Transcriptome analysis of low phosphate stress response in the roots of masson pine (Pinus massoniana) seedlings

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Abstract

Background: Masson pine (Pinus massoniana) is primarily present within subtropical and tropical areas in China, and a number of these regions have a severe deficiency in inorganic phosphate (Pi). As a macronutrient, phosphorus plays a crucial role in plant development. Although several studies have studied the responses of masson pine to Pi starvation at the global level using RNA-Seq and comparative proteomic analyses, the detailed features in the roots that primarily respond to low Pi stress have not yet been studied.

Results: This study examined the masson pine of the response of masson pine roots to a deficiency in Pi. Approximately 1,117 unigenes were shown to respond to Pi-deficiency by differential expression when analyzed using RNA-Seq. A total of 819 and 298 of these transcripts were up- and down-regulated, respectively. The results identified several phosphate transporters (PHT1, PHO88), ABC transporters and metal transporters. In particular, the ethylene response factor (ERF) was the most abundant transcription factor. Analyses of these genes, including gene ontology enrichment and the KEGG pathway analysis, indicated that the metabolic processes are the most enriched under abiotic stresses, including Pi-deficiency.

Conclusions: This study provided abundant transcriptomic information to functionally dissect the response of masson pine roots to a deficiency of Pi, which will provide additional aid to elucidate the biological regulatory mechanisms that the pines use to respond to low Pi stress.

Background

The masson pine (*Pinus massoniana)* is a native gymnosperm within the Southern portions of China. This tree has broad economic value for its pulp, resin, and timber production. The distribution area of this tree species is primarily in acid yellow soils, which have a critical lack of inorganic phosphate (Pi) (Zhang et al, 2010). Phosphorus, in the form of Pi, is an essential macronutrient in plants with a critical function in development and metabolic activities (Abel et al, 2002). Although the soil may contain large amounts of phosphorus, the phosphorus that is available for the plant to take up is often limited due to its tendency to complex with other soil constituents and then be slowly released (Shen et al, 2011). Thus, the deficiency of phosphorus in soils may constrain the growth and productivity of the plants that grow in this soil (Vance et al, 2003). Over the past several decades, Pi fertilizer has been widely used for crop and forestry plantations, annually decreasing the non-renewable phosphate rock reserves, which are likely to run out soon (Deng et al, 2018). Furthermore, much of the Pi fertilizer applied can result in marked environmental pollution, including the eutrophication of water systems (Cordell et al, 2009). Therefore, it would be significant to understand the strategies that plants use to absorb and utilize phosphorus and adapt the mechanisms of the plants that are highly efficient at using soil Pi.

Plants have evolved a number of different strategies that enable them to adapt and manage a deficiency of Pi, involving modifications in developmental programs and metabolic networks (Fan et al, 2014).

These adaptive strategies are thought to be mediated synergistically by a series of genes. Extensive research on the response of model plants to Pi starvation that have been conducted over the past several decades have considerably enhanced the knowledge of the phosphorus signaling and response pathway (Hammond et al, 2003; Woo et al, 2012; Gho et al, 2018). A series of genes enable the plant to adapt to a deficiency of Pi, primarily through the regulation of signal transduction, internal remobilization, phosphorus acquisition, and metabolic changes (Fang et al, 2009). Previous studies identified the key role of the PHOSPHATE STARVATION RESPONSE1 (PHR1) gene in transcriptional responses that are related to a deficiency of Pi (Zhao et al, 2018). This research also revealed that the proteins contained in the SYG1/PHO81/XPR1 (SPX) domain are vital for the plant to sense Pi and maintain homeostasis (Zhang et al, 2014; Wild et al, 2016; Zhao et al, 2018). The expression of the genes that encode the purple acid phosphatases (PAPs) and Pi transporters wer found to be up-regulated under conditions in which Pi is deficient (Duff et al, 1994; Poirier and Jung, 2018). In addition, a number of genes involved in the metabolism of lipids, including UDP sulfoquinovose synthases and nonspecific phospholipase, were induced under low-Pi stress (Lan et al, 2012).

