Proc. Natl. Acad. Sci. USA
Vol. 96, pp. 5868–5872, May 1999
Plant Biology

Transcriptional regulation of plant phosphate transporters

[antibodies/tomato (*Lycopersicon esculentum*)/plasma membrane]

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Communicated by John D. Axtell, Purdue University, West Lafayette, IN, March 16, 1999 (received for review December 15, 1998)

ABSTRACT **Phosphorus is acquired by plant roots primarily via the high-affinity inorganic phosphate (P_i) transporters. The transcripts for P_i transporters are highly inducible upon P_i starvation, which also results in enhanced P_i uptake when P_i is resupplied. Using antibodies specific to one of the tomato P_i transporters (encoded by *LePT1*), we show that an increase in the *LePT1* transcript under P_i starvation leads to a concurrent increase in the transporter protein, suggesting a transcriptional regulation for P_i acquisition. LePT1 protein accumulates rapidly in tomato roots in response to P_i starvation. The level of transporter protein accumulation depends on the P_i concentration in the medium, and it is reversible upon resupply of P_i. LePT1 protein accumulates all along the roots under P_i starvation and is localized primarily in the plasma membranes. These results clearly demonstrate that plants increase their capacity for P_i uptake during P_i starvation by synthesis of additional transporter molecules.**

Phosphorus availability is considered one of the major factors that limits growth of plants in natural ecosystems. The concentration of available phosphorus is generally in the micromolar range, which is below that of many micronutrients (1). Consequently plants have developed several adaptive mechanisms to overcome P_i deficiency. These include changes in root growth and architecture, increased production of phosphatases and RNases, altered activity of several enzymes of the glycolytic pathway (2, 3), and an increased P_i uptake rate of roots (4). The ultimate consequences of these modifications are increased P_i availability in the rhizosphere and enhanced uptake.

Phosphorus is acquired by the plant roots in an energy-mediated cotransport process driven by a proton gradient generated by plasma membrane H^+-ATPases (5). The kinetic characterization of the P_i-uptake system by whole plants and cultured cells indicates a high-affinity transport activity operating at low concentrations (micromolar range) and a low-affinity activity operating at higher concentrations. The very low concentration (micromolar) of P_i in soil solution suggests that high-affinity transporters are primarily involved in P_i uptake by plants (3). The low-affinity system is apparently expressed constitutively, whereas the high-affinity system is induced under P_i deficiency (6). The induction process appears to involve *de novo* protein synthesis, since inhibitors of protein synthesis drastically reduce the induction of high-affinity P_i transport. The increased synthesis of a high-affinity carrier system has been proposed to be responsible for enhanced P_i uptake observed under P_i-deficiency conditions (6).

High-affinity P_i transporter genes have been cloned and characterized from fungi and from several plant species, including *Arabidopsis*, tomato, potato, *Medicago,* and *Catharanthus* (7). All the cloned P_i transporters are integral membrane proteins containing 12 membrane-spanning regions, separated into two groups of 6 by a large hydrophilic charged region, a common feature shared by many proteins involved in transport of sugars, ions, antibiotics, and amino acids (8). The transcripts encoding these transporters are predominantly expressed in roots and are strongly induced upon P_i starvation (9, 10). Tomato (*Lycopersicon esculentum*) genes encoding P_i transporters (*LePT1* and *LePT2*) are induced in a temporal- and P_i-concentration-dependent manner (9). The induction of these genes is a rapid response to P_i starvation and is reversible upon replenishment of phosphorus. These responses point to the existence of a fine coordination between gene expression and increased uptake of P_i.

Although genes coding for major plant nutrient transporters have been isolated and their expression characterized at the level of mRNA accumulation, there is no evidence showing that these changes result in altered levels of transport proteins. In this study, using antibodies specific to one of the tomato P_i transporters (LePT1), we demonstrate that an increase in the transcripts under P_i starvation is accompanied by a concurrent increase in the transporter protein, suggesting a transcriptional regulation for P_i acquisition. To our knowledge, this is the first report describing the transcriptional regulation of any major nutrient transporter in plants. In addition, this study also shows that P_i transporters are enriched in the plasma membranes of P_i-deficient roots, a physiologically relevant location for the high-affinity P_i transporters.

