



Proton and anion transport across the tonoplast vesicles in bromeliad species

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Complete List of Authors:	Pereira, Paula; University of Sao Paulo, Botany Smith, Andrew; University of Oxford, Dept of Plant Sciences Purgatto, Eduardo; University of Sao Paulo, Department of Food and Experimental Nutrition Mercier, Helenice; Universidade de Sao Paulo Instituto de Biociencias, Departamento de Botânica
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1 **Helenice Mercier**

2 **e-mail:** hmercier@usp.br

3 **Phone number:** (55) (11) 30918066

4 **FAX number:** (55) (11) 30917547

5 **Address:** Department of Botany, Institute of Biosciences, University of São Paulo,
6 **CEP 05508-090, São Paulo, SP, Brazil**

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1 Title: **Proton and anion transport across the tonoplast vesicles in bromeliad species**

2 Running head: **Proton and anion transport in bromeliads**

3 **Paula Natália Pereira^a, James Andrew Charles Smith^b, Eduardo Purgatto^c and**
4 **Helenice Mercier^a**

5 ^a Department of Botany, Institute of Biosciences, University of São Paulo, CEP 05508-
6 090, São Paulo, SP, Brazil.

7 ^b Department of Plant Sciences, University of Oxford, Oxford, OX1 3RB, UK.

8 ^c Department of Food and Experimental Nutrition, Faculty of Pharmaceutical Sciences,
9 University of São Paulo, CEP 05422-970 São Paulo, SP, Brazil.

10

1 **Abstract**

2 Crassulacean acid metabolism (CAM) is one of the key innovations in the Neotropical
3 family Bromeliaceae that has enabled many of its species to occupy seasonally water-
4 limited terrestrial environments or microclimatically arid epiphytic niches. However,
5 the relationship between CAM activity and the transport processes responsible for
6 vacuolar organic-acid accumulation at night has not been systematically explored in this
7 family. In the present investigation, ATP- and PPi-dependent proton transport rates
8 were studied in tonoplast membrane vesicles isolated from leaves of six CAM and one
9 C₃ species of bromeliads. A consistent feature of these species was the high activity of
10 the tonoplast ATP-driven H⁺ pump which, averaged across the seven species tested,
11 showed a higher specific activity than the tonoplast PPi-driven H⁺ pump. For all CAM
12 species, the rate of ATP-dependent proton transport into the tonoplast vesicles was
13 strongly influenced by the nature of the balancing organic-acid anion, which displayed
14 the following order of effectiveness: fumarate > malate > citrate. Measurements of leaf
15 organic-acid content in six CAM bromeliads at dusk and dawn showed that nocturnal
16 accumulation of malate exceeded citrate by a factor of about 2.4-20.0 fold in five from
17 six bromeliad species used in this study, demonstrating a close correlation between the
18 CAM rhythm and the intrinsic properties of the vacuolar membrane across which these
19 organic acids are transported.

20
21 **Additional keywords:** Bromeliaceae, CAM plants, malate, proton pump, vacuoles.

22
23 **Introduction**

24 The Neotropical family Bromeliaceae represents a highly diverse group of
25 vascular plants containing well over 3000 species. Members of this family exhibit some
26 distinctive innovations associated with the wide variety of habitats they occupy
27 (Benzing 1980, 2000; Smith *et al.* 1986; Smith 1989; Givnish *et al.* 2011; Crayn *et al.*
28 2015). One key innovation in bromeliads is the absorptive epidermal trichomes, which
29 are responsible for uptake of water and nutrients through the leaf surface in epiphytic
30 species (Mez 1904; Smith and Till 1998). Another is the presence of a ‘tank’ structure
31 (phytotelm) formed by the overlapping basal portions of the rosulate leaves. This
32 structure is important because it collects the detritus and water that is ultimately

absorbed by the epidermal trichomes, which are present at higher density towards the basal compared with apical portion of the leaves, and which in the more extreme 'atmospheric' epiphytic forms in this family cover almost the entire shoot surface. The absorptive epidermal trichome and water-impounding tank are very likely two of the key adaptations that aided evolution of the epiphytic life-forms in Bromeliaceae (Medina 1974; Benzing 1980, 2000; Smith 1989; Givnish *et al.* 1997).

Crassulacean acid metabolism (CAM) is another physiological innovation found in many species of Bromeliaceae (Coutinho 1963; Medina 1974; Crayn *et al.* 2004). This mode of photosynthesis is characterized by nocturnal CO₂ fixation via the enzyme phosphoenolpyruvate carboxylase (PEPC), which represents a highly water-conserving photosynthetic pathway since it allows stomata to remain closed for much of the daytime (Kluge and Ting 1978; Winter and Smith 1996; Silvera and Lasso 2016). In a recent survey of Bromeliaceae encompassing nearly two-thirds of the family, Crayn *et al.* (2015) found that 43% of the species sampled showed carbon-isotope ratios indicative of obligate CAM photosynthesis. Among the eight constituent subfamilies there are five that contain CAM species, although in very different proportions. For example, from 792 sampled species in subfamily Tillandsioideae, only 28% showed $\delta^{13}\text{C}$ values indicative of obligate CAM, all of which were in the genus *Tillandsia*, with the remaining genera, including *Vriesea*, *Werauhia*, *Catopsis*, *Guzmania*, *Alcantarea* and *Racinaea*, showing C₃ photosynthesis. In contrast, in subfamily Bromelioideae, the majority of genera (including 90% of the 499 sampled species) exhibited the CAM pathway, with only a few genera, namely *Fernseea*, *Fascicularia*, *Greigia*, *Lapanthus* and *Ochagavia*, showing C₃ photosynthesis (Crayn *et al.* 2015).

