



Fine-tuning the transcriptional regulatory model of adaptation response to phosphate stress in maize (*Zea mays* L.)

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Abstract

The post green revolution agriculture is based on generous application of fertilizers and high-yielding genotypes that are suited for such high input regimes. Cereals, like maize (*Zea mays* L.) are capable of utilizing less than 20% of the applied inorganic phosphate (Pi) - a non-renewable fertilizer resource. A greater understanding of the molecular mechanisms underlying the acquisition, transportation and utilization of Pi may lead to engineering genotypes with high phosphorus use efficiency. In this study, we carried out functional domain similarity analysis, promoter analysis and comparative transcriptional expression profiling of 12 selected Pi responsive genes in the Pi stress tolerant maize inbred line HKI-163 under sufficient and deficient Pi conditions. Pi starvation led to significant increase in root length; marked proliferation of root hairs and lesser number of crown roots. Eleven genes were significantly up or down regulated in Pi deficient condition. The putative acid phosphatase, *ZmACP5* expression was up regulated by 162.81 and 74.40 fold in root and leaf tissues, respectively. The RNase, *ZmRNS1* showed 115 fold up regulation in roots under Pi deprivation. Among the two putative high affinity Pi transporters *ZmPht1;4* was found specific to root, whereas *ZmPht2* was found to be up regulated in both root and leaf tissues. The genes involved in

Pi homeostasis pathway (*ZmSIZ1*, *SPX1* and *Pho2*) were up regulated in root and leaf. In light of the expression profiling of selected regulatory genes, an updated model of transcriptional regulation under Pi starvation in maize has been presented.

Keywords Development · Gene expression · Growth · Maize · Phosphate

Introduction

Phosphorus (P) is one of the most important macronutrient for plant growth and development (Raghothama 1999; Bindraban et al. 2020). It is required for the constitution of many cellular components, including nucleic acids, membranes, etc. and participates in enzymatic reactions and signal transduction processes. In the soil, P exists in inorganic, organic and phytate forms. Plants acquire P by their roots as inorganic phosphate (Pi). Phytates, the major portion of organic phosphorus, often form salts with different ions and are found in less soluble or precipitated forms and hence cannot be utilized by plants. P is a major limiting factor in most agricultural systems. Modern high intensity agriculture is heavily dependent upon external inputs - phosphatic fertilizers being one of them (Chowdhury and Zhang 2021). Modern cereal cultivars, like hybrid maize, require a high dose of externally applied P-based fertilizers. However, it is estimated that more than 90% of the applied P remains unavailable to the crop, and results in environmental pollution via acidification, eutrophication, etc. Intake of phosphate contaminated water causes serious health problems. P is a non-renewable natural resource, mined as rock phosphate and its global reserves may be depleted in 50–100 years (Cordell et al. 2009). Thus, dependence of contemporary agriculture on P fertilizers, poses major food security and sustainability challenge (Udert 2018). Strategies to

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optimize P use in agriculture needs to be accorded high priority. Improvement of P use efficiency (PUE) in cereal crops could be a major tool to achieve this goal.

Plants have developed a wide range of strategies to adapt to Pi deficiency. These include changes in plant morphology (reduced primary root growth, enhanced number of lateral roots and root hairs); biochemical changes (anthocyanin and starch accumulation), and secretory proteins induction (induction of acid phosphatases, organic acid and RNase secretion). At molecular level, increased expression of Pi transporter genes, differential expression of transcription factors and expression of Pi responsive microRNAs (miRNAs) are also associated with adaptation response to Pi stress (Misson et al. 2005; Nguyen et al. 2015). In the last decade, significant progress has been made in identifying signaling components and their role in responses to Pi starvation in plants (Yuan and Liu 2008). Under Pi deprived condition, the transcriptional regulation of a set of Pi responsive genes occurs through various transcription factors by binding to their respective *cis*-targets present in the promoter region of these Pi responsive genes. Different transcription factors known to be involved in Pi responses include PHR1, ZAT6, WRKY75, WRKY6, MYB26, and BHLH32 in *Arabidopsis*; OsPHR2 and OsPTF1 in rice; ZmPTF1 in maize (Chen et al. 2009; Li et al. 2011) etc. Among these, PHR1 (PHOSPHATE STARVATION RESPONSE1), a MYB domain-containing transcription factor, is one of the most characterized. In recent years, some SPX domain-containing proteins (nuclear proteins) have also been shown to act as important feedback regulators of PHR2. The SPX domain-containing proteins are themselves activated by OsPHR2 under Pi starvation in both *Arabidopsis* and rice (Wang et al. 2009, 2014; Liu et al. 2010). PHR1 is itself regulated post-translationally through the action of SIZ1. SIZ1 is a SUMO E3 ligase that is localized in the nucleus. In *Arabidopsis*, it has been shown that PHR1 is the direct target of SIZ1 and the Phosphate Starvation Induced (PSI) genes were found repressed in *siz1* mutant even in Pi deficiency (Miura et al. 2005). Beside these adaptive responses, acid phosphatase (APase) is also an important participant in mobilization and utilization of organic P under Pi deprived condition (Tran et al. 2010; Tian and Liao 2015). These secreted APases are involved in release of Pi from organophosphates in the plant external environment and increase the availability of Pi to be absorbed by plant root. Several PSI secreted APases have been characterized in vascular plants, like lupin (Ozawa et al. 1995; Li and Tadano 1996; Miller et al. 2001), tobacco (Lung et al. 2008), common bean (Liang et al. 2010), tomato (Bozzo et al. 2006), and *Arabidopsis* (Veljanovski et al. 2006; Wang et al. 2011). In *Arabidopsis*, 11 out of 29 members of Purple Acid Phosphates (*AtPAP*)

are transcriptionally up-regulated by Pi starvation (Zhu et al. 2005; Wang et al. 2011).

Among the cereals, maize is the most widely produced and consumed cereal. Owing to its emergence as an industrial and feed crop; with rising worldwide demand, improvement of PUE in maize is likely to have a major impact on sustainability. Identification of key regulatory genes playing pivotal role in acquisition, transportation and utilization of Pi in maize could pave way for greater understanding of this phenomenon in this important crop. In the present study, apart from characterizing the physiological effects of Pi starvation, we have identified orthologs of *Arabidopsis* Pi-responsive genes in maize using *in-silico* approaches such as sequence homology and protein functional domain analysis. Now whole genome transcriptome data for Pi stress response in maize is also available (Du et al. 2017; Nie et al. 2021). A total of 246 genes were upregulated in a phosphate stress tolerant maize inbred line (Jian et al. 2017). Most of the Pi responsive genes identified through global transcriptome profiling in maize remain unconsolidated in mutant backgrounds. In *Arabidopsis*, the model for Pi homeostasis has been extensively tested using mutant and overexpression lines. We selected only these key *Arabidopsis* validated genes and extrapolated the model in a Pi stress tolerant tropical maize inbred line. In addition, expressions of identified Pi responsive genes have been analyzed in root and leaf under Pi starvation. Our study revealed the differential expression of identified Pi-responsive genes belonging to different metabolic pathways under phosphate deprived conditions in maize. To attribute a basis to observed differential expression of the Pi-responsive genes under phosphate deprived conditions, *cis*-regulatory elements present in the upstream promoter region of the selected two genes have also been predicted *in-silico*. Finally, a graphical abstract of transcriptional regulatory model for the Pi starvation responses in maize has been proposed on the basis of present study of selected maize regulatory genes and the previously reported literature (Fig. 1).

