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Phosphate-starvation response in plant cells: *De novo* synthesis and degradation of acid phosphatases

(enzyme induction/plant tissue culture/polyclonal antibodies/nutrient stress)

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ABSTRACT **Induction of phosphatase activity is an important component of the plant cell response to phosphate deficiency. Suspension cell cultures of *Brassica nigra* contain two major inducible acid phosphatase (APase) isozymes; vacuolar phospho*enol*pyruvate (PEP) APase and cell wall nonspecific APase. Polyclonal antibodies raised against purified PEP-APase crossreacted specifically with both isozymes. Furthermore, anti-(PEP-APase) IgG detected proteins from a wide range of higher plants, suggesting that the major plant APase isozymes have diverged from a common ancestral form. Quantification on immunoblots indicated that in *B. nigra* suspension cells experiencing transition from P_i sufficiency to deficiency or vice versa, the amount of total antigenic APase protein correlated closely with total enzyme activity. This was also shown in intact plant roots. Therefore, the activity was governed by the synthesis and degradation of APases. Increases in the amounts of both major APase isozymes occurred simultaneously following P_i deprivation of *B. nigra* suspension cells, suggesting the involvement of a common regulatory mechanism.**

Intracellular and extracellular acid phosphatases (APases) are integral components of the plant response to P_i deficiency (1–7), which commonly occurs in the wild. The regulation of these enzymes is therefore critical to a plant's survival.

The *de novo* synthesis of phosphatases in response to P_i deprivation has been demonstrated in bacteria (8) and yeast (9), where the enzymes either mobilize internal polyphosphate reserves or release P_i from exogenous esters for subsequent absorption. The nonspecific (NS) APase in plants may play this latter role in the cell wall (2), but since in higher plants excess phosphorus is stored in the vacuole as P_i, the presence of an inducible intracellular APase appears to be enigmatic. The specificity of a major APase isozyme for phospho*enol*pyruvate (PEP) in P_i-deprived *Brassica nigra* cells (10) has led us to postulate its critical role in the catalysis of one step of a series of glycolytic bypasses (1). These alternative reactions constitute a pathway that minimizes the requirements for adenylates and P_i, both pools of which are depressed under conditions of low P_i supply.

In the present work, immunological studies were undertaken to gain insight into the regulation of APase activity with respect to P_i status. *De novo* synthesis of both the vacuolar PEP-APase (11) and the cell wall NS-APase (2, 11) of *B. nigra* occurred in response to P_i deprivation. Furthermore, the level of enzymatic activity was a direct consequence of the quantity of enzyme protein present in the cells. Finally, despite distinct differences in their CNBr peptide maps (11), these isozymes were immunologically very similar and appeared to be representative of a broad class of structurally related plant enzymes.

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MATERIALS AND METHODS

Chemicals and Plant Material. Biochemicals, cell culture ingredients, coupling enzymes, *N,N'*-methylenebisacrylamide, and immunochemicals were obtained from Sigma. SDS was from Schwarz/Mann Biotech (ICN). Protein assay reagent was from Bio-Rad. All other reagents were of analytical grade and were obtained from BDH.

B. nigra suspension cells were grown as previously described (10). Transfer of cells into media containing different concentrations of P_i was performed using a low inoculum procedure (1, 2).

Seeds of maize (*Zea mays* L. cv. Earlivee), wheat (*Triticum aestivum* L. cv. Norstar), and alfalfa (*Medicago sativa* L.) were germinated on Whatman no. 1 filter paper soaked in sterile distilled water and then were transferred to washed silica sand, where they were irrigated with a 1:20 dilution of P_i-deficient Murashige and Skoog medium (12). Growth conditions were 25°C under a continuous photon flux of 64 $\mu E \cdot m^{-2} \cdot s^{-1}$ [1 E (einstein) = 1 mol of photons]. Seeds of potato [*Solanum tuberosum* (L.) cv. Explorer], *Arabidopsis thaliana* (L.) v. Schur (genotype Columbia), tobacco [*Nicotiana tabacum* (L.) cv. Delgold], and *B. nigra* (L.) cv. Koch were germinated and grown on Murashige and Skoog 1% agar plates containing no P_i. Plants were harvested when P_i-deficiency symptoms began to appear in the leaves (2–4 weeks old). *B. nigra* plants were also grown on plates containing normal (1.25 mM) P_i for comparative purposes and harvested after the same growth period as the P_i-starved plants.