Masson pine seedlings under low-Pi stress were the source of transcriptomes, which were analyzed using a combination of 8×60 K DNA microarrays and RNA-Seq (Fan et al, 2014). The results indicated a strong representation of genes and transcription factors that were associated with stress. In addition, genes whose functions were unknown were subjected to up- and down-regulation. The results suggested that Pi-deficiency stress involves the cumulative implication of a number of genes and multiple metabolic pathways. Later, Fan et al (2016) compared proteomes using seedlings of an elite line of masson pine and identified 98 proteins that were differentially expressed. These proteins that responded to Pi-starvation were involved in defense, photosynthesis, biosynthesis, cellular organization, secondary metabolism, energy metabolism, and signal transduction. Although these studies identified both global transcriptomic and proteomic responses to Pi-deficiency in masson pine, the roots that that exhibit primary responses to low-Pi stress have been studied less intensively. Therefore, functional analyses of candidate genes are merited. In this study, a transcriptomic profile of masson pine root responses to a deficiency of Pi was demonstrated and shows how the responsive genes for phosphate starvation bolster the efficiency of the acquisition, recycling, and remobilization of phosphorus in roots.

Results

Transcriptome dataset of masson pine roots

Six libraries were generated to reveal the gene expression profiles of the masson pine roots subjected the stress of Pi-deficiency. One was based on three Pi-sufficient biological replicates (CK36-R–1, CK36-R–2, CK36-R–3) and three Pi-deficient (P36-R–1, P36-R–2, P36-R–3) treatments. These libraries were used for further transcriptome sequencing analysis. After filtering the adaptor sequences and those that had a low base quality, 85.19, 83.72, 83.77, 77.07, 100.56 and 71.67 million high-quality paired end reads were generated, respectively (Table 1). The averages of Q20 and Q30 were more than 98.5% and 96.0%, respectively. A total of 95,658 unigenes were obtained, with an average length of 748 bp, and an N50

length of 1104 bp. The unigenes ranged from 201 to 12,246 bp in length. More specifically, the number of genes within the size ranges of 201–500, 501–1,000, 1,001–2,000, 2,001–3,000 bp and >3,000 bp were 50,653, 22,918, 15,080, 4,848 and 2,159 unigenes, respectively. These results indicated that the experiment was successful in producing high quality transcriptome data.

The resultant unigenes were queried in the NR, KOG, SwissProt, and KEGG databases according to the sequence similarity analysis. Over 45,000 unigenes were identified in the plants, and the NR database provided the largest number of annotations, with a value of 43,168 (45.72%).

Table1 Summary of the sequencing data quality of the masson pine roots

Sample	Raw reads Num.	Clean reads Num. (%)	Clean bases (bp)	Q20(%)	Q30(%)	GC content (%)
CK36-R-1	86366152	85188372 (98.64%)	12607545166	98.61%	96.40%	44.88%
CK36-R-2	84887286	83722708 (98.63%)	12397443754	98.63%	96.41%	44.77%
CK36-R-3	85046146	83770456 (98.5%)	12413404013	98.52%	96.15%	45.38%
P36-R-1	78177848	77070524 (98.58%)	11420947488	98.57%	96.31%	45.21%
P36-R-2	102130242	100556172 (98.46%)	14883863178	98.51%	96.18%	45.19%
P36-R-3	72818704	71666234 (98.42%)	10594449288	98.09%	95.19%	45.07%

DEGs in response to a low Pi treatment

DE-Seq with the FDR set to less than 0.05 enabled differential expression analysis to be conducted using the raw data obtained from the Illumina sequencing. A total of 1,117 unigenes responded to the treatment of Pi-deficiency and were significantly differentially expressed. A total of 819 and 298 of these transcripts were up- and down-regulated (Fig 1, Table S2), respectively. Previous research suggested that the DEGs related to phosphorus metabolism can be divided into six categories, including the genes that are involved in transcription, lipid metabolism, transport, phosphorylation/dephosphorylation, metabolism, and miscellaneous (Lan et al. 2015). In this study, several kinds of putative transcription factors (TFs) have been shown to be associated with low-Pi tolerance. Of these, the ethylene response factor (ERF) comprised the most abundant TFs that responded to low-Pi stress. Beyond that,theMYB family TF (Unigene0011607), WRKY TF (Unigene0042859), and bZIP TFs (Unigene0042142 and Unigene0054957) were significantly expressed under the low-Pi condition. These identified TFs were up-regulated with the exception of one bZIP TF (Unigene0042142).