Materials and methods

Plant material and growth conditions

Pi stress tolerant maize (*Zea mays* L.) inbred line HKI-163 was used in this study (Ganie et al. 2015). The seeds were soaked in distilled water for 4 to 6 h and rinsed 4 to 5 times with sterile water followed by surface sterilization by washing with 70% ethanol for 2 min. The seeds were again washed with sterile water (4 to 5 times) followed by treatment with 0.1% HgCl₂ for 10 min. Seeds were washed again five times with sterile water. The seeds were kept in

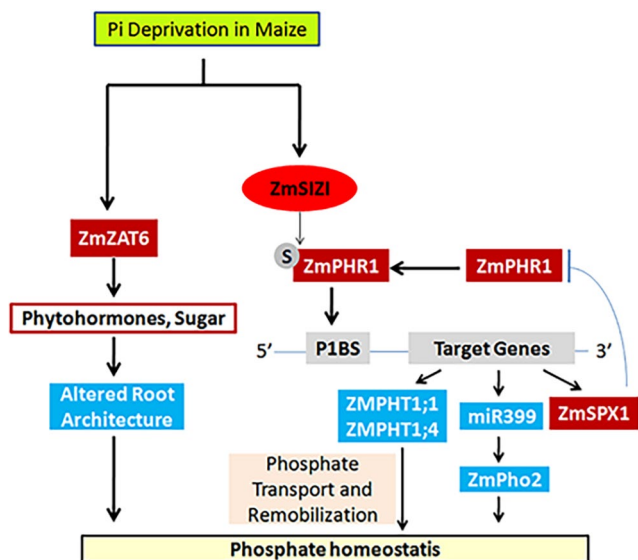


Fig. 1 Graphical abstract of the study. Diagram shows regulatory genes and their downstream outcome involved in phosphate deprived condition in maize plant. The hypothesis is derived from sequence and domain similarity of studied maize genes with *Arabidopsis* and their real time expression analysis in shoot and root tissues and the expression similarity with characterized genes. Red colored boxes show regulatory genes and blue boxes denote downstream Pi responsive genes studied in our work. Small gray round circle denotes for sumoylation of PHR1 (phosphate starvation response 1), a transcription factor. P1BS is the cis-regulatory element found on promoter region of many phosphate responsive genes, where PHR1 transcription factor binds and regulate their expression

a sterile germinating paper and then kept for germination at $30 \pm 2^\circ\text{C}$ for 4 days in dark. The resulting seedlings were transferred to a high-Pi (1 mM KH_2PO_4) nutrient solution and allowed to grow for 2–3 days, after which the seedlings were carefully removed and two sets were prepared for the study. The first set was transferred to modified Hoagland's hydroponic solution with low Pi (LP) - basal medium + 5 μM KH_2PO_4 and 1 mM KCl. The second set was transferred to modified Hoagland's hydroponic solution with high or sufficient Pi (SP) - basal medium + 1 mM KH_2PO_4 (Li et al. 2007; Jiang et al. 2017). Both the sets were allowed to grow for 21 days hydroponically under LP and SP conditions. The nutrient solution was replaced every 3 days. The plants were grown at $25\text{--}30^\circ\text{C}/20\text{--}25^\circ\text{C}$ (day/night) with a photoperiod cycle of 14 h of light ($500\text{--}600 \mu\text{mol m}^{-2} \text{s}^{-1}$) and relative humidity of 65% in a phytotron.

Phenotyping

Six plants from each of the two Pi treatments were harvested after 21 days of treatment and washed with water. The plants were placed in paper bags and dried at 60°C for 48 h. Fresh and dry weights of shoots and roots were determined using an analytical balance (Mettler Scientific, Highstown, NJ). The shoot and root lengths were measured with meter scale. The plants were photographed and the root architecture/ root hair density was studied using phase contrast microscope (Olympus CX41).

Estimation of plant Pi content

The Pi concentration accumulated in LP and SP grown plants was determined as per Murphy and Riley (1962). Briefly, 10 mg of plant part was crushed with the help of mortar and pestle and 8 ml sterile water was added. A total of 1.6 ml of the mixed reagent (125 ml of 5 N sulphuric acid, 1.5 g ammonium molybdate, 75 ml of 0.1 M ascorbic acid solution and 12.5 mg potassium antimonyl tartrate dissolved and made up the volume to 250 ml) was added and diluted to 10 ml volume with water. Standard curve was made by using 0.2 μg , 0.4 μg , 0.8 μg , 1.6 and 3.2 μg Pi/ml standard Pi solutions (0.1757 g of potassium dihydrogen phosphate in 1 l of sterile water amounting to 40 mg Pi/l) in 10 ml of final volume. The reaction was mixed well. After 10 min, the reaction mixture was centrifuged at 6000 rpm for 5 min. Supernatants were taken in other labeled tubes. Optical density was measured at 882 nm using 1 ml cuvette. Blank reading was determined by freshly distilled water with mixed reagent. Plant Pi content (expressed as μg Pi/mg of dry weight of the plant part) was calculated as per the standard graph.

Bioinformatic Analysis

Maize genome sequences were downloaded from www.maizesequence.org/index.html, and the National Center for Biotechnology Information (NCBI) genome databases. Sequences of *Arabidopsis* genes characterized for Pi responsiveness and the regulatory genes were used as queries to search against the maize protein database with BLASTP program. Hits with Expectation (E)-values below 0.0001 were selected for further domain analysis. All selected sequences from BLAST program were analyzed by functional domain similarity, as confirmed by SMART (<http://smart.embl-heidelberg.de>) and Pfam (<http://pfam.sanger.ac.uk/>) databases (Finn et al. 2006; Letunic et al. 2009). The PLACE website (<http://www.dna.affrc.go.jp/>)

[PLACE/signalscan.html](#)) was used to predict *cis*-elements in the probable promoter regions of Pi responsive genes in the 1000-bp genomic DNA sequences upstream of the initiation codon (Higo et al. 1999).

