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AN ACTIVE PROTON PUMP OF INTACT VACUOLES ISOLATED FROM *TULIPA* PETALS

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Anthocyanin pigments within *Tulipa* petal vacuoles provide the means for real-time spectrophotometric monitoring of vacuolar sap pH and for studying ATP-dependent proton transport in isolated, intact vacuoles. Spectra of petal extracts were used to select empirically those wavelengths giving an approximately linear variation in anthocyanin absorbance with pH over a pH range of interest. A sensitive single-beam spectrophotometer with vertical optics was used to minitor absorbance changes of intact, settled vacuoles. Substrates and inhibitors of vacuolar ATPase (Lin, W., Wagner, G.J., Siegelman, H.W. and Hind, Q. (1977) Biochim. Biophys. Acta 465, 110–117) were added to probe proton transport. Acidification of the vacuole sap occurred following addition of MgATP, but not CaATP. Proton accumulation was inhibited by 10 μM Dio 9, an inhibitor of tonoplast ATPase in vitro, and the proton gradient established by addition of MgATP was dissipated after addition of 10 μM CCCP. No pumping response was observed with intact protoplasts. Potential differences across the tonoplast were directly measured by impaling vacuoles with glass microelectrodes. Potential differences of 10–20 mV (inside positive) were recorded when vacuoles were suspended in 0.7 M mannitol/10 mM Hepes buffer (adjusted to pH 8.0 with KOH), and 0.5 mM dithiothreitol. Addition of MgATP increased the potential difference by 2–5 mV.

Introduction

It is well established that membrane-bound ATPases are involved in ion transport in animal tissues [1]. The role of ATPase in transport in plants is less well established [2,3]. It is clear that, in chloroplasts and plant mitochondria, ATP or electron transfer reactions, via ATPase, provide energy for ion transport [4,5]. The role of plant plasma membrane ATPase in ion transport is a controversial topic, but ATP-stimulated proton transport has been observed in plasma membrane vesicles from *Neurospora* [6]. Recently, ATP-dependent proton transport has been demonstrated in sealed vesicles obtained from microsome preparations of tobacco callus [7], pea [8], corn coleoptiles [9] and corn roots [10]. It is not known whether these vesicles originate from plasma membrane, tonoplast, endoplasmic reticulum, golgi or the outer membranes of other cytoplasmic organelles.

Little is known about the mechanisms which facilitate ion transport in higher plant vacuoles, yet this organelle is thought to be a primary ion reservoir in higher plants [11–13]. Sutcliffe [14] observed that cells plasmolyzed in hypertonic salt solutions recovered at a rate which was consistent with complete transfer of the absorbed ions into the vacuole. Nucleotide-specific, Mg-dependent

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Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

ATPase has been reported in association with *Tulipa* and *Hippeastrum* petal vacuoles and tonoplast [12,15], *Tulipa* leaf vacuoles [12], with vacuoles [16,17] and tonoplast [18] isolated from red beetroot, and with the membrane from lutoids of *Hevea* [19]. Marin et al. [20] recently described MgATP-dependent, uncoupler-sensitive proton transport in lutoids of *Hevea*. ATP-stimulated, uncoupler- or ionophore-sensitive transport of sucrose [17] and glucose [21] in plant vacuoles and of citrate in *Hevea* lutoids [22] has also been reported.

The mature plant cell vacuole generally has a low sap pH [12,23], and a high pH gradient across the tonoplast is expected. The tonoplast proton gradient may serve to energize secondary active transport of neutral and charged solutes across the membrane [13,20]. A tonoplast proton translocating ATPase may function in maintianing the proton gradient [13] and/or in regulating intracellular pH [24] in higher plant cells.

In this study we used endogenous anthocyanin pigments of *Tulipa* petal vacuoles to facilitate real-time spectrophotometric monitoring of proton fluxes across the tonoplast of intact vacuoles. Results obtained suggest that ATPase of *Tulipa* petal tonoplast functions in proton transport.

