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Transcriptome Analysis of *Vicia villosa* in Response to Low Phosphorus Stress at Seedling Stage

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Abstract: *Vicia villosa*, a high-quality green manure crop, helps to increase the content of soil phosphorus (P) by returning to the field. Soil P deprivation is a severe constraint on crop productivity that triggers the low P stress response in plants, which is controlled by various transcriptional regulatory network pathways. Identifying key genes from these transcriptional regulatory networks can help in developing low P-tolerant crops. In this study, we performed physiological observations and transcriptome sequencing of seedlings from the two *Vicia villosa* varieties, Xu Shao 3 and Soviet *Vicia villosa*, under P starvation conditions. The results showed that the main root length, plant height, shoot dry weight, root acid phosphatase activity, and inorganic P content of Xu Shao 3 were significantly higher than those of Soviet *Vicia villosa* under low P conditions. Based on transcriptome data analysis, 183 (shoot) + 144 (root) differential genes (DEGs) between the two varieties were identified; 144 (shoot) + 79 (root) were upregulated, and 69 (shoot) + 65 (root) were downregulated. KEGG analysis found that DEGs in shoots were significantly enriched in photosynthesis pathways, such as vitamin B6 and riboflavin metabolism. Meanwhile, DEGs in roots were enriched in plant signal transduction, fatty acid degradation, citric acid cycle, pentose, glucuronic acid conversion, etc. GO enrichment analysis suggested that DEGs in shoots were significantly enriched in biological processes, including cell response to P stress, intracellular ion homeostasis, etc., and molecular functions, including phosphate ester hydrolase, phosphatase, acid phosphatase activity, etc. Furthermore, DEGs associated with low P tolerance included three acid phosphatases, a phosphoesterase, a sulfoquinovosyl diacylglycerol synthase, a phosphoenolpyruvate carboxylase, six phosphate transporters and glycerol-3-phosphate transporters, eight *SPX*, and two *PHL* genes. In conclusion, Xu Shao 3 exhibited stronger inorganic P accumulation ability and a lesser effect on growth than Soviet *Vicia villosa* under low P conditions, which might result from photosynthesis, sugar, and P metabolism differences between the two varieties. Acid phosphatase, phosphoesterase, phosphoenolpyruvate carboxylase, sulfoquinovosyl diacylglycerol synthase, phosphate transporter, glycerol-3-phosphate transporter, and *SPX* were key DEGs leading to the difference in low P stress tolerance between the two varieties.

Keywords: *Vicia villosa*; transcriptome; low phosphorus stress; phosphate transporter; phosphorus

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1. Introduction

Phosphorus, a component element of many important biological macromolecules, such as adenosine triphosphate, phospholipid, and coenzymes, is an essential nutrient required in energy metabolism, enzymatic reactions, signal transduction, and other key life activities [1]. Poor mobility and easy fixation with metal ions limit the plant absorption of inorganic phosphate, which negatively affects plant growth and development [2]. Therefore, phosphate fertilizers are applied to increase the effective phosphorus (P) content of the soil. However, the application of excessive phosphate fertilizer is a "high input, low output"

approach, which not only causes the depletion of phosphate rock resources but also harms the environment [3,4]. Therefore, it is critical to study the biological mechanism of plant tolerance to low P stress, which can reduce the application of phosphate fertilizer and help improve the efficiency of P uptake and utilization.

Plants have evolved with a series of adaptive mechanisms to counter low P stress, such as changing root morphology, increasing the secretion of organic acids and acid phosphatases, and symbiosis with clumping mycorrhizae [5–8]. These adaptive responses are collectively known as phosphate starvation response (PSR) [9]. PSR is a complex regulatory mechanism that integrates internal and external P conditions involving local and system signal responses [10]. Under low P conditions, most of the plant systemic signaling responses are regulated by Phosphate Starvation Response 1 (*PHR1*), *PHR1*-like proteins (*PHL*), and other transcription factors [11]. For example, under low P conditions, (SPX domain gene) *SPX* positively regulates the *PHR1* gene; notably, PHR1 binds to the P1BS cis-element (GNATATNC) of the promoter of downstream phosphate starvation-induced genes (*PSI*) and then initiates the expression of phosphatase genes, *PHT1*, *PHF1*, *PAP,* and other *PSI* genes [12–16].

In recent years, transcriptome analysis of phosphate starvation response has been carried out in a variety of plants. In *Arabidopsis thaliana*, differentially expressed and highly phosphorus-specific putative regulatory genes were identified in the complex response to phosphorus deficiency and resupply, regulating phosphorus nutrients [17]. In maize (*Zea mays*), many differentially expressed phosphorus-responsive (Pi)-responsive genes associated with transcriptional response to phosphorus deficiency were identified in the root system. These Pi-responsive genes participate in sugar and nitrogen metabolic pathways, ion transport, signal transduction, transcriptional regulation, and other processes related to plant growth and development; additionally, it was predicted that miRNAs play an important role in determining the level of low phosphorus tolerance in different genotypes of maize [18–20]. In rice (*Oryza sativa*), transcriptome analysis identified that the *OsPI1* gene was the most significantly upregulated gene under P-deficient conditions and became downregulated after P supply, highlighting the role of this gene under specific low phosphorus conditions [21]. Transcriptome analysis in response to phosphorus starvation conditions in wheat (*Triticum aestivum*) revealed a general upregulation of an approximate 341 and 13 times increase in the expression of *TaIPS1* (a homolog of *IPS1*) in roots and shoots, respectively [22]. It was found that low phosphorus response genes were mainly involved in ion transport, transcriptional regulation, active oxygen scavenging, hormone signal transduction, and other metabolic processes [17–22].