MATERIALS AND METHODS

Isolation and Analysis of P_i Transporter Transcripts. Total RNA was isolated from the roots of tomato plants by extraction with hot phenol and precipitation with lithium chloride (11). Total RNA (10 μg) was electrophoretically separated on denaturing formaldehyde 1% agarose gels and blotted onto BA-S (Schleicher & Schuell) nitrocellulose membrane. The northern analysis conditions were similar to those described earlier (9).

Antibody Production. A synthetic peptide (C-VAEIRATS-GRTVPV) corresponding specifically to the C terminus of LePT1 was used as the antigen for preparation of LePT1-specific antibodies. An extra cysteine residue was added at the N terminus for coupling to a carrier protein. The peptide was coupled to activated keyhole limpet hemocyanin (KLH) carrier according to the supplier's instructions (Pierce). After the coupling reaction, excess peptide and other components of the reaction were removed by dialysis against phosphate-buffered saline (PBS). Approximately 200 μg of KLH-peptide conjugate was mixed with an equal volume (500 μl) of complete Freund's adjuvant and injected subcutaneously into laying hens. The chickens were given two booster injections (100 μg of conjugate and Freund's incomplete adjuvant) 2 and 4 weeks after the primary immunization. The antibodies (IgY fraction) were purified from eggs collected before the injections (pre-

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immune), and eggs collected 1 week after the final booster injection (immune), essentially as described by Song *et al.* (12)

Culture of Tomato Plants and Isolation of Total Proteins. Tomato plants were grown in an aeroponics facility as described earlier (13). The plants were subjected to various P_i treatments by spraying roots at regular intervals with a fine mist of half-strength modified Hoagland's solution containing indicated amounts of P_i. For P_i-replenishment studies, the plants were starved of P_i for 5 days and then either resupplied with 250 μM P_i or maintained in P_i-deficient conditions. Plant roots were harvested, frozen in liquid nitrogen, and stored at −70°C. The tissues were ground to a fine powder in a mortar and pestle chilled with liquid nitrogen. The ground powder was transferred to a vial containing cold acetone and stored at −20°C overnight. The acetone-insoluble precipitate was collected by filtration through Whatman no. 1 paper and washed several times with cold acetone to remove moisture. The powder was dried under vacuum, and the total proteins were extracted by boiling in SDS sample buffer (20 μl/mg of powder) for 10 min.

Western Blots. The proteins were separated on SDS/10% polyacrylamide gels and transferred to nitrocellulose membranes in Towbin buffer (14). The membranes were blocked with 3% gelatin in TBS (20 mM Tris·HCl/500 mM NaCl, pH 7.5) at room temperature for 30 min, and then washed twice with TTBS (TBS + 1% Tween-20) for 5 min each. The blots were incubated for 4 hr at room temperature with a 1:1000 dilution of LePT1 antibodies in TTBS containing 1% gelatin. The membranes were washed three times with TTBS and then incubated with secondary antibody (alkaline phosphatase-conjugated rabbit anti-chicken antibody, 1:5000 dilution, Jackson ImmunoResearch) for 1 hr at room temperature. After two washes with TTBS and one wash with alkaline phosphatase buffer (100 mM NaCl/100 mM Tris·HCl, pH 9.5/50 mM $MgCl_2$), the membranes were incubated in 0.01% 5-bromo-4-chloro-3-indolyl phosphate/0.01% nitroblue tetrazolium solution (in alkaline phosphatase buffer) for color development. The reaction was stopped by rinsing the membrane several times with water.

