

A Harpin Binding Site in Tobacco Plasma Membranes Mediates Activation of the Pathogenesis-Related Gene *HIN1* Independent of Extracellular Calcium but Dependent on Mitogen-Activated Protein Kinase Activity

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Harpin from the bean halo-blight pathogen *Pseudomonas syringae* pv *phaseolicola* (harpin_{PspH}) elicits the hypersensitive response and the accumulation of pathogenesis-related gene transcripts in the nonhost plant tobacco. Here, we report the characterization of a nonproteinaceous binding site for harpin_{PspH} in tobacco plasma membranes, which is assumed to mediate the activation of plant defense responses in a receptor-like manner. Binding of ¹²⁵I-harpin_{PspH} to tobacco microsomal membranes (dissociation constant = 425 nM) and protoplasts (dissociation constant = 380 nM) was specific, reversible, and saturable. A close correlation was found between the abilities of harpin_{PspH} fragments to elicit the transcript accumulation of the pathogenesis-related tobacco gene *HIN1* and to compete for binding of ¹²⁵I-harpin_{PspH} to its binding site. Another elicitor of the hypersensitive response and *HIN1* induction in tobacco, the *Phytophthora megasperma*-derived β -elicitin β -megaspermin, failed to bind to the putative harpin_{PspH} receptor. In contrast to activation by β -megaspermin, harpin_{PspH}-induced activation of the 48-kD salicylic acid-responsive mitogen-activated protein kinase (MAPK) and *HIN1* transcript accumulation were independent of extracellular calcium. Moreover, use of the MAPK kinase inhibitor U0126 revealed that MAPK activity was essential for pathogenesis-related gene expression in harpin_{PspH}-treated tobacco cells. Thus, a receptor-mediated MAPK-dependent signaling pathway may mediate the activation of plant defense responses induced by harpin_{PspH}.

INTRODUCTION

Phytopathogenic bacteria harbor a gene cluster (*HRP*, for hypersensitive reaction and pathogenicity) that controls pathogenicity in susceptible plants and the ability to elicit the hypersensitive reaction (HR) in nonhost plants or resistant cultivars of host plants (Lindgren et al., 1986; Galan and Collmer, 1999). Some *HRP* genes encode elements of a bacterial type III secretion system, by which effector proteins are exported and delivered into the cytosol of host plant cells (Galan and Collmer, 1999; Kjemtrup et al., 2000). Some of these effector proteins were found to interact with plant intracellular proteins and to activate the plant defense system.

Harpins constitute another group of effector proteins exported by the type III pathway of plant pathogenic *Erwinia*, *Pseudomonas*, and *Ralstonia* spp (Galan and Collmer,

1999). Although identified several years ago, the roles of these proteins during colonization of host plants and their site of action have remained unclear. However, when infiltrated into nonhost plants, harpins trigger disease resistance-associated responses, such as HR, transcript accumulation of pathogenesis-related (*PR*) genes, and systemic acquired resistance (Baker et al., 1993; He et al., 1993; Gopalan et al., 1996; Strobel et al., 1996; Dong et al., 1999; Galan and Collmer, 1999). Physiological target sites for harpin action, therefore, were suggested to reside at the plant cell surface. Immunolocalization studies revealed a Ca²⁺-dependent association of *Pseudomonas syringae* pv *syringae* harpin with tobacco cell walls (Hoyos et al., 1996), but harpin-induced K⁺/H⁺ exchange at the plant plasma membrane and subsequent plasma membrane depolarization (Hoyos et al., 1996; Pike et al., 1998) raised questions regarding the concept of a cell wall binding site mediating such responses. Alternatively, bacterial elicitors may be recognized by the plant just like elicitors derived from phytopathogenic fungi and oomycetes, which bind to plasma membrane proteins (Nürnberger et al., 1995; Mithöfer et al.,

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1996; Umemoto et al., 1997; Bourque et al., 1999). An example of this phenomenon is provided by the recent identification of a 115-kD tomato microsomal membrane protein that binds the bacterial flagellin-derived elicitor flg22 (Meindl et al., 2000). On the other hand, harpins may interact with membranes directly and trigger plant defense responses in an ionophore-like manner, because harpins from various phytopathogenic *P. s. syringae* pathovars were found recently to associate stably with synthetic bilayer membranes and to evoke cation currents of large unitary conductance (Lee et al., 2001).

Elicitor binding to cell surface binding sites initiates an intracellular signaling cascade that results in the activation of plant-specific defense responses (Yang et al., 1997; Scheel, 1998; Grant and Mansfield, 1999; Nürnberger, 1999). Changes in cytoplasmic free calcium concentration ($[Ca^{2+}]_{cyt}$) are implicated in elicitor-induced signal transduction chains in various plants (Yang et al., 1997; Scheel, 1998; Grant and Mansfield, 1999). Previous work had demonstrated the importance of extracellular Ca^{2+} for the activation of pathogen defense responses (Yang et al., 1997; Scheel, 1998; Grant and Mansfield, 1999). More recently, receptor-mediated influx of extracellular Ca^{2+} in elicitor-treated plant cells was shown to cause characteristic $[Ca^{2+}]_{cyt}$ signatures as a prerequisite for the activation of pathogen defense (Mithöfer et al., 1999; Blume et al., 2000). In addition, mitogen-activated protein kinase (MAPK) cascades constitute another common element of intracellular signal transduction chains in eukaryotic cells (Herskowitz, 1995; Hirt, 2000). In plants, MAPK activation has been implicated in the adaptation to various environmental stimuli, including pathogen infection or treatment with pathogen-derived elicitors (Hirt, 2000). Tobacco mosaic virus infection of tobacco plants (Zhang and Klessig, 1998a) or treatment of tobacco cells with elicitors (Lebrun-Garcia et al., 1998; Zhang et al., 1998) and *Trichoderma viride*-derived xylanase (Suzuki et al., 1999) stimulated transient activation of a 48-kD salicylic acid-inducible MAPK (SIPK) (Zhang and Klessig, 1997, 2000). When the *Cladosporium fulvum*-derived race-specific elicitor AVR9 was infiltrated into tobacco plants expressing the tomato resistance gene *Cf-9*, both SIPK and another tobacco mosaic virus and elicitor-responsive tobacco MAPK, WIPK (for wounding-induced protein kinase) (Zhang and Klessig, 1998b), were activated (Romeis et al., 1999). Moreover, elicitor-responsive parsley SIPK and WIPK and Arabidopsis SIPK orthologous enzymes have been reported (Ligterink et al., 1997; Nühse et al., 2000; Scheel et al., 2000). In addition to specific $[Ca^{2+}]_{cyt}$ signatures, differentially induced MAPK isoenzymes with characteristic activity profiles are assumed to encode signal specificity during the activation of pathogen defense in plants (Hirt, 2000).

P. s. syringae-derived harpins and elicitors from various *Phytophthora* species trigger the HR, *PR* gene expression, and systemic acquired resistance in the nonhost plant tobacco (Ricci et al., 1989; He et al., 1993; Baillieux et al., 1995; Gopalan et al., 1996). Using harpin from *P. s. phaseolicola*

(harpin_{P_{sph}}) and the *Phytophthora megasperma* β -elicitor β -megaspermin, we have attempted a comparative analysis of signal perception and transduction mechanisms induced by bacterial and oomycete elicitors. We provide evidence that tobacco plasma membranes harbor a specific binding site for harpin_{P_{sph}} that does not bind β -megaspermin. This binding site is likely to mediate harpin_{P_{sph}}-induced expression of the *PR* gene *HIN1* (Gopalan et al., 1996). In contrast to the β -elicitor-induced activation of *HIN1*, harpin_{P_{sph}}-induced *HIN1* expression was independent of extracellular Ca^{2+} . We further show that MAPK activity, which includes SIPK activity, is required for harpin_{P_{sph}}-induced *HIN1* expression.

RESULTS

Tobacco Cell Responses to Treatment with Harpin_{P_{sph}}

When infiltrated into tobacco leaves, purified recombinant harpin_{P_{sph}} elicited an HR (Figure 1A). At concentrations of 1 μ M, symptoms became visible 8 hr after infiltration. In addition, treatment with harpin_{P_{sph}} of cultured tobacco cells resulted in transcript accumulation of the *PR* genes *PR1*, *PR2*, acidic chitinase (*PR3*), and chitinase/lysozyme (Heitz et al., 1994) (Figure 1B). Transcripts derived from genes considered HR marker genes (*HSR203*, *HSR201*, *HSR515*, and *HIN1*) (Gopalan et al., 1996; Pontier et al., 1999) also accumulated in harpin_{P_{sph}}-treated tobacco cells (Figure 1B).

