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Original Article

Expression analysis of phosphate induced genes in contrasting maize genotypes for phosphorus use efficiency

Análise da expressão de genes induzidos por fosfato em genótipos de milho contrastantes para eficiência de uso de fósforo

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Abstract

Phosphorus is an essential nutrient for plant growth and development. The ability of plants to acquire phosphate (Pi) from the rhizosphere soil is critical in the Brazilian Cerrado characterized by acidic soil. The induction of Pi transporters is one of the earliest molecular responses to Pi deficiency in plants. In this study, we characterize the transcriptional regulation of six (*ZmPT*1 to *ZmPT*6) high-affinity Pi transporters genes in four Pi-efficient and four Pi-inefficient maize (Zea mays) genotypes. The expression analysis indicated that Pi-starvation induced the transcription of all *ZmPT* genes tested. The abundance of transcripts was inversely related to Pi concentration in nutrient solution and was observed as early as five days following the Pi deprivation. The Pi-starved plants replenished with 250 µM Pi for four to five days resulted in *ZmPT* suppression, indicating the Pi role in gene expression. The tissue-specific expression analysis revealed the abundance of *ZmPT* transcripts in roots and shoots. The six maize Pi transporters were primarily detected in the upper and middle root portions and barely expressed in root tips. The expression profiles of the six *ZmPTs* phosphate transporters between and among Pi-efficient and Pi-inefficient genotypes showed an absence of significant differences in the expression pattern of the *ZmPT*s among Pi-efficient and Pi-inefficient genotypes. The results suggested that Pi acquisition efficiency is a complex trait determined by quantitative loci in maize.

Keywords: phosphate transporters, Pi use efficiency, transcriptional regulation, Pi sufficiency.

Resumo

O fósforo é um nutriente essencial para o crescimento e desenvolvimento das plantas. A capacidade de adquirir fosfato (Pi) é crítica para as plantas crescidas nos solos ácidos do Cerrado brasileiro. A indução de transportadores de Pi é uma das primeiras respostas moleculares à deficiência de fosfato em plantas. Neste estudo, caracterizamos a regulação transcricional de seis (*ZmPT*1 a *ZmPT*6) genes transportadores de Pi em quatro genótipos de milho (*Zea mays*) Pi-eficiente e quatro Pi-ineficientes. A análise de expressão indicou que a privação de Pi induziu a transcrição de todos os genes *ZmPT*. A abundância de transcritos foi inversamente relacionada à concentração de Pi na solução nutritiva e foi observada cinco dias após a privação de Pi. Em plantas Pi-deprivadas ocorreu supressão gênica após terem sido reabastecidas com 250 µM de Pi por quatro a cinco dias, indicando o papel do Pi na regulação da expressão gênica. A análise de expressão tecido-específico revelou a abundância de transcritos *ZmPT* em raízes e brotos. Os seis genes transportadores de Pi testados foram detectados principalmente nas porções superior e média das raízes e fracamente nas pontas das raízes. Os perfis de expressão dos seis genes *ZmPT* transportadores de fosfato nos genótipos Pi-eficientes e Pi-ineficientes mostraram uma ausência de diferenças significativas no padrão de expressão entre os genótipos Pi-eficientes e Pi-ineficientes. Os resultados indicaram que a eficiência na aquisição de Pi é uma característica complexa que envolve a participação de outros genes no milho.

Palavras-chave: transportador de fósforo, eficiência de uso de fósforo, regulação transcricional, suficiência de fosfato.

1. Introduction

In Latin America, maize is grown in acidic soils with high levels of immobilized Pi (Fink et al., 2016). During the past three decades, the Brazilian Embrapa Maize and Sorghum enterprise has extensively exploited the natural maize genetic variation from different world regions to improve the adaptability and yield of corn in the Brazilian Cerrado (Parentoni et al., 1999).

Many studies have been carried out to understand the morphological, biochemical, and molecular responses of plants to Pi deficiency (Raghothama, 1999;

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Vasconcelos et al., 2020). Morphological and physiological studies on intra-specific variability for Pi acquisition demonstrated the adaptive mechanisms that have evolved in the Pi-efficient trait in sorghum and rice (Leiser et al., 2015; Irfan et al., 2020). Several crops have shown significant variability in root system architecture in response to phosphorus deficiency (Bates and Lynch, 2001; Doi et al., 2017). The root hair increases the Pi-absorbing area per unit root length, and its role in Pi acquisition is well documented under laboratory and field conditions (Gahoonia and Nielsen, 2004; Bernardino et al., 2019). Other evident modifications in plant architecture are decreased growth and dry matter accumulation (Bernardino et al., 2019).

The molecular mechanisms underlying the plant response to Pi starvation are highly complex and have been intensively studied and well-characterized (Raghothama and Karthikeyan, 2005; Jain et al., 2007; Leiser et al., 2015; Vasconcelos et al., 2018; Bernardino et al., 2019). Recent studies have identified major and minor QTLs associated with Pi-deficiency in rice (Jewel et al., 2019), wheat (Yang et al., 2018), and sorghum (Leiser et al., 2015; Bernardino et al., 2019).

A coordinated induction and suppression of various Pi-responsive genes involved in an intricate metabolic pathway, ion transport, signal transduction, transcriptional regulation, and other processes related to growth and development were also identified (Wu et al., 2003; Vasconcelos et al., 2018). Many root Pi-responsive genes are known in several species, such as high-affinity Pi-transporters, acid phosphatases, and purple acid phosphatases, phosphoenolpyruvate phosphate, and pyrophosphate-dependent phosphofructokinase, RNases, phosphodiesterases, β-glucosidase, and others with unknown functions (Raghothama, 2000b; Velasco et al., 2020). Among the Pi-starvation induced genes, those encoding high-affinity Pi transporters play a critical role in plant Pi acquisition (Raghothama, 1999; Nagy et al., 2006; Vasconcelos et al., 2018, 2021). Thus, Pi starvation-induced genes are considered to be involved in multiple metabolic pathways indicating a complex Pi regulation system in higher plants (Wu et al., 2003; Devaiah and Raghothama, 2007).

Many plant Pi transporter genes are induced by Pi deficiency, and their transcripts rapidly decrease upon Pi supply (Smith et al., 2003; Vasconcelos et al., 2021). Northern blot analysis has shown that almost high-affinity Pi transporter genes are expressed preferentially in roots suggesting a role for Pi uptake from the soil (Raghothama, 2000a). Studies based on in situ hybridization and immunolocalization showed that the high-affinity transporter genes are strongly expressed in root epidermis, root cap, and root hairs, and their encoded proteins have been localized in the plasma membrane (Muchhal and Raghothama, 1999; Chiou et al., 2001). The expression of Pi transporters in the root hair cells (trichoblasts) is of particular interest, as they are the primary site of Pi uptake in plants (Gahoonia and Nielsen, 1998; Shibata and Sugimoto, 2019). This study evaluates the expression pattern of the high-affinity phosphate transporters in Pi-efficient and Pi-inefficient maize genotypes.

