The Interaction of Harpin$_{Pss}$ with Plant Cell Walls

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Erwinia amylovora and *Pseudomonas syringae* pv. *syringae* produce elicitors of the hypersensitive reaction (HR), harpin$_{Ea}$ and harpin$_{Pss}$, respectively. Harpin$_{Ea}$ causes K$^+$ efflux and extracellular alkalinization in suspension-cultured cells of tobacco. These responses are associated with disease resistance. We treated living, fixed, and permeabilized cells and protoplasts from tobacco suspension cultures with harpin$_{Pss}$, antiharpin$_{Pss}$ antibody, and fluorochrome-tagged antibody and examined them with confocal laser microscopy. The fluorescent signal was localized in the outer part of the cell and was not observed in protoplasts. EGTA, a chelating agent that extracts Ca^{++} and pectins from cell walls, blocked harpin$_{Pss}$ binding, as evidenced by the absence of fluorescent signal. The pH of the external medium of suspension cultures alkalinized in response to harpin$_{Pss}$ and *P. s. syringae*. Bacteria and harpin$_{Pss}$-induced alkalinization of the extracellular medium were also completely blocked upon EGTA treatment. Protoplasts alkalinized the medium at much reduced levels in response to *P. syringae* pv. *syringae* and did not alkalinize the medium in response to harpin$_{Pss}$. These results suggest that the cell wall is crucial for HR induction in the suspension culture cell.

Additional keywords: confocal scanning laser microscope.

The hypersensitive response (HR), a highly localized defense response, is associated with incompatible bacterium-plant combinations and is characterized by the rapid death of plant cells at the site of pathogen invasion. In leaf tissue, HR induced upon bacterial infiltration at a concentration of at least 5×10^6 cells per ml, results in massive leakage of electrolytes, mainly K$^+$, followed by complete tissue collapse and desiccation within 24 h (Klement 1982). In suspension-cultured cells, HR induction has been associated with cell browning, pH changes in the extracellular medium, K$^+$ leakage and eventual cell death (Atkinson et al. 1985). Recently, elicitors of bacteria-induced HR, harpin$_{Ea}$ (Wei et al. 1992) and harpin$_{Pss}$ (He et al. 1993), have been isolated from *Erwinia amylovora* and *Pseudomonas syringae* pv. *syringae*, respectively. Though structurally different, both cause immedi-

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ate K$^+$ efflux and extracellular alkalinization in tobacco suspension-cultured cells (Wei et al. 1992; Baker et al. 1993; Hoyos 1994) and rapid collapse of tobacco leaves (Wei et al. 1992; He et al. 1993). These elicitors are being used to examine signal transduction, i.e., the cascade of host responses following exposure to a pathogen product that may eventually result in electrolyte leakage, cell death, and the transcriptional activation of defense-related genes. It has been suggested (Atkinson 1993) that this cascade bears similarity to the mammalian signal transduction pathways, which typically involve receptor binding, transiently increased cellular calcium levels, changes in protein phosphorylation and membrane potential, and de novo protein synthesis.

Consistent with the mammalian model, HR development in tobacco leaves induced by both harpin$_{Pss}$ and harpin$_{Ea}$ is prevented by inhibitors of calcium influx, gene activation, and ATPase activity (He et al. 1993; 1994). The application of harpin$_{Ea}$ or harpin$_{Pss}$ to the bathing solution of tobacco leaf segments causes membrane depolarization within minutes (S. Pike and X. A. Pu, unpublished). The harpin$_{Ea}$ depolarization requires calcium influx and can be delayed by the protein kinase inhibitor K252a (S. Pike, unpublished). In tobacco suspension cells, harpin$_{Ea}$-induced alkalinization of the extracellular medium, K$^+$ efflux, and membrane depolarization also are blocked by K252a (Popham et al. 1995), as is the active oxygen burst (Baker et al. 1993). The mechanisms underlying harpin$_{Pss}$ and harpin$_{Ea}$ perception by the plant cell, however, remain uncharacterized.

We have examined the interaction of harpin$_{Pss}$ with living, fixed, and permeabilized tobacco suspension cells using antiharpin$_{Pss}$ antibody, fluorochrome tagged antibody, and confocal laser microscopy. The pH changes in the extracellular media of suspension cells and protoplasts were measured concurrently as an indication of HR induction. Our results indicate that harpin$_{Pss}$ is localized in the outer portion of the plant cell, probably in the cell wall, and that the cell wall is crucial for the harpin$_{Pss}$-HR induction of tobacco suspension cells.

RESULTS

Harpin binding.

The three harpin$_{Pss}$ preparations used in this study were full length [*E. coli* DH5α (pSYH10)], truncated [*E. coli* DH5α (pSYH5)], and truncated gel-purified protein. These proteins were separated in a polyacrylamide gel along with protease-inactivated harpin$_{Pss}$, the protein products of *E. coli* DH5α and *E. coli* DH5α pBluescript SK (pBSK) without the *hrpZ* insert, and harpin$_{Ea}$, the protein HR elicitor produced by *Erwinia*

amylovora (Fig. 1A). An immunoblot of these proteins using antiharpin$_{Pss}$ antibody (He et al. 1993) preabsorbed only against tobacco proteins still recognized *E. coli* proteins (Fig. 1B). Further preabsorbtion against *E. coli* DH5α (pBSK) proteins eliminated binding to the *E. coli* bands but harpin$_{Pss}$ and other bands present in the active harpin$_{Pss}$ preparations were still recognized (Fig. 1C).

The fluorescent signal of tobacco cells treated with harpin$_{Pss}$ and both antibodies was localized on the cell surface (Fig. 2: C2, D2, E2). All are optical cross sections through the center of the cell. No fluorescence was seen in control cells treated with both antibodies but without harpin (Fig. 2, A2) or the protein products of *E. coli* DH5α (pBSK) which lacks harpin$_{Pss}$ (Fig. 2, B2). No fluorescence was found in the cytoplasm of cells that were fixed and permeabilized to allow antibody entry and binding to possibly internalized harpin (data not shown). Because these images do not permit discrimination between cell wall and plasma membrane, the experiments were repeated with isolated protoplasts.

