Upregulation of vacuolar H^+-translocating pyrophosphatase by phosphate starvation of *Brassica napus* (rapeseed) suspension cell cultures

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Abstract The influence of phosphate (Pi) deprivation on the vacuolar H^+-translocating pyrophosphatase (PPiase) and ATPase in tonoplast vesicles from *Brassica napus* suspension cells was assessed. Pi starvation significantly elevated the ratios of PPi-: ATP-dependent H^+ translocation rate and H^+-PPiase: H^+-ATPase hydrolytic activities. These increases were reversed 36 h following resupply of 2.5 mM Pi to the Pi-starved cells. Immunoblotting indicated that Pi starvation also induced a two-fold increase in the amount of H^+-PPiase protein, whereas the amount of H^+-ATPase remained unchanged. It is proposed that H^+-PPiase facilitates the conservation of limited ATP pools, and Pi recycling during Pi stress.

Key words: Phosphate starvation; Inorganic pyrophosphate; Proton pump; Vacuolar type H^+-pyrophosphatase; Vacuolar type H^+-ATPase; *Brassica napus*

1. Introduction

Although phosphate (Pi) plays a critical role in cellular metabolism and bioenergetics, it is one of the least available nutrients in many terrestrial and aquatic ecosystems. The extensive use of Pi fertilizers in agriculture attests to the suboptimal free Pi levels of most soils. Studies of the effects of Pi stress on plant metabolism and gene expression have revealed some remarkable adaptive mechanisms that contribute, within species-dependent limits, to ameliorate the negative impact of Pi deficiency [1,2].

ATP pools may be reduced by as much as 80% as a consequence of the significant decline in cytoplasmic Pi that accompanies severe Pi stress [1,3–6], thus diminishing ATP-dependent metabolic pathways. However, enhanced flux through alternative pathways that circumvent ATP- or Pi-limited reactions has been suggested to facilitate the metabolism of Pi-starved plant cells [1,3–11]. Since large amounts of inorganic pyrophosphate (PPi) are produced as a byproduct of macromolecule synthesis, reactions that utilize PPi in place of ATP provide an obvious bioenergetic advantage to plant cells subjected to diminished nucleoside-*P* pools. The plant cytosol lacks soluble inorganic alkaline pyrophosphatase (PPiase) and consequently contains PPi concentrations of up to about 0.5 mM [3–7].

At least three ATP-dependent reactions of the plant cytosol may use PPi preferentially during environmental stresses such as anoxia or Pi starvation that deplete cellular ATP pools. Firstly, the conversion of sucrose to hexose-*P*s can proceed via the ATP-dependent invertase pathway or via the PPi-dependent sucrose synthase pathway. Secondly, the phosphorylation of fructose-6-*P* to fructose-1,6-P_2 can be achieved by either the ATP-dependent ATP:fructose-6-*P* 1-phosphotransferase or PPi-dependent PPi:fructose-6-*P* 1-phosphotransferase (PFP). Upregulation of sucrose synthase and PFP activities has been reported for both O_2- and Pi-deprived plants [1,3,4,6,7,10–12]. Together with the selective maintenance of cytosolic PPi pools [1,3–7], this indicates that anaerobic or Pi-starved plants may preferentially utilize PPi-dependent glycolytic reactions, thereby conserving limited ATP pools.

The vacuolar H^+-PPiase (EC 3.6.1.1) represents a third enzyme that endows plants with the capability of utilizing PPi – to circumvent an ATP-consuming cytosolic reaction (the vacuolar H^+-ATPase; EC 3.6.1.3). Although these alternative H^+ pumps are ubiquitous in plant vacuolar membranes (the tonoplast), their expression varies amongst plants and tissues [13,14]. Rice seedling anoxia evoked a marked increase in H^+-PPiase protein and specific activity, but had much smaller influence on the H^+-ATPase [12]. It was concluded that V-PPiase plays a key role during anoxia to maintain the proton electrochemical gradient between the cytosol and vacuole. It has similarly been demonstrated that activity of PPi-dependent H^+ transport was elevated by about 3-fold in root tonoplast vesicles of rye plants cultivated for 10 days under conditions of mineral deficiency, while the activity of ATP-dependent H^+ transport only increased slightly [15]. Whether this response of rye roots was due to any or all of the different limiting nutrients was not determined.