Materials and Methods

Protoplasts and vacuoles were isolated from *Tulipa* petals (cv. Red Shine) essentially as previously described [12,25]. Modifications included the use of a 10–14 h digestion period (25°C) for protoplast isolation, brief (15 s) stirring in protoplasts in Na_2HPO_4/H_3PO_4, pH 8.0, to release vacuoles, and the use of 0.7 M mannitol/10 mM Hepes-NaOH (pH 8.0)/20 mM KCl/0.5 mM dithiothreitol (resuspending medium) to resuspend vacuoles.

Preparation of an anthocyanin extract for determining the relationship between pigment absorbance and pH was as follows. Petals were rapidly blended in cold distilled water (1:2, w/w), and the homogenate was filtered through cheesecloth and centrifuged at $12000 \times g$ for 10 min at 4°C. Absorption spectra of the supernatant solution were recorded at room temperature at various pH values. The spectra were used to select empirically those wavelengths which give an approximately linear variation in absorption with pH over the pH range close to that of isolated vacuoles (approx. pH 7.0) [12].

To monitor proton transport, 2.0 ml resuspended vacuoles (about $2 \cdot 10^6$ vacuoles) were transferred to a glass cuvette and allowed to settle to the bottom. Part of the solution (1.5 ml) was removed and replaced with fresh resuspending medium. Ammonium chloride and sodium malate were added to make the solution 0.25 mM and 0.125 mM with these substances, respectively. A sleeve with attached, curved syringe needles was lowered into the cuvette. Needle openings faced upward and were just below the solution surface. This arrangement allowed additions to be made without purturbing the fragile vacuoles at the bottom of the cuvette. Additions (10 μl) were made through microcapillary turbing attached to the needles, with microsyringe repeating dispensers. When a solution containing Evans blue dye was injected into resuspending medium with this arrangement, dye rapidly (10 s) became evenly distributed in the medium. The cuvette (with sleeve) was placed in the sample chamber of a single-beam spectrophotometer with vertical optics (similar to that described by Norris and Butler [26]. After approx. 10–15 min, equilibration with NH_4Cl was complete. The beam was turned on and, after the signal was stabilized, additions (MgATP, inhibitors, etc.) were made, and the resulting optical changes were measured as $\Delta T/T$ (transmission change over total transmission) and recorded on a Varian *X-Y* recorder through a Type 564 Tektronix oscilloscope.

The measuring system was calibrated as follows. Vacuoles (about $1.5 \cdot 10^6$) were suspended in 10 ml resuspension medium and counted [25], and an aliquot was taken for anthocyanin determination [27]. The anthocyanin content per vacuole was calculated. The suspension was centrifuged for 2 min at $80 \times g$ and the supernatant was removed. The pellet (about 0.2 ml) was gently resuspended in 10 ml of the resuspension medium minus the buffer and dithiothreitol, and vacuoles were again sedimented. The supernatant (about 1.7 ml) was removed and vacuoles were disrupted by repeated passage through a Pasteur pipette. No remaining anthocyanin-containing vesicles were observed by

means of light microscopy. The volume of the resulting suspension was determined, and the number of vacuole equivalents was determined from the anthocyanin content (corrected for anthocyanin contributed by contaminating protoplasts). Transmission changes of the suspension resulting from additions of standard HCl were recorded under conditions used to minitor proton pumping. In this way, a unit of $\Delta T/T$ at 578 nm was related to nmol H^+. Addition of gramicidin or valinomycin (10 μM final concentration) during disruption of vacuoles had little effect on the calibration. By a series of such experiments a relationship was established for nmol $H^+/(\Delta T/T)$ vs. vacuole number.

To measure potential differences across the tonoplast, a conventional glass microcapillary electrode filled with 3 M KCl and fixed on a micromanipulator [28] was inserted into intact vacuoles which had settled to the bottom of a tissue holder (similar to that described by Lin and Hanson [29] fixed on a microscope stage.