Live or fixed protoplasts isolated from tobacco suspension cells by our standard protoplast isolation or by the protocol described by Diekmann et al. (1994) did not exhibit any membrane fluorescence after treatment with antibodies alone (Fig. 3, A2) or harpin$_{Pss}$ and both antibodies at 25°C (Fig. 3, B2) or with either treatments at 4° (data not shown). The fluorescence in Fig. 3, B2 corresponds to cell wall debris in Fig. 3, B1. Similar results were obtained with protease-treated protoplasts (data not shown).

The presence of signal in the periphery of cells with intact walls treated with harpin$_{Pss}$ and the primary and secondary antibodies, but not protoplasts, suggested a possible interaction of harpin$_{Pss}$ with the plant cell wall rather than the plasma membrane. In further experiments we used EGTA, a chelating agent that removes Ca^{++} and pectins from cell walls (Fry 1986), to study the role of the plant cell wall in harpin$_{Pss}$ binding. When EGTA was added prior to (Fig. 4, B2) or together with harpin$_{Pss}$ (Fig. 4, C2), no fluorescent signal was detectable after treatment with secondary antibody, indicating that harpin$_{Pss}$ binding was completely blocked. When EGTA was added to the suspension cells 30 min (Fig. 4, D2) after harpin treatment, the fluorescent signal was partially dissipated, indicating disruption of harpin$_{Pss}$ binding (compare to Fig. 4, A2). However, this reduction in intensity of fluorescent signal was not observed in all experiments in which harpin$_{Pss}$ was applied prior to EGTA; at times the signal was as strong as in cells treated with harpin$_{Pss}$ alone.

pH changes.

In standard assay buffer, suspension cells alkalinized the extracellular medium in 2 to 4 h after treatment with wild-type *P. syringae* pv. *syringae*, but not with the harpin mutant (Fig. 5A). Alkalinization was induced immediately after addition of different concentrations of full-length harpin$_{Pss}$ (Fig. 5B). The pH change caused by the same concentrations of truncated harpin$_{Pss}$ is similar in magnitude to the changes caused by the full-length protein (data not shown). The protein product of *E. coli* DH5α carrying the pBluescript SK plasmid without the *hrpZ*, prepared and diluted as the harpin$_{Pss}$, caused a pH change of about half the magnitude as harpin$_{Pss}$ (Fig. 5C). At the same protein concentration, this protein and protease-inactivated harpin$_{Pss}$ caused similar but smaller alkalinization

than harpin$_{Pss}$ (Fig. 5C). Harpin$_{Pss}$ (200 µg/ml) induced a sustained pH change in suspension cell medium similar to that induced by the wild-type *P. syringae* pv. *syringae* (Fig. 5A and B) and caused necrosis in tobacco leaves, while the proteins from *E. coli* DH5α (pBSK) at the same concentration did not cause necrosis in tobacco leaves. Harpin$_{Pss}$ preparations at concentrations ≤10 µg protein per ml induced transient pH changes in suspension cell medium and never caused tobacco leaf necrosis.
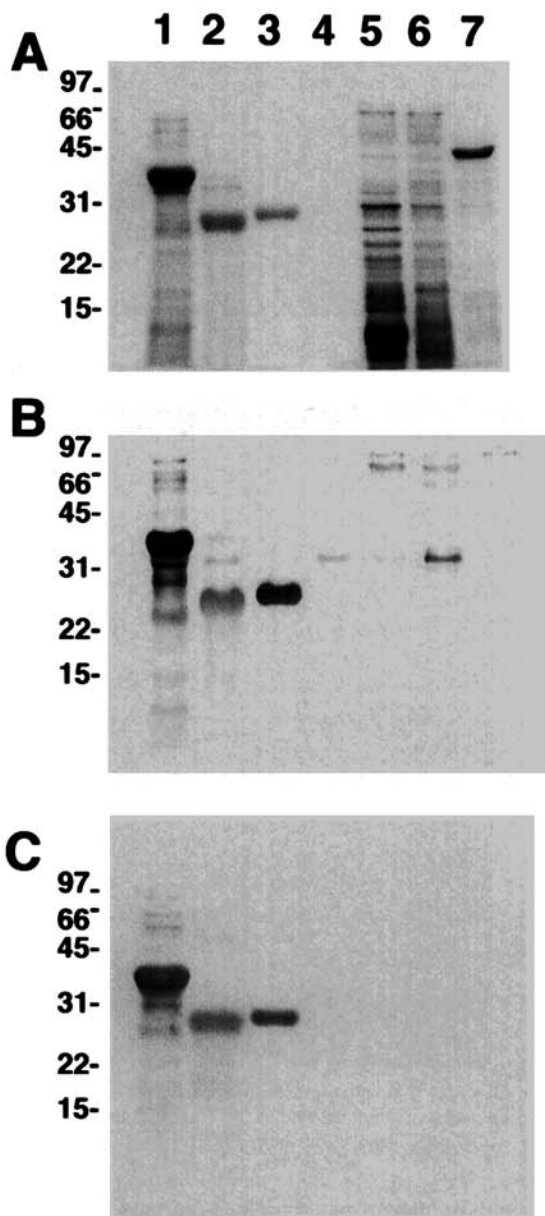