Over the years, numerous physiological and biochemical studies have been conducted on the terrestrial CAM bromeliad *Ananas comosus* (L.) Merr., the pineapple, which is one of the best-known species in the family because of its economic importance (Neales *et al.* 1968; Martin 1994; Ming *et al.* 2015). Methods for the isolation of vacuolar membrane have been reported for this species, and the H⁺-ATPase and H⁺-inorganic pyrophosphatase (PPiase) characteristic of the tonoplast have been partially characterized (Chen and Nose 2000; McRae *et al.* 2002). The tonoplast of pineapple also possesses a relatively low-affinity sucrose transporter, which is presumed to mediate transport of sucrose from the cytosol into the vacuole during the daytime (McRae *et al.* 2002; Ming *et al.* 2015). However, little has been studied concerning the

1 process of organic acid transport across the vacuolar membrane in this species or other
2 members from the family Bromeliaceae. White and Smith (1989) found in *Kalanchoë*
3 *daigremontiana* Hamet et Perrier de la Bâthie, a constitutive CAM plant, that the
4 highest rates of ATP- and PPi-dependent H^+ transport across the vacuolar membrane
5 could be observed in the presence of malate and certain other four-carbon dicarboxylate
6 anions. This was subsequently shown to be attributable to a distinctive inward-
7 rectifying anion channel (Hafke *et al.* 2003), which seems to be an inherent feature of
8 the vacuolar membranes of CAM plants. In studies with the inducible CAM plant
9 *Mesembryanthemum crystallinum* L., it has been shown that rates of ATP-dependent H^+
10 transport at the vacuolar membrane are higher in plants in the CAM mode compared
11 with the C_3 mode, and that CAM induction by salt stress appeared to be associated with
12 increased permeability of the tonoplast membrane to malate (Struve and Lüttge 1987;
13 Barkla *et al.* 1995; Lüttge *et al.* 2000).

14 Although studies have been done on proton transport and the permeability of
15 vacuolar membranes for malate and fumarate in CAM constitutive plants, no studies
16 have been conducted to check this transport within different CAM species from the
17 same family or even from the same subfamily. In addition, no studies have been
18 performed to evaluate the difference in the vacuolar transport between CAM plants kept
19 in the same growth conditions. Based on this scenario, this study aims to evaluate the
20 ATP-driven H^+ pump at the tonoplast compared with the PPi-driven H^+ pump, and
21 measure the organic acids, malate and citrate, accumulated during the night in six
22 bromeliad species, all of which perform CAM, from the subfamilies Bromelioideae or
23 Tillandsioideae (Fig. 1). All results were obtained through a combination of
24 biochemical approaches. This study also examines the possible correlation between
25 nocturnal malate and citrate accumulation, their transport across the tonoplast, and the
26 degree of CAM expression among bromeliad species kept in the same environmental
27 conditions. Finally, this study evaluates the relative activities of the tonoplast ATPase
28 and PPiase for each bromeliad species with respect to proton pumping into isolated
29 membrane vesicles, as well as the order of effectiveness of fumarate, malate and citrate
30 as balancing permeant anions in sustaining the activity of these electrogenic pumps.

31 **Material and methods**

32 *Plant material and growth conditions*

1 Adult plants of *Aechmea nudicaulis* (L.) Griseb., *Ananas comosus* (L.) Merr. var.
2 *ananassoides*, *Billbergia pyramidalis* (Sims) Lindl., *Nidularium billbergioides* (Schult.
3 & Schult.f.) L.B.Sm., *Tillandsia pohliana* Mez, *Tillandsia usneoides* (L.) L. and *Vriesea*
4 *sucrei* L.B.Sm & Read (used for comparison as a species that performs C₃
5 photosynthesis) (Fig. 1) were collected in the Institute of Botany of São Paulo, and were
6 then transferred to a controlled environment growth chamber, under a photosynthetic
7 flux density (PFD) of about 200 $\mu\text{mol m}^{-2} \text{s}^{-1}$ of photosynthetic active radiation
8 (measured at the surfaces of the uppermost leaves), a 12 h photoperiod, a day/night air
9 temperature of 25/20 °C, and a day/night relative humidity of 60/70%. Plants were
10 cultivated in pots containing fine sand, with one plant per pot. Over 10 days of
11 acclimation, all plants were watered with distilled water on a daily basis. After this
12 period, about 8 to 12 fully developed leaves, or stem internodes in the case of *T.*
13 *usneoides*, were collected for the biochemical assays.

14 *Tonoplast isolation*

15 Tissue was harvested from plants in the controlled environment chambers 1.0 to 1.5 h
16 after commencement of the light period. Tonoplast fractions from the mesophyll tissue
17 were isolated according to the method of White & Smith (1989) and McRae *et al.*
18 (2002) with minor modifications. The leaf tips and margins were removed from excised
19 leaves using a razor blade, and sections of leaf lamina or stem totaling approximately 80
20 g fresh mass were suspended in 250 mL of ice-cold extraction buffer containing the
21 following: 450 mM mannitol, 3.0 mM MgSO₄, 2.0 mM ethylenediaminetetraacetic acid
22 disodium salt (EDTA), 10 mM DL-dithiothreitol (DTT), 1.0% (w/v)
23 polyvinylpyrrolidone (PVP-40), 0.5% (w/v) bovine serum albumin, 100 mM
24 tris(hydroxymethyl)aminomethane (Trizma[®] base, adjusted to pH 8.0 with HCl), 1 mM
25 phenylmethanesulphonyl fluoride, 1.1 M glycerol, 0.5 mM 3,5-di-*tert*-4-
26 butylhydroxytoluene, 25.19 mM potassium disulfite and 1.0 mM benzamidine
27 hydrochloride. After precooling, the tissue was homogenized in a commercial blender
28 and the homogenate filtered through two layers of cheesecloth and then centrifuged at
29 18000 $\times g$ for 20 min. The resulting supernatant was centrifuged at 80000 $\times g$ for 60
30 min. The resulting pellet was layered over a 25% (w/v) sucrose cushion containing 1.1
31 M glycerol, 1.0 mM disodium EDTA, 10 mM Tricine (*N*-
32 [tris(hydroxymethyl)methyl]glycine), adjusted to pH 8.0 with BTP (1,3-

bis[tris(hydroxymethyl)methylamino]propane), and 2.0 mM DTT. The gradients were centrifuged at $100\,000 \times g$ for 70 min, after which tonoplast vesicles were removed from the interface using a Pasteur pipette. Vesicles were then pelleted at $100\,000 \times g$ for 50 min and finally resuspended in the same buffer as the first pellet. All steps were performed at 4°C. Preparations were stored at -80°C until required.