Isolation of Plasma Membrane Fractions. Plasma membranes were isolated from roots of P_i-starved tomato plants by an aqueous two-phase extraction procedure (15, 16). The root tissue was homogenized by blending in an ice-cold grinding buffer (4 ml/g) consisting of 250 mM sucrose, 3 mM EDTA, 2.5 mM dithiothreitol (DTT), and 25 mM Tris-Mes, pH 7.5. The homogenate was filtered through four layers of cheesecloth and centrifuged at 13,000 × *g* for 15 min at 4°C. The supernatant was recentrifuged at 80,000 × *g* for 60 min at 4°C to pellet the membranes. The microsomal pellet was resuspended in 6 ml of the resuspension buffer (5 mM KH_2PO_4, pH 7.8/250 mM sucrose/3 mM KCl) by repeated pipetting and added to a 30-g phase partitioning system (final concentrations: 6.2% dextran T-500, 6.2% PEG 3350, 5 mM KH_2PO_4, pH 7.8, 3 mM KCl, and 250 mM sucrose). After thorough mixing of the phases and centrifugation at 1,000 × *g* for 5 min in a swinging-bucket rotor, the upper and lower phases were collected. The phases were repartitioned twice in fresh phase buffers as described (15). The purified phases were diluted with grinding buffer and centrifuged at 150,000 × *g* for 60 min to pellet the membranes. The membrane pellets were resuspended in 1 ml of grinding buffer and used for protein analysis and enzyme assays.

Enzyme Assays. The purity of plasma membrane fractions isolated as described above was analyzed by marker enzyme assays for the plasma membrane (vanadate-sensitive ATPase, ref. 17), tonoplast (NO_3-sensitive ATPase, ref. 18), and mitochondrial membranes (oligomycin-sensitive ATPase, ref. 19). ATPase assays were performed in 400-μl reaction volumes containing 250 mM sucrose, 30 mM Tris-Mes at pH 6.5 (pH 8.0 for assaying NO_3-sensitive ATPase activity), 3 mM ATP (Tris salt), 3 mM $MgSO_4$, 1 mM sodium azide (omitted when testing for oligomycin sensitivity), 0.1 mM ammonium molybdate, 50 mM KCl (when present), and 5 μg of membrane protein. The specific inhibitors were added as follows; 0.1 mM sodium orthovanadate for vanadate-sensitive activity, 50 mM KNO_3 in place of KCl for NO_3-sensitive activity, and 10 μg/ml oligomycin. Orthovanadate was included in the reactions testing for NO_3-sensitive activity, KNO_3 was present in the reactions testing for vanadate-sensitive activity, and both KNO_3 and orthovanadate were included in the reactions testing for oligomycin-sensitive activity. Reactions were carried out at 37°C for 1 hr, and the released P_i was measured by the method of Ames (20).

RESULTS

Transcription and Translation of P_i Transporters Are Coordinated During P_i Starvation. Western blotting of total proteins isolated from roots of tomato plants (Fig. 1*B*) indicate that the antibodies react specifically with a 60-kDa protein present in P_i-starved plants. The observed size of the detected protein corresponds to the size of the LePT1 protein calculated from the amino acid sequence (9). Accumulation of LePT1 protein increased markedly in plants grown under P_i-limiting conditions. LePT1 protein was present primarily in the roots, with a small amount also detectable in the leaves and stems of P_i-starved plants (data not shown). Both the *LePT1* mRNA and protein increased during P_i starvation, indicating that transcription and translation are highly coordinated in increasing P_i uptake. Similar responses in message accumula-