Fig. 1. SDS-polyacrylamide gel electrophoresis of harpin$_{Pss}$ preparations and Western blots showing specificity of antiharpin$_{Pss}$ antibody. All protein preparations were partially purified by boiling. **A,** Gel stained with Coomasie Brilliant Blue. Lane 1, full-length harpin$_{Pss}$; lane 2, truncated harpin$_{Pss}$; lane 3, truncated gel-purified harpin$_{Pss}$; lane 4, truncated protease-inactivated harpin$_{Pss}$; lane 5, DH5α, lane 6 DH5α (pBSK); lane 7, harpin$_{Ea}$. **B** and **C,** Immunoblots with lanes corresponding to the gel shown in **A. B,** Antiserum was not preabsorbed against *Escherichia coli* and still recognized *E. coli* bands but not harpin$_{Ea}$. **C,** Antiserum preabsorbed against DH5α (pBSK) crude extract no longer recognizes *E. coli* products.
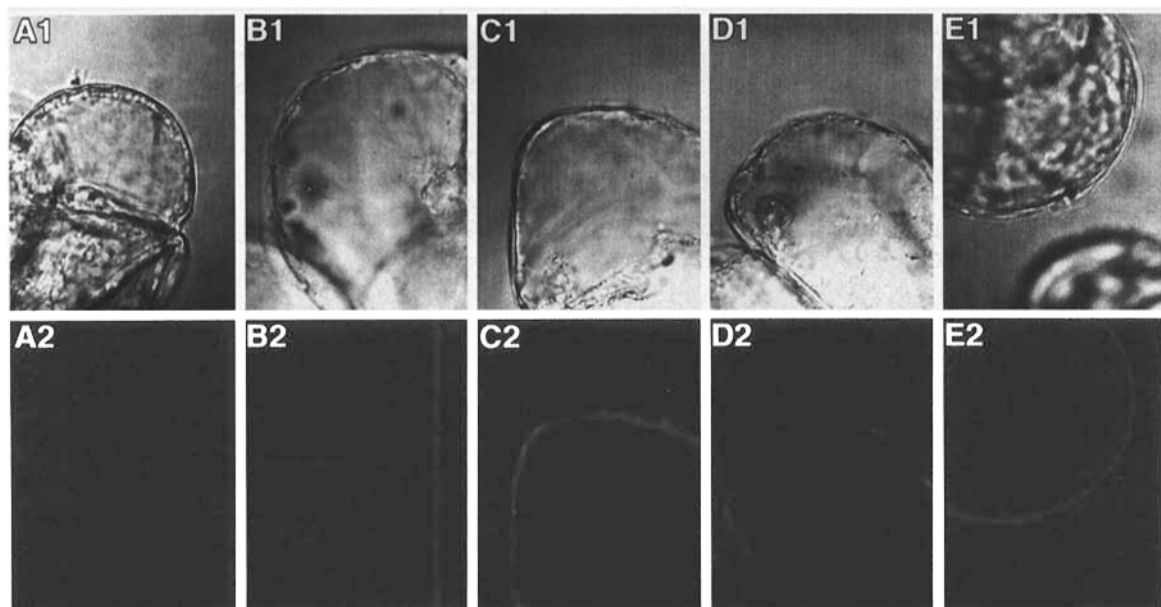
Fig. 2. Confocal laser micrographs of tobacco suspension-cultured cells treated with antiharpin$_{Pss}$ antibody, fluorochrome-tagged secondary antibody and **A,** buffer. **B,** Protein product of DH5α (pBSK) without *hrpZ* insert (140 μg per ml). **C,** Full-length harpin$_{Pss}$ (200 μg per ml). **D,** Truncated harpin$_{Pss}$ (140 μg per ml). **E,** Truncated gel-purified harpin$_{Pss}$ (140 μg per ml). No. 1 = transmitted image; 2 = fluorescent image.
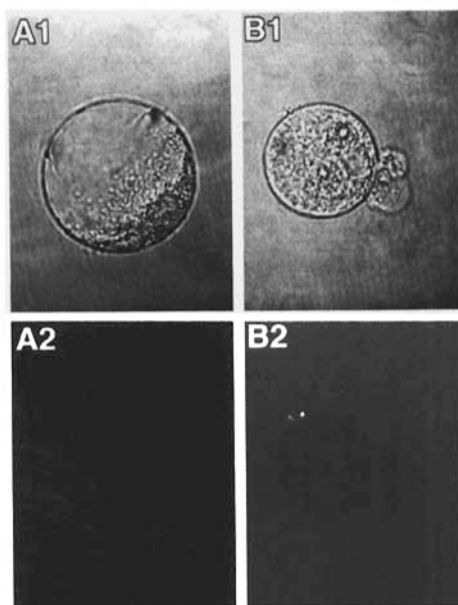

Fig. 3. Confocal laser micrographs of tobacco protoplasts treated with **A,** Primary and secondary antibodies. **B,** Truncated harpin$_{Pss}$ (140 μg per ml) and antibodies. No. 1 = transmitted image; 2 = fluorescent image.

Protoplasts treated with harpin$_{Pss}$ did not alkalinize the extracellular medium (Fig. 6A). However, treatment with *P. syringae* pv. *syringae* caused a constant increase in the pH of the extracellular medium throughout the experiment (Fig. 6A), though of less magnitude than that observed for suspension cells (Fig. 6B). However, suspension cells, incubated in assay buffer containing 0.7 M mannitol for comparison with the protoplasts experiments (Fig. 6B) produced less alkalinization than in tests using the normal assay buffer (Figs. 5 and 7 A and B). These results indicate that bacterial products other

than harpin$_{Pss}$ contribute to the residual pH change in the protoplast medium and suggest that the cell wall is important for the pH change induced by either harpin$_{Pss}$ or *P. syringae* pv. *syringae*.

Treatment of tobacco suspension cells with EGTA before (data not shown) or together with full-length harpin$_{Pss}$ (Fig. 7A) blocked the harpin$_{Pss}$-induced alkalinization of the external medium. The addition of EGTA even 30 min after application of harpin$_{Pss}$, reduced the alkalinization (Fig. 7A). The addition of EGTA 3, 4, or 5 h after bacterial treatment reversed the pH increase caused by *P. s. syringae* (Fig. 7B). In both harpin$_{Pss}$ and bacterial treatments, EGTA did not decrease pH below control level. EGTA alone acidified buffer-treated suspension cells (Fig. 7B).

DISCUSSION

Oligosaccharide fungal elicitors have been reported to induce pH changes, K$^+$ efflux, and plant defense responses in both plant suspension cells and protoplasts (Bach et al. 1993; Brisson et al. 1994; Renelt et al. 1993). Additionally, many fungal elicitors bind to the plasma membrane of plant cells (Cheong and Hahn 1991; Cheong et al. 1993; Cosio et al. 1988, 1990; Hahn et al. 1994; Nürnberger et al. 1994, 1995; Schmidt and Ebel 1987) and one has been imaged on the protoplast surface (Dieckmann et al. 1994). In contrast, our search for the binding site of harpin$_{Pss}$, a bacterial elicitor of HR, revealed the interaction of harpin$_{Pss}$ with the cell wall, and not with the plasma membrane of protoplasts. A harpin$_{Pss}$/cell wall interaction was demonstrated both in immunofluorescent experiments and pH measurements of the extracellular medium of suspension cells and protoplasts. This interaction may involve specific binding sites; however, we have not ruled out an ionic interaction of harpin$_{Pss}$ with cell wall pectic polysaccharides.

