J. AMER. SOC. HORT. SCI. 125(4):420–424. 2000.

PP*i* Formation by Reversal of the Tonoplast-bound H^+-pyrophosphatase from ‘Valencia’ Orange Juice Cells

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ADDITIONAL INDEX WORDS. vacuole, fruit development, *Citrus sinensis*

ABSTRACT. **Tonoplast vesicles isolated from juice cells of mature ‘Valencia’ oranges [*Citrus sinensis* (L.) Osbeck] showed similar tonoplast-bound vacuolar ATP*ase* (V-ATP*ase*) and inorganic pyrophosphatase (V-PP*iase*) activity as measured by product formation. Both proton pumps were able to generate a similar pH gradient, although steady-state was reached faster with ATP as substrate. When a ΔpH of 3 units was imposed (vesicle lumen pH of 4.5 and incubation medium of 7.5), tonoplast-bound PP*iase* was not able to significantly amplify the existing ΔpH. Although not able to function as a H^+ pump, V-PP*iase* effectively synthesized PP*i* in the presence of inorganic phosphate (P*i*). Formation of PP*i* by V-PP*iase* was enhanced by ATP but inhibited by NaF, gramicidin, and by antibodies raised against V-PP*iase* from mung bean [*Vigna radiata* (L.) R. Wilcz. (Syn. *Phaseolus aureus* Roxb.)]. Immunological analysis demonstrated an increase in V-PP*iase* protein with fruit maturity. Data indicate that under in vivo conditions, the V-PP*iase* of mature orange juice cells acts as a source of inorganic pyrophosphate (PP*i)* but not as a H^+ pump. We propose that synthesis of PP*i* provides a mechanism for recovery of stored energy in the form of the pH gradient across the vacuole during later stages of development and postharvest storage.**

The early stages in the development of citrus fruit (*Citrus* L. sp.) are characterized by a massive accumulation of citric acid and a parallel decline in vacuolar pH. In sweet oranges (*Citrus sinensis*), for example, the concentration of citric acid reaches levels of up to 115 mM (Clements, 1964) with vacuolar pH dropping to 2.8 or lower (Echeverria and Burns, 1989). A pH gradient (ΔpH) between the acidic vacuole and the neutral cytosol of such magnitude can only be generated by the V-ATP*ase* (Davies, 1994; Schmidt and Briskin, 1993) despite the existence of two H^+ pumps at the tonoplast of plant cells (i.e., V-ATP*ase* and V-PP*iase*; Rea and Sanders, 1987). Thermodynamic constraints prevent the V-PP*iase* from operating in the hydrolytic mode under these extreme physiological conditions (Schmidt and Briskin, 1993).

Later in citrus fruit development, and continuing throughout postharvest storage, vacuolar citric acid content declines with a concomitant increase in pH (Clements, 1964; Harding and Lewis, 1941; Ting and Vines, 1966; Yamaki, 1990). As the fruit matures, some anatomical and physical characteristics of the pericarp develop into effective gas barriers resulting in partial oxygen deprivation and decreased aerobic respiration in the interior juice cells (Bain, 1958; Hirai and Ueno, 1977). This increase in anaerobic respiration is evidenced by rising levels of ethanol and acetaldehyde in maturing (Davis, 1970; Roe et al., 1984) and stored fruit (Davis, 1970; Davis et al., 1973). In plant cells, in response to low oxygen pressure, cytosolic ATP content declines with a concomitant increase in the ADP/ATP ratio. A marked decline in the energy status (ATP content) of the juice cells has long been recognized to occur in mature citrus fruit (Bruemmer and Roe, 1985).

