PPi Formation by Reversal of the Tonoplast-bound H+-pyrophosphatase from ‘Valencia’ Orange Juice Cells

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ABSTRACT. Tonoplast vesicles isolated from juice cells of mature ‘Valencia’ oranges [Citrus sinensis (L.) Osbeck] showed similar tonoplast-bound vacuolar ATPase (V-ATPase) and inorganic pyrophosphatase (V-PPase) 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 PPase was not able to significantly amplify the existing ΔpH. Although not able to function as a H+ pump, V-PPase effectively synthesized PPi in the presence of inorganic phosphate (Pi). Formation of PPi by V-PPase was enhanced by ATP but inhibited by NaF, gramicidin, and by antibodies raised against V-PPase from mung bean [Vigna radiata (L.) R. Wilcz. (Syn. Phaseolus aureus Roxb.)]. Immunological analysis demonstrated an increase in V-PPase protein with fruit maturity. Data indicate that under in vivo conditions, the V-PPase of mature orange juice cells acts as a source of inorganic pyrophosphate (PPi) but not as a H+ pump. We propose that synthesis of PPi 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-ATPase (Davies, 1994; Schmidt and Briskin, 1993) despite the existence of two H+ pumps at the tonoplast of plant cells (i.e., V-ATPase and V-PPase; Rea and Sanders, 1987). Thermodynamic constraints prevent the V-PPase 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 (Brummer and Roe, 1985).

In plants with low ATP levels, PPi dependent phosphofructokinase (EC 2.7.1.90; PFP) acquires a dominant role in the glycolytic synthesis of fructose-1,6-P2 (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 PPi inasmuch as its cytosolic levels remain unchanged even during marked respiratory fluxes (Dancer and Rees, 1989; Weiner et al., 1987). It was observed recently that tonoplast-bound PPase 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-PPase in the supply of PPi was demonstrated for maize (Zea mays L.) seeds and coleoptiles under similar conditions (Rocha and de Meis, 1998). These observations suggest that V-PPase may play a role in the supply of PPi under anaerobic conditions (limited ATP) occurring during later stages of citrus fruit maturity. Preliminary studies have established the presence of PPase in tonoplast vesicles isolated from sweet orange juice cells. Given the fact that V-PPase 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 PPi formation during vacuole deacidification, therefore becoming a PPi source for PFP and glucose-1-P production.

In this report, we present evidence demonstrating the synthesis of PPi coupled to the efflux of protons by ‘Valencia’ orange juice cell tonoplast V-PPase.

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 limnetoides Tanaka) (Echeverria et al., 1997). After isolation, tonoplast vesicles were
Fig. 1. (A) Activity of tonoplast bound V-ATPase and V-PP\textsubscript{i}ase from mature ‘Valencia’ orange juice cells and (B) H\textsuperscript{+} gradient formation of similar vesicles in the presence of ATP, P\textsubscript{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\textsubscript{ase} and V-PP\textsubscript{i}ase 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\textsuperscript{+} pumping capability of V-PP\textsubscript{i}ase in tonoplast vesicles with interior pH of 4.5 and 7.5. After equilibrium was reached, 10\mu M gramicidin was added to collapse the pH gradient. Both samples contained the same V-PP\textsubscript{i}ase activity.

Fig. 4. PP\textsubscript{i} formation by tonoplast vesicles from mature ‘Valencia’ orange juice cells in the presence and absence of ATP. The vesicles (60\mu g 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\textsubscript{i} was coupled to the synthesis of fructose-1,6-P\textsubscript{2} using PP\textsubscript{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\textsubscript{i} was present at 2 mM.
increased 27% in vesicle samples treated with 8 mM CHAPS \{3-[(3-Opted for vesicles resuspended after three freeze-thaw cycles at
V-ATP
cactivity remained. Lower pHs had a more deleterious effect on the
increase in immunoreactivity with advanced maturity (Fig. 5).

**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 PPi (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-ATPase is
capable of generating a pH gradient of similar or greater magni-
tude than the V-PPiase (Giannini and Briskin, 1987; Rea and
Sanders, 1987; Rocha and de Meis, 1998).

To establish the limits at which V-ATPase and V-PPiase
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-ATPase and V-PPiase.
At pH 4.5, 100% of the V-ATPase and 90% of the V-PPiase
activity remained. Lower pHs had a more deleterious effect on the
V-ATPase 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-ATPase activity after
the corresponding freeze and thaw cycles. V-ATPase activity
increased 27% in vesicle samples treated with 8 mM CHAPS \{3-[(3-
cholamidopropyl) dimethylammonio]-1-propanesulfonate], indi-
cating that the vesicles were predominantly right side out.