Measurement of vesicle acidification

Rates of intravesicular acidification on energization of the tonoplast H⁺-ATPase or H⁺-PPiase were determined according to the method described by White & Smith (1989) with minor modification. Initial rates of H⁺ transport at 25°C were determined from the initial rates of fluorescence quenching upon the addition of 3.0 mM Tris-ATP or 500 µM Na₄PPi to the reaction medium. For assays of ATP-dependent H⁺ transport, the reaction medium contained approx. 2–9 µg protein, 3.0 µM quinacrine (6-chloro-9-[[4-(diethylamino)-1-methylbutyl]amino]-2-methoxyacridine dihydrochloride), 6.0 mM MgSO₄, 0.3 mM disodium EDTA, 150 mM mannitol and 25 mM BTP buffered to pH 8.0 with Mes (2-(N-morpholino)ethansulphonic acid). For measurements of PPi-dependent H⁺ transport, the reaction medium was identical except that the MgSO₄ concentration was increased to 7.5 mM and the medium also contained 100 mM K-Mes. Permeant anions to be tested were present at 50 mM (supplied as fumaric acid, malic acid, or citric acid, and buffered to pH 8.0 with BTP). Inhibitors tested (final concentrations) were 50 mM potassium nitrate (inhibitor of vacuolar H⁺-ATPase), 100 µM sodium orthovanadate (inhibitor of plasma membrane H⁺-ATPase), 100 µM sodium azide (inhibitor of mitochondrial ATP synthase), and 2.0 mM ammonium sulphate (uncoupler of transmembrane pH gradients). Fluorescence quenching was measured using a model LS-55 luminescence spectrometer (Llantrisant, UK) with excitation at 422 nm and emission at 495 nm, both with a slit width of 5 nm.

Protein determination

Protein concentration was measured according to Bradford (1976), using bovine serum albumin as the standard.

Organic acid quantification

Malate and citrate in tissue extracts were determined by HPLC in a chromatographic system (Hewlett-Packard®, series 110, Waldbronn, Germany) equipped with a Supelcogel C-610H (30 cm × 7.8 mm) column (at 30°C) and a diode-array detector (210 nm, for acid analysis) according to the method described by Amorós *et al.* (2003) and Pereira *et al.* (2013). All measurements were made in triplicate, and the results are expressed in dry weight basis (DW).

Statistical analysis

All data are presented as mean values ± standard deviation (s.d.). Significant differences among the bromeliad species or treatments were determined using the Tukey–Kramer test at $P < 0.05$. Differences between treatments with two inhibitors in the same species were evaluated using Student's *t*-test at $P < 0.05$.

Results

Proton transport rates in the presence of inhibitors of vacuolar or non-vacuolar ATPases

Initially, proton transport rates were tested using quinacrine fluorescence-quenching to assay rates of acidification of isolated tonoplast vesicles in the presence of specific inhibitors of non-vacuolar ATPases (NaN_3 plus Na_3VO_4) or in the presence of an inhibitor of the vacuolar H^+ -ATPase (KNO_3), in order to determine the relative contribution of different membranes to the ATP-dependent H^+ transport rates in preparations from each bromeliad species. As shown in Table 1, KNO_3 caused a significantly greater inhibition of ATP-dependent proton transport than did NaN_3 plus Na_3VO_4 for tonoplast preparations from all seven bromeliad species tested. In *Ananas comosus*, *Tillandsia usneoides* and *Vriesea sucrei*, the degree of inhibition by KNO_3 showed that the majority (74–88%) of ATP-dependent proton pumping was driven by the vacuolar H^+ -ATPase than by $\text{NaN}_3 + \text{Na}_3\text{VO}_4$. In tonoplast preparations from *Aechmea nudicaulis*, *Billbergia pyramidalis*, *Nidularium billbergioides* and *T. pohliana*, the inhibition of ATP-dependent proton pumping by KNO_3 was somewhat less (42–47%), but was still at least twice the degree of inhibition caused by $\text{NaN}_3 + \text{Na}_3\text{VO}_4$, suggesting that in all species the predominant fraction of ATP-driven proton transport was attributable to the activity of the vacuolar H^+ -ATPase.

ATP-dependent proton transport rates

Experiments were next conducted to investigate the relative activities of ATP- and PPi-dependent proton transport in the tonoplast vesicle fractions prepared from the seven species of bromeliads.

For all species tested, the initial rates of proton transport into the membrane vesicles were monitored by quinacrine-fluorescence quenching following addition of ATP or PPi in the presence of three different anions (fumarate²⁻, malate²⁻, or citrate³⁻) (Supplementary Figures 1 and 2). Recovery of quinacrine fluorescence to the level observed immediately prior to the addition of ATP or PPi occurred after adding 2 mM NH₄⁺ as an uncoupler of pH gradients (after 400–800 s, depending on the species), demonstrating that the fluorescence quenching was entirely attributable to the acidification of the vesicle interior (Supplementary Figure 1A).

In all of the experiments, fumarate supported the highest rates of vesicle acidification in tonoplast vesicles from all seven CAM bromeliad species (Table 2; Supplementary Figures 1 and 2). *B. pyramidalis* showed the highest rate of ATP-dependent proton transport, in the presence of fumarate as a charge-balancing anion (achieving a rate of approximately 3000% relative fluorescence quench min⁻¹ mg protein⁻¹, after subtracting the control value in the absence of any added anion), followed by *A. comosus*, *A. nudicaulis*, *N. billbergioides*, *T. pohliana* and *T. usneoides* (Table 2). For *B. pyramidalis*, the rate of ATP-dependent proton transport in the presence of fumarate was about 13 times higher than that of *T. usneoides* (Table 2). Malate and citrate as charge-balancing anions supported considerably lower rates of ATP-dependent proton transport than fumarate, but the relative rates of proton transport showed a similar trend across the seven species (Table 2). Thus, the order of effectiveness of the three organic-acid anions in supporting ATP-dependent proton transport was fumarate > malate > citrate in the six CAM species.

