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Research article

Comparative transcript profiling of maize inbreds in response to long-term phosphorus deficiency stress

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ABSTRACT

Maize (*Zea mays* L.) is an important food and energy crop, and low phosphate (Pi) availability is one of the major constraints in maize production worldwide. Plants adapt suitably to acclimate to low Pi stress. However, the underlying molecular mechanism of Pi deficiency response is still unclear. In this study, comparative transcriptomic analyses were conducted to investigate the differences of transcriptional responses in two maize genotypes with different tolerances to low phosphorus (LP) stress. LP-tolerant genotype QXN233 maintained higher P and Pi levels in shoots than LP-sensitive genotype QXH0121 suffering from Pi deficiency at seedling stage. Moreover, the transcriptomic analysis identified a total of 1391 Pi-responsive genes differentially expressed between QXN233 and QXH0121 under LP stress. Among these genes, 468 (321 up- and 147 down-regulated) were identified in leaves, and 923 (626 up- and 297 down-regulated) were identified in roots. These Pi-responsive genes were involved in various metabolic pathways, the biosynthesis of secondary metabolites, ion transport, phytohormone regulation, and other adverse stress responses. Consistent with the differential tolerance to LP stress, five maize inorganic Pi transporter genes were more highly up-regulated in QXN233 than in QXH0121. Results provide important information to further study the changes in global gene expression between LP-tolerant and LP-sensitive maize genotypes and to understand the molecular mechanisms underlying maize's long-term response to Pi deficiency.

1. Introduction

Phosphorus (P) is an essential macronutrient for plant growth and development, as well as a structural element of nucleic acids and phospholipids; it plays a crucial role in organic compound biosynthesis, photosynthesis and energy metabolism, gene expression or signal transduction, and regulation of enzyme reactions (Yang and Finnegan, 2010). Phosphate (Pi) is primarily taken up in its inorganic form as HPO_4^{2-} or $H_2PO_4^-$ ions, which are present in soil solution at extremely low concentrations, that is, generally <10 mM and typically around 2 μM; in plants, concentrations of over 40 mM can be achieved (Bollons and Barraclough, 1997). Thus, P acquisition is a crucial factor for the plant growth and development, and its availability is often a major constraint for agricultural productivity and crop yield.

Under Pi deficiency conditions, plants have evolved multiple morphological adaptations, such as adventitious rooting, aerenchyma formation, basal root elongation, basal root growth angle, lateral rooting, root hair density and root hair length (Ma et al., 2001; Miller et al., 2003). For example, *Medicago truncatula* increases the number and length of root hairs in response to Pi deficiency; additionally, plants can establish mycorrhizal associations or form cluster roots so as to acquire a large amount of Pi from soil for plant growth (Bucher, 2007). Plant roots commonly exude organic acids and enzymes, including ribonucleases (RNases),

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nucleases, phosphodiesterases, and acid phosphatases (APases), to facilitate enhanced Pi acquisition and utilization (Gaume et al., 2001; Nilsson et al., 2010). Under low phosphorus (LP) stress, tolerant maize inbred lines secrete more organic acids and APases in roots than those of the sensitive ones (Gaume et al., 2001). Similarly, *Apase* gene is strongly induced due to Pi deficiency in *Arabidopsis* (Haran et al., 2000). Furthermore, the genes encoding purple acid phosphatase (PAP) transcripts, namely, *PAP11* and *PAP12*, are identified to be up-regulated in white lupin (Wasaki et al., 2003).

Plants possess both low and high affinity Pi transport systems to acquire Pi in demand. Previously, four Pi transporters, i.e., *PHT1-PHT4*, which are located in different cellular parts, uptake Pi in *Arabidopsis* (Lin et al., 2009). The *Arabidopsis* genome exhibits nine *PHT1* members, and the expression of *AtPHT1;1* and *AtPHT1;2* increases considerably because of Pi deprivation (Mudge et al., 2002). In addition, the orthologous genes of *PHT1;1*, which is also induced under LP stress, are observed in tomato (Daram et al., 1998), barley (Smith et al., 1999), and *Lupinus albus* (Liu et al., 2001). In *Arabidopsis*, the Pi starvation response 1 (PHR1), as a myeloblast (MYB) transcription factor (TF), binds a *cis*-element 'GNATATNC' (PHR1-specific binding sequence, P1BS) in the promoter regions of other Pi-responsive genes, such as Pi transporters, protein kinases, RNases, phosphatases and metabolic enzymes (Li et al., 2009; Rubio et al., 2001). Similarly, the *PHR1* and *PHR2* genes are involved in response to Pi deficiency in *Oryza sativa* (Zhou et al., 2008). Several other TFs in plants, including MYB62, basic helix-loop-helix (bHLH32), zinc finger of *Arabidopsis* 6 and WRKY DNA-binding protein 75, also respond to Pi deficiency (Zhang et al., 2014). In addition, microRNAs are involved in Pi deficiency, and consequently regulate Pi homeostasis in plants (Chiou et al., 2006).

In recent years, several transcriptomic analyses of the response to Pi deficiency have been performed in maize (Calderon-Vazquez et al., 2008; Lin et al., 2013; Pei et al., 2013) and other species including *Arabidopsis* (Morcuende et al., 2007), rice (Wasaki et al., 2006), and wheat (Oono et al., 2013). The Pi-responsive genes are mainly involved in metabolic processes, ion transport, transcriptional regulation, reactive oxygen production and scavenging, protein synthesis and degradation, and hormone signal transduction. In maize, the expression patterns of maize inorganic Pi transporters, APases, phytase, 2-deoxymugineic acid synthase 1, peroxidase (POD), and MYB TF were validated in LP-tolerant inbred line 178 root response to LP stress (Calderon-Vazquez et al., 2008). Furthermore, the genome-scale transcriptome analysis of the Pi utilization efficient hybrid line L3 × 228-3 was performed, and the results indicated that gene expression profiles are correlated with changes in specific metabolites (Lin et al., 2013). Subsequently, a comparative metabolite profiles of two different LP-tolerant maize genotypes revealed that di- and trisaccharides and metabolites of ammonium metabolism accumulate obviously in leaves; by contrast, Pi-containing metabolites and organic acids decrease under LP condition (Ganie et al., 2015). A large-scale evaluation of diverse maize germplasm resources was carried out to identify and select maize materials with increased tolerance to LP stress (Zhang et al., 2015a). Although studies of the comparative transcriptome and metabonomics have achieved significant progress in exploring the maize response to Pi deficiency, the Pi regulatory mechanisms are highly complex and still unclear currently.

Recently, two contrasting LP-tolerant maize inbred lines, namely, QXN233 (LP-tolerant) and QXH0121 (LP-sensitive), were developed in our laboratory. The LP response of the two contrasting maize inbreds was compared, and the comparative RNA sequencing (RNA-Seq) analysis was further performed in these contrasting maize genotypes under sufficient phosphate or low phosphate conditions. This study focuses on gene expression in leaves and roots of these contrasting maize genotypes, importantly, identifying and analyzing some differentially expressed Pi-responsive genes, which may be key factors affecting their different capacities of LP tolerance. This study will help to further enrich our understanding of the response processes to LP stress in maize, and lay the foundation for elucidating the molecular mechanisms of maize tolerance to LP stress.

2. Materials and methods

2.1. Plant growth and treatments

The two genotypes, namely, maize inbred lines QXN233 and QXH0121, were derived from Reid inbred lines and Huanggai inbred lines, respectively. They were reserved at Maize Research Institute, Shandong Academy of Agricultural Science, and employed in this study. The seeds of these two inbred lines were surface-sterilized for 20 min with 10% sodium hypochlorite solution, rinsed with sterilized distilled water for five times and kept for three days over sterile wet filter paper at 30 °C and then planted in quartz sand for seven days, with individual pots full of 0.5 L of Hoagland's nutrient solution. The composition of the nutrient solution was as follows: 0.5 mM KH_2PO_4 (normal condition), 2 mM $Ca(NO_3)_2$, 0.65 mM $MgSO_4$, 25 μM Fe-EDTA, 5 μM $MnSO_4$, 50 μM KCl, 2 μM $ZnSO_4$, 0.5 μM $CuSO_4$, 0.005 μM $(NH_4)_6Mo_7O_{24}$, and 25 mM H_3BO_4. The solution was given every two days. When the seedlings had three full-grown leaves, they were treated by supplementing the low Pi solution (P1, 1 μM KH_2PO_4, LP stress), the optimum Pi solution (P2, 500 μM KH_2PO_4, Control) and the high Pi solution (P3, 1000 μM KH_2PO_4, sufficient Pi condition). The low Pi, optimum and high Pi solutions were applied every two days for each pot. The stressed and control seedlings were harvested for the analysis of physiological parameters after Pi starvation for twenty-fifth days, and 6–15 plants per replicate of each treatment group were harvested. Fresh harvested roots and leaves were rinsed with sterilized distilled water, frozen in liquid nitrogen, and stored at −80 °C for further gene expression analysis.

2.2. Anthocyanin measurement

The anthocyanin contents of whole maize seedlings were determined as described previously (Calderon-Vazquez et al., 2008). Leaves were harvested and frozen from both P1 and P2 conditions at 25 d after the onset of stress. Frozen samples were ground using an ice-cold mortar and pestle. Subsequently, the ground powder was then incubated in acidified methanolic extracts (5% HCl) for 4 °C overnight. After centrifugation at 12,000 rpm for 2 min, the absorbance (A) of the supernatant extract was measured at 530 and 657 nm, and the concentration of anthocyanin was calculated using the formula $A530-0.25A657$.

2.3. Growth parameter measurements

A total of 6–15 plants were randomly selected from each treatment group. The entire roots were spread appropriately, and scanned with an EPSON Transparency unit (EPSON, Beijing, China), and analyzed with WinRHIZO software version 5.0 (Regent Instruments, Inc., Canada) to calculate the total root length, total root surface area, total root volume, and total root tips.

2.4. Total P and Pi measurements

Fresh maize roots and shoots of samples were frozen and ground using an ice-cold mortar and pestle. The ground powder of roots or shoots was then incubated in 2% (v/v) glacial acetic acid at

42 °C for 30 min. After centrifugation at 12,000 rpm for 2 min, the inorganic Pi content of the supernatant was measured using the ammonium molybdate (Mo)–antimony potassium tartrate (Sb)–ascorbic acid (Vc) method as follows: 0.4% (w/v) Mo melted in 0.5 M H_2SO_4 was mixed with 0.05% Sb (solution A), and 10% Vc (solution B) was mixed at the ratio of 6:1. One milliliter of this working solution was added to 1 mL of the sample solution. The mixture was incubated at 37 °C for 1 h and cooled for 5 min at 4 °C. Finally, the absorbance of the mixture was measured at 820 nm. The Pi concentration was calculated by normalization of fresh weight (Nanamori et al., 2004).

Roots and shoots of samples were dried in an oven at 85 °C to a constant weight and subsequently weighed. The total plant P content was analyzed by using the MoSb-Vc method when the plant material was digested with H_2SO_4-H_2O_2 at 300 °C (Zhou et al., 2008). The P concentration was normalized by dry weight (Dw).