From the transport overview, phosphate transporter genes, including *PHT1* (Unigene0072509) and *PHO* (Unigene0093941), were dramatically up-regulated in Pi-limited roots. ATP-binding cassette (ABC)

transporters (Unigene0067284 and Unigene0073352) were strongly induced. Other transporter subfamily genes, including organic cation, potassium, metal, and sugar transporters, were also identified as being highly responsive to the low-Pi stress in the masson pine roots. In terms of lipid metabolism, some genes that may degrade the phospholipid were induced under low-Pi stress, such as short-chain dehydrogenase/reductase (Unigene0002082), glycerol kinase (Unigene0008107 and Unigene0020667), and lipoxygenase (Unigene0065294). One iron-superoxide dismutase gene (Unigene0040690) was dramatically down-regulated. Many of the genes that were identified as being up-regulated in response to phosphorus starvation were involved in metabolic processes and included pyruvate phosphate dikinase (Unigene0009113 and Unigene0065735), thioredoxin (Unigene0006520), cytochrome P450 (Unigene0008678), phosphoenolpyruvate carboxykinase (Unigene0036016), and phosphoglucomutase (Unigene0030703). In the class of phosphorylation/dephosphorylation, the DEGS included a subset of 26 kinases and one acid phosphatase (Table S2).

Previous studies indicated that abiotic stress conditions, including Pi-deficiency, can generate reactive oxygen species (ROS) (Fan et al, 2014). We identified some genes that are involved in scavenging ROS that were up-regulated, including glutathione peroxidase (Unigene0017511).

To validate the results of the transcriptome sequencing, 10 differentially expressed unigenes were chosen to be subjected to qRT-PCR analysis (Fig. 2). The results showed that the expression of all the unigenes selected was consistent between the transcriptomic and the qRT-PCR analyses. This consistency confirmed that the transcriptome data was highly reliable.

GO enrichment analysis of the DEGs

To obtain a more precise understanding of the DEG function in Pi-limited roots, GO term enrichment analysis was conducted, and the all up- and down-regulated transcripts were assessed based upon cellular component, molecular functions as well as biological processes (Fig. 3). For the biological process ontology, the dominant terms in this study included “metabolic process”, “cellular process”, and “single-organism process”, followed by “response to stimulus”. The dominant terms for the cellular component were “cell”, “cell part”, “macromolecular complex”, “organelle”, and “organelle part”. The processes represented by the “binding”, “catalytic activity”, and “structural molecule activity” GO terms accounted for the majority of the molecular function, followed by “transporter activity”.

KEGG enrichment analysis

To illustrate the function of the DEGs identified using RNA-Seq, a KEGG enrichment analysis was conducted. The results showed that 267 of the 1,117 DEGs could be assigned to 72 KEGG pathways (Table S3). Among them, four pathways (metabolic pathways, secondary metabolite biosynthesis, ribosome, and microbial metabolism in diverse environments) were the most abundant in the Pi-deficient roots. Furthermore, the KEGG terms that were over-represented (*Q<0.05)* were classified as the “ribosome”

(Fig. 4). However, as shown in Fig. 4, the degradation of aromatic compounds, carbon fixation in photosynthetic organisms, and glycolysis/gluconeogenesis pathways were over-represented among the DEGs, suggesting that these pathways could be involved in the response to low-Pi. Other pathways that were closely linked to phosphorus were further detected, such as oxidative phosphorylation, pyruvate metabolism, pentose phosphate pathway, phosphatidylinositol signaling system, citrate cycle, and plant hormone signal transduction.

Discussion

As a non-renewable nature resource, phosphate rock is being rapidly depleted because of the world's reliance on Pi fertilizers. Given this reason, a substantial amount of studies based on microarrays and RNA-Seq methods have documented certain crop species and various model plant responses under conditions in which P is deficient (Thibaud et al, 2010; O'Rourke et al. 2013; Gho et al, 2018). Previous research has reported global transcriptome responses to Pi-deficiency in masson pine seedlings. However, candidate root genes exhibiting primary responses to Pi-deficiency have been subjected to relatively few functional analyses. This study used RNA-Seq to examine whole-transcriptomic responses to low-Pi stress in masson pine roots. This study identified 1,117 DEGs that responded to Pi-deficiency conditions, of which 10 were chosen at random for qRT-PCR validation (Fig. 2). The results showed that qRT-PCR-based expression changes generally agreed with those of the RNA-Seq, and confirming that identified DEGs were reliable.