PPi-dependent proton transport rates

In contrast to the ATP-dependent proton transport, rates of PPi-dependent H⁺ transport into the isolated tonoplast vesicles were considerably lower and did not show such clear trends with respect to species or anion-dependence. Once again, the highest rate of PPi-dependent proton transport in the presence of fumarate was observed for *B. pyramidalis*,

1 and the lowest for *T. usneoides* (Table 3), but this maximum rate for *B. pyramidalis* was
2 4.2-fold lower than the rate of ATP-dependent proton transport observed in the same
3 species (cf. Table 2). In general, the rates of PPi-dependent vesicle acidification were
4 not significantly higher in the presence of fumarate than they were for malate, except in
5 the case of *T. pohliana* (Table 3). *T. usneoides*, as well as showing the lowest rates of
6 PPi-dependent vesicle acidification, was anomalous in showing relatively high rates of
7 proton transport in the presence of citrate, or even without added organic-acid anion
8 (Table 3). Thus, the order of effectiveness of the anions in supporting PPi-dependent
9 proton transport was fumarate \approx malate > citrate for *A. nudicaulis* and *N. billbergioides*,
10 fumarate > malate > citrate for *A. comosus* and *B. pyramidalis*, fumarate > citrate >
11 malate for *T. pohliana*, and citrate > fumarate \approx malate for *T. usneoides*.

13 *Nocturnal malate and citrate accumulation*

14 To relate the transport properties of the tonoplast membrane to patterns of organic-acid
15 metabolism occurring during the CAM cycle, nocturnal accumulation of malate and
16 citrate was measured in the leaves (and internodes of *T. usneoides*) of the six CAM
17 bromeliad species included in this study (Fig. 2). All CAM species showed a greater
18 nocturnal accumulation of malate compared with citrate when averaged across the six
19 species. *B. pyramidalis* showed the highest nocturnal malate accumulation, followed by
20 *A. nudicaulis*, *A. comosus* and *N. billbergioides* (Fig 2A). *A. comosus* and *N.*
21 *billbergioides*, followed by *B. pyramidalis*, were the species that showed the greatest
22 citrate accumulation during the dark period (Fig. 2B). As expected, *V. sucrei*, a C₃
23 species, did not accumulate any malate or citrate at night, but a small amount of diurnal
24 malate accumulation was observed, as is often found in C₃ plants (Fig. 2).

26 **Discussion**

27 Numerous studies have been undertaken to evaluate CAM expression and its ecological
28 significance in Bromeliaceae, which represents a particularly diverse family of
29 angiosperms. For example, Crayn *et al.* (2015) used stable carbon-isotope ratios to
30 investigate the photosynthetic pathways in 1893 bromeliad species covering nearly two-
31 thirds of the family. At a biochemical level, PEPC activity, combined with other
32 methods, was used to monitor CAM induction in the epiphytic bromeliad *Guzmania*

1 *monostachia* (L.) Rusby ex Mez (Pereira *et al.* 2013). At the subcellular level, although
2 there have been many studies of ATP- and PPi-dependent proton (H^+) transport in
3 tonoplast vesicles of CAM species, only a few have investigated the intrinsic
4 relationship between CAM photosynthesis and H^+ transport rates at the vacuolar
5 membrane (White and Smith 1989, 1992; Barkla *et al.* 1995; McRae *et al.* 2002).
6 Moreover, only one study has documented proton and sucrose transport rates across the
7 tonoplast in a bromeliad species, *Ananas comosus* (McRae *et al.* 2002). Thus, in the
8 present study we compared ATP- and PPi-dependent H^+ transport across the vacuolar
9 membrane, and their dependence on organic-acid anions capable of providing charge
10 balance, in tonoplast vesicles isolated from six species of CAM bromeliads (*Aechmea*
11 *nudicaulis*, *Ananas comosus*, *Billbergia pyramidalis*, *Nidularium billbergioides*,
12 *Tillandsia pohliana* and *T. usneoides*) and one C_3 species (*Vriesea sucrei*) used as a
13 control.

14 The ATP-dependent proton transport rates assayed in the presence of specific
15 inhibitors of non-vacuolar ATPases (NaN_3 plus Na_3VO_4) or of KNO_3 as an inhibitor of
16 the vacuolar H^+ -ATPase showed that the majority of the inhibitor-sensitive ATP-
17 dependent proton pumping observed in these fractions was attributable to the vacuolar
18 membrane (Table 1). The contribution from other membranes to ATP-dependent proton
19 pumping in this fraction was evidently rather minor, although this varied in extent
20 across the seven bromeliad species used in this study. McRae *et al.* (2000) also
21 observed in experiments with a crude microsomal membrane fraction isolated from
22 leaves of *Ananas comosus* that more than 90% of the observed ATP-dependent proton
23 transport activity could be attributed to the vacuolar H^+ -ATPase on the basis of its
24 inhibitor sensitivity. Even if these membrane preparations contain variable amounts of
25 non-tonoplast membrane, it appears that the propensity of the tonoplast to form well-
26 sealed, transport-competent vesicles (White and Smith 1989; McRae *et al.* 2002) results
27 in the major part of the ATP-dependent proton transport observed in these preparations
28 reflecting the activity of the vacuolar H^+ -ATPase.

29 Similar to the characteristics observed for the tonoplast of the CAM plant
30 *Kalanchoë daigremontiana* (White and Smith 1989), the rates of ATP-dependent proton
31 transport recorded here for the bromeliad tonoplast preparations in the presence of
32 different organic-acid anions as charge-balancing anions indicated a greater
33 permeability of the tonoplast for fumarate relative to malate and citrate. In contrast to *K.*

1 *daigremontiana*, however, in which rates of ATP- and PPi-dependent H⁺ transport
2 across the tonoplast are reasonably similar, in all of the bromeliad species studied the
3 ATP-dependent rates of proton transport were considerably higher than the PPi-
4 dependent rates for the six CAM bromeliad species (Tables 2 and 3). A similar
5 predominance of the tonoplast ATPase was observed in assays of the hydrolytic
6 activities of the vacuolar H⁺-ATPase and H⁺-PPiase in membrane preparations from
7 *Ananas comosus* (Chen and Nose 2000; McRae *et al.* 2002). A possible explanation for
8 this bias in favour of the tonoplast H⁺-ATPase over the H⁺-PPiase in bromeliads might
9 be connected with the high activity of pyrophosphate-dependent 6-phosphofructokinase
10 (PPi-PFK) in bromeliads, as first described in *Ananas comosus* (Carnal and Black
11 1983). The standard ATP-dependent phosphofructokinase (PFK) is responsible for
12 catalyzing the conversion of fructose 6-phosphate to fructose 1,6-biphosphate in
13 glycolysis (Pollack and Williams 1986), but it appears that in plants there are two
14 alternative enzymes for this step in glycolysis (Carnal and Black 1983; Mertens 1991;
15 Alves *et al.* 2001). Carnal and Black (1983) reported that angiosperms with PPi-PFK
16 activities 4 to 70 times higher than ATP-PFK tend to be succulent and exhibit CAM
17 photosynthesis. These same authors showed in *A. comosus* that PPi-PFK activity, but
18 not ATP-PFK activity, would be sufficient to support the rate of glycolytic carbohydrate
19 processing required for acid accumulation during the night in this CAM bromeliad
20 species (Carnal and Black 1989). Therefore, it is possible that a relatively low activity
21 of the tonoplast H⁺-PPiase in bromeliads helps to avoid competition for substrate with
22 PPi-PFK, which is required to catalyse high rates of glycolytic breakdown of storage
23 carbohydrate at night (Carnal and Black 1989; Holtum *et al.* 2005).