Harpin_{P_{sph}}-induced *HIN1* transcript accumulation was observed as early as 30 min after elicitation and persisted for a minimum of 5 hr (Figure 1C). Therefore, we chose *HIN1* as an exemplary gene to analyze elicitor-induced *PR* gene expression by single tube multiplex reverse transcription-polymerase chain reaction (RT-PCR). A constitutively expressed gene encoding the translation elongation factor *EF1 α* served as an internal standard in these assays. *HIN1* transcript accumulation in tobacco cell cultures did not differ quantitatively from that observed in harpin_{P_{sph}}-infiltrated tobacco leaves (Figure 1D), thus validating the use of cell suspensions for studies of harpin_{P_{sph}} perception and signal transduction.

The concentration of harpin_{P_{sph}} required to trigger half-maximum expression (EC_{50}) of *HIN1* and *HSR203* in tobacco cells was 120 and 82 nM, respectively (Table 1). This is in good agreement with concentrations of the elicitor required to stimulate a rapidly induced K^+/H^+ exchange and Cl^- efflux (Table 1). Similar ion fluxes have been associated with pathogen resistance responses in many plant systems (Yang et al., 1997; Scheel, 1998; Grant and Mansfield, 1999) and are assumed to be involved in signaling *PR* gene expression and HR. Such a correlation of EC_{50} values strongly suggests that harpin_{P_{sph}}-induced cellular responses are activated upon recognition of the elicitor at a signal-specific binding site.

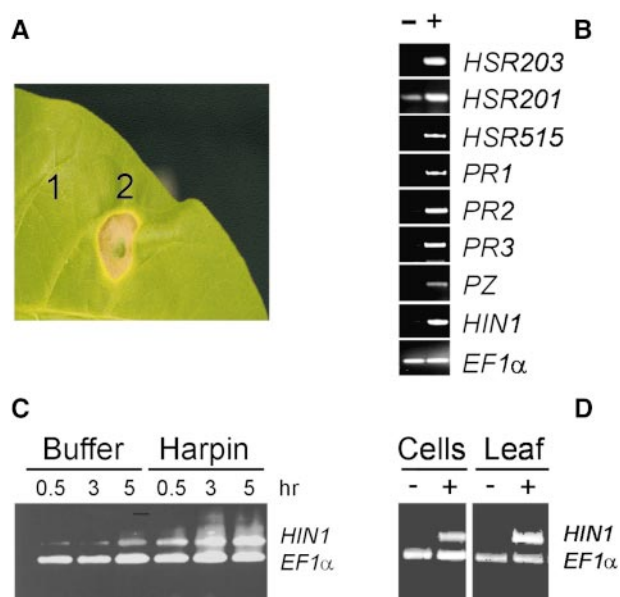


Figure 1. Harpin_{P_{sph}}-Induced Hypersensitive Cell Death and *PR* Gene Expression in Tobacco.

Harpin_{P_{sph}} (1 μ M) was infiltrated into tobacco leaves or added to suspension cultured tobacco cells.

(A) Tobacco leaf treated with 5 mM Mes buffer, pH 5.5 (1), or harpin_{P_{sph}} (2) 2 days after infiltration.

(B) Total RNA prepared from tobacco cells treated for 3 hr with buffer (-) or harpin_{P_{sph}} (+) was used as a template in RT-PCR assays with DNA primers derived from the tobacco genes indicated (see text). The tobacco gene encoding *EF1 α* served as an internal control. *PZ*, chitinase/lysozyme.

(C) Kinetics of *HIN1* expression in tobacco cells treated with buffer or harpin_{P_{sph}}. Total RNA from tobacco cells was prepared at the times after infiltration indicated and used in RT-PCR to simultaneously amplify *HIN1* and *EF1 α* transcripts.

(D) *HIN1* expression in tobacco cells or leaves treated for 3 hr with buffer (-) or harpin_{P_{sph}} (+). RT-PCR analysis was performed as described in (C).

Specific Binding of Harpin_{P_{sph}} to Tobacco Plasma Membranes

To characterize harpin_{P_{sph}} binding sites on tobacco membranes, the protein was radioiodinated (¹²⁵I) at the meta position of the phenoxyl ring of a tyrosine residue. Because native harpin_{P_{sph}} lacked tyrosine, PCR was used to attach this amino acid to the C terminus. Expression products were nonradioactively iodinated and separated by reverse phase HPLC. Matrix-assisted laser-desorption ionization time of flight mass spectrometry analysis of the reaction products confirmed complete iodination of harpin_{P_{sph}} and revealed that harpin_{P_{sph}} was not post-translationally modified during heterologous expression. Because iodination did not affect HR- or *HIN1*-inducing activities of harpin_{P_{sph}} in tobacco leaves (data not shown), ¹²⁵I-harpin_{P_{sph}} (specific radioactiv-

ity, 2200 Ci/mmol) was prepared and used as ligand in binding assays.

Binding of ¹²⁵I-harpin_{P_{sph}} to tobacco microsomal membranes was investigated by filtration to separate free from bound label, which ensured that any loss of ligand caused by rapid dissociation of the receptor-ligand complex was negligible. Specific binding of ¹²⁵I-harpin_{P_{sph}} to tobacco microsomes was not affected significantly by ionic strength (up to 1 M NaCl) and was greatest at pH 7.0 (84% at pH 6.0, 51% at pH 8.0, and 33% at pH 10.0). Therefore, binding assays were performed in neutral buffer containing 100 mM NaCl. The stability of the radioligand under binding assay conditions was confirmed by SDS-PAGE/autoradiography of aliquots taken from the binding mixture after various times of incubation (data not shown). In all experiments, specific binding constituted no more than 5% of the initially added ligand, ensuring that ligand depletion did not obscure binding assays.

Kinetic analysis of ¹²⁵I-harpin_{P_{sph}} binding demonstrated that ligand association with tobacco microsomal membranes was initially faster than dissociation (Figure 2A). Half-maximal binding was achieved within 15 sec after addition of the ligand, and equilibrium between association and dissociation was reached after 60 sec. Addition of a 100-fold molar excess of unlabeled harpin_{P_{sph}} 45 min after the addition of the radioligand to tobacco membranes resulted in the rapid dissociation of bound label (Figure 2B). Thus, binding of ¹²⁵I-harpin_{P_{sph}} was reversible.

Saturation analyses with increasing concentrations of ¹²⁵I-harpin_{P_{sph}} (50 to 700 nM) were performed. Specific binding increased exponentially at radioligand concentrations up to 250 nM. At higher concentrations, specific binding began to plateau (Figure 2C), suggesting that saturation of microsomal binding sites was approached. Nonspecific binding showed a linear increase with increasing ligand concentrations (Figure 2C). Unfortunately, experiments with higher radioligand/competitor concentrations were impeded by the tendency of harpin_{P_{sph}} to precipitate. Thus, the competitor

Table 1. EC₅₀ Values^a of Harpin_{P_{sph}}-Induced Responses in Tobacco Cells

Response	EC ₅₀ Value (nM)
Medium alkalization	100
K ⁺ efflux	50
Cl ⁻ efflux	120
Expression of <i>HIN1</i> ^b	120
Expression of <i>HSR203</i> ^b	82

^a Concentrations of harpin_{P_{sph}} required to stimulate 50% of the particular plant response as derived from dose-response curves.

^b Transcript accumulation was quantified by phosphorimaging RNA gel blots hybridized with α -³²P-dATP-labeled *HIN1* and *HSR203* cDNA, respectively. Hybridization of filters with α -³²P-dATP-labeled rDNA was performed to normalize RNA loading.

