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Effects of phosphorous supply on growth, phosphate distribution and expression of transporter genes in tomato plants

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Abstract

Phosphorus (P) is absorbed only as phosphate ions (Pi), which are often lower than the plant needs in cultivated soils. In this research, we have used various amounts of Pi for four weeks to feed hydroponically young tomato plants. Pi treatments influenced growth, biomass production, P levels in different tissues and the expression pattern of two Pi transporter genes, *LePT1*and *LePT2.* In a low Pi concentration density and length of root hairs, total root length, specific root length and root to shoot ratio increased while biomass, leaf area, and root density decreased. Root morphology was not changed significantly when the Pi supply was 0.5 mM or higher. The P level in roots or shoots did not increase significantly, when Pi level was above 1 mM. Correlation between roots, P content and two Pi transporter activities showed that transcription of *LePT2* is induced at 0.2 g P/100g^{-1} dry weight in roots while *LePT1*expression remains high, up to 0.5 g P/100g^{-1} of dry weight. In total, these results showed that the P content of roots and shoots are strongly correlated with supplied Pi concentrations. Allocating more P in shoots than in roots indicated that leaves are cumulating organ for P distribution at high Pi condition.

Keywords: Phosphorous Supply; Growth; Transporter Gene; Expression; RT-PCR; Tomato.
Abbreviations: RT-PCR_ Reverse Transcriptase-PCR; *LePT1_Lycopersicon esculentum Phosphate Transporter-1; LePT2_ Lycopersicon esculentum Phosphate Transporter-2,* SRL_ Specific Root Length; LA_Leaf Area; DW_Dry Weight.

Introduction

Most of phosphorous (P) sources in soils are in the form of inorganic and organic phosphate (Vance et al., 2003), while P is only taken up by plants as phosphate ions (Pi) in soluble form (Marschner, 1995). It is well known that soluble phosphate ions (Pi, HPO_4^{2-} and $H_2PO_4^-$) are the preferred absorbable forms for plants (Marschner, 1995). Many previous studies have clarified different mechanisms involved in Pi acquisition by plants and explained the related mechanisms that mediate the control of P allocation and distribution in the plant organs (Malboobi et al., 1995; Hammond et al., 2004; Lambers et al., 2006, Abdolzadeh et al., 2009). Gene expression plays a major role in physiological, biochemical and morphological procedures of plant adaptations to environmental conditions, including the availability and uptake of nutrients (Wu et al., 2003; Franco-Zorrilla et al., 2004; Bremer and Schenk, 2009). P uptake in plants like other processes occurs under the control of several genes that eventually regulate Pi homeostasis needed for chains of reactions involved in plant metabolism, growth and development (Wang et al., 2002; Hammond et al., 2003; Vance et al., 2003; Wu et al., 2003; Franco-Zorrilla et al., 2004). Previous studies have shown that two types of encoding genes are responsible for Pi transportation into tomato plants, a low affinity transporter system that often expressed in plants and a high affinity that is induced only when plants face severe P- deficiency (Kim et al., 1998; Daram et al., 1998; Mukatira et al., 1996; Liu et al., 1998; Raghothama, 1999). The expression patterns of the responsible genes, *LePT1* and *LePT2* have already been compared to discriminate the regulations of the two genes in deficient and sufficient Pi supply (Liu et al., 1998). Transcription of these genes may coincide with structural changes in the root system depending on the available Pi. For instance, they modify root architecture in response to available Pi levels. In this study, the effect of P supply by different Pi concentrations on several events related to growth, P allocation and the expression of Pi transporters were examined to describe correlations between molecular and physiological responses of tomato plants to accumulation of P in the plant tissue.

