Journal of Experimental Botany, Vol. 41, No. 223, pp. 193–198, February 1990

Nitrate-Sensitive ATPase Activity and Proton Pumping in Guard Cell Protoplasts of *Commelina*

M. D. FRICKER[1,2,3] and C. M. WILLMER[2]

[1] *Department of Botany, University of Edinburgh, The King's Buildings, Mayfield Road, Edinburgh EH9 3JH, Scotland, UK*

[2] *Department of Biological Sciences, University of Stirling, Stirling FK9 4LA, Scotland, UK*

Received 19 July 1989

ABSTRACT

ATPase activity was measured in crude homogenates of guard cell protoplasts of *Commelina communis* L. using a linked enzyme assay. A low level of azide-sensitive ATPase activity was detected with a pH optimum of 6·8. This activity was stimulated by 0·01% (v/v) Triton X-100, and the pH optimum shifted to pH 7·4. Nitrate-sensitive ATPase activity was measured in the presence of azide and showed a pH optimum around pH 8·0. Proton pumping activity in a mixed population of vesicles from GCP was monitored using fluorescence quenching of quinacrine. Mg-ATP dependent proton pumping was observed at pH 8·0, but not at pH 6·6. The activity at pH 8·0 was inhibited by nitrate and DCCD but not vanadate. These data indicate that activity of the tonoplast proton pump was being measured. There was, however, no evidence for a tonoplast cation (K^+)/proton antiporter under these assay conditions as potassium did not reduce the initial rate of pH gradient formation or increase the rate of collapse of a pre-formed gradient after inhibition of the pump.

Key words: Tonoplast ATPase, proton pump, guard cell protoplasts, *Commelina*.

INTRODUCTION

It is now well established that stomatal movements involve turgor changes generated by large changes in the amounts of osmotically active solutes within the guard cells. The bulk of the osmotic pressure change can be attributed to the varying concentration of potassium, where the balancing ion is either chloride or an organic acid anion, typically malate (Raschke, 1975; MacRobbie, 1988). To achieve the increase in osmotic pressure associated with stomatal opening the inorganic ions must be imported across the plasma membrane and the organic anions synthesized from less osmotically active precursors, principally starch (Raschke, 1975). Under certain conditions, particularly the early stages of opening, a requirement for additional osmotica has also been demonstrated (MacRobbie, 1988). In this respect sucrose concentration has been shown to increase during opening (Outlaw and Manchester, 1979), though other organic anions (e.g. citrate) or intermediates in the glycolytic pathway from starch to malate may be involved.

Whilst events at the plasma membrane have received considerable and justified attention (see reviews by Zeiger, 1983; Outlaw, 1983; MacRobbie, 1988; Hedrich and Schroeder, 1989), it has been recognized that intracellular compartmentation of these ions occurs (MacRobbie, 1977). Evidence from micro-electrode studies (Penny and Bowling, 1974; MacRobbie and Lettau, 1980) and tracer influx and efflux kinetics (see review, MacRobbie, 1988) indicate significant transport and accumulation of ions in the vacuole. However, until recently, the inaccessible nature of the tonoplast has prevented direct investigation

[3] To whom correspondence should be addressed at: Department of Plant Sciences, University of Oxford, South Parks Road, Oxford OX1 3RA, England, UK.

Abbreviations: ATP = adenosine triphosphate; BSA = bovine serum albumin; DCCD = *N,N'*-dicyclohexyl carbodiimide; DMSO = dimethylsulphoxide; DTT = dithiothreitol; EDTA = ethylenediaminetetra-acetic acid; GCP = guard cell protoplast; MES = 2-(*N*-morpholino)- ethanesulphonic acid; NADH = β-nicotinamide adenine dinucleotide, reduced form; PEP = phospho(enol)pyruvate; PK/LDH = pyruvate kinase/lactate dehydrogenase enzymes; PMSF = phenylmethylsulphonyl fluoride; PVP-40 = polyvinylpyrrolidone, MW 40 000; Tris = Tris(hydroxymethyl)aminoethane.