The absence of both fluorescent signal in protoplasts and pH change of the extracellular medium, could be due to the
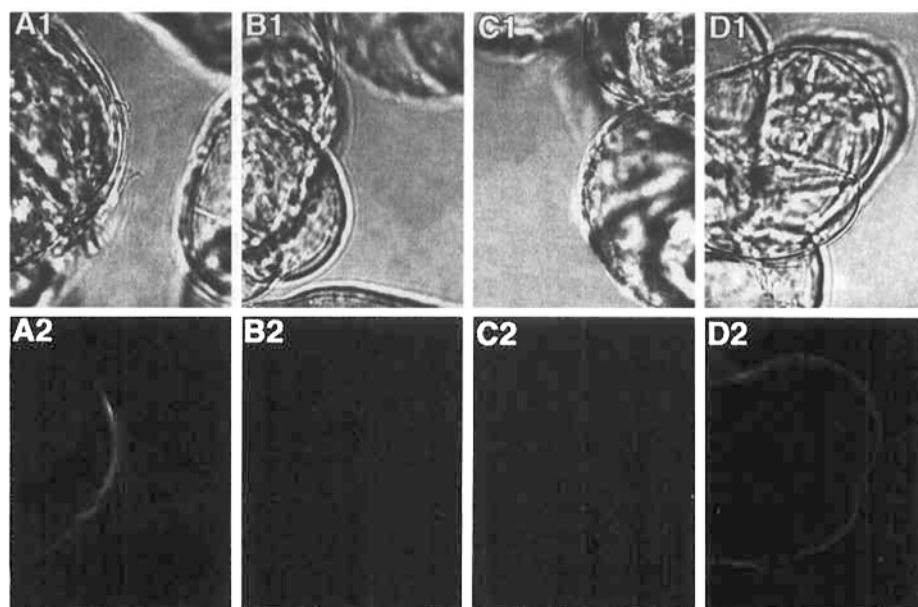
Fig. 4. Confocal laser micrographs of tobacco suspension-cultured cells treated with full-length harpin$_{Pss}$, antiharpin$_{Pss}$ antibody and fluorochrome-tagged secondary antibody. **A**, Control, no EGTA. **B**, EGTA applied 15 min prior to harpin$_{Pss}$. **C**, EGTA applied along with harpin. **D**, EGTA applied 30 min after harpin$_{Pss}$. No. 1 = transmitted image; 2 = fluorescent image.

removal of binding sites or cell wall/membrane connections during enzymatic protoplast isolation. However, the Dieckmann (1994) protocol, which was designed to reduce receptor proteolysis and internalization, did not lead to harpin$_{Pss}$ binding in protoplasts. It is also possible that the fluorescent method we used is not sensitive enough to detect low-abundance, high-affinity binding sites on the plant cell membrane.

Reversible binding may explain why a very high concentration of harpin$_{Pss}$ was required to produce adequate signal in confocal experiments. Reversible binding of harpins to the cell exterior is suggested by electrophysiological experiments with tobacco leaf tissue and harpin$_{Ea}$ or harpin$_{Pss}$. Membrane potentials were depolarized and slowly recovered following the topical application of high concentrations of harpin$_{Ea}$; however, removal of either harpin from the bathing solution allowed faster recovery of membrane potential (S. Pike and X.-A. Pu, unpublished). Alternatively, a high concentration of harpin$_{Pss}$ may be required for a significant biological response. Only a high harpin$_{Pss}$ concentration produced a pH change of a similar magnitude to that produced by bacteria. This high concentration was also required for tobacco leaf necrosis. Transient pH changes of less magnitude may reflect less specific stress responses.

We did not detect harpin$_{Pss}$ in the cytoplasm of fixed, permeabilized suspension cultured cells with fluorescent labeled antibody. Similarly, in tobacco leaves inoculated with 2×10^8 colony-forming units of *P. syringae* pv. *syringae*, immunogold-labeled antibodies were not detected in cytoplasm of leaf cells (S. Y. He, unpublished). However, we have not yet ruled out the possibility that fragments of harpin$_{Pss}$ which are not recognized by the antibody reach the plasma membrane and bind to the membrane or are internalized. One study of harpin$_{Pss}$ fragments reported that some fragments possessed elicitor activity (He et al. 1993), and in a subsequent report,

all fragments elicited the HR in tobacco leaves (Alfano et al. 1996). Elicitor activity is not associated with any consensus sequence and the primary sequence does not predict where harpin$_{Pss}$ may interact with the cell (Alfano et al. 1996). *E. coli* DH5α proteins also induce pH changes but of less magnitude than those induced by harpin$_{Pss}$ in tobacco suspension cells, but in our study, in concordance with the report by He et al. (1993), the *E. coli* proteins are unable to cause necrosis in tobacco leaves.

The results of our experiments with EGTA suggest that harpin$_{Pss}$ may directly associate with pectic molecules in the cell wall or that conformational changes in binding sites resulting from the removal of Ca^{++} may prevent binding. Harpin$_{Pss}$ binding, and consequently alkalinization of the extracellular medium in tobacco suspension cells, were eliminated by treatment with EGTA prior to, or together with harpin$_{Pss}$. Alkalinization caused by *P. syringae* pv. *syringae* was reversed subsequent to application of EGTA. Alternatively the removal of calcium from the medium may have prevented the calcium influx associated with the HR induction (Atkinson et al. 1990), thus inhibiting HR development. However, it also is possible that the acidification caused by EGTA alone prevented antibody binding and overcame the bacteria/harpin$_{Pss}$-caused alkalinization. It is not likely that EGTA-induced acidification was merely a result of toxicity because the EGTA/harpin$_{Pss}$ or bacteria treatments acidified the medium to, but not below, the buffer-treated control levels.

