Phosphate or phosphite addition promotes the proteolytic turnover of phosphate-starvation inducible tomato purple acid phosphatase isozymes

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Abstract Within 48 h of the addition of 2.5 mM phosphate (HPO_4^{2-}, Pi) or phosphite ($H_2PO_3^-$, Phi) to 8-day-old Pi-starved (−Pi) tomato suspension cells: (i) secreted and intracellular purple acid phosphatase (PAP) activities decreased by about 12- and 6-fold, respectively and (ii) immunoreactive PAP polypeptides either disappeared (secreted PAPs) or were substantially reduced (intracellular PAP). The degradation of both secreted PAP isozymes was correlated with the de novo synthesis of two extracellular serine proteases having M_rs of 137 and 121 kDa. In vitro proteolysis of purified secreted tomato PAP isozymes occurred following their 24 h incubation with culture filtrate from Pi-resupplied cells. The results indicate that Pi or Phi addition to −Pi tomato cells induces serine proteases that degrade Pi-starvation inducible extracellular proteins.

Keywords: Phosphite; Phosphate starvation; Purple acid phosphatase; Serine protease; *Lycopersicon esculentum*

1. Introduction

Although phosphate (HPO_4^{2-}, Pi) plays a pivotal role in cellular metabolism and bioenergetics, it is one of the least available nutrients in many natural terrestrial and aquatic ecosystems [1–5]. Plant Pi deprivation elicits a complex array of biochemical adaptations, including acid phosphatase (AP; EC 3.1.3.2) induction [1–9]. Pi-starvation inducible (PSI) APs function as intracellular (vacuolar) or extracellular (secreted) Pi scavenging systems that catalyze the hydrolysis of Pi from phosphate-monoesters. In both plants and yeast, PSI gene repression occurs in the presence of Pi or its analog, phosphite ($H_2PO_3^-$, Phi) [1–4,10–16].

Phosphite is a reduced form of Pi in which a non-acidic hydrogen replaces one of the oxygens bound to the P atom. Phi is an important but controversial agricultural commodity that is being widely marketed either as a crop fungicide or as a superior source of crop P nutrition. It is well established that fungicides based upon Phi effectively control plant pathogens belonging to the order Oomycetes (particularly *Phytophthora* sp.) that are responsible for a host of crop diseases [15]. However, evidence that plants (or yeast) can directly utilize Phi as their sole source of nutritional P is lacking [2,3,10–16]. Although plant and yeast cells readily assimilate Phi, it is relatively stable in vivo and does not appear to be readily oxidized or metabolized. Interestingly, low Phi concentrations are very deleterious to the development of Pi-starved (−Pi), but not Pi-sufficient (+Pi) plants and yeast. Phi negates their acclimation to Pi deficiency by specifically blocking the derepression of genes encoding PSI proteins (i.e., repressible AP, high-affinity plasmalemma Pi transporters, etc.) [2,3,10–16]. A recent study revealed that Phi treatment markedly accelerated the initiation of programmed cell death that otherwise occurs when *Brassica napus* (oilseed rape) suspension cells are subjected to one month of Pi-starvation [16]. While Phi is not a substrate in enzyme-catalyzed phosphoryl transfer reactions, plant and yeast Pi transporters participating in Pi uptake or signal transduction components involved in sensing cellular Pi status do not appear to efficiently discriminate between the Pi and Phi anions.

Although the influence of Pi and Phi on plant PSI gene expression has been well documented, few studies have considered the fate of plant PSI proteins when Pi stress is reversed by Pi-resupply [7,11]. Whether Phi can substitute for Pi in promoting the turnover of PSI proteins is unknown. The aim of the present study was to assess the impact of Pi- or Phi-addition on PSI intracellular and secreted AP isozymes (IAP and SAP, respectively) of 8-day-old −Pi tomato suspension cells. The Pi starvation response of these cells includes the pronounced induction of a heterodimeric IAP and two monomeric SAP isozymes (SAP1 and SAP2) [8,9]. All three PSI tomato AP isozymes are purple APs (PAPs). PAPs represent a distinct class of non-specific AP containing a bimetallic active centre that endows them with a characteristic purple or pink color. Here, we document a striking reduction in IAP and SAP activities and their corresponding immunoreactive polypeptides following Pi or Phi addition to 8-day-old −Pi tomato suspension cell cultures. The disappearance of SAP1 and SAP2 that occurred 48 h following Pi or Phi addition to the −Pi tomato cells was correlated with the de novo synthesis of two secreted serine proteases having M_rs of about 137 and 121 kDa. The results imply that Pi or Phi not only represses PSI genes in plants, but that either anion also promotes the proteolytic turnover of plant PSI proteins.

