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The Hypersensitive Reaction of Tobacco to *Pseudomonas syringae* pv. *pisi*[1]

ACTIVATION OF A PLASMALEMMA K^+/H^+ EXCHANGE MECHANISM

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ABSTRACT

Net electrolyte efflux from suspension-cultured tobacco cells undergoing the hypersensitive reaction to *Pseudomonas syringae* pv. *pisi* resulted from a specific efflux of K^+ which was accompanied by an equimolar net influx of H^+. These fluxes began 60 to 90 minutes after inoculation of tobacco cells with bacteria, reached maximum rates of 6 to 9 micromoles per gram fresh weight tobacco cells per hour within 2.5 to 3 hours, and dropped below 4 micromoles per gram per hour within 5 hours. Tobacco cells lost approximately 35% of total K^+ during this period, and average cellular pH declined by approximately 0.75 pH unit. These events were accompanied by a 30% decrease in cellular ATP. K^+ and H^+ fluxes were inhibited by the protonophore (*p*-trifluoromethoxy)carbonyl cyanide phenylhydrazone and by increasing the K^+ concentration of the external solution. Tobacco leaf discs inoculated with the bacterium also exhibited a specific net K^+ efflux and H^+ influx. These results suggest that induction of the hypersensitive reaction in tobacco proceeds through the activation of a passive plasmalemma K^+/H^+ exchange mechanism. It is hypothesized that activation of this exchange is a major contributing factor in hypersensitive plant cell death.

The hypersensitive reaction is characterized by the rapid death of individual plant cells which come into contact with pathogenic organisms, and is generally associated with disease resistance of the whole plant to the pathogen (11, 13, 14). The capacity to express the HR[3] appears to be universal among higher plants and can be triggered by bacterial, fungal, viral, and nematode pathogens. Despite its close association with resistance, the HR and its role in pathogen localization are not well understood. It has even been argued that hypersensitivity is a consequence rather than a cause of disease resistance (11). The clarification of these issues will require a thorough understanding of the molecular basis for and consequences of the HR.

This report deals with the HR of tobacco to an incompatible bacterial pathogen, *Pseudomonas syringae* pv. *pisi*. Our specific objective was to conduct a detailed study of electrolyte loss from hypersensitive tobacco cells. Several reports have demonstrated that electrolyte loss occurs during the early stages of the HR of various hosts to pathogenic bacteria (5, 6, 9, 18). Ion flux studies in our laboratories were facilitated by the use of suspension-cultured tobacco cells. We have determined that these cells express the HR when inoculated with incompatible bacteria, and that electrolyte loss begins within 1.5 h after inoculation (3). This symptom is induced weakly or not at all by saprophytic or compatible bacteria such as *P. fluorescens* and *Agrobacterium tumefaciens* or by nonviable *P. syringae* pv. *pisi*. In this report we demonstrate that electrolyte loss from hypersensitive tobacco cells results from a specific efflux of K^+ and net influx of H^+. We suggest that this K^+/H^+ exchange phenomenon provides a plausible physiological explanation for hypersensitive cell death, and we discuss its molecular basis. A preliminary report of this work has been given (4).

MATERIALS AND METHODS

Pseudomonas syringae pv. *pisi* Sackett, a pathogen of pea, was obtained from R. N. Goodman, University of Missouri. Bacteria were streaked onto nutrient agar plates and incubated at 25°C for 16 to 20 h before each experiment. Bacteria were suspended in standard assay medium (0.175 M mannitol, 0.5 mM K_2SO_4, 0.5 mM $CaCl_2$, 5 mM Mes adjusted to pH 6.0 with Tris), washed twice by centrifugation, and resuspended in this medium to an inoculum density of 8×10^9 viable bacteria/ml unless otherwise stated. Suspension-cultured tobacco cells were derived from *Nicotiana tabacum* var Hicks (10) and maintained as previously described (3). Cells were collected by filtration from logarithmically growing cultures, washed with 10 ml assay medium/g fresh weight tobacco, and resuspended in 28.5 ml assay medium/g tobacco. Cell suspensions were incubated in 50-ml beakers at 27°C on a reciprocal shaker at 150 oscillations/min for approximately 1 h. This preincubation period was necessary for consistent induction of the HR. Tobacco cell suspensions were then inoculated with 1.5 ml bacterial suspension/g tobacco. This procedure gave a final concentration of 4×10^8 viable bacteria/ml and induced a maximum HR response in tobacco cells. Inoculated tobacco cell suspensions were incubated for 30 min to allow attachment of bacteria to tobacco cells. Attachment of bacterial cells to plant cell walls is believed to be required for induction of the HR (12, 26). At the end of the 30-min period, tobacco cells were collected by filtration and washed with 50 ml assay medium/g to remove unattached bacteria. This step served also to synchronize HR induction in tobacco cells by attached bacteria. Washed cells were resuspended in fresh assay medium

