Phosphate Starvation Inducible Metabolism in 
*Lycopersicon esculentum*

I. EXCRETION OF ACID PHOSPHATASE BY TOMATO PLANTS AND SUSPENSION-CULTURED CELLS

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ABSTRACT

Both tomato (*Lycopersicon esculentum* cv VF 36) plants and suspension cultured cells show phosphate starvation inducible (psi) excretion of acid phosphatase (Apase). Apase excretion in vitro was proportional to the level of exogenous orthophosphate (Pi). Intracellular Apase activity remained the same in both Pi-starved and sufficient cells, while Apase excreted by the starved cells increased by as much as six times over unstressed control cells on a dry weight basis. At peak induction, 50% of total Apase was excreted. Ten day old tomato seedlings grown without Pi showed slight growth reduction versus unstressed control plants. The Pi-depleted roots showed psi enhancement of Apase activity. Severely starved seedlings (17 days) reached only one-third of the biomass of unstressed control plants but, because of a combination of both psi Apase excretion by roots and a shift in biomass to this organ, they excreted 5.5 times the Apase activity of the unstressed control. Observed psi Apase excretion may be part of a phosphate starvation rescue system in plants.

The utility of the visible indicator dye 5-bromo-4-chloro-3-indolyl-phosphate-p-toluidine as a phenotypic marker for plant Apase excretion is demonstrated.

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Phosphorus is an essential nutrient for all cells. For organisms that absorb their mineral nutrients directly from the external medium, the orthophosphate anion (Pi, H₂PO₄⁻ or H₃PO₄⁻) is the preferentially absorbed form of Pi. A macronutrient based on its contribution to biomass, Pi² is one of the least available mineral nutrients in many environments. The level of Pi in the solution phase of soils (1.0 μM or less) is often below those of many micronutrients (3, 10). In contrast to low levels of soluble Pi, ecosystems often contain large amounts of both organic and insoluble mineral Pi (6). This ecological paradox has resulted in the evolution of a number of gene systems that function to enhance the availability of Pi. These coordinately expressed psi operons, collectively called a psi regulon, have been studied extensively in both bacteria and yeast (12, 16, 17).

The best characterized psi gene systems are in *Escherichia coli* where many psi genes are part of a group of phosphate-regulated operons called the pho regulon. The pho regulon includes a minimum of 20 to 24 genes that share a common positive regulatory element encoded by the gene phoB. The PhoB protein (mol wt 29 kD), which is itself psi, binds specifically to DNA sequences containing the promoter regions of several genes in the pho regulon. It activates both the transcription of these genes (12). Many of the psi genes function to enhance Pi availability in, and uptake from, the external medium. For example, phosphate starvation induces *phaA*, the gene that codes for alkaline phosphatase. This hydrolytic enzyme is excreted into the periplasmic space where it acts to cleave extracellular organic P to Pi. A second psi gene system, the phosphate specific transport (Pst) operon uses energy to transport Pi across the *E. coli* membrane. The Pi transport efficiency of this system is much greater than that of the constitutive Pi shuttle (12). Under Pi starvation, the strategy is to solubilize organic P to Pi and take up the Pi with increased efficiency. A similar psi system has been characterized in yeast (7, 17).

Although phosphate is a major plant nutrient as well as a key regulatory component in both photosynthetic and respiratory metabolism, little is known about phosphate-regulated gene expression in higher plants. While many microorganisms can take up and metabolize low mol wt organic phosphates, plants depend almost exclusively on Pi absorbed from the external solution for Pi nutrition (2, 3). In 1971, Ueki and Sato (13) demonstrated that omitting inorganic phosphate from the medium resulted in an increase in the Apase activity in tobacco cells growing in suspension culture. In later studies, Ueki (14, 15) showed that excretion of acid phosphatase was the result of an energy-dependent transport system that was inhibited by exogenous Pi and could be regulated by divalent cations.

In this article, we report on the regulation of psi excretion of acid phosphatase in suspension cultured cells of two species of *Lycopersicon*. The data presented here clearly demonstrate that the amount of Apase excreted by these cells is proportional to the degree of Pi depletion in the media. In addition, even when the Pi-depleted cells are excreting six times the amount of Apase into the external medium as their +Pi counterparts, the intracellular Apase activity remains the same as in the +Pi cells. We further report the results of whole plant studies to show that during the early stages of Pi starvation, enhancement of Apase activity occurs primarily in root tissue. Under severe Pi starvation, up to 9% of the Apase produced by the roots is found in the external hydroponic medium.