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Abbreviations: AP, acid phosphatase; CCF, cell culture filtrate; CX, cycloheximide; IAP, intracellular acid phosphatase; PAP, purple acid phosphatase; Phi, phosphite; +Pi and −Pi, Pi-sufficient and Pi-deficient, respectively; PMSF, phenylmethylsulfonyl fluoride; PSI, phosphate-starvation inducible; SAP, secreted acid phosphatase

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2. Materials and methods

2.1. Plant material

A heterotrophic cell suspension of tomato (*Lycopersicon esculentum*, cv Moneymaker) was cultured on rotational shakers (125 rpm) in 50 ml of Murashige-Skoog media containing 0 or 2.5 mM Pi as previously described [8]. For the generation of experimental samples, 8-day-old −Pi cells were treated with filter-sterilized 2.5 mM KPi ± 2.5 μM cycloheximide (CX) or 2.5 mM KPhi and cultured for up to an additional 48 h. Cells were harvested on a Büchner funnel fitted with Whatman #1filter paper. Cells and cell culture filtrate (CCF) were frozen separately in liquid N_2 and stored at −80 °C until used.

2.2. Acid phosphatase extraction and activity assays

Clarified extracts were prepared from quick-frozen tomato cells as described previously [9]. AP activities were determined using a continuous coupled spectrophotometric assay [8,9]. Protein concentrations were determined with the Coomassie Blue G-250 dye-binding method [17] using bovine γ-globulin as the protein standard.

2.3. Immunoblotting

SDS–PAGE, electroblotting of mini-gels onto PVDF membranes, and immunoblotting were performed as described previously [8,9]. Immunoblots of CCF and clarified cell extracts were probed with rabbit anti-(*Arabidopsis thaliana* PAP)-immune serum (a gift from Dr. Tom McKnight, Texas A&M University) or rabbit anti-(tomato IAP) immune serum [9]. Immunological specificities were confirmed by immunoblots in which pre-immune serum was substituted for either anti-(*Arabidopsis* PAP) or anti-(tomato IAP) immune serum. The relative amounts of each PAP polypeptide in the clarified cell extracts or CCF were determined by quantification of the antigenic bands on immunoblots via laser densitometry [9].

2.4. In-gel protease detection via gelatin substrate polyacrylamide gel electrophoresis

Aliquots of CCF (concentrated 10-fold using a Centricon 30 ultrafilter) or clarified cell extracts were subjected to 10% (w/v) SDS–PAGE in gels polymerized with 0.1% (w/v) gelatin as previously described [18]. Aliquots (25 μl) of each sample were also incubated for 30 min at 37 °C in the presence of several class specific protease inhibitors in 25 μl of buffer A (100 mM K-phosphate, pH 6.0, 5 mM L-cysteine, 0.1% v/v Triton X-100). Following SDS–PAGE, proteases were renatured by incubating the gels in 2.5% (v/v) Triton X-100 for 30 min at 25 °C. This was followed by incubation in buffer A at 37 °C for 90 min. Protease activities appear as clear bands against a Coomassie Brilliant Blue R-250 stained background.