In plants with low ATP levels, PP*i* dependent phosphofructokinase (EC 2.7.1.90; PFP) acquires a dominant role in the glycolytic synthesis of fructose-1,6-P_2 (Mertens et al., 1990) thus becoming a mechanism for ATP conservation. Under similar anaerobic conditions, sucrose breakdown occurs seemingly through the sucrose synthase (SS) pathway (Perata et al., 1996; Ricard et al., 1991). Both the activity of PFP and conversion of UDPG (product of sucrose synthase) to glucose-1-P require a steady supply of PP*i* inasmuch as its cytosolic levels remain unchanged even during marked respiratory fluxes (Dancer and ap Rees, 1989; Weiner et al., 1987). It was observed recently that tonoplast-bound PP*iase* is over-expressed in response to energetic stress such as anoxia and chilling in rice (*Oryza sativa* L.) seedlings (Carystinos et al., 1995) and mung bean [*Vigna radiata* (syn. *Phaseolus aureus*)] hypocotyl (Darley et al., 1995). A role for V-PP*iase* in the supply of PP*i* was demonstrated for maize (*Zea mays* L.) seeds and coleoptiles under similar conditions (Rocha and de Meis, 1998). These observations suggest that V-PP*iase* may play a role in the supply of PP*i* under anaerobic conditions (limited ATP) occurring during later stages of citrus fruit maturity. Preliminary studies have established the presence of PP*iase* in tonoplast vesicles isolated from sweet orange juice cells. Given the fact that V-PP*iase* can not act in the hydrolytic direction under conditions found in mature citrus fruit, we examined the possibility of this tonoplast-bound H^+ pump acting in the direction of PP*i* formation during vacuole deacidification, therefore becoming a PP*i* source for PFP and glucose-1-P production. In this report, we present evidence demonstrating the synthesis of PP*i* coupled to the efflux of protons by ‘Valencia’ orange juice cell tonoplast V-PP*iase*.

Materials and Methods

PLANT MATERIAL. Mature ‘Valencia’ oranges were collected in early April 1999 from groves located at the Citrus Research and Education Center, Lake Alfred, Fla. Fruit were transported to the laboratory and used immediately for tonoplast extraction.

TONOPLAST VESICLE EXTRACTION. Tonoplast vesicles were isolated in a discontinuous sucrose gradient following the procedure described previously for sweet limes (*Citrus limmetioides* Tanaka) (Echeverria et al., 1997). After isolation, tonoplast vesicles were

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Received for publication 25 Oct. 1999. Accepted for publication 7 Apr. 2000. Florida Agricultural Experiment Station journal series R-07192. We are grateful to M. Maeshima for the gift of antibodies used in this study. The cost of publishing this paper was defrayed in part by the payment of page charges. Under postal regulations, this paper therefore must be hereby marked *advertisement* solely to indicate this fact.

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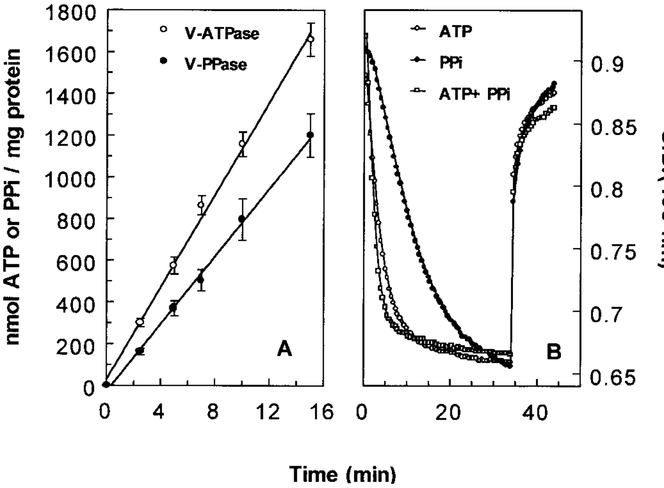

Fig. 1. (**A**) Activity of tonoplast bound V-ATP*ase* and V-PP*iase* from mature 'Valencia' orange juice cells and (**B**) H^+ gradient formation of similar vesicles in the presence of ATP, PP*i*, or both. Data in A are the average of three replicates and vertical bars = SE. B is a graphical representation of spectrophotometric data reading from a typical experiment.