MATERIALS AND METHODS

All experiments were carried out with either *Lycopersicon esculentum* (VF36) or *Lycopersicon pennellii* (ATICO 716, gen-
Callus cultures were initiated in 1987 using the method of Nover et al. (9) with slight modifications. Continuous cultures were maintained via transfer of 5 ml of 10 d old cells (late log phase, see Fig. 1) to 45 ml of liquid medium. All experiments with suspension cultures were conducted using cells derived from a single source of callus for each organ. Cells were subcultured a minimum of three times before the initiation of the research, and all experiments reported here were carried out within 3 months of each other. Apase (orthophosphoric-monooester phosphohydrolase, EC 3.1.3.2) activity was assayed using p-nitrophenyl-phosphate (Sigma 104). The spectrophotometric assay at 400 nm was conducted using the method recommended by the manufacturer (11). Suspension-cultured cells with different levels of Pi were sampled at 24 h intervals. A 0.5 ml aliquot was withdrawn and centrifuged at 15,000g for 10 min, and 0.2 ml of supernatant was added immediately to the assay medium. Dry weight determinations were made after 24 h at 80°C. For determination of total Apase, 1.5 ml of cells were disrupted (Brinkman Polytron, setting 7 for 15 s) on ice and then centrifuged. Apase in the supernatant was assayed, and dry weights were taken from a duplicate nondisrupted aliquot. Nonexcreted Apase activity was calculated as the difference between excreted and total Apase activity. One unit of Apase activity is defined as that amount of enzyme activity that will liberate 1 nmol of p-nitrophenol-phosphate per hour under the stated assay conditions. Water soluble Pi was determined by the spectrophotometric method of Murphy and Riley (8).

Whole plants were grown in hydroponic culture at a constant 28°C with a 12 h photoperiod (240 μE m⁻² s⁻¹). +Pi media was half-strength Hoagland solution (2). The -Pi treatment substituted 1 mM NH₄NO₃ for the 2 mM NH₄H₂PO₄. Solutions were changed every 3 d. Total Apase activity was determined by disruption of tissue in the growth medium -Pi (polytron, setting 5 for 30 s) followed by centrifugation at 12,000g and assay of the supernatant. Tissue dry weight determinations were made on a duplicate set of samples. To follow excretion of Apase into the external medium by roots, three plants were placed in an aerated 100 ml plastic beaker containing 15 ml of fresh growth media ± Pi. The top of the beaker was sealed around the stems with paraffin film, and Apase activity in the solution was assayed after 5 h.

For visualization of Apase activity with XP, seed of L. esculentum was surface-sterilized and germinated on water agar. Seedlings were transferred into sterile jars containing 30 ml of 1% agar with MS salts plus 3% w/v sucrose ± Pi. XP (0.004% w/v; 16) was added to both treatments. The jars were sealed and placed in the lighted incubator under the environmental conditions previously described.

**RESULTS**

Figure 1 shows the changes in excreted and intracellular Apase activity in suspension cultured L. esculentum cells in the presence or absence of exogenous Pi. This figure also shows phosphate uptake and biomass accumulation for the two treatments during the growth cycle. Biomass accumulation remained the same in both treatments for the first 8 d (Fig. 1C), which indicated that cells in late log phase had an internal P pool sufficient for this amount of growth. As shown in Figure 1b, the +Pi cells accumulated approximately 40% of the Pi available in the medium in the first 8 d. As a result, while growing at the same rate to the same total biomass, the +Pi cells accumulated 0.06 mmol Pi/g dry weight while the -Pi cells took up virtually no exogenous Pi. Figure 1 clearly demonstrates the psi nature of enhancement in excreted Apase activity. Figure 1a shows the psi excretion of Apase in the -Pi cells. The Pi-depleted cells showed enhancement in excreted Apase activity versus the Pi-sufficient cells within 24 h. This response increased throughout the initial 8 d period, when the -Pi cells were growing at the same rate as the +Pi cells. Maximum induction (d 9-11, Fig. 1a) in -Pi cells occurred after the onset of inhibition of growth as a result of phosphate starvation. At peak induction, the -Pi cells were excreting up to 6 times more Apase into the external medium than the +Pi cells on a dry weight basis. The +Pi cells showed little variation in the low level of excreted Apase during the entire growth cycle. Differences in the buffering capacity of the two media were not a factor since the decrease in pH during cell growth in the two treatments did not differ significantly (data not shown).