Purified potato tuber (PT) APase was purchased from Sigma.

Protein Extractions. Proteins for enzyme assays and immunoblots were extracted from all tissues and cells in phosphatase extraction buffer (10). For all samples except *B. nigra*, entire plants were thoroughly ground (1/1, wt/vol) in a chilled mortar and pestle and centrifuged at 13,000 rpm in a Beckman Microfuge II for 5 min. The supernatant was assayed for phosphatase activity and soluble protein concentration before being frozen in liquid nitrogen and stored at −80°C until required. PT-APase was dialyzed against phosphatase extraction buffer prior to use.

B. nigra plants were separated into three fractions: leaf, stem, and root. Each fraction was extracted as described above.

Protein Measurements. Soluble protein concentrations were measured by the method of Bradford (13) using the Bio-Rad reagent and bovine gamma globulin as standard.

Preparation of PEP-APase Antibodies. Rabbit anti-(PEP-APase) immune serum was obtained by following the suggested immunization protocol for the Ribi adjuvant system (product code R-730; Ribi Immunochem). The primary in-

Abbreviations: APase, acid phosphatase; NS, nonspecific; PEP, phospho*enol*pyruvate; PT, potato tuber.

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jection contained 200 μg of PEP-APase in 2 ml of adjuvant, and a secondary injection of 100 μg in 2 ml of adjuvant was administered after 28 days. Ten days later, blood was collected from the major aural artery and allowed to clot at room temperature for 30 min before centrifugation at 1500 × *g* for 10 min. The crude antiserum was affinity-purified against PEP-APase according to Lin *et al.* (14), with the exception that a poly(vinylidene difluoride) membrane (Immobilon-P, Millipore) was used in place of nitrocellulose.

Immunotitration of Phosphatase Activity. Immunotitrations of PEP-, NS-, and PT-APase activities were performed as described (14).

Polyacrylamide Gel Electrophoresis and Electroblotting. SDS/PAGE was performed as previously reported (10), using the Doucet and Trifaró (15) system and a Bio-Rad minigel apparatus. Electroblotting of proteins on to poly(vinylidene difluoride) membrane followed instructions in the Bio-Rad electroblotting manual.

Immunological Detection by Western Blotting. The method of Moorhead and Plaxton (16) was employed for the detection of immunoreactive proteins on poly(vinylidene difluoride) membranes. Affinity-purified rabbit anti-(PEP-APase) IgG and goat anti-(rabbit IgG) IgG conjugated to alkaline phosphatase (Sigma) were used. Affinity-purified PEP-APase antibodies were diluted 1:20 in blocking buffer.

Enzyme Assays. Phosphatase activities of the various plant tissue and cell culture extracts were estimated as described (10).

Laser Densitometry. Amounts of PEP- and NS-APase in crude extracts of *B. nigra* suspension cells were determined by quantification of the enzyme bands on immunoblots with an LKB Ultroscan XL enhanced laser densitometer and Gel Scan software (version 2.1). Serial dilutions of PEP- and NS-APase were used as calibration standards and total APase was estimated as the sum of the two respective signals.

RESULTS

Specificity of Anti-(PEP-APase) Antibodies. Rabbit anti-(PEP-APase) crude immune serum immunoprecipitated 100% of the activity from purified preparations of both PEP- and NS-APase, indicating its utility in the quantification of each of these isozymes (Fig. 1). Within the range of antiserum investigated, ≈70% of the activity of PT-APase was removed. The I_{50} values of anti-(PEP-APase) immune serum indicate that the order of antigenicity against PEP-, NS-, and PT-APase was 270, 520, and 1250 μl of serum per unit of enzyme, respectively.

Preliminary studies indicated that the antibodies detected numerous plant glycoproteins. Therefore, the oxidation of antigenic glycosylated side chains was routinely performed on all subsequent immunoblots by treatment with sodium periodate (ref. 17 and data not shown).