The H+ pumping capacity of V-PPiase 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 internal pH.
Although both samples contained similar PPi hydrolytic activity, a
marked reduction in H+ pumping capacity was observed for the V-
PPiase 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-PPiase to pump H+ is close to its thermodynamic limit at the
experimentally imposed ΔpH of 3 units.

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

| Condition                        | PPi synthesis \(\text{nmol·mg}^{-1}\) PPi 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 Pi           | 186.5 ± 11.4                                             |
| + 2.5 mM ATP + 2 mM Pi + 25 mM NaF| 15.4 ± 7.5                                               |
| + 2.5 mM ATP + 2 mM Pi + antibody PPiase | 162.2 ± 18.7                                       |
| + 2.5 mM ATP + 2 mM Pi + 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 Pi           | 145.5 ± 8.2                                              |

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

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

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

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

Fig. 5. Immunodetection of V-PPiase 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-PPiase. Lane numbers represent different stages of development. 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.

73 kDa
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$_{i}$ase 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$_{i}$ase in vesicles with an interior pH of 4.5 ($\Delta$pH 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$_{i}$ase from its $H^+$ pumping mode. Second, similar vesicles (with an imposed $\Delta$pH 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$_{i}$ase maintained a $\Delta$pH of sufficient magnitude to energize the V-PP$_{i}$ase. Inhibition of PP$i$ formation by the V-PP$_{i}$ase inhibitor NaF, by antibodies against V-PP$_{i}$ase, 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 $\Delta$pH.

Another observation that argues in favor of the involvement of PP$_{i}$ase in PP$i$ formation is the sharp increase in V-PP$_{i}$ase 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$_{i}$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$_{i}$ase 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 respira- tion and sucrose use such as SS, UDPG pyrophosphorylase, and PP$i$ dependent V-PP$_{i}$ase all increase during postharvest storage. The remarkable preponderance of V-PP$_{i}$ase 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$i$.

### Literature Cited

Bain, J.M. 1958. Morphological, anatomical and physiological changes in the developing fruit of the ‘Valencia’ orange Citrus sinensis (L.) Osbeck. Austral. J. Bot. 6:1-24.

Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72:248-254.

Brummer, J.H. and B. Roe. 1985 Pyruvate dehydrogenase activity during ripening of ‘Hamlin’ oranges. Phytochemistry 24:2105-2106.

Brune, A., P.C. Gonzalez, and E. Echeverria. 1998. Citrate uptake into tonoplast vesicles from acid lime (Citrus aurantifolia) juice cells. J. Membr. Biol. 166:197-203.

Carystinos, G.D., H.R. MacDonald, A.F. Monroy, R.S. Dhindsa, and R.J. Poole. 1995. Vacular $H^+$-translocating pyrophosphatase is induced by anoxia or chilling in seedlings of rice. Plant Physiol. 108:641-649.

Chifflet, S., A. Torriglia, A. Chisea, and S. Tolosa. 1988. A method for the determination of inorganic phosphate in the presence of labile organic phosphate and high concentrations of protein: Application to lens ATP$_{i}$ase. Anal. Biochem. 168:1-4.

Clements, R.L. 1964. Organic acids in citrus fruits. Varietal differences. J. Food Sci. 29:276-280.

Dancer, J. and T. ap Rees. 1989. The effect of 2,4-dinitrophenol and anoxia on the inorganic pyrophosphate content of the spadix of Arum maculatum and root apices of Pisum sativum. Planta 178:421-424.

Darley, C.P., J.M. Davies, and D. Sanders. 1995. Chill-induced changes in the activity and abundance of the vacuolar proton pumping pyrophosphatase from mung bean hypocotyls. Plant Physiol. 109:659-665.

Davis, J.M. 1994. The bioenergetics of vacuolar $H^+$ pumps. Adv. Bot. Res. 25:339-362.

Davis, P.L. 1970. Relation of ethanol content of citrus fruits to maturity and storage conditions. Proc. Fla. State Hort. Soc. 83:294-297.

Davis, P.L., B. Roe, and J.H. Brummer. 1973. Biochemical changes in citrus fruits during controlled atmosphere storage. J. Food. Sci. 38:225-229.

Echeverria, E. and J. K. Burns. 1989. Vacuolar acid hydrolysis as a physiological mechanism for sucrose breakdown. Plant Physiol. 90:530-533.