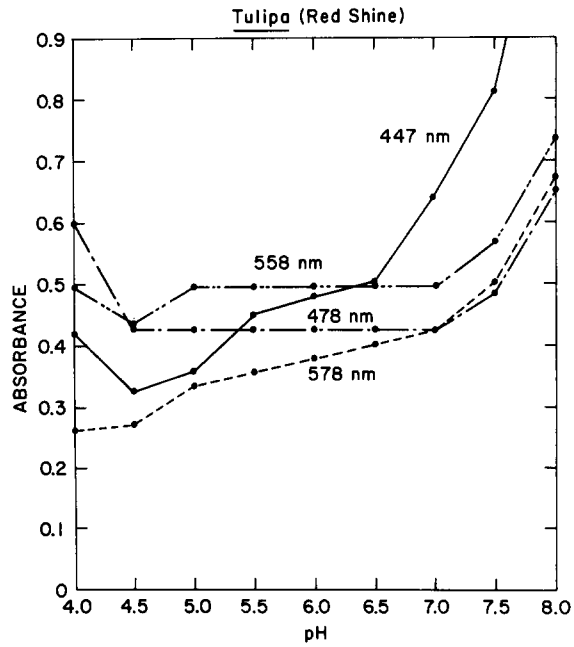

Fig. 1. The absorption of a *Tulipa* petal extract at selected wavelengths as a function of pH.

Results and Discussion

Anthocyanins are natural, endogenous plant pigments, the absorption characteristics of which are pH-dependent. The absorption spectrum in the red region (400–600 nm) of a fresh *Tulipa* petal extract varies as a function of pH both in peak position [12] and in absorbance (Fig. 1). Examination of the pH dependence at 447, 478, 558 and 578 nm revealed that at 578 and 447 nm a linear variation in absorbance occurred over the pH range 5–7 (Fig. 1). The presence of 0.25 mM NH_4Cl or 25 mM KCl (components of the pump reaction mixture) had little effect on anthocyanin absorption between pH 7.0 and pH 8.0 (data not shown). Since the internal pH of isolated, intact, *Tulipa* petal vacuoles in pH 8.0 buffer [12] is 7.0, 578 nm was chosen to monitor internal pH changes in isolated vacuoles.

It had been observed in early experiments that internal acidification on addition of MgATP was maximal after equilibration of vacuole sap pH from about 7 to 7.5 (medium pH was 8.0) by addition of 0.25 mM NH_4Cl. A higher concentration of NH_4Cl was required to collapse the gradient completely. In addition, higher responses were observed when 0.125 mM malate was present. After equilibration of vacuoles to about pH 7.5 in the presence of malate (about 12–15 min), internal acidification occurred upon addition of MgATP (Fig. 2). The time required for NH_4Cl equilibration was substantially greater than that of an ATP-induced acidification response (0.5 min). Therefore, short-term re-equilibration of an ATP-induced proton gradient due to the presence of NH_4Cl was minimal. Acidification could be induced several times, after which a relatively large pH gradient was re-established and little further response occurred with further addition of MgATP. Quantitatively, 63 nmol H^+ were accumulated by $0.2 \cdot 10^6$ vacuoles in resuspending medium containing 0.25 mM NH_4Cl and 0.125 mM malate upon addition of 600 nmol MgATP. Preliminary experiments indicate that introduced ATP was also hydrolyzed by soluble phosphatase and ATPase-bearing tonoplast fragments released from lysed vacuoles. The newly formed pH gradient was dissipated upon addition of 10 μM CCCP or FCCP in etanol, indicating that the accumulation of protons (and not other ions) was responsible for the observed acidification. Addition of ethanol alone

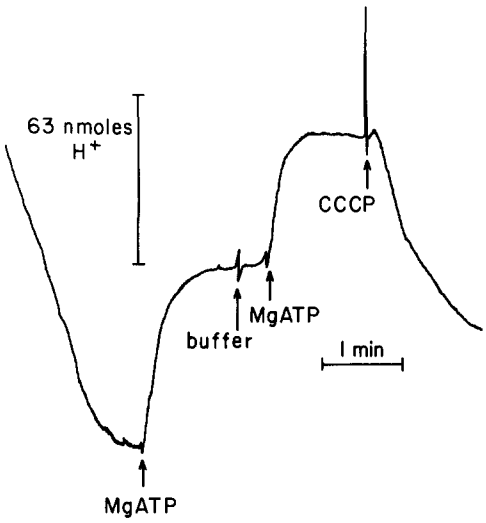

Fig. 2. MgATP stimulated acidification of intact *Tulipa* petal vacuoles. Additions of MgATP and buffer were made as described in Materials and Methods. CCCP was introduced directly after opening the sample compartment of the spectrophotometer.