24 Previous studies conducted with tonoplast vesicles from *K. daigremontiana* did
25 not consider the rates of ATP- and PPi-dependent H⁺ transport observed in control
26 solutions in the absence of added anions (White and Smith 1989; White *et al.* 1990;
27 Betty and Smith 1993; Lüttge *et al.* 2000; Lüttge 2000). The rates of proton transport
28 in the absence of supplementary anions were also not characterized in
29 *Mesembryanthemum crystallinum* (Barkla *et al.* 1995) or in *A. comosus* (McRae *et al.*
30 2002). In contrast, in the present study it was observed that significant rates of ATP-
31 and PPi-dependent proton transport occurred into tonoplast vesicles of all the bromeliad
32 species in the absence of fumarate, malate and citrate anions. Also, in the majority of
33 the species, the rate of ATP- and PPi-dependent H⁺ transport in the control (no added

anion) was higher than in the presence of citrate (this anion was also not tested in most of the studies previously described). This result implies that the tonoplast membrane of bromeliads may exhibit a relatively high background conductance to some other permeant ion, although the ions involved in providing charge balance under these conditions could not be positively identified. Nevertheless, the considerably higher rates of ATP-dependent H^+ transport, in particular, that are observed in the presence of fumarate and malate implies that these anions represent the most physiologically important permeant ions involved in transport across the tonoplast in bromeliads.

Besides proton transport into the vacuole, many studies have quantified the accumulation of organic acids, mainly malate and citrate, during the dark period in CAM plants as a measure of the degree of CAM activity (Maxwell *et al.* 1994; Pereira *et al.* 2013). Based on both ATP- and PPi-dependent vacuolar proton transport and the magnitude of nocturnal malate and citrate accumulation, we demonstrated the highest degree of CAM activity in *B. pyramidalis*, with the lowest recorded in *T. usneoides*. On the other hand, as expected, *V. sucrei*, used as a C_3 species as a control in these experiments, revealed a diurnal malate accumulation as well as low rates of proton transport into the vacuole, a characteristic response of C_3 species. For all six CAM species, Δ malate values were considerably higher than Δ citrate values (Fig. 2): the ratio averaged between Δ malate and Δ citrate was from 2.4 to 20.0-fold depending on five of the CAM bromeliad species used in this study. For example, *A. nudicaulis* presented an average between Δ malate and Δ citrate of 20.0-fold, while *A. comosus* showed an average between Δ malate and Δ citrate of 2.4-fold. In addition, these data reveal a correlation between the accumulation of malate and citrate during the night and the rates of ATP- and PPi-dependent proton transport observed in the presence of malate or citrate anions. The quinacrine fluorescence-quenching experiments performed for all CAM species in this study showed that the permeability of the tonoplast membrane for malate appears to be consistently higher than its permeability for citrate. This result correlates well with the higher nocturnal malate accumulation in the vacuole compared with a citrate accumulation.

In summary, this study has compared ATP- and PPi-dependent proton transport in tonoplast vesicles of one C_3 and six CAM bromeliad species from two subfamilies, Bromelioideae and Tillandsioideae. All the bromeliad species showed much higher rates of ATP-dependent than PPi-dependent rates of proton transport into the tonoplast

vesicles. Species from the subfamily Bromelioideae exhibited a higher accumulation of organic acids during the night and also a higher ATP-dependent proton transport rate compared with members of the subfamily Tillandsioideae. In addition, this study demonstrated the order of effectiveness of different organic-acid anions in providing charge balance for ATP-dependent H^+ transport was fumarate > malate > citrate in the six CAM bromeliad species, whereas rates of PPi-dependent H^+ transport were low irrespective of the balancing anion present. Finally, the average rate of nocturnal malate accumulation in the six CAM bromeliad species studied was five times greater than the citrate accumulation, which closely reflected the relative permeability of the tonoplast to these anions as shown by the ATP-dependent rates of H^+ transport across the vacuolar membrane.

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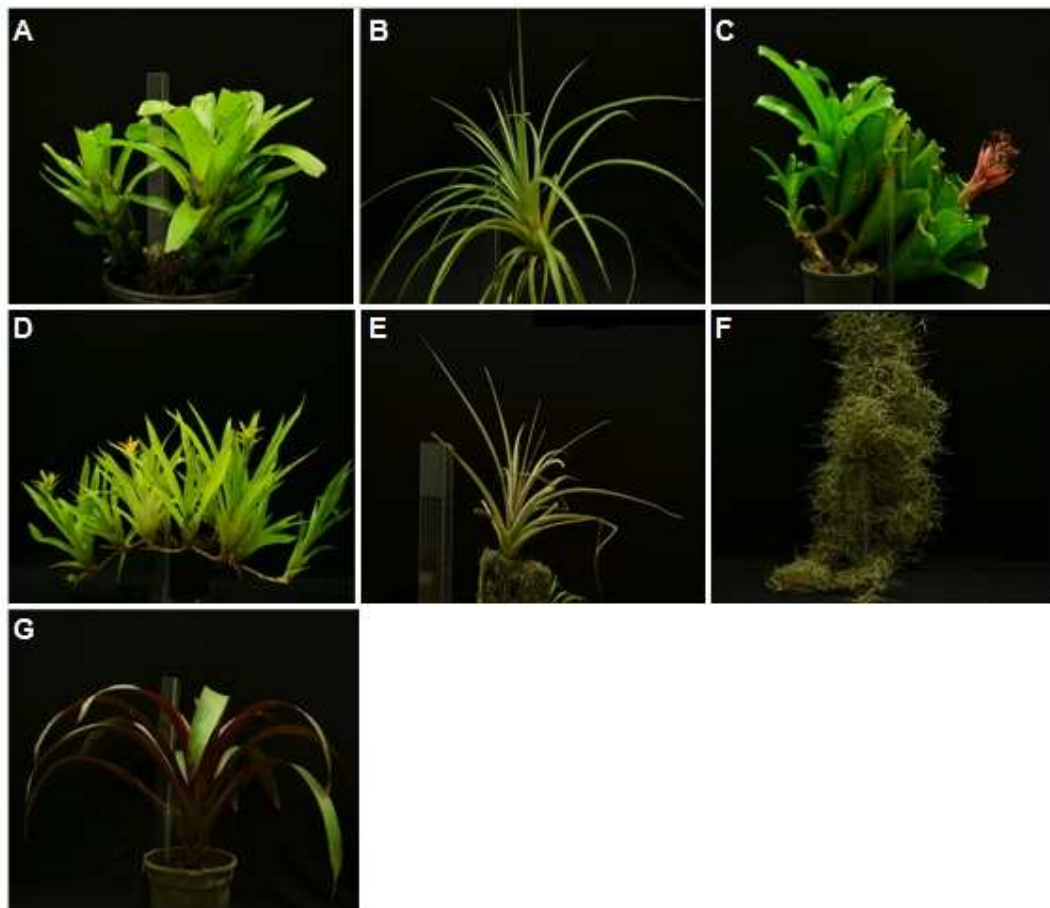


