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Physiology and Biochemistry

Concurrent Loss in Tn*5* Mutants of *Pseudomonas syringae* pv. *syringae* of the Ability to Induce the Hypersensitive Response and Host Plasma Membrane K^+/H^+ Exchange in Tobacco

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ABSTRACT

Baker, C. J., Atkinson, M. M., and Collmer, A. 1987. Concurrent loss in Tn*5* mutants of *Pseudomonas syringae* pv. *syringae* of the ability to induce the hypersensitive response and host plasma membrane K^+/H^+ exchange in tobacco. Phytopathology 77:1268-1272.

Stimulation of a plasmalemma K^+ efflux/H^+ influx exchange in tobacco by phytopathogenic bacteria has been shown to correspond with development of the hypersensitive response. To further test this relationship, mutants of *Pseudomonas syringae* pv. *syringae* were tested for loss of ability to induce the hypersensitive response or stimulate the K^+/H^+ exchange. Mutants were produced by using plasmid vector pGS9 to introduce transposon Tn*5* into *P. s.* pv. *syringae*. Ability to induce the hypersensitive response was assayed by infiltrating mutants, at a concentration of 5×10^7 cfu/ml, into tobacco leaves. Stimulation of the K^+/H^+ exchange was detected by incubating suspension-cultured tobacco cells with mutants at a concentration of 10^8 cfu/ml for 18 hr, then testing for an increase in pH of the medium with a pH indicator. Colonies that failed to stimulate H^+ influx were assayed quantitatively in a second assay that also confirmed the absence of K^+ efflux from suspension-cultured tobacco cells. All mutants that failed to induce one response, either the hypersensitive response or the K^+/H^+ exchange response, also failed to induce the second response. Of 1,600 mutants screened, about 1.7% were auxotrophic. Six prototrophic mutants completely failed to induce either response, and three were able to only partially induce either response; all other prototrophs induced both responses. The results demonstrate a close relationship between bacterial induction of the K^+/H^+ exchange and the hypersensitive response.

Additional key words: ion transport, *Nicotiana tabacum*.

The hypersensitive response is characterized by the rapid death of plant cells at the site of pathogen invasion. Bacterial pathogens causing this response are localized within the hypersensitive lesion and do not invade the surrounding tissue. One of the earliest detectable changes characteristic of tissues undergoing the hypersensitive response is an increased leakage of electrolytes that can be detected within 4–6 hr after inoculation (8,13). Atkinson et al (5) demonstrated that electrolyte loss during the hypersensitive response of tobacco to *Pseudomonas syringae* pv. *pisi* begins within 1–2 hr after inoculation as a specific plasmalemma K^+ efflux/H^+ influx exchange. Recently, Pavlovkin et al (22), studying membrane potential changes during a hypersensitive reaction involving cotton inoculated with *P. s.* pv. *tabaci*, also reported an early and specific loss of intracellular K^+ and proposed an increased plasmalemma permeability to H^+. Because of the fundamental roles played by K^+ and H^+ in eukaryotic cells

(16,17,20,24,30), rapid and prolonged K^+/H^+ exchange could be lethal to plant tissue and play a major role in the hypersensitive response (5).

From these studies, a close association appears to exist between induction of the hypersensitive response and stimulation of a net K^+ efflux/H^+ influx exchange. The objective of this study was to further test this relationship by determining whether the same attributes of a phytopathogenic bacterium are responsible for both responses and to generate bacterial mutants for subsequent exploration of the process. Mutants of *P. s.* pv. *syringae* (incompatible on tobacco) were tested for impaired ability to induce the hypersensitive response as well as the K^+ efflux/H^+ influx exchange in tobacco. Transposon mutagenesis was selected as the method for generating mutants because of its advantages in subsequent molecular manipulations (19).

MATERIALS AND METHODS

Bacterial strains and media. Antibiotic concentrations used were streptomycin sulfate at 50 μg/ml, nalidixic acid at 50 μg/ml, and kanamycin sulfate at 25 μg/ml.

The *P. s.* pv. *syringae* 61 isolate obtained from M. Sasser (University of Delaware) was originally isolated from wheat and is weakly virulent on bean. A nalidixic acid-resistant strain (Pss 61 Nal^R) was obtained as a spontaneous mutant by direct selection and was maintained at 30 C on King's B agar.

Escherichia coli strain WA803, carrying plasmid pGS9, a suicide vector for Tn*5* mutagenesis (15), was maintained at 37 C on LB agar (10 g of tryptone, 5 g of yeast extract, and 10 g of NaCl per liter) containing kanamycin.