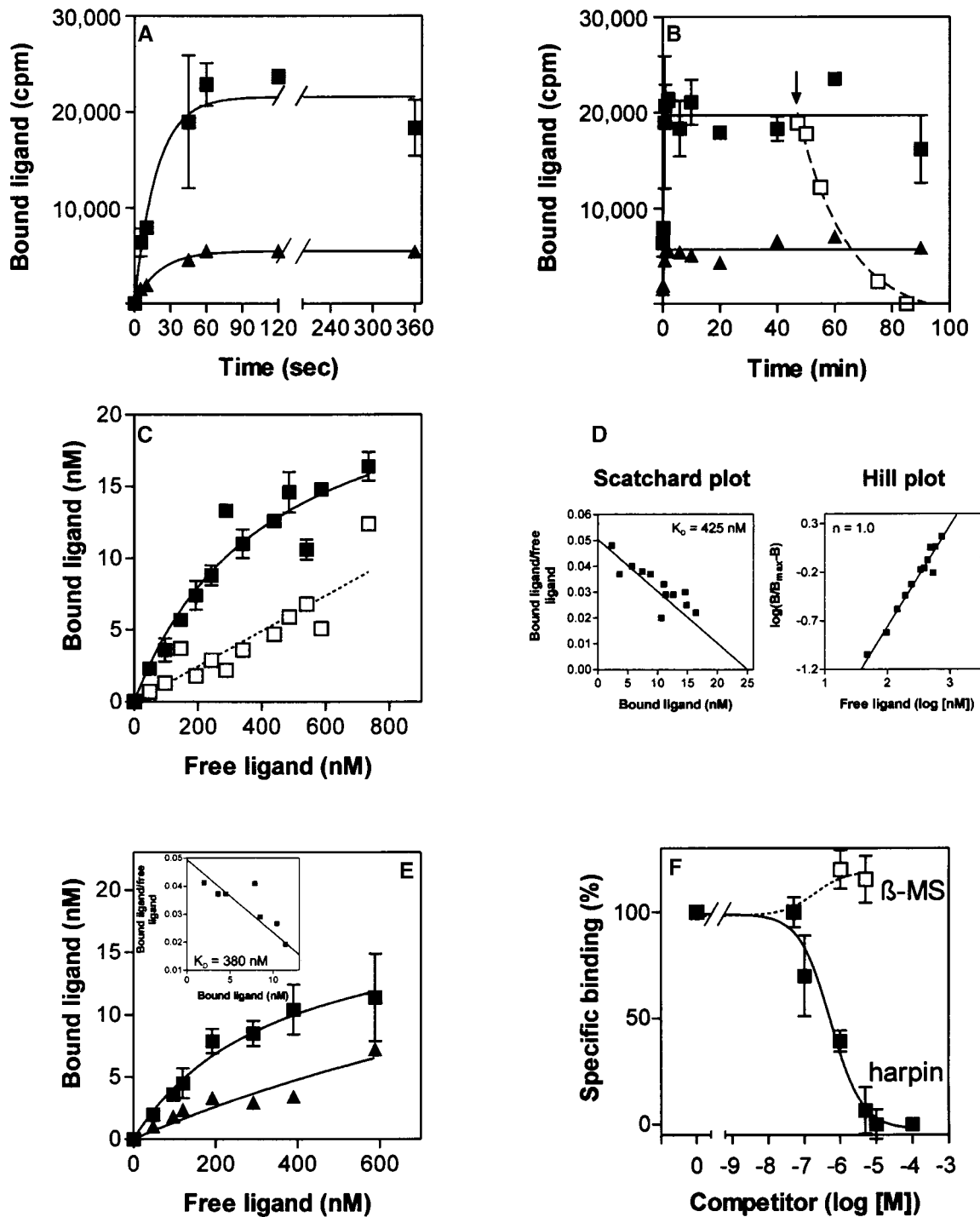


Figure 2. Binding of ^{125}I -Harpin_{PspH} to Tobacco Membranes.

Data points represent the average of triplicate experiments. Data points representing nonspecific binding are the average of duplicate experiments. **(A)** Time course of binding of 50 nM ^{125}I -harpin_{PspH} (1:10 dilution with unlabeled harpin_{PspH}) to tobacco microsomal membranes added at time 0. Membrane-bound radioligand was determined at the times indicated. Squares show the amount of radioligand that was bound specifically by the ^{125}I -harpin_{PspH} binding site. Nonspecific binding is indicated by triangles. Specific binding was obtained by subtracting nonspecific binding from total binding. Nonspecific binding was determined in the presence of 5 μM unlabeled harpin_{PspH}.

concentrations required to determine the degree of nonspecific binding at higher ^{125}I -harpin_{P_{sph}} levels could not be used. Linearization of the data in a Scatchard plot (Figure 2D) indicated the existence of a ^{125}I -harpin_{P_{sph}} binding site in tobacco microsomal membranes with a dissociation constant (K_D) of 425 nM and an apparent binding site concentration of 6.7 pmol/mg of protein. Hill plot analysis of the binding data yielded a Hill coefficient of 1 (Figure 2D), indicating that there is no cooperativity in the binding of ^{125}I -harpin_{P_{sph}} to tobacco membranes.

Binding of ^{125}I -harpin_{P_{sph}} to tobacco protoplasts revealed a saturable binding site with an apparent K_D of 380 nM and a binding site concentration of 1.5 pmol/10⁶ protoplasts (Figure 2E). Consistent with the experiments performed with microsomal membranes, ^{125}I -harpin_{P_{sph}} binding to protoplasts did not show any cooperativity (data not shown). Because binding experiments were performed under conditions believed to prevent radioligand endocytosis (15 min at 0°C; Hulme and Birdsall, 1990), our results indicated that the harpin_{P_{sph}} binding site is localized predominantly in the plasma membrane.

Competition experiments with increasing concentrations of unlabeled harpin_{P_{sph}} yielded a concentration resulting in 50% inhibition of specific binding of 550 nM (Figure 2F). This is consistent with the K_D value determined in ligand saturation analyses (Figures 2C and 2D). When added at a 50-fold molar excess over ^{125}I -harpin_{P_{sph}}, unlabeled harpin_{P_{sph}} reduced specific binding of the radioligand by 92%. In contrast, β -megaspermin, from the phytopathogenic oomycete *Phytophthora megasperma* (Baillieul et al., 1995), did not compete for binding of ^{125}I -harpin_{P_{sph}} when applied at the same concentration (Figure 2F). Therefore, the harpin_{P_{sph}} binding site and the recently characterized elicitor receptor (Bourque et al., 1998, 1999) are unlikely to be identical.

Treatment of tobacco microsomal membranes with proteinase E before binding assays did not abolish the binding of ^{125}I -harpin_{P_{sph}} (data not shown). In addition, use of the radioligand in chemical cross-linking assays with the

homobifunctional reagent, 3,3'-dithiobis[sulfosuccinimidyl propionate] (DTSSP), failed to identify one or more microsomal membrane proteins as possible constituents of the ^{125}I -harpin_{P_{sph}} binding site (data not shown). Thus, the ^{125}I -harpin_{P_{sph}} binding site may not be a protein.

Harpin_{P_{sph}} Fragments Are Sufficient for *HIN1* Activation and Competition of ^{125}I -Harpin_{P_{sph}} Binding

To identify the minimum portion of harpin_{P_{sph}} required to elicit *HIN1* expression, cDNAs encoding harpin_{P_{sph}} or portions of the polypeptide were expressed as His₁₀-tag fusion proteins in *Escherichia coli* (Figure 3A). The expression products were purified to apparent homogeneity on nickel-nitrilotriacetic acid agarose and subsequently assessed for their ability to induce *HIN1* transcript accumulation in tobacco cells. Deletion of 100 or 200 N-terminal amino acid residues of a total of 345 amino acids of harpin_{P_{sph}} did not adversely affect *HIN1*-inducing activity (Figure 3A, fragments II and III). Fragment V, corresponding to amino acids 100 to 300, also was elicitor active. Further C-terminal deletion of this fragment resulted in the complete loss of elicitor activity (Figure 3A, fragment VI). In addition, an N-terminal 80-amino acid peptide was found to be devoid of *HIN1*-inducing activity (Figure 3A, fragment VII). Fragment III represented the smallest elicitor-active polypeptide that could be expressed in *E. coli*.

Together, these data suggest that the elicitor activity of harpin_{P_{sph}} resided in a C-terminal fragment corresponding to amino acids 200 to 300 (Figure 3A, fragment VIII). Because attempts to produce this minimal fragment in *E. coli* proved unsuccessful, we chemically synthesized overlapping peptides covering amino acids 174 to 345 (amino acids 174 to 218, 200 to 224, 219 to 263, 225 to 257, 236 to 280, 264 to 300, and 301 to 345). However, neither alone nor in combination did these peptides exhibit *HIN1*-inducing activity

Figure 2. (continued).

(B) Time course of displacement of ^{125}I -harpin_{P_{sph}}. ^{125}I -harpin_{P_{sph}} (50 nM; 1:10 dilution with unlabeled harpin_{P_{sph}}) was incubated with tobacco microsomal membranes for the times indicated. To initiate the displacement of ^{125}I -harpin_{P_{sph}}, a 100-fold molar excess of unlabeled harpin_{P_{sph}} (open squares) was added 45 min (arrow) after the radioligand. Specific binding (closed squares) and nonspecific binding (triangles) are indicated.

(C) Saturability of ^{125}I -harpin_{P_{sph}} binding to tobacco microsomal membranes. Specific binding (closed squares) and nonspecific binding (open squares) were determined in the presence of a 100-fold molar excess of unlabeled harpin_{P_{sph}}.

(D) Scatchard plot and Hill plot of the binding data shown in **(C)**. The binding constant (K_D) and the Hill coefficient (n) were determined according to Hulme and Birdsall (1990).

(E) Saturability of ^{125}I -harpin_{P_{sph}} binding to tobacco protoplasts. Graphs for specific binding (squares) and nonspecific binding (triangles) are shown. The inset shows a Scatchard plot of the binding data. The dissociation constant (K_D) was determined according to Hulme and Birdsall (1990).