Vicia villosa is an important leguminous green manure crop in China, which provides nutrients to the next crop, enhances the stability of soil aggregate structure, improves the activities of soil peroxidase and acid phosphatase, promotes soil physical and chemical properties, and thus improves overall crop yield and quality. *Vicia villosa* can help reduce the application of chemical fertilizer after rolling over and returning to farmland [23–25]. However, different *Vicia villosa* varieties have different responses to low P stress. The molecular physiological mechanism of plant resistance to low P stress and how different P-efficiency varieties absorb and utilize P nutrients more efficiently are still unclear. Understanding such information can significantly enhance our ability to develop better varieties or strategies for efficient absorption and accumulation of P in plants, increasing the effectiveness of soil P returning to the field. The transcriptome analysis of *Vicia villosa* under low phosphorus conditions is rarely reported. Therefore, in this study, we identified key differentially expressed genes (DEGs) related to low P tolerance between the two varieties of *Vicia villosa* based on the FPKM (Fragments Per Kilobases per Millionreads) method and criteria p-adjust < 0.05 and $|\log_2 FC| \geq 1$. The DEGs were functionally annotated by GO (Gene Ontology) functional enrichment analysis and KEGG (Kyoto Encyclopedia of Genes and Genomes) metabolic pathway analysis for low P stress. Our results provide a theoretical basis for breeding low P tolerant and P-efficiency varieties of *Vicia villosa*.

2. Materials and Methods

2.1. Test Materials and Culture Conditions

The experimental materials (Xu Shao 3 and Soviet *Vicia villosa*) were provided by Professor Cao Weidong at the Institute of Agricultural Resources and Regional Planning, Chinese Academy of Agricultural Sciences, China.

The culture experiments were conducted in 2020; two treatments were set up: normal phosphorus (NP, 200 μM Pi) and low phosphorus (LP, 2 μM Pi), with 3 replicates each. We used the intelligent light incubator (model: PRX-1000D-LED-30) for culture. The floating seedling cultivation method was adopted in the Hoagland improved nutrient solution (pH 6.3 ± 0.1), which was changed every 3 days. The specific steps were as follows: full seeds of *Vicia villosa* were picked, disinfected with 75% alcohol, and then placed in a culture dish containing gauze. Seed germination was promoted in a 25 °C light incubator. After 3 cm of the radicle was exposed, it was cultured in 1/2 Hoagland nutrient solution for 7 days and then in nutrient solution with different P concentrations. The samples were taken out after continuous treatment for 12 and 18 days. The lighting incubator setting conditions were as follows: 12 h lighting, 25 °C, light intensity 30,000 LX; Darkness for 12 h, 22 °C; 60% humidity. The nutrient solution composition under normal phosphorus conditions was: 1.3 mM NH_4NO_3, 0.19 mM K_2SO_4, 200 μM $NaH_2PO_4{\cdot}2H_2O$, 0.37 mM $CaCl_2{\cdot}2H_2O$, 0.38 mM $MgSO_4{\cdot}7H_2O$, 50 μM H_3BO_3, 10 μM $MnCl_2{\cdot}4H_2O$, 0.76 μM $ZnSO_4{\cdot}7H_2O$, 0.32 μM $CuSO_4{\cdot}5H_2O$, 0.016 μM $(NH_4)6MO_7O_{24}{\cdot}4H_2O$, and 77 μM Na_2Fe-EDTA. For the low P treatment, $NaH_2PO_4{\cdot}2H_2O$ content in the nutrient solution was 2 μM, while the rest of the composition was the same.

2.2. Measurement of Physiological Indexes

Plants that were uniformly grown were chosen after 12 and 18 days of seedling culture. These plants were then cut at the rootstock junction and divided into shoot and root sections, respectively. The plant height and main root length of twelve plants were measured for the treatments with normal and low phosphorous levels. The plants were then killed green at 105 °C for 30 min, dried at 80 °C to constant weight, and the dry weights of the shoot and root each were measured. To test the inorganic phosphorus content using the molybdenum antimony anti-colorimetric method, the shoot and root systems of 10 fresh plants were chosen, ground into powders with the aid of liquid nitrogen, and extracted with H_2SO_4 [26]. Five shoot sections from fresh plants were ground in liquid nitrogen and then added to a solution of HCl-methanol mixture to measure anthocyanin content by UV-Vis spectrophotometry [27]. The p-nitrophenyl phosphate disodium method was used to determine the acid phosphatase activity in the roots of five fresh plants [28].

2.3. RNA Sample Extraction and Transcriptome Sequencing

In total, 24 libraries were constructed for the shoot and root systems of Soviet *Vicia villosa* and Xu Shao 3 under normal phosphorus and low phosphorus treatments for 12 days with three biological replicates per treatment. To eliminate the differences among individuals, 5 strains of the same variety were treated with low phosphorus for 12 days, mixed, and then sampled. Three biological repeats were performed for each material. The samples were frozen in liquid nitrogen and then stored at −80 °C for RNA extraction. The RNA extraction, library construction, and transcriptome sequencing were completed by Megi Biomedicine Technology Co., Ltd., Beijing, China.

After sequencing on the Illumina HiSeq4000 platform, low-quality reads were removed by fastp (v. 0.19.5) [29] to obtain more than 40 million clean reads for each sample; the error rate of all clean reads was <1‰. The GC content of 24 samples measured after filtering was 41.58–43.46%, the Q20 values were >98%, and the Q30 values were around 94%, indicating the high accuracy and suitability of the whole sequencing data for transcriptome analysis.

2.4. Analysis of Transcriptome Data

Expression levels of transcripts were quantified using the RSEM alignment software Version 1.3.1 (http://deweylab.biostat.wisc.edu/rsem/, accessed on 9 May 2023), and gene expressions were calculated using the FPKM: Fragments per kilobase of exon model per million mapped fragments (fragments obtained by interrupting the library construction, when sequenced by PE, the same fragment contains two reads). The specific formula is as follows: FPKM = the number of fragments uniquely compared to genes for X gene/(length of X gene $\times$ the total number of fragments uniquely compared to participating genomes) $\times$ 10^9.