HR is reportedly inhibited in tobacco plants and suspension cells treated with *P. syringae* pv. *syringae* and *P. syringae* pv. *pisi* by pretreatment with pectolytic enzymes (Baker et al. 1986, 1990). In addition, Bauer et al. (1995) report that an *Erwinia chrysanthemi* mutant deficient in the production of pectate lyase isozymes, PelABCE, induced HR, in contrast to the wild type, which did not. These results imply inhibition of HR by pectolytic enzymes. Proposed explanations for these
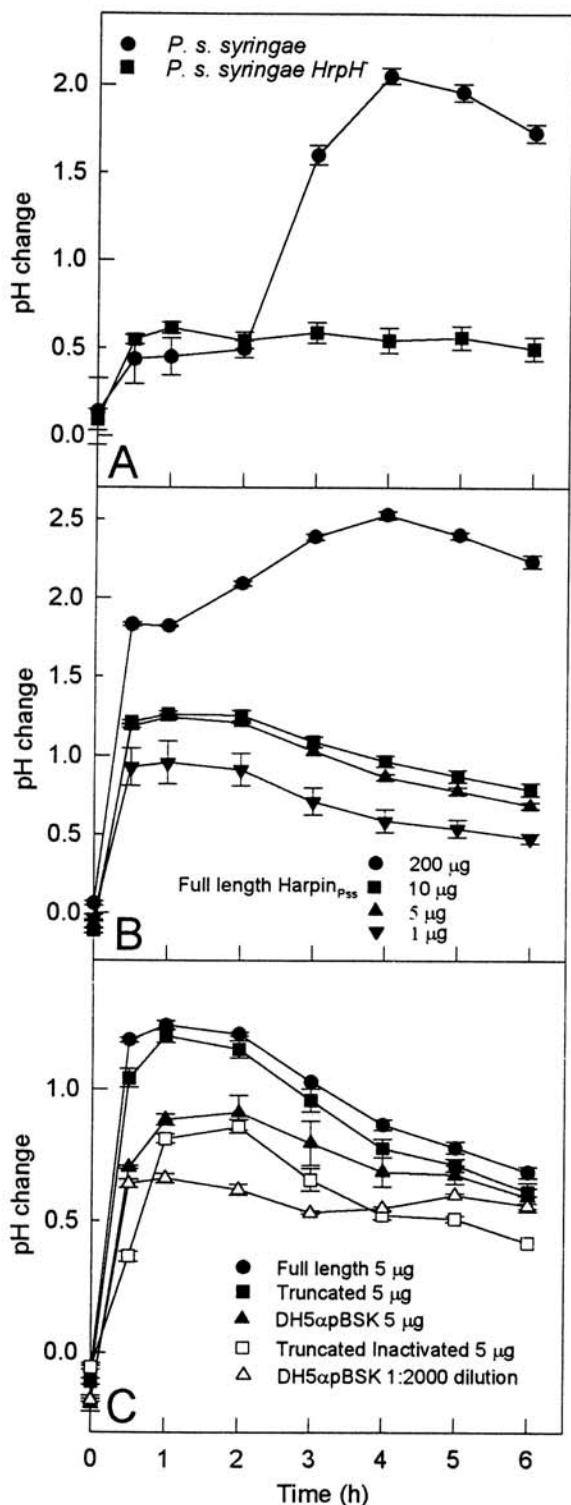
results range from effects on the host plant to effects on the bacteria: (i) decreased host sensitivity to the bacterial inducer of the hypersensitive response, and (ii) effects of the pectolytic enzymes on bacterial multiplication, bacterial metabolism, or rate at which a bacterial inducer of hypersensitive response is produced (Baker et al. 1986). In light of our results, several alternative explanations could be considered: competition of cell wall peptides released by the enzyme treatment with harpin for binding sites, or destruction of, or conformational changes in harpin binding sites by enzymatic treatment.

Harpin$_{Ea}$-induced responses in tobacco suspension cells such as K$^+$ efflux, extracellular alkalinization, active oxygen species production, and membrane depolarization are blocked

Fig. 5. pH changes in the external medium of tobacco suspension cells treated with **A**, 10^8 cells per ml *Pseudomonas syringae* pv. *syringae* 61 and *P. syringae* pv. *syringae hrp* H^- mutant. **B**, Different concentrations of full-length harpin$_{Pss}$ as shown. **C**, Different harpin$_{Pss}$ preparations as shown and the protein product of DH5α (pBSK) without the *hrpZ* insert at a protein concentration equivalent to harpin preparations and at the same dilution as the harpin$_{Pss}$ preparations (1:2,000). Since all preparations were prepared in parallel, the equivalent dilution most likely represents the actual DH5α (pBSK) background present in the harpin$_{Pss}$ preparations. Points are the means of 3 replications minus buffer-treated control values in one representative experiment. Error bars are standard deviation.
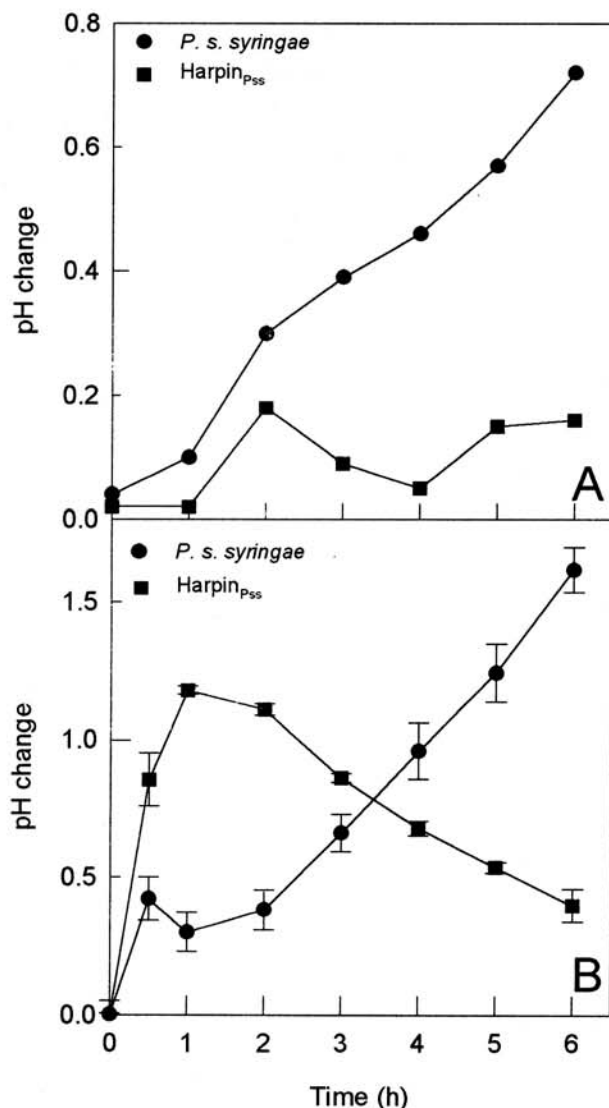
Fig. 6. pH changes in the external medium of **A**, tobacco protoplasts treated with 10^8 cells per ml *Pseudomonas syringae* pv. *syringae*, or 200 µg per ml full-length harpin$_{Pss}$ showing pH change only from bacterial treatment. Points are values minus buffer-treated control values in a representative experiment (n = 6 experiments). **B**, Tobacco suspension cultured cells treated as in **A**. Points are the means of 3 replications minus buffer-treated control values in a representative experiment. Error bars are standard deviation. In both **A** and **B** the normal assay buffer was supplemented with mannitol, final concentration 0.7 M, which reduced the pH response in suspension cells (see Figs. 5 and 7 A and B).

