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Plasma Membrane H^+-ATPase Activity in Spores, Germ Tubes, and Haustoria of the Rust Fungus *Uromyces viciae-fabae*

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Struck, C., Hahn, M., and Mendgen, K., 1995. Plasma membrane H^+-ATPase activity in spores, germ tubes, and haustoria of the rust fungus *Uromyces viciae-fabae*. *Fungal Genetics and Biology* 20, 30–35. Using plasma membrane-enriched vesicles, the properties of the H^+-ATPase (EC 3.6.1.35) from the rust fungus *Uromyces viciae-fabae* were studied. The enzyme is strictly Mg^{2+}-dependent and is inhibited by vanadate. The pH-optimum is at 6.7. By Western blot analysis using a monoclonal antibody against corn plasma membrane H^+-ATPase a polypeptide of approximately 104 kDa could be detected. The vanadate-sensitive H^+-ATPase activity of microsomal vesicles obtained from different stages of rust development was determined. Uredospores had only a very low enzyme activity (1.9 μmol Pi × mg^{-1} protein × h^{-1}). In germ tubes the ATPase activity was about twofold higher (4.0 μmol Pi × mg^{-1} protein × h^{-1}). An eightfold higher ATPase activity (16.1 μmol Pi × mg^{-1} protein × h^{-1}) was found in microsomal vesicles from haustoria which had been isolated from rust-infected *Vicia faba* leaves. These results suggest, that the electrochemical gradient generated by the H^+-ATPase of haustoria plays an important role for their function, possibly by promoting nutrient uptake from host cells.

Index Descriptors: **ATPase; biotrophic fungi; haustorium; nutrient uptake; plant pathogen.**

Biotrophic plant pathogens absorb their nutrients from living host cells. In powdery mildew and rust fungi, haustoria seem to play a major role in this process (Mendgen, 1981; Woods and Gay, 1987; Mendgen and Nass, 1988; Aist and Bushnell, 1991), but the mechanism of metabolite uptake is poorly understood. Active transport of nutrients across the host–haustorial interface has been suggested. This was founded on ultrastructural localization of ATPase activity in the fungal plasma membrane (Spencer-Phillips and Gay, 1981; Woods and Gay, 1987). The plasma membrane H^+-ATPase of fungi and plants generates the electrochemical proton gradient which is essential for active transport of solutes into cells. So far there was no experimental evidence for the activity of H^+-ATPase in dikaryotic haustoria of rust fungi.

A new method to isolate rust haustoria from infected plant material (Hahn and Mendgen, 1992) has opened the possibility to characterize ATPase in hyphae and haustoria and to study the haustorial function.

In this paper we present a procedure for the isolation of the plasma membrane-enriched fraction from *Uromyces viciae-fabae* and examine some properties of the plasma membrane H^+-ATPase, from *Uromyces* spores, germ tubes, and haustoria.

MATERIALS AND METHODS

Fungal Material

Uredospores of *Uromyces viciae-fabae* (Pers.) Schroet. were produced on *Vicia faba* cv. Con Amore as described by Deising *et al.* (1991).

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Development of Germ Tubes

Five-gram uredospores were washed in 5 liters of 0.01% Tween 20, filtered, and suspended again in approximately 3 liters of 0.01 % Tween 20. For induction of germ tube development in submerged culture the suspension was stirred in darkness for 3 h at 20°C. Germinated uredospores were then collected by filtration through a glass fiber filter and immediately frozen under liquid nitrogen for subsequent homogenization.

Isolation of Haustoria

Haustoria were isolated by binding on a ConA-Sepharose macrobead column as described by Hahn and Mendgen (1992) with the exception that BSA[2] was omitted from the homogenization and the suspension buffers. Pellets of approximately 1–3 × 10^7 haustoria contaminated with 1–2 times the number of chloroplasts were frozen under liquid nitrogen and stored at –70°C.