Differential gene analysis was performed using DEGSeq (http://bioconductor.org/packages/stats/bioc/DEGSeq/, accessed on 9 May 2023), and the Benjamini-Hochberg correction method was used to correct the significant p values, with p-adjust < 0.05 or $|\log_2 FC| \geq 1$ as the screening criteria. The ratio of gene expression between the two samples was expressed in terms of the fold change of difference (Fold Change). Six major databases, Pfam, Swiss-prot, GO, NR, KEGG, and eggNOG, were used for the functional annotation of DEGs. The GO (http://www.geneontology.org/, accessed on 9 May 2023) and KEGG (http://www.genome.jp/kegg/, accessed on 9 May 2023) databases were used to analyze the functional enrichment and metabolic pathways of DEGs.

2.5. Statistical Analysis

Statistical analysis of data was performed using IBM SPSS Statistics 22, bar graphs were constructed using GraphPad Prism 8, and transcriptome data analysis was performed using Major's cloud (https://cloud.majorbio.com/, accessed on 9 May 2023).

3. Results

3.1. Physiological Characteristics Differences between Two Species under Low Phosphorus Stress

After 12 days of low P treatment, compared with normal P treatment, the plant height of Soviet *Vicia villosa* and Xu Shao 3 decreased by 26.01 and 24.10%, respectively. The main root length of Soviet *Vicia villosa* exhibited no significant change, but that of Xu Shao 3 increased by 23.17%. The shoot dry mass of Soviet *Vicia villosa* and Xu Shao 3 decreased by 48.53 and 34.34%, respectively. There was no significant change in the root dry mass of the Soviet *Vicia villosa*, but it decreased by 30.43% in Xu Shao 3. The anthocyanin content of Soviet *Vicia villosa* and that of Xu Shao 3 increased by 12- and 5.26-fold, respectively (Table 1).

Table 1. Physiological characteristics of *Vicia villosa* under the normal and low Phosphorus treatments.

Index	Normal Phosphorus Treatment		Low Phosphorus Treatment	
	Soviet *Vicia villosa*	Xu Shao 3	Soviet *Vicia villosa*	Xu Shao 3
Plant height (cm)	44.48 ± 2.29 b	49.19 ± 1.92 a	33.38 ± 1.53 d	37.35 ± 0.98 c
Main root length (cm)	24.11 ± 1.27 b	23.95 ± 0.72 b	25.17 ± 1.33 b	29.50 ± 2.00 a
Shoot dry mass (g)	0.068 ± 0.003 b	0.099 ± 0.003 a	0.035 ± 0.001 c	0.065 ± 0.0001 b
Root dry mass (g)	0.019 ± 0.001 ab	0.023 ± 0.002 a	0.0191 ± 0.002 ab	0.016 ± 0.002 b
Anthocyanin content (μg/g)	0.99 ± 0.15 c	1.63 ± 0.27 c	12.91 ± 0.82 a	10.21 ± 0.58 b
Acid phosphatase activity (mmol PNP/h/g)	2.46 ± 0.31 c	2.21 ± 0.44 c	5.53 ± 0.48 b	9.82 ± 0.54 a

Note: Different lowercase letters after corresponding data indicate significant differences in physiological indices between different treatments and varieties ($p < 0.05$).

The inorganic P content and acid phosphatase activity of the two varieties were analyzed. As shown in Figure 1, after 12 days of normal P treatment, the inorganic P content in the shoots and roots of Xu Shao 3 were 30% and 15.6% higher than that in Soviet *Vicia villosa*, respectively. After 12 days of low P treatment, inorganic P content in shoots and roots of the two varieties exhibited no significant difference; however, inorganic P content in shoots and roots of Xu Shao 3 was significantly higher than that of Soviet *Vicia villosa* after 18 days of low P treatment (Figure 1). Meanwhile, no significant difference was found

in the activity of acid phosphatase in roots between the two varieties after 18 days of normal P treatment. However, after 18 days of low P treatment, the activity of acid phosphatase in the roots of Xu Shao 3 was significantly higher than that of Soviet *Vicia villosa* (Table 1).

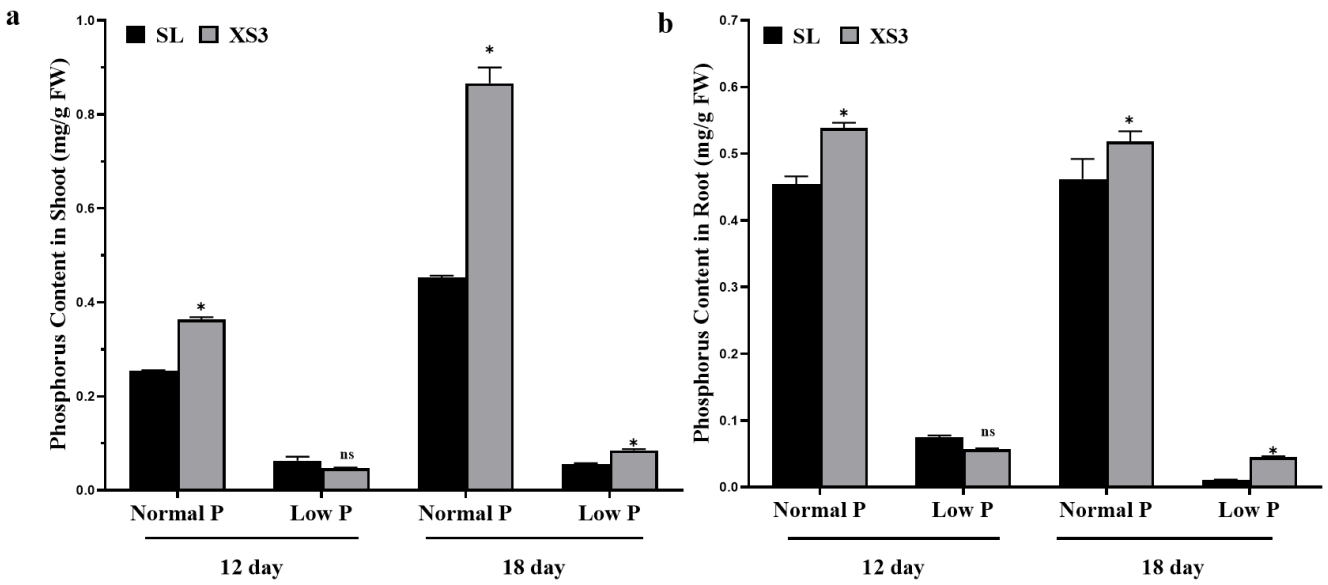

Figure 1. Inorganic P content in *Vicia villosa* (**a**) Shoot and (**b**) Root under normal and low P conditions. Note: * $p < 0.05$; ns, not significant.