by lanthanum chloride, a Ca^{++} channel blocker, and K252a, a protein kinase inhibitor (Baker et al. 1993; He et al. 1994; Popham et al. 1995). These events suggest that harpin$_{Ea}$ triggers a signal transduction pathway that involves active oxygen species production, protein phosphorylation, and Ca^{++} influx. Furthermore, membrane potential depolarization in tobacco leaves (S. Pike, unpublished) and suspension cells (Popham et al. 1995) induced by harpin$_{Ea}$ apparently affects the active, ATP-dependent, portion of the membrane potential which is directly related to function of the plasma membrane H^+-ATPase. Binding to the cell wall rather than the plasmalemma poses problems: How is a signal transduction pathway triggered from a distant cell-wall bound harpin, and how is this signal (s) transmitted to the plasma membrane?

Recently a protein has been identified that could function in such wall-to-membrane signal transduction namely, the wall-associated kinase, Wak1 (He et al. 1996). The predicted topology of Wak1, formerly called Elusin, includes an extracellular domain with homology to the epidermal growth factor family of proteins, a membrane spanning domain, and a cytoplasmic serine/threonin kinase (Kohorn et al. 1996). Wak1 is very difficult to solubilize, but is released by a combination of 4% SDS, 50 mM DTT, and boiling or enzymatic digestion of the cell wall. Its cell wall location has been confirmed by immunocytochemistry together with electron microscopy and light microscopy (He et al. 1996). Results from protease treatments of protoplasted cells were consistent with an extracellular EGF domain and a cytoplasmic kinase domain. Wak1's plasma membrane location was substantiated by enrichment of the band in the purified plasma membrane fraction and pelleting with the plasma membrane. Wak1 has kinase activity (Z-H. He, personal communication). Wak1 is found in all vegetative tissues of *Arabidopsis* and all higher plants tested including tobacco.

Another possibility is the plasmalemma control center (PCC) model proposed by Pickard (1994) to explain mechanical and biochemical interactions between plant cells and environment. These PCCs would transform secondary mechanical and biochemical signals via mechanical connections and second-messenger calcium. PCCs are visualized as the plant cell's equivalent of the mammalian cell's adhesion sites. They are described as clustered mechanosensory Ca^{++}-selective ion channels physically connected to clusters of integrin-like transmembrane proteins and associated wall linking proteins, cytoskeleton and regulatory molecules. In mammalian systems, integrins are involved in signal transmission, cytoskeletal action, and cell-to-cell adhesion events (Hynes 1992). Also, there are several processes in which integrin-mediated signaling has been implicated, including tyrosine phosphorylation, cytoplasmic alkalinization, activation of lymphocytes, activation of secretion, differentiation, and induction of gene expression (Hynes 1992; Werb et al. 1989). Integrin ligands are extracellular matrix glycoproteins such as vitronectin, fibronectin, and type I collagen (Hynes 1992).

Vitronectin- and fibronectin-like proteins have been reported in four species of flowering plants (Sanders et al. 1991) and in tobacco suspension cells (Zhu et al. 1993); however, the anti-vitronectin antibodies are not entirely specific for vitronectin-like (Zhu et al. 1994). Most recently, Gens and co-workers (1996) visualized co-localized anti-integrin, anti-vitronectin, and anti-fibronectin antibody pairs as *puncta* on

the surface of onion protoplasts with computational optical-sectioning microscopy. Anti-vitronectin and anti-fibronectin antibodies, but not anti-integrin antibodies, were similarly colocalized in depectinated wall fragments. These results are consistent with the PCC model.