When the antibodies were affinity-purified against PEP-APase and used to probe immunoblots prepared from crude extracts of P_i-starved *B. nigra* suspension cells, bands corresponding to the two major isozymes were clearly discernable (Figs. 2*A* and 3*A*). Purified PT-APase could also be easily detected (see Fig. 6, lane E). The relative intensities of the signals corroborate the immunoprecipitation results with respect to the order of antigenicity. Affinity-purified antiserum could detect as little as 0.1 ng of purified *B. nigra* PEP- or NS-APase.

Inducibility of *B. nigra* APases. Fig. 2 shows the immunoblot (*A*) as well as the activity profile and immunological quantification (*B*) of APases in suspension cells during transition from P_i sufficiency to deficiency incurred through the withdrawal of exogenous P_i. Induction of APase activity and the synthesis of both PEP- and NS-APase began at 6–8 days and were complete by day 18, when the APases comprised ≈0.25% of the total soluble cellular protein. Activities varied with total APase protein quantities (Fig. 4), thereby invoking *de novo* synthesis as the main regulatory process in the induction transition phase.

Fig. 2*C* shows the corresponding increases in PEP- and NS-APase protein during induction, as quantified by laser

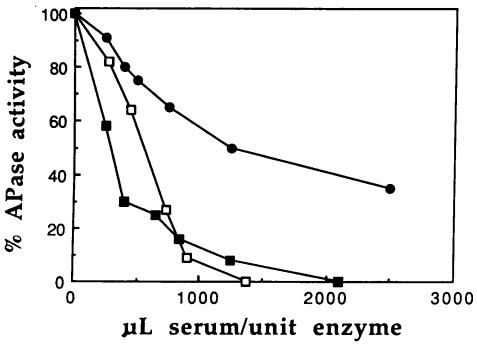

FIG. 1. Immunotitration of APases by anti-(PEP-APase) crude immune serum. Immunoprecipitation (14) was performed on black mustard PEP-APase (■) and NS-APase (□) and on PT-APase (●).

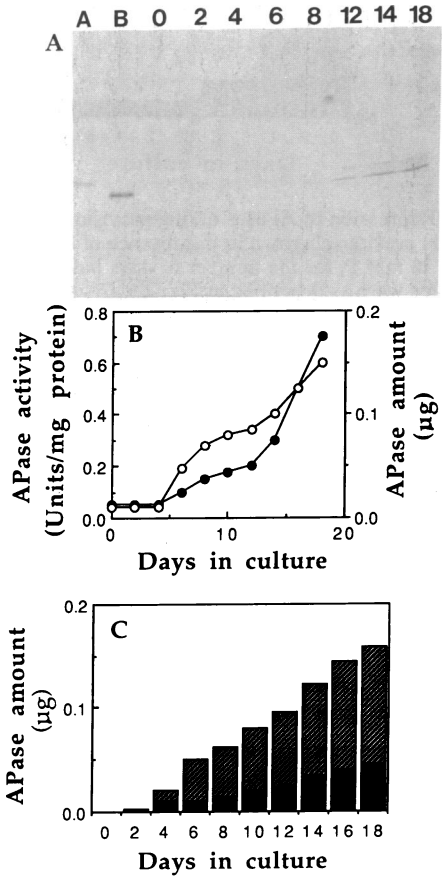

FIG. 2. Induction of APases in response to P_i deprivation. (*A*) Immunoblot. Cells previously grown in 10 mM P_i were cultured in the absence of P_i for the number of days indicated above each lane. Lane A, 10 ng of NS-APase (60-kDa subunit); lane B, 10 ng of PEP-APase (55-kDa subunit); all other samples consisted of 60 μg of extractable protein. (*B*) Cellular APase activity (○) compared with total immunoreactive APase (●). Immunological quantification of PEP- and NS-APase was performed by laser densitometry on the immunoblot shown in *A*. (*C*) Cellular quantity of PEP- and NS-APase. Solid bars, PEP-APase; striped bars, NS-APase. Immunoblot was probed with anti-(PEP-APase) IgG.