The aim of the present study was to assess the impact of Pi deprivation on the activities and amounts of the vacuolar H^+-PPiase and H^+-ATPase in *Brassica napus* suspension cell cultures. Here we report that Pi starvation of *B. napus* suspension cells resulted in a significant increase in the H^+-PPiase protein levels. This increase was correlated with increased PPi-dependent tonoplast H^+ transport and PPi-dependent hydrolytic activity.

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Abbreviations: +Pi and −Pi, cultured in the presence and absence of 2.5 mM KPi, respectively; PPi, inorganic pyrophosphate; PFP, PPi:fructose-6-*P* 1-phosphotransferase

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2. Materials and methods

2.1. Plant material

An embryogenic microspore-derived heterotrophic cell suspension of winter oilseed rape (*B. napus* L. cv. Jet Neuf) was cultured in liquid NLN media as previously described [6,12]. Cells were maintained as 50 ml cultures on a rotational shaker (125 rpm) at 22°C. For the generation of experimental samples, two 7-day-old 50 ml cultures were added to 700 ml of fresh NLN media containing either 0 or 2.5 mM KPi (−Pi and +Pi cells, respectively). After 10 days, cells were harvested on a Buchner funnel fitted with Whatman #1 filter paper.

2.2. Isolation of tonoplast vesicles

All procedures were performed at 4°C. Freshly harvested −Pi or +Pi cells (approximately 50 g fresh weight) were homogenized with four 30 s pulses of a Biospec Bead Beater in 200 ml of 30 mM Tris–HCl (pH 7.5), 0.5% (w/v) bovine serum albumin, 10% (v/v) glycerol, 5% (w/v) polyvinylpyrrolidone, 5 mM EGTA, 5 mM $MgSO_4$, 0.25 M mannitol, 2 mM dithiothreitol, 26 mM potassium metabisulfite, and 0.2% (v/v) of a plant protease inhibitor cocktail (Sigma, cat. #P-9599). The homogenate was filtered through four layers of cheesecloth and centrifuged at $8000 \times g$ for 20 min. Supernatants were then centrifuged at $100\,000 \times g$ for 45 min. The resulting microsomal pellets were resuspended in 10 ml of 6 mM Tris–MES (pH 8.0), 10% (v/v) glycerol, 0.25 M mannitol, 1 mM EDTA, and 2 mM dithiothreitol using a Potter–Elvehjem homogenizer. The suspension was centrifuged for 2 h at $100\,000 \times g$ in a discontinuous sucrose density gradient as previously described [16]. Tonoplast vesicles were collected from the 0/16% (w/w) interface, washed with 40 ml suspension buffer, resedimented at $100\,000 \times g$, and resuspended in 0.2 ml of the same buffer.

2.3. Measurement of PPi- and ATP-dependent H^+ transport into tonoplast vesicles

Formation and dissipation of acid-interior pH gradients was monitored by the fluorescence quenching of the permeant amine dye acridine orange [16]. Tonoplast vesicles containing 40 μg of membrane protein were added to a reaction mixture (0.7 ml final volume) containing 30 mM Tris–MES (pH 7.5), 1 μM acridine orange, 20 mM KCl, and either 0.6 mM PPi or 1.4 mM ATP. Proton translocation was initiated by the addition of either 1.6 or 3.2 mM $MgSO_4$ (for PPi- and ATP-dependent assays, respectively). The fluorescence decrease with time was measured at 25°C with a Perkin-Elmer LS-50B Spectrofluorometer, using excitation and emission wavelengths of 495 and 540 nm, respectively, and a slit width of 5 nm for both excitation and emission. The FL-Winlab program Version 2.0 from Perkin-Elmer was used to acquire and analyze the data.

2.4. Enzyme assays

Acid phosphatase activity of clarified homogenates from 10-day-old +Pi and −Pi *B. napus* cells was determined as previously described [6]. H^+-PPiase and H^+-ATPase specific activities were calculated from the rates of PPi or ATP hydrolysis, respectively, by measuring the amount of Pi released [17]. The reaction was initiated by the addition of tonoplast vesicles (containing 10 μg membrane protein) isolated from +Pi or −Pi cells to 0.25 ml of a reaction mixture containing 30 mM Tris–MES (pH 8.0), 50 mM KCl, and 3 mM MgATP or 1.5 mM MgPPi. After 30 min at 37°C the reaction was terminated by the addition of 0.75 ml of 1.4% (w/v) ascorbic acid containing 0.36% (w/v) ammonium molybdate and 2.5% (v/v) H_2SO_4. The A_{820} was measured after 20 min and compared to standards of known Pi concentration.