Bacterial matings. Tn*5* mutagenesis of *P. s.* pv. *syringae* 61 was carried out as previously described (15,27). Bacteria from 24- to 48-hr-old plate cultures were used to start antibiotic-free cultures of *P. s.* pv. *syringae* 61 Nal^R in nutrient broth at 30 C and *E. coli* WA803 (pGS9) in LB broth at 37 C. After overnight incubation, cultures were diluted with three volumes of fresh broth. After an additional 5 hr of incubation, the cultures were adjusted to 10^9 bacteria per milliliter and 2 ml of each were mixed together. The mixtures were drawn down onto 0.45-μm membrane filters (type A, Nalgene) under gentle vacuum, ensuring that the membranes remained very wet to avoid desiccation of cells. The filters were then aseptically transferred to nonselective TY agar medium (7) to incubate overnight at 30 C. Controls consisted of unmixed cultures treated in the same manner.

Bacterial matings were interrupted by resuspension of the cells from the membrane filters in 4.5 ml of HM salts (29) containing 0.01% Tween 20, followed by high-speed vortexing for at least 10 sec. Mating mixtures were then serially diluted with HM salts plus Tween and plated onto TY agar medium containing streptomycin (25,28) to select for Tn*5*-containing transconjugants and to counterselect donor cells. Background mutation frequencies were determined by plating aliquots of undiluted controls onto the same medium. Controls were also plated onto either LB agar plates containing kanamycin or nutrient agar containing nalidixic acid and incubated under appropriate conditions to determine viable donor and recipient cell counts, respectively.

Bona fide Tn*5* transconjugates were confirmed by replica plating from TY streptomycin plates onto TY medium containing kanamycin. Tn*5* mutants were maintained on King's B agar containing nalidixic acid and streptomycin. Auxotrophs were determined by replica plating onto minimal medium (10).

Hypersensitive response bioassay. The ability of Tn*5*-containing *P. s.* pv. *syringae* 61 Nal^R colonies to cause a hypersensitive response was determined on tobacco (*Nicotiana tabacum*) cultivar Hicks grown under conditions previously described (6). The second and third fully expanded leaves of plants were inoculated with bacterial suspensions containing about 5×10^7 bacteria per milliliter as determined by optical density. The inoculation site was punctured with a 26-gauge needle and the inoculum introduced with a needleless syringe held perpendicular to the leaf, gently supported on the opposite side by a finger. A 2 cm^2 area was infiltrated and outlined with a marking pen. Two leaves were inoculated per mutant. These areas were observed 24 hr later for hypersensitive necrosis.

Net proton influx bioassay. For the primary screening, bromcresol purple, a pH indicator, was used to indicate net proton influx in tobacco suspension cultures incubated with the bacterial mutants. Net H^+ influx by tobacco cells increases the pH of the external medium by approximately 1 pH unit relative to uninoculated cells and shifts the color of the pH indicator from yellow to purple. Characteristics and care of tobacco callus suspension cultures have been previously described (4). Cells from logarithmically growing cultures were collected on Miracloth (Calbiochem-Behring, La Jolla, CA 92037) filters and rinsed with 10 ml of assay buffer (0.5 mM MES-Tris, pH 6.0, 0.175 M mannitol, 0.5 mM $CaCl_2$, 0.5 mM K_2SO_4) per gram of fresh weight callus. Callus cells were resuspended in fresh assay buffer at 1 g/15 ml and incubated for 1 hr at 27 C at 180 rpm. A 0.1-ml aliquot of this suspension was transferred into a sterile 6.4-mm polystyrene flat-bottom microtiter well and inoculated with 0.1 ml of a bacterial suspension in the same buffer containing 10^8 cfu/ml. Fifty transconjugates along with appropriate controls (bacteria only and callus only) were assayed in a single microtiter plate. Plates were covered and incubated at 25–28 C with shaking at 200 rpm. After 16 hr, 10 L of aqueous 10% bromcresol purple was added to each well and the plate agitated to mix the dye and cell suspension. Cell suspensions turning bright yellow (below pH 5.8) were rated negative for net H^+ influx and those turning gray-purple (above pH 6.3) were rated positive. Controls remained yellow.