Fig. 1 The seven bromeliad species used in these experiments: (A) *Aechmea nudicaulis*; (B) *Ananas comosus*; (C) *Billbergia pyramidalis*; (D) *Nidularium billbergioides*; (E) *Tillandsia pohliana*; (F) *Tillandsia usneoides*; (G) *Vriesea sucrei*.

1 **Table 1. Initial rates of ATP-dependent proton transport into isolated tonoplast**
2 **vesicles from leaves of seven species bromeliads.**

3 Proton transport was measured as the initial rate of quinacrine-fluorescence quenching
4 in the presence of 50 mM fumarate following addition of 3 mM ATP to the suspension
5 of tonoplast vesicles as described in Materials and methods. Rates of proton transport
6 were quantified as % relative fluorescence quenching $\text{min}^{-1} \text{mg protein}^{-1}$.
7 Measurements were made in the presence of inhibitors of vacuolar H^{+} -ATPase (50 mM
8 KNO_3) or non-vacuolar H^{+} -ATPases (0.1 mM NaN_3 plus 0.1 mM Na_3VO_4) in
9 comparison with the control (no inhibitors). Results are expressed as means (\pm s.d.) for
10 three independent preparations. Values in parentheses give the percentage inhibition
11 relative to the control for each species; asterisks indicate significant differences between
12 each inhibitor (KNO_3 or $\text{NaN}_3 + \text{NaVO}_3$) and the control within a species by Student's
13 *t*-test at 5% significance level.

Specific activity (% quench $\text{min}^{-1} \text{mg protein}^{-1}$) (inhibition relative to control)			
Species	Control	+ KNO_3	$\text{NaN}_3 + \text{Na}_4\text{VO}_3$
<i>Aechmea nudicaulis</i>	1074 \pm 2	604 \pm 8 (44 %) *	829 \pm 9 (23 %) *
<i>Ananas comosus</i>	627 \pm 6	140 \pm 7 (78 %) *	463 \pm 9 (26 %) *
<i>Billbergia pyramidalis</i>	1200 \pm 1	675 \pm 4 (44%) *	1146 \pm 3 (5%) *
<i>Nidularium billbergioides</i>	546 \pm 5	291 \pm 7 (47%) *	431 \pm 9 (21%) *
<i>Tillandsia pohliana</i>	1045 \pm 9	612 \pm 3 (42%) *	788 \pm 3 (25%) *
<i>Tillandsia usneoides</i>	432 \pm 1	114 \pm 3 (74%) *	310 \pm 3 (28%) *
<i>Vriesea sucrei</i>	927 \pm 6	116 \pm 16 (88%) *	681 \pm 8 (27%) *

Table 2. Initial rates of ATP-dependent proton transport into isolated tonoplast vesicles from leaves of seven species of bromeliads in dependence on the balancing carboxylate anion.

Proton transport was measured as described in Table 1 in the presence of three different carboxylate anions (fumarate, malate, or citrate, each supplied as their BTP-salt at 50 mM) in comparison with the control (no added carboxylate anion). Rates of proton transport were quantified as % relative fluorescence quenching $\text{min}^{-1} \text{mg protein}^{-1}$; values are expressed as means (\pm s.d.) for three independent preparations. Different capital letters indicate values that were significantly different among species using the same anion (Tukey–Kramer test; $P < 0.05$). Different lower case letters indicate values that were significantly different among different carboxylate anions in the same species (Tukey–Kramer test; $P < 0.05$).

Species	Specific activity (% quench $\text{min}^{-1} \text{mg protein}^{-1}$)			
	Fumarate	Malate	Citrate	Control
<i>Aechmea nudicaulis</i>	2235 \pm 19	512 \pm 19	116 \pm 10	465 \pm 15
<i>Ananas comosus</i>	2573 \pm 33	503 \pm 3	37 \pm 1	112 \pm 1
<i>Billbergia pyramidalis</i>	3542 \pm 1	632 \pm 33	208 \pm 17	577 \pm 4
<i>Nidularium billbergioides</i>	1983 \pm 9	339 \pm 8	44 \pm 8	266 \pm 9
<i>Tillandsia pohliana</i>	1635 \pm 22	444 \pm 6	114 \pm 12	336 \pm 27
<i>Tillandsia usneoides</i>	271 \pm 10	65 \pm 3	33 \pm 2	54 \pm 9
<i>Vriesea sucrei</i>	89 \pm 4	25 \pm 3	46 \pm 4	27 \pm 2

Table 3. Initial rates of PPi-dependent proton transport into isolated tonoplast vesicles from leaves of seven species of bromeliads in dependence on the balancing carboxylate anion.