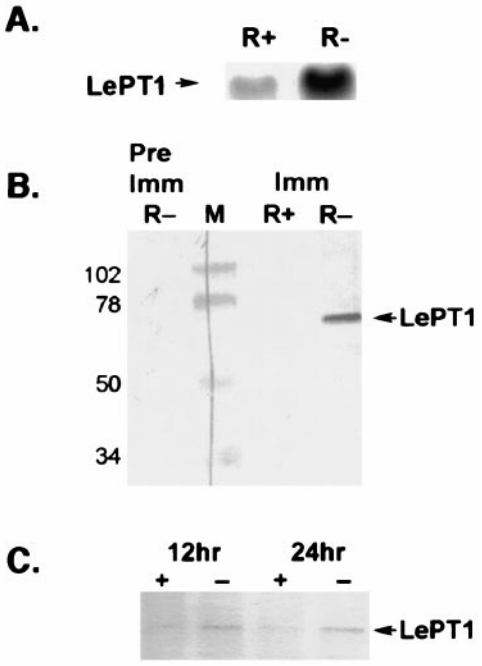

FIG. 1. (*A*) *LePT1* expression under P_i starvation. Northern blot analysis of total RNA (10 μg) isolated from the roots of tomato plants grown aeroponically and misted with a solution containing 250 μM P_i (R+) or no P_i (R−) for 5 days. (*B*) Accumulation of LePT1 protein in roots of tomato plants. Western blot of total proteins (20 μg per lane) isolated from the roots of tomato plants treated as described above. The antibodies isolated from eggs of immunized hens (Imm) react with a protein of ≈60 kDa, corresponding to the predicted size of LePT1 protein, in the roots of P_i-starved plants. This protein is not detected when antibodies from preimmune eggs (Pre imm) are used. M, molecular mass markers. (*C*) LePT1 protein accumulates rapidly in response to P_i starvation. Western blot of total proteins (20 μg per lane) isolated from roots of plants sprayed with 250 μM (+) or no (−) P_i for 12 or 24 hr. M, molecular mass markers.

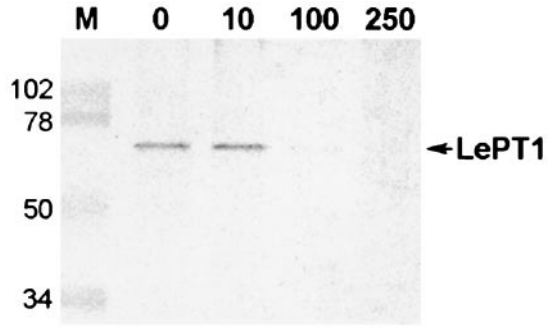

FIG. 2. Accumulation of LePT1 protein in response to decreasing concentration of P_i in the medium. Western blot of total proteins (20 μg per lane) isolated from roots of plants sprayed with nutrient solution containing the indicated concentration (μM) of P_i for 5 days. M, molecular mass markers.

tion during P_i starvation have been reported in other plants (10, 21–25). The accumulation of LePT1 protein is evident within 24 hr of P_i starvation (Fig. 1*C*), suggesting a rapid induction of the synthesis of this protein in response to P_i starvation.

Accumulation of LePT1 Protein in Roots Is Controlled by P_i Availability. Earlier studies showed that expression of *LePT1* transcript increased in roots of plants provided with 100 μM P_i or less, suggesting a relation between the concentration of P_i in the medium and the level of *LePT1* gene expression (9). The increased accumulation of *LePT1* transcript resulted in increased accumulation of LePT1 protein (Fig. 2). A strong accumulation of LePT1 protein was evident in roots of tomato plants sprayed with nutrient solution containing 10 μM or less of P_i. Lower levels of LePT1 protein were also observed in roots of plants provided with 100 μM P_i. The negative correlation between P_i availability in the medium and LePT1 protein accumulation was further examined by resupplying P_i to plants that were P deficient and strongly expressing the *LePT1* gene (Fig. 3). When 250 μM P_i was resupplied to these plants, the LePT1 protein level decreased within 24 hr, and it reached the uninduced level by 5 days. These observations, along with the earlier data on mRNA accumulation (9), clearly demonstrate a direct correlation between increased transcript levels of the P_i transporter gene and increased accumulation of transporter molecules.