2.5. cDNA library construction and sequencing

Library construction and sequencing were performed according to the method described previously (Zhang et al., 2015b). mRNA was isolated from the total RNA samples using oligo (DT) magnetic beads (Invitrogen, CA, USA). Purified mRNA was first fragmented by using the RNA fragmentation kit (Ambion, USA), and a one paired-end library was prepared for each sample according to the manufacturer's instructions. mRNA libraries were individually sequenced for the 16 samples (two from each of the P1 and P2 groups in the leaves of QXN233, two from each of the P1 and P2 groups in the roots of QXN233, two from each of the P1 and P2 groups in the leaves of QXH0121, and two from each of the P1 and P2 groups in the roots of QXH0121) using the Illumina High-seq 2000 sequencing system at the Beijing Genomics Institute (Shenzhen, China).

2.6. RNA-seq data analysis

After the removal of the sequencing adapt and low-complexity reads, all RNA-Seq reads were aligned with the maize B73 RefGen_V2 genomic DNA sequence using the SOAP 2 software (Li et al., 2009) and related to known genes through a BLAST (BLAST 2.2.23) analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Transcript abundance was calculated using the reads per kb per million reads method (Mortazavi et al., 2008). The trimmed mean of M-values (TMM) was used to normalize gene expression levels. All TMM values of the two samples each group were integrated, and NOISeq (version: 2.8.0) was used to detect the differentially expressed genes (DEGs) between two groups (Tarazona et al., 2011). Differentially expressed transcripts were required to achieve a > 2-fold change in expression between samples and the probability (P) thresholds of differential expression $\geq$ 0.8. Higher the probability resulted in the remarkable change in expression between the two groups.

2.7. Functional enrichment analysis of DEGs

Gene Ontology (GO) functional enrichment analysis (http://www.geneontology.org/) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis (http://www.genome.jp/kegg/) were performed for the DEGs. DEG lists were submitted to the NCBI-Nr database for annotation by using Blast2GO software and for functional classification by using WEGO software (Kanehisa et al., 2008). The significantly enriched metabolic pathways or signal transduction pathways in DEGs were identified via pathway enrichment analysis using KEGG (Hooper and Bork, 2005). In all tests, P values were calculated using Benjamini-corrected modified Fisher's exact test and $\leq$0.05 was considered a threshold of significance, with the calculation formula as described previously (Benjamini and Yekutieli, 2001).

2.8. Quantitative real-time PCR (qRT-PCR) of candidate genes

The results from RNA-Seq data analysis were validated by using qRT-PCR. The total RNA samples from the control and LP-treated groups were used for first strand cDNA synthesis and subsequent qRT-PCR. cDNA synthesis was performed with 5 × All-in-One RT MasterMix (AccuRT Genomic DNA Removal Kit included) (ABM, Canada) according to the manufacturer's protocol. Afterward, qRT-PCR was performed on ABI 7500 Real-time PCR system (ABI, USA) using Bestar qPCR Master Mix (SYBR Green) (DBI Bioscience, Germany), following the manufacturer's instructions. The forward and reverse primers used for qRT-PCR are listed in Table S1. The thermal cycling conditions were as follows: 2 min at 95 °C, 40 cycles at 95 °C for 15 s, and 1 min at 60 °C. The 18S rDNA of maize was used as an internal control to evaluate the levels of selected gene transcripts, Each sample was analyzed thrice, and the relative transcript abundance was calculated with the $2^{-\Delta\Delta Ct}$ method (Schmittgen and Livak, 2008).

2.9. Statistical analysis

The data were reported as mean values ± standard error in all figures from at least three independent experiments with three replicates. All data obtained were subjected to ANOVA, and the significant difference between P treatment and genotypes was compared via the LSD test at the P value (* means $P < 0.05$).

3. Results and discussion

3.1. Plant growth and P and Pi contents in contrasting maize inbreds under LP stress

Previously, many maize Pi uptake/use efficient genotypes were reported, and transcriptomic analyses of maize identified some key genes involved in LP stress (Calderon-Vazquez et al., 2008; Lin et al., 2013; Pei et al., 2013). Nevertheless, the regulatory mechanisms of maize response to Pi deficiency are complex and should be further clarified. In the present study, two contrasting maize inbreds (QXH0121 and QXN233) that exhibit significantly different anthocyanin and Pi contents under Pi limitation (Fig. S1) were selected from 10 maize inbreds for further study. To investigate their response to different Pi levels, three Pi concentrations (low Pi, optimum and high Pi, as described above) were used to test their phenotypic differences according to the previous report (Pei et al., 2013; Zeng et al., 2016). In addition, the plants in the three- or six-leaf seedling stage, as a vulnerable and rapid growth stage, were considered. As shown in Figs. 1 and 2, the quartz sand assay showed that vegetative growths of QXN233 and QXH0121, including the shoot sizes and the dry matter yield, were both considerably inhibited under LP stress than those of the control or sufficient Pi condition. Notably, the newly developed shoots of QXN233 remained green, whereas QXH0121 showed a purple brown symptom with higher anthocyanin content in leaves under LP condition (Fig. 1). This result indicated that QXN233 was more tolerant to LP stress than QXH0121. Moreover, under Pi-deficient conditions, QXN233 still maintained heavier shoot Dw than QXH0121 (Fig. 2A), despite that no significant difference existed in their root Dws (Fig. 2B). The shoot-to-root ratio, as an important indication of plants suffering from Pi deficiency, of QXN233 was also higher than that of QXH0121 under LP stress (Fig. 2C). Thus, QXN233 was more tolerant than QXH0121, as indicated by its

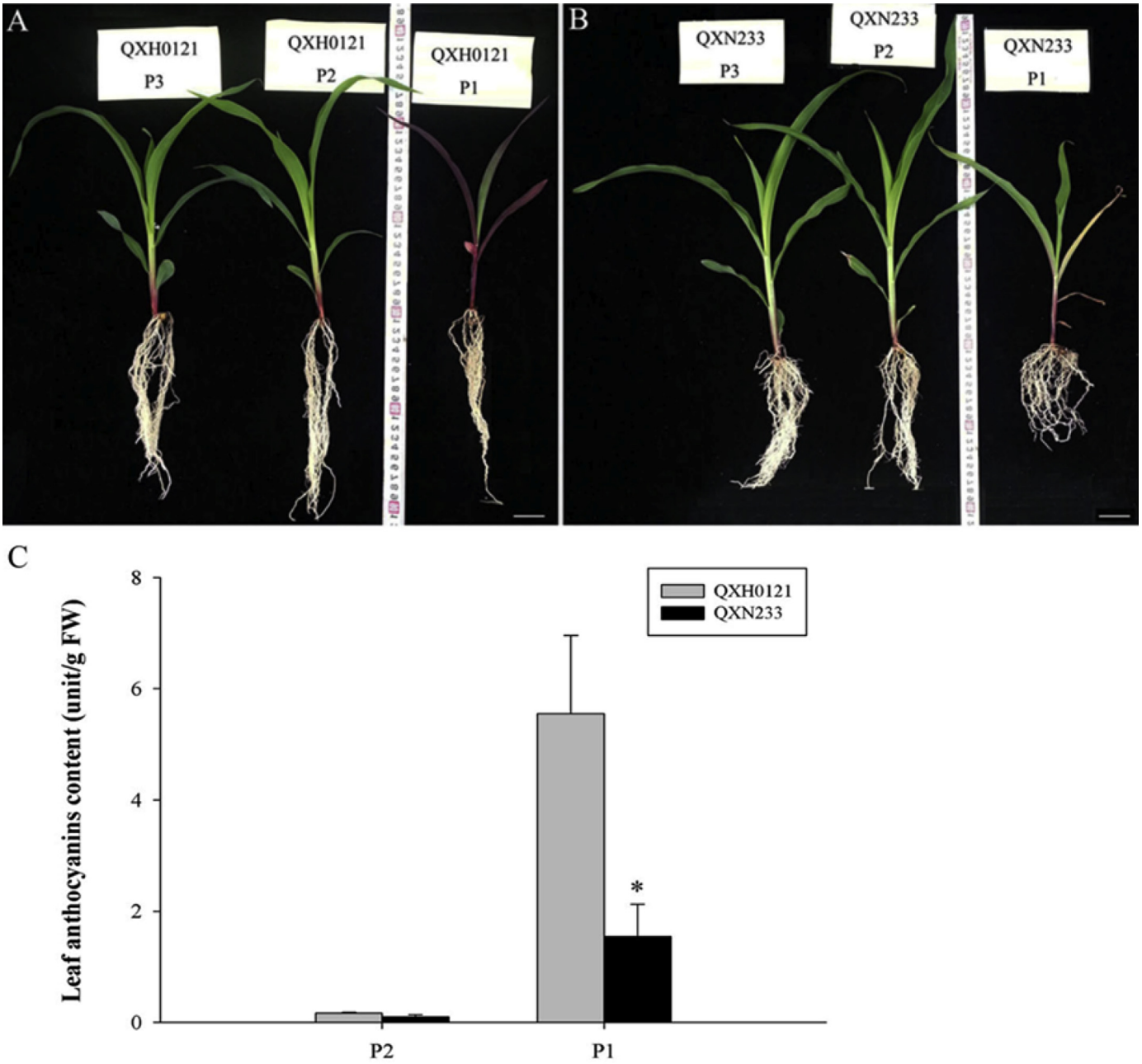

Fig. 1. Phenotypic responses and changes in anthocyanin levels of contrasting maize inbreds to phosphate (Pi) deficiency. (A) Phenotypic responses of QXH0121. (B) Phenotypic responses of QXH0121. (C) Anthocyanin contents of QXH0121 and QXN233. Maize seedlings were grown under 1 μM (P1, low phosphorus (LP) stress), 500 μM (P2, optimum Pi condition), and 1000 μM (P3, high Pi condtion) Pi concentrations after 25 d of treatments. Bar = 2 cm.

superior tolerant phenotype and smaller induction of biomass under LP stress (Figs. 1 and 2). Consequently, QXN233 exhibited better growth than QXH0121 plants.

Total P contents include organic P and free Pi contents. Plants acquire free Pi from the external environment. The total P and Pi contents in shoots and roots were measured to investigate what was total P contents and the distribution of Pi in contrasting maize inbreds, respectively (Figs. 3 and 4). Under LP condition, Pi deficiency caused a significant decrease in P and Pi accumulations in both shoot and roots (Figs. 3 and 4). Significantly, total P contents were relatively higher in the shoots and roots of QXN233 than those in QXH0121 plants (Fig. 3A and B), and the level of Pi accumulation in the shoots of QXN233 was also almost twice that in QXH0121 under LP condition with significant difference ($P < 0.05$, Fig. 4A). No significant difference in Pi accumulation was observed in the roots between both genotypes (Fig. 4B). These results suggested that QXN233 plants may possess a stronge ability of Pi translocation from roots to shoots, which was attributed to an increased Pi content accumulated in shoots to achieve its higher resistance level compared with QXH0121 plants under LP condition.