of the mechanisms and energetics underlying these vacuolar fluxes. In other plant cell types an inside-positive membrane potential is set up across the tonoplast by the action of either a proton pumping ATPase and/or a proton pumping pyrophosphatase (Sze, 1984, 1985; Blumwald, 1987). Under these conditions anion movement can occur in response to the electrical component of the pH gradient via suitable carriers or channels, but cation accumulation is thermodynamically unfavourable without a co-transport system (Blumwald, 1987; Poole, 1988). With the advent of patch clamp techniques direct measurement of the electrical properties of some tonoplast transport events has been possible. Two types of voltage-dependent vacuolar ion channel have been described. Both have relatively large conductances and limited ion selectivity, but differ in their activation by calcium and their response to membrane potential (Schroeder and Hedrich, 1989; Hedrich and Schroeder, 1989). One may be involved in anion accumulation and is activated by low (100–200 μmol m^{-3}) calcium and polarization of the vacuolar potential to positive values. The other is triggered by depolarization of the tonoplast to inside-negative values and elevated calcium levels (> 300 μmol m^{-3}). Unfortunately, electrophysiological approaches cannot resolve electrically silent co-transport systems, e.g. cation/proton antiports, which are coupled to the chemical component of the tonoplast proton motive force. The formation of a proton chemical gradient can be directly measured in tonoplast vesicle preparations by determination of the equilibrium distribution of a weak base, e.g. quinacrine. The presence of co-transport systems can be inferred by measuring the uncoupling effect of added ions (Blumwald and Poole, 1985). This technique has been used to monitor both primary electrogenic proton pumping and secondary co-transport events in several systems (see reviews by Sze, 1985; Blumwald, 1987).

In this paper we report on both the detection of nitrate-sensitive ATPase and proton pumping activities in purified guard cell protoplasts (GCP) isolated from *Commelina communis* and in vesicles prepared from GCP, respectively.

MATERIALS AND METHODS

Plant material

The growth of *Commelina communis* L., selection of epidermis and preparation of purified guard cell protoplasts (GCP) were as described previously (Fricker and Willmer, 1987).

Enzyme assays

ATPase activity was measured by coupling ATP hydrolysis to oxidation of NADH using the linked enzyme assay described previously (Fricker and Willmer, 1987), with minor modifications. Aliquots containing 5×10^4 purified GCP were collected by centrifugation (11 600 *g* for 30 s using an MSE microfuge) and resuspended in 50 mm^3 ice-cold homogenization buffer (3·0 mol m^{-3} DTT, 3·0 mol m^{-3} EDTA, 0·1% w/v BSA, 0·5% w/v PVP-40 and 50 mol m^{-3} MES/Tris, pH 6·5). Homogenization was achieved by ten passes through a 26 gauge needle. The assay medium (1·0 cm^3) contained 50 mm^3 crude GCP homogenate, 30 mol m^{-3} MES/Tris, 50 mol m^{-3} KCl, 3·0 mol m^{-3} $MgCl_2$, 2·0 mol m^{-3} PEP, 0·33 mol m^{-3} NADH, 3·0 mol m^{-3} DTT, 0·01% w/v BSA, 0·5 mol m^{-3} PMSF (1·0 mm^3 of a 500 mol m^{-3} stock in DMSO), 10 units of pyruvate kinase and 14 units of lactate dehydrogenase (14 mm^3 PK/LDH enzymes, Sigma). Ammonium molybdate (0·2 mol m^{-3}) and sodium vanadate (0·1 mol m^{-3}) were included in the assay to inhibit non-specific phosphatases and P-type ATPases respectively. To obtain pH curves, the pH was adjusted with Tris base or HCl added to the cuvettes during the course of the experiment. pH values were measured *in situ* with a M1-410 Micro combination pH probe (Microelectrodes, Inc., USA). The reaction was started by the addition of ATP (final concentration 3·0 mol m^{-3}). Nitrate-sensitive ATPase and azide-sensitive ATPase activities were measured as the difference in rates of NADH oxidation in the presence and absence of 50 mol m^{-3} KNO_3 and 1·0 mol m^{-3} NaN_3, respectively. Triton X-100 (0·01% v/v) was added as indicated in figure legends. Measurements were made in a Pye Unicam SP1800 dual beam spectrophotometer at 30 °C. The activities of the linking enzymes, with respect to K_m, V_{max} and lag time ($3{\cdot}5 \times K_m/V_{max}$), were examined over the pH range used in the ATPase assays and were not rate limiting using the criteria suggested by Lowry and Passoneau (1972) (data not shown).