Primer design and specificity checking

The primers were designed using Primer 3 software provided by NCBI online tools. The specificity of the primer pair sequence was checked against maize genome transcripts (CDS) from the NCBI database using the BLAST program. The primers used are listed in Supplementary Table S1.

RNA isolation and DNase Treatment

Total RNA was isolated from the root and the leaf samples from LP and SP grown plants harvested at 21 days after treatment (DAT). Approximately, 100 mg of root and leaf samples were crushed in liquid N₂ using mortar pestle separately. After evaporation of liquid N₂, 700 µl of TRIzol reagent (Thermo Fisher Scientific, USA) was added directly to the mortar pestle and allowed to thaw. After liquefaction of the sample, another 700 µl of TRIzol was added and transferred to a 2 ml centrifuge tube and kept for 2–3 min at room temperature. Further, 300 µl of chloroform was added and the tubes were capped securely and shaken vigorously. The samples were centrifuged at 12,000 rpm for 15 min at 4 °C. The aqueous phase was carefully transferred to a fresh RNase-free centrifuge tube. RNA was precipitated by adding equal volume of isopropyl alcohol. This mixture was incubated at room temperature for 10 min followed by centrifugation at 12,000 rpm at 4 °C for 5 min. The supernatant was discarded and the pellet obtained was washed with 1 ml of 75% ethanol per 1 ml of TRIzol reagent used by tapping only. Subsequently, the samples were vortexed briefly and centrifuged at 12,000 rpm for 5 min at 4 °C. The RNA pellet obtained was air dried for 5–10 min. The RNA was dissolved in 40 µl RNase-free diethyl pyrocarbonate (DEPC) treated water. About 1 µl of DNase solution (8 µl of 10X DNase buffer, 3U DNase mixed in H₂O) was added to 10 µl of the RNA sample and kept at 37 °C for 10 min. The digested RNA was column purified before reverse transcription reaction.

Reverse transcription-polymerase chain reaction (RT-PCR)

The DNase treated RNA was used for the synthesis of first strand cDNA using Superscript III reverse transcriptase kit

(Invitrogen, USA). Before performing the RT reaction, 4 µg total RNA was mixed with 1 µl of dNTPs and 1 µl of OligodT primer and incubated at 65 °C for 5 min. The tubes were immediately transferred on ice and kept for at least 1 min. The cDNA synthesis mix (1X RT buffer, 1.25mM MgCl₂, 10 Mm dithiothreitol (DTT), 1 U RNaseOUT recombinant ribonuclease inhibitor and 1U SuperScript III RT) was added to the RNA. The reaction was incubated at 50 °C for 50 min, followed by heat treatment at 85 °C for 5 min. Finally, the samples were treated with 1 µl RNase H at 37 °C for 20 min. These cDNA samples were used for quantitative real-time PCR (qRT-PCR) and semi-quantitative RT-PCR analyses.

Quantitative real-time PCR (qRT-PCR) conditions and analyses

The qRT-PCR was carried out to know the relative transcript levels of the Pi responsive genes in maize in response to high Pi and low Pi conditions treatments. The qRT-PCR was performed in triplicates using the Brilliant II SYBR Green QPCR Master mix (Agilent) in real time PCR (Agilent Technologies, USA) detection system. A 20 µl reaction- mixture containing 10 µL of SYBR[®] Green premix, 0.25 µM of each primer pair (Supplementary Table S1) and 2.0 µl cDNA template was gently mixed in 96-well Real-Time PCR plate, and centrifuged at 200 rpm for 2 min to spin down all reaction components in the plate. The reactions were carried out with the following thermal profile: 50 °C for 2 min, 95 °C for 3 min, followed by 40 cycles of 95 °C for 10 s, 60 °C annealing for 20 s. After 40 cycles, the specificity of the amplifications were tested by heating from 65 to 95 °C with a ramp speed of 1.9 °C/min, resulting in melting curves. The reference control genes were measured with three replicates in each PCR run, and their average cycle threshold (CT) was used for relative expression analyses. The *Actin* gene from maize was used as reference gene to normalize the expression values. The log₂ fold change value was calculated based on 2^{-(ΔΔCt)} method.

Semi-quantitative RT-PCR

The optimum numbers of cycles for semi-quantitative RT-PCR were worked out by using real time PCR amplification curve, so that optimum difference between control and treated could be visualized in semi-quantitative PCR amplification. For semi-quantitative analysis, PCR amplifications were performed in 25 µl of total volume reaction containing 100 ng template cDNA, 1X Taq Polymerase buffer, 1.5 mM MgCl₂, 1 mM dNTPs, 0.4 µM of each forward

and reverse primer pair and 2U of Taq DNA polymerase. The PCR amplification was achieved in a BioMetra thermal cycler with amplification conditions of 94 °C for 3 min (1 cycle) followed by 30 cycles of 94 °C for 30 s; 60 °C for 30 s; 72 °C for 30 s and finally 72 °C for 5 min extension. PCR product (10 µl) was analyzed by electrophoresis on 3% agarose gel along with 50 bp DNA ladder. The bands were detected by ethidium bromide staining and photographed under UV light using the Gel Documentation system (Alpha Innotech).

Statistical analysis

Analysis of variance (ANOVA) was performed using OPSTAT analysis software (<http://14.139.232.166/opstat/default.asp>). One-way analysis of variance was used to evaluate whether significant difference existed at $P \leq 0.05$ between the SP and the LP treatments; among leaves and roots. The means in all these analyses were separated using the least significant difference test at $P < 0.05$. The relative expression levels of genes were calculated as per Livak & Schmittgen (2001). Standard errors and standard deviation were calculated from replicates and significance was measured at the level of $P \leq 0.05$.

Results

Effect of Pi starvation on plant growth and Pi accumulation

After 21 days of phosphate treatment, the plants grown under LP conditions developed observable symptoms of Pi starvation with respect to shoot growth, root architecture, lateral roots, root hairs, leaf coloration and stems (Fig. 2; Table 1). Under Pi starvation, the shoot fresh and dry weight decreased by 33.2 and 29.9%, respectively, while the root fresh and dry weight decreased by 26.1 and 8.1%, respectively, corroborating the role of Pi in biomass accumulation. The overall plant fresh and dry weight reduced by 29.9 and 26.8%, respectively. The shoot length and stem girth were reduced by 14.7 and 21.0% respectively. There was a pronounced increase in root length due to Pi starvation. The average root length increased significantly by 85% ($P < 0.05$). Also, as discerned by phase-contrast microscopy (Fig. 2e), there was a proliferation of root hairs in the roots of Pi starved plants. Pi starvation also led to lesser number of crown roots (Fig. 2c; Table 1) and lengthier lateral roots (Fig. 2d). Further, the Pi stress treatment was confirmed by measuring the levels of endogenous Pi content in leaves and roots of the plant grown under Pi sufficient and deficient conditions. Under Pi stress (LP) conditions, both shoots and