The potential ability of different parts of roots to absorb P_i was examined by analysis of LePT1 protein accumulation in roots (Fig. 4). Interestingly, all the examined parts of P_i-deficient roots, except for extreme tip regions, had markedly higher levels of the protein. The data suggest that P_i starvation leads to enhanced expression of the P_i transporter in all parts

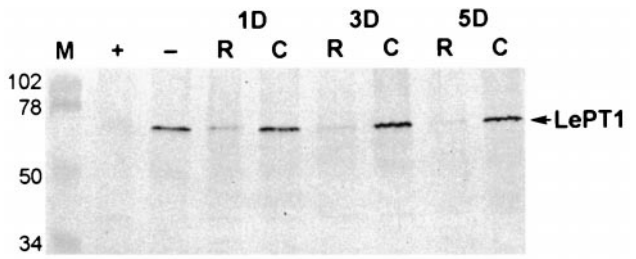

FIG. 3. Phosphate-starvation-induced accumulation of LePT1 protein in roots can be reversed by resupplying P_i to the starved plants. Tomato plants were sprayed with nutrient solutions containing 250 μM (+) or no (–) P_i. After 5 days the P_i-starved plants were replenished with 250 μM P_i (R) or continued to grow in P_i-deficient medium (C) for 1, 3, or 5 days as indicated. Total proteins (20 μg per lane) isolated from roots were analyzed by Western blotting. M, molecular mass markers.

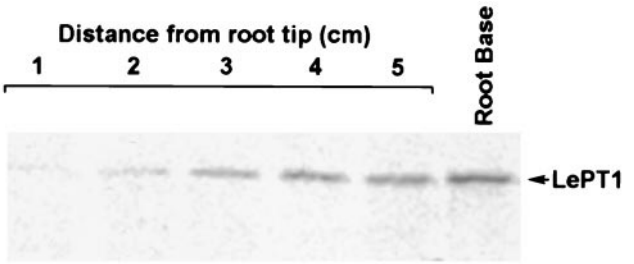

FIG. 4. Accumulation of LePT1 protein in different parts of P_i-starved tomato roots. Tomato plants were grown under P_i-starvation conditions for 5 days. Roots were divided into 1-cm sections. Proteins isolated from these tissue samples (20 μg per lane) were analyzed by Western blotting.

of the plant root, supporting the hypothesis that under P_i deficiency the ability of the entire root system to absorb P_i increases (26).

Phosphate Transporters Are Enriched in the Plasma Membrane. On the basis of the deduced amino acid sequence of P_i transporters, LePT1 protein is predicted to be an integral membrane protein. The sequence also lacks any discernible signal peptide motif at the N terminus, suggesting that it is likely to be associated with the plasma membrane. This prediction was confirmed by the observation that LePT1 protein is enriched in the plasma membrane fractions isolated from roots of P_i-starved tomato plants (Fig. 5*A*). The total membranes isolated from these roots were fractionated into plasma membrane and other membrane fractions by repeated aqueous two-phase extraction. This procedure yields highly

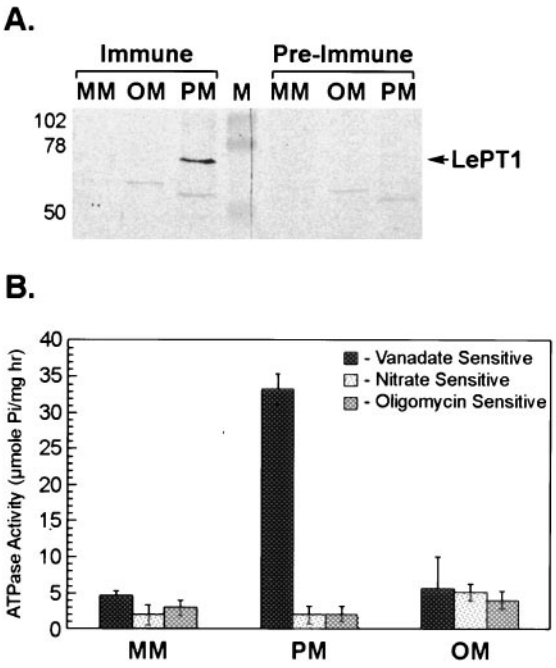

FIG. 5. (*A*) LePT1 shows enrichment in the plasma membrane of roots. The microsomal membranes (MM) obtained from roots of P_i-starved tomato plants were further fractionated into plasma membranes (PM) and other membranes (OM) by the aqueous two-phase extraction procedure (15). Equal quantities (5 μg) of membrane proteins from these fractions were analyzed by Western blotting. M, molecular mass markers. (*B*) Activities of marker enzymes in different membrane fractions. Specific activities of vanadate-sensitive ATPase (a plasma membrane marker), nitrate-sensitive ATPase (a tonoplast membrane marker), and oligomycin-sensitive ATPase (a mitochondrial membrane marker) were determined for each of the membrane fractions to assess their purity. The PM fraction is enriched in vanadate-sensitive ATPase activity.