Assays for proton transport

Proton transport into membrane vesicles was measured by the rate of quenching of quinacrine fluorescence, essentially as described by White and Smith (1989). Aliquots containing 10^5 purified GCP were collected by centrifugation (11 600 *g* for 30 s, MSE Microfuge) and resuspended in 50 mm^3 assay buffer (150 mol m^{-3} mannitol, 25 mol m^{-3} MES/Tris, pH 8·0, 0·3 mol m^{-3} Tris–EGTA, 0·2 mol m^{-3} sodium molybdate, 2·0 mol m^{-3} DTT) supplemented with 5·0 μg α-macroglobulin and 0·5 mm^3 of 500 mol m^{-3} PMSF dissolved in DMSO. A mixed population of vesicle types was obtained from GCP by passing them through a 26 gauge needle ten times. The assay medium (0·5 cm^3) contained 50 mm^3 crude homogenate and 450 mm^3 assay buffer supplemented with 60 mol m^{-3} Tris–Cl, 0·05 mol m^{-3} quinacrine, 1·0 mol m^{-3} NaN_3 and 3·6 mol m^{-3} Tris–ATP. Experiments were started by the addition of $MgSO_4$ to give a final concentration of 7·2 mol m^{-3}. Tris–NO_3 (50 mol m^{-3}), K–MES (50 mol m^{-3}), Tris–EDTA (20 mol m^{-3}), sodium vanadate (0·1 mol m^{-3}), Gramicidin (10 μg) and DCCD (0·05 mol m^{-3}) were added as indicated in figure legends. Ethanol from the Gramicidin and DCCD stock solutions was present at 1% and had no effect on the fluoresence assays (data not shown). Measurements were made at room temperature (23 °C) on a Perkin-Elmer model LS-5B spectrofluorimeter with excitation at 427 nm and emission at 495 nm, both with a 5·0 nm band width.

Protein determination

Protein levels were measured using the micro-adaption of the Bradford dye-binding assay using BSA as standard (Bradford, 1976).

Chemicals

All chemicals were from Sigma Chemical Company Ltd., Poole, Dorset, UK or Aldrich Chemical Company Ltd., Poole, Dorset, UK.

RESULTS

Enzyme activities and proton transport capability both declined rapidly after homogenization ($t_{1/2}$ *c.* 20 min), despite the presence of protease inhibitors and reducing agents, unless the extract was diluted rapidly into the assay buffer. Therefore, aliquots of GCP were homogenized individually for each assay and diluted into the assay buffer within 20 s. Under these conditions NADH oxidation rates remained stable for at least 60 min and initial rates of proton transport were similar in duplicate aliquots over at least a 5 h period. The reasons for the time-dependent inactivation are unknown, but may explain our failure to detect active proton pumping after attempts to purify a tonoplast enriched fraction using differential centrifugation and flotation on sucrose or Ficoll cushions (data not shown).

The pH profile of azide-sensitive ATPase activity was fairly broad with an optimum at pH 6·8 in the absence of detergent (Fig. 1). Maximum activity corresponded to 0·66 ± 0·12 pmol protoplast^{-1} h^{-1} (2·64 ± 0·48 μmol h^{-1} mg^{-1} protein). In the presence of 0·01% Triton X-100 the pH optimum shifted to 7·4 and the maximum activity increased to 1·55 ± 0·05 pmol protoplast^{-1} h^{-1} (6·20 ± 0·20 μmol h^{-1} mg^{-1} protein). Nitrate-sensitive ATPase activity, measured in the presence of azide, increased with increasing pH to a plateau above pH 8·0 (Fig. 2). The maximum activity corresponded to 2·27 ± 0·29 pmol protoplast^{-1} h^{-1} (9·08 ± 1·16 μmol h^{-1} mg^{-1} protein), and accounted for 56% of the total ATPase activity. Vanadate-sensitive ATPase activity was virtually absent (5%) at this pH, whilst azide-sensitive activity accounted for *c.* 8% of total. The remaining ATPase activity could not be attributed to specific enzymes.

At pH 8·0 crude homogenates of GCP showed proton transport activity into vesicles as judged by the quenching of quinacrine fluorescence (Fig. 3A, B). No transport occurred in the presence of ATP alone but was initiated by the addition of an excess of Mg^{2+} ions (Fig. 3A, B). The initial rate of fluorescence quench was completely inhibited by Tris–NO_3 and DCCD. Gramicidin rapidly reversed the fluorescence quench to a level greater than the initial fluorescence (Fig. 3A). The pH gradient was also slowly dissipated after inhibition of the pump by Tris–NO_3, DCCD or EDTA (to chelate Mg^{2+} ions), but not vanadate (Fig. 3A). Potassium had no statistically significant effect on the initial rate of fluorescence quench, the steady-state quench level or the rate of pH gradient collapse induced by DCCD, EDTA and NO_3 (Fig. 3B; Table 1).