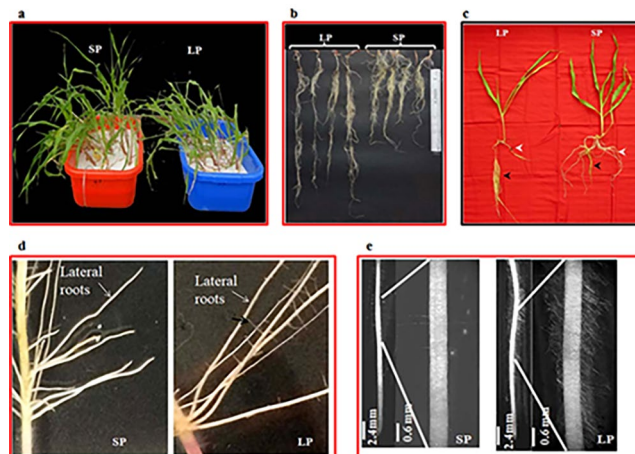


Fig. 2 Effect of phosphate availability on plant growth and root architecture in a phosphate stress tolerant maize genotype HKI-163 grown in two growth conditions- deficient (LP; 5 µM phosphate) and sufficient (SP, 1mM phosphate). (a) Plant growth under hydroponics in SP and LP conditions. (b) Root length under SP and LP conditions. (c) Root architecture. Black arrow: main root with lengthy lateral roots (in LP condition) and less lengthy lateral roots (in SP condition). White arrow: crown roots, more in numbers under SP condition and less in LP. (d) Close-up of roots under LP and SP treatment. White arrow: main root with lengthy lateral roots (in LP condition) and less lengthy lateral roots (in SP condition). (e) Phase contrast microscopy images of root hairs- main root with short and less root hairs (in SP condition) and lengthy and more root hairs (in LP condition). Photographs were taken after 21 days of treatment

Table 1 Physiological parameters of maize genotype HKI 163 grown under low phosphate (LP) and sufficient phosphate (SP) conditions for 21 days

Parameter	SP	LP
Plant fresh weight (g/plant)	10.21 ± 0.91	7.16 ± 0.59****
Plant dry weight (g/plant)	1.02 ± 0.75	0.75 ± 0.06 ^{ns}
Root fresh weight (g/plant)	1.11 ± 0.12	0.82 ± 0.14**
Root dry Weight (g/plant)	0.16 ± 0.01	0.15 ± 0.03 ^{ns}
Shoot fresh weight (g/plant)	9.12 ± 0.82	6.09 ± 0.43****
Shoot dry weight (g/plant)	0.86 ± 0.06	0.60 ± 0.05****
Root: Shoot Ratio	0.12 ± 0.01	0.13 ± 0.02 ^{ns}
Root length (cm)	30.5 ± 1.45	56.33 ± 1.97****
Shoot length (cm)	58.00 ± 1.03	49.50 ± 1.63****
Stem girth (mm)	23.83 ± 1.85	18.83 ± 1.14***
Number of crown roots	10.17 ± 1.01	0.17 ± 0.17****

All the values shown here represent the mean ± SD of six plants

**** $P < 0.0001$ Extremely significant

*** $P = 0.0001$ to 0.001 Extremely significant

** $P = 0.001$ to 0.01 Very significant

* $P = 0.01$ to 0.05 Significant

^{ns} $P \geq 0.05$ Not significant

roots accumulated $< 0.1 \mu\text{g Pi mg}^{-1}$ of dry weight biomass, in contrast to accumulation of approximately $1 \mu\text{g Pi mg}^{-1}$ of dry weight biomass under Pi sufficient (SP) conditions

(Fig. 3). This confirms the efficacy of the hydroponic experiment in imposing quantifiable Pi stress on the plants.

Comparative sequence and domain analysis of potential Pi- starvation responsive genes in maize

For identification of potential Pi-responsive genes, three lines of evidences were used in this study. First, a comparative sequence analysis was carried out to identify potential maize orthologs of the reported Pi-responsive genes from *Arabidopsis thaliana* with 52 to 93% sequence similarity and up to 99% query coverage. Utilizing minimum E-value criteria, most of the selected genes from *A. thaliana* had clear orthologs in *Z. mays* (Supplementary Table S2). Secondly, the isolated sequences were subjected to Pfam and SMART databases for functional domain analysis. We found that the corresponding maize genes had largely the same domain architecture, position wise and structure wise, as seen in their counterpart in *Arabidopsis* (Supplementary Fig S1-S3). The genes could be grouped into three major classes on the basis of their functions, viz., the genes involved in regulation; the major secretory proteins; and the Pi transporters. Four major regulatory genes were included in the analyses. The *PHR1* gene is a transcription factor involved in regulating a subset

of the genes which are responsive to Pi starvation (Rubio et al. 2001; Bari et al. 2006). The maize *PHR1* gene has the myb-DNA binding domain like *Arabidopsis*, in between 200 and 400 amino acid residues at C terminal of the protein. The maize SPX1 domain containing protein, also has the same domain architecture as AtSPX1 protein at amino terminus, with only difference that the domain size was slightly larger as compared to AtSPX1. SIZ1, a E3 SUMO-protein ligase has domain architecture like their counterpart in *Arabidopsis* i.e. presence of all the functional domains viz. the SAP domain, the DNA-binding domain, the plant homeodomain (PHD), a ubiquitin ligase and Zinc finger (Znf) domain containing multiple finger-like protrusions for salt bridge formation. Another important regulatory protein ZmPho2- an ubiquitin-conjugating enzyme E2, catalytic (UBCc) domain containing protein was found identical to corresponding *A. thaliana* protein at C terminal. ZAT6- the Zinc finger domain containing transcription factor was also found to possess same domain architecture (Supplementary Fig S1). Among the secretory proteins, PAP10 and PAP17 possessed the entire metallophos domain at proper position as AtPAP10 and AtPAP17 with only a slight difference in PAP17 metallophos domain. The maize PAP2 and ZmRNS1 had the same SANT domain and ribonuclease_T2 domain respectively as in AtPAP2 and AtRNS1 (Supplementary Fig S2). Both the high affinity transporters (the MFS: major facilitator superfamily) studied had the similar sugar transporter-like domain and 12 trans-membrane domains as other MFS transporters (Supplementary Fig S3). The third and the final line of evidence towards identification of Pi starvation induced genes were based on actual transcript expression analysis of the genes, as presented further.

Expression pattern of regulatory genes involved in Pi starvation response in a Pi stress tolerant maize genotype.