Figure 1 (d-f) compares the relative levels of internal and excreted Apase activity in the +Pi and -Pi cells. In the +Pi treatment (Fig. 1d), nonexcreted Apase was apparently intracellular since the high levels of Apase activity released by the +Pi cells upon disruption were never seen in the external media. In the -Pi treatment, 50% of total Apase activity was excreted at maximum induction. This maximum in induced extracellular Apase activity corresponds approximately with the time when cell growth has ceased (9 d, Fig. 1c). Figure 1f shows that little difference existed between these two treatments with respect to intracellular levels of this enzyme. As a result, the -Pi treatment had 2 times the total Apase activity of the +Pi control with this difference being in the excreted activity. While not unequivocal, these data strongly suggest de novo synthesis of the excreted Apase. This in turn suggests induction of a gene with psi regulatory sequences to control induction and some form of regulation to target this enzyme for excretion into the apoplast.

The data presented in Figure 2 show the effect of five different levels of exogenous Pi on the activity of Apase excreted by suspension-cultured cells of L. pennelli derived from cotyledon, hypocotyl, and root tissue. L. pennelli was chosen for this experiment because suspension-cultured cells from all three tissues grew at approximately the same rate. By comparison, cul-

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**Fig. 1.** a, Phosphate starvation inducible excretion of Apase by L. esculentum cells growing in medium +Pi (●) or -Pi (△); b, water-soluble Pi in the growing medium of +Pi and -Pi cells; c, dry weight accumulation by +Pi and -Pi cells; d, comparison of intracellular (*) and excreted (○) Apase in +Pi cells; e, comparison of intracellular (■) and excreted (▲) Apase in -Pi cells; f, comparison of intracellular Apase in +Pi and -Pi cells.
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Fig. 2. Effect of different levels of initial soluble Pi on excreted Apase, Pi uptake, and dry weight accumulation in root (a, b, c), hypocotyl (d, e, f), and cotyledon derived (g, h, i) cells of L. pennelli. Initial level of Pi in the medium is 0.0 mm (△), 0.31 mm (□), 0.94 mm (○), 1.25 mm (■), or 1.56 mm (●).

Fig. 3. Effect of Pi starvation on tissue Apase activity, dry weight accumulation, and excreted Apase activity in tomato seedlings growing in hydroponics. Total Apase activity was assayed for root, stem, and leaf tissue at 17 (a), 14 (b), and 10 (c) days of age. The enhancement in Apase activity in -Pi versus +Pi treatments is shown in d, e, and f. At each time point, seedlings were transferred to fresh growth medium -Pi and Apase excretion into the external solution assayed after 5 h. Total solution activity was normalized per g dry weight of root tissue.

versus slight starvation (cf. Fig. 3, a and c), the total excreted Apase activity per plant increased 5.5 times in older severely stressed plants versus the unstressed controls.

In the Pi-deficient plants, biomass accumulation occurred mainly in the root tissue (Fig. 4; see also Ref. 1). Root biomass in the moderately Pi-starved (14 d) seedlings was 100% of the Pi-sufficient seedlings, while both stem and leaf biomass were reduced to 50% of control. Severely stressed seedlings showed an almost complete inhibition of growth. These whole plant data suggest that Pi stress acts pleiotropically to enhance the absolute amount of Apase in the root zone by increasing both the relative root mass and Apase activity. Calculations from the data in Figures 3 and 4 show that the combination of 7-times enhancement in excreted Apase activity (per g dry weight of roots) and the relatively high proportion of root biomass in the -Pi plants resulted in a 5.5-times enhancement in total excreted Apase activity from the initial 3.2 in +Pi plants versus the -Pi treatment. This occurs even though the +Pi plants were over 3.2 times the size of the -Pi plants.