3.2. Root morphological changes in response to LP stress

LP stress could commonly cause significant changes in root morphology, including altering the root-to-shoot ratio, total root length and root surface area, to increase the contact area with the soil; these changes improve the absorptive capacity of roots (Ma et al., 2001; Miller et al., 2003). However, in the present study, no

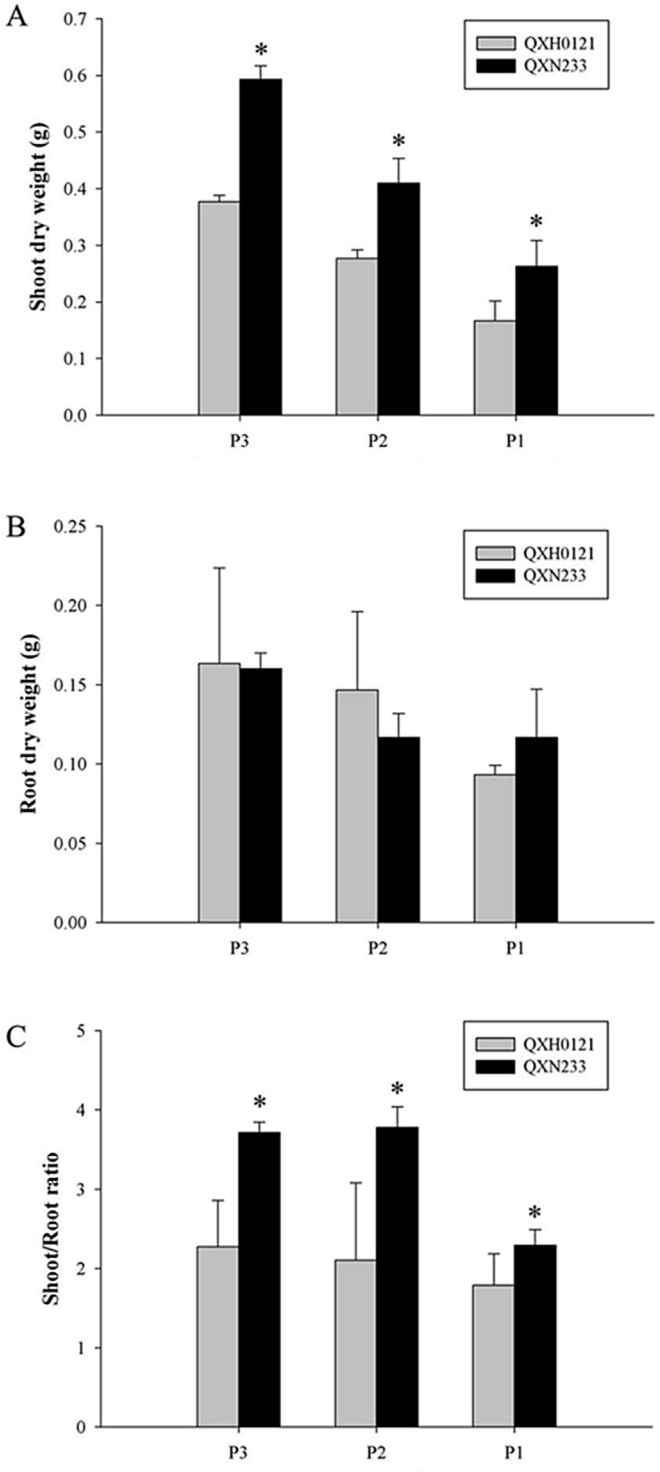

Fig. 2. Physiological responses of contrasting maize inbreds to Pi starvation. Maize seedlings were grown under 1 μM (P1, LP stress), 500 μM (P2, optimum Pi condition) and 1000 μM (P3, high Pi condtion) Pi concentrations after 25 d of treatments. (A) Shoot dry weights. (B) Root dry weights. (C) Shoot-to-root weight ratios. Experiments were run in triplicate and used at least 20 seedlings. Values were given as mean ± standard error (SE) (n = 3). Asterisks indicate significant difference at $P < 0.05$.

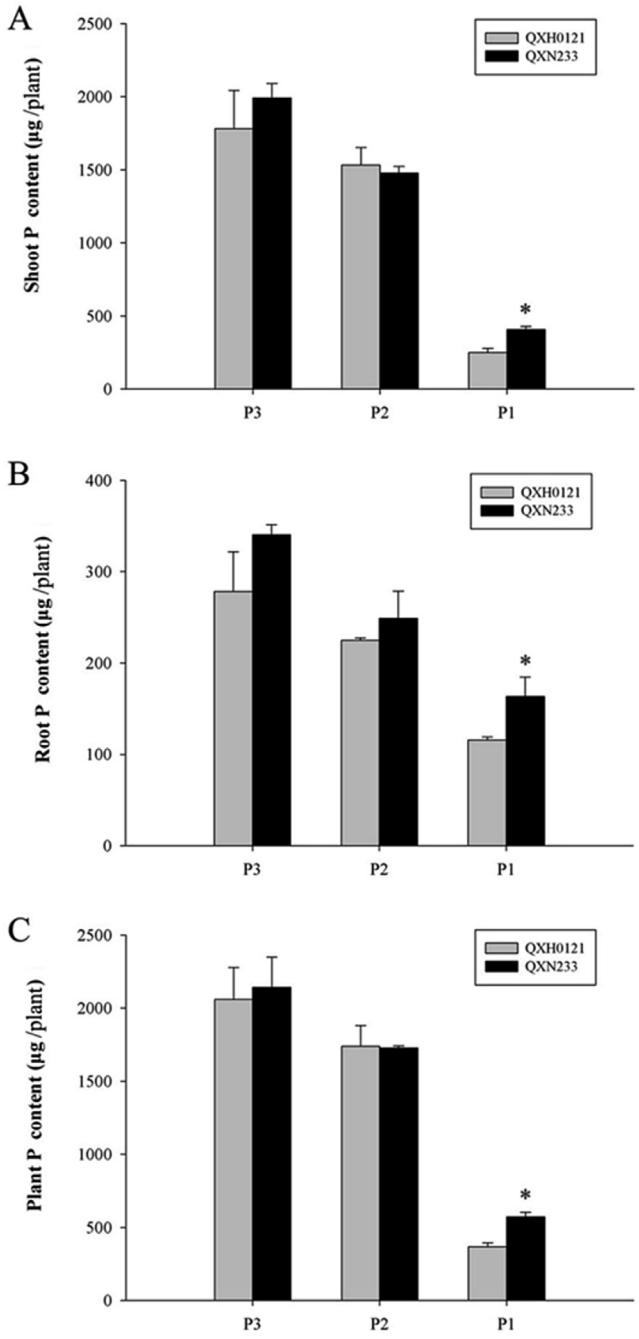

Fig. 3. Changes in total P contents in shoots and roots of contrasting maize inbreds. Maize seedlings were grown under 1 μM (P1, LP stress), 500 μM (P2, optimum Pi condition) and 1000 μM (P3, high Pi condtion) Pi concentrations after 25 d of treatments. (A) Shoot P contents. (B) Root P contents. (C) Total P contents of the whole plant. Values are mean ± SE (n = 6–15 seedlings) from three independent experiments. Asterisks indicate significant difference at $P < 0.05$.

significant differences were observed between QXN233 and QXH0121 under LP treatment (Figs. 5 and 7); this result was possibly caused by their similar vigorous root growth at the younger seedling stage, which allowed them to recover rapidly from Pi deficiency, and also consistent with their dry matter yield results (Fig. 2). In addition, almost no significant differences between the root growth of three-leaf plants and the control groups (Fig. 5) existed for each genotype, whereas the root growth of six-leaf plants was inhibited obviously when subjected to LP stress (Figs. 6 and 7). These results indicated that maize plants possessed a more severe inhibition of root growth at six-leaf stage than that of three-leaf stage when confronted with LP stress.

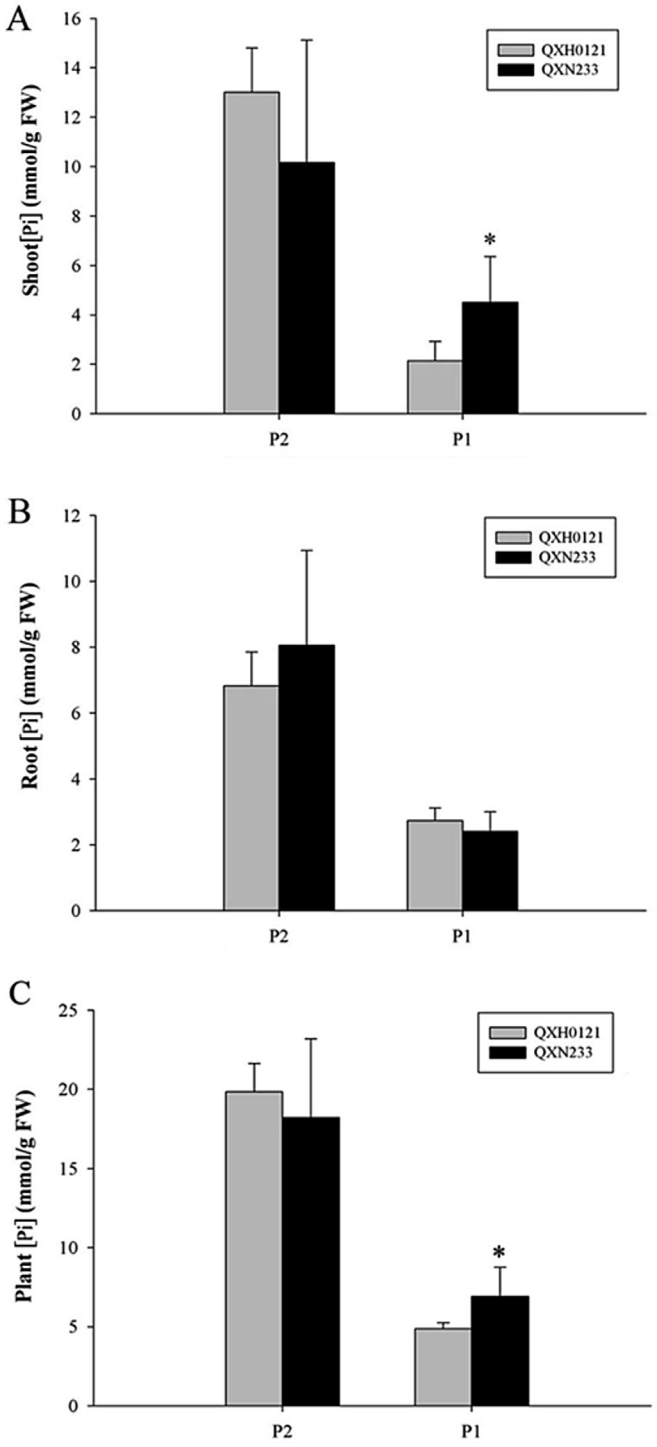

Fig. 4. Distribution of free Pi in shoots and roots of contrasting maize inbreds. Maize seedlings were grown under 1 μM (P1, LP stress) and 500 μM (P2, optimum condition) Pi concentrations after 25 d of treatments. (A) Pi accumulation in shoot. (B) Pi accumulation in root. (C) Total Pi accumulation of the whole plant. Values are mean ± SE (n = 6–15 seedlings) from three independent experiments. Asterisks indicate significant difference at $P < 0.05$.