Isolation of Membranes

To isolate microsomal membranes of *Uromyces viciae-fabae* a modified technique of Serrano (1988) was used. Microsomes of uredospores, germ tubes, and haustoria were isolated in a homogenization medium containing 25 m*M* Tris/HCl (pH 8.0), 0.5 m*M* PMSF, 0.2 m*M* EDTA, and 0.4 *M* sucrose and separated by differential centrifugation. Uredospores were homogenized in a mortar at 4°C using sea sand. Germinated spores and haustoria were homogenized in the same way but under liquid nitrogen. In the case of germ tubes this procedure largely prevents the breaking of spores. The following steps were performed at 4°C. Homogenates of spores and germ tubes were filtered through two layers of miracloth and centrifuged at 1,500*g* in a Sorvall SS34 rotor for 10 min. Haustorial homogenate was centrifuged in the same manner but without filtration. Each pellet was suspended in a small volume of fresh homogenization buffers using a glass-teflon homogenizer and pelleted again at 1,500 *g*. The supernatants of both steps were then centrifuged at 10,000*g* for 10 min in the same rotor. To obtain the microsomal pellets, the resulting supernatants of spore and germ tube homogenates were centrifuged at 50,000*g* for 1 h in a Sorvall SS34 rotor. To pellet haustorial microsomes the supernatant was centrifuged in a Sorvall RP80 rotor at 50,000*g* for 1 h. Each membrane pellet was suspended in suspension buffer (10 m*M* Tris/HCl, pH 7.5, 0.2 m*M* EDTA, 0.2 m*M* dithioerythritol) containing 20% glycerol using a glass-teflon homogenizer. The suspensions were frozen under liquid nitrogen and stored at –70°C.

Isolation of Plasma Membrane-Enriched Fraction

Discontinuous sucrose gradients were prepared in 4-ml ultracentrifuge tubes consisting of 0.6 ml 40% (w/w) sucrose and 1.3 ml each of 32% and 25% (w/w) sucrose in suspension buffer. Gradients were overlayered with 0.4 ml microsomal membranes in suspension buffer and centrifuged at 100,000*g* for 1 h in a Sorvall RP80 rotor. Material accumulated in the 25/32% interface was collected and diluted with 3 vol of suspension buffer without glycerol and again centrifuged at 105,000*g* for 1 h in the same rotor. The resulting pellets were suspended in a small volume of suspension buffer.

Protein Determination

Protein determination was based on the method of Bradford (1976), by using the Bio-Rad microassay adapted for microtiter plates.

ATPase Assay

The measurements of ATPase-activity were carried out in microtiter plates, in a final volume of 60 μl. The reaction buffer was prepared as described at Hodges and Leonard (1974) with some modifications. Except when otherwise stated it consisted of 3 m*M* ATP, 5 m*M* $MgSO_4$, 50 m*M* Tris/MES (pH 6.5), 50 m*M* KCl, 50 m*M* KNO_3, 5 m*M* NaN_3, 0.2 m*M* $(NH_4)_6MO_7O_{24}$, and 5 μg membrane protein. The assay was performed in the presence and absence of 50 μ*M* Na_3VO_4. The reaction was allowed to proceed for 10 min at 36°C and stopped by adding 100 μl of 1% (w/v) SDS solution. The released inorganic phosphate was quantified photometrically as described by Ames (1966) using an ELISA reader. Unless otherwise stated the results are the means obtained from three to five independent membrane preparations, with all measurements being performed in two or three replications.

SDS–PAGE and Immunodetection

SDS–polyacrylamide gel electrophoresis was carried out according to Schägger and von Jagow (1987). Protein staining was done with silver nitrate.

[2] Abbreviations used: BSA, bovine serum albumin; DES, diethylstilbestrol; PVDF, polyvinylidene fluoride; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel; TBS, Tris-buffered saline.

Electroblotting onto PVDF membranes (Millipore, U.S.A.) was performed according to Kyhse-Andersen (1984) and immunological detection was done as described by Blake *et al.* (1984). The membrane was blocked in TBS (20 m*M* Tris, 150 m*M* NaCl, pH 7.6) containing 2% nonfat dried milk for 1.5 h. Monoclonal antibodies raised against corn plasma membrane H^+-ATPase (Villalba *et al.*, 1991) were used as hybridoma supernatant diluted 1:100 in TBS. The second antibody conjugated to alkaline phosphatase (anti-mouse lgG, Sigma) was diluted 1:5000 in TBS.