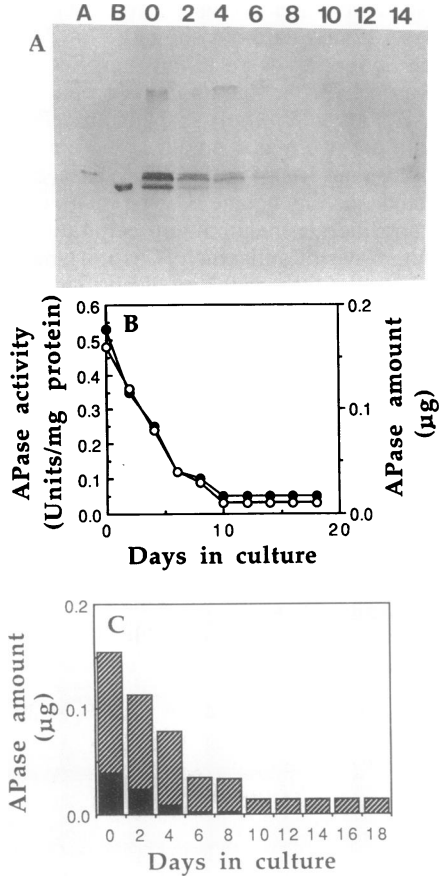

FIG. 3. Repression of APases during provision of P_i. (*A*) Immunoblot. Cells previously grown in the absence of P_i for 1 week were cultured in 10 mM P_i for the number of days indicated above each lane. Samples were as described in Fig. 2*A*. (*B*) As for Fig. 2*B*. (*C*) As for Fig. 2*C*.

densitometry of the immunoblot in Fig. 2*A*. The timing of induction was the same for both isozymes and coincided with the enhancement of potential P_i absorption by the cells (data not given).

Fig. 3 presents the results with P_i-starved cells that became P_i-sufficient when supplied with 10 mM P_i. Instead of a single band coinciding with NS-APase at ≈60 kDa (Fig. 3*A*, lane A), a prominent doublet was detected (lanes 0–6). Both of these as well as the protein detected at 55 kDa degraded at a similar rate. A complete suppression of APase activity was in effect and no further APase degradation occurred beyond 10 days (Fig. 3*B*). Here again enzyme activities and quantities correlated during the repression transition phase (Fig. 4); the two isozymes were degraded similarly (Fig. 3*C*). At day 10

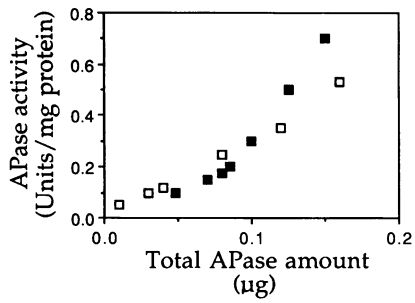

FIG. 4. Correlation between APase quantity and extractable activity (from data in Figs. 2*B* and 3*B*). ■, Induction transition phase [linear regression correlation coefficient (r) = 0.92]; □, repression transition phase (r = 0.99).

Table 1. Specific activities of extractable APase

Brassica tissue	APase, units/mg of protein	
	P_i sufficient	P_i deficient
Leaf	0.026 ± 0.003	0.047 ± 0.003
Stem	0.070 ± 0.004	0.120 ± 0.009
Root	0.038 ± 0.006	0.174 ± 0.007
Suspension cells	0.045 ± 0.002	0.530 ± 0.024

Activities were determined from *B. nigra* suspension cells and intact plant tissues grown under P_i-sufficient or P_i-deficient conditions. One unit of APase activity equals 1 μmol of PEP hydrolyzed per min. Values are means ± SE of three replicates.

PEP-APase was no longer detectable, whereas some NS-APase was present in the cells even at 18 days.

The difference in total APase activity and total APase protein from a fully induced to a fully repressed state was ≈12-fold, indicating that overall changes in the amount of activity were caused by changes in the amount of enzyme protein. Since it is difficult to clearly distinguish between the activities of the two APases in crude extracts, comparisons between the activities and amounts of the respective APases were not made.