2. Material and Methods

2.1. Evaluation of maize genotypes for P efficiency

The evaluation of maize genotypes for phosphorus use efficiency was performed at the Embrapa Maize and Sorghum Research Center, Sete Lagoas, MG, Brazil. The experiments were conducted on dark red dystrophic latosol with two ppm of phosphorus under the Cerrado vegetation. The experimental area was divided into two parts, one half receive superphosphate (broadcast) to increase the P level to 15 ppm, and the other part was kept under two ppm of P. In both areas, the soil pH was raised to 5.0 and supplied with adequate sources of Ca, Mg and K. More than 500 maize inbred lines were previously evaluated in these areas. A set of eight contrasting maize inbred lines with more than eight generations of self-pollination was evaluated in these two P levels during 2001 and 2002. The plot size was two-row, 4 m long, with an inter-row spacing of 90 cm. The selection criteria were yield (ears kg/ha) under each P level.

The model used in the analysis was (Equation 1):

$$Y_{itjk} = m + t_i + l_j + a_k + b_{t(jk)} + tl_{ij} + ta_{ik} + la_{jk} + tla_{ijk} + e_{(ijkt)} \quad (1)$$

Where Y_{itjk} is the effect of treatment i in rep t, in P level j and year k; μ is the general mean; t_i is the effect of treatment i, and i= 1 to 8; l_j is the effect of P level j and j=1 and2; a_k is the effect of year k and k=1 and 2; $b_{t(jk)}$ is the effect of rep t within P level j in year k, and t=1 to 4; tl_i is the interaction of treatment i by location j; ta_{ik} is the interaction of treatment i by year k; la_{jk} is the interaction of location j by year k; tla_{ijk} is the interaction of treatment i by location j by year k; $e_{(ijkt)}$ is the experimental error.

The data were analyzed using SAS 8e for Windows (SAS Institute Inc., Cary, NC), considering a fixed model. The Pi-use efficiency was defined as the genotype ability to yield under a low nutrient supply and Pi yield responsiveness under low and high Pi conditions according to Gerloff (1977), who classified the genotypes based on their nutrient efficiency and responsiveness: 1. Efficient/responders – High-yield plants under low nutrient levels respond to nutrient addition (E/R); 2. Inefficient/responders – Plants with a low yield at a low level of the nutrient have a high response to nutrient addition (I/R); 3. Efficient/non-responder – Plants with high yield in low nutrition levels do not respond to nutrient addition (E/NR); 4) Inefficient/non-responders – Plant with a low yield at low nutrient level, which does not respond to nutrient additions (I/NR). In this work, according to the ability of a given inbred to yield under low P, they were classified as highly Pi-efficient (HE), Pi-efficient (E), Pi-intermediate (I), and Pi-inefficient (IN). Based on their response to Pi supplementation (yield 15-2ppm), inbred were classified as highly responsive (HR), responsive (R), intermediate responsive (IR), and non-responsive (NR). Eight genotypes with contrasting responses to Pi-use efficiency were used in physiological and molecular studies to identify the mechanisms controlling Pi acquisition efficiency in maize (Table 1).

Table 1. P-efficient and P-inefficient maize genotypes selected at the Embrapa Maize and Sorghum, Sete Lagoas, MG, Brazil.

Maize genotypes	
Pedigree	Responsiveness to P
L-03	Efficient - HE / HR
L-11	Inefficient - I / IR
L-16	Inefficient - IN / HR
L-22	Inefficient - I / NR
L-36	Efficient - E / HR
L- 53 (723)	Inefficient - IN / NR
L-161-1	Efficient - E / R
L-5046	Inefficient - I / NR

2.2. Plant growth conditions

The experiments were designed to assess the biochemical, morphological, and molecular analysis of high-affinity phosphate transporters of maize and their transcriptional regulation by phosphorus. The maize seeds were germinated in seedling trays containing Scott's ready earth plug mix (Scotts Co., Marysville, OH) and grown in the greenhouse for one week. Then, the seedling roots were washed in water to remove the entire medium, and transferred to one half-strength modified Hoagland's nutrient solution (Liu et al., 2001). After one week, the plants were transferred to Hoagland nutrition solution containing different concentrations of Pi (0, 5, 10, 25, 50, 100 and 250 µM). During the treatments, the nutrient solutions were renewed on alternate days. After 15 days of treatment, the roots were harvested, frozen in liquid nitrogen, and the total RNA extracted for Northern analyses. Since the expression of different phosphate transporters in maize showed induction at 0 µM of Pi and complete suppression at 250 µM of Pi, these two concentrations were treated as P^- and P^+, respectively. For the time-course study, roots from P^- and P^+ treatments were harvested sequentially after 1, 3, 5, 6, 7, 8, 12, and 15 days of growth. Furthermore, after 15 days of growth under P^- and P^+ conditions, roots, stem, young leaves, and old leaves were harvested separately for evaluating the spatial expression of different Pi transporters in these tissues. In another set of experiments, Pi starved plants for 15 days were replenished with 250 µM of Pi and harvested after 1, 2, 3, 4, and 5 days of treatment.

2.3. Dry weight, root/shoot ratios, and phosphorus content

The maize plants were grown in hydroponics culture in the presence (250 µM Pi) or absence (0 µM Pi) of phosphate and harvested 15 days after the treatment. The roots and shoots were separately dried at 50ºC until a constant weight for comparison purposes. For phosphorus content determination, the samples were ground and digested with concentrated nitric acid at 95ºC overnight. The nutrient content was determined by inductively coupled plasma-optical emission/mass spectroscopy (ICP/OES/MS) according to Lahner et al. (2003).

2.4. Measurement of anthocyanins production

The total anthocyanin quantification was done as described by Abdel-Aal and Hucl (1999). Fresh root samples from the two treatments were ground separately in liquid nitrogen. Approximately one gram (the exact weight was recorded) of each powdered sample was mixed with 10 ml acidified ethanol. The acidified ethanol was prepared by mixing 85 ml of 100% ethanol and 15 ml of 1.0 N HCl, and adjusted to pH 1.0 with 4 N HCl. The samples in acidified ethanol were mixed and then centrifuged at 10,000 rpm for 5 minutes at room temperature. The supernatant was transferred to a 50 ml volumetric flask, and the volume was made up with acidified ethanol. The absorbance of the anthocyanin pigments in the samples was measured at 535 nm against a reagent blank. The cyanidin 3-glucoside was used as a standard for quantifying the total anthocyanin. A concentration series between 0 and 27 µg of cyanidin 3-glucoside was prepared in 3 ml of acidified ethanol, and the total anthocyanin content was expressed on a fresh weight basis.