Proton transport was measured as described in Table 1 in the presence of three different carboxylate anions (fumarate, malate, or citrate, each supplied as their BTP-salt at 50 mM) in comparison with the control (no added carboxylate anion). Rates of proton transport were quantified as % relative fluorescence quenching min⁻¹ mg protein⁻¹; values are expressed as means (± s.d.) for three independent preparations. Different capital letters indicate values that were significantly different among species using the same anion (Tukey–Kramer test; *P* < 0.05). Different lower case letters indicate values that were significantly different among different carboxylate anions in the same species (Tukey–Kramer test; *P* < 0.05).

Specific activity (%. min ⁻¹ quench mg protein ⁻¹)				
Species	Fumarate	Malate	Citrate	Control
<i>Aechmea nudicaulis</i>	627 ± 4	668 ± 38	99 ± 6	194 ± 20
<i>Ananas comosus</i>	266 ± 0.5	197 ± 1	37 ± 2	49 ± 1
<i>Billbergia pyramidalis</i>	848 ± 19	729 ± 10	3 ± 0.2	327 ± 18
<i>Nidularium billbergioides</i>	383 ± 8	407 ± 8	1 ± 0.2	194 ± 8
<i>Tillandsia pohliana</i>	345 ± 14	48 ± 5	73 ± 4	39 ± 3
<i>Tillandsia usneoides</i>	13 ± 1	13 ± 2	31 ± 3	49 ± 2
<i>Vriesea sucrei</i>	25 ± 3	77 ± 6	26 ± 1	21 ± 3

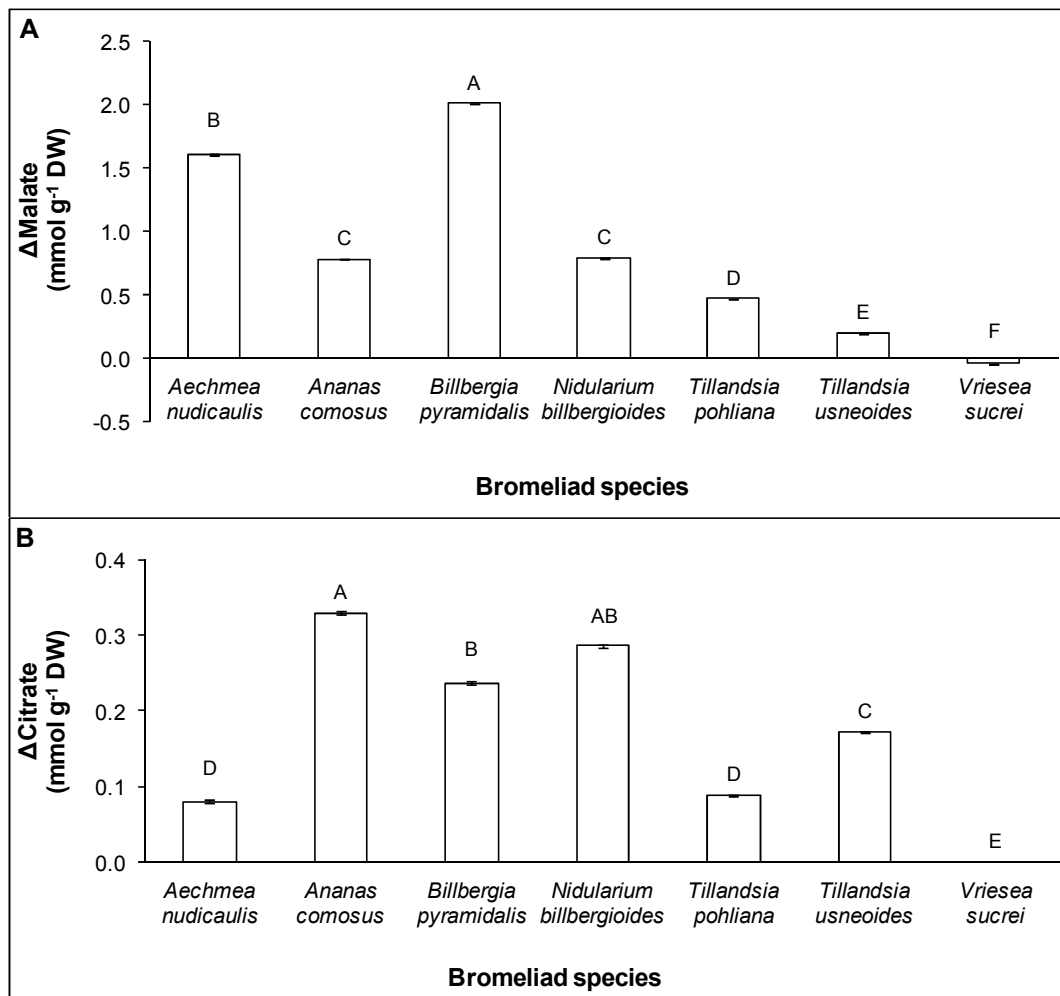
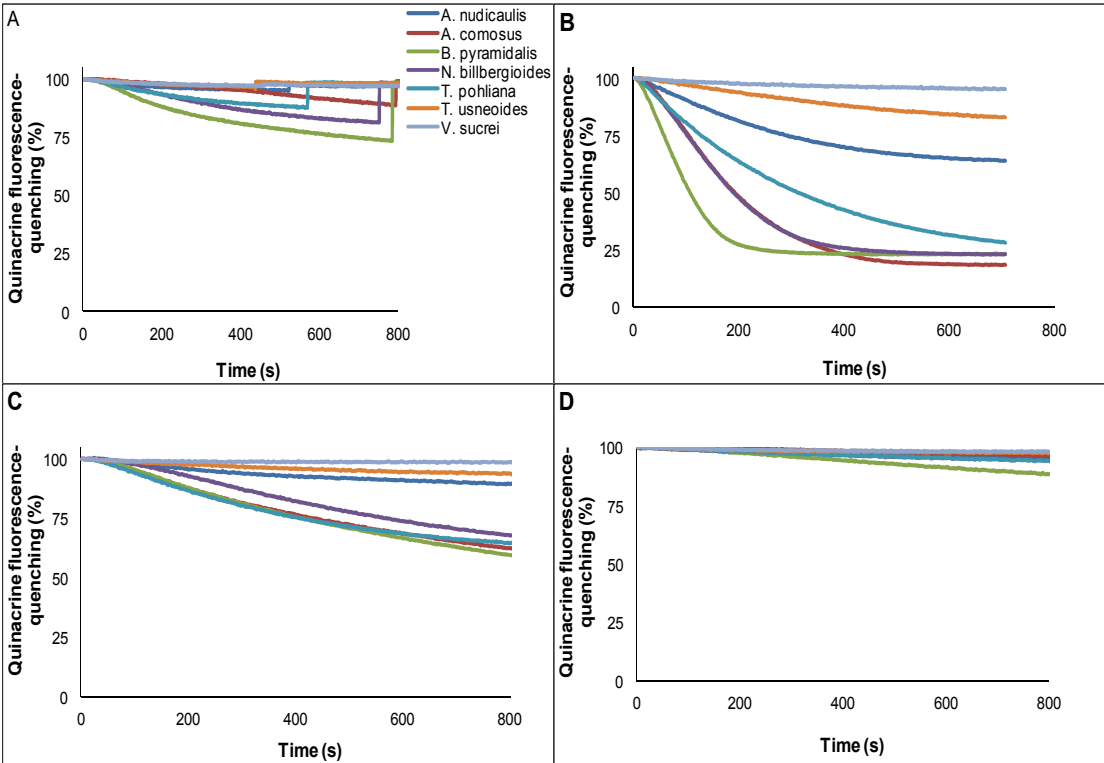
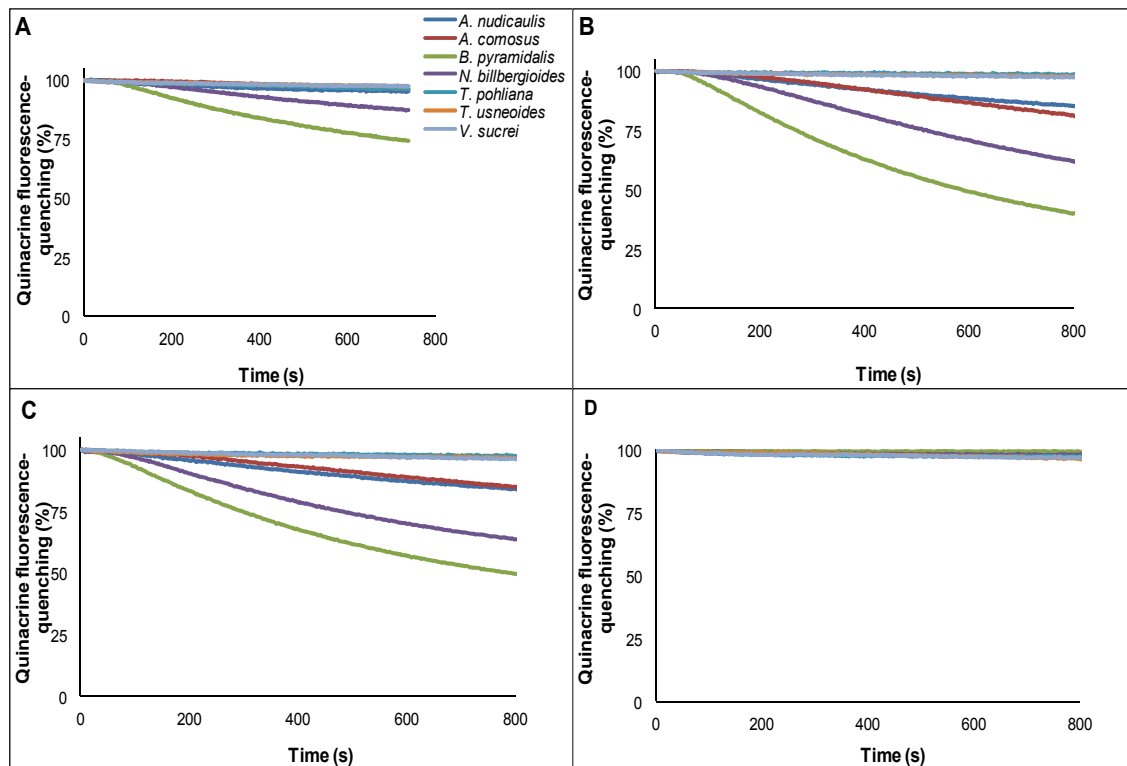