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[3] Abbreviations: HR, hypersensitive reaction; Mops, morpholinopropane sulfonic acid; FCCP, (*p*-trifluoromethoxy)carbonyl cyanide phenylhydrazone.

and returned to incubation. Experimental determinations were carried out for up to 5 h after inoculation. Control (uninoculated) cells were treated as above except that an aliquot of assay medium was substituted for the bacterial inoculum. Exceptions to the above procedures were made for several experiments: the concentration of Mes in the assay medium was reduced from 5 mM to 0.5 mM for H^+ influx assays, K_2SO_4 and $CaCl_2$ were omitted from the assay medium for net cation efflux assays reported in Figure 1, and bacterial inoculum density varied from 0 to 1.2×10^{10} viable bacteria/ml for the dose:response experiments.

The number of viable bacterial cells attached/g of tobacco cells was determined as follows. At the end of the 30-min attachment period, tobacco cells were washed with 50 ml sterile assay medium/g and ground with a mortar and pestle containing sterile assay medium and 0.2-mm glass beads. The concentration of viable bacteria in the homogenate was determined by dilution plate count on nutrient agar. The number of tobacco cells/g fresh weight tobacco was estimated by making serial dilutions of suspended tobacco cells and counting (with a light microscope) the number of cells/unit volume.

Net H^+ fluxes were determined by titration of assay medium containing tobacco cells to pH 6.0 with 10 mM NaOH or HCl at 15- to 30-min intervals. Since uninoculated cells normally exhibited a H^+ efflux, hypersensitive H^+ influx was defined as the difference between net H^+ fluxes determined simultaneously in control and inoculated tobacco cells. A combination pH electrode with a calomel reference was used for pH measurements.

For determination of net K^+ efflux, 0.5-ml aliquots of assay medium were removed from tobacco cell suspensions at 15- to 30-min intervals. An automatic pipet tip with Miracloth taped over the aperture to exclude cells was used to draw samples. Each sample was replaced with a like volume of assay medium. K^+ content of the collected samples was determined by atomic absorption spectroscopy. Net effluxes of other cations (Na^+, Ca^{2+}, and Mg^{2+}) were also determined in this manner. K^+ influx was indirectly determined by the rate of $^{86}Rb^+$ uptake by tobacco cells. $^{86}Rb^+$ (approximately 10^5 dpm) was added to 7.5 ml assay medium containing 0.25 g tobacco. Immediately or after a 15- or 20-min uptake period, cells were collected by suction filtration and washed with 60 ml cold (0 to 4°C) assay medium supplemented with 4.5 mM $CaCl_2$ and 0.5 mM K_2SO_4. The data given are the differences between the uptake values at 15 or 20 min and the values determined immediately after isotope addition. The $^{86}Rb^+$ content of the cells was determined with a Geiger counter. Preliminary experiments indicated that the rate of $^{86}Rb^+$ uptake was constant for up to 60 min. The data exhibited a linear regression which extrapolated to zero at zero time. Therefore, any error due to isotope exchange was minimal.

Total K^+ was determined on 0.5-g samples of tobacco cells washed in K^+-free assay medium. Cells were drained for about 1 min on absorbent paper and transferred into 9.5 ml 1% (v/v) Triton X-100. These suspensions were subjected to gentle shaking for at least 2 h and then filtered to remove cell debris. The K^+ content of the filtrates was determined by atomic absorption spectroscopy.

The pH of the total cell sap was determined by a modification of a previously published procedure (15). A 2.0-g sample of callus cells was placed in a Miracloth filter, washed with 10 ml of distilled H_2O, and blotted dry. The cells were then ruptured by pressing the sap through a syringe, and sap pH was measured immediately using a combination microelectrode.

For the ATP experiments, a modified inoculation procedure was employed. Tobacco cell cultures (0.2 ml packed cell volume/ml) were inoculated directly with bacteria to give a bacterial density of 3×10^7 bacterial/ml. In contrast to the standard protocol, the bacteria cells were not removed and the tobacco cells remained in the culture media throughout the experiments. Under these conditions, the ATP levels in the controls (uninoculated) remained constant.