3.2. Number of Differentially Expressed Genes between Two Varieties under Low Phosphorus Stress

DEGseq was used to identify DEGs between the two varieties of *Vicia villosa*. 2808 and 10442 DEGs caused by low P stress were detected in shoots (Figure 2a), and 9238 and 5039 DEGs in roots of Soviet *Vicia villosa* and Xu Shao 3, respectively (Figure 2b). There were 480 common DEGs in shoots (Figure 2a) and 1026 common DEGs in roots between the two varieties (Figure 2b). These common DEGs were mainly caused by low P stress but not due to the differences between the two varieties.

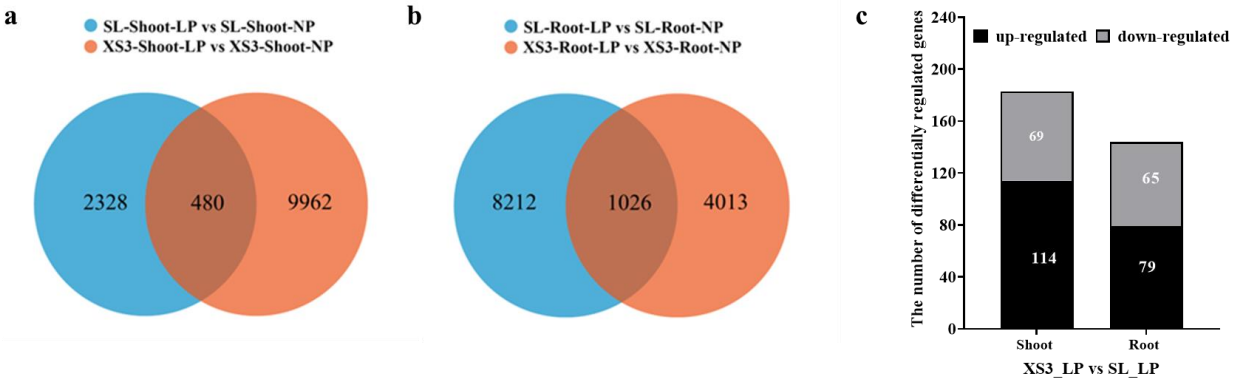

Figure 2. Differentially expressed genes between Xu Shao 3 and Soviet *Vicia villosa* under low P conditions. Note: SL, Soviet *Vicia villosa*; XS3, Xu Shao 3. Venn diagram of differential expression gene in the shoot (**a**) and root (**b**) of Xu Shao 3 and Soviet *Vicia villosa* under low P conditions. (**c**) Differential expression genes in Xu Shao 3 under low P conditions, compared with those in Soviet *Vicia villosa*.

Further analysis of DEGs between the two varieties revealed that there were 183 DEGs in shoots, including 114 upregulated and 69 downregulated DEGs (Figure 2c), and 144 DEGs in roots, including 79 upregulated and 65 downregulated DEGs (Figure 2c).

3.3. Biological Function Analysis of Differentially Expressed Genes between Two Varieties under Low Phosphorus Stress

Compared with those of Soviet *Vicia villosa*, there were 114 and 79 upregulated genes in shoots and roots of Xu Shao 3 under low P conditions, respectively (Figure 2c). GO enrichment analysis showed that these upregulated genes in the shoots of Xu Shao 3 were enriched in key biological processes and molecular functions (Figure 3a). The key biological processes included the following two aspects: (1) the direct response to stress, such as cell response to extracellular and external stimuli, cell response to nutrient levels, and cell response to phosphate starvation, and (2) intracellular ion homeostases, such as intracellular phosphate ion homeostasis and inorganic anion homeostasis. The molecular functions also included the following two aspects: (1) enzyme activity related to P metabolisms, such as phosphate ester hydrolase activity, phosphatase activity, lipid bond hydrolase activity, acid phosphatase activity, and (2) cell communication. The upregulated genes in the root of Xu Shao 3 were enriched in key biological processes and cell components (Figure 3b). The key biological processes included peptide metabolism, translation, biosynthesis of peptides and amides compounds, biosynthesis of nitrogen-containing compounds, and macromolecular biosynthesis. The cell components mainly refer to the ribosome and its components and various organelles that do not bind to the cell membrane.