2.5. Phosphatase activity

A colorimetric method for phosphatase activity determination was, according to Abdel-Aal and Hucl (1999). Prior to anthocyanin measurements, 500 mg of ground plant powder was mixed with a protein extraction buffer (Na acetate 50 mM pH 4.8). The samples were vortexed and then centrifuged at 12,000 rpm for 5 minutes at 4ºC to obtain the protein extract. The supernatant was transferred to a new tube, and then 10 µL of the protein solution was mixed with 190 µL of sodium acetate buffer (50 mM, pH 4.8) containing 0.4 mg of p-Nitrophenyl Phosphate (pNPP). The samples were incubated at 37ºC for 30 minutes, and the reaction was terminated by adding 600 µL of 0.1 N NaOH. The concentration of pNP was determined spectrophotometrically at 405 nm. A series of pNP standard solutions (0.1, 0.2, 0.4, 0.8, and 1.0 µg/ml of pNP) was prepared to obtain a standard curve. One unit of acid phosphatase activity was defined as one µmol nitrophenol released per minute, as Hubel and Beck (1996). The total protein normalization was made by the Bradford assay (Bio-Rad) using BSA (Sigma) as standard (Bradford, 1976).

2.6. RNA isolation and Northern blot analyses

Total RNA of contrasting genotypes of maize and sorghum were extracted with hot phenol, followed by lithium chloride precipitation (Pawlowski et al., 1994). Ten micrograms of total RNA were electrophoretically separated on 1.2% (w/v) denaturing formaldehyde agarose gel and blotted onto a nylon membrane (MAGNA Osmonics Inc., Minnetonka, MN) following the manufacturer's instructions. After blotting, the RNA was immobilized by UV cross-linking in Stratalinker (Stratagene, La Jolla, CA, USA). The pre-hybridization was carried out for 2 to 4 hours at 42ºC in a solution containing 50% (v/v) formamide, 5X Denhardt's solution, 0.1% (w/v) SDS, 6X SSPE and 150 µg/ml denatured salmon sperm DNA. DNA fragments labeled with ^{32}P-dCTP using the DECA prime II™ DNA labeling kit (Ambion, Austin, TX) was used to probe the membranes. Hybridization was carried out with 10^6 cpm

of probe/ml in a buffer identical to the pre-hybridization buffer at 42°C for 16 hours. The filters were initially washed twice, ten minutes each, in a low stringency solution (2X SSC (0.3M NaCl/30mM sodium citrate) and 0.2% sodium dodecyl sulfate (SDS), followed by a stringent high wash with 0.1X SSC and 0.1% SDS (v/v) at 42°C for ten minutes. The membranes were exposed to Kodak XAR-5 films, and the blots were reused by stripping off the probes in boiling water for five minutes.

2.7. Data analyses

The comparative analyses of the genomic and amino acid composition of high-affinity Pi transporters were carried out using Genetic Computer Group (GCG, Madison, WI) software available through the ACLCB (AIDS Center Laboratory for Computational Biotechnology version 6.1), Purdue University software.

Table 2. Mean squares for yield (kg ears/ha) of eight maize inbred lines evaluated in a Red Oxisol at two levels of phosphorus (2 ppm and 15 ppm) in 2001 and 2002.

Source	df	MS	F
Rep (E)	6	78,585.45	0.61
Inbreds (I)	7	5,083,180.48	39.17**
P levels (E)	1	17,285,730.03	133.22**
Year (Y)	1	4,348,300.50	33.51**
I x E	7	491,671.44	3.79**
I x Y	7	1,336,355.63	10.30**
E x Y	1	9,492,813.78	73.16**
I x E x Y	7	512,207.12	3.95**
Error	90	129,757.54	

**Significant at 1% probability level.

3. Results

3.1. Maize responds differently to P under field conditions

The effects of the treatments, i.e., Pi levels, years, and the respective interactions were significant at 1% probability ($p < 0.01$). The hybrid response to Pi was influenced by the year (Table 2). The means of inbreds in each Pi level in two years are shown in Table 3. Under low Pi level (2 ppm), the yield ranged from 338 to 1,773 kg ears/ha with a mean of 760 LSD (5%) detected significant differences among inbred lines. The inbred L-03 clearly showed the best Pi-use efficiency. Thus, it was classified as highly efficient (HE), followed by inbreeds L-36 and L-161-1 (efficient). L-11, L-22, and L-5046 formed a group of intermediate (I) inbred lines. Inbreeds L-53 and L-16 were considered inefficient (IN).

Under high Pi level (15 ppm), the yield ranged from 892 to 2,898 kg ears/ha with a mean of 1,495. The mean reduction in yield across the Pi levels was close to 50%. The yield differences between the Pi levels of 15 and 2 ppm were used to classify inbreeds response to Pi. The inbreds L-03, L-16, and L-36 were considered highly responsive (HR). Inbred L-161-1 was responsive (R), inbred L-11 was intermediate responsive (IR), and inbreds L-22, L-5046, and L-53 were non-responsive (NR). The inbred L-03 was superior in terms of Pi use and responsiveness.

The maize genotypes were chosen due to contrasting differences in phosphorus deficiency conditions (Figure 1A). The Pi-use efficient plants grow better under phosphorus deficiency and accumulate more biomass and phosphorus due to their superior ability to extract available phosphorus from the soil resulting in more crop yield. Phosphorus stress results in a progressive reduction in plant growth and shoot biomass (Figure 1B-1C). Regardless of genotype, in phosphorus-deficient conditions, the maize plants have less dry weight than under sufficient conditions. Phosphorus limits the growth of maize as well as other crops. Plant

Table 3. Origin and means for eight maize inbred lines contrasting for P-use efficiency and P-response, evaluated under two levels of phosphorus in the soil (2 and 15 ppm) during two years (2001, 2002). The inbreds were classified as highly efficient (HE), efficient (E), intermediate (IE), and inefficient (I) based on their yield under low P (2 ppm). The inbreds were also classified as highly responsive (HR), responsive (R), intermediate responsive (IR), and non-responsive (NR) based on their response to phosphorus (yield 15-2ppm).