Protein concentrations of 0.1% (v/v) Triton X-100 solubilized tonoplast vesicles were determined by the Coomassie blue G-250 dye-binding method of Bollag et al. [18] with bovine γ-globulin as the standard.

2.5. Immunoblotting

SDS–PAGE and electroblotting of gels onto poly(vinylidene difluoride) membranes were performed as described previously [11]. Western blots were probed with antibodies raised against the red beet vacuolar H^+-ATPase or the *Arabidopsis thaliana* vacuolar H^+-PPiase. Immunoreactive polypeptides were visualized using an alkaline phosphatase-conjugated secondary antibody and chromogenic staining [11]. Immunological specificity was confirmed by performing immunoblots in which rabbit preimmune serum was substituted for the respective antisera. The relative amount of H^+-PPiase or H^+-ATPase protein in tonoplast extracts from 10-day-old +Pi vs. −Pi *B. napus* cells was determined by quantification of the respective antigenic polypeptides on immunoblots (in terms of A_{633}) using a LKB Ultroscan XL laser densitometer and Gel Scan software (ver. 2.1). Derived A_{633} values were linear with respect to the amount of immunoblotted protein.

2.6. Statistical analyses

Means ± S.E.M. were computed using Datadesk for Windows Version 6.0.2 ISE. Where applicable, means were compared using a paired two-sample Student's *t*-test.

3. Results

3.1. Effect of Pi starvation on growth, acid phosphatase activity, and relative ATP- and PPi-dependent vacuolar H^+ transport

B. napus suspension cells cultured for 10 days in the absence of exogenous Pi displayed a significant (50%) decrease in fresh weight, but a 4-fold increase in extractable acid phosphatase activity (not shown). These results are consistent with our previous studies on the response to Pi starvation of *B. napus* [6,10,11], and indicate that the 10-day-old −Pi and +Pi cells were in fact Pi-starved and Pi-sufficient at the time of harvest.

The rate and magnitude of ATP- and PPi-dependent H^+ translocation into tonoplast vesicles of +Pi and −Pi cells were estimated by monitoring fluorescence quenching of acridine orange (Fig. 1). ATP-dependent H^+ transport did not change following Pi deprivation, whereas the initial rate and steady-state levels of PPi-dependent H^+ transport increased markedly upon Pi starvation, suggesting an increased capacity for PPi-dependent H^+ transport (and the concomitant vacuolar acidification) during Pi stress. Resupply of 2.5 mM Pi to 10-day-old −Pi cells for 36 h resulted in a reduction of PPiase H^+ translocation rate and magnitude to that of +Pi cells (not shown).

3.2. Effect of Pi starvation on H^+-PPiase and H^+-ATPase hydrolytic activities

The influence of Pi starvation on the hydrolytic activities of the vacuolar H^+-ATPase and H^+-PPiase was determined in tonoplast vesicles isolated from +Pi and −Pi *B. napus* cells (Fig. 2). Confirmation that the phosphohydrolase activities

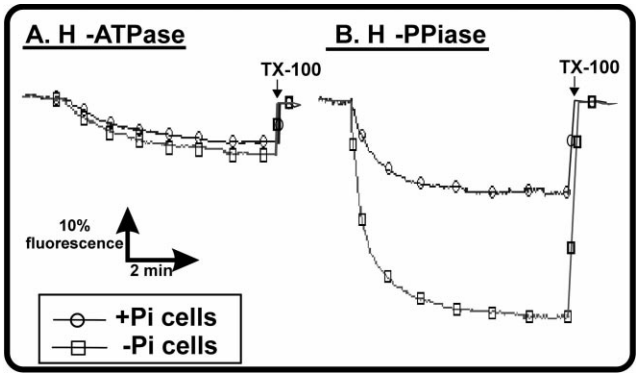

Fig. 1. ATP- (A) and PPi-dependent (B) H^+ transport into tonoplast vesicles isolated from 10-day-old +Pi and −Pi *B. napus* suspension cells. H^+ transport was estimated by monitoring the fluorescence quenching of acridine orange in tonoplast vesicles isolated from freshly harvested +Pi and −Pi cells. The reactions were initiated by the addition of 3.2 and 1.6 mM $MgSO_4$ for ATP- and PPi-dependent H^+ transport, respectively. At the indicated times, 0.05% (v/v) Triton X-100 was added.