RESULTS

Enrichment of Plasma Membrane Vesicles

Experiments were started with germinated spores because they provided the most abundant source of rust plasma membranes. Enrichment of the plasma membrane fraction was performed with a discontinuous sucrose density gradient. As marker the activity of the vanadate-sensitive H^+-ATPase was measured in presence of azide (5 m*M*), nitrate (50 m*M*), and molybdate (0.2 m*M*). A twofold increase of the vanadate-sensitive ATPase activity in the plasma membrane-enriched fraction compared with crude microsomal membranes was achieved (Fig. 1). Inhibition of the ATPase activity by increasing vanadate concentrations was significantly stronger in the plasma membrane-enriched fraction than in microsomes (Fig. 2).

On Western blots of SDS–PAGE separated membrane proteins of rust germ tubes a heterologous monoclonal antibody against the corn plasma membrane H^+-ATPase detects two polypeptides with 98 and 104 kDa (Fig. 3). The smaller band could be due to proteolytic degradation products. The bands recognized by the antibody were stronger in the plasma membrane-enriched fraction than in the crude microsomal fraction, confirming the enrichment of H^+-ATPase.

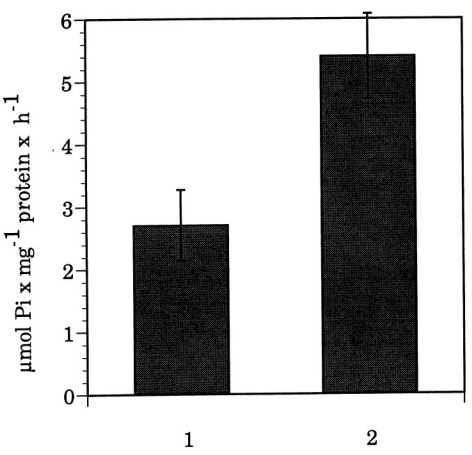

FIG. 1. Activity of vanadate-sensitive ATPase in germ tubes of *Uromyces viciae-fabae*. Column 1, crude microsomal fraction. Column 2, plasma membrane-enriched fraction. Standard deviations are indicated.

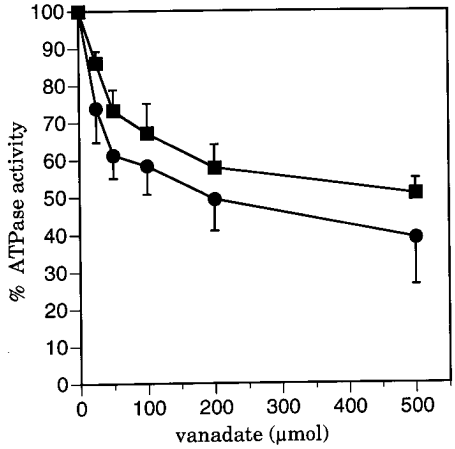

FIG. 2. Effect of vanadate on ATPase activity. ■, Crude microsomal fraction. ●, Plasma membrane-enriched fraction.

Characterization of Vanadate-Sensitive H^+-ATPase

To determine the characteristics of the vanadate-sensitive H^+-ATPase we used the plasma membrane-

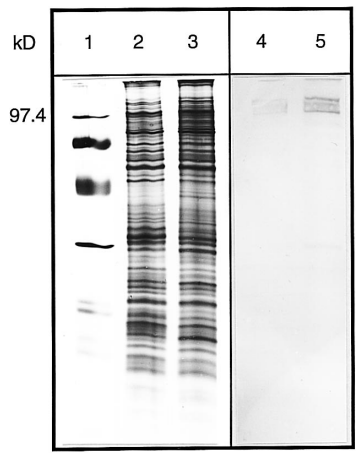

FIG. 3. SDS–PAGE and Western blot of rust germ tube membrane proteins. Lanes 1–3, silver stained; 4, 5, Western blot: 1, molecular mass standards from Bio-Rad; 2, 4, crude microsomal fraction; 3, 5, plasma membrane-enriched fraction.

enriched fractions of uredospores and germ tubes produced in submerged culture.

The influence of pH on the ATPase activity is shown in Fig. 4. A broad pH optimum ranging from pH 6.5 to pH 7.0 with a peak at pH 6.7 was observed.

Further characteristics of the enzyme of germ tubes are shown in Table 1. The ATPase activity is strictly dependent on Mg^{2+}. The remaining, Mg^{2+} independent enzyme activity was not inhibited by vanadate (not shown). A slight stimulation of ATPase activity could be observed in the presence of potassium. Therefore, all enzymatic measurements were done with 5 m*M* $MgSO_4$ and 50 m*M* KCl.