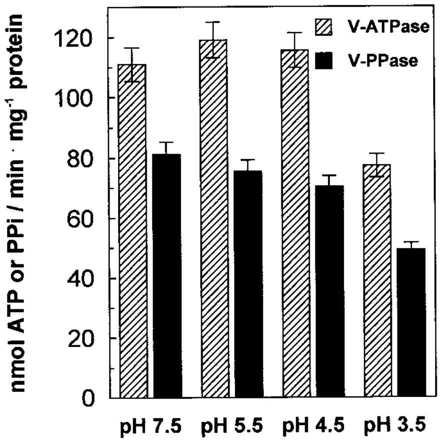

Fig. 2. V-ATP*ase* and V-PP*iase* activities in tonoplast samples from mature 'Valencia' orange juice cells equilibrated at different pHs. Tonoplast samples were frozen and thawed three times in MES buffer at the indicated pH before enzyme assay. The enzymatic determinations were performed at pH 7.5 as explained in Materials and Methods. Data are an average of three experiments.

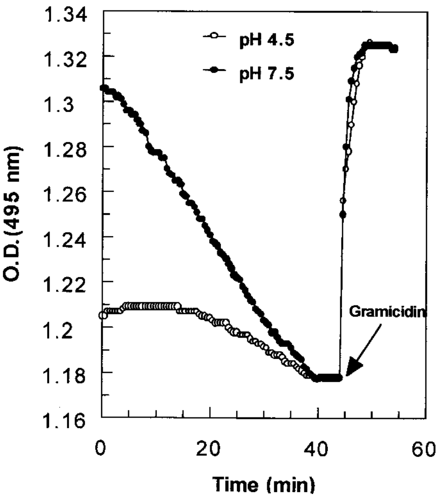

Fig. 3. The H^+ pumping capability of V-PP*iase* in tonoplast vesicles with interior pH of 4.5 and 7.5. After equilibrium was reached, 10 µM gramicidin was added to collapse the pH gradient. Both samples contained the same V-PP*iase* activity.

resuspended in a solution containing 10 mM Mes [2-(N-Morpholino) ethane-sulfonic acid] (pH 7.5-3.5), 250 mM sorbitol, and 2 mM dithiothreitol (DTT), and frozen at –80 °C until use. Exchange of the internal pH of the tonoplast vesicles was accomplished by three freeze–thaw cycles applied to resuspended vesicles in the desired buffered solutions (Bush and Langston, 1988).

ATPASE AND PPIASE ACTIVITY. Both V-ATP*ase* and V-PP*iase* activities were measured colorimetrically following the production of free P*i*. ATP*ase* reaction contained 50 mM BTP/Mes (pH 7.5), 4 mM DTT, bovine serum albumin (BSA) at 4 mg·mL^{-1}, 250 mM sorbitol, 50 mM KCl , 10 µM gramicidin, 4 mM ATP, 4 mM $MgSO_4$, and vesicles in a total volume of 500 µL. V-PP*iase* activity was measured in a solution similar to that of ATP*ase* except 1.0 mM PP*i* was added instead. The P*i* released was determined as described by Chifflett et al. (1988). Activity was measured in nanomoles of product hydrolyzed per minute per milligram protein. Protein was determined as described by Bradford (1976). Formation of an acid interior pH gradient across the tonoplast was measured by the decrease in absorbance of acridine orange at 495 nm as in a reaction above for ATP*ase* and PP*iase* except that gramicidin was omitted and 10 µM acridine orange was added (Palmgren, 1990). Reactions were carried out in a Shimadzu UV-160 spectrophotometer at 30 °C.

PPI SYNTHESIS AND DETERMINATION. To study the synthesis of PP*i*, tonoplast vesicles with internal pH of 4.5 were used. Determination of PP*i* was achieved by coupling its synthesis with the production of fructose-1,6-P_2 by PFP and ensuing triose-phosphates in a solution containing 100 mM HEPES buffer (pH 7.5), 250 mM sorbitol, 2 mM DTT, 5 mM fructose-6-P, 2.5 mM P*i*, 5 mM $MgCl_2$, 10 µM fructose-2,6-P_2, 0.5 mM NADH, 0.25 units PFP, 1 unit aldolase, 0.5 unit each triose-P isomerase and glyceraldehyde-3-P dehydrogenase, and following the continuous change in NADH absorbance at 340 nm (VanSchaftingen et al., 1982).