INBRED	ORIGIN	YIELD (kg/ha) 2 ppm P	YIELD (kg/ha) 15 ppm P	YIELD (kg/ha) 15-2 ppm	CLASS
L-03	P-efficient synthetic	1,773 **A***	2,898 **A**	1,125	**HE / HR**
L-36	P-efficient synthetic	868 **B**	1,872 **B**	1,004	**E / HR**
L-161-1	BR 106-Synthetic	863 **B**	1,726 **BC**	863	**E / R**
L-11	CMS 14C-Pool 25	786 **BC**	1,360 **CD**	574	**I / IR**
L-22	BR 106	627 **BC**	809 **E**	182	**I / NR**
L-5046	CMS 50	448 **BC**	884 **DE**	436	**I / NR**
L-53(723)	BR 111-Pool 21	338 **C**	892 **DE**	554	**IN / NR**
L-16	BR 106	378 **C**	1,519 **BC**	1,141	**IN / HR**
Mean		760	1,495	735	
LSD 5%		477	509		

*Means followed by the same letter at a column are not different at 0.05 probability level.

dry weight (DW) was less in the treatments with low Pi levels in the nutrition solution. Efficient and inefficient inbreds grown in the absence of Pi (0 µM of Pi) showed a reduction in growth average of 22% and 28%, respectively, compared to their growth in the presence of 250 µM Pi after 15 days of treatment (Figure 1B). The decrease of plant dry weight in the absence of Pi was more severe in inefficient than in the efficient genotypes. Plant growth was influenced by genotype and phosphorus conditions (Figure 1B). Considerable variation in plant biomass was observed for P+ and P- treatments among all genotypes.

3.2. Root/shoot ratios

In phosphorus-deficient conditions, plants change the root to shoot ratio. Roots grow more, and shoots decrease their growth in phosphorus stress. The root/shoot ratio was influenced by genotype and P level in the nutrition solution. Genotypes differed in the root/shoot ratio; for example, the maize efficient line L-36 had a root/shoot ratio of 1.05 in phosphorus stress conditions while the inefficient line L-5046 had 0.5 in the same condition. The maize root/shoot coefficient exhibited a slight increase in Pi-efficient genotype than the Pi-inefficient (Figure 1C).

3.3. Phosphorus content

Phosphorus accumulation under phosphorus stress was almost the same for all maize genotypes, i.e., less than 0.2 mg/g (Figure 1D-1E). However, the amount of phosphorus accumulated in each genotype grown in 250 µM of Pi varied among the maize genotypes. Phosphorus accumulation was significant for genotypes in 250 µM of Pi. When plants were grown in the presence of Pi (250 µM)

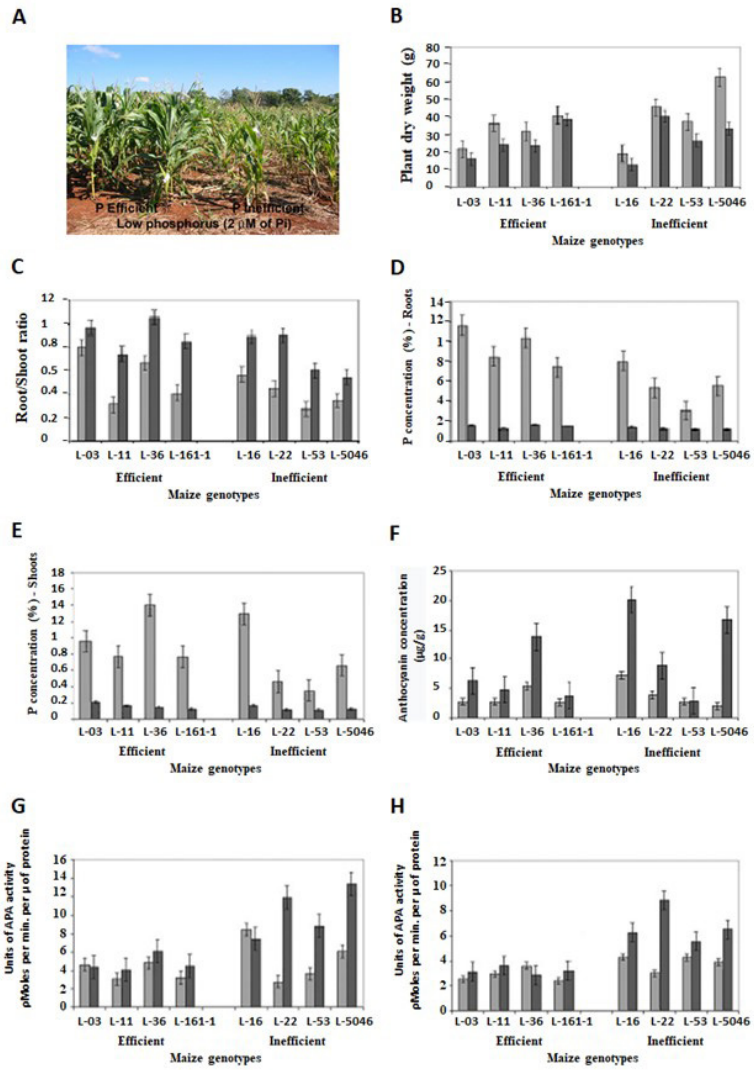

Figure 1. A – P-efficient and P-inefficient maize plants grown in the Cerrado under low Pi conditions. B – Dry weight of maize genotypes. C – Root/shoot ratio of maize plants. D and E – Phosphorus content. F – Anthocyanin concentration. G and H – Units of APA activity. B to H, The maize plants were grown in hydroponics culture in the presence (250 µM Pi - gray bar) or absence (0 µM Pi – black bar) of phosphate harvested after 15 days in treatment. Each bar is the mean of three replicates with a standard deviation.

in the nutrient solution, the phosphorus content ranged from 0.4 to 1.4% in maize genotypes (Figure 1D-1E). In general, phosphorus accumulation was associated with higher phosphorus levels in the nutrition solution. However, the mean phosphorus content in P-efficient and inefficient maize genotypes showed that efficient genotypes accumulated more phosphorus than inefficient in both roots and shoots (Figure 1D-1E).

3.4. Anthocyanin accumulation as a response to phosphorus deficiency

Anthocyanin accumulation could be associated with environmental stress responses in plants. Several reports have shown the production of anthocyanin in plants in response to phosphorus stress. The accumulation of anthocyanin in roots of maize genotypes was assessed by growing the plants in hydroponic culture P+ and P- were used to quantify anthocyanin. The results showed significant differences in the production of anthocyanin among the maize genotypes. The difference was observed in the P-efficient and P-inefficient genotypes. However, anthocyanin accumulation is, on average higher for the maize inefficient lines (Figure 1F).

3.5. Acid phosphatase activity in maize as a response to phosphorus deficiency

Acid phosphatase (APase) is essential for many physiological processes, including phosphorus efficiency regulation. The exuded and internal APase activity (APA) changed in response to phosphorus availability. The APAs were quantified in plants grown in the presence (250 µM) and absence (0 µM) of phosphorus to characterize efficient and inefficient maize genotypes. In maize, no significant differences were observed in P-efficient genotypes either for roots or shoots, but significant differences were noticeable among inefficient genotypes in roots and shoots (Figure 1G-1H).