An examination of all the enriched GO terms of biological process, "metabolic process", particularly terpenoid biosynthetic processes, was the term that was induced the most significantly in masson pine roots deficient in Pi. A previous study showed that diterpene phytoalexins and allelochemicals increased in plant roots as a response to various stress conditions (Zhao et al, 2005; Kong et al, 2006). In *Arabidopsis* and rice roots, strigolactone resulting from terpenoid biosynthetic processes accumulated under Pi starvation and is known to suppress the growth of shoots (Gomez- Roldan et al, 2008) and to enhance the development of roots (Arite et al, 2012). These results suggested that secondary metabolites in masson pine may play an essential role in enhancing the rate of survival in conditions of low Pi. Furthermore, the KEGG pathway analysis showed that the ribosome, the degradation of aromatic compounds, carbon fixation in photosynthetic organisms, and the glycolysis/gluconeogenesis pathways were over-represented among the DEGs, suggesting that these pathways may be involved in the response to low Pi, and these annotations provide a valuable source to investigate the specific processes by examining the functions of the DEGs.

Previous studies suggested that the plant transcription factors were essential for the regulation of the downstream genes under low-Pi conditions. Fan et al (2014) demonstrated that the MYB TF family represented the largest class of TFs that was induced by a deficiency of Pi in masson pine seedlings. As a MYB TF, PHR1 was previously identified for its involvement in low-Pi stress that activates a series of genes, including those that contained SPX domains that were activated by their binding to promoter regions (Nilsson et al, 2007; Secco et al, 2012). In this study, only one putative MYB TF was dramatically

induced under Pi-deficiency in roots. Twelve years ago, Hernández et al. (2007) identified 4 TFs up-regulated in Pi-deficient common bean roots, and three of them were members of the MYB family. Later, increasing numbers of plant MYBs were identified as regulators involved in Pi starvation. Examples include AtMYB2 that regulates the response of the plant to a deficiency in Pi by controlling miR399 expression in *Arabidopsis* (Baek et al, 2013). As is well known, several morphological processes can be affected under Pi-starvation conditions, including a change in the root system architecture (RSA). Ethylene had previously been shown to be involved in remodeling the RSA, and it participates in other types of plant responses to a deficiency in Pi (Song and Liu, 2015). As a group of AP_2 ($APETALA_2$) domain-containing transcription factors, ERFs serve as either repressors or activators of ethylene-mediated transcription. Chen et al (Chen et al, 2018) showed that the down-regulation of the *JcERF035* gene contributed to the regulation of RSA and both the biosynthesis and accumulation of anthocyanins in aerial tissues of plants under Pi-starvation conditions. Some transcriptomic analyses have shown marked gene expression changes in plant roots (Thibaud et al, 2010; Kang et al, 2014; Zhao et al, 2018). This study identified ERFs as the most abundant up-regulated TFs responding to low-Pi stress. Whether these confer the tolerance of masson pine to Pi-deficiency merits investigation.

Previous studies showed that the uptake, transport and translocation of phosphorus in plants are generally performed by phosphorus transporters (PHT1 and PHO1) (Nussaume et al, 2011; Hamburger et al, 2002). Most transporters that are in the PHT1 group are known to be differentially expressed in the roots of *Zea mays,* rice and *Brachypodium distachyon* (Nagy et al, 2006; Gho et al, 2018; Zhao et al, 2018). In this study, only one high affinity phosphate transporter gene (*PHT1)* was up-regulated (Table S2), and one putative *PHO88* rather than *PHO1* was differentially expressed in masson pine roots. It has been suggested that PHO88 plays a role in the phosphate transport pathway via the regulation or maturation of secretory proteins in yeast (Hurto et al, 2007). As a putative membrane protein, it was rarely reported in plant roots that were deficient in Pi, and additional genetic analyses will be necessary to determine the role of PHO88 in the phosphorus homeostasis in plant roots. In addition to phosphate transporters, some other transporter families were significantly changed under Pi-deficiency. ABC transporters, which are known to be induced under various abiotic stress conditions (Huang et al, 2010; Xi et al, 2012), were dramatically induced in this study. A norganic cation/carnitine transporter was also up-regulated, suggesting that it might be involved in the response to a Pi-deficiency in plants, as was previously documented in soybean roots (Zeng et al. 2016). Under Pi-deficiency, plants over-accumulated some metallic cations, including Fe^{3+}, K^{+} or Al^{3+} (Hirsch et al, 2006; Abel, 2017). In this study, one gene that encoded a metal transporter (Unigene0067271) was also up-regulated in the roots.