(F) Competitive inhibition of ^{125}I -harpin_{P_{sph}} (100 nM) binding to tobacco microsomal membranes by increasing concentrations of harpin_{P_{sph}} or β -megaspermin (β -MS). One hundred percent specific binding corresponded to the binding detected in the absence of competitor (1,600,000 cpm), whereas 0% specific binding corresponded to the binding detected in the presence of a 100-fold molar excess of competitor (10 μM) (nonspecific binding, 374,000 cpm).

Error bars indicate standard error.

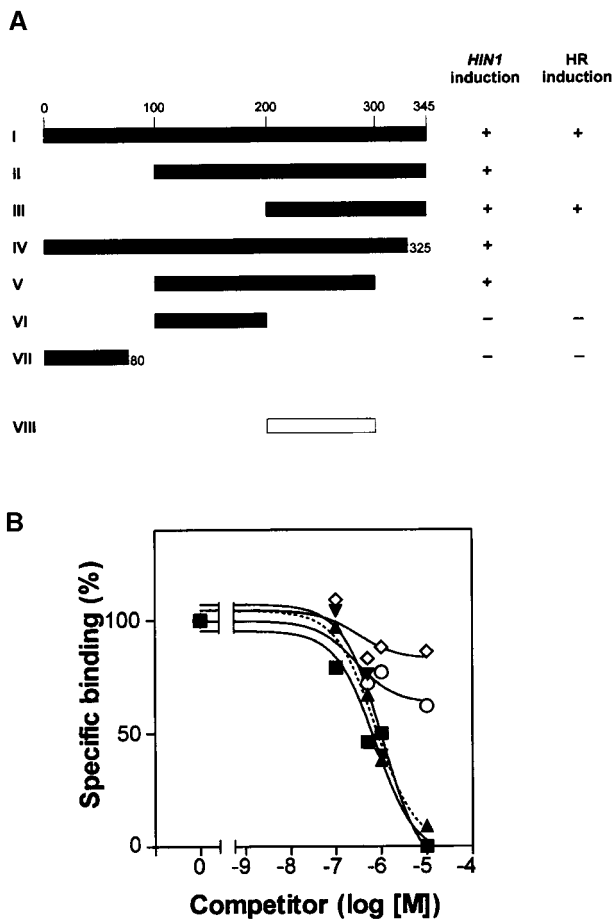


Figure 3. Structure/Activity Relationship of Harpin_{P_{sph}} and Harpin_{P_{sph}} Deletion Derivatives Expressed in *E. coli*.

(A) cDNAs encoding harpin_{P_{sph}} (fragment I) and deletion derivatives (fragments II to VII) fused to a His₁₀-encoding tag were expressed in *E. coli*. Expression products were purified to apparent homogeneity on nickel-nitrilotriacetic acid agarose and tested for their ability to induce *HIN1* expression or the HR in tobacco cells at a concentration of 1 μ M. Total RNA was prepared from tobacco cells treated with harpin_{P_{sph}} for 3 hr, and *HIN1* expression was monitored by RT-PCR as described in the legend to Figure 1C. The white bar (fragment VIII) denotes the overlapping region of two active derivatives (fragments III and V) and defines the smallest fragment deduced to harbor elicitor activity. +, full activity relative to that of the full-length expression product; -, no detectable activity.

(B) Competitive inhibition of ¹²⁵I-harpin_{P_{sph}} (100 nM) binding to tobacco microsomal membranes by recombinant deletion derivatives shown in **(A)** and harpin_{P_{ss}} from *P. s. syringae*. Increasing concentrations of fragment I (closed squares), fragment III (closed triangles), harpin_{P_{ss}} (closed inverted triangles), fragment VI (open circles), or fragment VII (open diamonds) were used as competitors of binding of 100 nM ¹²⁵I-harpin_{P_{sph}}. The graphs show the amount of specific binding as described in the legend to Figure 2A. One hundred percent specific binding corresponded to the binding detected in the absence of competitor (1,850,000 cpm), whereas 0% specific binding corresponded to the binding detected in the presence of a 100-

(data not shown). In radioligand competition assays, the elicitor-active fragment III proved to be as active as the canonical harpin_{P_{sph}} in blocking the binding of ¹²⁵I-harpin_{P_{sph}}, whereas the two elicitor-inactive fragments (VI and VII) failed to do so (Figure 3B). Consistently, neither alone nor in combination did the synthetic peptides encompassing amino acids 174 to 345 compete for binding of ¹²⁵I-harpin_{P_{sph}} as effectively as canonical harpin_{P_{sph}}. Hence, a qualitative and quantitative correlation was found between the abilities of harpin_{P_{sph}} fragments to bind to the receptor and to elicit *HIN1* expression. Residual binding activity of 35 to 50% of the radioligand was detected when one of three synthetic peptides (amino acids 219 to 263, 236 to 280, or 301 to 345) was used as a competitor at a 100-fold molar excess over ¹²⁵I-harpin_{P_{sph}} (data not shown).

Recombinant harpins from *P. s. syringae* (harpin_{P_{ss}}; Figure 3B) and *P. s. tomato* (harpin_{P_{st}}; data not shown), which are structurally related to harpin_{P_{sph}} (77 and 53% identical at the amino acid level; GenBank accession number AF268940), exhibited competitor activity similar to that of harpin_{P_{sph}}, indicating that they targeted the same binding site in tobacco. Harpin_{P_{ss}} and harpin_{P_{st}} also were found to induce *HIN1* expression in tobacco (Gopalan et al., 1996; our unpublished data). Fragment VIII of harpin_{P_{sph}} (Figure 3A) exhibited 60% identity to both harpin_{P_{ss}} and harpin_{P_{st}}, which is not very different from the overall identity observed between the three proteins. Thus, a secondary structure motif, rather than a highly conserved peptide fragment, is likely to represent the recognition determinant for the stimulation of *HIN1* expression.

Harpin_{P_{sph}} Activates a Salicylic Acid-Inducible MAPK in Tobacco Cells Independent of Extracellular Calcium

Recent studies have provided evidence that rapidly induced influxes of extracellular Ca²⁺ and subsequent changes in the [Ca²⁺]_{cyt} contribute to the activation of defense-associated responses in various plants (Xu and Heath, 1998; Mithöfer et al., 1999; Blume et al., 2000). However, decreasing the extracellular free [Ca²⁺] to 20 nM using the membrane-impermeable chelator 1,2-bis(o-aminophenoxy)ethane-*N,N,N,N*-tetraacetic acid (BAPTA) did not affect harpin_{P_{sph}}-induced *HIN1* transcript accumulation (Figure 4). The Ca²⁺ influx inhibitors La³⁺ and Gd³⁺ also failed to block harpin_{P_{sph}}-induced *HIN1* activation (Figure 4). In contrast, extracellular Ca²⁺ and Ca²⁺ influx proved indispensable for β -megaspermin-induced *HIN1* activation, because BAPTA and lanthanides inhibited this response in elicited tobacco cells (Figure 4).

A myelin basic protein (MBP)-phosphorylating protein ki-

fold molar excess of competitor (10 μ M) (nonspecific binding, 525,000 cpm). Each data point represents the average of duplicate experiments.

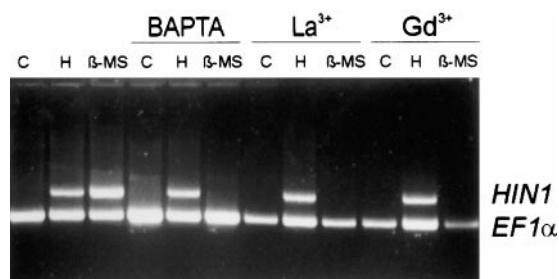


Figure 4. Harpin_{P_{sph}}-Induced *HIN1* Expression Is Independent of Extracellular Calcium.