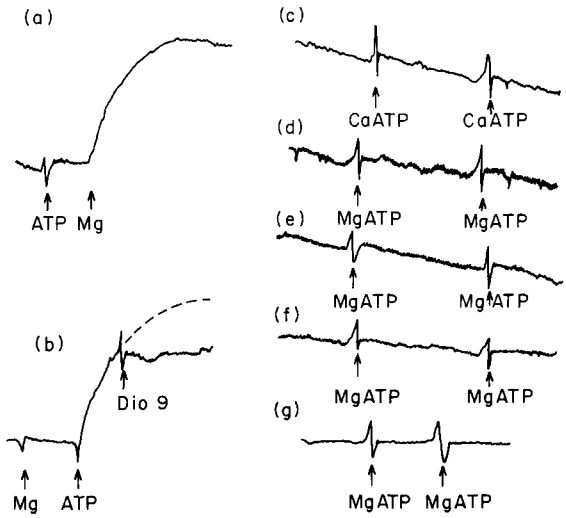

Fig. 3. Responses of intact *Tulipa* vacuoles, vacuole fractions and protoplasts in the proton pump monitoring system. (a) Addition of 600 nmol Na_2ATP and subsequent addition of equimolar Mg (as $MgSO_4$); (b) addition of 600 nmol Mg and subsequent addition of equimolar Na_2ATP – introduction of Dio 9 inhibited the response; (c) addition of 600 nmol CaATP; (d,e,f,g) addition of 600 nmol MgATP to intact *Tulipa* petal protoplasts, mechanically broken vacuoles, vacuoles after addition of 0.5% (v/v) Triton X-100, and intact vacuoles monitored at 535 nm, respectively.

had no effect and addition of uncouplers before adding MgATP inhibited the response (not shown).

Since an optical change in the measuring system used might be brought about by factors other than proton transport into vacuoles, a number of control experiments were performed. As shown in Fig. 2, additions caused only a minor perturbation. Addition of neutralized ATP alone had no effect, but subsequent addition of equimolar Mg gave rise to a typical acidification response (Fig. 3a). Similarly, addition of Mg alone had no effect, but subsequent addition of equimolar ATP resulted in proton pumping (Fig. 3b). ATPase associated with *Tulipa* tonoplast is Mg-dependent [12,30]. Addition of Dio 9 to 10 μM final concentration inhibited acidification. This energy transfer inhibitor has a similar effect on tonoplast ATPase [12]. Addition of CaATP (600 nmol) had no effect (Fig. 3c). Tonoplast ATPase of *Tulipa* vacuoles does not hydrolyze CaATP [30]. Intact protoplasts gave no response in this system (Fig. 3d), which indicated that acidification responses observed cannot be ascribed to transport across the plasma membrane of protoplasts which are residual in the vacuole preparations used. Generally, protoplasts made up about 1–3% of the structures in the preparations used. No response was observed when vacuoles were broken by repeated pipetting prior to addition of MgATP (Fig. 3e); however, the resulting suspension retained Mg-dependent ATPase activity (not shown). This result indicates that only intact vacuoles containing a high concentration of anthocyanin can produce the observed response upon addition of MgATP. Addition of 0.5% Triton X-100 to intact vacuoles caused their disruption and resulted in no response after addition of MgATP (Fig. 3f). No response of intact vacuoles to MgATP was observed at 535 nm, a wavelength at which anthocyanin absorption is unchanged in going from pH 7.5 to pH 6.5 (Fig. 3g).

These observations strongly suggest that the optical changes observed with MgATP were due to internal pH changes in vacuoles. Qualitatively similar internal acidification responses were observed at appropriate wavelengths with *Streptocarpus* and *Hippeastrum* petal vacuoles (data not shown).