ATP was extracted from 0.5-g samples of tobacco cells plunged into 5 ml boiling ethanol for 3 min. Cell suspensions were cooled on ice and ground thoroughly in a ground glass homogenizer. Homogenates were evaporated to dryness under a stream of nitrogen and resuspended in 5 ml 120 mM Mops, 5 mM $MgCl_2$ (pH 7.4). ATP was determined by a luciferin-luciferase assay as described by White and Knopp (28).

Tobacco plants (*Nicotiana tabacum* var Hicks) were grown in a greenhouse for 8 to 12 weeks after transplanting. Fully expanded upper leaves were inoculated by injecting bacterial suspensions of 1×10^8 viable bacteria/ml into the intercellular spaces. Net K^+, H^+, Mg^{2+}, and Cl^- fluxes were determined on tobacco leaf halves inoculated with bacteria or with water as control. Inoculated or control leaf halves were allowed to air-dry for approximately 2 h and then 1-cm discs were cut from the appropriate areas. Discs (approximately 0.3 g) were vacuum infiltrated with assay medium and transferred to 25 ml fresh assay medium. Net cation fluxes were measured as described for suspension-cultured tobacco cells. Net Cl^- efflux was measured with a chloridometer. All reported values represent the difference between inoculated and control leaf disc measurements.

RESULTS

Analysis of net cation efflux from inoculated tobacco callus cells (Fig. 1) showed that K^+ was rapidly lost from cells whereas other cations (Na^+, Mg^{2+}, Ca^{2+}) were not. This specificity of cation efflux was maintained for at least 6 h after inoculation of tobacco cells with bacteria, after which a net efflux of other cations was observed. Inoculated tobacco cells also increased the pH of the assay medium. This was interpreted as a net H^+ influx by these cells. Control (uninoculated) cells exhibited a net efflux of H^+. Because of this inherent and opposite H^+ flux, hypersensitive H^+ influx was first observed as a decreased efflux relative to the control.

The HR response of suspension-cultured tobacco cells, as measured by hypersensitive H^+ uptake, reached a maximum when tobacco cells were inoculated with 2×10^8 viable bacteria/ml for a 30-min attachment period (Fig. 2). A maximum K^+ efflux response was observed at essentially the same bacterial inoculum density (data not shown). This inoculum density resulted in an average of approximately 100 bacteria attached/tobacco cell. However, a significant response was observed with

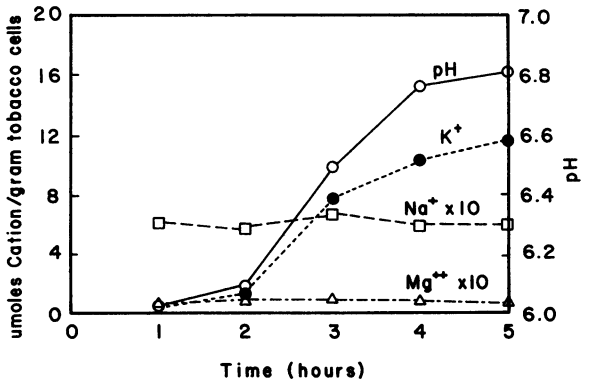

FIG. 1. Cumulative cation loss from, and increase in the assay medium pH, tobacco callus cells inoculated with *P. syringae* pv. *pisi*. Tobacco cells (0.5 g, fresh weight) were incubated in 15 ml assay medium. Data ($n = 3$) represent the concentrations of cations or the pH of the medium at intervals after inoculation of tobacco cells with bacteria. Na^+ and Mg^{2+} and are shown at 10 times actual concentrations. The data for Ca^{2+} is superimposable with that for Mg^{2+} and is not shown.