3.6. Plant Pi transporters have high amino acid sequence similarity

Phosphate transporters from herbaceous plant species (*Arabidopsis thaliana*; *Lycopersicum esculentum*, *Medicago truncatula; Solanum tuberosum*) and cereals (*Zea mays*, *Sorghum bicolor*) revealed a significant degree of conservation in their amino acids domains, which ranged from 60 - 97% (Figure 2, Table 4). The homology was high amongst the Pi transporters of maize (79-97%) and sorghum (94%).

3.7. ZmPTs are regulated in response to changes in Pi concentration

For this study, Pi efficient and Pi inefficient genotypes of tropical maize were grown hydroponically in half-strength Hoagland's nutrient medium supplemented with 250 µM Pi (P+) or without Pi (P-) 15 days to evaluate the variability within the *ZmPT*s expression, among and between Pi-efficient and Pi-inefficient genotypes. The purpose of this study was to determine the molecular mechanism underlying the high Pi acquisition and utilization efficiency

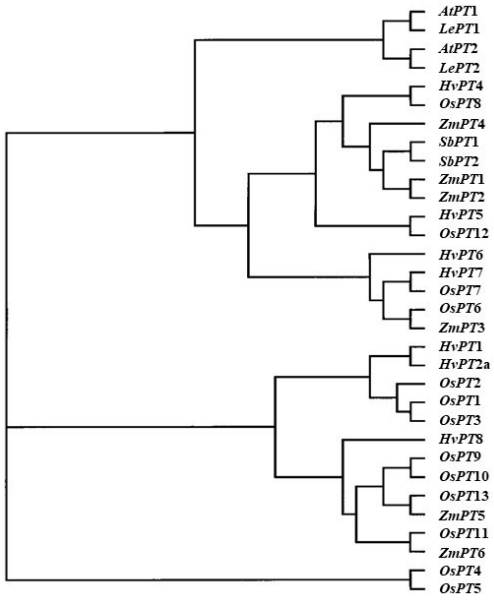

Figure 2. Phylogenetic analysis based on nucleotide sequences of plant phosphate transporters. Plant phosphate transporters were assembled using ClustalX, and NJ-plot was used to develop the tree. Abbreviations are shown for respective transporters: *ZmPT*s: *Zea mays* phosphate transporters; *AtPT: Arabidopsis thaliana* phosphate transporters; *LePT: Lycopersicon esculentum* phosphate transporters; *OsPT: Oryza sativa* phosphate transporters; *HvPT: Hordeum vulgare* phosphate transporters; *SbPT: Sorghum bicolor* phosphate transporters.

in the Pi-efficient and Pi-inefficient genotypes. RNA blot analysis of different genotypes, comprising efficient and inefficient types, grown under Pi deficiency (0 µM) exhibited significant accumulation of the *ZmPT* transcripts in roots (Figure 3A). However, no appreciable variation was observed in the abundance of any of the *ZmPT* transcripts among different Pi-efficient and Pi-inefficient genotypes.

Furthermore, only basal level expression was detected for different transporters across all the genotypes grown under Pi sufficient conditions (250 µM). The study indicated that the expression of high-affinity phosphate transporters is induced in response to Pi deficiency in both Pi-efficient and Pi-inefficient genotypes. However, no apparent correlation could be drawn between the Pi utilization efficiency of different maize genotypes and the *ZmPT*s transcripts abundance.

In order to evaluate the phosphate concentration range, which would be amenable for the induction of different *ZmPT*s in P-efficient and inefficient genotypes, representatives of efficient (L-161-1) and inefficient (L-5046) genotypes were selected and grown hydroponically under different Pi concentrations (0 to 250 µM) for 15 d (Figure 3B). It was interesting to note the induction of transcription for all the *ZmPT*s genes of P-efficient genotypes in a wide range of Pi concentrations (0 µm – 50 µM) and the accumulation of *ZmPT* 3 and *ZmPT* 6 transcripts at 50 µM Pi concentration. Conversely, the P-inefficient genotype showed a very narrow range of Pi concentration

Table 4. Homology among the Pi transporters of five plant species.

	ZmPT1	*ZmPT2*	*ZmPT3*	*ZmPT4*	*ZmPT5*	*ZmPT6*	*AtPT1*	*AtPT2*	*LePT1*	*LePT2*	*MtPT1*	*MtPT2*	*StPT1*	*StPT2*
ZmPT1	100													
ZmPT2	94.0	100												
ZmPT3	88.0	82.5	100											
ZmPT4	93.0	97.0	82.0	100										
ZmPT5	81.1	80.8	82.0	80.2	100									
ZmPT6	82.1	82.1	79.0	81.0	83.0	100								
AtPT1	76.3	77.2	72.7	76.6	80.3	78.5	100							
AtPT2	74.0	74.0	73.0	73.5	77.5	88.5	78.4	100						
LePT1	71.0	73.0	72.3	74.0	82.0	78.5	77.1	79.6	100					
LePT2	73.3	73.7	71.7	72.0	82.75	78.0	78.1	76.8	79.0	100				
MtPT1	76.0	71.0	69.0	71.0	55.0	65.0	79.1	77.1	75.9	81.0	100			
MtPT2	76.0	71.0	69.0	71.0	55.0	65.0	79.2	77.7	76.3	81.6	98.3	100		
StPT1	79.0	83.0	75.0	83.0	65.0	75.0	80.2	83.2	76.7	81.3	78.2	78.2	100	
StPT2	79.0	82.0	75.0	83.0	64.0	76.0	75.2	75.0	76.7	93.8	79.0	79.6	78.9	100

The similarity between plant phosphate transporters was determined using the FASTA program in GCG (Madison, WI) package. Protein sequences were retrieved in FASTA format using BLAST. Abbreviations are shown for respective transporters: *ZmPT: Zea mays* phosphate transporter; *AtPT: Arabidopsis thaliana* phosphate transporter; *LePT: Lycopersicon esculentum* phosphate transporter; *MtPT: Medicago truncatula* phosphate transporter; *StPT: Solanum tuberosum* phosphate transporter; *SbPT: Sorghum bicolor* phosphate transporter.