Perhaps more relevant to our data is the report that binding to proteins with vitronectin-like antigenicity may play a role in bacterial pathogenicity. Vitronectin is bound both by some
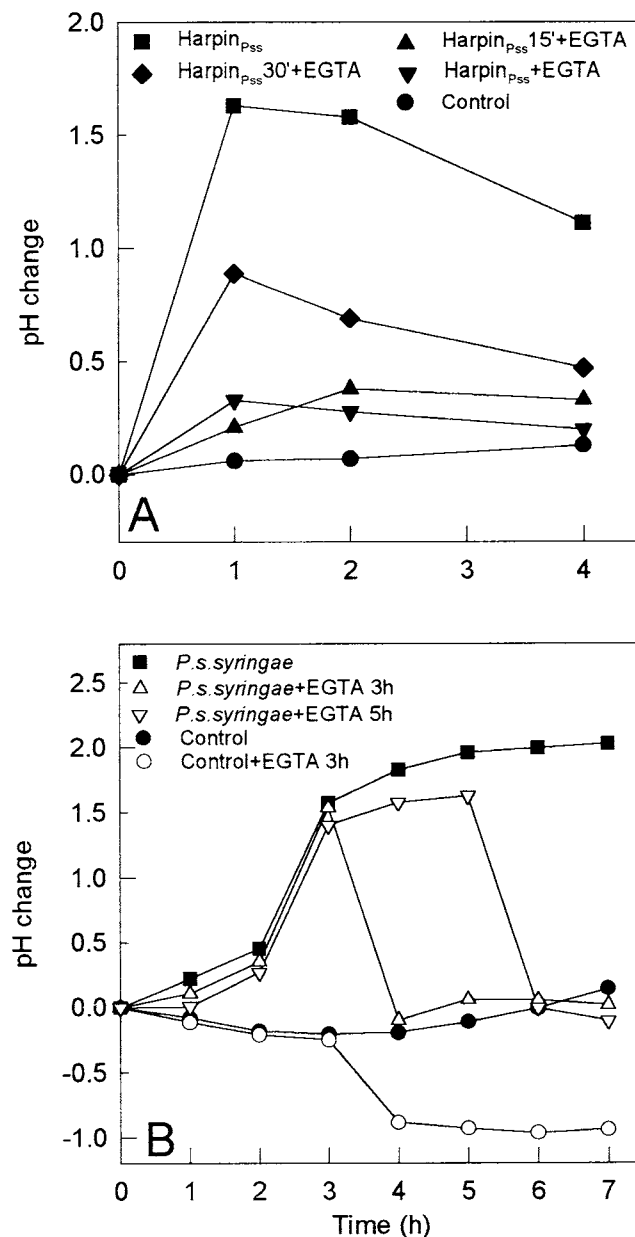

Fig. 7. pH changes in the external medium of tobacco suspension cells treated with A, harpin$_{Pss}$ full-length protein (200 µg per ml protein), full-length harpin$_{Pss}$ + 10 mM EGTA (applied at different times as shown), buffer control and B, *Pseudomonas syringae* pv. *syringae* 10^8 cells per ml alone, *P. syringae* pv. *syringae* + EGTA (added 3 and 5 h after bacterial treatment), buffer (control), or buffer + EGTA (added 3 h after buffer treatment). Addition of EGTA 4 h after bacterial treatment produced the same result as addition at 3 and 5 h. Points are values obtained in a representative experiment (n = 3 experiments). In A, the pH changes caused by addition of EGTA alone has been subtracted from all points resulting from treatment with harpin$_{Pss}$ + EGTA.

mammalian pathogens (Fuquay et al. 1986; Chatwal et al. 1987) and a wild-type strain of the plant pathogen, *Agrobacterium tumefaciens* (Wagner and Matthysse 1992). Binding to the plant cell is required for pathogenesis by *Agrobacterium tumefaciens*, a bacterium that transfers DNA into the plant cell. Mutant *Agrobacterium tumefaciens* with reduced ability to bind vitronectin were much less able to bind to carrot cells and were avirulent. Binding by the wild-type bacteria was inhibited by anti-vitronectin antibodies and added vitronectin, but not by BSA. Cell wall proteins extractable with dilute detergent mediate bacterial binding and cross-react with human vitronectin. These results suggest that vitronectin-like binding sites are functional in plant cells.

A new and exciting horizon has been opened with results presented in this study, but additional experiments involving protoplasts and cell wall fractions are required to define the role of the cell wall in HR induction.

MATERIALS AND METHODS

Materials.

Tobacco suspension-cultured cells derived from *Nicotiana tabacum* L. 'Hicks' (obtained from M. Atkinson, University of Wisconsin, Madison), were maintained as previously described (Atkinson et al. 1985). Four days after transfer to fresh medium (log phase), cells (0.1 g per ml) were collected by filtration and resuspended in 5 ml of standard assay medium (0.175 M mannitol, 0.5 mM K_2SO_4, 0.5 mM $CaCl_2$, 0.5 mM MES adjusted to pH 6.0 with NaOH). Cell suspensions were equilibrated in the assay medium in 30-ml beakers at 27°C on a rotatory shaker at 150 rpm for 2 h. All chemicals were purchased from Sigma (St. Louis, MO) unless otherwise stated. All experiments were repeated at least twice, and results from a representative experiment are shown.

Protoplast isolation.

In the first experiments, protoplasts were isolated from tobacco suspension cells in the standard assay medium containing in addition 0.7 M mannitol, 0.2% BSA, 1 mM ascorbic acid, 1% cellulysin, and 0.5% macerase (Calbiochemical, La Jolla, CA). Tobacco suspension cells were incubated for 4 h in this enzyme solution on a rotatory shaker, collected and washed (assay medium + 0.7 M mannitol) by centrifugation (100 g, 4 min), and resuspended in assay medium containing 0.7 M mannitol to a final concentration of 1×10^5 protoplasts per ml. Protoplasts were treated with harpin$_{Pss}$ and antibodies at 25°C. In later experiments protoplasts were prepared following the procedure by Diekmann et al. (1994). In these experiments the enzyme solution was heated at 45°C for 10 min in order to inactivate proteases, and protoplasts were treated with harpin$_{Pss}$ and incubated with antibodies at 4°C to minimize reduction in the number of binding sites, e.g., through endocytosis. Protoplasts were also incubated for 30 min with 1% chymotrypsin at 25°C, washed five times by centrifugation, and resuspended in 0.4 M mannitol + 1 mM $CaCl_2$ to ascertain the role of proteins in harpin$_{Pss}$ binding. pH experiments with protoplasts were performed six times with a single replication each and a representative experiment is shown.

Elicitor preparation and analysis.