Effects of Ca²⁺ chelator and Ca²⁺ channel inhibitors on elicitor-induced *HIN1* expression in tobacco cells. Tobacco cells were treated for 1.5 hr with buffer (C), 1 μM harpin_{P_{sph}} (H), or 50 nM β-megaspermin (β-MS) in the absence or presence of BAPTA (8 mM), LaCl₃ (0.25 mM; La³⁺), or GdCl₃ (0.25 mM; Gd³⁺). MgSO₄ (20 mM) was included in the buffer together with BAPTA to prevent membrane destabilization due to Ca²⁺ depletion. Total RNA prepared from elicited tobacco cells was analyzed by RT-PCR as described in the legend to Figure 1C.

nase of 48 kD was activated within 5 min after treatment of tobacco cells with harpin_{P_{sph}} (Figure 5A). Protein kinase activation was transient and decreased to nearly background levels within 60 min after elicitation. In immunoblot analyses performed with a MAPK-specific antibody that recognized the dually phosphorylated threonine-glutamic acid-tyrosine tripeptide motif _pTE_pY, a 48-kD protein was detected in protein extracts from harpin_{P_{sph}}-treated cells (Figure 5B). Thus, harpin_{P_{sph}} activated a tobacco MAPK that was likely SIPK (Zhang and Klessig, 1997). To verify this, we used a monospecific antiserum raised against a unique N-terminal peptide of SIPK (Zhang et al., 1998) for immunoprecipitation and subsequent *in vitro* protein kinase assay with MBP as a substrate. As shown in Figure 5C, the antiserum precipitated a MBP kinase activity from extracts of harpin_{P_{sph}}-induced cells (lane 2) that was not precipitated from buffer-treated cells (lane 1). Most importantly, immunoprecipitation of this kinase could be inhibited with an excess of the SIPK-specific peptide used for antibody production (lane 3). Thus, harpin_{P_{sph}} stimulated SIPK activity in tobacco cells.

The activation of *HIN1* expression by harpin_{P_{sph}}, but not by β-megaspermin, was independent of extracellular Ca²⁺ (Figure 4). Because the activation of SIPK by elicitors was reported recently for the *Phytophthora parasitica*-derived α-elicitor parasiticein and the *Phytophthora cryptogea* β-elicitor cryptogein (Zhang et al., 1998), we wondered if extracellular Ca²⁺ was required for SIPK activation in response to harpin_{P_{sph}} or β-megaspermin. Protein extracts prepared from tobacco cells treated with elicitor either in the absence or presence of BAPTA were fractionated by SDS-PAGE, blotted, and analyzed with the anti-_pTE_pY antiserum. Harpin_{P_{sph}}-mediated activation of the 48-kD MAPK (SIPK) was not affected by BAPTA treatment (Figure 6), indicating that MAPK

activation was independent of extracellular Ca²⁺. In contrast, β-megaspermin-induced activation of this enzyme was sensitive to BAPTA treatment and thus dependent on extracellular Ca²⁺.

Harpin_{P_{sph}}-Induced MAPK Activation Is Required for *PR* Gene Expression in Tobacco

MAPK cascades are assumed to constitute an element of elicitor-induced signal transduction cascades in various plant systems (Hirt, 2000). However, the direct involvement of activated MAPK in triggering plant defense responses has yet to be demonstrated. To causally link harpin_{P_{sph}}-induced MAPK activity and *HIN1* expression in tobacco, we used the inhibitor of MAPK kinases (MAPKK) U0126 (Favata et al., 1998). This inhibitor is believed to be more specific and more active against MAPKK than inhibitor PD98059 (Favata et al., 1998), which was reported recently to block the MAPK pathway in Arabidopsis and tobacco (Desikan et al., 1999; Romeis et al., 1999). Although PD98059 blocks MAPKK activity by binding directly to the inactive (nonphosphorylated) form of the enzyme, U0126 does not affect MAPKK activation by phosphorylation but inhibits the activated (phosphorylated) MAPKK at the catalytic site (Favata et al., 1998). When tobacco cells were pretreated with U0126, harpin_{P_{sph}}-mediated activation of the 48-kD MBP kinase was strongly reduced (at 50 μM) or compromised (at 100 μM) (Figure 7A, top section). SIPK activation was reduced by 50% in the presence of 5 μM U0126 (not shown). Similar U0126 concentrations (25 μM) have been found to efficiently block activation of the human MAPK, ERK2, by the MAPKK MEK1 *in vitro* (Favata et al., 1998; Goueli et al., 1999). Incubation of tobacco cells with U0126 for 4 hr had no apparent effect on the viability of the cells, which was determined in a combined viability/lethality assay using fluorescein diacetate and propidium iodide (Blume et al., 2000). In addition, the harpin_{P_{sph}}-induced oxidative burst remained unaffected in tobacco cells treated with 100 μM U0126, suggesting that MAPK activation and reactive oxygen species production are not linked functionally (not shown).

The level of harpin_{P_{sph}}-induced *HIN1* transcript accumulation in U0126-treated tobacco cells was diminished compared with that in DMSO-treated control cells (Figure 7A, bottom section). To quantify the effect of the inhibitor on harpin_{P_{sph}}-induced *HIN1* activation, RT-PCR products (Figure 7A) were blotted for hybridization with α-³²P-dATP-labeled cDNA and relative *HIN1* expression levels were determined by phosphorimaging. This was necessary because the inhibitor itself slightly induced the transcript accumulation of *HIN1* and other *PR* genes in nonelicited tobacco cells (Figure 7A). Pooled data from three independently performed experiments revealed that 100 μM U0126 reduced *HIN1* expression by 78% compared with control levels (Figure 7B). Interestingly, albeit to a variable extent, transcript accumulation of *PR* genes such as *PR1*, *PR2*, and acidic chitinase

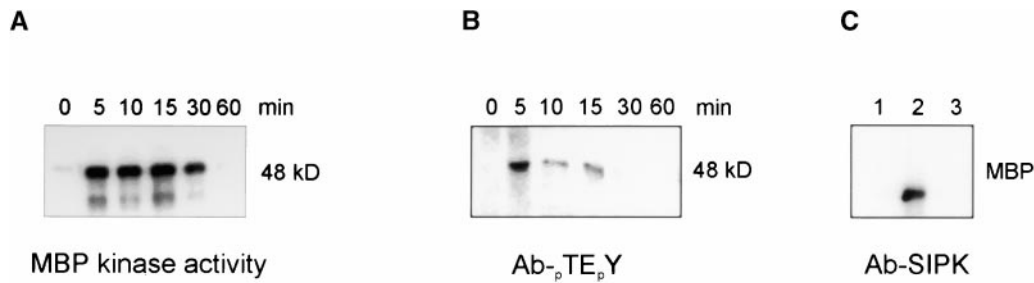


Figure 5. Activation of the SIPK in Tobacco Cells Treated with Harpin_{P_{sp}h}.

Tobacco cells treated with 1 μ M harpin_{P_{sp}h} were harvested at the times after infiltration indicated and used to prepare protein extracts.

(A) Kinase activity determined by an in-gel kinase assay using MBP as the substrate.

(B) Protein extracts analyzed by immunoblotting using an antiserum (Ab) recognizing the p_{TE}p_Y motif of activated MAPK.

(C) Protein extracts were prepared from tobacco cells treated with buffer (lane 1) or harpin_{P_{sp}h} (lanes 2 and 3) for 5 min. For immunoprecipitation, the tobacco SIPK-specific antibody (Ab-p48N) (Zhang et al., 1998) was added alone (lanes 1 and 2) or together with the competitor peptide used for antibody production (lane 3). Kinase activity of immunoprecipitated material was assayed using MBP as the substrate. The phosphorylated MBP was visualized by autoradiography.

(*PR3*) or of the HR marker genes *HSR201*, *HSR515*, and *HSR203* also was affected by the inhibitor (Figure 7A). A similar reduction in *HSR203* expression was found when RNA gel blots were probed with *HSR203* cDNA (Figure 7A). Thus, a harpin_{P_{sp}h}-activated MAPK cascade appears to be involved in signaling *PR* gene expression in tobacco.

DISCUSSION

A Harpin Binding Site Involved in *PR* Gene Expression in Tobacco

We have shown that tobacco plasma membranes harbor a binding site for *P. s. syringae*-derived harpins (Figures 2 and 3). Binding of ¹²⁵I-harpin_{P_{sp}h} was inhibited by excess of unlabeled harpin_{P_{sp}h} and binding was saturable (Figure 2), as was expected of an authentic receptor (Hulme and Birdsall, 1990). Ligand saturation analyses performed with tobacco protoplasts revealed a dissociation constant of the ligand/binding site interaction of 380 nM, which is in good agreement with the EC₅₀ value obtained for a number of harpin_{P_{sp}h}-induced responses of tobacco cells (Figure 2D, Table 1). Moreover, use of a series of deletion derivatives of harpin_{P_{sp}h} showed a close quantitative and qualitative correlation between the abilities of ligands to inhibit binding of the radioligand and to induce *HIN1* expression (Figure 3). Thus, the harpin_{P_{sp}h} binding site detected in the plasma membrane is likely to mediate the activation of defense responses in tobacco.