3.3. RNA-seq analysis

The RNA-Seq data analysis in this study contained the following eight RNA libraries: P2-QXN233-leaves, P1-QXN233-leaves, P2-QXN233-roots, P1-QXN233-roots, P2-QXH0121-leaves, P1-QXH0121-leaves, P2-QXH0121-roots, and P1-QXH0121-roots. The DEGs between QXN233 and QXH0121 were analyzed under the normal or LP condition. When the low-quality reads were removed, the total reads of maize genes were over 4×10^9 mer oligonucleotides, which represented $>5.1 \times 10^7$ distinct identifiable maize genes; these data are provided in Table S2.

The transcripts exhibiting a difference between QXN233 and QXH0121 plants of more than two-fold at the probability (P) value ≥ 0.8 were identified as differentially expressed transcripts and used to search for genes induced or inhibited by Pi. Whether the alterations of the global genome were associated with their response to LP stress was determined, and all the detailed data are shown in Tables S3 and S4.

3.4. Differentially expressed genes (DEGs) between QXN233 and QXH0121

A total of 1391 transcripts were differentially expressed between QXN233 and QXH0121 plants when subjected to LP stress (Fig. 8A). Among these transcripts, 468 (321 up- and 147 down-regulated) genes were identified in leaves of QXN233, and 923 genes were identified in roots (626 transcripts up- and 297 down-regulated) (Fig. 8B). In addition, Under normal condition, there were a total of 7910 transcripts were differentially expressed because of genotypic differences.

Furthermore, as shown in Fig. 8B, a total of 597 DEGs (176 in leaves and 421 in roots) were up-regulated similarly trends in the control and LP-treated groups, and a total of 187 (62 in leaves and 125 in roots) DEGs were down-regulated. Under LP stress, 350 (145 in leaves and 205 in roots) DEGs were up-regulated in QXN233 compared with QXH0121, and these DEGs were possibly important determinant factors contributing to the higher LP tolerance of QXN233 relative to QXH0121.

3.5. Functional classifications of DEGs between QXN233 and QXH0121

According to the GO analysis, those DEGs between QXN233 and QXH0121 represented a large range of functional categories (Fig. 9; Tables S3 and S4). For cellular location (Fig. 9A and B), the cell (25.7% in leaves and 24.0% in roots), cell part (25.7% in leaves and 24.0% in roots) and organelle, were mainly affected with LP stress, with 22.2% in leaves and 21.2% in roots. For cellular function (Fig. 9C and D), LP stress caused notable effects on the catalytic activity (46.5% in leaves and 40.8% in roots), binding (39.6% in leaves and 40.1% in roots), transporter activity (3.3% in leaves and 5.8% in roots), nucleic acid binding TF activity (3.0% in leaves and 2.4% in roots) and antioxidant activity (1.3% in leaves and 4.1% in roots). Additionally, the main effects of stress on cellular processing were directed at metabolic processes (23.2% in leaves and 21.0% in roots), cellular processes (17.8% in leaves and 17.5% in roots), single-organism processes (9.7% in leaves and 11.2% in roots) and response to various stimuli (10.6% in leaves and 10.4% in roots) (Fig. 9E and F).

When these DEGs between QXN233 and QXH0121 were further subjected to the KEGG analysis, the major pathways regulated by LP stress were associated with various metabolic pathways and biosyntheses of secondary metabolites and plant–pathogen interaction in leaves and roots of them (Fig. 10); this result is consistent with the important roles in metabolism in planta.

3.6. Expression analysis via relative qRT-PCR

The expression pattern of 16 genes with different transcript abundances observed in the RNA-Seq analysis were randomly used for validation through qRT-PCR. As shown in Figs. 11 and 12, and

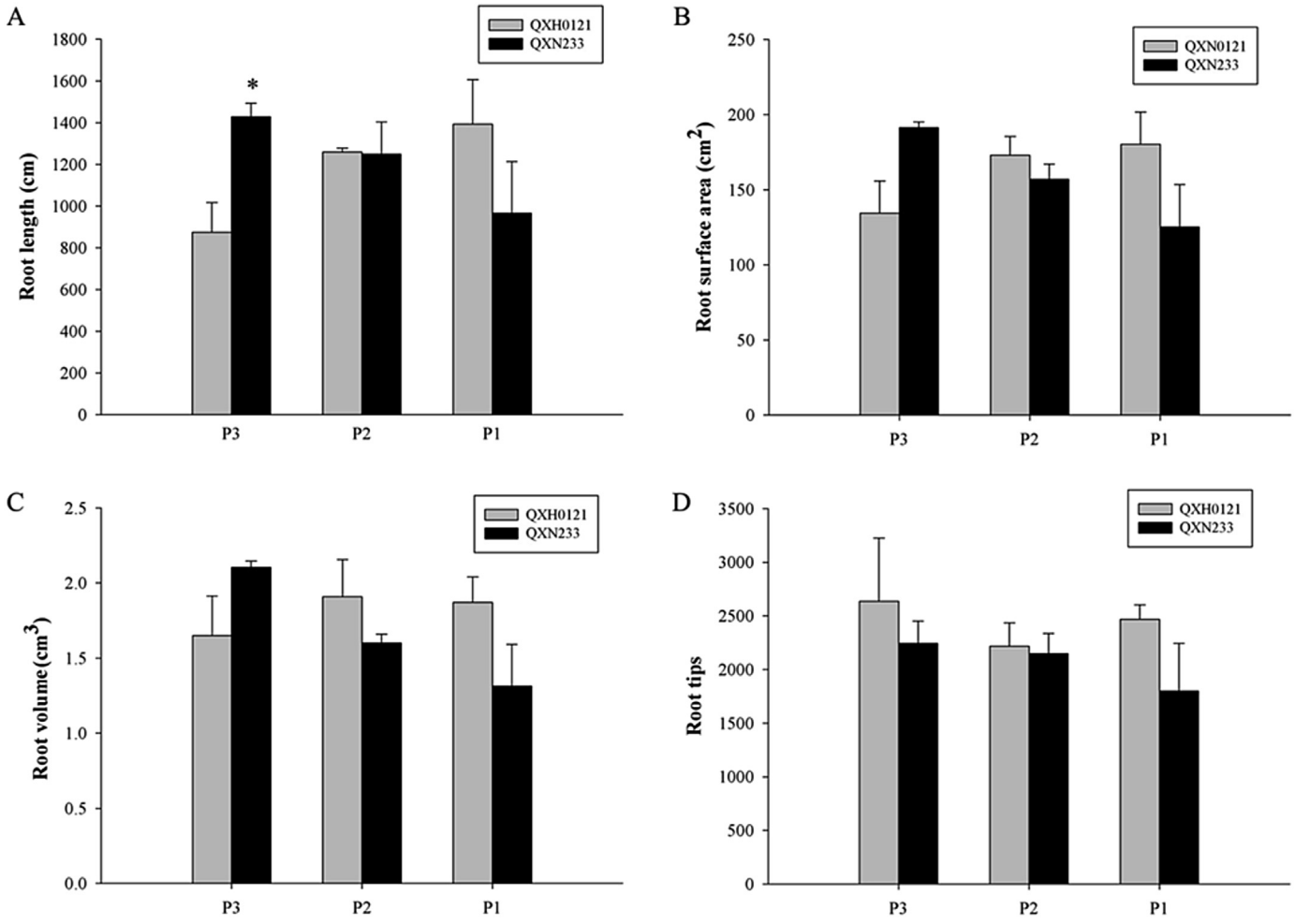

Fig. 5. Root modifications of contrasting maize inbreds in response to Pi deficiency. Maize seedlings were grown under 1 μM (P1, LP stress), 500 μM (P2, optimum Pi condition) and 1000 μM (P3, high Pi condtion) Pi concentrations after 25 d of treatments. (A) Total root length. (B) Total root surface area. (C) Total root volume. (D) Total root tips. Values are mean ± SE (n = 6–15 seedlings) from three independent experiments. Asterisks indicate significant difference at $P < 0.05$.

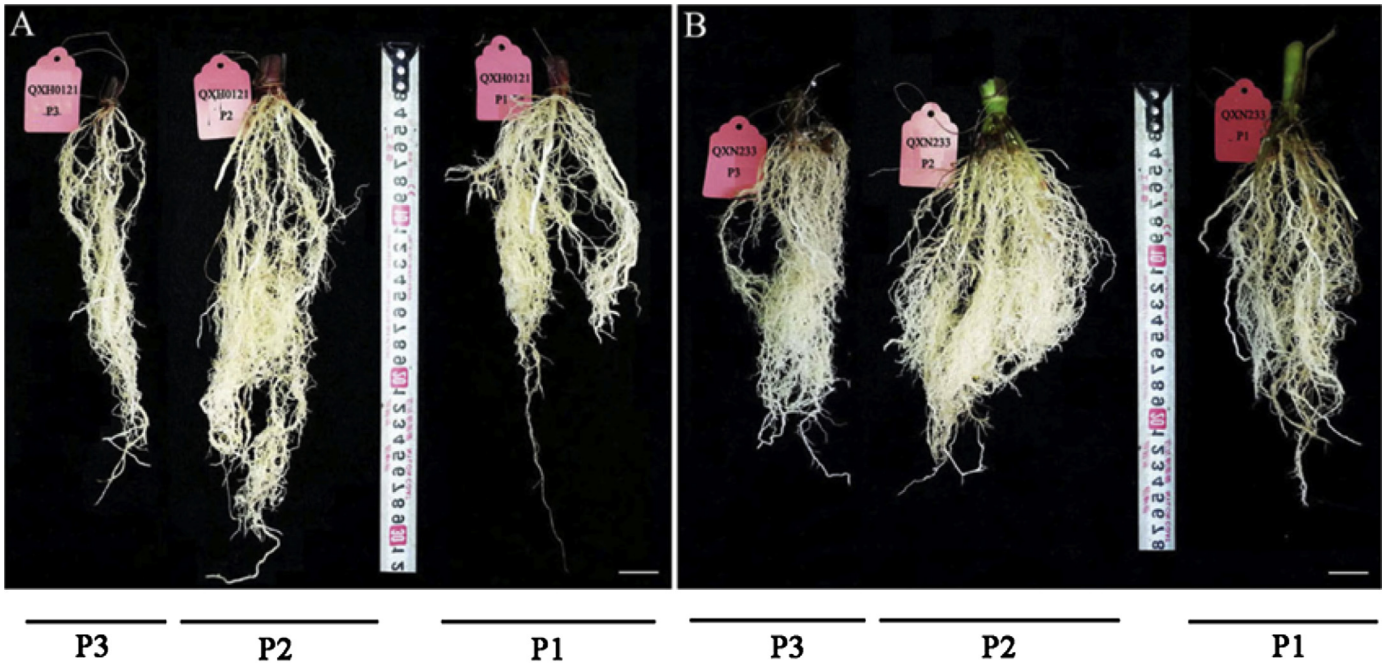

Fig. 6. Phenotypic responses of contrasting maize inbreds in roots during Pi starvation. Maize seedlings were grown under 1 μM (P1, LP stress), 500 μM (P2, optimum Pi condition) and 1000 μM (P3, high Pi condtion) Pi concentrations after 40 d of treatments. (A) QXH0121. (B) QXN233. Bar = 2 cm.