Materials and methods

Plant culture and Pi treatments

Tomato seeds (*Lycopersicon esculentum.* L. Mill. 'Ailsa Craig') were sterilized by soaking in 70% ethanol for 2 min and germinated in seedling trays filled with coco peat mixture. When plants reached the four-leaf-stage (28 days after sowing), uniform seedlings were selected and transplanted into plastic boxes contained 4 litres of liquid medium. Before transplanting, roots were cleaned free of

Table1. Mean values of growth indices affected by P supply.

P supply (mM)	Total fresh weight (g/plant)	Shoot fresh weight (g/plant)	Root fresh weight (g/plant)	Total dry weight (g/plant)	Shoot dry weight (g/plant)	Root dry weight (g/plant)	Root/shoot dry weight ratio
0	13.92c	8.409d	5.545c	3.314d	1.598d	1.716c	1.089a
0.1	20.13b	13.677c	6.511c	4.552c	2.599c	1.951bc	0.750ba
0.5	26.60a	18.44b	8.185b	6.083b	3.871b	2.210b	0.571b
1	30.74a	20.88a	9.815a	7.246a	4.593a	2.650a	0.578c
2	30.32a	20.573ab	9.728a	7.358a	4.732a	2.623a	0.352d
LSD	3.46	2.3804	1.3742	0.620	0.493	0.383	0.224

Plants were grown in nutrient solution at four levels of P supply. 4-week-old plants were harvested and values are means of three replications. Mean comparison was performed by LSD method at P< 0.01. Means with no significant differences are shown with the same letters.

-Pi 0.1mMPi 0.5 mM Pi 1mM Pi 2 mM Pi

Fig 1. The effect of different concentrations of Pi on morphology and structure of roots in hydroponically grown tomato plants. 4-week-tomato plants cultured in nutrient solutions are shown. Plants were exposed to 16 hr light period at 25°C and an 8 hr dark period at 20°C, 14000 lux light intensity and 65% relative humidity. The highest average root length was obtained in –Pi treatment.

residuals and washed several times with distilled water. The basic nutrient solution was made of the following macro and micro nutrients (Wang et al., 2002). For different Pi treatments, various concentrations of $(NH_4)_2 HPO_4$ were used and equivalents of missing Pi replaced with $(NH_4)_2 SO_4$ to a sum of 2 mM, in order to compensate the lack of nitrogen in the solutions. Medium was refreshed every 3 days and adjusted for EC=0.8 dS m^{-1} and pH=5.8 every day and it was aerated with an air pump to supply oxygen for roots. Having 4 weeks of treatments, samples were taken 6 hrs after refreshing the medium (Wang et al, 2002). Roots and leaves were separated, frozen in liquid N_2, and stored at -80°C for RNA isolation and measurements (Keskin et al., 2010).

Growth measurements

Leaf area (LA) per plant was measured with the Bio-scientific Ltd Area Meter (ADC, UK). Leaf greenness measured by chlorophyll meter (SPAD-502, Minolta, Japan). Then the average of five readings for each sample was calculated. Leaf, shoot, and root samples were subsequently dried at 70°C for total dry weight, shoot and root dry weights and root length measurements according to the method of Tennant (1975). Root volume was taken on fresh roots of each plant by the water displacement method, using graduated cylinder method (Saleh and Gritton, 1988). The length to dry mass ratio of roots for each sample was used as an estimation of specific root length (SRL).

P measurements

Total P contents of root and leaf samples were determined colorimetrically through assay for phosphor-vanado-molybdic complex (Jackson, 1967) using spectrophotometer (Cary 50, varian, Australia) at 410 nm.

Root digital image analysis

Two plants were taken from each treatment of three replicates and used for measurements. Root volume, root length, root hair length and density were measured for the main root. Root hairs were essentially segmented from the recovered roots as described by Bucher et al. (1997). A digital image of the segmented root hair fragment (50 µm) was recorded for hairy root density and also for root hair length using an image analyzing system (Leica, DMBL, 40X lens, Germany), fitted with a DMBL 40X lens. Image processing was carried out by Leica@Win550 software (Leica, Germany).