Regulation and Tissue Specificity of APases in *B. nigra* Plants. Table 1 shows the total extractable APase activity from the various organs of *B. nigra* plants and cell suspensions that had been grown under either P_i-sufficient or P_i-deficient conditions. In all cases P_i deprivation caused increases in activity, and of the plant organs, differences were most pronounced in the roots. This, however, was only a 4.6-fold increase compared with the 12-fold increase in the cultured cells.

Fig. 5 presents an immunoblot performed on the same samples as those for which the APase activities are given in Table 1. A strong single band corresponding to PEP-APase was detected in stem tissue from both P_i-sufficient and P_i-deficient plants (lanes D and G, respectively), whereas no signal corresponding to either isozyme could be detected in the leaf samples (lanes F and I). Instead, a single immunoreactive band of low molecular mass was observed in both leaf samples.

The single band detected in the deficient root samples (lane H) was intermediate in molecular mass between PEP- and NS-APase. A minor signal of this type was also seen in the cultured cell extract (Fig. 5, lane C, and Fig. 3*A*), and P_i deprivation induced its formation in both the root and the cells. Laser densitometry of the immunoreactive bands indicated that, in the root, APase activity could be attributed directly to enzyme quantity.

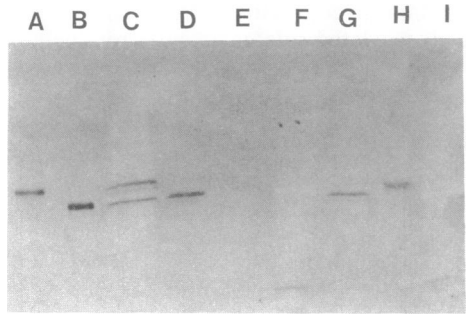

FIG. 5. Immunoblot of *B. nigra* plant tissue extracts (cf. Table 1). Plants were grown in either P_i-sufficient (1.25 mM) or -deficient (0 mM) conditions. Lanes A and B were as described in Fig. 2*A*; all other samples consisted of 60 μg of crude protein extract. Lane C, P_i-starved suspension cells; lane D, P_i-sufficient stem; lane E, sufficient root; lane F, sufficient leaf; lane G, P_i-deficient stem; lane H, deficient root; lane I, deficient leaf.

Immunological Survey of APases. Immunoblots were performed on samples from a variety of diverse taxonomic groups. Phosphatases from *Escherichia coli*, yeast, algae, *Drosophila*, and human prostate were not detected by anti-(PEP-APase) IgG. Significant crossreactivity was observed, however, in samples from all the investigated higher plant taxa (Fig. 6), the degree of which decreased with the evolutionary divergence between the organism and *B. nigra*.

DISCUSSION

The two major APase isozymes of *B. nigra* are different in several of their catalytic and physical characteristics, including their CNBr peptide maps (10, 11), and each contributes to the amelioration of P_i deprivation by its own particular role within the cell (1, 2, 11). They are, however, similar enough to be closely related immunologically (Fig. 1). In *Saccharomyces cerevisiae* functionally distinct APases are also structurally similar (18). For example, gene duplication supports there being a common ancestry despite promoter-element divergence between the P_i-repressible *PHO5* and the constitutive *PHO3*. In our suspension cells there was no evidence of an antigenically related constitutive APase, although in a previous study evidence for a constitutive enzyme was seen in activity-stained gels after nondenaturing electrophoresis (2).

These isozymes appear to be part of a large interspecific group of plant proteins (Fig. 6), implying divergence from a common ancestry. This is entirely consistent with there being strong evolutionary pressure to maintain the efficiency of the ameliorative processes in which these enzymes are involved. This places them into the growing number of plant enzyme groups for which epitopic conservation has been demonstrated (19–21). Although proteins from distant monocotelydonous species were detected by anti-(PEP-APase) IgG, no cross-reactive proteins were seen in samples from other than angiosperms. Therefore, the P_i-starvation-inducible phosphatases of microbes (8, 9) are not antigenically related to the plant APases. Thus far, attempts to employ *S. cerevisiae* and *Schizosaccharomyces pombe* APase genes as heterologous probes have failed to identify comparable DNA sequences from higher plants (K. S. Gellatly and D.D.L., unpublished work).

