling pathways in plants. Overall, the above seven genes were identified as candidate genes related to P efficiency in soybean.

Table 4. Genes located within 130 kb upstream and downstream of significant SNPs AX-93897192 and AX-93897200.

Gene ID	Define in Phytozome	Homologous Genes in *Arabidopsis*
Glyma.19g192600	NTKL-binding protein 1	*At2g36410*: transcriptional activator (DUF662)
Glyma.19g192700	growth regulating factor 3-related	*At3g52910*: growth regulating factor encoding transcription activator
Glyma.19g192800	glycogen branching enzyme	*At5G03650*: encodes starch branching enzyme similar to SBE2 from maize and rice
Glyma.19g192900	ATP-binding cassette transporter	*At1g66950*: encodes a plasma membrane-localized ABC transporter
Glyma.19g193000	/	*At2g36370*: ubiquitin-protein ligases
Glyma.19g193100	protein kinase-related	*At3g52890*: KCBP-interacting protein kinase
Glyma.19g193200	60S acidic ribosomal protein P2	*At3g44590*: cytosolic ribosomal protein gene
Glyma.19g193300	/	*At3g52870*: IQ calmodulin-binding motif family protein
Glyma.19g193400	bZIP transcription factor-like protein	*At1g19490*: putative bZIP transcription factor
Glyma.19g193500	zinc finger FYVE domain containing protein	*At5g03610*: GDSL-motif esterase/acyltransferase/lipase
Glyma.19g193600	LysM domain	*At3g52790*: peptidoglycan-binding LysM domain-containing protein
Glyma.19g193700	inosine nucleosidase/ Inosinase	*At2g36310*: encodes a cytoplasmic nucleoside hydrolase
Glyma.19g193800	AN1-type zinc finger protein	*At2g36320*: A20/AN1-like zinc finger family protein
Glyma.19g193900	purple acid phosphatase 21-related	*At3g52820*: purple acid phosphatase 22
Glyma.19g194000	purple acid phosphatase 20-related	*At3g52780*: purple acid phosphatases superfamily protein
Glyma.19g194100	protein little zipper 3	*At3g52770*: small-leucine zipper containing protein (ZFR3)
Glyma.19g194200	integral membrance YIP1 family protein	*At3g52760*: integral membrane YIP1 family protein

Table 4. *Cont.*

Gene ID	Define in Phytozome	Homologous Genes in *Arabidopsis*
Glyma.19g194300	protein terminal flower 1	*At5g03840*: PEBP (phosphatidylethanolamine-binding protein) family protein
Glyma.19g194400	alpha/beta-hydrolases super family protein-related	*At3g48410*: alpha/beta-hydrolases superfamily protein
Glyma.19g194500	protein abscisic acid-insensitive 5	*At2g36270*: encodes a member of the basic leucine zipper transcription factor family
Glyma.19g194600	F-box-like	*At1g67190*: F-box/RNI-like superfamily protein
Glyma.19g194700	iron-sulfur cluster assembly protein	*At2g16710*: iron-sulfur cluster biosynthesis family protein
Glyma.19g194800	cell division protein FTSZ-related	*At2g36250*: tubulin/FtsZ family protein
Glyma.19g194900	/	*At3g52740*: plant specific protein
Glyma.19g195000	/	/
Glyma.19g195100	small nuclear ribonucleoprotein	*At2g23930*: putative small nuclear ribonucleoprotein G
Glyma.19g195200	auxin responsive protein	*At2g36210*: SAUR-like auxin-responsive protein family
Glyma.19g195300	125 KDA kinesin-related protein-related	*At2g36200*: P-loop containing nucleoside triphosphate hydrolases superfamily protein
Glyma.19g195400	beta-fructofuranosidase, insoluble isoenzyme CWINV2-related	*At3g52600*: cell wall invertase 2
Glyma.19g195500	ubiquitin/60s ribosomal protein L40 fusion	*At2g36170*: 60S ribosomal protein L40-1
Glyma.19g195600	alpha/beta hydrolase fold-containing protein	*At3g52570*: alpha/beta-Hydrolases superfamily protein
Glyma.19g195700	senescence regulator	*At3g15040*: senescence regulator