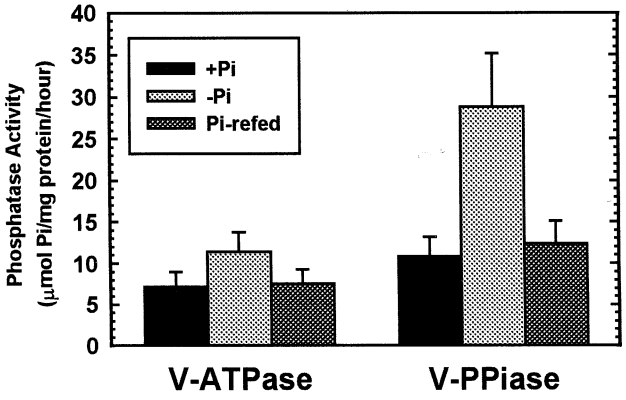

Fig. 2. Effect of Pi deprivation on the hydrolytic activities of vacuolar H^+-ATPase and H^+-PPiase in tonoplast vesicles from 10-day-old +Pi and −Pi *B. napus* cells. 'Pi-refed' denotes 10-day-old −Pi cultures that were resupplied with 2.5 mM Pi and cultured for an additional 36 h. All values represent means ± S.E.M. of $n = 4$ separate cultures.

reported in Fig. 2 were solely due to the V-PPiase or V-ATPase was provided by determining their respective sensitivities to the type-specific inhibitors aminomethylenediphosphonate and bafilomycin A_1 [12,14]. The V-PPiase activity of *B. napus* tonoplast vesicles was completely suppressed by 20 μM aminomethylenediphosphonate, whereas at least 95% of the V-ATPase activity was inhibited by 100 nM bafilomycin A_1.

Pi-starved cells displayed a moderate increase in H^+-ATPase activity (1.6-fold) and a much greater increase in H^+-PPiase activity (3-fold) (Fig. 2). Thus, in response to Pi starvation the H^+-PPiase to H^+-ATPase specific activity ratio significantly increased from 0.67 ± 0.10 to 1.26 ± 0.21 (Student's paired *t*-test, $P < 0.01$). This was largely reversed following 36 h of resupply of 2.5 mM Pi to the 10-day-old −Pi cells (Fig. 2).

3.3. Effect of Pi starvation on H^+-ATPase and H^+-PPiase protein levels

Tonoplast proteins from +Pi and −Pi cells were solubilized and resolved by SDS–PAGE, blotted and probed with antibodies raised against vacuolar H^+-ATPase and H^+-PPiase (Fig. 3). While the amount of the immunoreactive 57 kDa H^+-ATPase subunit was identical in tonoplast extracts from both +Pi and −Pi *B. napus* cells (Fig. 3A), the amount of immunoreactive 65 kDa H^+-PPiase subunit was twice as abundant in tonoplast extracts from −Pi cells (Fig. 3B).

4. Discussion

B. napus is a 'non-mycotrophic' plant whose roots do not form symbiotic associations with mycorrhizal fungi to facilitate Pi uptake from Pi-deficient soils [10]. Recent evidence suggests that, relative to mycorrhizal associating or 'mycotrophic' plants, the endogenous metabolism of non-mycotrophic plants is geared to allow a more efficient acclimation to Pi deprivation [1,10]. We have previously determined that Pi starvation of *B. napus* suspension cells results in 4.5-, 2.5- and 2.8-fold increases in the extractable activities of acid phosphatase, phosphoenolpyruvate carboxylase, and PFP, respectively [6,10,11]. All three enzymes have been suggested to contribute to the survival of Pi-starved *B. napus* in which significant reductions in cellular pools of Pi and adenine nucleotides, but not PPi, occur [6]. Here we provide evidence suggesting that the Pi starvation response of *B. napus* also involves the upregulation of the vacuolar H^+-PPiase.

