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LOCALIZATION OF A PHOSPHATASE (ATPase) ON THE PLASMALEMMA OF THE MAIZE SCUTELLUM*

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Key Word Index—*Zea mays*; Gramineae; maize scutellum; phosphatase; ATPase; plasmalemma.

Abstract—A phosphatase (ATPase) was demonstrated on the surface of the maize scutellum cell by showing that (1) when exogenous ATP was hydrolysed by intact scutellum cells, ADP, AMP and P_i appeared in the bathing solution in stoichiometric amounts, (2) the rate of hydrolysis was sensitive to bathing solution pH; (3) exogenous Mg^{2+} increased the rate of hydrolysis and (4) when the ATPase reaction was carried out in the presence of lead nitrate, TEM photographs showed lead phosphate deposits located almost exclusively in the plasmalemma. The ATPase was tightly bound to the plasmalemma and was not destroyed by freezing and thawing scutellum slices, a treatment which disrupted the plasmalemma. Acid treatment (10 mM HCl) of fresh or frozen-thawed scutellum slices destroyed acid phosphatase activity but had little effect on ATPase activity at pH 6.5. Following acid treatment of the scutellum slice preparations, a definite Mg^{2+} requirement for ATPase activity could be demonstrated.

INTRODUCTION

There have been many reports of phosphatase (ATPase) activity localized on the plasmalemma of plant cells [1–5], including sieve cells of *Pinus* [1] and *Cucurbita* [2], and root cells of *Nicotiana* [3] and maize [4]. The impetus for these studies was the supposed link between membrane transport of inorganic ions, amino acids and sugars and the activity of an ATPase on the plasmalemma [3, 6–9].

In the maize scutellum, sucrose uptake appears to be driven by the pH difference and electrical potential difference across the plasmalemma [10]. According to Mitchell's chemiosmotic hypothesis, these forces are generated by a proton pump (a reversible ATPase or a redox pump of the respiratory chain) in the coupling membrane [11]. Maize scutellum slices secrete protons and can acidify their bathing solution to a pH well below 5 [10] which suggests that their cells contain proton pumps. In the plasmalemma of a eukaryotic cell, the proton pump is most likely a reversible ATPase. The work reported in this paper was undertaken to establish the existence of an ATPase on the plasmalemma of the maize scutellum.

RESULTS

Cell-surface phosphatase activity of fresh scutellum slices

Scutellum slices were incubated in distilled water for 1 hr and then placed in a buffered ATP solution. Phosphatase activity of these slices was determined by measuring P_i in the bathing solution. The effect of pH on phosphatase activity is shown in Fig. 1. Addition of Mg^{2+} stimulated activity at all pH's tested and peak activity was at pH 5.7.

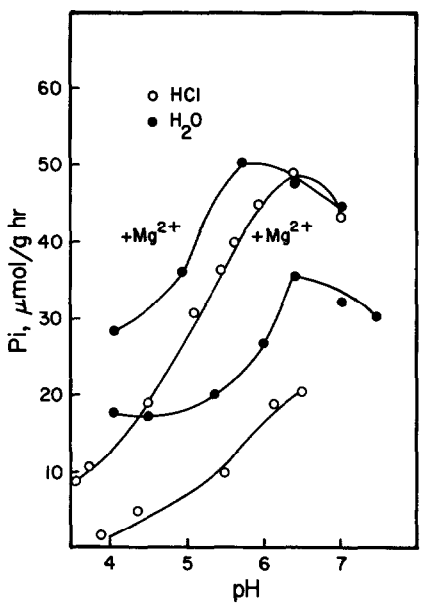

Fig. 1. Phosphatase activity of fresh scutellum slices. The vessels contained 50 mM buffer (MES, pH 3.5–6.5; MOPS, pH 6.5–7.5), 3 mM ATP and, in some cases, 20 mM $MgCl_2$. Experiments were run for 60 min. Each point is an average of the results of 5 experiments. Standard deviations were less than 10% of the mean for all points above 5 μmol P_i/g/hr.