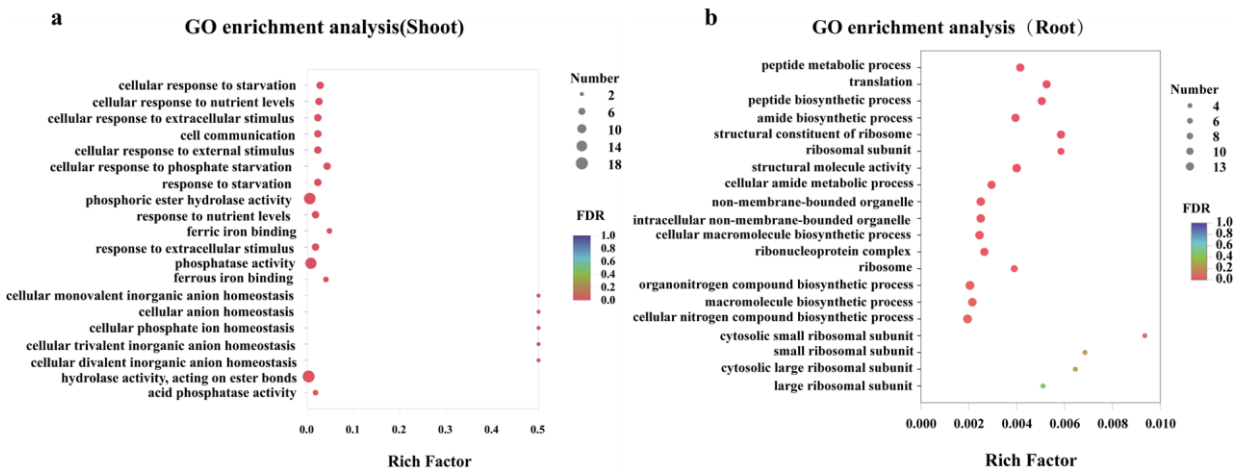

Figure 3. Go enrichment analysis of upregulated genes in the (**a**) shoot and (**b**) root of Xu Shao 3 compared with Soviet *Vicia villosa*.

3.4. Metabolic Pathways Analysis of Differentially Expressed Genes between Two Varieties under Low Phosphorus Stress

KEGG metabolic pathway analysis was conducted on DEGs between Xu Shao 3 and Soviet *Vicia villosa* under low P conditions (Figure 4). The upregulated genes in the shoots of Xu Shao 3 were mainly enriched in pathways related to the photosynthesis pathways, such as vitamin B6 metabolism and riboflavin metabolism (Figure 4a). The upregulated differential genes in roots were mainly enriched in metabolic pathways such as the ribosome, plant hormone signal transduction, spliceosome, fatty acid degradation, oxidative phosphorylation, citrate cycle, pentose, and glucuronate interconversions (Figure 4b).

3.5. Identification of Differentially Expressed Genes Related to Phosphorus Metabolism between Two Varieties under Low Phosphorus Stress

3.5.1. Genes Involved in Soil Phosphorus Activation

Acid phosphatase, phosphatase, and pyrophosphatase secreted by plant roots hydrolyze intracellular and extracellular organophosphates, which leads to the release of organic phosphorus compounds in soil for plant growth [30,31]. Under low P conditions, three genes encoding acid phosphatase were upregulated in both two varieties, two genes were expressed in shoots (transcript_101704 and transcript_163306), and another one was

expressed in roots (transcript_114615). Moreover, the expression of these three genes was significantly increased in Xu Shao 3 than that in Soviet *Vicia villosa*. Especially, the expression of the gene encoding purple acid phosphatase (transcript_114615) was almost one-fold higher in the roots of Xu Shao 3 than that in Soviet *Vicia villosa* (Table 2).

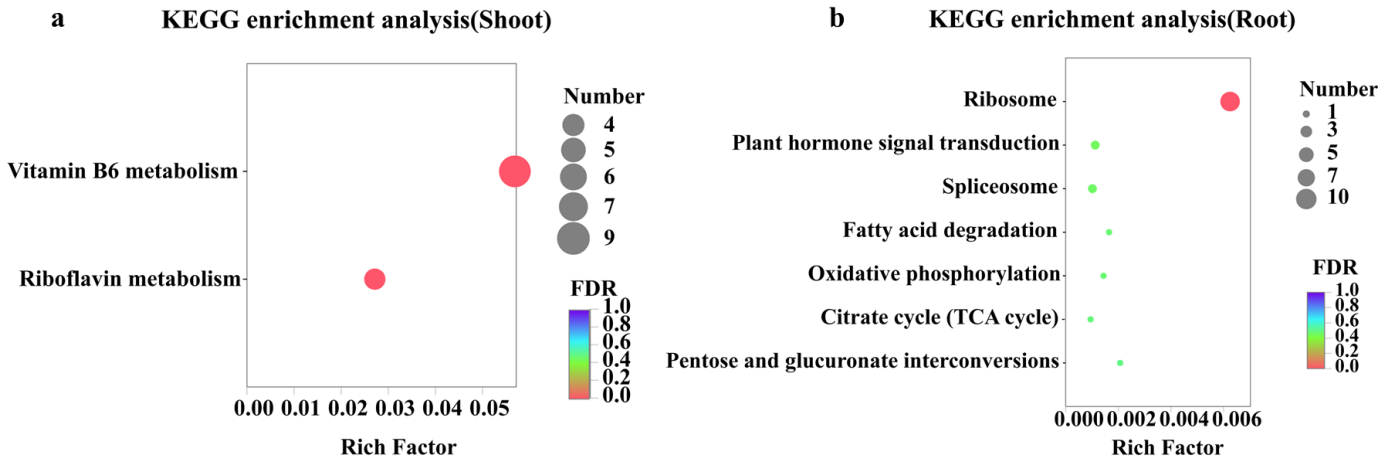

Figure 4. KEGG enrichment analysis of upregulated genes in the shoot (**a**) and root (**b**) of Xu Shao 3 compared with Soviet *Vicia villosa*.

Table 2. Differentially expressed genes involved in soil phosphorus activation between Xu Shao 3 and Soviet *Vicia villosa*.

Tissue	Gene ID	log_2FC (SL_LP/SL_NP)	log_2FC (XS3_LP/XS3_NP)	Annotation
Shoot	transcript_101704	7.199	7.939	acid phosphatase
	transcript_163306	8.857	9.098	acid phosphatase
	transcript_126553	−8.317	7.265	phosphoesterase
	transcript_157201	7.439	9.441	inorganic pyrophosphatase
	transcript_30883	−9.642	10.041	Phosphoenolpyruvate carboxylase
	transcript_4101	−4.683	−5.036	malic dehydrogenase
Root	transcript_4101	−9.572	−6.534	malic dehydrogenase
	transcript_76499	−4.293	−8.188	malic dehydrogenase
	transcript_114615	2.135	4.529	Purple acid phosphatase

Note: Fold change (FC) = gene expression under low phosphorus conditions/gene expression under normal phosphorus conditions.