A comparably high degree of correlation between elicitor and displacement activities of ligand derivatives was found for other elicitors and their binding sites as well (Cheong and Hahn, 1991; Nürnbergger et al., 1994; Bourque et al., 1998; Kooman-Gersmann et al., 1998; Meindl et al., 2000). Unfor-

tunately, the preparation of tobacco protoplasts caused *PR* gene expression in the absence of harpin_{P_{sp}h} (data not shown); hence, it was impossible to determine if protoplasts would respond to harpin_{P_{sp}h} treatment in a manner similar to intact cells. This would have been most desirable, because Hoyos et al. (1996) reported binding of harpin_{P_{ss}} exclusively to the uppermost layer of tobacco cell walls but not to tobacco protoplasts; this binding was detected by confocal laser microscopy using an anti-harpin_{P_{ss}} antibody and a fluorochrome-tagged anti-IgG antibody. This association was Ca²⁺ dependent and detectable only at harpin_{P_{ss}} concentrations of \sim 5 μ M, which exceeded by far the concentrations required for stimulation of harpin_{P_{sp}h}-induced plant cell responses (Table 1).

Important questions regarding whether binding was saturable and reversible were not addressed in that study. Therefore, we performed radioligand saturation assays with intact tobacco cells. Scatchard plot analysis of the data yielded a scattered distribution of data points (data not shown), which prevented meaningful analysis but led us to conclude that ¹²⁵I-harpin_{P_{sp}h} may bind to tobacco cells at multiple sites with very different ligand affinities. Although it cannot be excluded that harpins may interact with the plant cell wall in addition to the plasma membrane, it is difficult to reconcile a cell wall binding site with a harpin_{P_{ss}}-induced K⁺/H⁺ exchange response and plasma membrane depolarization (Hoyos et al., 1996; Pike et al., 1998). Signal perception remote from the plasma membrane and initiation of an intracellular signaling cascade would require the involvement of extracellular matrix receptor-like molecules, as in mammalian cells (Turner and Burrige, 1991). Although such proteins appear to exist in plants, their function has yet to be elucidated (Kohorn, 2000). Thus, it is not clear if the study by Hoyos et al. (1996) identified a physiological target implicated in the activation of plant defense responses or if this

association was due to an ionic interaction with cell wall pectic polysaccharides.

Using harpin_{P_{sph}} fragments, we identified regions within the protein that were capable of binding to the binding site to some extent but were unable to elicit *HIN1* expression in tobacco cells. When added at a 100-fold molar excess over ¹²⁵I-harpin_{P_{sph}}, two elicitor-inactive peptide fragments (covering amino acids 219 to 280) showed residual competitor activity, suggesting that different regions of the elicitor are implicated in elicitor perception and generation of an intracellular signaling cascade. This is similar to the address-message concept according to which the yeast invertase-derived glycopeptide elicitor gp8 or the bacterial elicitor flg22 activates defense responses in tomato cells (Basse et al., 1992; Meindl et al., 2000). Similar to our findings, biologically inactive fragments of these elicitors partially retained the ability to interact with their binding sites. A somewhat surprising observation in our studies was that a peptide fragment (amino acids 301 to 345) that was apparently dispensable for the elicitor activity of harpin_{P_{sph}} showed partial competitor activity. However, it is possible that this region contributed only marginally to the binding of ¹²⁵I-harpin_{P_{sph}} to tobacco membranes and that deletion of this fragment had so little impact on its elicitor activity that we did not detect it in *HIN1* expression assays.

Recent studies that complement our analyses of harpin_{P_{sph}}-induced signal perception and transduction in tobacco cells attempted to elucidate the role of harpin_{P_{sph}} in bacterial pathogenicity. Like structurally related YopB from the mammalian pathogen *Yersinia enterocolitica* (Tardy et al., 1999), harpin_{P_{sph}} and homologous proteins from *P. s. tomato* or *P. s. syringae* were found to integrate into protein-free bilayer membranes and to form an ion-conducting pore in vitro (Lee et al., 2001). Binding of membrane-interacting proteins is most often mediated by specific membrane phosphoglycer-

olipids, such as negatively charged phosphatidic acid or neutral phosphatidylethanolamine (Thevissen et al., 2000a, and references therein). Interestingly, the association of harpin_{P_{sph}} to lipids was strongly enhanced in the presence of negatively charged phosphoglycerolipids and phosphatidylethanolamine (Lee et al., 2001), both of which are constituents of plant plasma membranes (Staehelin and Newcomb, 2000). Direct interaction of harpin_{P_{sph}} with lipids seems to be consistent with a protease-insensitive binding site detected in tobacco membranes. In addition, activation of plant defense responses by ionophore-like compounds has been reported in many plant systems (Yang et al., 1997; Scheel, 1998). Because binding of protein ligands to specific lipids may resemble very closely the molecular interactions between proteinaceous ligands and receptors, it appears likely that the harpin_{P_{sph}} binding site detected in tobacco plasma membranes is not a protein.

Precedent for this may be provided by high affinity binding sites for plant defensins, which were detected on fungal plasma membranes (Thevissen et al., 2000b). Defensins trigger ion fluxes similar to those observed in harpin_{P_{sph}}-treated tobacco cells. Recently, Thevissen et al. (2000a) reported that defensin binding to fungal plasma membranes was strongly dependent on mannose-(inositol-phosphate)₂-ceramide, the major sphingolipid in membranes of *Saccharomyces cerevisiae*. Their data support a model in which membrane patches containing sphingolipids act as specific binding sites for defensins or, alternatively, are required to anchor membrane- or cell wall-associated proteins, which themselves interact with defensins (Thevissen et al., 2000a). Similarly, the protein antibiotic nisin Z from *Lactococcus lactis* binds with high affinity to the membrane-anchored cell wall precursor lipid II of Gram-positive bacteria (Breukink et al., 1999). Like harpin_{P_{sph}} (Lee et al., 2001), nisin Z is an amphipathic, highly charged protein. Remarkably, nisin Z exerted its biological function through high affinity binding to lipid II and subsequent formation of an ion-conducting pore (Breukink et al., 1999).

At present it is unknown if activation by harpin_{P_{sph}} of pathogen defense responses in tobacco is mediated by a specific (non)proteinaceous receptor, by direct insertion of the protein into membranes, or by receptor-mediated membrane insertion (Figure 8). It is also conceivable that membrane insertion and receptor-mediated recognition of harpin_{P_{sph}} occur independently in tobacco membranes, and that either or both pathways could lead to activation of defense responses in tobacco. The latter case seems to be supported by our observations that harpin_{P_{sph}}-induced ion pore formation was detectable at concentrations as low as 2 nM (Lee et al., 2001), whereas binding of harpin_{P_{sph}} (Figures 2D and 2E) and transcriptional activation of *PR* genes (Table 1) required significantly higher elicitor concentrations. On the other hand, pore formation sufficient to trigger plant defense responses may require a threshold concentration of harpin_{P_{sph}} higher than 2 nM. However, specific receptors would explain why plant species respond differently to harpin_{P_{sph}}

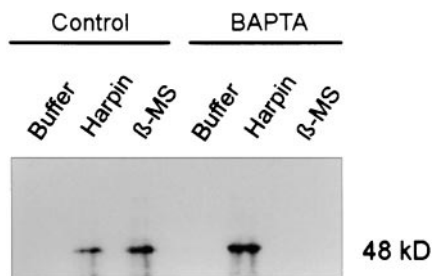


Figure 6. Harpin_{P_{sph}}-Induced SIPK Activation Is Independent of Extracellular Calcium.

Protein extracts prepared from tobacco cells treated for 5 min with buffer, 1 μ M harpin_{P_{sph}}, or 50 nM β -megaspermin (β -MS) in the absence (Control) or presence of the Ca²⁺ chelator BAPTA were analyzed for MAPK phosphorylation by immunoblotting using the anti-pTE_pY-antiserum.

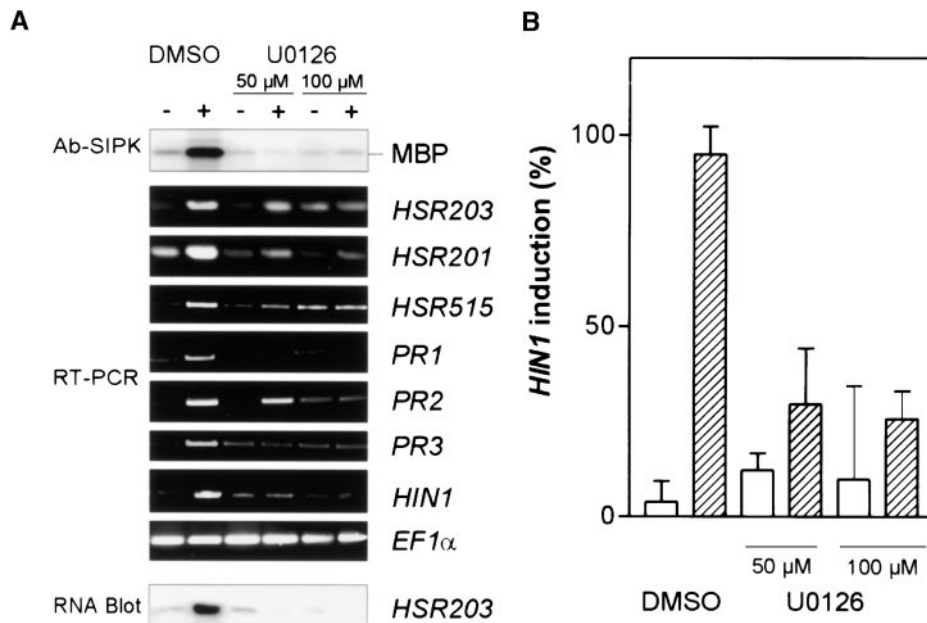


Figure 7. The MAPKK-Specific Inhibitor U0126 Compromises Both Harpin_{PspH}-Induced SIPK Activation and *PR* Gene Expression in Tobacco.