H^+ influx/K^+ efflux exchange bioassay. Tn*5* mutants that were negative in the net proton influx bioassay were assayed for H^+/K^+ exchange as previously described (5) except that bacteria were incubated with callus cell suspensions for 1 hr instead of 30 min before rinsing; this incubation period allowed attachment of bacteria to tobacco cells. Unattached bacteria were removed by filtration, and tobacco cells were resuspended in fresh assay buffer. Net K^+ and H^+ fluxes were then assayed for up to 8 hr. Net H^+ fluxes were determined by acid-base titration of assay buffer and net K^+ fluxes by atomic absorption spectroscopy of assay solutions.

Characterization of virulence mutants. Mutants that failed to induce the hypersensitive response or K^+efflux/H^+ influx exchange were compared with the wild-type strain for any overt differences. Colony characteristics were observed over a 5-day period on King's B agar containing nalidixic acid with or without streptomycin, as appropriate. Standard terminology was used to describe the colony morphology (12). Culture growth rates were compared on minimal broth media (10). Bacterial populations were monitored by absorbance at 600 nm. Fatty acid profiles were prepared by Microbial ID, Inc. (Barksdale Road, Newark, DE 19711) using a HP 5898A microbial identification system and the aerobic bacteria library (Hewlett-Packard Co., Palo Alto, CA 94304). The use of this technique is based on the finding that differences in fatty acid profiles often indicate differences in bacterial relatedness (26).

Symptom development in tobacco was examined after infiltration of different bacterial concentrations ranging from 10^5 to 10^{10} as described above. Bacterial concentrations were confirmed by dilution plating. Symptom development on beans was compared by infiltration of 5×10^7 cfu/ml into the unifoliate leaf by the method of Klement (14).

RESULTS

Transposon-mediated mutagenesis. Transposon Tn*5* was introduced into nalidixic acid-resistant sublines of *P. s.* pv. *syringae* 61 with a frequency of about 3.2×10^{-7} per recipient cell. Selection for streptomycin resistance and subsequent screening for kanamycin resistance by replica plating were found to provide clear identification of transconjugants. Auxotrophic mutants, which failed to develop colonies on minimal medium, were found at a frequency of 1.7%. Auxotrophic requirements were not determined.

Bioassay of mutants. The primary screens to determine ability to cause a hypersensitive response in tobacco and/or ability to stimulate proton uptake in suspension-cultured tobacco cells were

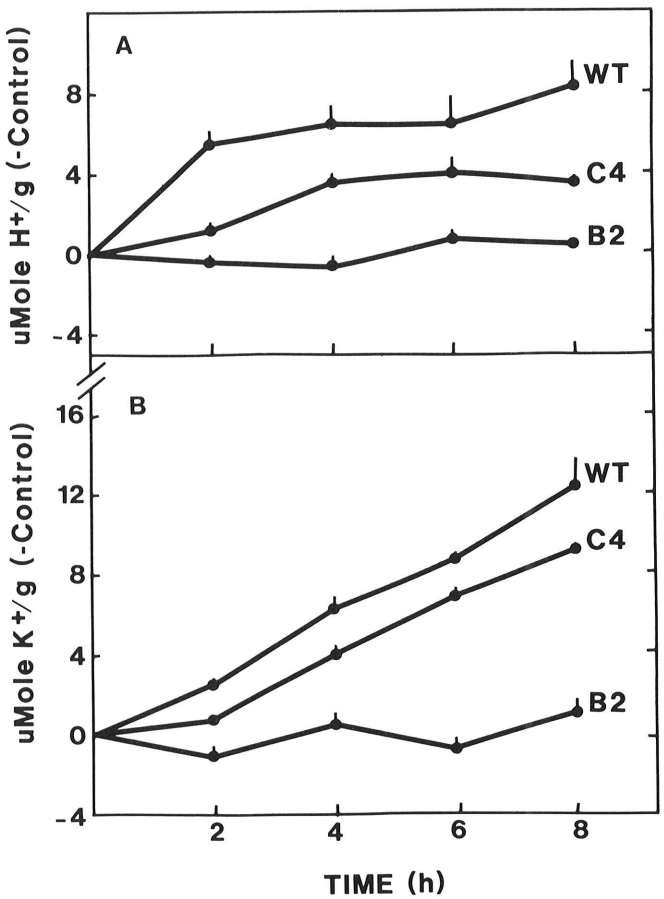

Fig. 1. Effect of *Pseudomonas syringae* pv. *syringae* wild-type (WT) and transconjugant (B2, no hypersensitive response; C4, variable response) strains on net H^+ and K^+ transport in suspension-cultured tobacco cells relative to controls (uninoculated suspension-cultured tobacco cells). Values greater than 0 indicate net H^+ influx or net K^+ efflux, respectively, relative to the control. Values less than 0 indicate net H^+ efflux or net K^+ uptake relative to the control. Data are means of three replicates, and bars represent 1 SD.