2.5. Fluorimetric protease assay using casein and fluorescamine dye

CCF protease activity was also assessed using a fluorimetric assay with casein as the substrate [19]. Aliquots (50–200 μl) of CCF were diluted with 50 mM NaPi (pH 6.0) to a final volume of 0.4 ml, and preincubated at 37 °C for 5 min. The caseinolytic reaction was initiated by the addition of 0.1 ml of 20 mg ml^{-1} casein. Reactions were incubated at 37 °C for 2 h and terminated by the addition of 50 μl of 50% (w/v) trichloroacetic acid. Following incubation at 4 °C for 20 min and centrifugation at 2000 × *g* for 20 min, 200 μl aliquots were neutralized with 200 μl of 0.3 M KOH. This was followed by the addition of 400 μl of 0.4 M potassium borate (pH 9.7) and 400 μl of 0.3 mg ml^{-1} fluorescamine in acetone. A Perkin–Elmer LS-50B Luminescence Spectrophotometer was employed to quantify the fluorescence of fluorescamine adducts at 390/470 nm (excitation/emission), using 25–200 nmol L-aspartate as standard. All assays were linear with respect to time and enzyme concentration. One unit of protease activity was defined as the amount releasing an equivalent of 100 nmol of aspartate h^{-1}.

3. Results and discussion

3.1. Influence of Pi or Phi addition on PAP isozymes of −Pi tomato suspension cells

Tomato suspension cells cultured for 8 days in the absence of exogenous Pi exhibited a marked increase in IAP and SAP activities, as compared to +Pi cells (Fig. 1). The enhanced IAP activity of the −Pi cells (Fig. 1A) arises from the de novo synthesis of a single 142 kDa PAP heterodimer, composed of an equivalent ratio of structurally dissimilar 63 and 57 kDa subunits [9]. By contrast, the elevated SAP activity present in CCF of the −Pi tomato cells (Fig. 1B) is due to the upregulation and secretion of a pair structurally unrelated monomeric PAP isozymes (84 kDa SAP1 and 57 kDa SAP2) [8].

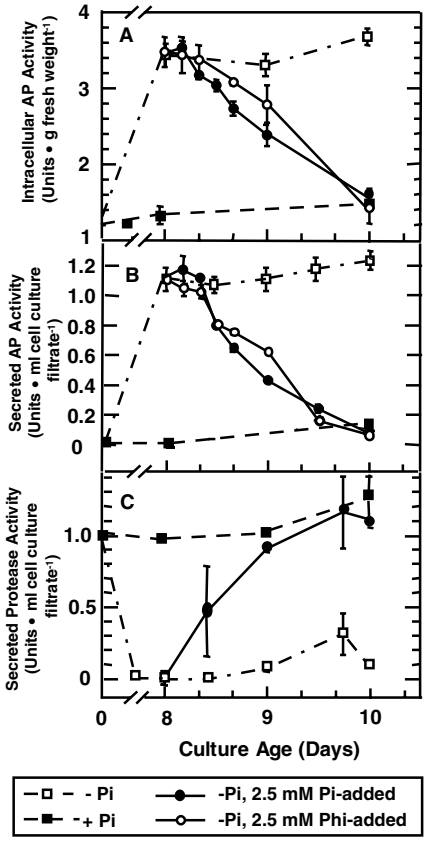

Fig. 1. Time course for activities of (A) IAP, (B) SAP, and (C) CCF protease activity from tomato suspension cells cultured in 0 or 2.5 mM Pi for up to 10 days. Eight-day-old −Pi cultures were supplied with 2.5 mM Pi or Phi and cultured for an additional 2 days as indicated. IAP and SAP activities were determined using phosphoenolpyruvate as the substrate and a coupled spectrophotometric assay [8,9], whereas protease activity was determined using casein as the substrate and the fluorimetric assay described in Section 2.5 [19]. All values represent the means ± S.E.M. of $n = 3$ separate cultures.