Echeverria, E., P.C. Gonzalez, and A. Brune. 1997. Characterization of proton and sugar transport at the tonoplast of sweet lime (Citrus limnetoides) juice cells. Physiol. Plant. 101:291-300.

Giannini, J.L. and D.P. Briskin. 1987. Proton transport in plasma membrane and tonoplast vesicles from red beet (Beta vulgaris L.) storage tissue. Plant Physiol. 84:613-618.

Giannini, J.L. and D.P. Briskin 1988 Pyridine nucleotide oxidation by plasma membrane fraction from red beet (Beta vulgaris L. storage tissue. Arch. Biochem. Biophys. 260:653-660.

Harding, P.L. and W.E. Lewis. 1941. The relation of size of fruit to solids, acid and volume of juice in the principal varieties of Florida oranges. Proc. Fla. State Hort. Soc. 54:52-66.

Hirai, M. and I. Ueno. 1977. Development of citrus fruits: Fruit development and enzymatic changes in juice vesicle tissue. Plant Cell Physiol. 18:791-799.

Laemmli, U.K. 1970. Cleavage of structural proteins during assembly of the head of the bacteriophage T4. Nature. 227:680-685.

Mertens, E., Y. Larondelle, and H-G. Hers. 1990. Induction of pyrophosphate/fructose 6-phosphate 1-phosphotransferase by anoxia in rice seedlings. Plant Physiol. 93:584-587.

Müller, M.L., U. Irkens, D. Kramer, and L. Taiz. 1997. Purification and reconstruction of the vacuolar $H^+$-ATP$_{i}$ase from lemon fruits and epicotyls. J. Biol. Chem. 272:12762-12770.

Müller, M.L., U. Irkens, B.D. Rubinstein, and L. Taiz. 1996. On the mechanism of hyperacidification in lemon: Comparison of the vacuolar $H^+$-ATP$_{i}$ase of fruits and epicotyls. J. Biol. Chem. 271:1916-1924.

Palmgren, M.G. 1990. An $H^+$-ATP$_{i}$ase assay: Proton pumping and ATP$_{i}$ase activity determined simultaneously in the same sample. Plant Physiol. 94:882-886.
Perata, P., L. Guglielminetti, and A. Alpi. 1996. Anaerobic carbohydrate metabolism in wheat and barley, two anoxia intolerant cereal seeds. J. Expt. Bot. 301:999-1006.

Rea, P.A. and D. Sanders. 1987. Tonoplast energization: Two pumps one membrane. Physiol. Plant. 71:131-141.

Ricard, B., J. Rivoal, A. Spiter, and A. Pradet. 1991. Anaerobic stress induces the transcription and translation of sucrose synthase in rice. Plant Physiol. 95:669-674.

Rocha, A. and L. de Meis. 1998. Reversibility of H+-ATPase and H+-pyrophosphatase in tonoplast vesicles from maize coleoptiles and seeds. Plant Physiol. 116:1487-1495.

Roe, B., P.L. Davis, and J.H. Bruemmer. 1984. Pyruvate metabolism during maturation of Hamlin oranges. Phytochemistry 23:713-717.

Schmidt, A.L. and D. Briskin. 1993. Energy transduction in tonoplast vesicles from red beet (Beta vulgaris) storage tissue: H+/substrate stoichiometries for the H+-ATPase and H+-PPase. Arch. Biochem. Biophys. 301:165-173.

Takasu, A., Y. Nakanishi, T. Yamauchi, and M. Maeshima. 1997. Analysis of the substrate binding site and carboxyl terminal region of vacuolar H+-pyrophosphatase of mung bean with peptide antibodies. J. Biochem. 122:883-889.

Ting, S.V. and H.M. Vines. 1966. Organic acids in the juice vesicles of Florida ‘Hamlin’ orange and ‘Marsh’ grapefruit. J. Amer. Soc. Hort. Sci. 88:291-297.

Van Schaftingen, E., B. Ledarer, R. Bartrons, and H.G. Hers. 1982. A kinetic study of pyrophosphate: fructose-6-phosphate phosphotransferase from potato tubers: Application to a microassay of fructose 2, 6-bisphosphate. European J. Biochem. 129:191-195.

Weiner, H., M. Stitt, and H.W. Heldt. 1987. Subcellular compartmentation of pyrophosphate and alkaline phosphatase in leaves. Biochem. Biophys. Acta 893:13-21.

Yamaki, Y. 1990. Seasonal changes in the organic acids in juice of citrus fruits. J. Jpn. Soc. Hort. Sci. 58:895-898.