Pi deficiency of *B. napus* significantly increased the initial rate and steady-state levels of PPi-dependent acridine orange fluorescence quenching in tonoplast vesicles (Fig. 1), suggesting that −Pi *B. napus* have a larger PPi-dependent H^+-pumping capacity than +Pi *B. napus*. These results were corroborated by assessing the tonoplast H^+-PPiase hydrolytic activity (Fig. 2). The H^+-PPiase activity was about 3-fold greater, and the H^+-PPiase to H^+-ATPase activities ratio almost 2-fold greater in −Pi cells relative to +Pi cells. Resupply of Pi to −Pi cells for 36 h resulted in return of ATPase and PPiase H^+ translocation and phosphatase activities to nearly the levels observed in +Pi cells. Western blots revealed a good correlation between the H^+-PPiase activity and the relative amount of the enzyme protein (Fig. 3). Thus, the increased capacity for PPi-dependent tonoplast H^+ transport and H^+-PPiase hydrolytic activity that accompanied Pi stress of *B. napus* arose, at least partially, from an increased expression of H^+-PPiase protein. The activity of H^+-ATPase was about 1.5-fold greater in −Pi cells relative to +Pi cells (Fig. 2), whereas the relative amount of immunoreactive 57 kDa H^+-ATPase subunit appeared to remain unchanged (Fig. 3).

Upregulation of tonoplast H^+-PPiase has also been observed in anoxic or chilled rice seedlings [12], which showed the same pattern of reduced adenylate levels and maintained PPi levels as do Pi-starved plants [13]. The induction of rice H^+-PPiase by anoxia or chilling stress was similarly reflected by corresponding increases in PPi-dependent tonoplast H^+ transport, and H^+-PPiase hydrolytic activity and protein levels [12].

Thermodynamic considerations indicate that PPi would be

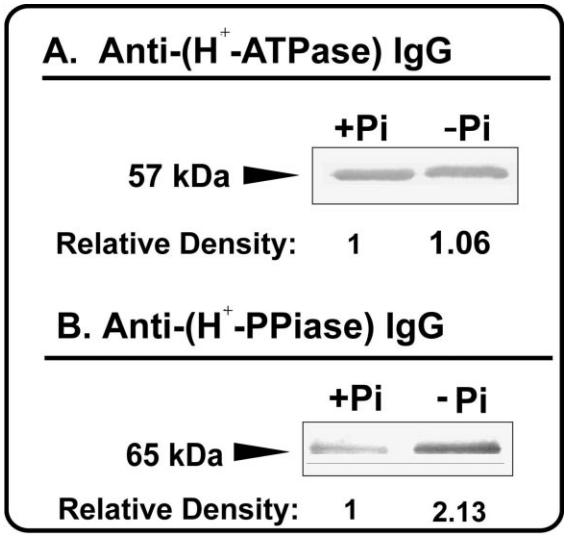

Fig. 3. Immunoblot analysis of tonoplast H^+ pumps in 10-day-old +Pi and −Pi *B. napus* suspension cells. Tonoplast proteins (4 μg/lane) were resolved by SDS–PAGE and blotted as described in Section 2. Immunoblotting was performed using antibodies raised against the vacuolar H^+-ATPase (A) or H^+-PPiase (B), and antigenic polypeptides were visualized using an alkaline phosphatase-tagged secondary antibody [12]; phosphatase staining was for 5–10 min at 30°C. Relative amounts of antigenic polypeptides were determined via laser densitometry. Values represent the average of two different tonoplast extracts.

particularly favored as a phosphoryl donor, relative to ATP, under cytosolic conditions known to accompany stresses such as anoxia or nutritional Pi deprivation [19]. The upregulation of H^+-PPiase by anoxia in rice [12] or Pi starvation in *B. napus* is therefore consistent with the utilization of this alternative mechanism of maintaining the vacuole acidification, employing PPi as an energy donor to conserve diminishing pools of ATP. The use of PPi versus ATP by PFP or H^+-PPiase also facilitates Pi recycling during Pi stress, since two and one Pi molecules are produced per PPi and ATP hydrolyzed, respectively.

In conclusion, our results support the notion that the Pi stress response of *Brassica* sp. includes the upregulation of PPi-dependent enzymes to circumvent ATP-limited reactions of the plant cytosol while simultaneously conserving limited cellular pools of ATP, and recycling of valuable Pi. Recent studies have revealed that PPi-dependent H^+ pumps are not necessarily confined to the tonoplast, but may also be localized in the plasma membrane of plant cells [14]. It would be of interest to determine the influence of anoxia or Pi stress on plant plasma membrane PPi- vs. ATP-dependent H^+ pumps.

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