3.5. Expression Analysis of Candidate P-Efficiency Related Genes

To further identify the functions of candidate P-efficiency-related genes, we detected the expression levels of candidate genes based on low-P induced transcriptome analysis in the NCBI database. *Glyma.19g192900* (*GmABCG39*) was induced by P deficiency in the roots of the low-P tolerant soybean accession "B20" [16] and nodules of "YC03-3" [31]. *Glyma.19g193900* and *Glyma.19g194000* were induced by P deficiency in the roots and leaves of "B20" [16]. *Glyma.19g193900* was also upregulated by P deficiency in the nodules of "YC03-3" [31], and *Glyma.19g195200* was induced by P deficiency in the nodules of "YC03-3" [31].

Based on these results, the expression profiles of *Glyma.19g192900* (*GmABCG39*), *Glyma.19g193900*, *Glyma.19g194000,* and *Glyma.19g195200* in different tissues were analysed based on RNA-seq data available in SoyBase. As shown in Figure 3a, *GmABCG39* was expressed only in the roots. Given that roots play vital roles in conferring tolerance to low-P stress, *GmABCG39* was chosen as the P-efficiency-related gene for further study.

Induced expression analysis of *GmABCG39* in the low-P-tolerant accession "Kefeng No.1" and the low-P-sensitive accession "Nannong 1138-2" revealed that *GmABCG39* was significantly upregulated under -P condition compared with +P condition in "Kefeng No.1" and "Nannong 1138-2", with ratios corresponding to 5.14 and 16.95, respectively (Figure 3b). Overall, *GmABCG39* might act as a positive regulatory element involved in soybean P metabolism.