Table 2. The effect of P supply on root characteristics of tomato plants.

P supply (mM)	Root hair density (No/50 µm)	Root hair length(µm)	Root length (cm)	Root volume (ml)	Root DW/ volume (g/ml)	SRL (cm/gr)
0	1.32c	0.2c	433.6a	3.97a	0.442b	18.987a
0.1	241.40a	127.80a	298.0b	3.62a	0.542ab	16.863b
0.5	27.08b	16.25b	164.3c	3.27a	0.679a	7.513c
1	20.25b	4.05c	125.6c	3.85a	0.692a	6.993cd
2	17.67b	2.12c	122.0c	3.62a	0.736a	5.993d
LSD	10.004	4.968	70.164	1.069	0.235	1.251

Specific root dry mass, root dry weight to volume ratio. SRL, specific root length, as described in material and method. The seedlings were exposed to 16 hours light and 8 hours night in a hydroponic culture system. Mean comparison was performed by LSD method at $P< 0.01$. Means with no significant differences are showed with the same letters.

Fig 2. Differences in root hair density and length of tomato plants root tips exposed to five concentrations of phosphorous in liquid medium. Images were fitted by Leica, DMB and 10X lens. Bar= 300µm. There was no root hairs formation in the -Pi plants (no Pi). The longest root hair length and the highest density were formed in 0.1 mM Pi-fed plants which were decreased as the supplied Pi was increased.

RNA extraction and Semi-quantitative RT-PCR

Total RNA was extracted from tomato root and leaf samples using RNX-Plus™ (Cinnagen, Tehran, Iran) according to manufacturer's manual. DNA contamination was checked, conducting regular PCR. Nucleotide sequences of two phosphate transporter mRNAs, *LePT1* and *LePT2*, from the GenBank (accession No. AF022873.1 and AF022874.1), were used for primer design by Oligo5 software (Molecular Biology Insights Inc. USA). The specific primer pair for the *LePT1* was 5'-TTACTACCATCATGACGGTGCA-3' and 5'-GGACATGTCGTTAGCTGCCT-3', and for *LePT2* 5'-TTCAGGGCTCTCCTTTGGTAG-3' and 5'- GTTCGATTT-TGGCTTCCTCGCT-3'. Samples were checked to avoid hairpins and complimentarily between primers (Charkazi et al., 2010). Additionally, two primer sequences, 5'- GCTTTC-AACAACTTCTTCAG - 3' and 5'- GGGGCGTAGGAGG-AAAGC -3, were used for amplification of α-tubulin gene as an internal control. First strand cDNA was synthesized using 5 µg of total RNA, 20 pmol oligo (dT) and 1 µl reverse transcriptase using RevertAid kit (Fermentas, Litvania), following the manufacturer's instructions. The amplification conditions were pre-denaturation for 5 min at 94ºC and 35 cycles of 55 sec at 94ºC, 55 sec at 59ºC, and 55 sec at 72ºC, and an extension for 5 min at 72ºC. The PCR products were separated on 1.2% agarose gels and visualized under UV light by ethidium bromide staining. For semi-quantitative expression analysis, TotalLab V1.10 software (Phoretix, Newcastle, UK) was used to process gel images. The ratio of expression values of target gene to α-tubulin was computed to standardize the expression data.

Statistical analysis

Experiments were based on randomized complete block design with three replications, with eight plants samples for each treatment. Data were subjected to analysis of variance (GLM) using and mean comparison by LSD method and SAS 9.1 software (SAS Institute, Cary, NC).