In order to confirm the genes identified by sequence homology and domain analysis, semi-qRT and real-time PCR analyses were conducted in parallel to verify the validity of these genes. Five regulatory genes that play central role in the Pi homeostasis- *ZmPHR1*, *ZmSPX1*, *ZmSIZ1*, *ZmPho2* and *ZmZAT6* were selected for further validation. These genes were chosen because they had been previously implicated to be involved in regulatory pathways modulating Pi starvation response, or because of their possible contribution to Pi homeostasis in plants under Pi deficiency (Rouached et al. 2010). The transcription levels of mRNA were significantly higher for all the genes at LP conditions compared with SP (control) conditions in both root and leaf, except *ZAT6*, which was specifically up regulated in leaves only. Real-time PCR analysis (Fig. 4) revealed that under Pi starvation, *ZmPHR1*, *ZmSPX1*, *ZmSIZ1*, *ZmPho2* and *ZmZAT6* genes were 4.28, 55.01, 3.57, 5.41 and 4.18

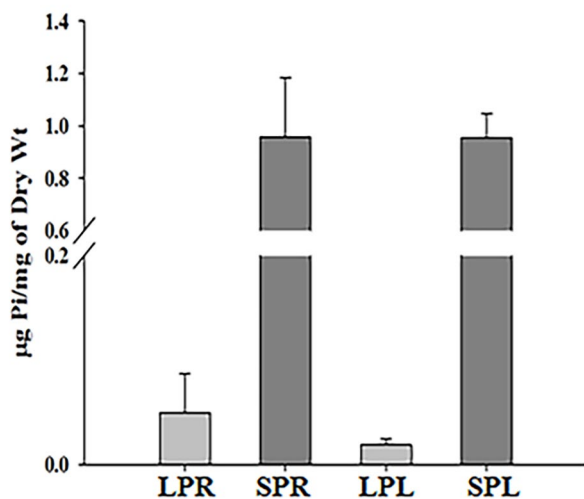


Fig. 3 Effect of phosphate availability on maize Pi accumulation. To determine the phosphate content of plants, plants were harvested, dried, and total phosphorus was assayed by method described by Murphy and Riley. Total Pi content is given in $\mu\text{g Pi/mg}$ of dry weight of the plant part. LPR, Maize root under Low Phosphate condition; SPR, Maize root under Sufficient Phosphate condition; LPL, Maize leaf under Low Phosphate condition; SPL, Maize leaf under Sufficient Phosphate condition. Values shown represent the mean of six plants and triplicate observation from each sample. Error bars represents standard deviation (SD) at $P < 0.05$

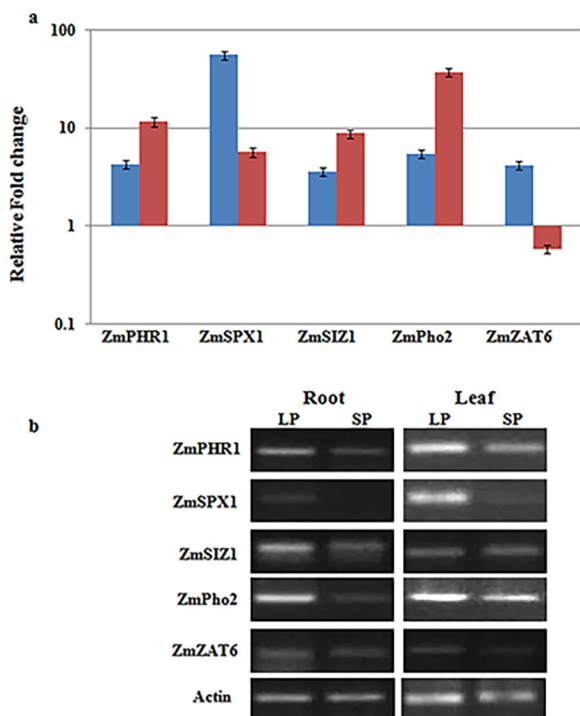


Fig. 4 Expression analysis of regulatory genes involved in phosphate responsive pathway. (a) The qRT-PCR based expression analyses of predicted regulatory genes at phosphate deprived conditions in HKI-163 inbred line of maize grown under phosphate deprived ($5 \mu\text{M KH}_2\text{PO}_4$) and sufficient phosphate conditions ($1 \text{ mM KH}_2\text{PO}_4$). Y-axis represents the relative fold change values at low phosphate condition as compared to respective genes at sufficient phosphate conditions. Significance was measured at the level of P value ≤ 0.05 . (b) Semi-quantitative RT-PCR expression analyses using same set of primers at 30 PCR cycles of same genes for re-confirmation

times overexpressed in the leaf. In the roots, the *ZmPHR1*, *ZmSPX1*, *ZmSIZ1*, *ZmPho2* genes were 11.49, 5.64, 8.76, 36.79 times over expressed, while *ZmZAT6* was under expressed 0.58 times. The above results also correlate to the band intensities of the respective amplicons as visualized in the semi-qRT-PCR.

Pi starvation mediated modulation of PAPs and RNase genes involved in P solubilization and remobilization

As an initial characterization of gene expression in maize plants, an expression pattern of 4 selected genes (*ZmPAP2*, *ZmPAP10*, *ZmACP5* and *ZmRNS1*) were examined using qRT-PCR and RT-PCR in leaf and root tissues (Fig. 5). The differential/varied expression levels of these genes were found in the leaves and roots as expected, except *PAP10* gene which was down regulated in the roots. As expected, the expression of *ZmPAP2* was slightly upregulated in both leaf and root tissues. The expression level of putative *PAP10*

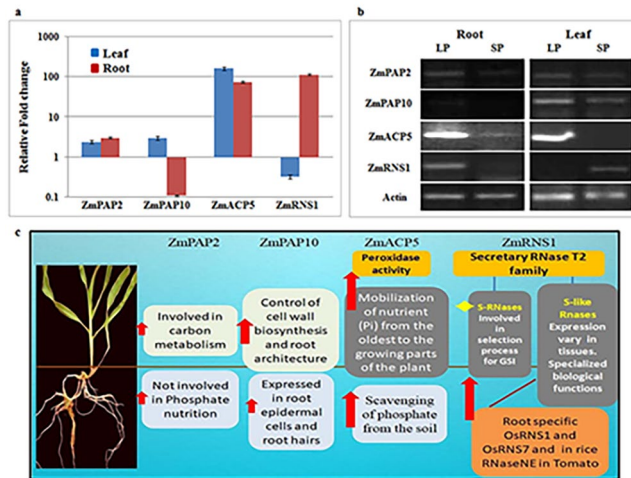


Fig. 5 Expression analysis of purple acid phosphatases (PAPs) and ribonuclease (RNase) genes involved in phosphate responsive pathway. (a) The qRT-PCR based expression analyses of predicted PAPs and RNase genes at phosphate deprived conditions. Y-axis represents the relative fold change values at low phosphate condition as compared to respective genes at sufficient phosphate conditions. Significance was measured at the level of P value ≤ 0.05 . (b) Semi-quantitative RT-PCR expression analyses using same set of primers at 30 PCR cycles of the same genes for re-confirmation. (c) A schematic representation of function and expression on known PAPs and RNase. Red arrow mark shows expression of respective known genes

was slightly high (2.97 fold) in leaf but extremely low (0.11 fold) in root tissue at Pi deprived condition as compared to sufficient Pi condition. RT-PCR showed that both *ZmACP5* and *ZmRNS1* mRNA levels were strongly upregulated in the root tissue (74.40 and 115.09 fold respectively). As expected, the expression level of *ZmACP5* in leaf tissue was high, while the *ZmRNS1* was down regulated in leaves.