The lipid composition of the plant membranes may be dramatically altered under Pi-starvation conditions, with a decrease in phospholipids and an increase in lipids that do not contain phosphorus (Russo et al, 2007). Generally the phospholipids of the plasma membrane and tonoplast are replaced by galactolipid digalactosyldiacylglycerol or glycolipids (Mehra et al, 2019; Tawaraya et al, 2018). In this study, some genes that may degrade the phospholipid were induced under low-Pi stress, including a short-chain dehydrogenase/reductase, glycerol kinase and lipoxygenase. The induction of these genes

suggested that the membrane lipid composition might change when subjected to the stress of low levels of Pi.

Conclusion

Herein we produced a whole transcriptomic profile of the roots of the masson pine under conditions of Pi-deficiency, allowing for detection of phosphate transporter genes, ERF TFs, and genes linked with lipid remodeling among 1,117 DEGs. The results of this study revealed some different DEGs and enriched pathways from previous research in seedlings (Fan et al, 2014), and provided new perceptions on the responses of masson pine to a deficiency of Pi.

Methods

Materials and treatment

Masson pine seeds were collected from an elite tree, which confers a tolerance to Pi-deficiency in our previous study, in the Duyun Forest Farm in the Guizhou Province in China. The seeds were disinfected with a 5-minute treatment of 5% sodium hypochlorite followed by a 10-minute 70% ethanol treatment. Next, the seeds were soaked in room temperature water. The germinated seeds were planted in plastic pots and grown at a day/night temperature of 28ºC/18ºC (14 h day/10 h night) in a plant growth chamber with 70% relative humidity and a light intensity of 2000 lux. At 15 days after emergence, the seedlings were treated with a modified Hoagland nutrient solution (Fan et al, 2014) that was deficient in Pi (0.01 mM KH_2PO_4) and sufficient in Pi (0.5 mM KH_2PO_4) as the treatment and control, respectively. The experiment utilized six seedlings per treatment that were replicated three times. The nutrient solution was added at three-day intervals. The seedling roots were harvested 36 days after treatment, snap frozen with liquid nitrogen followed by storage at –80ºC.

RNA isolation, cDNA library construction and sequencing

Total root RNA was isolated with the Invitrogen Plant RNA Isolation Kit based on provided directions. RNA was assessed for quality and was quantified through a combination of agarose gel electrophoresis together with a Agilent 2100 Bioanalyzer (Agilent Technologies, CA, USA). mRNA was specifically isolated from the extracted RNA through the use of Oligo (dT) magnetic beads, followed by fragmentation and cDNA generation. The short fragments were connected using adapters following their purification, and agarose gel electrophoresis was performed. Fragments of 200 bp in length were isolated for PCR amplification followed by sequencing on the Hi-Seq 2000 platform (Illumina, San Diego, CA, USA). The software for the platform was used to conduct the primary analysis of the data and the base calling.

Transcriptome assembly and assessment of the expression

To generate a set of transcripts that were non-redundant, adaptor sequences and empty reads were removed, and high-quality reads were selected using Q20 and Q30 as the basis. Trinity software was used for clean read assembly after filtering to construct the unigenes. The levels of expression for each unique transcript were calculated by quantifying the reads based on the RPKM approach (reads per kilobase per million reads) (Mortazavi et al, 2008). DEG-Seq was used to judge the differentially expressed genes (DEGs) with an FDR (false discovery rate) < 0.05 and the absolute value of log2 RPKM ratio ≥1 as the threshold (Anders and Huber, 2010).

Gene Enrichment Analyses

The GO and KEGG analyses were conducted with the Blast2GO program. Biological process, molecular function, and cellular component were graphed with the second level of the GO hierarchy. The adjusted standard of significantly substantiated GO terms and enriched pathway was a *p*-value set to less than 0.05.

Validation of transcriptome sequencing using quantitative real-time PCR (qRT-PCR)

Ten DEGs were randomly selected and quantitatively analyzed using qRT-PCR to confirm RNA sequencing expression profiles Primers were designed using Primer Premier 5.0 (Table S1). SYBR Select Master Mix Kit (Applied Biosystems, CA, USA) was used for triplicate qRT-PCR reactions with a 7500 Fast Real-Time PCR System (Applied Biosystems) based on provided directions. The cycle threshold (Ct) was utilized to assess relative transcript levels for each gene, which were normalized using *18s-rRNA* and *UBQ* as internal controls (Fan et al, 2014).