A gene encoding phosphatase (transcript_126553) was upregulated in the shoot of Xu Shao 3 but was downregulated in the shoot of Soviet *Vicia villosa*; meanwhile, a gene encoding inorganic pyrophosphatase (transcript_157201) was upregulated in the shoots of both varieties (Table 2). A gene encoding phosphoenolpyruvate carboxylase (transcript_30883) was upregulated in the shoot of Xu Shao 3 but was downregulated in the shoot of Soviet *Vicia villosa*; two genes encoding malate dehydrogenase (transcript_4101 and transcript_76499) were downregulated in both varieties (Table 2).

3.5.2. Genes Related to Phosphorus Absorption and Transport

Plants absorb inorganic soil P through the roots. Since the cytoplasmic content of inorganic P is higher than that in the solution outside the cell membrane, the uptake of soil inorganic P into plants requires the aid of phosphate transporters. There were six differentially expressed phosphate transporters between the two varieties, mainly expressed in the roots; transcript_157065 was expressed in both roots and shoots. All of them were upregulated under low P stress. Three of the genes (transcript_157065, transcript_102952, and transcript_121036) were more upregulated in Xu Shao 3 than in Soviet *Vicia villosa*. The

expression of transcript_157065 in the shoot and transcript_102952 in the root of Xu Shao 3 was one-fold higher than those in Soviet *Vicia villosa* (Table 3).

Table 3. Differentially expressed genes related to phosphorus uptake and transport between Xu Shao 3 and Soviet *Vicia villosa*.

Tissue	Gene ID	log_2FC (SL_LP/SL_NP)	log_2FC (XS3_LP/XS3_NP)	Annotation
Shoot	transcript_157065	5.711	10.362	Phosphate transporter 1–4
	transcript_131639	3.958	5.073	Glycerol-3-phosphate transporter 4
	transcript_124589	4.710	5.180	Glucose-6-phosphate
Root	transcript_102952	3.603	6.179	Phosphate transporter 1–4
	transcript_103286	7.245	6.902	Phosphate transporter 1–7
	transcript_121036	5.205	6.158	Phosphate transporter 1–4
	transcript_157065	6.203	6.305	Phosphate transporter 1–4
	transcript_3647	7.919	7.661	Phosphate transporter 1–4
	transcript_96710	5.553	5.340	Phosphate transporter 1–4
	transcript_108545	9.076	4.742	Glycerol-3-phosphate transporter 1
	transcript_131639	5.326	5.340	Glycerol-3-phosphate transporter 4
	transcript_142355	4.688	4.140	Glycerol-3-phosphate transporter 1
	transcript_77913	4.142	4.640	Glycerol-3-phosphate transporter 1
	transcript_93091	5.293	6.130	Glycerol-3-phosphate transporter 4
	transcript_94957	5.948	6.860	Glycerol-3-phosphate transporter 4
	transcript_166118	1.963	5.890	Sulfoquinovosyl diacylglycerol synthase

Note: Fold change (FC) = gene expression under low phosphorus conditions/gene expression under normal phosphorus conditions.

Glycerol-3-phosphate transporters play an important role in the redistribution of P in different plant organs, such as in the uptake of glycerol-3-phosphate into the cell, simultaneously excreting Pi outside the cell [32]. There were six differentially expressed glycerol-3-phosphate transporters between two varieties of *Vicia villosa*, all of them were upregulated under low P stress. The transcript_131639 was expressed in both roots and shoots, and the other five genes were mainly expressed in the roots. Four genes (transcript_131639, transcript_77913, transcript_93091, and transcript_94957) were more upregulated in Xu Shao 3 than in Soviet *Vicia villosa*, and the expression of transcript_108545 in Soviet *Vicia villosa* was one-fold higher than in Xu Shao 3 (Table 3).

In plants, P is transported into chloroplasts and mitochondria through the reverse exchange of triose-phosphoric acid and pentose phosphate. A gene involved in pentose phosphate synthesis (transcript_124589) was significantly upregulated under low P in the shoots of two varieties, and its upregulation was higher in Xu Shao 3 than in Soviet *Vicia villosa* (Table 3).

Sulfoquinovosyl diacylglycerol synthase (SQD) is a key enzyme in the synthesis of Sulfoquinovosyl diacylglycerol (SQDG), a major component of the thylakoid membrane of chloroplasts in plants. SQDG is an anionic lipid that is converted into other anionic lipids (mainly phospholipids) and enables the plant quickly adapt to the P deficiency environment [33]. A gene related to lipid remodeling, transcript_166118, was upregulated in the roots of both varieties under low P stress; however, its expression was almost double in Xu Shao 3 compared with Soviet *Vicia villosa* (Table 3).

3.5.3. Genes Related to Phosphate Starvation Response

The proteins with SPX structural domain, acting as phosphate receptors in plants, can sense external P concentration by binding inositol polyphosphate signaling molecules and participate in the regulation of P signal pathways [34]. As shown in Table 4, there were 8 DEGs with *SPX* domain between two varieties; transcript_36102 was expressed only in roots, and the other 7 DEGs were expressed in both roots and shoots. Under low

P stress, these eight genes were upregulated in both Soviet *Vicia villosa* and Xu Shao 3. The expression of six genes (transcript_132532, transcript_165540, transcript_180595, transcript_184361, transcript_24611, and transcript_24648) was higher in the shoot of Xu Shao 3 than in the shoot of Soviet *Vicia villosa*. The expression of four genes (transcript_180595, transcript_24611, transcript_24648, transcript_36102) was higher in the roots of Xu Shao 3 than in the roots of Soviet *Vicia villosa*. However, the expression of transcript_99245 was higher in both shoots and roots of Soviet *Vicia villosa* than that in Xu Shao 3.

Table 4. Differentially expressed genes related to phosphate starvation response between Xu Shao 3 and Soviet *Vicia villosa*.