Within 48 h of the addition of 2.5 mM Pi or Phi to the culture media of the 8-day-old −Pi cells, IAP and SAP activities were, respectively, reduced by about 6- and 12-fold, to levels equivalent to that of 8- or 10-day-old +Pi cells (Fig. 1A and B). By contrast, IAP and SAP activities of 8- to 10-day-old −Pi cells remained elevated and relatively constant. Rabbit immune serum raised against a recombinant *Arabidopsis* PAP cross-reacted strongly with homogeneous tomato SAP1 and SAP2 (Fig. 2A). Immunoblots of CCF probed with the anti-(*Arabidopsis* PAP) immune serum demonstrated the absence of immunoreactive 84 kDa SAP1 and 57 kDa SAP2 polypeptides within 48 and 24 h, respectively, of the addition of 2.5 mM Pi or Phi to the culture media of the 8-day-old −Pi cells (Fig. 2A). The disappearance of a third immunoreactive PSI polypeptide of about 65 kDa was also apparent following Pi or Phi addition to the 8-day-old −Pi cells (Fig. 2A). The identity of this is

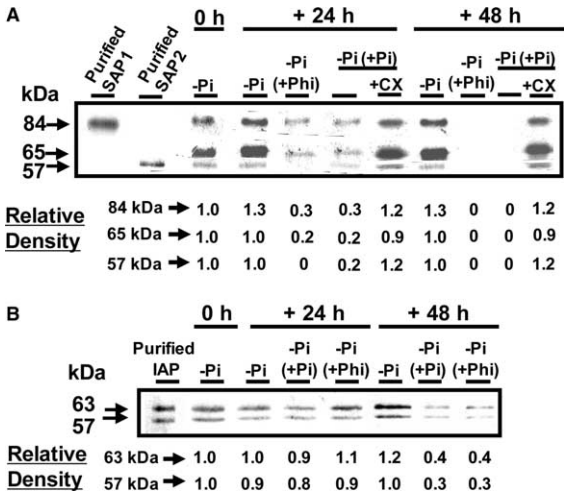

Fig. 2. Immunoblot analysis of tomato PAPs. CCF proteins (1.5 μg/lane), clarified cell extract proteins (4 μg/lane), or homogeneous SAP1, SAP2 and IAP (100, 25, and 50 ng, respectively) [8,9] were resolved by SDS–PAGE and blotted as described in Section 2.3. Time following addition of 2.5 mM Pi or Phi to the 8-day-old −Pi cells is indicated in the upper portion of each panel. CCF protein immunoblots are shown in panels A, whereas an immunoblot of intracellular proteins is shown in panel B. Immunoblotting was performed using a 2500-fold dilution of rabbit anti-(*Arabidopsis* PAP) immune serum (A), or a 2000-fold dilution of rabbit anti-(tomato IAP) immune serum (B). Antigenic polypeptides were visualized using an alkaline phosphatase-tagged secondary antibody [8,9]. Relative amounts of antigenic polypeptides were determined via laser densitometry. Values represent the average of two different experiments.

unknown. However, it appears to be a bonafide PSI protein since it was not detected on immunoblots of CCF from d-0 to d-10 +Pi tomato cells (not shown).

Quantification of immunoblots of clarified tomato cell extracts probed with rabbit anti-(tomato IAP) immune serum indicated a progressive 60–70% decrease in the densities of the antigenic 57 and 63 kDa IAP subunits within 48 h of Pi or Phi addition to the −Pi cells (Fig. 2B). Lower M_r immunoreactive bands never became apparent on immunoblots of the CCF or clarified cell extracts that were, respectively, probed with the anti-(*Arabidopsis* PAP or tomato IAP) immune serum (not shown). This suggests that Pi or Phi addition to the −Pi cells triggered the degradation of all three PSI PAP isozymes into non-immunoreactive peptides. The degradation of tomato SAP and IAP polypeptides appears to be specifically due to Pi or Phi addition to the −Pi cells, since no differences in their relative amounts was observed on immunoblots of 8-day versus 10-day-old −Pi cells (Fig. 2). These results further support the hypothesis that the sensing machinery that perceives and initiates plant cellular responses to nutritional Pi status cannot discriminate between the Pi and Phi anions [2,3,10–16].

3.2. Influence of Pi or Phi addition on protease activity of −Pi tomato suspension cells

Pi starvation of cultured tomato cells elicits the secretion of PSI ribonucleases, phosphodiesterases and APs which collectively facilitate Pi scavenging from extracellular nucleic acids [1,2,8,9,14,20,21]. Conversely, the coordinated repression of a PSI gene (*LEPS2*) and degradation of the corresponding mRNA transcripts occurred within 12 h of Pi-resupply to −Pi tomato seedlings [22]. To date, however, there is no information on the proteolytic machinery that mediates PSI protein turnover following Pi stress reversal due to Pi resupply.