enriched plasma membrane vesicles as judged by the activities of marker enzymes in the fractions (Fig. 5*B*). Vanadate-sensitive ATPase activity, a marker enzyme for plasma membrane, showed severalfold enrichment in the plasma membrane fractions but little or no enrichment in other fractions. The nitrate- (tonoplast marker) and oligomycin- (mitochondrial membrane marker) sensitive ATPase activities did not show any significant enrichment in the plasm membrane fractions. The presence of LePT1 protein in the plasma membranes correlates well with its proposed function in nutrient uptake.

DISCUSSION

The uptake of P_i by plants under natural conditions proceeds through an energy-dependent proton/phosphate symport system. Because of the persistently low concentration of P_i in soil solution, the high-affinity P_i transport is considered the primary mode of P_i uptake in plants. This transport system is composed of the high-affinity P_i transporters that are inducible during P_i starvation (3, 10). The ability of plants to increase P_i uptake under P_i starvation is well documented (4, 27, 28). The results of studies on P_i transporter gene expression in tomato have shown that the transcripts for P_i transporters (*LePT1* and *LePT2*) are highly induced under P_i-deficient conditions (9, 21). Similar increases in the transcript levels of genes encoding P_i transporters in other plant species, including *Arabidopsis*, potato, *Medicago,* and *Catharanthus,* have been reported (10, 22–25).

The increased P_i absorption following an episode of P_i starvation is thought to be associated with a higher capacity of roots for P_i transport, possibly by formation of additional carriers of P_i (4, 28). This increased P_i uptake occurs with little or no change in the apparent K_m but significant increase in V_{max} because of increased synthesis of P_i transporters (6). Results of many P_i uptake experiments confirm that plants adjust their P_i uptake on the basis of internal P_i status, particularly by increasing I_{max} (maximum influx), whereas change in K_m is of minor importance in this process (29). Although allosteric regulation of P_i uptake under P_i starvation is suggested (30), it is becoming evident that the capacity for P_i uptake is regulated by increasing the total number of transporter molecules (3). Inducible expression of P_i transporters is vital for growth of plants under varying concentrations of P_i. The induction of LePT1 protein in response to P_i starvation correlates with the observed increase in P_i uptake rate of roots and cell cultures (27, 31). Addition of protein synthesis inhibitors suppressed P_i starvation-induced P_i uptake in tobacco cell cultures, indicating that *de novo* synthesis of P_i transporters is required for the transport process (28). Increased accumulation of LePT1 protein in tomato plants provides direct evidence for altered synthesis of the transporters during P_i starvation. Expression of P_i transporter genes is not only induced rapidly in response to P_i deficiency, but is also reversible upon resupply of P_i. Both the induction and repression of P_i transporters are detectable within 24 hr of P_i removal or resupply, respectively. Similarly, enhanced P_i uptake rates in P_i-starved tomato plants returned to the normal rate 30 hr after P_i replenishment (32), indicating down-regulation of the uptake process. Regulated expression of P_i transporters will allow plants to obtain the required amount of P_i without leading to toxic effects. These data support the hypothesis that plants modulate their capacity for P_i uptake primarily by synthesis/turnover of the transporter molecules. However, under certain experimental conditions, some P_i-starved plants exposed to high concentration of P_i may accumulate toxic levels of the nutrient (27). This response is likely caused by rapid accumulation of P_i before the turnover of transport proteins and inhibition of transcript accumulation. A coordinated, rapid induction of both the mRNA (9) and protein after P_i removal is likely a reflection of transcriptional regulation of gene expression. Transcriptional regulation of P_i uptake will provide plants with a rapid and responsive mechanism to regulate P_i uptake in the rhizosphere. A similar mechanism is known to function in other organisms, such as yeast and bacteria (33, 34).