Besides vanadate, another inhibitor of fungal and plant plasma membrane H^+-ATPases is diethylstilbestrol (DES). This inhibitor was also active against the rust enzyme.

In the presence of GTP, instead of ATP as substrate of the enzyme a hydrolyzing activity was also observed which, however, was unaffected by vanadate. This shows that the vanadate-sensitive ATPase activity of *U. viciae-fabae* has a strong preference for ATP as substrate.

TABLE 1
Characteristics of ATPase Activity from Germ Tubes of *Uromyces viciae-fabae*

Assay medium[a]	ATPase activity (μmol Pi × mg^{-1} protein × h^{-1})
Control	14.05
$-Mg^{2+}$	2.28
$-K^+$	13.37
+50 μM vanadate	5.95
+1% EtOH	14.00
+100 μM DES in EtOH	8.44
GTP instead of ATP	12.21
GTP + vanadate	11.61

[a] The assay medium contained 5 m*M* NaN_3, 50 m*M* KNO_3, and 0.2 m*M* $(NH_4)_6MO_7O_{24}$.

Comparison of ATPase Activity in Rust Spores, Germ Tubes, and Haustoria

We examined the H^+-ATPase activity of uredospores, germ tubes, and haustoria isolated from infected leaves. Due to the tiny amounts of plasma membrane vesicles obtained from haustoria we used crude microsomes of all three fungal stages for comparison. The results are shown in Fig. 5. In uredospores only a low ATPase activity could be observed and an approximately twofold increase of activity in germ tubes. By far the highest ATPase activity was shown by the microsomal vesicles of haustoria: it was more than eightfold in comparison with the spores and nearly fourfold compared with the germ tubes. Due to the chloroplast contamination of the haustoria pellet control assays with chloroplasts isolated from uninfected leaves were performed. Only a very low value of vanadate-sensitive ATPase activity was detectable (Fig. 5).

DISCUSSION

The main problem for the isolation of plasma membranes from *Uromyces viciae-fabae* is the contamination with vesicles from intracellular membranes. We used a sucrose gradient similar to the protocol used by Giannini

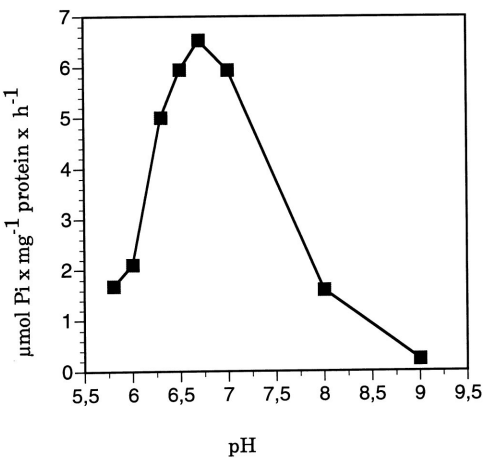

FIG. 4. pH dependence of vanadate-sensitive ATPase activity.

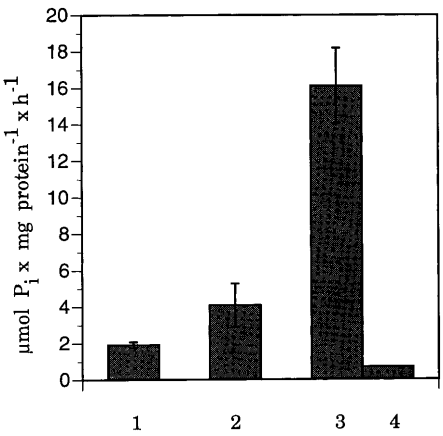

FIG. 5. Activity of vanadate-sensitive ATPase at different stages of rust developments. Enzyme assays were performed with crude microsomal vesicles. 1, spores; 2, germ tubes; 3, haustoria; 4, chloroplasts.

et al. (1988) for the preparation of putative plasma membrane vesicles (20/35% sucrose interface) from *Phytophthora megasperma.* By collecting the 25/32% interface we achieved a twofold enrichment of vanadate-sensitive ATPase compared with the crude microsomes. Inhibition by vanadate represents a specific trait of plasma membrane ATPases and thus served as a marker for the plasma membrane fraction.