No change in SAP activity or amount of immunoreactive SAP1 or SAP2 polypeptides was evident: (i) 48 h following resupply of 8-day-old −Pi cells with 2.5 mM Pi in the presence of the protein synthesis inhibitor, CX (Fig. 2A), or (ii) when CCF harvested from 8-day-old −Pi cells was incubated at room temperature in the presence or absence of 2.5 mM Pi for up to 1 week (not shown). Overall, the aforementioned results imply that the disappearance of PSI tomato SAPs due to Pi- or Phi-addition might be dependent upon the de novo synthesis of extracellular protease(s) in response to cellular sensing of altered Pi status. Zymography was therefore employed to assess a potential proteolytic mechanism underlying the disappearance of the PSI tomato PAPs following Pi- or Phi-addition to the culture media of −Pi tomato cells.

Gelatin substrate PAGE of clarified extracts indicated the presence of an intracellular 79 kDa protease whose abundance was relatively unaffected 48 h following the addition of 2.5 mM Pi or Phi to the culture media of the 8-day-old −Pi cells (not shown). PAGE analysis also revealed two protease isoforms with M_rs of approximately 121 and 137 kDa in CCFs of 10-day-old +Pi cells, or 8-day-old −Pi cells that had been treated with 2.5 mM Pi or Phi and cultured for an additional 48 h (Fig. 3). By contrast, no protease activity staining bands were observed following gelatin substrate PAGE of CCF from: (i) 10-day-old −Pi cells, (ii) 48 h Pi-resupplied cells that had been treated with 2.5 μM CX (upon addition of 2.5 mM Pi to the 8-day-old −Pi cells), or (iii) 48 h Phi-treated cells that was pre-incubated with 1 mM phenylmethylsulfonyl fluoride (PMSF) (Fig. 3), an irreversible serine protease inhibitor. In-gel proteolysis was unaffected when the CCFs were pre-incubated with the cysteinyl protease inhibitors, 1 mM 2,2′-dipyridyl disulfide or 10 μM *trans*-epoxysuccinyl-L-leucylamido (4-guanidino) butane (not shown).

The protease activity of tomato CCFs was also assessed using a sensitive fluorimetric assay with casein as the substrate. Caseinolytic activity of CCF harvested from d-8 to d-10 −Pi cells was very low (Fig. 1C). However, the disappearance of SAP activity and SAP1/SAP2 immunoreactive polypeptides due to Pi-resupply of the −Pi cells (Figs. 1B and 2A) was correlated with a marked enhancement in CCF caseinolytic activity (Fig. 1C). Within 24 h of the addition of 2.5 mM Pi to 8-day-old −Pi tomato suspension cells, CCF caseinolytic activity was comparable to that of CCF from 0-, 8- or 10-day-old +Pi tomato cells (Fig. 1C). By contrast, negligible caseinolytic activity was detected in CCF of 48 h Pi-resupplied cells that had been: (i) treated with 2.5 μM CX (upon addition of 2.5

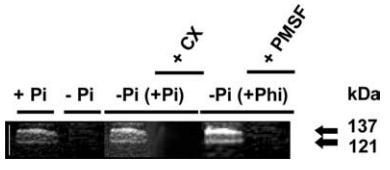

Fig. 3. PAGE analysis of secreted proteases of tomato suspension cells. CCF proteins (5 μg/lane) were resolved by gelatin SDS–PAGE, renatured, and in-gel proteolysis detected as described in Section 2.4. CCFs were from 10-day-old +Pi or −Pi cells, as well as 8-day-old −Pi cells that had been treated with 2.5 mM Pi ± 2.5 μM CX or 2.5 mM Phi and cultured for an additional 48 h. Samples from the 48 h Phi-treated cells were also pre-incubated with 1 mM PMSF for 30 min prior to PAGE.