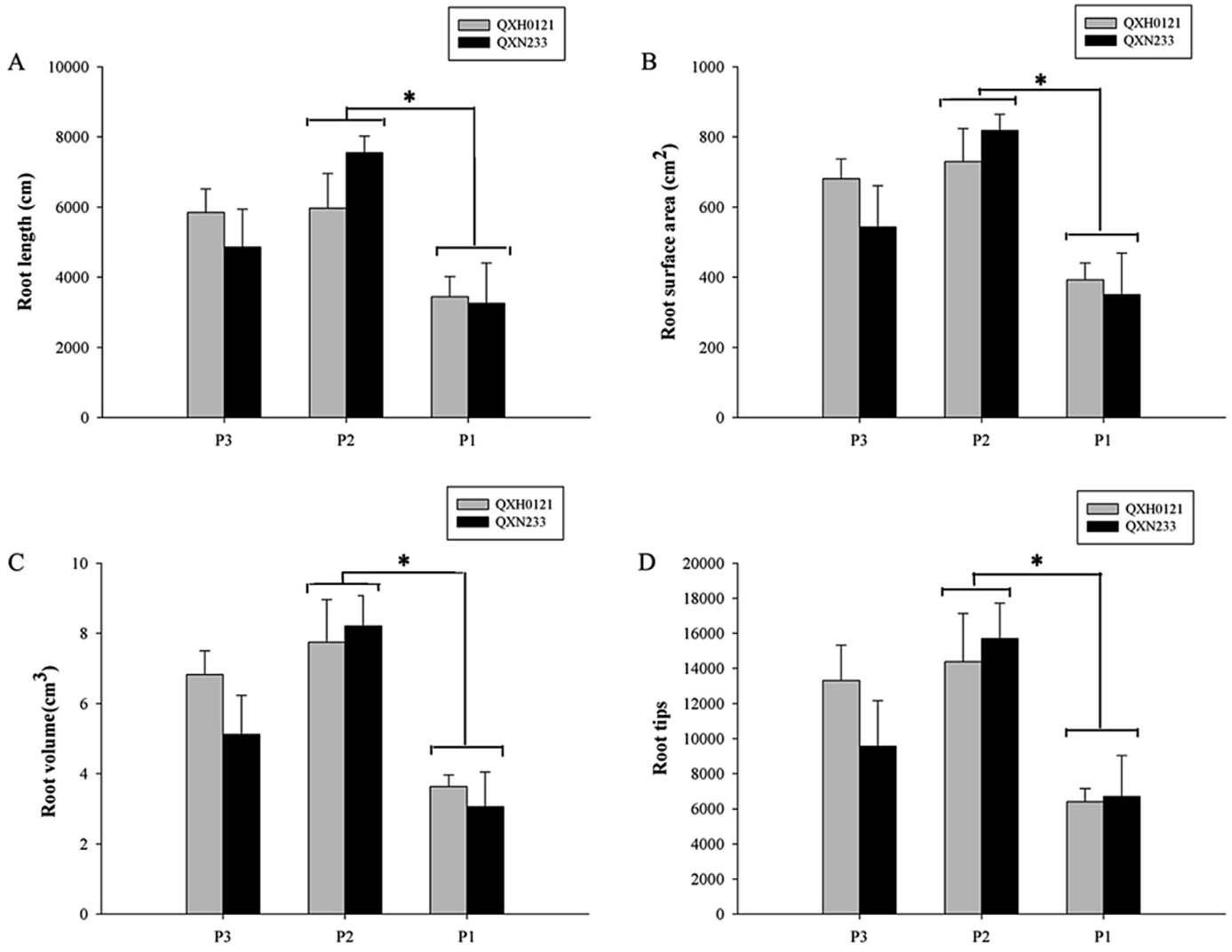

Fig. 7. Root modifications of contrasting maize inbreds in response to Pi starvation. Maize seedlings were grown under 1 μM (P1, LP stress), 500 μM (P2, optimum Pi condition) and 1000 μM (P3, high Pi condtion) Pi concentrations after 40 d of treatments. (A) Total root length. (B) Total root surface area. (C) Total root volume. (D) Total root tips. Values are mean ± SE (n = 6–15 seedlings) from three independent experiments. Asterisks indicate significant difference at $P < 0.05$.

Table 1, the qRT-PCR output of about 70% of 16 genes confirmed the RNA-Seq-based identification of DEGs in leaves and roots under LP stress. Among them, under LP stress, the expression pattern of GRMZM2G009779_T01 (an inorganic Pi transporter, *ZmIPT1*) was up-regulated more in leaves of QXN233 than that of QXH0121. However, four other inorganic Pi transporters, namely, GRMZM2G310175_T01 (*ZmIPT3*), GRMZM2G112377_T01 (*ZmIPT2*), GRMZM2G070087_T01 (*ZmIPT4*) and GRMZM2G326707_T01 (*ZmIPT5*), were induced with higher expression levels in leaves or roots of QXN233 than those of QXH0121; this result was not in accordance with the RNA-Seq data (Figs. 11 and 12; Tables S5 and S6). Some LP-responsive genes, including GRMZM2G402862_T01 (ABA-responsive element binding factor, *ZmABA-REBF*), GRMZM2G427815_T01 (peroxidase, *ZmPOD1*), and GRMZM2G052571_T01 (glutathione S-transferase, *ZmGST1*), were also up-regulated differentially in the qRT-PCR detection (Figs. 11 and 12).

3.7. DEGs related to metabolic pathways under LP condition in contrasting maize inbreds

As mentioned above, metabolic pathways or processes played a main role in plant growth under LP stress (Figs. 9 and 10). With respect to metabolism, these DEGs in leaves and roots were mainly involved in sugar synthesis, protein synthesis, amino acid degradation, and secondary metabolic pathway (Table S5 and S6). Previously, Pi deficiency typically leads to high starch and sucrose levels in shoots and roots, and many reports are in favor of the presence of a systemic control of plant Pi deficiency responses by a carbohydrate signal, most likely sucrose (Ciereszko and Barbachowska, 2000; Karthikeyan et al., 2007). In the present study, GRMZM5G840560_T01 (*ZmGS1*) and GRMZM2G553532_T01 (*ZmGS2*), which were both involved in 1,3-beta-D-glucan synthase activity, as well as GRMZM2G046117_T02 (*ZmSS1*) encoding a starch synthase and GRMZM2G031057_T01 (*ZmSS2*) related to starch synthase activity, were all induced strongly in QXN233 than in QXH0121 under LP stress or normal condition. The up-regulations of these DEGs in QXN233 synthesized a large amount of sugar to supply the metabolic demand; this result is related with root phenotype (Figs. 5–7) and in agreement with the previous report (Jain et al., 2007). Moreover, GRMZM2G058358_T01 (*ZmSEI1*) and GRMZM2G156632_T01 (*ZmSEI2*), which were associated with serine-type endopeptidase inhibitor activity, and GRMZM2G420870_T01 (*ZmUDPC*), which exhibited with ubiquitin-

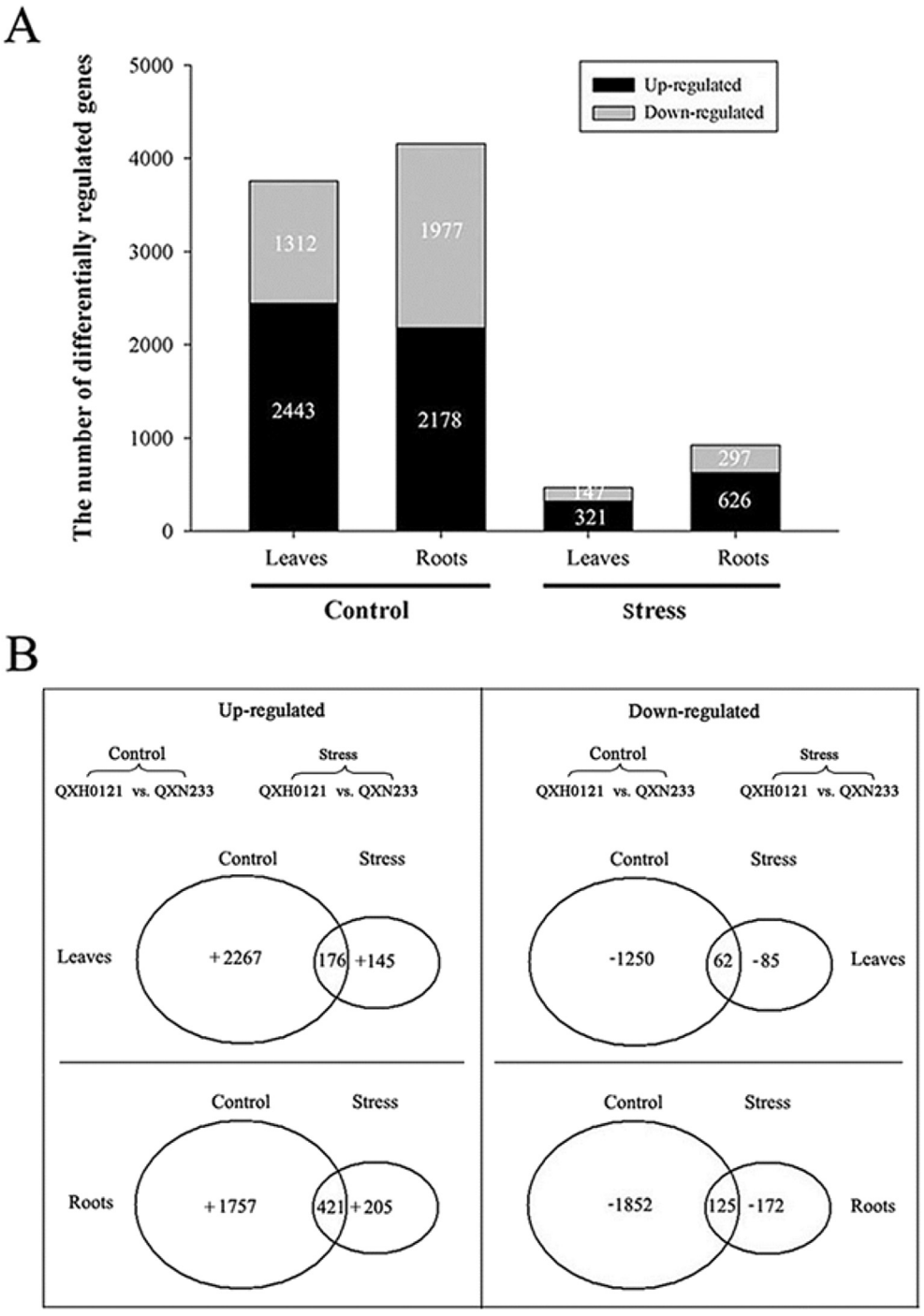

Fig. 8. Overview of differentially expressed genes (DEGs) between QXH0121 and QXN233 under the normal and LP conditions. A total of 7910 and 1391 genes in the control and LP-treated groups, respectively, were identified to be expressed differentially between QXH0121 and QXN233 plants according to our thresholds fold change of at least ± 2 and FDR ≤ 0.001. (A) Number of DEGs in leaves and roots of QXN233 compared with QXH0121 at the control and LP-treated groups, respectively. (B) Distribution of DEGs up-regulated or down-regulated in leaves and roots of QXN233 compared with QXH0121 in the control and LP-treated groups, respectively.

dependent protein catabolic processes, were all involved in amino acid degradation; furthermore, their transcript levels were obviously higher in QXN233 than in QXH0121 (Tables S5 and S6). The up-regulations of these genes related to metabolic processes indicated a metabolic adaptation of maize grown under LP condition, and these metabolic changes were advantageous for regulating and enhancing the growth under LP stress, as discussed in previous studies (Calderon-Vazquez et al., 2008; Lin et al., 2013). The induction of these DEGs is likely to promote carbon and nitrogen metabolisms to maintain their cellular Pi balance under LP stress. Amino acids are also used as a source of C, particularly in barley roots under Pi-deficient conditions (Huang et al., 2008). In addition, Pi deficiency affects anthocyanins accumulation, electron transport, glycolysis, and lipid metabolism (Zhang et al., 2014; Lin et al., 2013; Fang et al., 2009). The changes in metabolic mechanisms facilitated the mobilization of other metabolic resources to maintain energy supply and Pi homeostasis in maize during Pi deficiency.