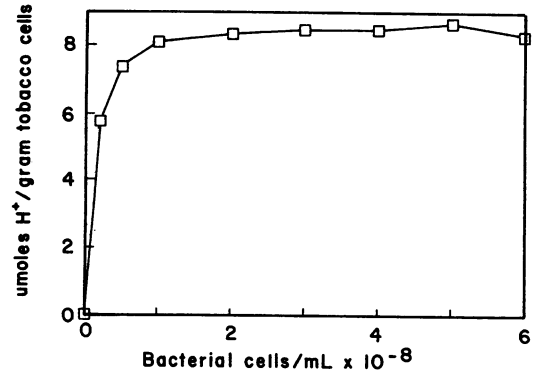

FIG. 2. Effect of bacterial inoculum density on H^+ uptake in tobacco callus cells. Tobacco cells were incubated in assay medium containing *P. syringae* pv. *pisi* cells at the indicated density for 30 min, washed to remove unattached bacteria, and resuspended in fresh assay medium. Data (n = 2) represent the difference between net proton transport in inoculated and control tobacco cells during the first 3.5 h after inoculation.

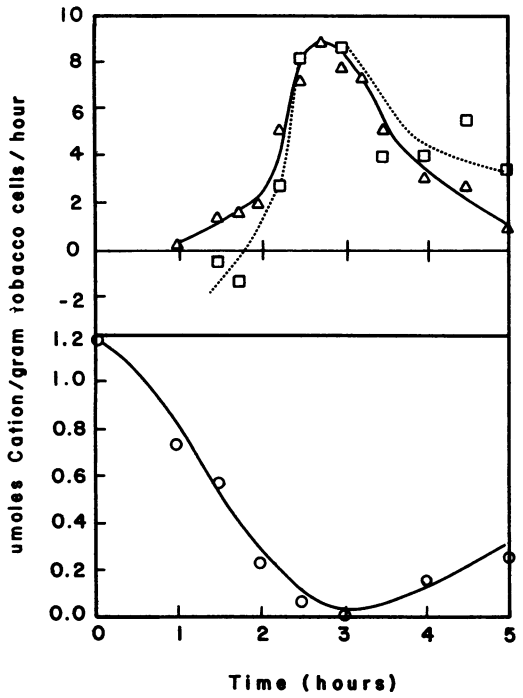

FIG. 3. Upper, Net K^+ and H^+ fluxes in tobacco callus cells after inoculation with *P. syringae* pv. *pisi*. H^+ fluxes were measured by intermittent titration of assay medium to pH 6.0 with acid or base. Net H^+ influx (Δ) represents the difference between H^+ transport in inoculated and control tobacco cells. K^+ efflux (□) represents the net K^+ efflux from inoculated tobacco cells. Lower, K^+ influx (○) was determined by measurement of Rb^{86} uptake rate by tobacco cells. Data are means of three determinations.

relatively low inoculum levels. The ratio of bacteria attached/tobacco cell at half maximum dose response was approximately one.

K^+ efflux and net H^+ influx in tobacco cells began 60 to 90 min after inoculation with bacteria (Fig. 3). Transport rates increased rapidly, reaching 6 to 9 μmol/g·h between 2.5 and 3 h after inoculation. K^+ efflux declined very rapidly between 3 and 4 h after inoculation and then exhibited a slow decline to 3 to 4 μmol/g·h within 5 h after inoculation. H^+ influx declined rapidly between 3 and 5 h after inoculation, reaching 1 to 2 μmol/g·h within 5 h after inoculation. A stoichiometry of approximately 1 K^+:1 H^+ was observed for experimental points up to 3.5 h after inoculation. After this time, K^+ efflux exceeded H^+ influx.

The rate of K^+ uptake in tobacco cells just prior to inoculation with bacteria was aproximately 1 μmol/g·h (Fig. 3). Influx declined rapidly between 1 and 3 h after inoculation and remained near zero or increased slighty thereafter.

Net K^+ loss from tobacco cells reflected primarily the rapid efflux but also a decreased influx of this ion. Cells lost up to 35% of total K^+ between 1 and 5 h after inoculation (Fig. 4). Total K^+ content of control (uninoculated) cells was 50 to 60 μmol/g and did not decrease during the 5-h experimental period. H^+ uptake was evidenced by an acidification of the cell sap (Fig. 4). Examination by light microscopy indicated that tobacco callus cells were highly vacuolated, with cytoplasm making up only about 10% of total cell sap volume. Analysis of K^+ compartmentation by treatment of tobacco cells with 5% DMSO (7) indicated that K^+ content of the cytoplasm was approximately 8% of total cellular K^+ (data not shown). Average values for K^+ loss and changes in cell sap pH are therefore most representative of vacuolar conditions. Cytoplasmic conditions during the HR may deviate more or less than is indicated by these average values. ATP content of control (uninoculated) tobacco cells was approximately 40 nmol/g. The ATP content of inoculated cells declined approximately 30% within 5 h after inoculation (Table I).