Fig. 2. Nocturnal accumulation of (A) malate and (B) citrate in the leaves of seven bromeliad species. Values for Δ malate and Δ citrate were calculated as the difference in malate and citrate concentration, respectively, in samples harvested at dawn minus those harvested at dusk as described in Materials and methods. Values are means (\pm s.d.) of 3 samples, with each sample consisting of material pooled from three individual plants. Different letters indicate values that were significantly different among bromeliad species (Tukey–Kramer test; $P < 0.05$). DW = dry weight.

1 **Supplementary Material**



2 **Supplementary Fig. 1.** ATP-dependent proton transport into isolated tonoplast
3 vesicles monitored by quinacrine-fluorescence quenching (expressed as a percentage of
4 the starting value) in (A) control conditions (no added carboxylate anion), or in the
5 presence of (B) 50 mM fumarate, (C) 50 mM malate, or (D) 50 mM citrate. Tonoplast
6 vesicles were isolated as described in Materials and methods from leaves of *Aechmea*
7 *nudicaulis*, *Ananas comosus*, *Billbergia pyramidalis*, *Nidularium billbergioides*,
8 *Tillandsia pohliana*, *T. usneoides* and *Vriesea sucrei*. Proton transport was initiated by
9 the addition of ATP (3.0 mM) at the start of the time-course. In A, 2.0 mM NH_4^+ was
10 added as an uncoupler at a time between 400 s and 800 s (depending on the species) to
11 demonstrate abolition of the pH gradient established by ATP-dependent proton
12 transport. Results are from one experiment representative of a total of three.
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Supplementary Fig. 2. PPi-dependent proton transport into isolated tonoplast vesicles monitored by quinacrine-fluorescence quenching (expressed as a percentage of the starting value) in (A) control conditions (no added carboxylate anion), or in presence of (B) 50 mM fumarate, (C) 50 mM malate, or (D) 50 mM citrate (D). Tonoplast vesicles were isolated as described in Materials and methods from leaves of *Aechmea nudicaulis*, *Ananas comosus*, *Billbergia pyramidalis*, *Nidularium billbergioides*, *Tillandsia pohliana*, *T. usneoides* and *Vriesea sucrei*. Proton transport was initiated by the addition of PPi (3.0 mM) at the start of the time-course. Results are from one experiment representative of a total of three.