(A) Tobacco cells were treated for 3 hr with buffer (–) or 1 μM harpin_{PspH} (+) in the absence (DMSO) or presence of 50 or 100 μM U0126, a MAPKK-specific inhibitor (Favata et al., 1998). The highest concentration of DMSO used was 0.2%. Protein extracts from elicited tobacco cells were analyzed for SIPK activity as described in the legend to Figure 5C. The expression of *PR* genes was analyzed by RT-PCR with total RNA and specific DNA primers for the indicated genes or, alternatively, by RNA gel blot analysis using total RNA and α-³²P-dATP-labeled *HSR203* cDNA.

(B) Quantification of the inhibitory effect of U0126 on harpin_{PspH}-induced *HIN1* expression. RT-PCR products obtained from three independent elicitation experiments as described in **(A)** were separated electrophoretically, blotted onto nylon membranes, and probed with α-³²P-dATP-labeled *HIN1* cDNA. The signal intensity was quantified by phosphorimaging and normalized to 100% for the maximum induction in each experiment. Buffer treatment (blank bars); harpin_{PspH} treatment (shaded bars). Error bars indicate ±SE.

treatment, whereas insertion into membranes may reflect the role of harpin_{PspH} during bacterial infection of host plants.

MAPK Activity but Not Extracellular Calcium Is Required for Harpin_{PspH}-Induced *HIN1* Expression

Extracellular Ca²⁺ is important for the induction of pathogen defense in various plants (Yang et al., 1997; Scheel, 1998; Grant and Mansfield, 1999). However, we found that BAPTA or lanthanide inhibitors of Ca²⁺ influx did not affect harpin_{PspH}-induced *HIN1* expression but abrogated β-elicitin-induced *HIN1* expression (Figure 4). Thus, structurally diverse elicitors, which target different receptors (Figure 2F), trigger *PR* gene expression dependent on or independent of extracellular Ca²⁺ in the same plant. Moreover, because harpin_{Pss}-induced HR has been shown to depend on extracellular Ca²⁺ (He et al., 1993), several emerging signaling cascades appear to be used for the activation of a complex plant defense response triggered by a single elicitor.

We have provided evidence that harpin_{PspH} stimulated SIPK rapidly and transiently (Figure 5). Similar activation ki-

netics have been reported for a 49-kD MBP-phosphorylating protein kinase that was induced in tobacco leaves upon treatment with harpin_{Ea} from *Erwinia amylovora* (Adam et al., 1997) and that is likely SIPK (Zhang and Klessig, 2000). In contrast, activation of SIPK by β-elicitins (Lebrun-Garcia et al., 1998; Zhang et al., 1998; Zhang and Klessig, 2000; our unpublished data) or the β-elicitin parasiticein (Zhang et al., 1998) was much more prolonged. Similarly prolonged activation of SIPK activity has been reported in tobacco cells treated with *Trichoderma viride*-derived xylanase (Suzuki et al., 1999) or in *Cf-9*-transformed tobacco cells treated with the race-specific *C. fulvum* elicitor AVR9 (Romeis et al., 1999). Moreover, elicitors but not harpin_{PspH} induced prolonged activation of the 44-kD MAPK, WIPK, and another yet undefined tobacco MAPK of 40 kD (Zhang et al., 1998; Zhang and Klessig, 2000). These findings demonstrate that not only elicitor- or pathogen-induced MAPK activity but signal-specific MAPK activity profiles and isoenzyme patterns may be key features of the signal transduction cascades involved in the activation of plant pathogen defense. In addition, MAPK activity is controlled both post-translationally and transcriptionally, which is considered another regulatory

mechanism through which the specificity of signal transduction cascades is maintained (Hirt, 2000).

In those cases investigated, MAPK activation by elicitor was dependent on extracellular Ca^{2+} (Ligterink et al., 1997; Lebrun-Garcia et al., 1998; Romeis et al., 1999; Suzuki et al., 1999; Fellbrich et al., 2000). Particularly, β -elicitor-induced SIPK activity (Lebrun-Garcia et al., 1998; Figure 6) and *HIN1* expression (Figure 4) were dependent on extracellular Ca^{2+} . In contrast, SIPK activation by harpin_{P_{sph}} was independent of extracellular Ca^{2+} and Ca^{2+} influx (Figures 4 and 6). Recently, Ca^{2+} -independent activation of SIPK was observed in tobacco cells undergoing hyperosmotic stress (Hoyos and Zhang, 2000), thus exemplifying the central role of this enzyme in stress adaptation as well as its implication in differentially regulated signaling chains.

Harpin_{P_{sph}}-induced Ca^{2+} -independent SIPK activation and *HIN1* expression prompted us to investigate a possible causal link between the two responses. Specific inhibition of MAPKK activity by U0126 (Favata et al., 1998) not only abolished harpin_{P_{sph}}-induced SIPK activation but also suppressed the expression of numerous *PR* genes in tobacco cells (Figure 7). This is novel in that it suggests the functional involvement of MAPK (SIPK) activity in plant defense activation. Romeis et al. (1999) showed inhibition of elicitor-induced tobacco SIPK activity by another MAPKK inhibitor, PD98059, which, however, did not block the elicitation of defense-related

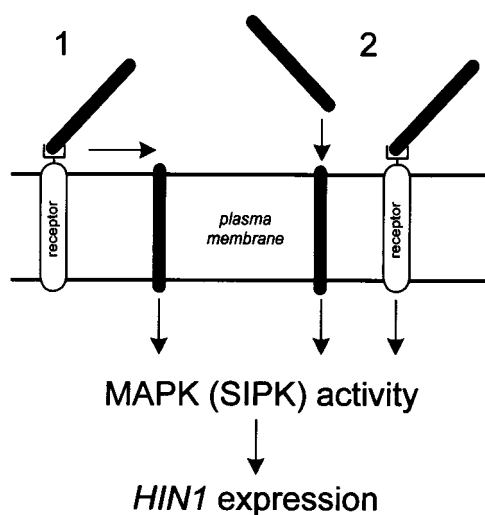


Figure 8. Hypothetical Model for Harpin_{P_{sph}}-Induced Signal Transduction in Tobacco.

Activation by harpin_{P_{sph}} of pathogen defense responses in tobacco may be mediated by receptor-mediated membrane insertion (1), by direct insertion of harpin_{P_{sph}} into membranes, or by a specific (non)proteinaceous receptor (2). Alternatively, membrane insertion and receptor-mediated recognition of harpin_{P_{sph}} may occur independently, with either or both pathways activating MAPK-dependent *HIN1* expression in tobacco.

responses, such as production of reactive oxygen species. Furthermore, PD98059 inhibited the activation of two MAPKs of 39 and 44 kD and of *PR* gene expression in harpin_{P_{sph}}-treated Arabidopsis cells (Desikan et al., 1999), but because the Arabidopsis SIPK ortholog AtMPK6 is a 49-kD protein (Nühse et al., 2000), it is not certain if either one of the PD98059-sensitive MAPKs represented AtMPK6.

Taken together, harpin_{P_{sph}} and β -elicitor-induced *HIN1* expression in tobacco cells was shown to be mediated through different receptors (Figure 2F). Subsequently, Ca^{2+} -independent and Ca^{2+} -dependent signaling cascades are initiated, which merge upstream of MAPK activity and give rise to expression of *PR* genes, such as *HIN1*.