FCCP, a proton ionophore and uncoupling agent, eliminated hypersensitive H^+ uptake (Table II). K^+ efflux declined significantly during this treatment but did not stop, indicating that some K^+ efflux was possible in the absence of net H^+ influx. K^+ efflux could also be decreased by increasing the K^+ concentration

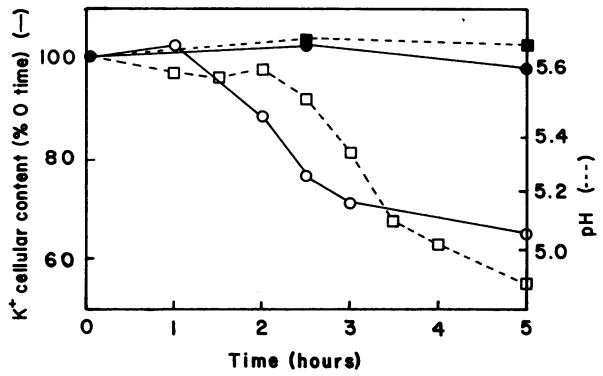

FIG. 4. Net loss of K^+ from tobacco callus cells and acidification of tobacco callus cell sap during the first 5 h of the HR. Total K^+ content and pH of cell homogenates were determined at intervals after inoculation with *P. syringae* pv. *pisi*. (○, □), Inoculated tobacco cells; (●, ■), control tobacco cells. Data are means of three determinations.

Table I. *ATP levels in Tobacco Callus Cells Inoculated with P. syringae pv. pisi*

Total ATP content was determined in 0.5-g tobacco cell samples taken at intervals after inoculation of tobacco cells with bacteria. The zero time sample was taken just prior to inoculation and represents the control. Data represent means and SD of three determinations.

Time after Inoculation	ATP Levels	
h	*nmol ATP/g*	*% controls*
0	38.2 ± 2.4	100 ± 6
1.5	38.0 ± 1.4	99 ± 4
3	29.7 ± 2.0	78 ± 5
5	27.2 ± 0.8	71 ± 2

Table II. *Effect of FCCP on H^+ and K^+ Fluxes in Tobacco Callus Cells Inoculated with P. syringae pv. pisi*

FCCP was added to tobacco cell suspensions 2 h and 25 min after inoculation of tobacco cells with bacteria. Ion fluxes were measured for the period between 2.5 and 3 h after inoculation. Data are means and SD of three determinations.

Treatment	Net H^+ Uptake	Net K^+ Efflux
	% control	
None (control)	100 ± 7	100 ± 9
5 μM FCCP	−4.6 ± 4.7	33 ± 19

Table III. *Effect of External [K^+] on K^+ and H^+ Fluxes in Tobacco Callus Cells Inoculated with P. syringae pv. pisi*

The [K^+] of the assay medium was adjusted from 1 mM to 5 or 10 mM 2 h and 25 min after inoculation of tobacco cells with bacteria. Ion fluxes were measured for the period between 2.5 and 3 h after inoculation. Data are means of two determinations.

[K^+]	Net K^+ Efflux	Net H^+ Uptake
mM	*% control*	
1.0 (control)	100	100
5.0	91	85
10.0	85	74

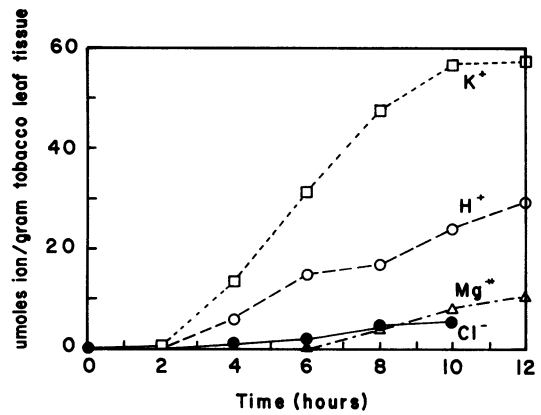

FIG. 5. Total K^+ loss and H^+ uptake by tobacco leaf tissue infiltrated with *P. syringae* pv. *pisi*. Data (n = 3) represent the difference between net transport in inoculated and control tobacco leaf discs. Cl^- and Mg^{2+} losses are also shown.

of the assay medium from 1 to 10 mM (Table III). Under these conditions, H^+ uptake rates exhibited a similar decline.