Results

Supplied Pi affected growth rate

The effect of Pi supply on growth and biomass accumulation was significant. As shown in Table 1. All growth indices including whole plant (fresh and dry weights) as well as shoot or root biomass accumulations were significantly increased when Pi supplied for 0.1, 0.5 and 1 mM. However, the differences in biomass indices between 1 and 2 mM Pi were not significant. It was remarkably noticed that, while dry and fresh weights of roots decreased about two folds, the root to shoot ratios increased significantly to more than three folds in Pi deficient plants. No-Pi treatments formed much longer roots in comparison with higher Pi levels (Table 2 and Fig 1). However, the root volume remained unchanged (Table 2). No significant change in root volume, reduced root biomass, and at the same time, increase in root length and SRL, all indicate a gross architectural changes in the roots of plants grown in limited Pi supply (Table 1 and 2; Fig 1). As shown in Table 2, the highest root hair density and length was obtained at 0.1 mM Pi supply, while root hair formation

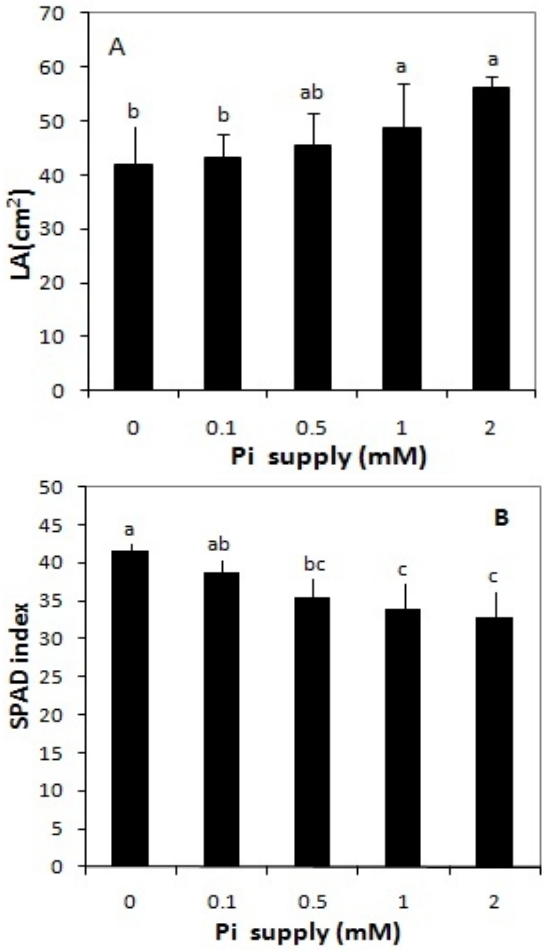

Fig 3. The effect of Pi supply on leaf area (LA; A), top and leaf greenness (SPAD Index; B). Details of experiments and statistical analysis are as Table 1. Also, see material and methods for the measurement of the above indices