Tissue	Gene ID	log_2FC (SL_LP/SL_NP)	log_2FC (XS3_LP/XS3_NP)	Annotation
Shoot	transcript_132532	3.636	5.690	SPX1
	transcript_165540	8.035	12.23	SPX3
	transcript_180595	7.426	12.32	SPX3
	transcript_184361	7.693	11.15	SPX3
	transcript_24611	4.794	9.15	SPX2
	transcript_24648	3.456	5.580	SPX1
	transcript_99245	5.285	4.050	SPX2
Root	transcript_132532	6.485	5.890	SPX1
	transcript_180595	9.973	10.940	SPX3
	transcript_184361	11.945	10.540	SPX3
	transcript_24611	7.355	7.980	SPX2
	transcript_24648	5.704	5.920	SPX1
	transcript_36102	5.978	6.340	SPX1
	transcript_99245	6.126	3.070	SPX2
	transcript_14839	−4.316	−4.960	PHL5
	transcript_131799	−1.522	−1.922	PHL1

Note: Fold change (FC) = gene expression under low phosphorus conditions/gene expression under normal phosphorus conditions.

PHR1 and *PHL* are important transcription factors involved in phosphate starvation response. Under low P stress, we found no DEG related to *PHR1* between the two varieties; notably, only two *PHL* genes were found in this transcriptome analysis. *PHL5* (transcript_14839) and *PHL1* (transcript_131799) were downregulated in the roots of both varieties; however, their downregulation was slightly higher in the roots of Xu Shao 3 than in the roots of Soviet *Vicia villosa* (Table 4).

4. Discussion

Low P stress can significantly reduce plant height and biomass and increase anthocyanin accumulation. There was a 13.24 and 16.85% decrease in height and root length of *Medicago sativa*, respectively, after 12 days of P deficiency [35]. The average plant height and biomass among 8 *Stylosanthes* varieties showed a downward trend under low soil P conditions [36]. Meanwhile, the growth of low P-tolerant varieties is less affected due to their ability to efficiently absorb and use P under low P conditions. The shoot biomass of both low-P-tolerant and low-P-sensitive rice varieties was reduced under the low P treatment, but the reduction in low-P-tolerant varieties was much less than that in P-sensitive varieties [37]. Our study showed that under low P stress, the plant height and shoot dry mass of Soviet *Vicia villosa* decreased by 26.01 and 48.53%, respectively, while the plant height and shoot biomass of Xu Shao 3 decreased by 24.1 and 34.34%, respectively. Meanwhile, the root acid phosphatase activity of Xu Shao 3 was significantly higher than that of Soviet *Vicia villosa*. This indicated that the growth inhibition effect of low P stress on Xu Shao 3 was comparatively lower than that on Soviet *Vicia villosa* (Table 1). It is generally believed that anthocyanin content increases in plants under low P stress, and the low-P-tolerant varieties have less accumulation of anthocyanins compared with P-sensitive varieties. Under low

P conditions, the anthocyanin content of low-P-tolerant variety CCM454 of *Zea mays* was significantly lower than that in low-phosphorus sensitive variety 31778 of *Zea mays* [38]. The anthocyanin content of the low-P-tolerant variety Xin Zi 1 of *Astragalus sinicus* was not affected by low P stress, while the same was significantly increased in P-sensitive varieties Yu Jiang macro-leaf of *Astragalus sinicus* under low P conditions [39]. It was found that anthocyanin accumulation in shoots of Soviet *Vicia villosa* was significantly higher than that in Xu Shao 3 under low P conditions, indicating the better low-P-tolerance of Xu Shao 3 compared to Soviet *Vicia villosa*.

Most of the low P response genes are mainly involved in ion transport, transcriptional regulation, active oxygen scavenging, hormone signal transduction, and P and sugar metabolisms [21–25]. Transcriptome analysis of *Oryza sativa* under low and normal P conditions showed that P nutrition affects diverse metabolic pathways mostly related to glucose, pyruvate, sucrose, starch, and chlorophyll [40]. In this study, GO analysis showed that the upregulated DEGs in Xu Shao 3 were enriched in phosphate hunger response, phosphatase activity, ribosome, and translation processes in cells (Figure 3), indicating that Xu Shao 3 responds to low P stress through phosphatase secretion and protein synthesis while Soviet *Vicia villosa* failed to do so. The GO transcriptome analysis of *Arachis hypogaea* under P deficiency showed that genes related to metabolism categories included cell response to phosphate starvation, peroxidase activity, and plant-type secondary cell wall biogenesis [41], which is partially consistent with our results in response to phosphate starvation. *Glycine max* varieties with high-P efficiency have a strong net photosynthetic rate and photosynthetic carbon assimilation ability and are less affected by low P treatment [42]. In this study, the significantly upregulated DEGs in shoots of Xu Shao 3 were mainly involved in the metabolism of vitamin B6 and riboflavin (Figure 4a). Vitamin B6 contains pyridyl phosphate, which participates in the synthesis of chlorophyll and starch and improves plant resistance to stress [43,44]. Meanwhile, the total biomass (shoot and root dry mass) of Xu Shao 3 was significantly higher than that of Soviet *Vicia villosa* (Table 1). Those results indicated that Xu Shao 3 has a stronger photosynthesis ability and could synthesize more starch and accumulate more dry matter under low P conditions, which partly explains the phenotypic characteristics of Xu Shao 3 with larger biomass under low P conditions. Under low P stress, plant root elongation requires more carbon sources to meet the demand of root cells for energy and sugar metabolism [45]. KEGG enrichment analysis revealed that compared with the Soviet *Vicia villosa*, DEGs in roots of Xu Shao 3 were mainly involved in the metabolic pathways of the ribosome, plant hormone signal transduction, and spliceosome (Figure 4b), indicating that roots of Xu Shao 3 were able to tolerate low P stress by synthesizing response proteins in ribosomes and promoting signal transduction. The elongation of plant roots requires more allocation of carbon sources to meet the needs for energy and sugar metabolism. Interstingly, KEGG analysis found that DEGs in roots were enriched in fatty acid degradation, citric acid cycle, pentose, and glucuronic acid conversion (Figure 4b), indicating that roots of Xu Shao 3 can manage the required energy and materials for growth and development by strengthening citric acid cycle and fatty acid degradation. In short, the physiological phenotypic differences between the two varieties may be related to their capacity for photosynthesis and energy production through P and sugar metabolism and hormone signal transduction. Xu Shao 3 has a strong photosynthetic, citric acid cycle and sugar metabolism, which provides enough energy and substances for normal growth and development, improves carbohydrate redistribution, and reduces the damage from low P stress.