Humphreys [12] found that maltase activity on cell surfaces of fresh maize scutellum slices could be almost eliminated by washing the slices for 30–60 min in 10 mM HCl. Acid treatment did not interfere with sucrose

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Table 1. Effect of H_2O or HCl treatment on phosphatase activity of frozen-thawed scutellum slices*

No.	Treatment	P_i, μmol/g/hr
1	None	153
2	30 min in H_2O	114
3	60 min in H_2O	54
4	30 min in HCl	67
5	60 min in HCl	11
6	60 min in HCl; + Mg^{2+}	40
7	60 min in HCl; no ATP	0

* At the end of the treatment, slices were washed twice with 10 ml H_2O. Then the phosphatase reaction was started by the addition of 10 ml ATP (3 mM) in 50 mM MES, pH 6.5. In No. 6, $MgCl_2$ (20 mM) was also added, and in No. 7, ATP was deleted from the phosphatase reaction solution.

synthesis, cellular respiration, or transport of maltose or sucrose across the plasmalemma [12, 13]. When fresh scutellum slices were treated with 10 mM HCl before testing for phosphatase activity, there was a drop in activity over the entire pH range from 4 to 6.5 (Fig. 1). When Mg^{2+} was added, however, HCl-washed and H_2O-washed slices showed *ca* equal phosphatase activities at pH 6.5. Acid treatment decreased phosphatase activity at lower pH's (Fig. 1), but appeared to have no effect on the phosphatase having peak activity at pH 6.5 when Mg^{2+} was added.

The following results indicate that the phosphatases are at the surfaces of the scutellum cells. Phosphatase activity was sensitive to bathing-solution pH; the substrate was added to the bathing solution and the product appeared in the bathing solution; the addition of Mg^{2+} stimulated activity and HCl treatment destroyed part of the activity.

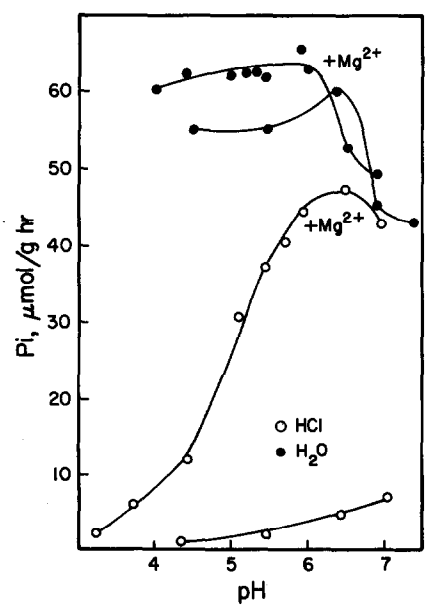

Fig. 2. Phosphatase activity of frozen-thawed scutellum slices. Frozen tissue was thawed with H_2O or 10 mM HCl and incubated for 60 min (see Experimental). Reaction conditions are given in Fig. 1. Each point is an average of the results of 5 experiments. Standard deviations were less than 10% of the mean for all points above 10 μmol P_i/g/hr. In the lowest curve, standard deviations ranged from 19 to 39% of the mean.

Phosphatase activity of frozen-thawed scutellum slices

In an attempt to allow the substrate access to both sides of the plasmalemma, frozen scutellum slices were thawed by the addition of either H_2O or 10 mM HCl, and then incubated at 30° for 30 or 60 min before measuring phosphatase activity. Frozen-thawed slices that were tested without pretreatment with either H_2O or HCl showed the highest phosphatase activity (Table 1). Phosphatase activity decreased 25% after the slices were H_2O-treated for 30 min and 65% after 60 min. HCl-treated slices showed greater decreases in enzyme activity. After 30 min in HCl, the activity decreased from 153 to 67 μmol/g/hr. After 60 min in HCl, the phosphatase activity was only 11 μmol/g/hr. However, when Mg^{2+} was added to the reaction mixture, the phosphatase activity of the HCl-treated slices rose to 40 μmol/g/hr, a rate *ca* equal to that obtained with fresh slices at the same pH (Fig. 1).