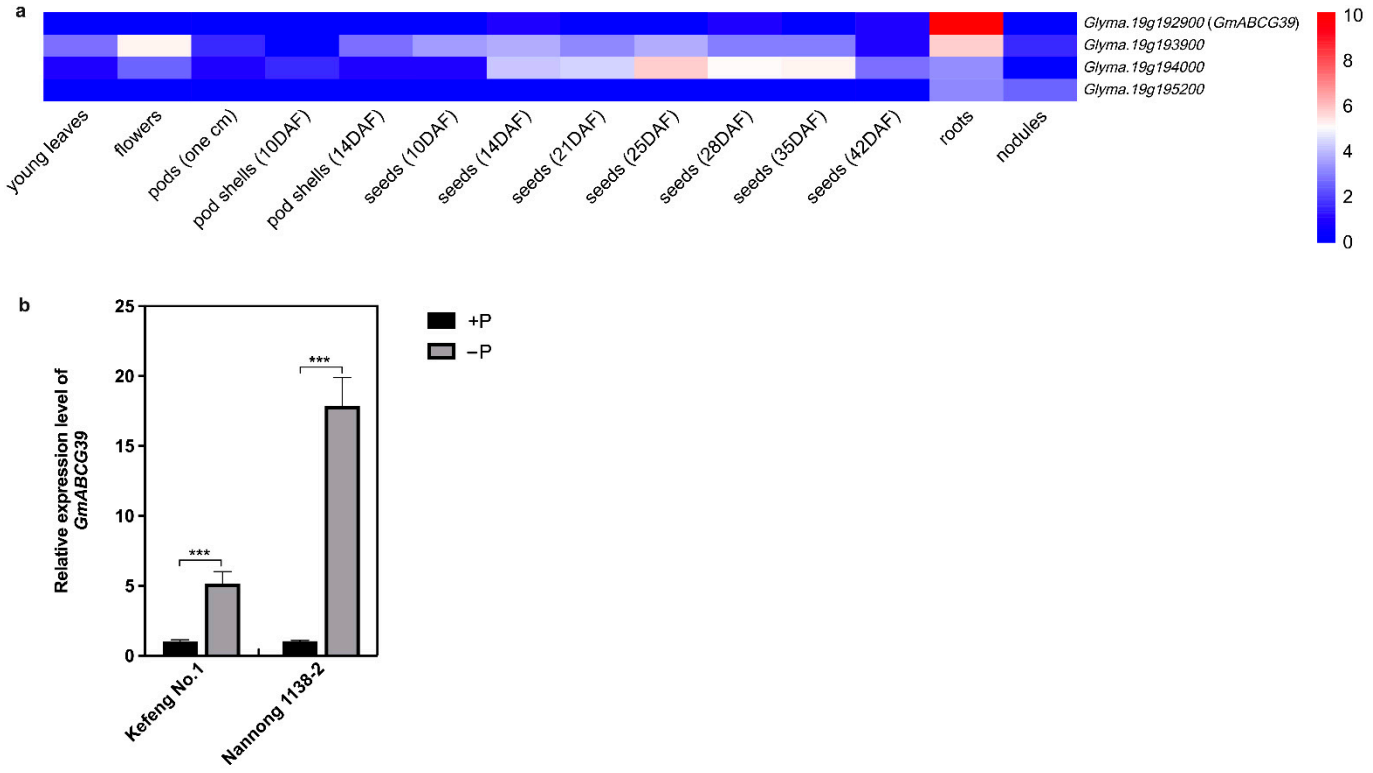

Figure 3. Expression profile of candidate genes. (**a**) Expression profile of four candidate P-efficiency-related genes in different soybean tissues. (**b**) Induced expression analysis of *GmABCG39* in "Kefeng No.1" and "Nannong 1138-2" under low-P stress. "Kefeng No.1": the low-P-tolerant accession; "Nannong 1138-2": the low-P-sensitive accession; +P: treatment including 1/2 Hoagland solution with 0.5 mM KH_2PO_4; −P: treatment including 1/2 Hoagland solution with 0.5 mM KCl; DAF: days after flowering; ***: significance at the 0.001 probability level (Student's *t*-test).

3.6. Bioinformatic Analysis of the P-Efficiency-Related Gene GmABCG39

The coding DNA sequence (CDS) of *GmABCG39* was 4,365 bp, and it encoded 1455 amino acid residues. In addition, two PHR1-binding sequence (P1BS; GNATATNC) elements, which were demonstrated to be involved in responses to low-P stress through binding high-P-efficiency transcription factors in plants [32], were present in the promoter region of *GmABCG39*.

Predictions of interactions revealed that ten proteins might interact with GmABCG39 (Figure 4). Interestingly, among the ten interacting proteins, three, namely, Glyma04g40591.1 (encoded by the *Glyma04g40591* gene), Glyma06g14210.1 (encoded by the *Glyma06g14210* gene, which was named *Glyma.06g137200* in Wm82.a2.v1), and Glyma18g13610.3 (encoded by the *Glyma18g13610* gene, which was named *Glyma.18g111000* in Wm82.a2.v1), were associated with iron (Fe) deficiency in soybean. Both *Glyma04g40591* and *Glyma06g14210* were induced in soybean accession "PI548533" after experiencing Fe deficiency for 1 h [33]; *Glyma18g13610* was predicted to encode 2-oxoglutarate and Fe(II)-dependent dioxygenase superfamily protein, whose homologous gene *At3g13610* was involved in Fe uptake in *Arabidopsis* [34]. Moreover, the P-efficiency-related gene *GmABCG39* was upregulated in response to Fe deficiency [33] as well as P deficiency (Figure 3b).