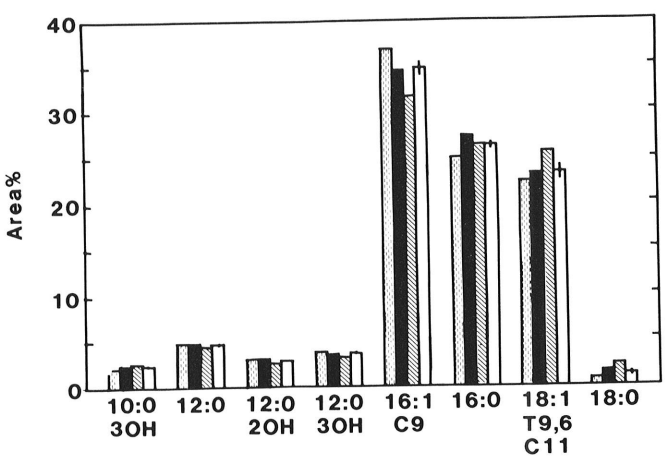

Fig. 2. Profile fatty acids found in wild-type (far left bar) and mutant (B2 left center, C4 right center) strains of *Pseudomonas syringae* pv. *syringae*. Far right bar represents mean of one replicate of all 10 strains (mutants and wild-type), and error bar represents 1 SD. Fatty acids (left to right): 3-hydroxydecanoic acid; dodecanoic acid; 2-hydroxydodecanoic acid; 3-hydroxydodecanoic acid; *cis*-9-hexadecenoic acid; hexadecanoic acid; *trans*-9, *trans*-6, and *cis*-11 isomers of octadecenoic acid; octadecanoic acid.

carried out with 1,600 mutants. The wild-type strain caused a hypersensitive response in tobacco within 18 hr after inoculation with 5×10^7 cfu/ml. The six prototrophic mutants designated B2 through B7 consistently failed to cause chlorosis or necrosis in tobacco. These same mutants were also the only prototrophic transconjugates consistently unable to stimulate net proton uptake in tobacco suspension-cultured cells, as indicated by a pH indicator dye. The dye turned purple when added to tobacco cells incubated with the wild-type strain for 18 hr or yellow when added to uninoculated tobacco cells or cells inoculated with B2–B7. The three mutants designated B1, C3, and C4 caused variable symptoms consisting of different degrees of chlorosis and necrosis in tobacco. These three mutants also stimulated net proton uptake to varying degrees. All auxotrophic mutants failed to induce either response.

To confirm that these mutants were impaired in ability to stimulate the H^+ influx/K^+ efflux response associated with the wild-type strain, H^+ and K^+ concentrations were measured quantitatively. Figure 1A shows that the wild-type strain stimulated H^+ uptake within 2 hr after incubation with the suspension-cultured tobacco cells and reached a maximum between 6 and 8 hr. Mutant B2, which typified mutants B2 through B7 (Table 1), failed to stimulate H^+ uptake, and the tobacco cultures continued to acidify the bathing solution similarly to the uninoculated control. The response to C4, similar to that to C3 and to B1 (Table 1), was intermediate between the control response and that to the wild-type strain. There was a delayed stimulation of proton uptake, which increased before 4 hr and reached a level significantly higher than that for the control but less than that for the wild-type strain by 8 hr. At concentrations of 4×10^8 cfu/ml, mutant B1 occasionally stimulated the K^+/H^+ exchange to a rate equal to that of the wild-type strain (*data not shown*). When bacterial inoculum density was reduced to 2×10^8 cfu/ml, the difference between the wild-type and B1 strains was greater.

The effect of the mutants on K^+ efflux was similar to their effect on proton uptake (Fig. 1B). Mutant B2 completely failed to stimulate K^+ efflux, and mutants B1, C3, and C4 induced a delayed K^+ efflux that achieved rates comparable to that of the wild-type strain within 8 hr.