List Of Abbreviations

Pi: inorganic phosphate; ERF: ethylene response factor; PHR1: phosphate starvation response1; SPX: SYG1/PHO81/XPR1; PAPs: purple acid phosphatases; DEGs: differentially expressed genes; FDR: false discovery rate; ABC: ATP-binding cassette; ROS: reactive oxygen species; AP_2: apetala$_2$

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

The datasets supporting the conclusions of this article is included within its additional files.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

PX performed the experiments, and was a major contributor in writing the manuscript. HH analyzed the datas of this article and helped writing the manuscript. All authors read and approved the final manuscript.

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Figures

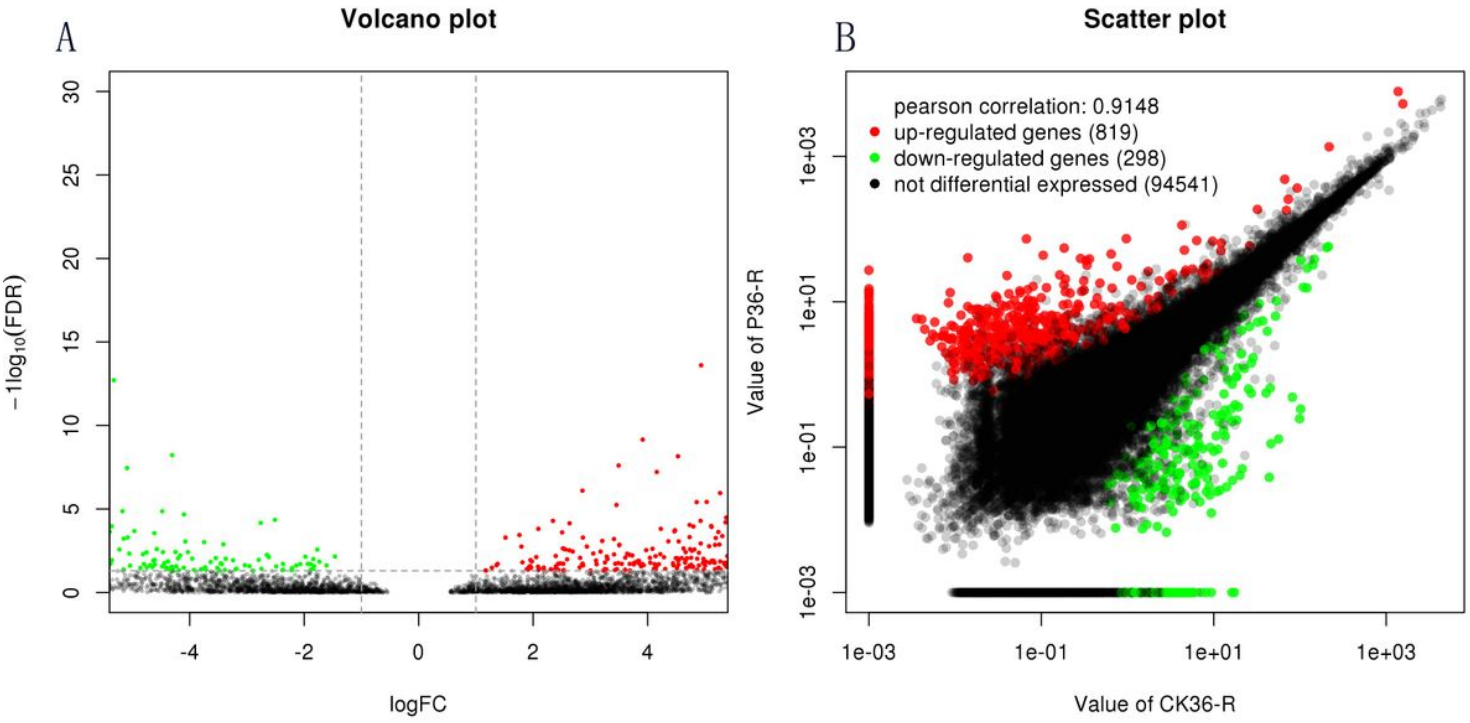

Figure 1

Differentially expressed genes under low-Pi stress. A: volcano plot represents fold change versus false discovery rate. The horizontal dashed line indicates a cutoff of statistical significance at 0.05. B: scatter plot x-axis displays the average signal intensity for the control sample, while the y-axis displays the average signal intensity for the treatment sample.