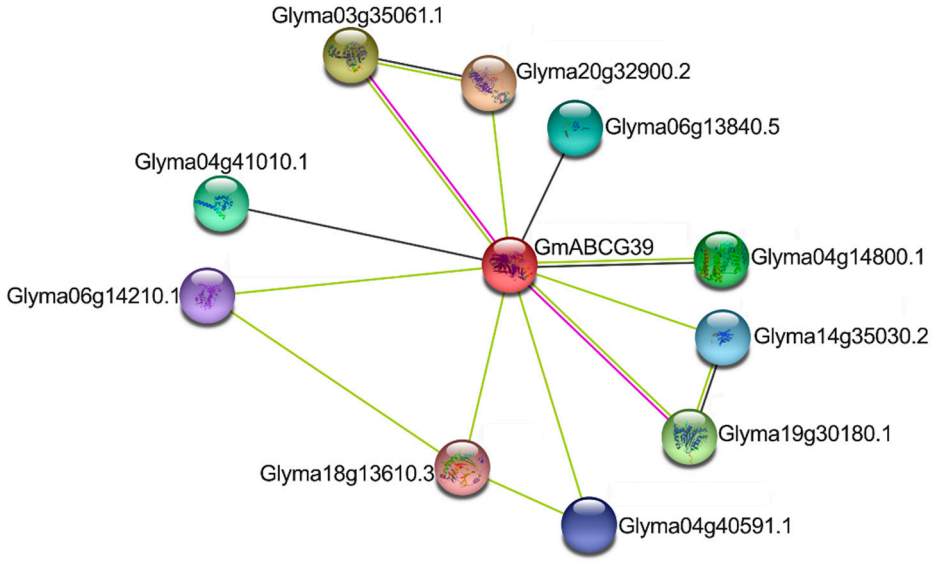

Figure 4. Predicted interacting proteins of GmABCG39. The fuchsia solid lines indicate experimentally determined proteins, the light green solid lines indicate gene neighbourhoods, the black solid lines indicate coexpression, the coloured nodes indicate query proteins and the first shell of interactors, the empty nodes indicate proteins of unknown 3D structure, and the filled nodes indicate that some 3D structure is known or predicted.

4. Discussion

4.1. Significant Correlations Were Detected among Soybean P-Efficiency-Related Traits at Maturity

The GWAS on soybean P efficiency has always focused on traits at the seedling stage [11,12,14]. In this study, however, we evaluated six P-efficiency-related traits at maturity and found that soybean P-efficiency was a complex quantitative trait that was controlled by multiple genes as all the six soybean P-efficiency-related traits, namely, PH, NN, BN, PN, 100SW, and SY, showed wide genetic variants and appeared to exhibit normal or approximately normal distributions (Table 1 and Figure 1).

Different from the significant positive correlations observed among PH, NN, BN, PN, and SY under both NP and LP conditions, PN and 100SW showed significant negative correlations under both NP and LP conditions (Table 2). Similarly, the significant negative correlations were found between PN and 100SW under NP condition in previous studies [35,36], which might be caused by supply competition between them.

In addition, we noticed that the correlations among the six P-efficiency-related traits at maturity under NP and LP conditions were consistent. However, the correlations among P-efficiency-related traits at the seedling stage always showed the opposite results under NP and LP conditions. For example, root dry weight and the root-to-shoot ratio showed a significant positive correlation under NP condition but showed a significant negative correlation under LP condition [11]. Similarly, the correlation between shoot P concentration and shoot Mg concentration [22] and the correlation between quantum efficiency of photosystem II and nonphotochemical quenching [14] under NP and LP conditions also showed the opposite results. These results might suggest that using traits at maturity as indexes to evaluate soybean P efficiency is reliable and stable.

Furthermore, the correlation coefficient between PH and NN was the highest compared with those of the others under NP ($r = 0.89$, $p < 0.001$) and LP ($r = 0.87$, $p < 0.001$) conditions (Table 2). These results were further verified by the GWAS results. For example, two SNPs, AX-93897192 and AX-93897200, were associated with both PH and NN (Table 3).

4.2. Novel P-Efficiency-Related Loci in Soybean at Maturity Were Identified

Through our GWAS of the six P-efficiency-related traits at maturity, a total of 27 SNPs were identified, and 9 SNPs were identified repeatedly (Table 3, Figures 2 and S1). Among

the nine repeated SNPs, AX-94111943, AX-93815278, and AX-94287407 on chromosome 13 were all located within the interval of QTL *qPC-F-1*, which was related to the P content of whole soybean plants at the seedling stage [37]; AX-94139280 on chromosome 15 was located within the interval of QTL *cqFARLPE-06*, which was related to flower abscission rate under LP condition [38]; and AX-93897192, AX-93897200, and AX-93659142 on chromosome 19 were located within the interval of QTL *q19-2*, which was related to P use efficiency, P uptake, P concentration, biomass yield, chlorophyll content, intercellular carbon dioxide concentration, and transpiration rate [39]. Taken together, these results suggested that the GWAS results concerning soybean P-efficiency-related traits at maturity are reliable. However, the significant markers identified in this study were environment-specific, which could be due to the fact that the traits at maturity were affected greatly by environments.