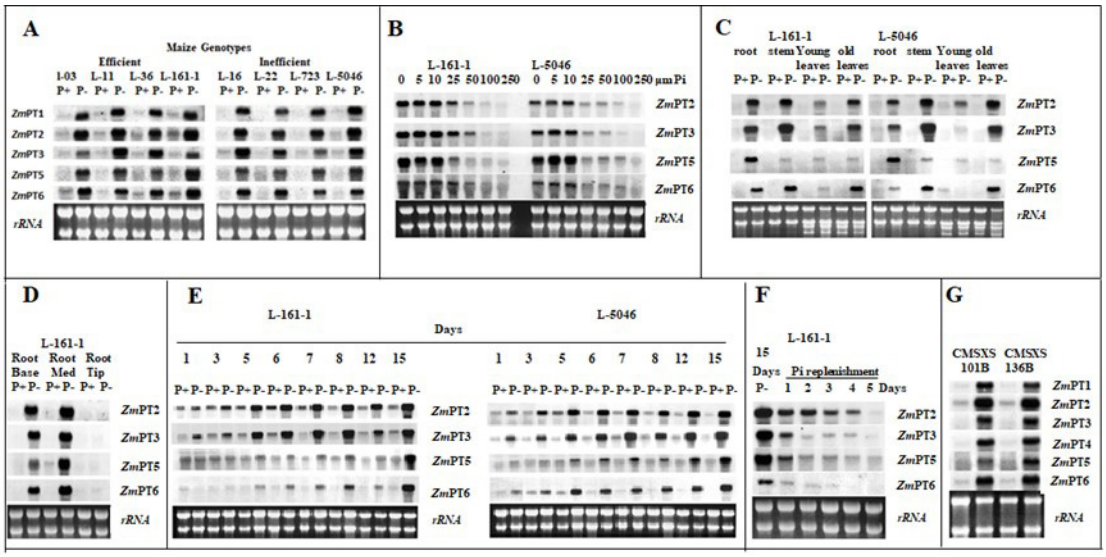

Figure 3. A – Northern blot analysis of phosphate starvation-induced *ZmPT*s genes in maize genotypes. B – Expression of *ZmPT*s in a plant grown in different phosphorus concentrations. C – Suppression of the *ZmPT*s expression by Pi resupply. D – Expression of *ZmPT* genes using RNA isolated from different root parts. E – Expression of *ZmPT*s in different maize plants under Pi starvation. F – Effect of duration of phosphate starvation on *ZmPT*s genes expression in maize genotypes. G – Expression of *ZmPT* homologs in roots of two sorghum genotypes. Total RNA isolated from different times of hydroponically grown plants supplied with half-strength modified Hoagland's solution containing 250 µM phosphate (+) or no phosphate (-) for different days or different concentrations as indicated. All the blots were probed with ^{32}P labeled *ZmPT*s. The panel below the Northern blots is the ethidium bromide-stained gel prior to blotting showing the RNA integrity and uniformity of loading.

(0 to 10 µM) under which the *ZmPT*s genes were induced. The study indicated the potential of a broad spectrum of Pi concentrations in the soil to induce different types of high-affinity Pi transporters in the P-efficient genotypes. Whether the efficient genotypes attribute contributes to their higher Pi utilization efficiency and, consequently, better yield could be answered by QTL analysis.

3.8. ZmPTs are expressed in diverse tissues

Since there was no information available on the tissue-specific expression of Pi transporters in maize, we carried out a detailed evaluation of efficient (L-161-1) and inefficient (L-5046) genotypes grown hydroponically with (+) or without (-) Pi for 15d. After the treatments, different plant parts, i.e., roots, stem, young and old leaf, were harvested, and the tissues used for RNA isolation and used for RNA blot analysis (Figure 3C). It was interesting to note a significant accumulation of the transcripts for *ZmPT*s 2, 3, and 6 in L-161-1, not only in the roots but also in the stem and old leaves.

Conversely, the expression of *ZmPT*5 was explicitly observed in the roots, and the expression was barely detectable in other parts of the plant (Figure 3C). The differential expression of *ZmPTs* in L-161-1 suggested a broader role for them in P uptake and mobilization. A similar trend in the expression profiles of different *ZmPT*s was observed for L-5046 (Figure 3C).

Therefore, to determine whether *ZmPT*s also exhibit any functional specificity for their expression in different parts of the roots, L-161-1 was grown hydroponically for 15 d under P (+) and P(-) conditions. After the treatment, different segments of the roots, i.e., 1 cm each from the shoot/root junction (base root), the middle section of the root, and the root tip were harvested and used for Northern analysis. Interestingly, the expression of different *ZmPT*s could be detected only in the basal and the middle part of P (-) roots, whereas their expression was barely detectable in the root tip (Figure 3D).

3.9. ZmPTs are temporally and transcriptionally regulated by Pi deficiency

A time-course study was carried out to evaluate how rapidly the expression of various *ZmPTs* is induced in the efficient and inefficient genotypes grown under Pi deficient conditions. Roots of maize genotypes grown hydroponically in P (+) and P(-) nutrient solutions were harvested at different time intervals starting from 1 d on wards up to 15 d and used for Northern analysis (Figure 3E). Different *ZmPT*s in the efficient genotype (L-161-1) showed a significant accumulation of their transcripts after 5 d of Pi starvation, and their abundance increased concurrently with the longer duration of Pi deficiency treatment. A similar trend was also observed for the expression of these transporters in the inefficient genotype (L-5046). The results indicated that phosphate transporters induction in the Pi-efficient and Pi-inefficient maize genotypes require at least five days of starvation.

To determine whether induction of *ZmPT*s is regulated transcriptionally by Pi deficiency, one of the efficient genotypes (L-161-1) was Pi-starved for 15 d and subsequently replenished with 250 µM Pi for different time intervals ranging from 1 d onwards up to 5d. Roots

were harvested from the plants replenished with Pi for different time intervals and were used for Northern analysis (Figure 3F). The study indicated the role of Pi in the transcriptional regulation of the different members of *ZmPT*s.

3.9.1. Homologs of maize phosphate transporters are induced in sorghum genotypes

The deduced peptides for different high-affinity transporters exhibited high sequence homology among diverse plant species (Table 4). However, it was not known whether the Pi transporters from maize and sorghum share any significant homology. Thus, to determine this, *ZmPT*s cDNAs were used as probes on the RNA blots prepared from the P (+) and P (-) roots of hydroponically raised efficient and inefficient genotypes of sorghum (Figure 3G). Hydroponics conditions used for raising sorghum genotypes were identical to those described earlier for maize. Different phosphate transporters from maize (*ZmPT*s) revealed an abundance of transcripts in the roots of efficient and inefficient sorghum genotypes exposed to P (-) conditions. This data confirmed that the maize and sorghum Pi transporters shared high genomic homology.