The effect of pH on phosphatase activity of frozen-thawed slices is shown in Fig. 2. With H_2O-treated slices, activity between pH 4 and 6 remained relatively high, then decreased above pH 6. Addition of Mg^{2+} increased the activity less than 15% in the pH range 4–6, and the maximum phosphatase activity was shifted from 6.5 to 5.9. Addition of Mg^{2+} to fresh H_2O-treated slices also shifted the pH optimum to a lower value (Fig. 1). With HCl-treated slices, phosphatase activity in the absence of added Mg^{2+} was low at all pH's tested. In the presence of Mg^{2+}, however, there was a sharp rise in activity near pH 5 and a peak activity at 6.5.

Since P_i was measured as the indicator of phosphatase activity, the amounts of P_i leakage and uptake by HCl-treated fresh slices and the amount of P_i leakage from HCl-treated frozen-thawed slices were measured (Table 2). There was no uptake of P_i into fresh slices, and P_i leakage was only 1–2 μmol/g from fresh or frozen-thawed slices.

Protein nitrogen and total phosphorus were determined for HCl-treated, fresh and frozen-thawed slices. HCl-treated fresh slices contained 9 mg N/g fr. wt and 150 μmol P/g fr. wt. HCl-treated frozen-thawed slices contained 8 mg N/g fr. wt and 126 μmol P/g fr. wt. Thus, most of the protein and phosphorus was not removed by acid treatment of frozen-thawed slices. Since scutella contain only *ca* 16 μmol/g fr. wt of inorganic phosphorus [14], the remaining phosphorus must be in the organic form, much of which is probably phytic acid. These organic phosphorus compounds were not used as substrates by the phosphatase in the frozen-thawed slices (Table 2).

Table 2. P_i leakage and uptake*

Tissue	P_i in bathing solution, μmol		pH
	Initial	After 60 min	
Fresh	0	1	6.5
	10	12	6.5
	20	21	6.5
	30	31	6.5
Frozen-thawed	0	1	4.5
	0	1	5.5
	0	1	6.5
	0	0	8.0

* Reaction vessels contained 0.5 g of HCl-treated scutellum slices in 10 ml of a solution containing 50 mM buffer (MES, pH 4.5–6.5; MOPS, pH 8.0), 20 mM $MgCl_2$ and (in some cases) Na phosphate, pH 6.5.