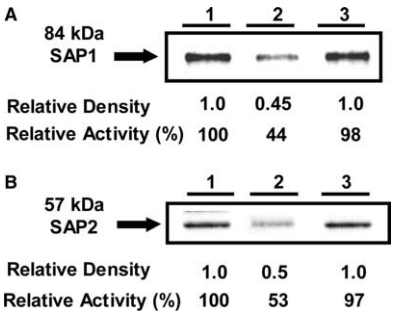

Fig. 4. Immunoblot analysis of SAP1 (A) and SAP2 (B) isozymes purified from CCF of 8-day-old −Pi tomato suspension cells [8,9]. Homogeneous SAP1 (1 μg) or SAP2 (0.5 μg) was incubated for 24 h at 25 °C in the presence (lanes 2 and 3) or absence (lane 1) of 1 μg of CCF proteins from 48 h Pi-resupplied tomato cells. Prior to SAP addition, the CCF was pre-incubated for 30 min in the presence (lane 3) or absence (lane 2) of 1 mM PMSF. SAP1 (50 ng/lane, panel A) and SAP2 (25 ng/lane, panel B) were resolved by SDS–PAGE and blotted as described in Section 2.3. Immunoblotting and visualization of antigenic SAP polypeptides were performed as described in Fig. 2. Relative amounts of antigenic polypeptides were determined via laser densitometry. Values represent the average of two different experiments.

mM Pi to the 8-day-old −Pi culture), or (ii) pre-incubated with 1 mM PMSF. These results corroborate those of Fig. 3 indicating that a pair of secreted serine peptidases (EC 3.4.21) were synthesized de novo in response to Pi-resupply to −Pi tomato cells. It will be of interest to determine their relatedness with an extracellular 75 kDa subtilisin-like serine protease recently isolated from CCF of *Arabidopsis* suspension cells [23]. Plant subtilisin-like proteases display endoproteolytic activity and are regulated by various developmental and environmental cues in a tissue-specific manner.

In vitro proteolysis of homogeneous SAP1 and SAP2 was evident following their 24 h incubation with CCF from the 48 h Pi-resupplied tomato cells (Fig. 4A and B, lane 2), but not when the same CCF was pre-incubated with 1 mM PMSF (Fig. 4A and B, lane 3), or boiled for 3 min (not shown). The approximate 2-fold decrease in the relative amounts of immunoreactive 84 kDa SAP1 and 57 kDa SAP2 polypeptides that followed their 24 h incubation with CCF from the 48 h Pi-resupplied cells was correlated with a similar reduction in their respective activities (Fig. 4A and B).

3.3. Concluding remarks

SDS–PAGE analyses have revealed that the CCF of +Pi, −Pi, and Pi-resupplied tomato suspension cells contain up to 50 different polypeptides, several of which are unique to −Pi cultures [20,24] (also this study, not shown). This suggests that the pair of Pi-inducible secreted serine protease isoforms of cultured tomato cells (Fig. 3) may display limited substrate selectivities. Confirmation that SAP1 and SAP2 are their genuine physiological substrates awaits the purification and kinetic characterization of both proteases. It will also be of interest to assess whether extracellular Pi-inducible proteases degrade additional hydrolytic enzymes that are specifically secreted into the CCF by −Pi tomato cell cultures, namely PSI ribonucleases and phosphodiesterases [2,14,20,21]. Although the functional importance of Pi-inducible secreted proteases in response to Pi-sensing and turnover of extracellular PSI proteins remains to be determined, the current study presents the first evidence for protease upregulation in response to Pi-resupply of −Pi plants.

The design of biotechnological strategies to enhance crop Pi acquisition from organic and inorganic pools of soil P is of great interest to scientists wishing to reduce the overuse of non-renewable and polluting Pi fertilizers in agriculture. Characterization of the structural and kinetic properties of intra- and extracellular PSI tomato PAP isozymes indicated their probable involvement in Pi-scavenging by −Pi tomato [8,9]. Our discovery of Pi-inducible proteases that appear to target PSI tomato SAPs implies that stable overexpression of extracellular PSI proteins in transgenic plants could be enhanced by modified protease expression and/or the design of protease-resistant PSI proteins.

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