Characterization of Tn*5* mutants. To determine if any overt differences existed between the mutant and wild-type strains, cultural characteristics, fatty acid profiles, and growth rates on minimal media were compared. When grown on King's B agar

TABLE 1. Effect of *Pseudomonas syringae* pv. *syringae* wild-type and transconjugant strains on net H^+/K^+ transport in suspension-cultured tobacco cells relative to controls[a]

Experiment	Bacterial strain	Net H^+ influx[b]		Net K^+ efflux[c]	
		μmol/g tissue	Percent of wild-type	μmol/g tissue	Percent of wild-type
1	Wild-type	4.44 ± 0.33	100	5.47 ± 0.83	100
	C3	3.08 ± 0.79	69	3.50 ± 0.30	64
	B5	0.86 ± 0.44	19	−0.24 ± 0.65	−4
	B6	0.89 ± 0.35	20	−0.44 ± 0.42	−8
	B7	0.69 ± 0.19	16	−0.21 ± 0.56	−4
2	Wild-type	7.74 ± 0.05	100	7.40 ± 0.95	100
	B2	0.71 ± 0.25	9	−0.17 ± 0.57	−2
	B4	0.78 ± 0.40	10	−0.74 ± 0.91	−10
3	Wild-type	7.49 ± 0.73	100	10.12 ± 1.02	100
	B3	1.03 ± 0.41	14	−0.3 ± 1.41	−3
4[d]	Wild-type	8.38 ± 1.21	100	12.3 ± 1.36	100
	B1	6.94 ± 0.36	83	9.77 ± 1.38	79
	C4	3.67 ± 0.25	44	9.13 ± 0.10	74

[a] Measurements were made 8 hr after inoculation of suspension-cultured tobacco cells with 4×10^8 bacteria per milliliter. Data are means of three replicates ± 1 SD.

[b] Values greater than 0 indicate net H^+ influx relative to the control. Values less than 0 indicate net H^+ efflux relative to the control.

[c] Values greater than 0 indicate net K^+ efflux relative to the control. Values less than 0 indicate net K^+ uptake relative to the control.

[d] Inoculum concentration of 2×10^8 cfu/ml.

during a 5-day period, all mutant and wild-type strains appeared to have similar cultural characteristics. All were fluorescent, circular, entire and undulate, raised, and smooth.

The fatty acid profiles of the wild-type and mutant strains were very similar (Fig. 2). The major fatty acids found were *cis*-9-hexadecenoic acid, hexadecanoic acid, and *trans*-9, *trans*-6, and *cis*-11 isomers of octadecenoic acid. This profile was typical of *P. syringae* based on the Hewlett-Packard aerobic bacteria library, which has been demonstrated to differentiate bacterial plant pathogens according to gross fatty acid profiles.

When grown on minimal media, wild-type and mutant strains had very similar growth rates. After a 17-hr lag period, bacterial multiplication increased at similar rates, with an average generation time of 2.8 ± 0.3 hr.

Mutant and wild-type strains were infiltrated into bean leaves to determine their virulence. The area infiltrated with 1×10^7 cfu/ml of wild-type strain became chlorotic after 48 hr; with 5×10^7 cfu/ml, the area became necrotic. Inoculations with the mutants at 1×10^7 and 5×10^7 cfu/ml resulted in no visible symptoms after 7 days, with the exceptions of B1, C3, and C4, which caused chlorosis and occasionally partial necrosis at the higher concentration; C4 also caused slight necrosis at the lower concentration.

Tobacco plants were inoculated with various concentrations of each mutant to determine if the efficiency of each to induce the hypersensitive response varied. The wild-type strain at concentrations greater than 1×10^7 cfu/ml caused confluent necrosis in tobacco within 24 hr; below this concentration to about 1×10^6 cfu/ml, inoculated tissue became chlorotic after 5–7 days. Leaf tissue inoculated with mutants B2 through B7 at 1×10^6 to 5×10^9 cfu/ml showed no symptom development after 24 hr; at the higher concentrations, however, a slight chlorotic symptom developed after 5–7 days. Inoculations with mutants B1, C3, and C4 resulted in a variable response, frequently with hypersensitive necrosis and/or chlorosis in a portion of the leaf area inoculated at concentrations greater than 1×10^8 cfu/ml.

DISCUSSION

The suicide vector pGS9 was successfully used to generate Tn*5* mutants of *P. s.* pv. *syringae*. The yield of kanamycin-resistant transconjugants in matings with *E. coli* WA803 (pGS9) was about 3×10^{-7} per recipient cell, which is slightly lower than previous reports of transposon mutagenesis with pseudomonads (1,9,15,21). However, the ability to select for streptomycin resistance, which is unexpressed in *E. coli* (25,28), and subsequently for kanamycin resistance by replica plating provides clear identification of bona fide transconjugates over background spontaneous mutants. Screening of the transconjugates revealed that 1.7% were auxotrophs, similar to previous reports (1,9,15,21). None of the auxotrophs elicited any response, presumably because all were metabolically inactive. Southern blot analysis (*data not shown*) of mutant chromosomal DNA digested with EcoR1 and probed with ^{32}P pGS9 revealed that mutants B2, B4, B5, B6, B7, C4, and B1 contained single Tn*5* insertions and B3 and C3 contained multiple insertions. Physical characterization and complementation analysis of the mutants are being presented elsewhere.