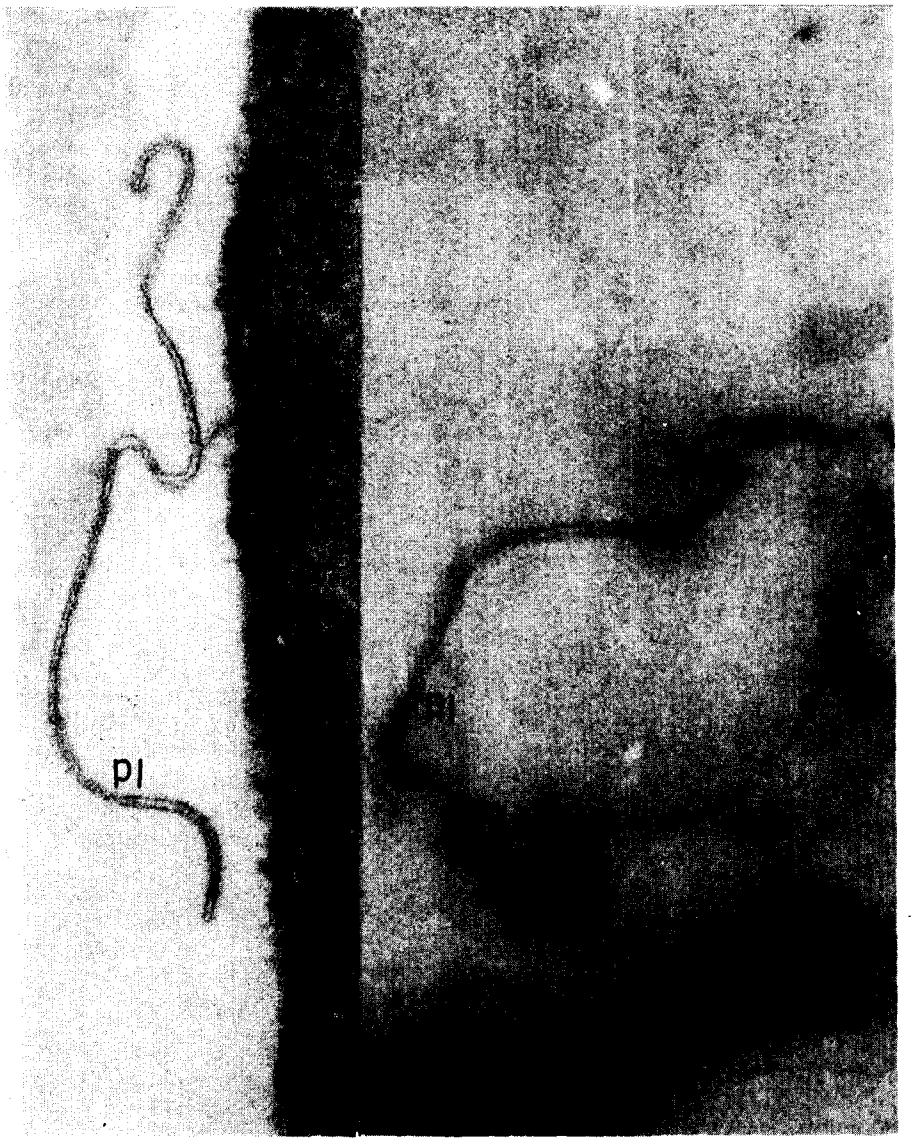

Fig. 3. Phosphotungstic acid stain for plant plasmalemma. Sections of HCl-treated, frozen-thawed scutellum slices stained with phosphotungstic-chromic acid, and showing plasmalemma (pl), cytosplasm (cy), and cell wall (CW). × 160 000.

Electron micrographs of the HCl-treated, frozen-thawed slices showed no distinct organelles. There were no distinguishable mitochondria, ER, dictyosomes nuclei or vacuoles. There were some lipid globules and patches of cytoplasm. When these cells were specifically stained for plasmalemma by phosphotungstic acid–chromic oxide [15], many vesiculated and non-vesiculated plasmalemma fragments were observed (Fig. 3).

From the above results, it is concluded that there is an acid-stable, tightly bound phosphatase located at the cell surfaces of the maize scutellum. The phosphatase is activated by Mg^{2+} and has a peak activity at pH 6.5. The nearly identical results obtained with HCl-treated, fresh and frozen-thawed slices strongly suggest that the same phosphatase was being observed in both cases.

Cytological localization of the phosphatase

A cytological study was done to establish the location of the phosphatase reaction within the cell. HCl-treated, fresh slices were fixed in glutaraldehyde, and then placed in a reaction mixture containing buffered ATP, Mg^{2+} and lead nitrate. A 1% glutaraldehyde treatment for 30 min inhibited the phosphatase 56%. When glutaraldehyde treatment was followed by a 1% lead nitrate treatment for 30 min, the phosphatase was inhibited 72%. Thus, there remained sufficient enzyme activity for a cytological localization study.

Precipitates of lead phosphate were deposited on the plasmalemma (Fig. 4). There were a few scattered lead phosphate deposits in the cytoplasm, but they were not associated with any particular organelle. The control, without ATP, showed no such localization. These results indicate that the plasmalemma was the site of ATPase activity.

Phosphatase localization in the frozen-thawed slices was not attempted because freezing and thawing released the vacuolar contents and disrupted other organelles (Fig. 3), causing the appearance of electron-dense