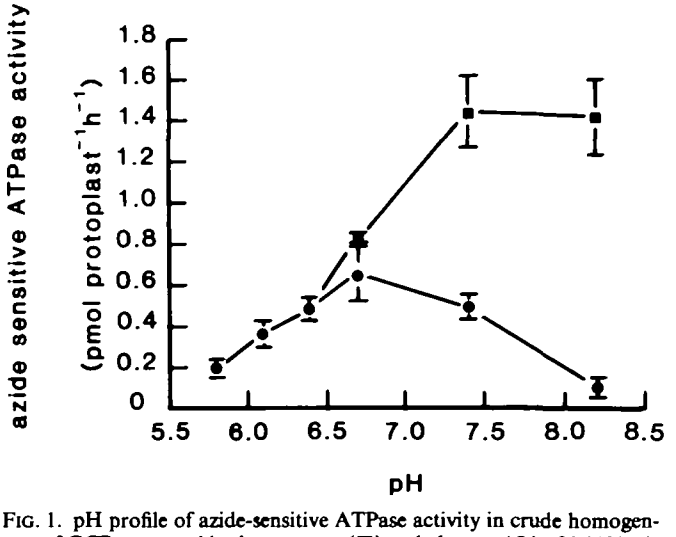

FIG. 1. pH profile of azide-sensitive ATPase activity in crude homogenates of GCP measured in the presence (■) and absence (●) of 0·01% v/v Triton X-100. Each point represents the mean ± s.e.m. ($n = 3$ and $n = 6$, respectively).

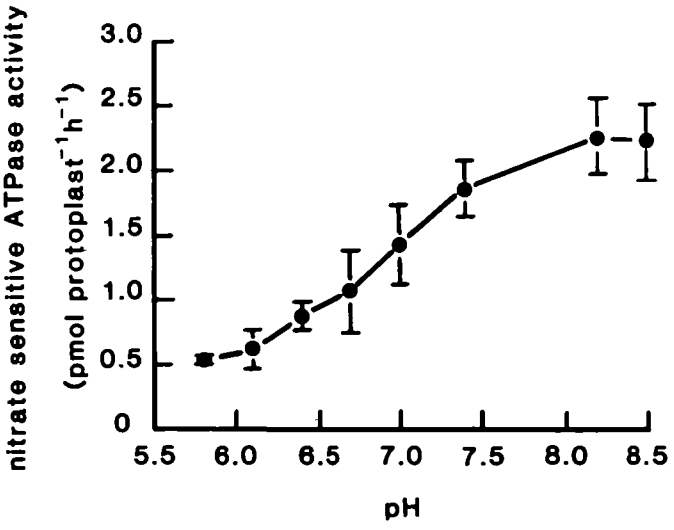

FIG. 2. pH profile of nitrate-sensitive ATPase activity in crude homogenates of GCP. Each point represents the mean ± s.e.m. ($n = 7$).

DISCUSSION

The tonoplast proton pumping ATPase has an alkaline pH optimum and is characteristically inhibited by nitrate (Sze, 1984). However, nitrate has been reported to inhibit the mitochondrial F_1–F_0 ATPase, which also has an

TABLE 1. *Effect of potassium on the properties of Mg–ATP-dependent quenching of quinacrine fluorescence*

Mg–ATP-dependent quenching of quinacrine fluorescence was measured in the presence and absence of 50 mol m^{-3} K–MES. Values are given for the initial rate, the steady-state level and the rate of collapse after inhibition of the pump activity with Tris–NO_3, EDTA or DCCD. (values are given as the mean ± s.e.m., $n = 6$).