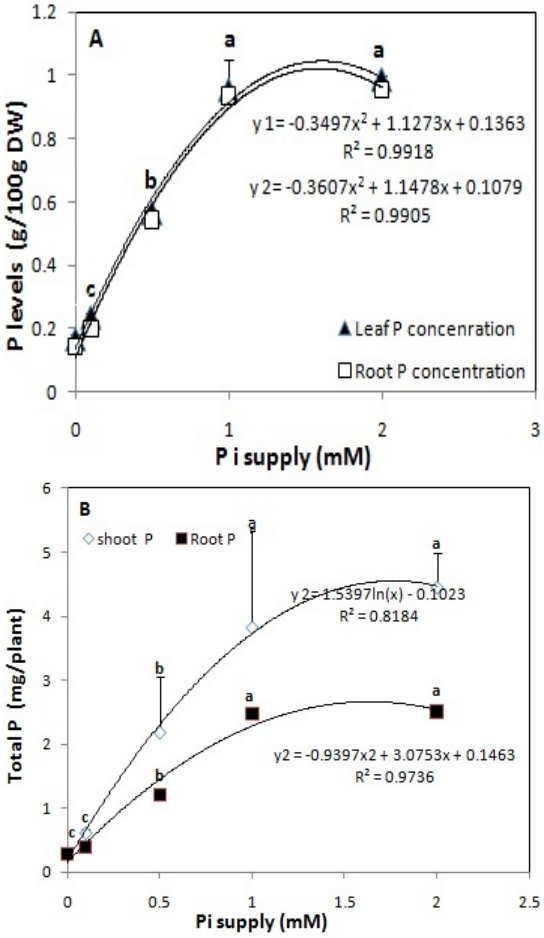

Fig 4. The effect of Pi supply on total P contents per unit of dry weight, in leaf and root tissue (A) and per plant (B). Means and standard deviations for 3 replicates are shown. Means with no significant are shown with the same letters as compared with LSD test method. Also, see material and methods for the measurement of the above indices.

was interrupted to almost zero in no-Pi plants. Also, there were significant differences between 0.1 mM Pi and 0.5 mM Pi plants. Over ten times higher root hair density was observed in the low Pi than the well fed plants (Table 2 and Fig 2). The effect of Pi supply on shoot was clearly reflected on leaf area (Fig 3A). Leaf area reduced significantly when Pi supply was below 0.5 mM. In reverse, the greenness intensity of leaves increased as the Pi supplies decreased (Fig 3B).

P accumulation upon Pi supply

As expected, there were correlations between Pi concentration in the medium and P contents of both leaf and root tissues, linearly below 1 mM of Pi concentration (Fig 4A). P accumulations in the roots and shoots exposed to no or 0.1 mM Pi solutions were five times less than those in the P-fed plants grown in higher Pi concentrations. The storage rates of P content per weight units of dry weight for P-starved leaves and roots were almost equal. As a result, there was a higher total P content of well-fed leaves in comparison with the roots of each plant (Fig 4B) due to increased biomass accumulation in the shoots (Table 1). These data also indicate that supplied Pi above 1 mM do not cause any significant increase in P accumulation or shoot biomass (Table 1 and Fig 4B).

Expression patterns of Pi transporter genes

The expression visualization and semi-quantification of *LePT1* and *LePT2* showed that both genes are differentially induced in root and leaf depending on Pi levels (Fig 5A to D), though the expression intensity for *LePT1* was higher than *LePT2* among the treatments. *LePT1* transcriptions were up-regulated in both Pi-starved leaves and roots. Similarly, when Pi supply was over 0.5 mM, *LePT1* was repressed sharply. In contrast, transcription of *LePT2*, encoding a high affinity Pi transporter, was detected only in roots in severe Pi starvation and interrupted in concentarations more than 0.1 mM Pi (Fig 5). Logarithmic correlation (R^2=0.80) between root's internal P and *LePT1* expression (Fig 5E and F) and $LePT_2$ indicated that total P content of the roots had effective influence on induction of gene expression at less than 0.2 g P $100g^{-1}$ DW in root tissue. In detail, at 0.2 g P 100 g^{-1} DW or higher, there was no detectable *LePT2* transcription in roots while *LePT1* expression was continued up to 0.5 g P $100g^{-1}$ DW.