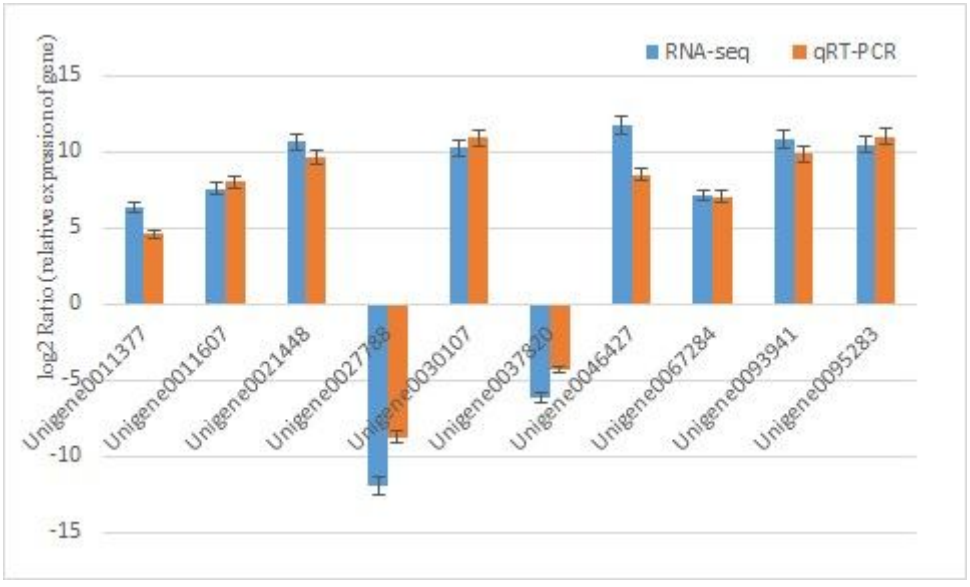

Figure 2

qRT-PCR and RNA-seq expression profiles for 10 randomly selected unigenes.