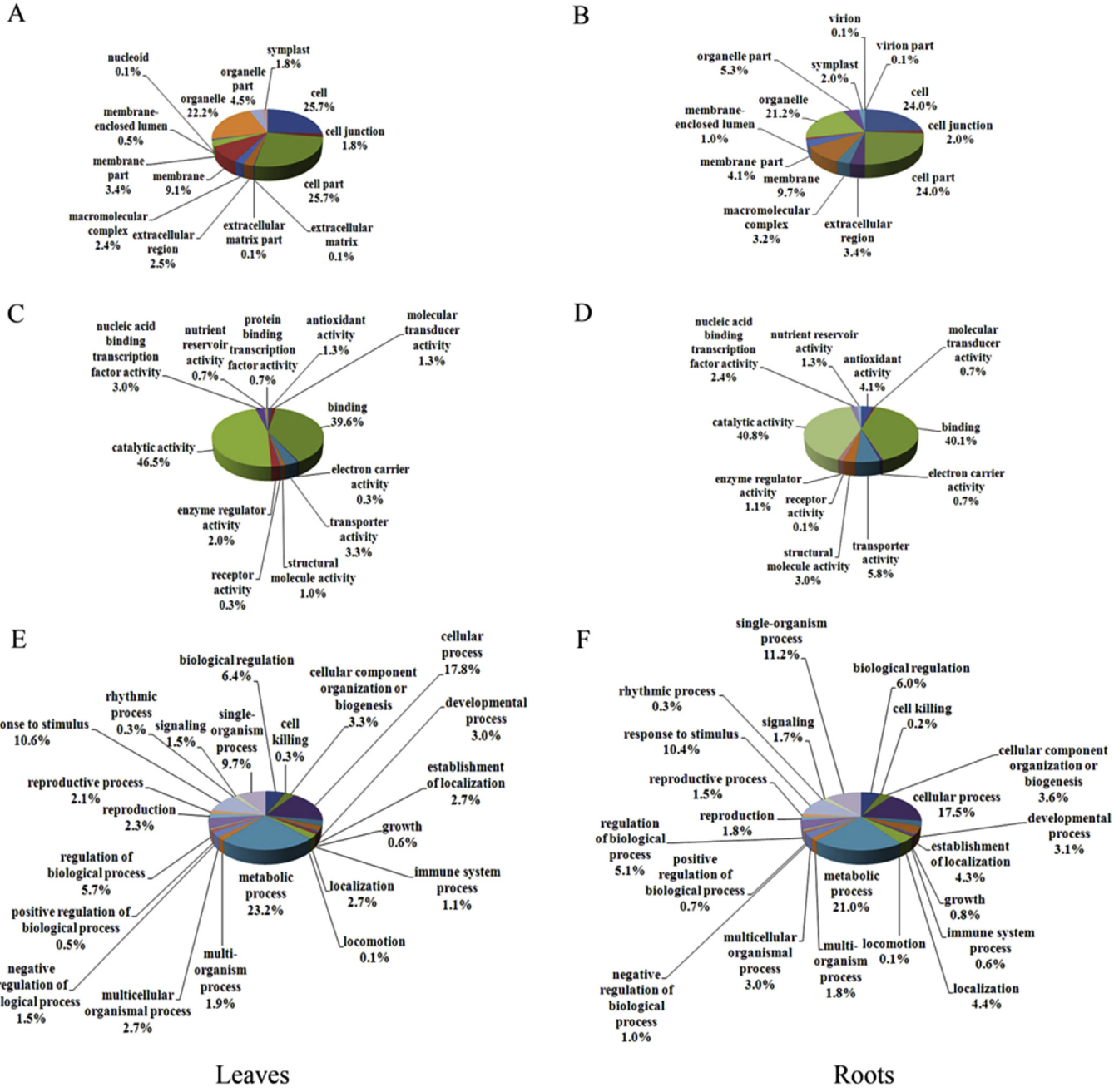

Fig. 9. Distribution of Gene Ontology (GO) in cellular components (A and B), molecular functions (C and D), and biological processes (E and F) of DEGs between QXH0121 and QXN233 leaves and roots under Pi-deficient condition.

3.8. DEGs related to Pi transporter activity under LP condition in contrasting maize inbreds

In plants, the inorganic Pi is taken up by roots from soil via some Pi transporters. Several previous reports have identified many early Pi deficiency inducible genes and late genes encoding Pi-responsive transporter in maize (Nagy et al., 2006), including *PHR1*, *SPX* proteins, and *PHT*s. For instance, two maize inorganic Pi transport factors 1 and 2 (*ZmPT1* and *ZmPT2*) in the Pi-efficient inbred line 178 are up-regulated under LP stress (Calderon-Vazquez et al., 2008). In the present study, no early regulatory DEGs was observed between QXN233 and QXH0121, which might be due to their long-term adaptation processes of low Pi limitation. However, two inorganic Pi transporters, namely, GRMZM2G009779_T01 (*ZmIPT1*) and GRMZM2G112377_T01 (*ZmIPT2*), were both induced in the leaves of these two lines under Pi-deficient conditions (Fig. 11 and Table 1). Specifically, *ZmIPT1* was more highly up-regulated in the leaves of QXN233 than in those of QXH0121 under LP stress, albeit with no significant difference, irrespective of Pi conditions (Fig. 11). *ZmIPT2*, as an inorganic Pi transporter, was also with a higher expression level in the leaves of QXN233 than that in QXH0121 under LP stress (Fig. 11). Moreover, GRMZM2G070087_T01 (*ZmIPT4*) and GRMZM2G326707_T01 (*ZmIPT5*) were both increased more highly in the roots of QXN233 vs QXH0121 under LP stress, despite of a decreased expression shown in QXN233 relative to the control (Fig. 12 and Table 1).

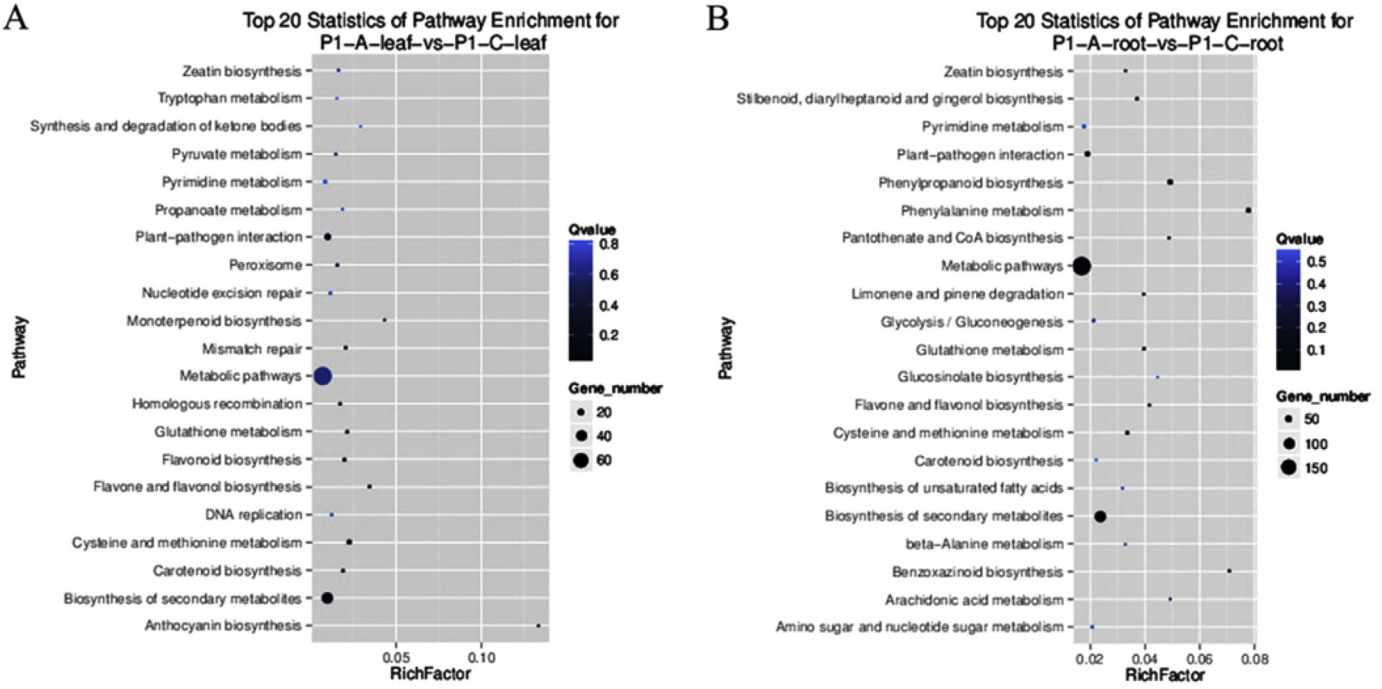

Fig. 10. Pathway analysis (Kyoto Encyclopedia of Genes and Genomes) of DEGs between QXH0121 and QXN233 leaves (A) and roots (B) under Pi-deficient condition.