To cope with low P stress, plants induce the expression of high-affinity phosphate transporters [43,46] and secrete acid phosphatase and organic acids [47] to increase P absorption and recycling. Meanwhile, to reduce the consumption of phosphate and ATP, plants reuse P through inorganic pyrophosphatase [48]. Phosphate transporters are carriers that can directly absorb inorganic phosphate from soil and redistribute it [49]. In this study, we found that three phosphate transporters were significantly upregulated in Xu Shao 3 under low P stress. Among the three transporters, the expression of transcript_157065

in shoots and transcript_102952 in roots of Xu Shao 3 was almost one-fold higher than that in Soviet *Vicia villosa* (Table 3). Six glycerin-3-phosphate transporters in Xu Shao 3 were upregulated under low P stress. Among them, the expression of transcript_131639 was higher in both shoots and roots of Xu Shao 3 than that in Soviet *Vicia villosa*, and the other five transporters were upregulated only in roots; notably, the expression of three transporters (transcript_77913, 93091, and 94957) was higher in Xu Shao 3 than in Soviet *Vicia villosa*, (Table 3). In addition, after 18 days of low P stress, P content in the shoots and roots of Xu Shao 3 was significantly higher than that in Soviet *Vicia villosa* (Figure 1). These results indicated that Xu Shao 3 has better resistance to low P stress and can accumulate more inorganic phosphorus than Soviet *Vicia villosa*. In this study, three genes encoding acid phosphatase and one encoding pyrophosphatase were upregulated in both varieties of *Vicia villosa*, but their expression was higher in Xu Shao 3 than in Soviet *Vicia villosa*. In particular, the expression of purple acid phosphatase, transcript_114615, in the roots of Xu Shao 3 was almost one-fold higher than that in Soviet *Vicia villosa* (Table 2). Phosphoenolpyruvate carboxylase catalyzes phosphoenolpyruvate to produce oxaloacetic acid and inorganic phosphate. Under P deficiency conditions, the expression of the gene encoding phosphoenolpyruvate carboxylase becomes upregulated and thereby promotes the synthesis of organic acids [50]. The genes encoding phosphatase (transcript_126553) and phosphoenolpyruvate carboxylase (transcript_30883) were upregulated in shoots of Xu Shao 3 but were downregulated in shoots of the Soviet *Vicia villosa* (Table 2). These results suggested that phosphate transporters, glycerol-3-phosphate transporter, purple acid phosphatase, pyrophosphatase, and phosphoenolpyruvate carboxylase, played an important role in Xu Shao 3 (low P-tolerant variety) adaptation to low P stress.

The SPX gene family plays a critical role in plant development and growth as well as in response to P stress. Transcriptome analysis of *Arabidopsis thaliana* showed that 558 genes were significantly enriched in leaf senescence, Pi starvation response, and salicylic acid signaling pathway in *AtSPX1* overexpressing *Arabidopsis thaliana* compared to the wild-type, demonstrating the involvement of *AtSPX1* in leaf senescence and Pi starvation response in *Arabidopsis thaliana* [51]. In this study, we found eight differentially expressed SPX domain proteins between the two varieties of *Vicia villosa*. Under low P stress, these eight genes were upregulated in both Soviet *Vicia villosa* and Xu Shao 3. However, among the eight genes, six genes had higher expression in the shoot and four genes in the roots of Xu Shao 3 compared with Soviet *Vicia villosa* (Table 4). Transcriptome and real-time quantitative PCR analysis in *Triticum aestivum* revealed that *TaSPX* genes were significantly upregulated after Pi starvation [52]. The identified members of the *SPX* gene family in this study will provide resources for genetic improvement and promote P use efficiency in plants. Additionally, the phenotypic analysis found that P content in Xu Shao 3 was significantly higher than that in Soviet *Vicia villosa* in low P stress (Figure 1). These results suggested that *SPX* genes may be the key genes responsible for such differences among varieties.

Phosphate starvation response (*PHR*) and PHR-Like (*PHL*) are important transcription factors in P regulatory network. Under low P conditions, the *SPX* gene can regulate the expression of *PHR* genes, and then PHR binds to the P1BS sequence of the downstream functional genes (such as *PHT* and *PHO* family) to initiate the P signaling pathway [53]. The in-depth study of the rice phosphorus signaling pathway identified *OsPHR3* and *OsPHR4* as phosphate starvation response genes whose overexpression increased P content in the transgenic plants along with activation of *PSI* genes [54]. In our study, transcription factors *PHL5* (transcript_14839) and *PHL1* (transcript_131799) were downregulated in the roots of both varieties under low P stress, but their expression was slightly more in Xu Shao 3 than in Soviet *Vicia villosa* (Table 4), indicating that the two genes were negative regulatory transcription factors in Pi starvation response pathways.

5. Conclusions

Under low P conditions, Xu Shao 3 exhibited stronger inorganic P accumulation ability and suffered a lesser effect on growth than Soviet *Vicia villosa*, which might result

from photosynthesis, sugar, and P metabolism differences between the two varieties. Acid phosphatase, phosphoesterase, phosphoenolpyruvate carboxylase, sulfoquinovosyl diacylglycerol synthase, phosphate transporter, glycerol-3-phosphate transporter, and *SPX* are key DEGs that determine the difference in tolerance to low P stress between the two varieties.

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