The induction of APase activity by P_i deprivation in *B. nigra* roots and suspension cells is a direct consequence of protein synthesis (Figs. 2, 4, and 5). Changes in *in vivo* protein synthesis profiles between different P_i nutritional states of these cells have been documented (22), but no specific proteins were identified. Akiyama *et al.* (23) presented circumstantial evidence in support of *de novo* APase synthesis under gibberellic acid treatment, and Goldstein *et al.* (3, 24, 25) reported that the secretion of APases from cultured tomato cells was induced by low exogenous P_i. Now our study has unequivocally confirmed the *de novo* synthesis of APases in response to P_i starvation in plants. In addition, the simultaneous synthesis of PEP- and NS-APase coincides with the enhancement of cellular P_i absorptive capacity (data not shown and ref. 2), indicating the possibility of a common regulatory pathway that governs all of these processes.

Organ-specific analysis showed very little APase activity in leaf tissue of P_i-deficient or -sufficient plants, whereas the stems always had elevated activities (Table 1). By comparison, root tissue responded positively to P_i deprivation, though the magnitude of induction was only half that of the cultured cells. Here, as in the cultured cells, APase quantity paralleled that of the extractable activity (densitometric measurements of Fig. 5). The APase present in stem tissue had a subunit molecular weight identical to that of PEP-APase whereas the inducible root APase appeared to be slightly larger and similar to the intermediate signal detected in samples of fully induced cultured cells (Fig. 5). This third protein may represent the product of a separate inducible gene or a difference in the posttranslational modification of APases between the roots and the cultured cells.

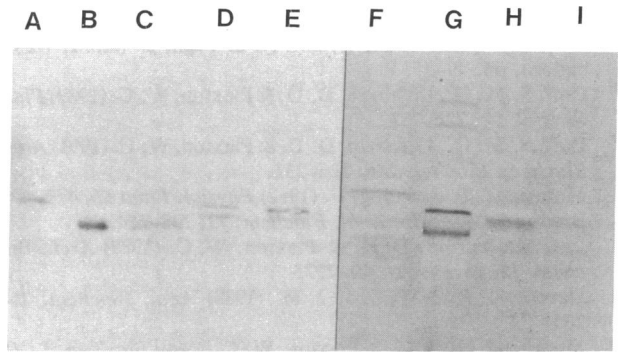

FIG. 6. Immunological survey of higher plant species. Immunoblot was probed with anti-(PEP-APase) IgG. Lanes A and B were as described in Fig. 2*A*. All other samples except lane E contained 60 μg of extractable protein from whole plants. Lane C, *A. thaliana*; lane D, potato; lane E, 10 ng of purified PT-APase; lane F, tobacco; lane G, alfalfa; lane H, maize; lane I, wheat. Last four lanes were from a separate gel run.

There is no requirement for rapid APase degradation during cellular release from P_i deficiency, because P_i itself is a potent competitive inhibitor of APases in higher plant cells (10, 11). In addition, P_i provision to previously deprived plants has revealed that cellular P_i absorptive capacity drops quickly enough to infer an allosteric feedback by internal [P_i] on the uptake process (26). This avoids any toxic effects of excessive P_i accumulation. During the refeeding experiment with *B. nigra*, APase degradation happened over a period of days in an apparently linear fashion (Fig. 3). It is most likely, then, that APase synthesis ceased coincident with or very soon after P_i deficiency was alleviated.

The common role of P_i in signal transduction (27) makes it an especially attractive candidate as the primary signal for its own assimilatory pathways. Rao and Torriani (28) have hypothesized that in *E. coli* a plasma membrane process involving protein kinases monitors extracellular P_i and signals derepression of the *pho* regulon accordingly. In plants, during the onset of P_i starvation there is a delay period between the removal of exogenous P_i and the *de novo* synthesis of APases (e.g., Fig. 2). This delay probably coincides with the depletion of the vacuolar P_i pool (29), thus implicating the tonoplast as a possible site of signal transduction.

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