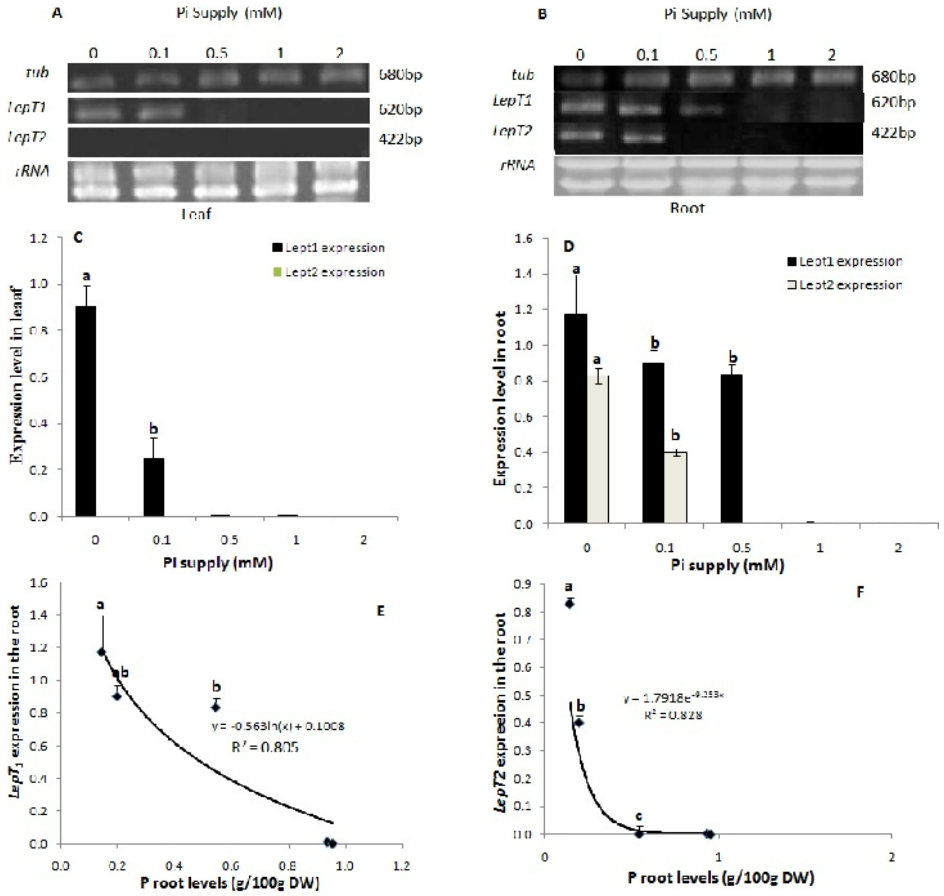

Fig 5. Relative expression level of *LePT1* and *LePT2* transcripts in various Pi supplies in leaf (A) and root (B). Tissue samples were harvested after six hours of refreshing the media. Semi-quantitative analysis of assays for *LePT1* and *LePT2* were conducted through RT-PCR with gene specific primers and normalizing data based on expression of an constitutively expressed gene, (α-*tubulin*). Uniform loading of RNA is shown by rRNA at the bottom (A and B). The effect of Pi supply concentrations on expression of *LePT1* and *LePT2* in leaf and root of tomato plants (C and D). Relationship between *LePT1* and *LePT2* expression in the roots and different levels of P in the root tissue (E and F).

Discussion

It is well known that all plants exposed to different Pi concentrations show correlated responses to internal P status (Biddinger et al, 1998; Bucher et al, 2001; De Groot et al, 2001; Lynch and Brown, 2001; Marschner et al., 2010). Root morphological traits are influenced by low Pi conditions while growth and total biomass increase in sufficient Pi conditions (Li et al., 2001; zobel et al., 2005; Hill et al., 2006; Louw-Gaume et al., 2010). Limited supply of Pi led to increased root to shoot ratio, root length, root volume and hairy root density and length. These adaptive alternations increased sharply when the P content decreased to less than 0.2 g P 100 g^{-1} DW in root or 0.5 g $100g^{-1}$ DW in the leaf. The outcome of these modifications is to increase Pi absorption ability of roots in deficient conditions (Raghothama, 1999; Lynch and Brown, 2001; Marschner et al., 2010). These results are consistent with previous findings that indicated Pi limitations increased length of primary roots, increased branching of lateral roots and increased density of root hairs (Lo´pez-Bucio et al., 2002, 2003; Hammond et al., 2004; Hill et al., 2006; Bremer and Schenk, 2009). In low-Pi tomato plants, the root area is enhanced by the formation of long root hairs (Foehse and Jungk, 1983; Bates and Lynch, 1996; Gahoonia and Nielsen, 1997; Bremer and Schenk, 2009). It has already been shown that the best correlated parameter to Pi uptake is root hair length rather than others traits such as density and radius of the roots (Itoh and Barber, 1983; Jungk, 2001). Foehse and Jungk (1983) found that sufficient Pi concentration lead to no or rudimentary root hairs in rapeseed, tomato and spinach. In this study, we have shown that the root hair density significantly increased when Pi supplied at 0.5 mM or less. Interestingly, when grown in no-Pi condition, root hairs disappeared (Table 2). Similarly, when *Arabidopsis* plants exposed to low Pi, the number of root hairs increased. However, if grown in no Pi medium, root hairs disappeared entirely (Lohrasebi and Malboobi, personal communication). This shows that plants need a minimum Pi resource to form root hairs. Our data also suggest that Pi stress increased the length of primary roots and specific root length indicating similar root adaptation mechanism in all Pi deficient plants (Fitter, 1985; Eissenstat et al., 2000). In low Pi condition roots dry mass reduced and simultaneously the root volume did not increase significantly. Wahl and Ryser (2000) concluded that lower root density in