Modulation of high affinity Pi transporters

Particularly high affinity Pi transporters are expected to perform crucial functions in Pi acquisition and remobilization at Pi deficiency. With a particular interest in these genes, we chose two genes (*ZmPht1;1* and *ZmPht1;4*) on the basis of sequence similarity with *A. thaliana* and the domain analysis. Both these genes were upregulated in leaves and roots under Pi deprived condition with varied level of expression. *ZmPht1;4* showed a higher level of expression in root tissues (fold expression in root was 22.61 fold whereas 5.34 fold in leaf) whereas *ZmPht1;1* was expressed more in leaf tissues (169.55 fold expression in leaf and 30.45 fold in roots) in response to Pi stress (Fig. 6).

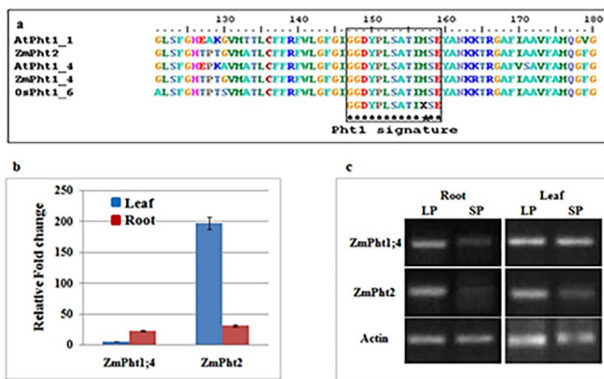


Fig. 6 Expression analysis of high affinity Pi transporters in maize. (a) Alignment of the peptide sequences of known high affinity Pi transporters from *Arabidopsis*, and rice with identified high affinity phosphate transporters of maize; identical peptides are highlighted in the same color. The *Pht1* signature sequence is shown in the box. (b) Semi-quantitative RT-PCR expression analyses using the same set of primers at 30 PCR cycles of the same genes for re-confirmation. (c) The qRT-PCR based expression analyses of the predicted high affinity Pi transporters genes at phosphate deprived conditions. The Y-axis represents the relative fold change values at low phosphate condition as compared to respective genes at sufficient phosphate conditions

Discussion

Maize is a widely cultivated grain crop and a major source of feed, food and industrial raw material. The identification of Pi starvation-induced genes and delineating the fundamental molecular-genetic mechanisms in Pi stress response are important steps towards engineering high PUE in this crop. A few regulatory genes and a many other Pi responsive genes have been identified as adaptation components of Pi starvation in plants, especially in model plants like *Arabidopsis* (Lopez-Arredondo et al. 2014). However, identities and functional biology of majority of Pi stress responsive genes and processes in maize, especially so in the tropical and the sub-tropical genotypes, remained sketchy. In this study, we chose a tropically adapted maize inbred line (HKI-163) that is tolerant to Pi stress and determined its physiological and molecular response to Pi starvation under controlled conditions.

As roots are the major tissues involved in Pi uptake, root growth and morphology is dramatically changed when Pi is deprived in the root zone. These changes lead to increase in root length, increase in the root to shoot ratio and lateral root length. All these measures are the adaptive response to Pi deficiency. The relationship between Pi assimilation efficiency and root morphology has been analyzed in maize (Peret et al. 2011). Consistent with these findings, our studies demonstrate increased root length, reduced total biomass, proliferation in root hair, increase in the length of the lateral and nodal roots, increased root: shoot ratio and reduced level of total Pi uptake in root and shoot

significantly (P value < 0.05) at low Pi condition compared to sufficient Pi condition (Figs. 2 and 3; Table 1). Our physiological data with respect to Pi starvation were consistent with previous studies on maize plants subjected to low Pi stress for 17 days (Li et al. 2007). The HKI-163 inbred line has been reported to exhibit higher shoot dry weight, total plant biomass, root length, root dry weight, leaf area, total P uptake and PUE as compared to Pi stress sensitive maize genotypes (Ganie et al. 2015). Survival adaptation by reinforcing genetic variation has been observed in nutrient deficiency response in other cereal crops (Sharma et al. 2021) and needs to be studied with reference to Pi deficiency in maize as well.

At a molecular level, Pi deficiency is regulated both at the transcriptional and post-transcriptional levels. So far, the major actor coordinating these various regulations is PHR1, via the PHR1-PHO2-miRNA399 pathway, conserved amongst flowering plants. Beside this transcription factor, few other regulators have also been identified including ZAT6, WRK75, bHLH32 and MYB62 (Yi et al. 2005; Chen et al. 2007; Devaiah et al. 2009). In this study, we selected ortholog of PHR1, SPX1, SIZ1 and PHO2-genes contributing in PHR1 regulation pathway and another less studied TF, ZAT6. Study of these genes with their sequence similarity with *Arabidopsis*, functional domain analysis and finally expression analysis in root and leaf tissues by semi-quantitative RT-PCR and quantitative real-time PCR (qRT-PCR) reveal that these selected accessions might be involved in Pi stress response in maize and might work as previously described regulators.

From the maize genome, we took a highly similar sequence ortholog to *Arabidopsis* PHR1 gene followed by domain analysis (Supplementary Table S2 and Supplementary Fig S1-S3). Interestingly, the rice genome contains two PHR1-like genes, both reported as involved in Pi starvation (Zhou et al. 2008). In the present study, we also found two PHR1 like gene sequences in maize but we selected only one highly similar sequence for further study. The identified ZmPHR1 had 62% sequence similarity with AtPHR1. Domain analysis showed MYB domain at C terminal end of the protein as previously characterized in *Arabidopsis* PHR1 and the real time expression was observed as expected. MYB-like domain of PHR1 binds to a DNA motif GNATATNC, termed P1BS (Rubio et al. 2001), which is present in the promoter of many Pi starvation-induced genes and regulate their expression (Mission et al. 2005; Müller et al. 2007). Furthermore, it has been shown that PHR1 affects expression of miRNA399 and consequently expression of PHO2 which is involved in Pi homeostasis (Fujii et al. 2005; Bari et al. 2006; Chiou et al. 2006). Therefore, *Pho2* is an indirect target of PHR1 TF via miRNA399. Bioinformatically, we analyzed the miRNA 399 target site on *ZmPho2*

gene and P1BS motif on the probable promoter region of miRNA 399 conjecturing that *ZmPho2* might be the indirect target for PHR1 and may have possible role in PHR1 signaling. However, our real time expression data did not support this. So it might be possible that there is another *Pho2* like element present in maize or some other sequence in the maize genome that works as *Pho2*.