The results of this study confirm that one function of the mutated gene(s) must involve activation of the K^+/H^+ exchange (5). Of 1,600 mutants tested, nine were found to be unable to induce either response in preliminary screening tests, while all other prototrophic mutants except auxotrophs stimulated both responses similar to the wild-type strain. Further testing of these mutants revealed two different phenotypes; six mutants were completely unable to stimulate either the hypersensitive or the K^+/H^+ exchange response, whereas three were able to cause only a partial or delayed stimulation of both responses. Comparison of the mutants showed no overt differences in cultural, chemical, or metabolic characteristics, suggesting that the alterations in the ability to induce host responses were not the result of major pleiotropic mutations. That we were not able to separate stimulation of K^+/H^+ exchange and the hypersensitive response provides further evidence that K^+/H^+ exchange is an early and key step in the hypersensitive response.

The generation of mutants that have either completely or partially lost the ability to induce wild-type symptoms on bean or tobacco is similar to the observations of others with *P. syringae* pathovars. Anderson and Mills (1) found that certain Tn*5* mutants of *P. s.* pv. *syringae* required 20- to 2,000-fold greater inoculum densities to induce necrotic symptoms in beans. Cuppels (9) reported generation of Tn*5* mutants of *P. s.* pv. *tomato* that either were avirulent or produced mild symptoms on tomato seedlings. Our data further indicate that at least two categories of mutations affecting induction of the hypersensitive and K^+/H^+ exchange responses were generated—one blocks induction completely and the other results in a delayed or altered induction. In addition, our present and companion (2) studies, as well as other reports (19), suggest that a correlation often exists between loss of ability to induce compatible and incompatible symptom development by phytobacterial mutants.

Another association between induction of the K^+/H^+ exchange and the hypersensitive response was demonstrated in recent reports from our laboratory. When tobacco leaves were infiltrated with nonmacerating levels of pectate lyase prior to inoculation with *P. s.* pv. *syringae*, the hypersensitive response was inhibited (6). In a parallel study, pectate lyase activated a transient K^+/H^+ exchange in tobacco that lasted about 1 hr, after which cells recovered and resumed the net H^+ efflux and K^+ influx shown by the controls. However, additional doses of the enzyme failed to reactivate this response (3). This suggests that the pectate lyase treatment may inhibit the hypersensitive response in the tissue by interfering with the prolonged K^+/H^+ exchange normally induced by plant pathogens.

The correlation between the two phenotypes—the ability to induce the hypersensitive response and K^+/H^+ exchange—demonstrated in this and previous studies (3,5,6) suggests that the same bacterial factor is responsible for both host responses. The simplest hypothesis based on these findings is that a bacterial elicitor either directly or indirectly, through a single or a cascade of membrane events, triggers a net K^+ efflux/H^+ influx exchange that severely alters the cytoplasmic and intercellular pH as well as the cytoplasmic K concentration.

As previously discussed (5), significant and rapid changes in H^+ and K^+ content can be fatal to plant cells (18,23). The most damaging consequence of hypersensitive H^+ uptake is probably an acidification of the cell interior (18,20), which would have widespread effects, including alteration of enzymatic activities and changes in macromolecular conformations (30). In addition to these changes, hypersensitive H^+ uptake would destroy or reduce the normal plasmalemma H^+ gradient (23) required for active transport and maintenance of the cell membrane potential. Similarly, there are numerous consequences of hypersensitive K^+ efflux, including inhibition of enzymes involved in protein synthesis, respiration, oxidative phosphorylation, and photophosphorylation. Potassium serves as an activator for many enzymes, and a concentration of about 50 mM is frequently required for maximal activity (11,30).

This study confirms a close association between the hypersensitive response and a specific physiological response that occurs early in host/pathogen recognition. The mutants developed in this study will provide a valuable tool for further investigations of the molecular basis of the hypersensitive response. In addition, the ability to quantitatively measure the K^+/H^+ exchange will provide a useful means by which to quantitate the effect of various factors on the early events related to the hypersensitive response.

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