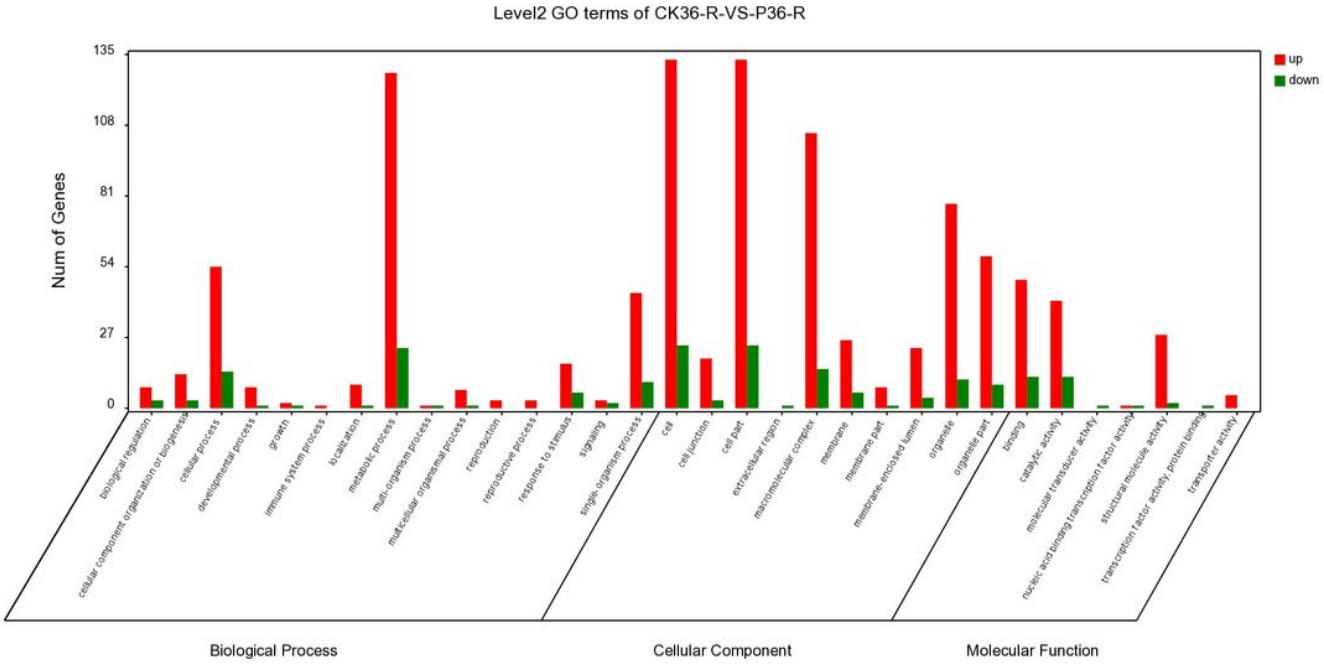

Figure 3

GO classification of the unigenes associated with phosphate deficiency in the masson pine roots.

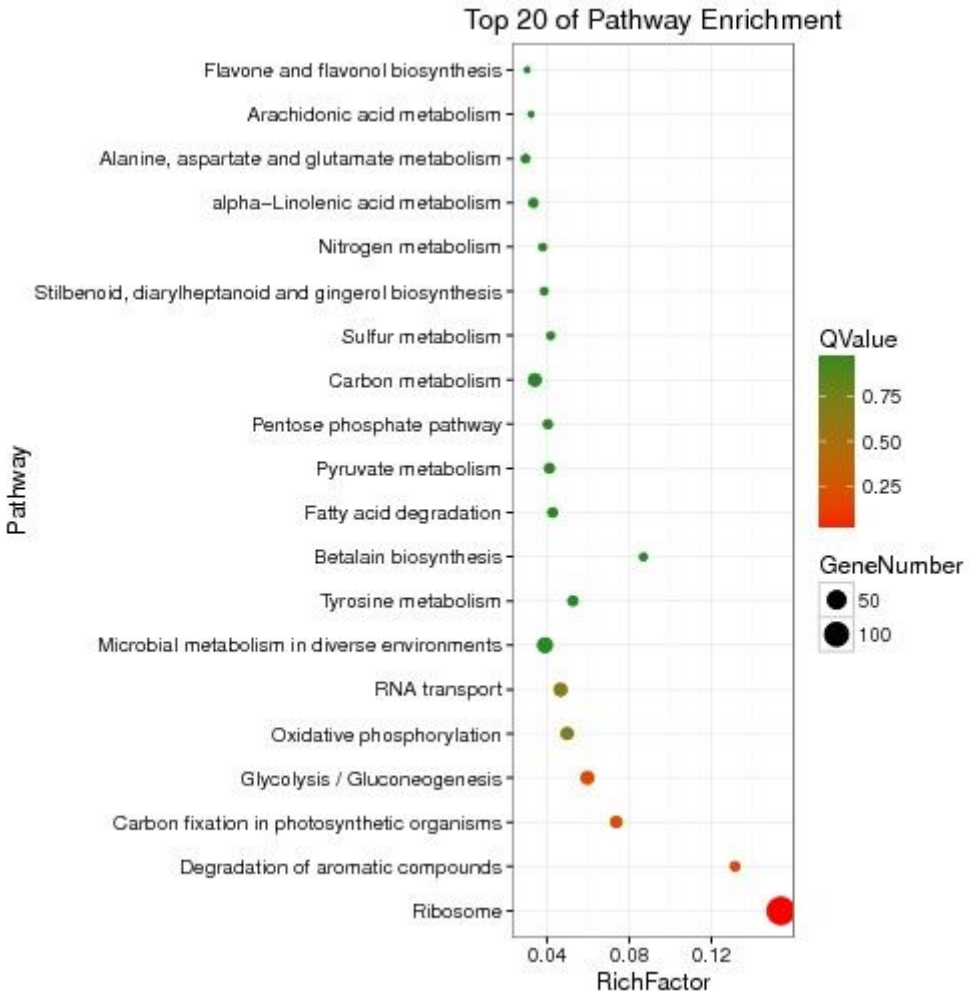

Figure 4

KEGG enrichment analysis diagram of DEGs. Q values correspond to p-values corrected for multiple hypothesis testing for KEGG analyses, with values from 0 – 1, with 0 indicting more significant enrichment

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- TableS3CK36RVSP36R.path.xls
- TableS2differentialexpressiongenes.xlsx
- TableS1qRTPCRprimers.xlsx