Similarly, GRMZM2G310175_T01 (*ZmIPT3*) was also induced significantly higher both in leaves and roots of QXN233 than those in QXH0121 under LP stress (Figs. 11 and 12 and Table 1). Previously, in *Arabidopsis*, *PHT1;5* plays a specific role in Pi translocation between roots and shoots (Nagarajan et al., 2011), and *PHT1;4*, *PHT1;8*, and *PHT1;9* might be restricted to Pi absorption when subjected to Pi deficiency (Misson et al., 2004). In the present study, high homology and similarity were exhibited between PHT1; 5 and the proteins encoded by ZmIPT2 and ZmIPT5 (Fig. S2); this result suggested that ZmIPT2 might play important role in Pi translocation from roots to shoots. *ZmIPT1* and *ZmIPT3*, which were up-regulated in the leaves of QXN233 (Fig. 11), might also participate in this process. Consequently, the up-regulations of these genes in the leaves of QXN233 were related to a relatively higher Pi levels in the shoots of QXN233 than those of QXH0121 (Figs. 3 and 4). Thus, QXN233 could possess stronger Pi translocation capacity from roots to shoots to increase the higher Pi level in shoot cells than that of QXH0121, which may improve plant growth and gain large Dws (Fig. 2). Furthermore, *ZmIPT4*, *ZmIPT5* and *ZmIPT3*, up-regulated in the roots of QXN233 (Fig. 12), were probably related to the enhancement of Pi uptake in its roots. Therefore, these results indicated that these Pi transporter genes were up-regulated in the tolerant line QXN233, thereby corresponding to a relatively higher P levels in the whole plants of QXN233 than those of QXH0121. Such genes are probably key determinates of the different Pi translocations from roots to shoots between QXN233 and QXH0121, and may be used as candidate genes for application in plant molecule breeding project to breed relatively low Pi-resistant plant. Whether the overexpress or knockdown of these inorganic Pi transporters leads to the increase or decrease of Pi levels should be further studied.

Additionally, other transporter genes, such as zinc transporters (GRMZM2G702923_T01, GRMZM2G134248_T02 and GRMZM2G047262_T01, namely, *ZmZNT1*, *ZmZNT2* and *ZmZNT3*), iron transporter (GRMZM2G378771_T01, *ZmIRT1*), and peptide/histidine transporter (GRMZM2G064091_T01, *ZmPT/HT1*) were identified in this study (Tables S5 and S6), These genes could play a potential role for regulating other ion homeostases when maize is confronted with Pi deficiency. Interestingly, at least eight genes encoding aquaporin, such as GRMZM2G168439_T01 (*ZmAQP1*), GRMZM2G136032_T01 (*ZmAQP2*) and GRMZM2G093090_T01 (*ZmAQP3*), were induced strongly in QXN233 under LP condition (Tables S5 and S6), which may be attributed to the fact that the osmosis balance in root cells should be maintained during Pi deficiency in QXN233.

3.9. DEGs related to phytohormone regulation under LP condition in contrasting maize inbreds

Several studies had demonstrated the implication of many phytohormones in the response and adaptation of plants to Pi deficiency, such as the role of auxin and ethylene in modulating the developmental adaptations of roots under Pi limitation (Jain et al., 2007; Fang et al., 2009); moreover, cytokinin signals the plant Pi or sugar status (Martin et al., 2000). In the present study, a set of transcripts related to the response to several phytohormone stimuli were up-regulated; these transcripts included GRMZM2G364320_T01 (*ZmCAB1*) and GRMZM2G063717_T01 (*ZmCAB2*) associated with calcium ion binding in response to ABA stimulus, GRMZM5G835747_T01 (*ZmUBQB*) with polyubiquitin binding, GRMZM2G020231_T01 (*ZmZNB1*) with zinc ion binding and GRMZM2G165354_T02 (*ZmGSHS*) with glutathione synthase activity in response to cytokinin, auxin and jasmonic acid (JA) stimulus, respectively (Tables S5 and S6). Furthermore, various TFs or signaling proteins related to hormone signaling pathway were identified (Tables S5 and S6). For instance, GRMZM2G177340_T02 (*ZmETHB*) participated in the ethylene biosynthetic process; additionally, GRMZM2G475185_T01 (*ZmETH-MSP*) and GRMZM2G040481_T01 (*ZmEI3*), as an ethylene-insensitive protein 3, positively and negatively regulated ethylene signaling pathway, respectively. GRMZM2G402862_T01 (*ZmABA-ERBF*), as an abscisic acid (ABA)-responsive element binding factor, and

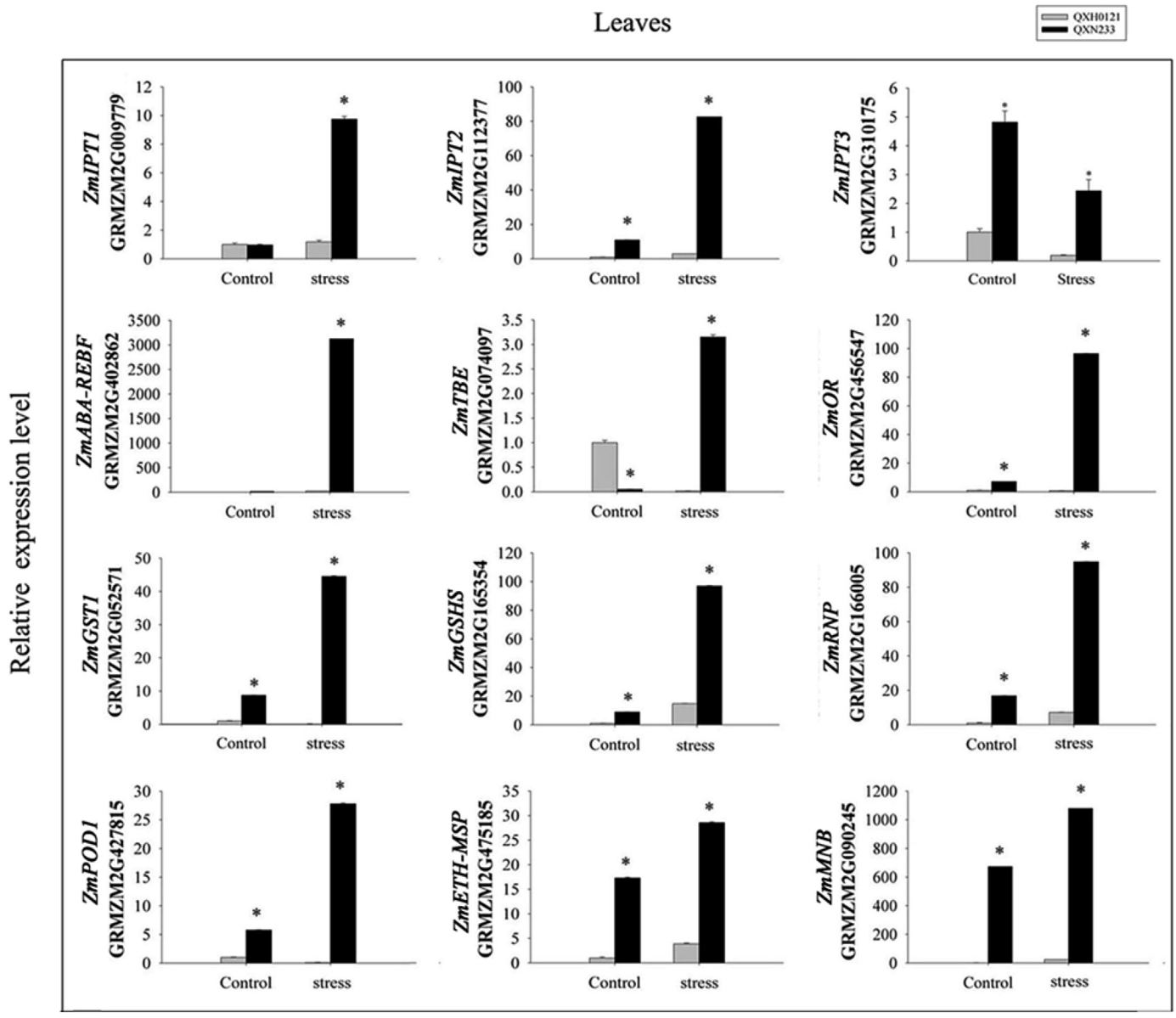

Fig. 11. Validation of the expression profiles in maize leaves identified by RNA-Seq analysis using quantitative real-time PCR (qRT-PCR). Relative expressions of 12 tested Pi-responsive genes in leaves are shown. The fold changes observed by RNA-Seq analysis were also included (Table 1). Values given as mean ± SE (n = 3) from three independent experiments. Asterisks indicate significant difference at $P < 0.05$.

GRMZM2G166005_T04 (*ZmRNP*), as a small nuclear ribonucleoprotein, were involved in the ABA- and JA-mediated signaling pathway. A large number of TFs are involved in plant response to Pi deficiency, and the effects of TFs on systemic responses are possibly mediated through one or more signaling pathways (Nilsson et al., 2010; Rubio et al., 2001; Morcuende et al., 2007). Consequently, the expression levels of these TFs were also more abundant in QXN233 than in QXH0121 under Pi deficiency condition, which may contribute to their regulatory mechanism of different LP tolerances (Figs. 11 and 12; Tables S5 and S6). The above information supported that plant hormones play a central role in regulating plant growth and development to alleviate environmental stresses (Zhang et al., 2014; Rubio et al., 2009). Nonetheless, direct evidences is still needed to confirm the role of phytohormones in response to Pi starvation.

3.10. DEGs related to redox homeostasis under LP condition in contrasting maize inbreds

Environmental stresses, including Pi deficiency, commonly cause oxidative stress, which produces a large number of harmful substances, thereby resulting in cell damage (Zhang et al., 2014). Moreover, plants have evolved a number of enzymatic and non-enzymatic mechanisms to detoxify toxic of reactive oxygen species (ROS) in cells (Gill and Tuteja, 2010). In the present study, at least 21 genes encoding POD and glutathione S-transferase protein involved in ROS scavenging, including GRMZM2G427815_T01 (*ZmPOD1*), GRMZM2G313184_T01 (*ZmPOD2*), GRMZM2G427954_T01 (*ZmPOD3*), and GRMZM2G504757_T01 (*ZmPOD4*), were involved in hydrogen peroxide catabolic process. GRMZM2G052571_T01 (*ZmGST1*), GRMZM2G127789_T01