Tobacco leaf tissue undergoing the HR exhibited an exchange of approximately 2 K^+/H^+ (Fig. 5). K^+ efflux was equivalent to or greater than that observed in cultured cells, but net H^+ uptake was only about half as rapid. Cl^- efflux was slightly greater in hypersensitive tissue than the control but was much less than K^+ or H^+ fluxes. Nonspecific efflux of cations such as Mg^{2+} was observed 6 or more h after inoculation.

DISCUSSION

We have demonstrated that electrolyte loss during the HR of tobacco to *Pseudomonas syringae pv. pisi* begins as a specific K^+ efflux which is accompanied by net H^+ influx. Within 5 h after inoculation K^+/H^+ exchange resulted in the loss of 35% of total K^+ and an acidification of the tobacco cell sap by approximately 0.75 pH unit. K^+ efflux and net H^+ influx slowed but did not stop within this time period, suggesting that K^+ loss and cell sap acidification continue beyond this point. This is in agreement with our report that electrolyte loss from these cells is most rapid at 3 h but continues at a lower rate for at least 12 h after inoculation (3). Most cells lost viability between 10 and 15 h after inoculation.

In view of the fundamental roles played by K^+ and H^+ in plant cells and the general requirement for ionic homeostasis (8, 16, 19, 29), the consequences of K^+/H^+ exchange may be severe. This is particularly true if significant changes occur in cytoplasmic pH and K^+ content. Because of specific K^+ requirements for activation of many enzymes and for energy conservation across membranes (29), low cytoplasmic K^+ concentrations as well as low pH may contribute to declines in respiration (3, 21), RNA synthesis (1), and ATP levels in hypersensitive cells or tissues.

We also suggest that net H^+ influx effectively reduces or negates the ATPase-mediated H^+ efflux across the plasma membrane (25). This would have at least two major consequences. Since this mechanism is believed to provide the driving force for active transport across the plasmalemma (24, 25), active transport should be inhibited. The sharp inhibition of K^+ influx which we observed is consistent with this prediction. Second, plasmalemma H^+ efflux would be dissociated or 'uncoupled' from ATP hydrolysis, leading to a stimulation of the ATPase and respiration. This prediction is supported by the respiratory stimulation which occurs during the early stages of the HR (3, 21).

For the reasons discussed above, we believe that the HR of tobacco to *P. syringae* pv. *pisi* proceeds through the activation of a plasmalemma K^+/H^+ exchange mechanism and that the consequences of this event may be severe enough to account for cell death. In general, our results are consistent with previous work indicating that the HR to bacterial pathogens involves early changes in membrane transport (5, 6, 9, 18) and polarization (22). Evidence for altered plasmalemma H^+ transport in association with the HR (22) and a bacterial leaf spotting disease (23) has been previously reported.

The capacity for rapid K^+ efflux/H^+ influx exchange has been demonstrated in other plant systems including rose (20) and corn root cells (17) and everted membrane vesicles of tobacco (27). These activities, as well as the hypersensitive exchange, appear to be driven by passive movements of K^+ and H^+ across the plasmalemma. It has been proposed that this mechanism functions in the regulation of cytoplasmic pH, [K^+], and the cell membrane potential of higher plant cells (17). Exchange rates can apparently be stimulated to greatly exceed physiological levels. For example, K^+/H^+ exchange in corn root and rose cells was not detected until activated by sulfhydryl compounds or UV radiation. Similarly, we observed bacterial activation in tobacco. Although we have no direct evidence that these activities are related to hypersensitive K^+/H^+ exchange, their existence is supportive of our results.

The data presented here raise a number of questions about the molecular basis of K^+/H^+ exchange and its induction. For example, is the exchange mediated by a specific plasmalemma transport protein(s)? The specificity and rapidity of the fluxes suggest this, but we have no direct evidence for it. Other questions concern the bacterial molecule(s) which elicits the response. It has recently been reported that a purified bacterial pectate lyase rapidly induces K^+/H^+ exchange in cultured tobacco cells (2). However, it remains to be determined whether this or other cell wall degrading enzymes are involved in HR induction.

In summary, we have proposed that activation of a plasmalemma K^+/H^+ exchange mechanism is an early step in the development of the HR of tobacco to *P. syringae* pv. *pisi*. Although we have not looked for this response in other plant-pathogen interactions, the possibility of its existence warrants further investigation.

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