Harpin$_{Pss}$, the HR elicitor from *P. syringae* pv. *syringae*, was prepared according to He et al. (1993). *E. coli* DH5α

transformants expressing the full-length protein, carried in pSYH10, and a truncated protein with the first 125 amino acids deleted, carried in pSYH5, were generous gifts from A. Collmer, Department of Plant Pathology, Cornell University. Bacteria were grown overnight in 1 liter of Terrific Broth at 30°C in the presence of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and ampicillin (100 μg per ml). Bacteria were harvested by centrifugation, washed once in 10 mM phosphate buffer (pH 6.5), and resuspended in 0.2 volume of the same buffer supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF). The bacterial suspension was immediately sonicated as described (Wei et al. 1992). The sonicate was boiled at 100°C for 15 min followed by centrifugation at 10,000 × g for 20 min. The supernatant containing harpin was dialyzed overnight against 5 mM $NaPO_4$ buffer, pH 6.5, and preserved in the presence of 0.1 mM PMSF in aliquots at − 20°C and used for treatment of cells and protoplasts. Negative controls, proteins of *E. coli* DH5α and DH5α (pBSK) were also extracted in the same way. The bacterial extract containing truncated harpin$_{Pss}$ was also fractionated by 12% SDS-PAGE and the band corresponding to the truncated elicitor was purified as described by Krishnan and Okita (1986). Truncated harpin$_{Pss}$ was inactivated with protease as previously described (Wei et al. 1992). Harpin$_{Ea}$ was obtained from S. V. Beer, Department of Plant Pathology, Cornell University, and was prepared according to Wei et al. (1992). All preparations were tested in the lower leaves of 8-week-old *Nicotiana tabacum* (Samsun NN) plants grown under fluorescent lights.

The bacterial extracts containing crude and gel-purified harpin$_{Pss}$ were fractionated by 12% SDS-PAGE through the Mighty Small II Gel Electrophoresis Unit following the manufacturer's instructions (Hoefer, San Francisco, CA). After electrophoresis, the gels were either stained with Coomassie Blue R-250 or immunoblotted and probed with anti-harpin$_{Pss}$ antibody. The proteins were transferred from the acrylamide gel to a nitrocellulose membrane in the electrode buffer (20 mM Tris, 150 mM glycine, 20% methanol) through the Mighty Small II Transfer Electrophoresis Unit following the manufacturer's instructions (Hoefer). Anti-harpin$_{Pss}$ antibody was used at 1:500 dilution. A second antibody, goat anti-mouse IgG-horseradish peroxidase conjugate, was used at 1:3,000 dilution as the manufacturer instructed (Bio-Rad, Richmond, CA).

Harpin$_{Pss}$ treatment.

Harpin$_{Pss}$ (final concentration 200, 140, 10, 5, or 1 μg per ml) or assay medium was added to tobacco suspension cells after equilibration in assay media on a rotary shaker at 150 rpm. pH measurements were recorded every hour for 6 h after treatment. Parallel samples for confocal laser microscopy were treated with harpin$_{Pss}$ for 30 min. Concentrations lower than 140 μg per ml did not produce sufficient fluorescent signal for confocal laser microscopy. Protoplasts were treated similarly except that they were gently shaken by hand during the equilibration time and were maintained without agitation during harpin$_{Pss}$ treatment.

EGTA treatment.

Tobacco suspension cells were incubated in assay medium containing 5 or 10 mM EGTA 15 min before the addition of harpin$_{Pss}$, together with harpin$_{Pss}$, and 30 min after harpin$_{Pss}$

treatment. Ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic (EGTA) was also added 3, 4, and 5 h after addition of *P. syringae* pv. *syringae* 61. pH changes in the extracellular medium were measured hourly during the 6-h period after harpin$_{Pss}$ treatment. Aliquots (250 μl) of harpin$_{Pss}$ treated cells were removed and prepared for examination under the confocal laser scanning microscope (CLSM).

Bacteria treatment.

P. syringae pv. *syringae* 61, an HR-inducing bacterium in tobacco, and *P. syringae* pv. *syringae* 61-2090, an *hrpH⁻* mutant that is defective in harpin$_{Pss}$ export, were used to treat suspension cells and protoplasts. Both bacterial strains were obtained from A. Collmer, Department of Plant Pathology, Cornell University. Bacteria were streaked onto a nutrient agar plate and grown overnight. Bacteria were washed from plates and grown in nutrient broth at 25°C on a rotary shaker overnight before the inoculation. For the *hrpH* mutant, kanamycin (50 μg per ml) was added to the culture medium. Bacterial concentration was adjusted turbidimetrically. Bacteria were added to equilibrated suspension cells, final concentration 10^8 cells per ml, and pH was measured hourly.

Primary and secondary antibodies and their dilutions.

Primary antibody used in this study was a polyclonal IgG that had been raised in rabbit against the full-length harpin$_{Pss}$ molecule (He et al. 1993) and was used at a dilution of 1:50 in washing solution. Secondary antibody, donkey anti-rabbit IgG, labeled with Cy 5 fluorescent dye (excitation: 647 nm; emission: 660 to 680 nm), was diluted 1:200 in washing solution. Nonspecific binding was eliminated by preabsorbing both antibodies with a protein extract from tobacco plants, a lyophilized extract from tobacco suspension cells, and an *E. coli* DH5α pBSK protein extract.

Sample preparation for confocal laser scanning microscopy.

Following harpin treatment, cells and protoplasts were washed 3 times by centrifugation and resuspended in harpin-free assay medium. Subsequently, they were incubated for 3 h in blocking solution (0.5 mM K_2SO_4, 0.5 mM $CaCl_2$, 1% bovine serum albumin [BSA], 1 mM N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid [HEPES] buffer, pH 7.4, and 1:50 normal donkey serum) before exposure to the primary antibody solution. After 60 min., samples were washed 3 times by centrifugation (0.5 mM K_2SO_4, 0.5 mM $CaCl_2$, 0.5 % BSA, 0.05% Tween 20, 1 mM HEPES buffer, pH 7.4) and exposed to the secondary antibody solution for 60 min. Periodically, cells and protoplasts were gently shaken by hand during antibody treatments. Cells and protoplasts were washed again by centrifugation 3 times in washing solution with final resuspension in washing solution. Samples fixed (6 h) with 4% paraformaldehyde and permeabilized with Triton ×100 (10 min) after harpin$_{Pss}$ treatment were prepared for CLSM using the same procedures. Stained sections were imaged using a krypton/argon confocal laser scanning microscope (MRC-600, BIO-RAD, Hercules, CA) attached to a Nikon Diaphot using 60× (N.A. = 1.4) PlanApo lens.

ACKNOWLEDGMENTS

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