4. Discussion

Considering the non-renewable resources of phosphate and several implications of using excessive fertilizers, it has almost become mandatory for the selection and development of cultivars capable of using a higher proportion of the fixed P already present in the soils for the long-term sustainability of the agricultural systems. Therefore, to alleviate the predicted adverse effects of agricultural expansion, scientists use classical breeding techniques to improve crop plants to improve crop yields with a lower fertilizer (Poirier and Bucher, 2002). Strategies to improve agricultural production on P-deficient soils have focused on making the most efficient use of available P in the soil so that crop production can be sustained with minimum P applications. A vital component of these strategies is the selection and development of species and cultivars that grow well at lower levels of available soil P (Coltman et al., 1987). Genetic variability for P-use efficiency and P-inefficiency was identified within a set of Brazilian maize inbred lines. Maize genotypes standards for more efficient P acquisition and utilization have been developed using field evaluations for grain yield in soils with contrasting P levels. Therefore, to elucidate the mechanisms underlying the phosphorus use efficiency (PUE), there is a need for the integrated use of quantitative genetic studies associated with morphological, biochemical, and molecular approaches to identify the complex array of genes associated with this trait. In this regard, in the last three decades, researchers at the Brazilian Embrapa Enterprise have identified maize cultivars adapted to acid soils with low phosphorus availability. Research efforts have primarily focused on screening available cultivars and advanced breeding lines for above-average yields on P-deficient soils rather than developing new cultivars specially adapted to Pi deficiency. Similar approaches have been successfully exploited in developing efficient rice cultivars capable of utilizing a higher proportion of the fixed P in the soil (Wissuwa and Ae, 2001). The P-efficient and P-inefficient maize genotypes developed at Embrapa provided excellent material for understanding the PUE molecular mechanism.

Since high-affinity Pi transporters in different plant species are specifically induced in response to Pi stress (Raghothama, 1999), it was logical to assume their potential role in higher Pi use efficiency of Pi efficient genotypes under low phosphorus soil conditions. The differential expression of Pi transporter genes in various plant tissues suggests their P acquisition role from the soil and P remobilization throughout the plant. This observation could be further substantiated by the low abundance of these transporter transcripts in young leaves. These results indicated a potential mechanism by which the maize genotypes would have evolved to acquire the Pi from the soil on the one hand and mobilize the available pool of Pi from old and senescing leaves, where it may not be required, to the sink, which in the present study could be the young leaves. It has been well documented in different plant species that genes for high-affinity transporters are expressed in diverse tissues such as leaves and different parts of the reproductive organs but preferentially in roots (Daram et al., 1998; Raghothama, 1999). The expression pattern observed for *ZmPT*s in the basal and middle section of the root was similar to that reported earlier for the *AtPT*1 gene of *Arabidopsis thaliana* (Karthikeyan et al., 2002), and also in *Sorghum bicolor* CMSXS101B and CMSXS136B in this study.

Tropical maize has been grown for generations in highly weathered soil, which works as a selective force for plant adaptation, particularly under phosphate deficiency. Attempts at Embrapa Maize and Sorghum research center identified genotypes that respond better to available or applied phosphorus. It is reasonable to presume that P-efficiency in those genotypes is a result of complex morphological, biochemical, and molecular modifications, since there is no single dominant factor associated with the Pi-efficient or Pi-inefficient genotypes among the multiple traits tested. However, distinct differences can be noted in specific pairwise comparisons. As anticipated, Pi deficiency significantly increased the root-to-shoot ratio in maize, and was quite variable among the Pi-inefficient and Pi-efficient genotypes. However, there was no clear correlation between the root-to-shoot ratio and Pi efficiency or inefficiency. These observations confirm the complex nature of plant adaptation to Pi deficiency.

Phosphorus use efficiency in tropical maize could also be due to efficient accumulation of phosphate from the soil solution by plants. This ability was tested in vitro by determining the total P content in plants grown in the presence and absence of phosphate for 15 days. The phosphorus content increased significantly in plants supplied with 250µM phosphate. Except for the Pi-inefficient line L-16, other Pi-inefficient lines had significantly lower Pi content compared with Pi-efficient lines. The data suggests a more robust trend among Pi-efficient lines to acquire and sequester phosphorus

from the medium. This information may be one of the parameters that allow Pi-efficient genotypes to produce more grain than their Pi-inefficient counterparts. However, this observation needs further validation by studies on plant Pi uptake and gene expression profiles.

The field data showed that grain yield under Pi deficiency depends on the Pi amount taken up by the plant and its efficient use. In addition, the hybrid response to Pi was influenced by the year. This effect may be due to water supply and temperature variation, which can influence the available Pi concentration in the soil solution (George et al., 2012). Analysis of rice genotypes led to the conclusion that modern rice varieties had higher grain yield regardless of Pi supply (Liu et al., 2001). Similar results were obtained by more detailed studies of diverse wheat genotypes by Grün et al. (2018). These studies showed that the higher yield of modern crop varieties was mainly due to their Pi-use efficiency for grain production, and efficient mobilization of P from vegetative to reproductive tissues. This parameter may also be valid for maize genotypes evaluated in this study. Although there was a marked difference in P accumulation between Pi-sufficient and Pi-deficient plants, the differences in growth (dry matter accumulation) were not due to Pi content in tissues. An overuse of Pi by plants during the treatment with 250 uM Pi and an efficient remobilization of the internal Pi by plants transferred to Pi deficient medium may explain this result.

In addition to differences in growth and phosphate accumulation, Pi deficient plants accumulated a significant amount of anthocyanin in their roots and shoots. Increases in anthocyanin in leaves during Pi stress have been attributed to protection of the photosynthetic apparatus. Indeed, anthocyanin reduces photoinhibition damage to the photosynthetic system under Pi-limited conditions (Hernández and Munné-Bosch, 2015). Two different scenarios regarding anthocyanin accumulation and phosphate use efficiency could be envisaged: first, Pi-efficient plants may accumulate less anthocyanin suggesting that they are more tolerant of phosphorus stress, thereby repressing the anthocyanin biosynthesis. This result reflects the plant ability to increase both the uptake and internal utilization of acquired phosphate, while inefficient plants accumulate more anthocyanin to protect the photosynthetic apparatus during Pi deficiency.

Interestingly the maize genotypes accumulated significant quantities of anthocyanin in roots of Pi deficient plants. However, the physiological relevance of anthocyanin accumulation in roots is not clear. This may suggest that anthocyanin has an additional protective role during Pi deficiency other than reducing the light damage to photosynthetic machinery. As noted with other parameters in this study, anthocyanin content did not correlate with Pi efficiency or Pi-inefficiency.

Considering the Pi-inefficient genotypes, although the significant increase in phosphatases activity during Pi deficiency, they showed distinct and significant differences relative to phosphatases activity in both shoot and root extracts. Phosphate-efficient genotypes produced similar amounts of acid phosphatases irrespective of Pi sufficiency or deficiency. Intra-specific variation in excreted phosphatases has been correlated with the depletion of organic P in the rhizosphere, and presumably, Pi uptake by plants (Li et al., 2016). The significant increase in phosphatases activity have been linked to a possible role of phosphatases in the Pi starvation rescue mechanism (Duff et al., 1994; Crombez et al., 2019). The significant increase the intracellular and extracellular activity of phosphatases during Pi deficiency is a distinct and universal plant response to obtain Pi from organic compounds found in the extracellular matrix (Duff et al., 1994). In our study, the enhanced intracellular activity of APA in Pi-inefficient genotypes in both roots and shoots lead us to hypothesize that the efficiency in phosphorus acquisition in tropical maize may not be entirely due to phosphatase activity, since the maize genotypes in this study were selected in highly weathered and organic Pi-depleted soils. However, there are many conflicting reports regarding the role of APA in phosphorus nutrition, and interpretations may depend on the plant species and experimental conditions under which the enzyme was measured (Clark, 1975; Maharajan et al., 2018). Therefore, how APA relates to plant response to phosphorus stress may require plant material similar in general characteristics or isogenic lines contrasting specifically for APA activity.