	+K	−K
Initial rate (% quench min^{-1})	19·0 ± 1·2	17·5 ± 1·5
Steady-state level (% quench)	64·1 ± 2·5	71·8 ± 5·3
Collapse rate (% quench min^{-1})	11·9 ± 4·2	8·4 ± 4·1

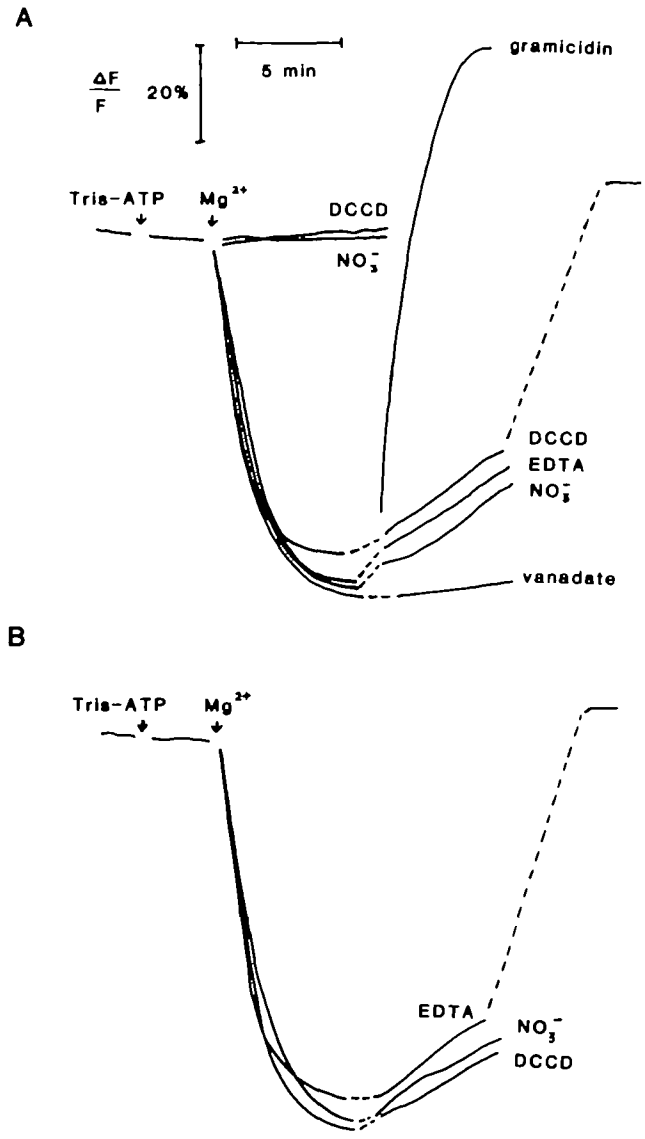

FIG. 3. Mg-ATP dependent quenching of quinacrine fluorescence at pH 8·0 in crude homogenates of GCP (equivalent to 10^5 GCP per assay). Typical traces are shown from one experiment in the absence (A) and presence (B) of 50 mol m^{-3} K-MES. Inhibitors were added at the following concentrations Tris-NO_3 (50 mol m^{-3}), DCCD (0·05 mol m^{-3}), Tris-EDTA (20 mol m^{-3}), sodium vanadate (0·1 mol m^{-3}), gramicidin (10 μg).

alkaline pH optimum (Randall, Wang, and Sze, 1985). The corresponding effects on the chloroplastic CF_1-CF_0 are not known. In crude homogenates of GCP the levels of both these enzymes should be high as guard cells are known to have high rates of respiration (Birkenhead, Laybourne-Parry, and Willmer, 1985) and exhibit both cyclic and non-cyclic photophosphorylation (Shimazaki and Zeiger, 1985). The activity of these F-type ATPases (terminology according to Pedersen and Carafoli, 1987) were not identified separately in this study, but were distinguished from the tonoplast ATPase by their sensitivity to azide (Randall *et al.*, 1985; Randall and Sze, 1986). F-type ATPase activity was low in crude homogenates of GCP but activated 2·5-fold by 0·01% v/v Triton X-100. This increase probably represents release of latent activity through disruption of sealed mitochondrial/chloroplastic vesicles surviving the gentle homogenization conditions used here. Triton X-100 did not increase the activity of nitrate-sensitive ATPase activity (data not shown).

The tonoplast ATPase was, therefore, routinely assayed in the presence of azide, but without detergent, to minimize interference from F-type ATPases. The pH dependence of activity was similar to that reported for *Beta vulgaris* (Bennett, O'Neill, and Spanswick, 1984) and *Kalanchoë daigremontiana* (Aoki and Nishida, 1984; Smith, Uribe, Ball, Heuer, and Lüttge, 1984*a*). Nitrate-sensitive ATPase activity accounted for 56% of total ATPase activity at pH 8·0, decreasing to 42% at pH 6·8. This is comparable to results from a 12 000 × *g* pellet from GCP of *Vicia faba*, where nitrate inhibited 37% of the total ATPase activity at pH 6·8, (Shimazaki and Kondo, 1987) but, in contrast to a recent report using a 6 000–100 000 × *g* pellet from GCP of *C. communis*, where no nitrate-sensitive ATPase was detected at pH 7·5. (Nejidat, Roth-Bejerano, and Itai, 1986).