Notably, compared with reported studies on the identification of soybean P-efficiency-related QTLs [15,16,37–43], two SNPs in this study, AX-93699741 on chromosome 3 and AX-94207363 on chromosome 20, were not colocated within the interval of reported QTLs associated with P efficiency in soybean. Thus, these two SNPs might be novel SNPs related to soybean P efficiency.

4.3. New P-Efficiency-Related Genes Were Identified at Maturity

Two repeated SNPs AX-93897192 and AX-93897200 on chromosome 19 were associated with two P-efficiency-related traits (PH and NN) and were considered as stable SNPs for subsequent searches for candidate P-efficiency-related genes.

Combining gene annotation information and data from the low-P-induced transcriptome analysis [16,31], we identified four candidate genes, namely, *Glyma.19g192900*, *Glyma.19g193900*, *Glyma.19g194000,* and *Glyma.19g195200*. Among these four genes, *Glyma.19g193900* and *Glyma.19g194000* encoded soybean purple acid phosphatase, and the expression of *Glyma.19g193900* was reported to be upregulated by 40–60-fold in two high-P-efficiency soybean accessions, "Nannong 94-156" and "Kefeng No.1", after seven days of P deficiency [39].

Glyma.19g195200 was identified as encoding an auxin responsive protein, and auxin signalling had been found to be associated with changes in root architecture caused by P deficiency [44,45]. In soybean, the endogenous indole-3-acetic acid (IAA) content in the roots was shown to increase in response to P deficiency, and the application of exogenous IAA promoted the activity of plasma membrane H^+-ATPase and P uptake [46]. Liu et al. predicted target sites of microRNAs that were differentially expressed in two soybean varieties with different P efficiencies under NP and LP conditions and found auxin transcription factors were among the main targets [47], indicating that *Glyma.19g195200* could be involved in responses to soybean P deficiency regulated by auxin signalling.

Glyma.19g192900, a member of the ABCG subfamily, was named *GmABCG39* [25]. *L.albABCG36s* and *L.albABCG37s* in white lupin were found to promote cluster root formation through the regulation of indole-3-butyric acid (IBA) transport, thus contributing to adaptation to low-P stress [48]. Moreover, *L.albABCG29* improved low-P tolerance by improving root growth [49]. Interestingly, *GmABCG39* was expressed only in the roots (Figure 3a), and its expression levels in roots were regulated by both P deficiency (Figure 3b) and Fe deficiency [33]. Two P1BS elements were present in the promoter region of *GmABCG39*, and three (*Glyma04g40591*, *Glyma06g14210,* and *Glyma18g13610*) genes encoding three of ten predicted interacting proteins were associated with Fe deficiency in soybean (Figure 4). Previous studies had demonstrated that P and Fe functioned dependently to maintain ion homeostasis [50], and *GmABCG39* might be involved in complex regulatory networks underlying P and Fe homeostasis in soybean roots.

5. Conclusions

In conclusion, a total of 27 SNPs were identified through a GWAS of six soybean P-efficiency-related traits at maturity across two environments, and two stable P-efficiency-related SNPs that were associated with both plant height and node number of the main

shoot were identified. Furthermore, a candidate gene, *GmABCG39*, that was upregulated in soybean roots in response to P deficiency was identified. This work enriches our understanding of the genetic mechanism underlying P-efficiency, which will be helpful in breeding high-P-efficient soybean accessions.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy12092031/s1, Figure S1: QQ plots of the GWAS results of phenotypic values of the six soybean P-efficiency-related traits at maturity under NP and LP conditions in E1 and E2.

Author Contributions: Conceptualization, Q.W., L.N., D.Y. and H.C.; validation, Q.W., W.Y. and W.Z.; formal analysis, Q.W. and W.Y.; investigation, Q.W. and L.N.; writing—Original draft preparation, Q.W.; writing—Review and editing, Q.W., D.Y. and H.C.; visualization, Q.W.; supervision, H.W. and H.C.; project administration, F.H. and D.Y. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported in part by National Natural Science Foundation of China (32090065, 31871649, 32072080), Ministry of Science and Technology (2017YFE0111000), Jiangsu Natural Science Foundation (BK20220996), EUCLEG Horizon 2020 of European Union (727312).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available in the Supplementary Materials.

Acknowledgments: We would like to thank the Bioinformatics Center of Nanjing Agricultural University.

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

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