Besides *Pho2*, SPX1 is direct target of PHR1. In the presence of Pi, SPX1 displays high binding affinity to PHR1 and sequesters it, so that binding of PHR1 to its target genes via P1BS is inhibited, and their transcription (including that of SPX1) is just at basal level. While in the absence of Pi, the affinity of SPX1 to interact with PHR1 is reduced, so PHR1 is free to interact with its targets, resulting in induced expression of the target genes including SPX1 (Puga et al. 2014; Yao et al. 2014; Zhang et al. 2016). As a result, there is increased expression of SPX1 at low Pi condition. High SPX1 protein levels allow rapid shutdown of PHR1 target gene expression after Pi re-feeding but at low Pi, the affinity of SPX1 is very low, while at constant Pi stress there is high level expression of SPX1 but in inactive form (Puga et al. 2014). Evidence for the importance of the P1BS motif in the promoter region of PHR1 targeted genes has been observed in monocot species (Schünmann et al. 2004). In the present study, beside the sequence similarity and domain analysis of the ZmSPX1 protein, the P1BS motif GNATATNC (PHR1 binding site on the target gene promoter) was also found on the –151 bp position of the ZmSPX1 promoter (Supplementary Table S2, Supplementary Fig S1 and Fig. 7). So it is a good indicator that the selected sequence encode ZmSPX1 gene. The expression pattern of the ZmSPX1 gene correlates with ZmPHR1 expression data in both root and leaf (Fig. 4). Hence, our study is in agreement with previous studies in *Arabidopsis* with respect to its expression pattern (Puga et al. 2014).

Under low Pi conditions, PHR1 TF is sumoylated by SIZ1, a SUMO E3 ligase that is localized in the nucleus, and this post-translational modification is likely important for PHR1 activity. Sumoylation of PHR1 controls expression of Pi starvation-responsive genes (Miura et al. 2005), although the mechanism of this regulation is still unknown. So the expression of PHR1 and its target genes should be directly correlated with the expression of SIZ1 gene. The up-regulation of the expression pattern of the ZmSIZ1 gene in both root and leaf tissue in the present study also supports this in maize (Fig. 4). Pi stress is known to trigger a significant increase in SUMO-protein conjugate levels (Kurepa et al. 2003; Miura et al. 2007). SIZ1 is also involved in the regulation of root growth in response to Pi starvation (Miura et al. 2005).

Beside, PHR1 microarray analysis revealed that ZAT6, a Cys-2/His-2 (C2H2) zinc finger transcription factor, has also

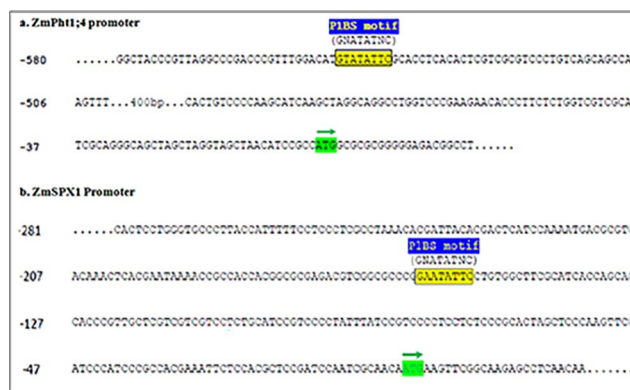


Fig. 7 ZmPht1;4 and ZmSPX1 promoter containing P1BS motif required for ZmPHR TF binding. 1000 bp region upstream to the ATG start site of ZmSPX1 gene was scanned for the presence of P1BS (GNATATNC) motif, a SPR1 binding site, by using a MEME online motif finder software. The P1BS motif were found at -542 and -151 bp of the promoter of ZmPht1;4 and ZmSPX1 respectively. Translational start site (ATG) is shown as green highlighted, ATG and green arrow indicates direction of translation. P1BS motif is shown as yellow colored box

been implicated in the regulation of Pi starvation responses and strongly induced during Pi deprivation (Rubio et al. 2001). This gene plays a vital role in seedlings by regulating growth and Pi homeostasis. ZAT6 is a gene induced during Pi starvation that responds rapidly and specifically to the altered Pi status of plants (Devaiah et al. 2007). In the present study, we observed increased expression of ZmZAT6 in maize leaf (Fig. 4) that is in agreement with the *Arabidopsis* microarray data. On the other hand, its reduced expression in roots as the plants grow older allows the primary roots to elongate (Devaiah et al. 2007). In the present study, the expression of the same gene was slightly reduced in root tissue at 25 days of Pi stress (Fig. 4). So the present data in root tissue at 25 days suggest that this gene is reduced to allow root elongation at low Pi at this stage and participate in the root development whereas an increased expression in the leaf suggest its role in Pi-homeostasis in the shoot part. These selected Pi responsive genes make two different regulatory pathways, first PHR1 regulated and second ZAT6 regulated mechanisms for Pi homeostasis and Pi management under Pi stress condition.

Pi uptake by plants operates via two systems, high and low affinity Pi transporter system (Furihata et al. 1992). The high affinity transport system is largely mediated under Pi deprived conditions by plasma membrane-localized Pi transporters belonging to the *Pht1* family (Raghothama 2000). PHT1 belongs to major facilitator super family (MFS) proteins, encoding high-affinity H⁺/Pi co-transporters. It has been previously reported that PHT1 Pi transporters play a critical role in Pi acquisition from soil solution and Pi remobilization within the plant (Gu et al. 2016). In the present study, *ZmPht1;1* and *ZmPht1;4* sequences were