Due to the well-known structural and functional similarities between the fungal and plant plasma membrane H^+-ATPase (Sussman, 1994) we used a monoclonal antibody directed toward a plant enzyme. We found a stronger cross-reactivity between the corn plasma membrane antibody and the rust enzyme of the plasma membrane-enriched fraction than with the rust microsomal fraction. This result represents a further indication for the enrichment of plasma membrane H^+-ATPase in the plasma membrane fraction.

The characteristics of the plasma membrane H^+-ATPase of *Uromyces viciae-fabae,* such as the requirement for Mg^{2+} and the substrate specificity for ATP, agree well with those of other fungi. The pH value of 6.7 is identical to that of *Neurospora crassa* (Bowman and Slayman, 1979) and close to that of *Phytophthora megasperma* (pH 6.5; Giannini *et al.,* 1988) and *Candida albicans* (pH 6.4; Hubbard *et al.,* 1986). These parameters distinguished the vanadate-sensitive plasma membrane ATPase from other ATPases found in mitochondria and in vacuoles and from unspecific phosphatases (reviewed by Bowman and Bowman, 1986). A slight stimulation by K^+ which is typical for fungal plasma membrane ATPases (Serrano, 1988) was also found for the *Uromyces viciae-fabae* enzyme.

Previous ultrastructural studies with the lead/phosphate precipitation technique did not detect ATPase activity in the haustorial plasma membrane of *Uromyces appendiculatus* but in the plasma membrane of *Erysiphe pisi* haustoria (Spencer-Phillips and Gay, 1981). This cytochemical technique, however, has been critized as being not specific for the H^+-ATPase (Katz *et al.,* 1988).

Our studies are the first direct demonstration for the presence of H^+-ATPase activity in a rust fungus. Comparing the H^+-ATPase activity of different developmental stages of the rust fungus we observed distinct differences. A very low value in uredospores, a twofold higher activity in germ tubes, and, compared to these two stages, a drastic increase of ATPase activity—approximately fourfold higher than in germ tubes—was observed in haustoria. These results suggest that the activity of the H^+-ATPase in rust fungi is developmentally regulated. This is supported by the observation that the addition of 2% glucose to germinated spores did not stimulate their ATPase activity (data not shown). This is in contrast to the situation found in *Saccharomyces cerevisiae,* where glucose was found to stimulate both the specific activity and gene expression of the H^+-ATPase (Rao *et al.,* 1993). In *Candida albicans,* ATPase activity was increased in germ tube forming cells as compared to yeast-like cells, and also a stimulation was observed by glucose (Monk *et al.,* 1993).

Our finding that the rust H^+-ATPase shows the highest activity in haustoria suggests an involvement of this enzyme in the nutrition of the fungus. A proton gradient across the haustorial membrane generated by this enzyme could drive the active transport of nutrients derived from the plant cell. This mechanism would explain the heavy metabolite losses of the infected leaves occurred after haustoria formation (Farrar and Lewis, 1987). In contrast, no or little nutrient uptake takes place in resting and germinating rust spores (Maclean, 1982), which were shown here to have only very low ATPase activity.

Our current studies are directed toward a further characterization of the electrogenic properties of the ATPase and the nature of possible carrier proteins which are dependent on its activity. In addition we have cloned the gene for this enzyme from *Uromyces viciae-fabae* in order to study the regulation of the plasma membrane H^+-ATPase on a molecular level (Siebels, Hahn, and Mendgen, unpublished data).

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REFERENCES

Aist, J. R., and Bushnell, W. R. 1991. Invasion of plants by powdery mildew fungi, and cellular mechanisms of resistance. In *The Fungal Spore and Disease Initiation in Plants and Animals.* (G. T. Cole and H. C. Hoch, Eds.), pp. 321–345. New York/London.

Ames, B. N. 1966. Assay of inorganic phosphate, total phosphate and phosphatase. *Methods Enzymol.* **8:** 115–118.

Blake, M. S., Johnston, K. H., Russel-Jones, G. J., and Gotschlich, E. C. 1984. A rapid, sensitive method for detection of alkaline phosphatase-conjugated anti-antibody on Western blots. *Anal. Biochem.* **136:** 175–179.

Bowman, B. J., and Bowman, E. J. 1986. H^+-ATPases from mitochondria, plasma membranes, and vacuoles of fungal cells. *J. Membrane Biol.* **94:** 83–97.