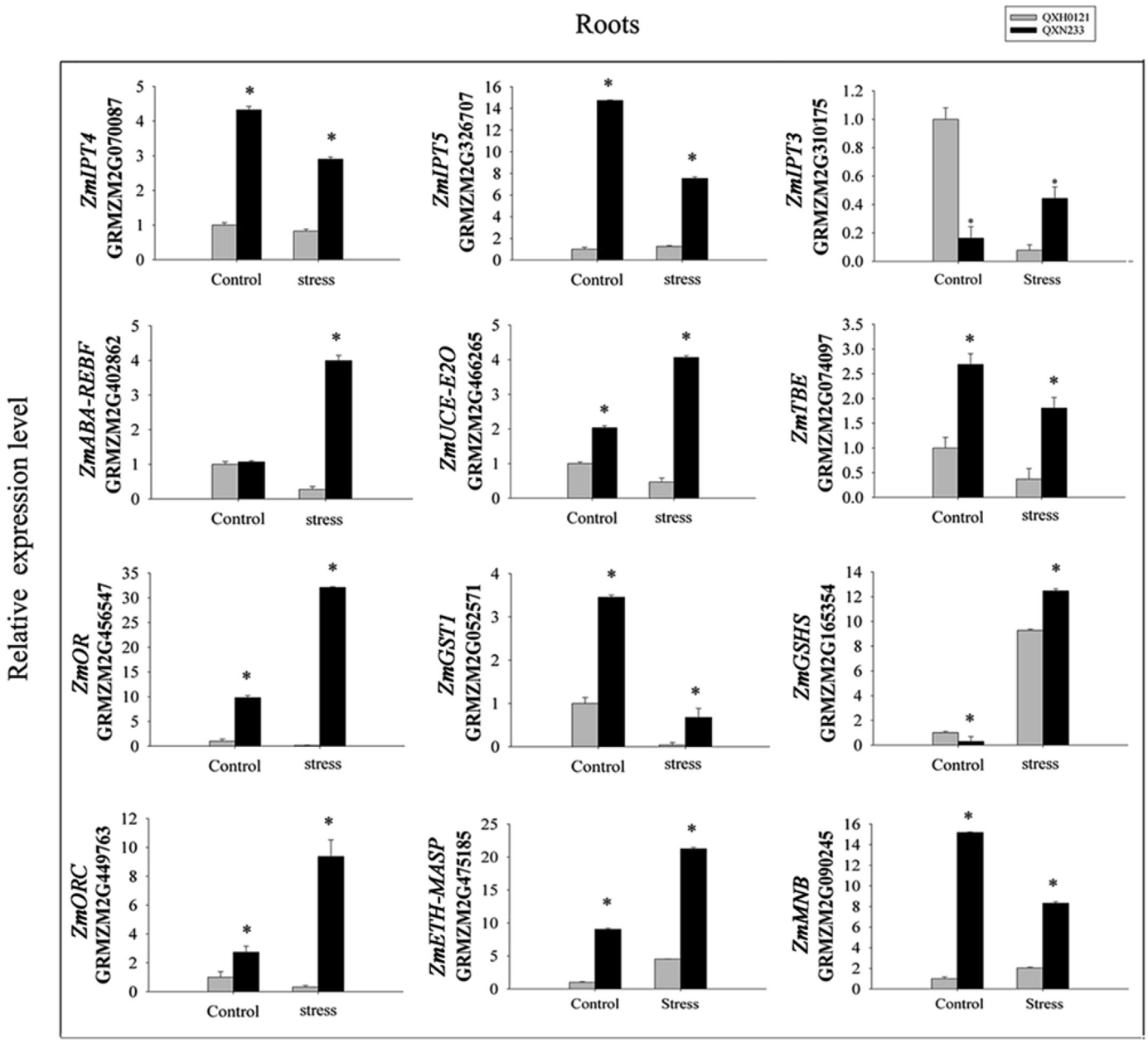

Fig. 12. Validation of the expression profiles in maize roots identified by RNA-Seq analysis using qRT-PCR. Relative expressions of 12 tested Pi-responsive genes in roots are shown. The fold changes observed by RNA-Seq analysis were also included (Table 1). Values given as mean ± SE (n = 3) from three independent experiments. Asterisks indicate significant difference at $P < 0.05$.

(*ZmGST2*) and GRMZM2G129357_T01 (*ZmGST3*) were involved in glutathione-S-transferase activity; GRMZM2G449763_T01 (*ZmORC*) and GRMZM2G169005_T03 (neomenthol dehydrogenase, *ZmNDH*) both participated in oxidoreductase activity, and both GRMZM2G456547_T01 (*ZmOR*) and GRMZM2G434572_T01 (1,2-dihydroxy-3-keto-5-methylthiopentene dioxygenase, *ZmMTD*) were also involved in oxidation–reduction process (Tables S5 and S6). These DEGs were all up-regulated in both lines (QXN233 and QXH0121) under LP stress. Importantly, the expression levels were considerably enhanced in QXN233 compared with QXH0121, specifically in their roots. Consequently, QXN233 showed a stronger ability to remove excessive ROS and mitigate ROS damage in its roots than QXH0121 when subjected to LP stress.

3.11. DEGs related to other stress responses under LP condition in contrasting maize inbreds

Multiple genes were also relevant to various abiotic and biotic stress responses (Tables S5 and S6). These DEGs were also up-regulated at the higher levels in QXN233 than in QXH0121, and possibly contributed for the LP tolerance of QXN233. Among them, several genes were involved in the response to other stresses, such as salt stress (GRMZM2G435244_T01 as a triosephosphate isomerase and GRMZM2G399338_T02 involved in histone acetyltransferase activity, namely, *ZmTIM* and *ZmHAT*), water deprivation (GRMZM2G410700_T01, hydroxylase, *ZmHD*), cadmium ion (GRMZM5G856076_T01 as a 2-oxoglutarate dehydrogenase E1

Table 1
Genes expressed differentially between QXH0121 and QXN233 leaves and roots under normal and LP stress. All indicated genes were selected to corroborate the expression patterns obtained in the RNA-Seq analysis and also within the threshold limits (fold change of at least ± 2 and P≧ 0.8). Fold values of DEGs between QXH0121 and QXN233 for a log2ratio scale are shown.

Tissue	GeneID	Putative_Annotation (Gene abbreviation)	Fold value (QXN233/QXH0121)		Probability (Stress)
			Control	Stress	
Leaves	GRMZM2G009779_T01 (GK000032.3)	inorganic phosphate transporter (*ZmIPT1*)	–	9.77	0.82
	GRMZM2G112377_T01 (GK000031.3)	inorganic phosphate transporter (*ZmIPT2*)	4.48	–	0.99
	GRMZM2G310175_T01 (CM000784.3)	inorganic phosphate transporter (*ZmIPT3*)	5.60	–	0.95
	GRMZM2G402862_T01 (CM000782.3)	ABA responsive element binding factor (*ZmABA-REBF*)	–	9.84	0.83
	GRMZM2G074097_T01 (GK000033.3)	thiamine biosynthetic enzyme (*ZmTBE*)	–	7.47	0.95
	GRMZM2G456547_T01 (CM000786.3)	oxidation-reduction process (*ZmOR*)	3.03	13.96	0.99
	GRMZM2G052571_T01 (GK000033.3)	glutathione S-transferase (*ZmGST1*)	–	11.22	0.92
	GRMZM2G165354_T02 (CM000784.3)	glutathione synthase (*ZmGSHS*)	4.63	9.77	0.82
	GRMZM2G166005_T04 (CM000780.3)	a small nuclear ribonucleoprotein (*ZmRNP*)	–	9.90	0.83
	GRMZM2G427815_T01 (GK000034.3)	peroxidase activity (*ZmPOD1*)	−4.93	11.49	0.93
	GRMZM2G475185_T01 (GK000032.3)	ethylene mediated signaling pathway (*ZmETH-MSP*)	3.01	13.17	0.98
	GRMZM2G090245_T01 (CM000782.3)	manganese ion binding (*ZmMNB*)	3.53	6.20	0.80
Roots	GRMZM2G070087_T01 (GK000031.3)	inorganic phosphate transporter (*ZmIPT4*)	−2.67	–	0.99
	GRMZM2G326707_T01 (CM000781.3)	inorganic phosphate transporter (*ZmIPT5*)	−1.05	–	0.86
	GRMZM2G310175_T01 (CM000784.3)	inorganic phosphate transporter (*ZmIPT3*)	4.48	–	0.95
	GRMZM2G402862_T01 (CM000782.3)	ABA responsive element binding factor (*ZmABA-REBF*)	–	5.10	0.89
	GRMZM2G466265_T01 (CM000781.3)	ubiquitin-conjugating enzyme E2O (*ZmUCE- E2O*)	4.34	5.60	0.90
	GRMZM2G074097_T01 (GK000033.3)	thiamine biosynthetic enzyme (*ZmTBE*)	–	3.95	0.83
	GRMZM2G456547_T01 (CM000786.3)	oxidation-reduction process (*ZmOR*)	–	5.49	0.83
	GRMZM2G052571_T01 (GK000033.3)	glutathione S-transferase (*ZmGST1*)	3.31	10.70	0.94
	GRMZM2G165354_T02 (CM000784.3)	glutathione synthase (*ZmGSHS*)	3.05	6.08	0.85
	GRMZM2G449763_T01 (CM000782.3)	oxidoreductase activity (*ZmORC*)	3.64	10.54	0.94
	GRMZM2G475185_T01 (GK000032.3)	ethylene mediated signaling pathway (*ZmETH-MSP*)	3.13	9.9	0.91
	GRMZM2G090245_T01 (CM000782.3)	manganese ion binding (*ZmMNB*)	5.20	10.49	0.93

component and GRMZM2G170689_T01 as a S-formylglutathione hydrolase, namely, *ZmOGDH* and *ZmSFGH*), cold (GRMZM2G074097_T01, thiamine biosynthetic enzyme, *ZmTBE*), hypoxia (GRMZM2G099092_T01, pectinesterase, *ZmPE*), and red light (GRMZM2G105518_T01 with chlorophyll binding, *ZmCB*) (Tables S5 and S6). In addition, numerous genes were relevant to plant disease resistance to virus (GRMZM2G117942_T01 and GRMZM2G117971_T01, involved in ribonuclease activity, namely, *ZmRNAase1* and *ZmRNAase2*), fungus (GRMZM2G094165_T04, carbonic anhydrase, *ZmCA*), and nematode (GRMZM2G095861_T01 with voltage-gated potassium channel activity, *ZmVGKC*) (Tables S5 and S6). The above results also supported a crosstalk of plant response to various environmental stresses, and intrinsic and synergetic interactions among cell response pathways attenuate cell injury caused by different stress conditions (Lin et al., 2013; Fujita et al., 2006).

4. Conclusion

Two contrasting maize inbreds showed a distinct long-term response to LP stress. QXN233 was more stress tolerant than QXN0121. The RNA-Seq analysis identified a large number of differentially expressed Pi-responsive genes, which were mostly involved in various metabolic pathways and biosyntheses of secondary metabolites; among these genes, five maize inorganic Pi transporter genes (GRMZM2G009779_T01, GRMZM2G310175_T01, GRMZM2G112377_T01, GRMZM2G070087_T01 and GRMZM2G326707_T01, namely, *ZmIPT1-5*) were more highly expressed in QXN233 than in QXH0121, which may contribute to the regulatory mechanism of different Pi sensitivities in contrasting maize inbreds. Corresponding to the high P and Pi levels of QXN233, these inorganic Pi transporters were most likely to the key regulators of their different LP tolerances. In addition to ion transporters, those related to hormone signaling pathway, ROS scavenging, and coping with various environmental stresses were also identified. These findings deepen our understanding of molecular processes in long-term response to LP stress and help in further exploration of the regulated mechanism of Pi-responsive genes in maize.

Contributions

YS, CM, XK, ZD and XL conceived the work and designed experiments. YS, YC, YCX and HXZ performed experiments and analyzed the results. HZ, YFX, QW, ZL, CM and XL contributed reagents, materials and analysis tools. YS, XK and ZD wrote the paper. All authors reviewed the manuscript and approved it for publication.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.plaphy.2016.10.017.

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