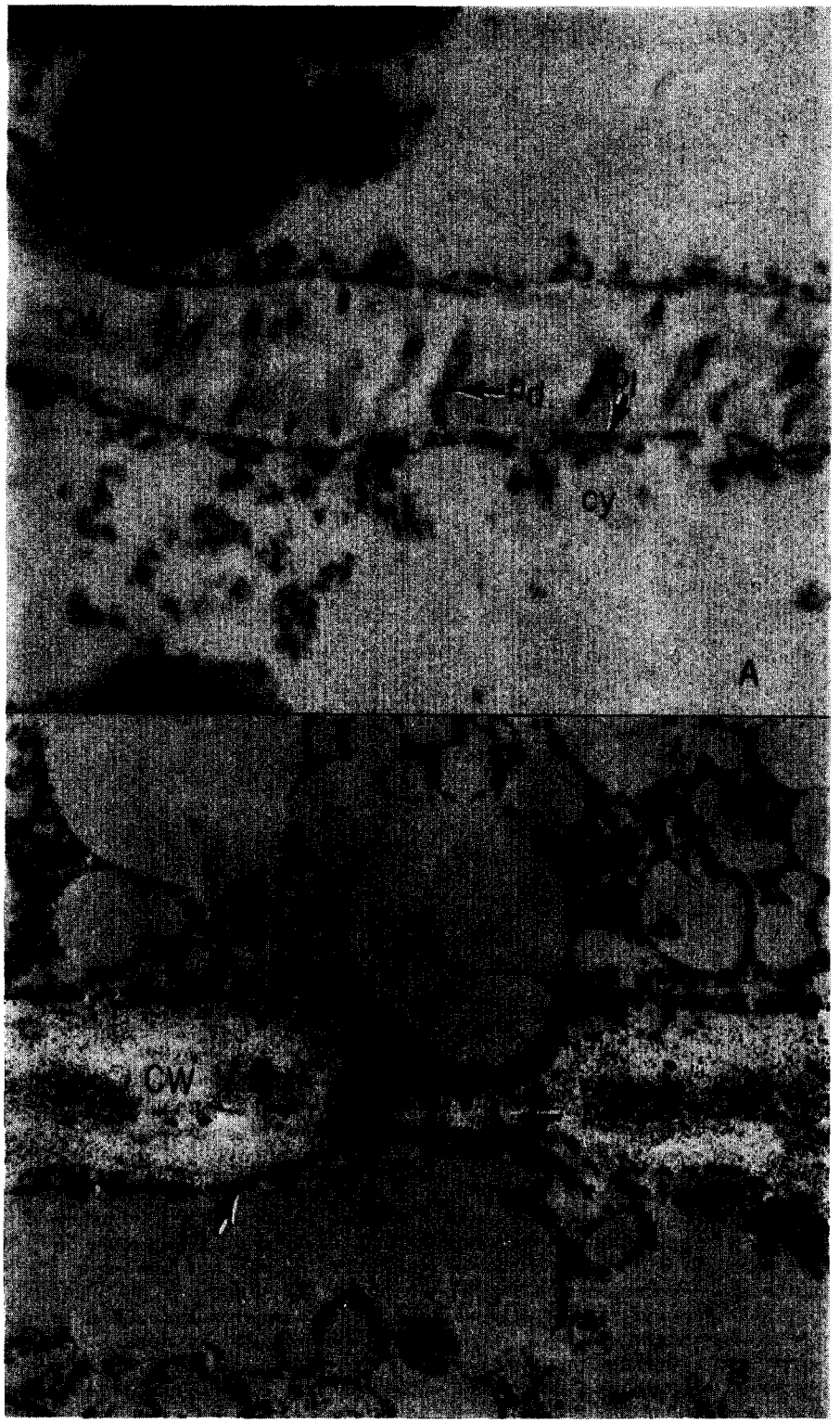

Fig. 4. Electron micrograph of ATPase localization in fresh scutellum slices. A. Control, without ATP, showing plasmalemma (pl), cytoplasm (cy), plasmodesma (pd), lipid (L), and cell wall (CW). × 20400. B. Enzyme localization, showing lead phosphate on the plasmalemma (pl) and along the plasmodesma (pd). × 18000.

granules throughout the cell. Presumably, these granules were Mg and K salts of phytic acid [16] that resembled lead phosphate deposits.

Mitochondrial ATPase

In the frozen-thawed slices, mitochondrial ATPase was a possible source of phosphatase activity. To test this, mitochondrial preparations from fresh maize scutella were assayed for ATPase activity with the same concentrations of Mg^{2+} and ATP that were used in whole-tissue experiments. Mitchondrial ATPase activity was 40% greater at pH 8.5 than at 6.5. This is in agreement with pH optima for mitochondrial ATPases from other plants (e.g. [7]). The mitochondria were then treated in the same way the slices had been (e.g. Fig. 2). Aliquots of the mitochondrial preparation were frozen for 48 hr at −5°, thawed, and washed with 10 mM HCl for 30 min. The mitochondria were then rinsed with buffer and tested for ATPase activity at pH 6.5 and 8.5 in 20 mM $MgCl_2$. There was no phosphatase activity at either pH.