The maximum ATPase activity on a protein basis in this study was close to that obtained in partially purified tonoplast fractions from tobacco (Briskin and Leonard, 1980), red beet (Walker and Leigh, 1981) and corn roots (O'Neill, Bennett, and Spanswick, 1983), even though an unfractionated, crude homogenate was used here. The activity of the tonoplast ATPase at cytoplasmic pH values (*c.* pH 7·0) was comparable to or greater than the activity of the vanadate sensitive (plasma membrane) ATPase activity reported earlier (Fricker and Willmer, 1987). If this trend is reflected in the transport capacity of the two system, then ion accumulation in the vacuole would represent a realistic means of cytoplasmic ion homeostasis in guard cells.

Proton pumping activity at pH 8·0, near the maximum for the ATPase activity, required Mg^{2+} ions and was inhibited by both nitrate and DCCD, similar to reports in other cell and tissue types (Sze, 1985; Poole, 1988). The collapse of the pH gradient induced by gramicidin to a level greater than the initial fluorescence, suggests that there were vesicles with a pH gradient, acid interior, already present. It seems likely that these were mitochondrial or chloroplastic in origin, remaining unbroken after the gentle homogenization, in a similar fashion to the latency observed in the azide-sensitive ATPase assays.

No Mg-ATP-dependent fluorescence quenching was observed at pH 6·6, the optimum for the plasma membrane ATPase (Fricker and Willmer, 1987) and vanadate did not collapse the pH gradient formed at pH 8·0, indicating that the plasma membrane H^+ pump was not active in this mixed vesicle preparation. This may be due

to genuine inactivation of the pump, but most probably results from a high proportion of 'right-side out' or leaky plasma membrane vesicles that are incapable of generating or sustaining a pH gradient (Sze, 1985).

We attempted to identify a potassium/proton antiporter through the uncoupling effect of added potassium on the Mg–ATP generated pH gradient, but could detect no statistically significant change in either the initial rate of fluorescence quench, the steady-state level reached or the rate of uncoupling after inhibition of the pump. This type of approach has been successfully employed with the halophyte, *Beta vulgaris*, where a sodium/proton antiporter was detected in tonoplast vesicles by the uncoupling effect of added cations after a pH gradient was externally imposed (Blumwald and Poole, 1985). It was not possible in this study to use pH jump studies to investigate co-transport events in GCP further due to the diverse vesicle populations present in the crude homogenate. Moreover, the isolation of intact vacuoles or tonoplast preparations suitable for such studies are particularly difficult to obtain from GCP. Attempts to prepare intact vacuoles from GCP of *Commelina* using either the DEAE–Dextran gradient technique (Smith, Uribe, Ball, and Lüttge, 1984*b*) or controlled osmotic lysis combined with flotation through Ficoll gradients (Boller and Kende, 1979) only gave recoveries of 3–7% (data not shown).

Integration of this tonoplast proton pump activity into models of guard cell behaviour must account for physiological changes in vacuolar pH observed during stomatal movements. If an inwardly directed H^+-pump energized the tonoplast during salt accumulation the vacuole would be expected to acidify. However, pH electrode work (Penny and Bowling, 1975) and pH indicator dyes (Pekarek, 1934) indicate that the vacuole becomes less acidic during opening. This apparently contradictory data can be reconciled if the level of co-transport via proton/cation antiport and intra-vacuolar buffering capacity are sufficient to mask the proton transport events initially energising the tonoplast (Lüttge and Smith, 1988).

The data presented here represent the first preliminary characterization of a tonoplast proton pumping ATPase from guard cells. Detailed biochemical description is almost impossible on account of the small amount of material available, but the high levels of proton pumping observed are encouraging and indicate that realistic experiments on tonoplast transport processes are feasible with this tissue.

ACKNOWLEDGEMENTS

One of us (M. D. F.) would like to thank S.E.R.C. for financial support during much of this work. our thanks to Dr P. J. White and Dr. J. A. C. Smith for discussion and helpful comments.

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