IMMUNOLOGICAL DETECTION OF V-PPIASE. Immunoblot analyses were performed after electrophoresis according to Laemmli et al. (1970). All lanes contained 10 µg of tonoplast protein. Proteins were transferred to a cellulose nitrate membrane and

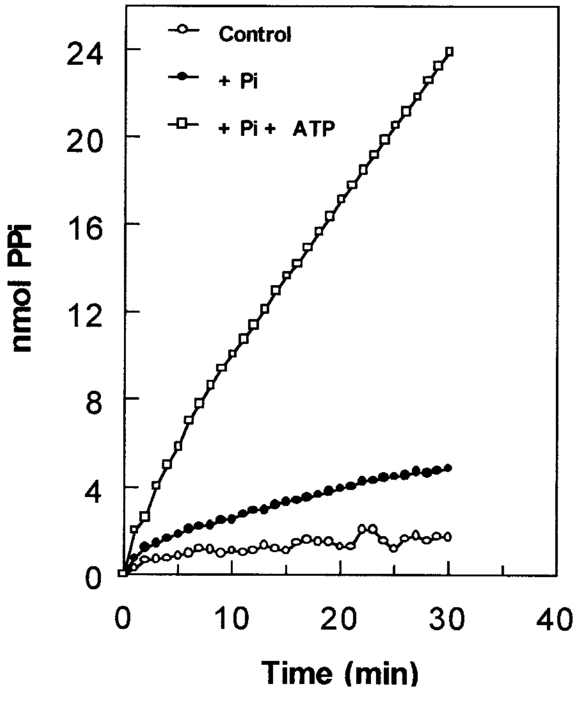

Fig. 4. PP*i* formation by tonoplast vesicles from mature 'Valencia' orange juice cells in the presence and absence of ATP. The vesicles (60 µg protein) had an initial interior pH of 4.5. Control tonoplast samples were boiled for 2 min before the start of the reaction. Formation of PP*i* was coupled to the synthesis of fructose-1,6-P_2 using PP*i* dependent phosphofructokinase. Data are a graphical representation of spectrophotometric output and converted to nmoles. Control samples contained boiled membrane aliquots. ATP was present at a final concentration of 2.5 mM, whereas P*i* was present at 2 mM.

Table 1. Synthesis of PP*i* by tonoplast vesicles isolated from 'Valencia' orange juice cells. PP*i* formation was measured by following the continuous decline in absorbance at 340 nm in a coupled reaction with PP*i*-dependent phosphofructokinase, aldolase, triose-phosphate isomerase-glyceraldehyde-phosphate dehydrogenase. Values presented are means ± SE (n = 3).

	PP*i* synthesis nmol·mg^{-1} PP*i* protein/30 min
Control (boiled vesicle sample)	0
Vesicles (interior pH 4.5) + 2 mM *Pi*	65.2 ± 6.7
+ 2.5 mM ATP	54.2 ± 5.1
+ 2.5 mM ATP + 2 mM P*i*	186.5 ± 11.4
+ 2.5 mM ATP + 2 mM P*i* + 25 mM NaF	15.4 ± 7.5
+ 2.5 mM ATP + 2 mM P*i* + antibody PP*iase*	162.2 ± 18.7
+ 2.5 mM ATP + 2 mM P*i* + 10 µM gramicidin	9.9 ± 3.1
Vesicles (interior pH 7.5)	
+ 2 mM *Pi*	8.7 ± 2.1
+ 2.5 mM ATP + 2 mM P*i*	145.5 ± 8.2

immunostained with a polyclonal antibody against a peptide that corresponds to the catalytic site of V-PP*iase* from mung bean (Takasu et al., 1997). Antibodies were a gift from M. Maeshima.

Results

Tonoplast vesicles isolated from mature 'Valencia' orange juice cells exhibited properties very similar to those already characterized for acid limes (*Citrus aurantifolia* Swingle) (Brune et al., 1998) and lemons (*Citrus limon* Burmif) (Müller et al., 1996, 1997). The vesicles were able to catalyze the hydrolysis of both ATP and PP*i* (Fig. 1A) and the hydrolysis was coupled to H^+ translocation as evidenced by the decrease in absorbance of acridine orange (Fig. 1B). The balance of activities between the two tonoplast H^+ pumps was consistent with patterns already established for most vegetative tissues in which the V-ATP*ase* is capable of generating a pH gradient of similar or greater magnitude than the V-PP*iase* (Giannini and Briskin, 1987; Rea and Sanders, 1987; Rocha and de Meis, 1998).