Several studies have been undertaken to decipher the morphological, physiological, and molecular responses of a plant to Pi deficiency (Raghothama, 1999; Ha and Tran, 2014; Li et al., 2019). At the molecular level, most studies had considered the effect of phosphate deprivation on its acquisition from the root/soil interface. For example, many studies have shown that Pi deficiency triggers the induction of genes encoding various high-affinity Pi transporters (Shin et al., 2004; Lan et al., 2017), RNases, and phosphatases (Bariola et al., 1994). The array of molecular responses has been associated with the increased soil exploration by the plant, the liberation of Pi from P-complexed forms, and its acquisition. Though most of these studies have indicated the plant molecular response to Pi deficiency, it is unknown whether these molecular attributes also contribute to the overall phosphate use efficiency (PUE). Since high-affinity Pi transporters are induced in response to Pi stress in different plant species (Raghothama, 1999; Vasconcelos et al., 2018; Li et al., 2019), we were interested in characterizing the genes encoding Pi-transporters in maize. The cDNA clones of the maize Pi transporters family (*ZmPTs*) were obtained from Pioneer Hybrid Co. Earlier studies reported that almost all plant Pi transporters share high sequence similarities with the yeast and *Neurospora crassa* high-affinity Pi transporters (Raghothama, 1999). Preliminary characterization of Pi transporters from maize revealed that they are induced when plants are grown under either Pi deficient conditions or low Pi concentrations (0-25 µm) in the nutrient medium. Since Pi-starvation or low Pi concentrations up-regulate the genes encoding Pi transporters, it is reasonable to assume that the maize Pi transporters play a critical role in plant Pi acquisition (Raghothama, 1999; Vasconcelos et al., 2018; Li et al., 2019). The temporal expression of the maize Pi transporters revealed a similar pattern. The longer duration required by maize to perceive Pi stress and consequent induction of different Pi transporters could be attributed to its physiology, growth, and development. Furthermore,

the results indicated that both P-efficient and P-inefficient genotypes respond identically to Pi stress concerning the time necessary to induce their different Pi transporters.

Furthermore, the Pi role in the Pi transporters' transcriptional regulation could be demonstrated in maize Pi-starved plants resupplied with Pi. The present study agrees with earlier reports in different plant species showing that Pi regulates the expression of the high-affinity Pi transporters (Daram et al., 1998; Liu et al., 2001; Karthikeyan et al., 2002; Vasconcelos et al., 2018; Li et al., 2019). Furthermore, variations in the period of Pi replenishment required for the complete suppression of the expression of different *ZmPT* family members suggest the potential functional specificity existing amongst them. The delayed suppression of *ZmPT2*, in particular, upon Pi replenishment, could be due to the abundance of its transcripts as indicated by the expression of this transporter not only in the roots but in different aerial parts as well (Figure 3F). However, it is intriguing to note that for *ZmPT3,* which exhibited an expression profile similar to that of *ZmPT2)* transcripts were significantly reduced upon Pi replenishment for more than one day. This result highlighted the functional differences between the *ZmPT*s *2* and *3* for their Pi regulation despite having similar expression profiles in different plant parts. Muchhal et al. (1996) reported a concomitant reduction in the abundance of high-affinity transporter proteins with the repression of the corresponding genes in plants grown under Pi sufficient conditions. Various studies indicated that the expression of genes encoding high-affinity Pi transporter is tightly controlled and responds to the plant Pi status. Since nutrient interactions are common in plants, particularly P interactions with Zn and Fe would be of further interest in the selected maize genotypes

The genes encoding the maize Pi transporters (*ZmPT* 2, 3, 5, and 6) were highly expressed in roots of five days Pi starved plants. This data is consistent with the role of the Pi transporters in plant phosphate acquisition from the root-soil interface. In the present study, high levels of *ZmPT*2, 3, and 6 transcripts were detected in stem and old leaves. These expression profiles of Pi transporters in different parts of the plants suggested their potential role in Pi mobilization across different tissues.

Furthermore, the abundance of the phosphate transporters transcripts in maize was significantly lower in the young leaves. Likely, *ZmPT*s may also be involved in the Pi redistribution from old leaves, where it is no longer required, to the sink in young leaves. It has been shown that almost 80% of Pi is remobilized out of senescing leaves in *Arabidopsis thaliana* (Himelblau and Amasino, 2001), and this was further corroborated by a report which showed a strong expression of the *Pht*1;4 promoter-driven reporter gene in senescing leaves of Pi-starved plants (Karthikeyan et al., 2002; Roch et al., 2019).

Earlier studies in several crop species such as rice, barley, and soybean had reported genotypic differences for Pi deficiency (Gahoonia and Nielsen, 2004). Most of these studies have correlated the higher Pi-use to various root traits (Gahoonia and Nielsen, 2004; Long et al., 2019). However, at present, the molecular basis of efficient Pi uptake has not been elucidated. In the last decades, extensive studies have demonstrated the role of Pi transporters in Pi acquisition and remobilization. However, how much they contribute towards phosphate use efficiency and yield potential, it poorly understood. In the present study, the expression of *ZmPT*s in plants grown hydroponically with or without Pi showed no differences between Pi-efficient and Pi-inefficient genotypes. This result suggest that phosphate efficiency in tropical maize is a complex trait possibly mediated by various physiological and biochemical factors. Differences observed in some genotypes could be due to gene pools of parents used in crosses to obtain the P-efficient and P-inefficient maize. It is reasonable to assume that maize genotypes growing in P-deficient soils of the Brazilian Cerrado developed enhanced responsiveness to phosphorus input and increased internal phosphorus use efficiency through different physiological and biochemical traits leading to different degrees of adaptation to Pi deficiency. Thus, one genotype may use Pi uptake and root morphology to attain phosphorus, whereas another may be better adapted to protect itself against oxidative damages by increasing anthocyanin content or modifying the phosphatases activity. These questions may be addressed using near-isogenic lines for these traits.

5. Conclusions

The expression profiles of the six *ZmPT*s phosphate transporters between and among Pi-efficient and Pi-inefficient genotypes showed an absence of significant differences in the expression pattern of the *ZmPT*s among Pi-efficient and Pi-inefficient genotypes. The results suggested that Pi acquisition efficiency is a complex trait determined by quantitative loci in maize.

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