Transcriptional regulation of P_i transporters in plants may not preclude other, as-yet-uncharacterized, control mechanisms, including posttranslational modification of proteins. In yeast, expression of structural genes encoding high-affinity P_i transporters and repressible phosphatases is controlled at the transcription level by an intricate cascade involving both positive and negative regulatory proteins (33). Protein–protein interaction is also involved in regulation of P_i uptake of yeast cells (35). There is a growing evidence supporting the existence of similar regulatory mechanisms in plants (7). Further studies should reveal interactions of P_i transporters with other proteins as observed in regulation of the yeast high-affinity P_i transporter. However, the complexity of tissues and organs in plants and the required transport of P_i throughout the plant suggest that additional regulatory mechanisms are likely to function in regulating P_i uptake and metabolism in plants.

There is a continuing debate on the ability of different parts of root system to absorb nutrients. The P_i transporter accumulation in different parts of roots shows that plants are able to absorb P_i all along the roots during P_i starvation. A similar observation on the ability of the root system to acquire P_i was made earlier (26). It has been shown that the entire root system retains the potential to transport P_i at an increased rate, despite anatomical and physiological changes associated with aging (4). Furthermore, under P_i starvation, influx of P_i was first evident in the older root zone close to the shoot, and it spreads along the root toward the apical region (26). This type of physiological and biochemical flexibility will allow any part of the root system to acquire P_i as and where the nutrient becomes available. It is becoming clear that plants respond to P_i starvation not only by increasing root growth but also by changing biochemistry of roots to increase P_i availability and enhance uptake (2, 7). Detailed *in situ* hybridization and immunolocalization studies of different members of the P_i transporter family should help in understanding the complexity of P nutrition in higher plants.

Localization of LePT1 protein in plasma membranes strongly suggests its primary role in high-affinity P_i transport in cells. The deduced amino acid sequence of LePT1 did not show the presence of any organellar targeting sequence, indicating that insertion of LePT1 into the plasma membrane may be due to its innate structural properties. The plasma membrane is the physiologically relevant target for the high-affinity P_i transporters. A steep P_i concentration gradient between the cytoplasm (millimolar) and soil solution (micromolar) and the negative inside transmembrane electrical gradient requires that P_i transport occurs via an energy-dependent high-affinity transport system located at the plasma membrane. Uptake kinetics studies in both yeast (21) and tobacco cell cultures (36) expressing the cloned P_i transporters showed that K_m of the high-affinity P_i transporters is within the range of available phosphorus in the soil solution. Furthermore, the presence of millimolar concentrations of P_i inside plant cells precludes the need for high-affinity P_i transporters in other organellar membranes. This is particularly true for vacuoles, the primary site for P_i storage in cells. The P_i transport across the tonoplast is presumed to occur through the low-affinity (high V_{max}) transport systems, whose biochemical and biophysical properties are not well understood at present. The P_i fluxes across the tonoplast could play a significant role in P_i homeostasis in the cytoplasm, and consequently in expression of P_i transporters, leading to altered uptake. The localization of P_i transporter proteins to plasma membranes and their expression in the root epidermis and root hairs (9, 21) are

indicative of the role of P_i transporters in the nutrient acquisition.

The results presented here show that an increase in the LePT1 mRNA under P_i starvation directly correlates with an increase in the transport protein. These data, in combination with studies of mRNA accumulation (9) and P_i uptake (4, 27), provide strong evidence for the transcriptional regulation of P_i uptake in plants.

We thank Drs. Peter B. Goldsbrough and David Rhodes for critical review of the manuscript. This work was supported by a grant from the U.S. Department of Agriculture National Research Initiative Competitive Grants Program (97-35100-4211) to K.G.R. This is journal paper no. 15964 of the Purdue University Agriculture Research Program.

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