To establish the limits at which V-ATP*ase* and V-PP*iase* would operate and, at the same time, create a ΔpH of the largest possible magnitude, we established a series of pH jumps with vesicles equilibrated at different internal pHs (7.5 to 3.5). Figure 2 shows the effect of pH used for vesicle equilibration by freeze and thaw cycles on the activity of both V-ATP*ase* and V-PP*iase*. At pH 4.5, 100% of the V-ATP*ase* and 90% of the V-PP*iase* activity remained. Lower pHs had a more deleterious effect on the V-ATP*ase* as seen by the rapid decline in activity. Therefore, we opted for vesicles resuspended after three freeze-thaw cycles at pH 4.5, which showed little damage to either of the two H^+ pumps.

Tonoplast vesicles were tested for latent V-ATP*ase* activity after the corresponding freeze and thaw cycles. V-ATP*ase* activity increased 27% in vesicle samples treated with 8 mM CHAPS {3-[(3-cholamidoprophyl) dimethylammonio]-1-propanesulfonate}, indicating that the vesicles were predominantly right side out.

The H^+ pumping capacity of V-PP*iase* was determined in vesicles with internal pH of 7.5 and 4.5. It is noteworthy that the difference in the initial quenching of acridine orange between both vesicle samples was due to the difference in the initial internal pH. Although both samples contained similar PP*i* hydrolytic activity, a marked reduction in H^+ pumping capacity was observed for the V-PP*iase* in vesicles at pH 4.5. Ultimately, the final pH was similar for both sets of vesicles (Fig. 3). This demonstrates that the capacity of the V-PP*iase* to pump H^+ is close to its thermodynamic limit at the experimentally imposed ΔpH of 3 units.

The H^+ gradient established by the pH jump was used to promote the reversal of the V-PP*iase*. Formation of PP*i* in the presence of 2 mM P*i* was hyperbolic and reached a steady-state level within 5 min (Fig. 4). Cessation of PP*i* formation was due likely to dissipation of the ΔpH used to energize PP*i* synthesis. Under the same conditions, addition of 2.5 mM ATP resulted in a significant increase in PP*i* formation which remained linear up to over 30 min (Fig. 4). It is evident that the additional H^+ pumped by the V-ATP*ase* maintained a stronger H^+ gradient and allowed for the additional formation of PP*i*. Addition of 10 µM gramicidin completely abolished the ΔpH-dependent PP*i* synthesis as expected by the ensuing collapse in ΔpH (Table 1). Vesicles with internal pH of 7.5 were unable to synthesize PP*i* in the absence of ATP (Table 1) demonstrating that coupling between $\Delta\mu H^+$ and PP*i* formation was required. Addition of ATP to vesicles at 7.5 resulted in the significant synthesis of PP*i* as the V-ATP*ase* was able to generate a pH gradient of sufficient enough magnitude to drive the PP*iase* in the reverse direction. Similar results were obtained by Rocha and de Meis (1998) with vesicles from maize coleoptiles and seeds.

Additional evidence for the in vitro formation of PP*i* by isolated tonoplast vesicles resulted from experiments performed using antibodies against the V-PP*iase* and NaF, an inhibitor of the V-PP*iase* (Rocha and de Meis, 1998). In both instances, formation of PP*i* was reduced, with NaF showing the highest degree of inhibition (Table 1). The percent inhibition by the antibodies on the capacity of V-PP*iase* to synthesize PP*i* was similar to that observed by Takasu et al. (1997) on H^+ pumping by mung bean V-PP*iase*.

Immunological detection of tonoplast samples from 'Valencia' orange juice cells with antibodies against V-PP*iase* revealed an increase in immunoreactivity with advanced maturity (Fig. 5). Tonoplast samples from mature fruit showed a much stronger reaction to V-PP*iase* antibodies than those of younger fruit at equal protein concentrations. The increase in V-PP*iase* specific activity occurs at a time when fruit deacidification is taking place.