found to share a high level of similarity to known *Arabidopsis* and rice high affinity Pi transporters and also have the GGDYPLSATIxSE signature sequence (Fig. 6a). Promoter scan by using MEME (Lescot et al. 2002) revealed that Pht1;4 transporter had the P1BS motif GNATATNC (PHR1 binding site on the target gene promoter) at -542 (Fig. 7a) whereas the promoter of *ZmPht2* does not have any P1BS motif which indicated that the expression of these transporters might be PHR1 dependent and independent respectively. *ZmPht1;4* might be regulated by PHR1 transcription factor in a similar manner as described previously for known high affinity Pi transporters (Bustos et al. 2010; Nilsson et al. 2010; Oropeza-Aburto et al. 2012). Functional characterization shows that some of the *Pht1* members, such as *AtPht1;1*, *AtPht1;4* and *OsPht1;6* are high-affinity transporters while others are low-affinity transporters, such as *OsPht1;2* (Shin et al. 2004; Ai et al. 2009). Previously, it was confirmed that *HvPht1;1*, *CmPht1* and a number of *Pht1* family transporters are strongly expressed in the root, and are induced by Pi starvation (Ai et al. 2009; Jia et al. 2011; Wu et al. 2011; Liu et al. 2014). Like *AtPht1;1*, *HvPht1;1* and other root specific high affinity transporters, *ZmPht1;4* also exclusively expressed in the root tissues (Fig. 6b, c) suggesting that they might play important role in Pi acquisition from soil. Whereas, *HvPht1;6* and other transporters show enhanced expression in both leaves and roots (Huang et al. 2008); localize in the leaf phloem tissue; and are involved in Pi re-translocation (Rae et al. 2003). Like *HvPht1;6*, *ZmPht1;1* (*ZmPht1;4*) were expressed in both root and leaf (Fig. 6b, c) suggesting that *ZmPHT1;1* is a high affinity transporter that might be participating in Pi re-translocation when Pi is limited in the leaf tissues.

Plant Apases (EC 3.1.3.2) are hydrolase enzymes which catalyze Pi from a group of phosphomonoesters and anhydrides (Duff et al. 1994). Among the Apases, purple acid phosphatase (PAP) has distinctive character of producing purple or pink color in aqueous solution and the presence of seven invariant residues in five conserved metal ligating motifs (Bozzo et al. 2006; Matange et al. 2015; Tian and Liao 2015). Despite low homology between PAPs from different kingdoms, five conserved motifs have been identified, including DXG, GDXXY, GNH (D/E), VXXH, and GHXH (Flanagan et al. 2006; Schenk et al. 2013). Studies show that plant PAP members are involved in P scavenging, recycling and utilization of different forms of extracellular organic P under conditions of P deficiency (Wang et al. 2011; Robinson et al. 2012; González-Muñoz et al. 2015; Liu et al. 2016). Sequence alignment using COBALT, a Constraint-based Multiple Alignment Tool, showed all the 5 PAPs conserved domain in selected maize PAPs (Supplementary Fig S4). In this study, we have selected two PAPs one is *AtPAP10* and second *AtPAP17* (*AtACP5*) on the basis

of sequence, domain and motif similarity. It has been shown that *AtPAP10* mRNA levels were increased 5- and 4-fold in Pi-starved leaves and roots of *Arabidopsis*, respectively (Wang et al. 2011). This suggests that *ZmPAP10* may function like *AtPAP10* when plants are stressed by Pi deficiency. Pi starvation induces *AtACP5* not only in roots but also in aerial parts of the plant. *AtACP5* displayed two type of activities viz. peroxidase activity and phosphatase activity. Pozo et al. (1999) suggested its probable role in recycling of the Pi from the Pi ester pool of the plant and its participation in the scavenging of Pi from the soil. Similarly, in the present study, the strong over expression of *ZmACP5* in both root and leaf under low Pi conditions (Fig. 5) suggests its role in Pi recycling in shoot and Pi scavenging in root surface. *AtPAP2*, a MYB family protein participate in flavonoid biosynthesis in *Arabidopsis* plant organs (Borevitz et al. 2000). *Arabidopsis* plants overexpressing PAP1 or PAP2 show intense purple pigmentation in many vegetative organs throughout development, and more detailed analysis of PAP1 over-expressing plants shows that some flavonoid biosynthetic genes are expressed constitutively, and the accumulation of anthocyanins is markedly enhanced (Borevitz et al. 2000; Tohge et al. 2005). Previously, it was shown that *AtPAP2* is involved in carbon metabolism but not in phosphorus nutrition and it expresses under Pi deprived conditions in both root and shoots (Feng and Lim 2011; Sun et al. 2012). Our results also show slight up regulation of *ZmPAP2* in both root and leaf.

Ribonucleases (RNases) found in cellular compartments of secretory pathway belong to the RNase T2 family of endoribonucleases. These RNases are secreted directly from the cell to produce phosphomonoesters by RNA degradation (Desspande and Shankar, 2002). T2 families of RNases are of two types in plants, S-RNases and S-like RNases (Fig. 5c). The S-RNases are involved in selection process for gametophytic self-incompatibility and expressed in the gametic tissues only (Roalson and McCubbin 2003). S-like RNases are expressed in many different organs or tissues to achieve specialized biological functions under different stresses including Pi starvation, pathogen infection, wounding and different biotic and abiotic stress. In the present study, a particular RNS was strongly expressed in the root tissue (115 fold expression in low Pi condition as compared to sufficient Pi) but not in the leaf tissues suggesting that it is a root specific RNS (Fig. 5a, b). Transcript level of RNS1 and RNS2 in *Arabidopsis*, RNaseNE in *Nicotiana glauca* and RNaseLX and RNaseLE in tomato are induced during Pi limitation (Bariola et al. 1994; Kock et al. 2006). RNaseNE mRNA is expressed in the roots, but not in vegetative tissue (Dodds et al. 1996). Tissue specific expression analysis of RNase T2 transcripts by RT-PCR revealed the root specific expression of *OsRNS1* and *OsRNS7* and show that these two

RNases are specifically expressed in roots (MacIntosh et al. 2010). So, ZmRNS1 is an S-like RNase in maize exclusively expressed in root tissue under Pi deprived conditions.

Conclusions

This study analyzed Pi responsive genes and promoters in maize by *in silico* tools and characterized transcriptional profile of these genes in a Pi stress tolerant genotype (HKI-163) using two different methods under Pi deficient conditions. Based on the results obtained here, the model of transcriptional regulation under Pi starvation in maize has been updated and fine-tuned. Future studies may investigate if over-expression of some of these Pi-responsive genes can improve the PUE in tropically- adapted maize.

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Authors' contributions PY and TK conceived the idea and provided overall supervision to the study. VD and AA performed the experiments and analyzed the data with guidance from PY, IS and KK. RV helped establish the hydroponics. VD and AA wrote the primary draft, which was further augmented, edited and improved by PY. All the authors read and approved it for publication.

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Availability of data and material All the data reported here are provided in tables, figures and text in the manuscript. Additional data files have been provided in Supplementary Online Materials. Request for detailed protocols, materials etc. should be addressed to the corresponding author.

Code Availability Not applicable.

Declarations

Conflicts of interest/Competing interests The authors declare that they have no conflict of interest.

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