P-deficient plants must be related to thinner cell walls, which could transport more mineral to root cells (Wahl and Ryser, 2000). Pi-fed plants accumulated higher rates of P in shoots than roots, when grown in sufficient Pi condition, though early rapid growth led to similar P amount per weight unit in comparison with P-deficient plants (Fig 4B). Relative allocating of more P into shoots than roots indicates that shoots are strong sources for P or essential parts for Pi as photosynthesis requires intensive phosphorylation process (Rao and Terry, 1989; Louw-Gaume et al., 2010). It is believed that plants adapted to have more inflow rate of P into shoots than roots (Cogliatti and Santa Maria, 1990; Burleigh and Harrison, 1999; Raghotama 1999). Some others researchers reported that low Pi supply reduced leaf area in P-deficient plants (Zobel et al., 2005; Khavarinejad et al., 2009). Less photosynthetic products in Pi deficiency causes negative effect on leaf cell expansion which is the result of carbohydrate deficiency while roots are stronger sink than leaves to allocate internal carbohydrate (Fig 3) (Rao and Terry, 1989; Stitt and Quick, 1989; Mollier and Pellerin, 1999; Vance et al., 2003; Louw-Gaume et al., 2010). As a molecular approach to P uptake in tomato plants, expression pattern of two Pi-transporter encoding genes showed deliberate responses of plants to Pi deficient conditions. A Partial correlation (R^2=0.80) between low P content in the roots and low affinity transporter system (*LePT1*) activity was also estimated (Fig 5A, B, C and D). Under mild Pi stress, low affinity system expressed in both roots and shoots while high affinity system was triggered only in the roots when internal P concentration was lower than 0.2 g P 100 g^{-1} DW (Fig 5F). These results are in line with Liu et al., (1998), suggesting *LePT2* is induced in ≤ 0.1mM Pi and moderate deficiency could not trigger *LePT2*, although other modifications in root occurred (Fig 1 and 2). This point is on the critical level of internal P status for these mechanisms. Moreover, when total P content of roots is less than 0.2 g P 100 g^{-1} DW, at least one of two transporter systems in the root cells was up-regulated (Fig 5A and B). According to Mukatira et al. (1996), P deficiency in the shoots is the main precursor to induce high affinity system (*LePT2)*. This could be the reason that plants adapted to raise P allocation in the shoots rather than in the roots (Fig 4B). Reuter and Robinson (1997) suggested that 0.65 g P 100 g^{-1} DW was approximately the sufficiency range for P level in tomato leaf. In this experiment, average P leaf content of 0.5 mM Pi-fed plants was 0.564 g P 100 g-1 DW which is just below that range (Fig 4A). As a conclusion, this research showed that P content of roots and shoots are strongly correlated with Pi supply. Tomato plants have evolved adaptive mechanism to absorb Pi as much as possible by changing root structure and by up-regulating the expression of Pi transporters.

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