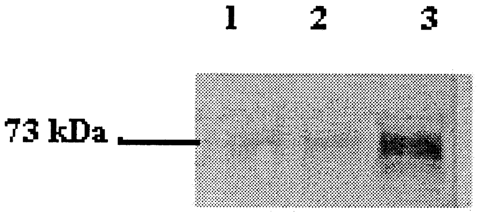

Fig. 5. Immunodetection of V-PP*iase* in tonoplast samples from 'Valencia' orange juice cells at different times of fruit maturity. The antibodies were raised against the H^+ catalytic site of mung mean V-PP*iase*. Lane numbers represent different stages of dev-elopment. Each lane contained 10 µm membrane protein; 1 = early stage of development, 3-month-old fruit; 2 = middle stage of development, 6-month-old fruit; 3 = mature fruit, 12-month-old fruit.

Discussion

Results herein demonstrate that under steep trans-tonoplast H^+ gradients (similar but smaller to those found in mature citrus fruit) citrus juice cell tonoplast-bound V-PP*iase* effectively synthesizes PP*i* but appears not to be involved in H^+ pumping. This conclusion is based on two lines of evidence. First, V-PP*iase* in vesicles with an interior pH of 4.5 (ΔpH of 3 units) showed significantly lower rates of H^+ pumping (Fig. 3) despite considerable hydrolysis of PP*i* (Fig. 2). Although the rates on Fig. 3 are affected by higher H^+ fluxes from tonoplast vesicles at 4.5, lower rates of H^+ pumping were also estimated by taking into account H^+ leakage from tonoplast vesicles at pH 4.5 in the absence of ATP or PP*i*. The constraints imposed by a steeper trans-tonoplast H^+ gradient, such as those found in orange juice cells of over 4 pH units, would virtually suppress the V-PP*iase* from its H^+ pumping mode. Second, similar vesicles (with an imposed ΔpH of 3 units) effectively synthesized PP*i* in the presence and absence of ATP. Formation of PP*i* in the presence of ATP remained linear for >30 min as the H^+ pumping of V-ATP*ase* maintained a ΔpH of sufficient magnitude to energize the V-PP*iase*. Inhibition of PP*i* formation by the V-PP*iase* inhibitor NaF, by antibodies against V-PP*iase*, and by the disruption of the H^+ gradient by gramicidin verifies this contention. It is noteworthy that PP*i* production by vesicles with an interior pH of 4.5 was higher than those at pH 7.5 as the result of a higher initial ΔpH.

Another observation that argues in favor of the involvement of PP*iase* in PP*i* formation is the sharp increase in V-PP*iase* enzyme transcript specific activity with increasing fruit maturity (Fig. 5). This increase in activity occurs during stage 3 of development (Bain, 1958) at a time where deacidification of citrus juice vacuoles is taking place. Vacuolar acidification, on the contrary, occurs earlier during stage 2 of growth (Bain, 1958) during the period of juice cell expansion.

The hyperacidification that occurs in the vacuole of citrus juice cells early in development requires the presence of an unconventional type of V-ATP*ase* that operates near the thermodynamic equilibrium (Müller et al., 1996, 1997). The resulting low vacuolar pH acts as the driving force for accumulation of high concentrations of citric acid which, in some cultivars, reach values of over 300 mM (Brune et al., 1998). Later in development and during postharvest storage, use of citric acid results in an increase in vacuolar pH. Based on the mechanisms of citrate transport across the tonoplast (Brune et al., 1998), efflux of citrate^{-3} would result in a further acidification of the vacuole by leaving behind 3 H^+. Instead, an increase in pH is observed commonly indicating a removal of H^+ from the vacuole as the fruit matures and during postharvest life. In this respect, the tonoplast-bound V-PP*iase* can prove particularly useful in recovery of free energy present in the H^+ gradient by synthesizing PP*i* at a time where anaerobic respiration becomes more prominent. All biochemical indications in mature citrus fruit favor the shift from aerobic to anaerobic respiration and the preference for PP*i* as substrate. Enzymes associated with increased anaerobic respiration and sucrose use such as SS, UDPG pyrophosphorylase, and PP*i* dependent V-PP*iase* all increase during postharvest storage. The remarkable preponderance of V-PP*iase* in maturing and harvested citrus fruit, and the fact that its activity increases at these stages when it can not operate as a H^+ pump, argues favorably for its role in the formation of PP*i*.

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