Figure 5 shows the utility of the chromogenic substrate XP for visualization of Pi starvation inducible enhancement of Apase activity in root tissue. Seedlings were grown for 15 d during which time the blue color of the cleaved XP was developed on the roots of the -Pi seedlings. This phenotypic marker will be useful in identifying mutants for the Apase excretion trait.

DISCUSSION

When viewed as a whole, these data provide strong preliminary evidence for the existence of a psi phosphate starvation rescue system in higher plants. One component of this system is psi production and excretion of Apase in order to access and solubilize organic P in the external medium (the rhizosphere in vivo). Ueki and Sato (13) demonstrated that Apase activity in suspension-cultured cells of tobacco increased upon omission of Pi from the culture medium. These workers also showed that this effect was reversible by the addition of Pi to the medium and that accumulation of Apase activity was inhibited by addition of
The data presented in Figures 1 and 2 clearly demonstrate that tomato cells regulate the amount of Apase excreted into the external medium in response to the concentration of Pi in the medium. Intermediate levels of Pi depletion resulted in intermediate levels of Apase activity in the external medium. Apase excretion appears to be regulated in a manner that allows for a fine tuning of the response to Pi availability. Previous workers have shown that the pleiotropic plant response to Pi starvation involves enhanced root growth, Pi uptake, and bidirectional transport of Pi between roots and shoots (1).

The whole plant studies presented here demonstrate the potential utility of psi excretion of Apase as one component of a phosphate starvation rescue system in vivo. At 10 d of age, there is little difference between the two treatments in terms of biomass. In spite of this, root tissues responded to Pi depletion and showed 2.6 times enhancement in Apase activity. At 14 d, Pi starvation drastically inhibited plant growth, and Apase activity increased in all tissues of the -Pi plants.

The data presented in Figures 3 and 4 demonstrate both the complexity and utility of the whole plant response to Pi starvation. Figure 4 shows clearly that, as Pi starvation progresses, the plant retained root growth at the expense of other organs. This phenomenon resulted in an increase in root biomass from 11 to 24% of the total biomass of 10- and 14-d-old -Pi plants, respectively. In +Pi plants, the root component increased from 7 to 12% in the same time period. The psi enhancement of excreted Apase activity was magnified by this distribution of biomass. After 17 d, the -Pi roots had over 80 units of Apase per mg dry weight versus 40 for the unstressed control (Fig. 3a). In a 5 h period, 7.27 units per mg dry weight of root tissue was excreted into the external solution. It appears that the organismal strategy under Pi starvation is to channel biomass to the roots and to increase excretable tissue Apase activity. The result of these two responses was enhancement of total extracellular Apase activity. The 7-times enhancement in excretion (per g root dry weight) by the 17-d-old roots combined with a relatively unpressed root growth rate resulted in a 5.5-times enhancement of Apase activity excreted by the -Pi versus the +Pi plant. Meanwhile, the total biomass of the -Pi plant was reduced to 31% of the +Pi plant.

In a natural plant environment, the extent of Apase induction and excretion will undoubtedly be affected by a number of variables including the developmental stage prior to onset of Pi stress and conditions within the rhizosphere. Studies with 19-d-old +Pi tomato plants showed that psi enhancement of Apase activity occurred within 24 h of transfer to Pi depleted medium (data not shown). This enhancement reached a peak at 10 d after transfer to Pi depleted medium. It is possible that psi Apase activity is restricted to certain tissues so that calculations of enhancement based on normalization to total organ biomass will greatly underestimate changes in gene expression on the cellular level. This might explain the difference in enhancement seen with whole plants versus cell cultures and could also explain the dramatic effects seen with XP, which also appeared to show much more than a 2- to 3-times enhancement.

Excreted tomato Apase has two characteristics that support a possible role in scavenging organic P in the rhizosphere. First, it has a pH optimum in the range of 4 to 6. Since the root surfaces of many plants are known to be acidified, an acid (versus alkaline) phosphatase would be of greater utility in this region. Second, we have found this enzyme to be stable for hours in the hydroponic solution at 28°C. This type of stability would be expected for an enzyme that functions in the rhizosphere. Studies of this system will enhance our understanding of gene regulation in plants and may play a role in the development of biorational approaches to P fertilization in agriculture (4, 5).
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LITERATURE CITED


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