Bowman, B. J., and Slayman, C. W. 1979. The effects of vanadate on the plasma membrane ATPase of *Neurospora crassa*. *J. Biol. Chem.* **254:** 2928–2934.

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

Deising, H., Jungblut, P. R., and Mendgen, K. 1991. Differentiation-related proteins of the broad bean rust fungus *Uromyces viciae-fabae*, as revealed by high resolution two-dimensional polyacrylamide gel electrophoresis. *Arch. Microbiol.* **155:** 191–198.

Farrar, J. F., and Lewis, D. H. 1987. Nutrient relations in biotrophic infections. In *Fungal Infection of Plants.* (G. F. Pegg and P. G. Ayres, Eds.) pp. 92–132. Cambridge.

Giannini, J. L., Holt, J. S., and Briskin, D. P. 1988. Isolation of sealed plasma membrane vesicles from *Phytophthora megasperma* f.sp. *glycinea* I. Characterization of proton pumping and ATPase activity. *Arch. Biochem. Biophys.* **265:** 337–345.

Hahn, M., and Mendgen, K. 1992. Isolation by ConA binding of haustoria from different rust fungi and comparison of their surface qualities. *Protoplasma* **170:** 95–103.

Hodges, T. K., and Leonhard, R. T. 1974. Purification of a plasma membrane-bound adenosine triphosphatase from plant roots. *Methods Enzymol.* **32:** 392–406.

Hubbard, M. J., Surarit, R., Sullivan, P. A., and Shepherd, M. G. 1986. The isolation of plasma membrane ATPase from the yeast *Candida albicans*. *Eur. J. Biochem.* **154:** 375–381.

Katz, D. B., Sussman, M. R., Mierzwa, R. J., and Evert, R. F. 1988. Cytochemical localization of ATPase activity in oat roots localizes a plasma membrane-associated soluble phosphatase, not a proton pump. *Plant Physiol.* **86:** 841–847.

Kyhse-Andersen, J. 1984. Electroblotting of multiple gels: A simple apparatus without buffer tank for rapid transfer of proteins from polyacrylamide to nitrocellulose. *J. Biochem. Biophys. Methods* **10:** 203–209.

Maclean, D. J. 1982. Axenic culture and metabolism of rust fungi. In *The Rust Fungi* (K. J. Scott and A. K. Chakravorty, Eds.), pp. 37–120. Academic Press, London.

Mendgen, K. 1981. Nutrient uptake in rust fungi. *Phytopathology* **71:** 983–989.

Mendgen, K., and Nass, P. 1988. The activity of powdery-mildew haustoria after feeding the host cells with different sugars, as measured with a potentiometric cyanine dye. *Planta* **174:** 283–288.

Monk, B. C., Niimi, M., and Shepherd, M. G. 1993. The *Candida albicans* plasma membrane and H^+-ATPase during yeast growth and germ tube formation. *J. Bacteriol.* **175:** 5566–5574.

Rao, R., Drummond-Barbosa, D., and Slayman, C. W. 1993. Transcriptional regulation by glucose of the yeast PMA1 gene encoding the plasma membrane H^+-ATPase. *Yeast* **9:** 1075–1084.

Schägger, H., and von Jagow, G. 1987. Tricine-sodium dodecyl sulfate-polyacrylamide gel elctrophoresis for the separation of proteins in the range from 1 to 1000 kDa. *Anal. Biochem.* **166:** 368–379.

Serrano, R. 1988. H^+-ATPase from plasma membrane of *Saccharomyces cerevisiae* and *Avena sativa* roots: Purification and reconstitution. *Methods Enzymol.* **157:** 533–544.

Spencer-Phillips, P. T. N., and Gay, J. L. 1981. Domains of ATPase in plasma membranes and transport through infected plant cells. *New Phytologist* **89:** 393–400.

Sussman, M. R. 1994. Molecular analysis of proteins in the plant plasma membrane. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **45:** 211–234.

Villalba, J. M., Luetzelschwab, M., and Serrano, R. 1991. Immunocytolocalization of plasma-membrane H^+-ATPase in maize coleoptiles and enclosed leaves. *Planta* **185:** 458–461.

Woods, A. M., and Gay, J. L. 1987. The interface between haustoria of *Puccinia poarum* (monokaryon) and *Tussilago farfara*. *Physiol. Mol. Plant Pathol.* **30:** 167–185.