Maximal internal acidification was observed in the presence of malate malonate or maleate. To

determine whether internal acidification was due to influx of organic acid, [^{14}C]malonate and [^{3}H]inulin (the latter being a nonpenetrant macromolecule useful as a monitor of the external fluid) were supplied to vacuoles under conditions which result in internal acidification. Neither ^{14}C nor ^{3}H was enriched in intact vacuoles or tonoplast recovered after the reaction. The $^{14}C/^{3}H$ ratio of the trace levels of isotopes recovered with vacuoles after addition of MgATP was unchanged from that of the reaction mixture before addition of MgATP. This result suggests that organic acid does not serve as penetrant anion during internal acidification and supports the conclusion that internal acidification is a result of proton transport.

Nucleotide specificity studies showed that, listed in order of decreasing rates, the magnitudes of the acidification response produced by MgATP, MgCTP, MgGTP, MgUTP, MgADP, MgAMP and MgPNPP were 1.0, 0.2, 0.42, 0.1, 0.1, 0 and 0, respectively. *Tulipa* tonoplast MgATPase has a similar nucleotide specificity [30].

Attempts were made in two preliminary experiments to determine the stoichiometry of MgATP-induced proton transport in vacuoles. After the completion of a typical pumping response, the amount of unhydrolyzed ATP remaining in the reaction mixture was determined. In a parallel experiment the consumption of ATP by the solution above settled vacuoles was determined in order to establish the level of ATP hydrolysis by tonoplast fragment ATPase and soluble phosphatase. When these components were considered, a stoichiometry of H^+/ATP of about 2 was calculated. These results are preliminary but are in general agreement with ratios observed in well studied proton translocating systems [4,5].

Results described here for proton transport in intact petal vacuoles are similar to those reported by Marin et al. [20] for lutoids of *Hevea* in that proton transport in both systems is inward, Mg-dependent, inhibited by uncouplers and relatively insensitive to MgADP. Marin et al. [20] used the weak base methylamine to monitor lutoid pH.

Potential differences across the tonoplast were directly measured here by impaling intact vacuoles with glass microelectrodes. Potential differences of 10–20 mV (inside positive) were recorded for vacuoles suspended in 0.7 M mannitol 10 mM Hepes adjusted to pH 8.0 with KOH 0.5 mM dithiothreitol. Under these conditions MgATP increased the potential difference by 2–5 mV. Positive tonoplast potentials have been reported by Rona [31] and Reinhold et al. [32]. Marin et al. [20] used the lipophylic cation tetraphenylphosphonium to monitor membrane potential in lutoids of *Hevea* and observed a shift of 60 mV (to a more positive interior) in response to addition of MgATP.

There are four major advantages in using endogenous, pH-sensitive anthocyanin pigments to monitor proton fluxes in isolated vacuoles.

(1) The organelles are intact and are thought to contain all the sap components present in protoplasts [12,13,27] and probably have a sap composition similar to that of vacuoles in situ.

(2) Isolated vacuoles of petals have been shown to contain most if not all of the anthocyanin in protoplasts [27]. Only membrane-bound enclaves which have a high concentration of anthocyanin pigment can show the expected absorbance changes. Therefore, vesicles of plasma membrane, golgi, endoplasmic reticulum, etc., even if present as contaminants in vacuole preparations, cannot interfere.

(3) Protoplasts do not respond to the addition of MgATP in the system, presumably because they are impermeable to ATP. Therefore, there is no interference from protoplasts which are residual in vacuole preparations.

(4) Proton transport can be monitored in real time.

Concluding remarks

A system is described for monitoring proton transport in intact vacuoles containing only endogenous constituents by monitoring pH-dependent absorbance changes of anthocyanin pigments. Results obtained with this system support the conclusion that tonoplast of mature petal cell vacuoles contains a proton-transporting system which is energized by MgATP. Both proton transport in intact vacuoles as measured here and tonoplast ATPase measured in vitro are Mg-dependent, are inhibited by Dio 9, do not respond to CaATP, and have similar nucleotide specificity. A tonoplast proton pump may maintain the vacuole/cytosol pH gradient which exists in most plant cells.

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