Stoichiometry of the phosphatase reaction with ATP as the substrate

End point stoichiometric experiments were run in triplicate with HCl-treated, fresh slices. Slices (0.5 g) were incubated in a solution containing 2.5 mM ATP, 20 mM $MgCl_2$ and buffer, pH 6.5. After 60 min, 12.1 μmol ATP had disappeared from the bathing solution and P_i (22 μmol), ADP (3.2 μmol) and AMP (6.9 μmol) had appeared in the bathing solution. Thus, 83% of the ATP lost was recovered in the bathing solution as ADP and AMP. ADP was a good substrate and AMP was a poor substrate for the scutellum plasmalemma ATPase [17].

DISCUSSION

The results of this study indicate that the phosphatase in HCl-treated preparations of both fresh and frozen-thawed scutellum slices is a plasmalemma-bound ATPase. This is supported by the following results. (1) Cytological localization clearly showed that the areas of ATPase activity were on the plasmalemma (Fig. 4). There are several reports of similar results obtained with phloem sieve cells [1] and sieve tube members [18, 19]. (2) In both fresh and frozen-thawed slices, HCl treatment for 1 hr did not destroy the phosphatase activity (Figs. 1 and 2). This rigorous treatment would denature most proteins. However, the plasmalemma proteins of the maize scutellum appear to be resistant to acid treatment. After treating scutellum slices in HCl, rates of sucrose and maltose uptake [12], of sucrose synthesis from exogenous fructose, and of respiration [13] are not changed. The levels of ATPase activity at pH 6.5 in fresh and frozen-thawed slices were nearly identical (Figs. 1 and 2), and HCl treatment decreased the activity between pH 4 and 5, presumably by destroying acid phosphatases. Nurminen *et al.* [20] studied enzymes on the cell wall and plasmalemma of yeast. When the wall was enzymatically digested and the released enzymes assayed, saccharases and acid phosphatases were released, but Mg^{2+}-ATPase was not. This was interpreted to mean that the ATPase was bound to the membrane. (3) There was no uptake of P_i and very little leakage of P_i from HCl-treated, fresh slices (Table 2). It is unlikely, therefore, that ATP entered the cell, was hydrolysed and P_i leaked out into the bathing solution. The results indicate that the reaction occurred in or on the cell surface, ATP was hydrolysed outside the cell and P_i remained in the bathing solution. (4) The stoichiometric studies confirmed that the substrate and products remained in the bathing solution. The phosphatase seems to be able to recognize substrate coming from outside the cell. Similar results were reported with intact ascites tumor cells [21]. The tumor cells hydrolysed exogenous ATP to produce AMP and P_i, and both products were found in the bathing solution. Under normal conditions, the ATPase would only encounter ATP from the inside.

Most ATPases are considered to be composed of two functional parts. The part that is associated with the synthesis or hydrolysis of ATP is called the F_1 portion, and the part thought to be involved in proton transport is called the F_0 portion [22, 23]. The F_0 portion is embedded in the lipid of the membrane, whereas the F_1 portion protrudes from the membrane. Thus, ATPases are thought to be vectorially oriented in the membrane. Models of ATPases are drawn with the substrate recognition portion (F_1) protruding into the cytoplasm, the matrix of the mitochondrion, or the stroma of the chloroplast [22, 23]. Bacterial membranes obtained by osmotic lysis yield essentially all right-side-out vesicles [24]. Such vesicles showed high ATPase activity when ATP was introduced from the outside. A high rate of ATP hydrolysis was not expected because the model presumed a vectorial orientation with the substrate recognition portion of the ATPase on the inside. However, the model is supported by results using spheroplasts of *E. coli*, indicating that the ATPase is localized on the inner part of the cytoplasmic membrane [25].

Although the vectorial model described is considered to be the best model for mitochondrial and chloroplastic ATPases [22, 23], not as much is known about ATPases in the plasmalemma of higher plants, and the model may not accurately describe the plasmalemma enzyme. Most information about plasmalemma ATPase comes from work with partially purified plant plasma membranes and the structure of the enzyme is not known [26–29]. It is possible that plant plasmalemma ATPases are different in orientation from mitochondrial or bacterial ATPases. In the maize scutellum, results with fresh and frozen-thawed slices were very similar (Figs. 1 and 2). Since freezing and thawing ruptured the plasmalemma which then formed both vesicles and patches of membrane (Fig. 3), it appears that the scutellum plasmalemma ATPase is tightly bound to the membrane, and is capable of substrate recognition from either side of the plasmalemma.

There is evidence for the existence of a proton pump in the maize scutellum plasmalemma [10, 30]. The ATPase on the plasmalemma may be acting as the pump, setting up a proton gradient and an electrical potential difference which would drive cation and sugar transport across the membrane [6–8, 10, 31].

EXPERIMENTAL

Preparation of scutellum slices. Maize grains (*Zea mays* L., cv McNair 508) were soaked in running H_2O for 24 hr, then placed on moist paper towels and grown in the dark at 24–25° for 72 hr. Scutella were exised and cut transversely with a razor blade into slices 0.5 mm or less in thickness. Slices were washed in H_2O until the washings were clear, blotted on filter paper and weighed into groups of 0.5 g (80–90 slices). The thickness of the

scutellum slices, in one sample, was measured under a dissecting microscope fitted with an ocular micrometer. A single slice was then sectioned and the cells making up the thickness were counted. Scutellum cells are nearly uniform, and each cell was *ca* 50 μm in diameter. From measurements of the thicknesses in a random sample of slices, it was determined that the slices were 3–7 cells in thickness. There were many intercellular spaces.

Treatment of scutellum slices. For some expts, slices were frozen at −4° to −5° and these could be stored for at least 27 days with no loss of phosphatase activity. Fresh and frozen slices were incubated in 10 ml of H_2O or 10 mM HCl for 1 hr at 30° before use in phosphatase assay. The bathing solns were replaced with fresh H_2O or HCl after 30 min and at the end of the treatment slices were rinsed twice in H_2O. All incubations were carried out with 0.5 g of slices in 10 ml bathing soln at 30° with orbital shaking.

Analysis of ATP, ADP, AMP and P_i. P_i was measured by the modified Fiske–Subbarow method of ref. [32]. ATP was assayed by reacting it with glucose and hexokinase (EC 2.7.1.1) and measuring the G6P formed with G6P dehydrogenase (EC, 1.1.1.49) [33]. ADP was assayed by reacting it with PEP and pyruvic kinase (EC 2.7.1.40) and measuring the pyruvate formed with lactic dehydrogenase (EC 1.1.1.27) [34]. AMP was phosphorylated with ATP and myokinase (EC 2.7.4.3) and the resulting 2 equivalents of ADP were determined as above [34].

Mitochondrial preparation. Mitochondria were isolated from fresh whole scutella according to the method of ref. [35]. The purity of the prepn was checked by TEM. Many intact as well as partially broken mitochondria were seen.

Organic nitrogen and total phosphorus. N was determined by Nesslerization [36] and total P was determined by the method of ref. [32], using H_2SO_4 digests of scutellum slices.

ATP product localization. The method of ref. [37] was modified for use in the ATPase localization study. Fresh scutellum slices were HCl-treated and then incubated in buffered 1% glutaraldehyde (50 mM MES, pH 6.5) for 30 min. The slices were rinsed twice with buffer and incubated for 30 min at 30° in a soln containing $Pb(NO_3)_2$ (1%), $MgCl_2$ (20 mM), MES (50 mM, pH 6.5) and ATP (3 mM). Four controls (minus ATP, $MgCl_2$ alone, $Pb(NO_3)_2$ alone, and buffer alone) were run. After the incubation, the slices were rinsed with buffer, fixed in 4% buffered OsO_4 for 30 min, and then dehydrated in a series of EtOH solns, followed by 100% Me_2CO. The slices were embedded in an Epon-Araldite mixture, sectioned and observed under a TEM.

Phosphotungstic-chromic oxide stain for plasmalemma. Frozen-thawed slices were prepared for EM as described above, but without the incubation in $Pb(NO_3)_2$ and ATP. The sections were specifically stained for plant plasmalemma with PTA–CrO_3 as described in ref. [15].

Biochemicals. All biochemicals were purchased from Sigma Chemical Co., St. Louis, Mo. ATP, Sigma grade II, was used in this study [38].

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