METHODS

Plant Growth, Maintenance of Plant Cell Cultures, and Elicitor Application

Tobacco (*Nicotiana tabacum* cv Samsun NN) plants were grown in a greenhouse at 22°C with a 14-hr-light/10-hr-dark cycle. Six- to 8-week-old plants were used for infiltration experiments. Test substances were infiltrated into the apoplast using a 1-mL disposable plastic syringe. Tobacco BY2 cell lines were maintained as described (Nagata et al., 1992). For subculturing, 2 mL of cells was transferred weekly into 50 mL of fresh medium. Experiments with cultured cells were performed 3 days after subculture. To maintain uniform conditions for all experiments, cultured cells were filtered and resuspended in incubation buffer (0.5 mM Mes, 4% [v/v] B5 salts, and 3% sucrose, pH 5.7) at a density of 5 g/100 mL. Diluted cells (5 mL) were transferred into Petri dishes and equilibrated for a minimum of 1 hr at 22°C with shaking (125 rpm) before the addition of effectors. Inhibitors were added 30 min before elicitor treatment. Harpin from *Pseudomonas syringae* pv *phaseolicola* (harpin_{P_{sph}}) and β -megaspermin were added at final concentrations of 1 μM and 50 nM, respectively. Cells were collected by filtration 3 hr after elicitor treatment or at the indicated times and stored in liquid nitrogen. For experiments with Ca^{2+} channel blockers or 1,2-bis(o-aminophenoxy)ethane-*N,N,N,N*-tetraacetic acid, the elicitation time was reduced to 1.5 hr. Cell viability was determined routinely by fluorescein diacetate/propidium iodide staining (Blume et al., 2000). Tobacco BY2 protoplasts were prepared using the protocol described previously for the preparation of parsley protoplasts (Dangl et al., 1987).

Elicitor Preparation

For the production of recombinant harpins, a DNA fragment encoding harpin_{P_{sph}} (GenBank accession number AF268940) or a polymerase chain reaction (PCR) fragment of harpin from *P. s. tomat* amplified from genomic DNA (Preston et al., 1995) was placed under the control of a T7 promoter (pT7-7 or pJC40) (Clos and Brandau, 1994; Lee et al., 2001). For the expression of harpin from *P. s. syringae*, the plasmid pSYH10 was used (He et al., 1993). DNA constructs were transformed into BL21 (DE3) pLysS *Escherichia coli* cells, and protein expression in bacteria grown to midlogarithmic phase was induced with 1 mM isopropyl- β -D-thiogalactoside for 5 hr. Subsequently, cells were harvested and lysed by sonication in extraction

buffer (50 mM Tris, 100 mM NaCl, 1 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride, pH 8.0). The sonicated material was boiled (100°C, 10 min) and centrifuged to remove denatured proteins, and heat-stable proteins were precipitated by ammonium sulfate fractionation (45% saturation). The precipitated protein was resuspended in 5 mM Mes buffer, pH 5.5, and desalted using PD-10 columns (Amersham Pharmacia, Freiburg, Germany). This procedure allowed purification to more than 95% homogeneity according to SDS-PAGE/silver staining and reverse phase HPLC analysis (Nürnberg et al., 1994).

To verify the integrity of the recombinant protein, the N terminus was sequenced using a G1050 Protein Sequencer (Hewlett-Packard, Palo Alto, CA). For the expression of truncated forms of harpin_{PspH}, DNAs encoding the fragments shown in Figure 3A were amplified by PCR from plasmid pT7-7/hrpZ (Lee et al., 2001). Primers were designed from the harpin_{PspH}-encoding DNA sequence and modified to construct appropriate restriction sites. NdeI–BamHI fragments (except fragment IV, which was encoded by a NdeI–EcoRI DNA fragment) were introduced into the modified pET vector pJC40 encoding an N-terminal His₁₀ tag (Clos and Brandau, 1994). The clone encoding harpin_{PspH} fragment VII (Figure 3A) was obtained by removal of an internal XhoI DNA fragment. Expression in BL21 (DE3) pLys E. coli cells was initiated by 1 mM isopropyl-β-D-thiogalactoside, and expressed proteins were isolated on nickel–nitrilotriacetic acid agarose using the Qiagen (Hilden, Germany) protocol. Harpin_{PspH} fragments were dialyzed against 5 mM Mes, pH 5.5. The homogeneity of purified recombinant harpin_{PspH} was tested by SDS-PAGE/silver staining. β-Megaspermin was prepared as described (Baillieul et al., 1995). Protein concentrations were determined with the bicinchoninic acid assay kit (Pierce, Sankt Augustin, Germany) using BSA as the standard. Solid phase peptide synthesis with N-α-9-fluorophenylmethoxycarbonyl (Fmoc)-protected amino acids was performed with an Economy Peptide Synthesizer EPS 221 (ABIMED, Langen, Germany) according to the manufacturer's instructions. Synthesized peptides were purified to homogeneity as described (Nürnberg et al., 1994).

RNA Extraction and Analyses

RNA was extracted by the hot phenol/chloroform/LiCl precipitation method (Sambrook et al., 1989). RNA prepared from 250 mg of frozen tobacco cells was resuspended in 50 μL of water. Routinely, 1 to 2 μL of RNA solution was used in reverse transcription (RT)-PCR analyses. RT was initiated in the presence of oligo(dT) primers (42°C, 30 min), and after inactivation of the reverse transcriptase (95°C, 5 min), the appropriate primers were added for PCR cycling (25 cycles of 15 sec at 95°C, 1 min at 55°C, and 1 min at 72°C). Initially, reverse transcriptase and Taq polymerase were added separately, but subsequently, a single tube reaction was used (Ready-To-Go RT-PCR beads; Amersham Pharmacia). Each rehydrated bead was used for one reaction of 50 μL. Amplification of a constitutively expressed gene (translation elongation factor 1α [*EF1α*]) served as an internal control in RT-PCR assays. For analysis of *HIN1* expression, multiplex PCR was used to amplify simultaneously transcripts of *HIN1* and *EF1α*. The RT-PCR products were analyzed by agarose gel electrophoresis. For quantification, RT-PCR products were transferred to nylon membranes and hybridized to α-³²P-dATP-labeled *HIN1* cDNA (Sambrook et al., 1989). Signal intensity was determined by phosphorimaging.

For amplification of tobacco genes by RT-PCR, the following primers were used: *HSR203* (forward, 5'-TGTACTACTGCTACAC-

CGC-3'; reverse, 5'-GATAAAAGCTATGTCCACTCC-3'); *HSR201* (forward, 5'-CATCACGAATACGATGAAGTACG-3'; reverse, 5'-CAG-GCAAACAAATTGGAACC-3'); *HSR515* (forward, 5'-AACTCTCCCTTAAGTACGGAC-3'; reverse, 5'-CAATAGTCCATACACTCACGA-3'); *PR1* (forward, 5'-GATGCCATAACACAGCTCG-3'; reverse, 5'-TTT-ACAGATCCAGTTCTTCAGAGG-3'); *PR2* (forward, 5'-CTGCCCTGTACTTGTGGG-3'; reverse, 5'-TCCAGGTTTCTTTGGAGTTCC-3'); *PR3* (forward, 5'-GGTTCTATTGTAACGAGTGAC-3'; reverse, 5'-TTC-TATGTAACGAAGCCTAGC-3'); chitinase/lysozyme (forward, 5'-TCT-CATGTTTCTTCTCCGG-3'; reverse, 5'-CAAAGTAACCTAGCA-ATCCTCTACC-3'); *HIN1* (forward, 5'-GAACGGAGCCTATTATGG-CCCTTCC-3'; reverse, 5'-CATGTATATCAATGAACACTAAACGCC-3'); and *EF1α* (forward, 5'-TCACATCAACATTGTGGTCAT-TGGC-3'; reverse, 5'-TTGATCTGGTCAAGAGCCTCAAG-3'). RNA gel blotting with total RNA from tobacco cells and α-³²P-dATP-labeled DNA was performed as described (Sambrook et al., 1989).

Iodination of Harpin_{PspH}, Microsomal Membrane Preparation, and Radioligand Binding Assays

A tyrosine residue was added to the C terminus of harpin_{PspH} for radioiodination. PCR with corresponding primers was performed to change the TGA stop codon by the deletion of one nucleotide into a tyrosine-encoding TAC codon, which was followed by a TGA stop codon created by the frameshift. The introduced mutation and the integrity of the coding sequence were verified by DNA sequencing. Nonradioactive iodination of harpin was performed by the addition of IODO-BEADS (Pierce) and NaI according to the supplier's instructions. Labeling with Na¹²⁵I to a specific radioactivity of 2200 Ci/mmol was performed by Biotrend Chemikalien (Köln, Germany). Tobacco BY2 cells (6 days old) were used for the preparation of microsomes as described (Nürnberg et al., 1994). The microsomal pellet recovered from 150 g of frozen tobacco cells was resuspended in 5 mL of 20 mM sodium phosphate and 100 mM NaCl, pH 7.0. Microsomal protein (200 μg) was resuspended in a total volume of 200 μL of buffer and kept on ice during the course of the experiment. If not indicated otherwise, binding was initiated by the addition of 100 nM ¹²⁵I-harpin_{PspH}. The binding reaction was terminated by adding 5 mL of ice-cold buffer, and membranes were harvested by filtration on Whatman GF/B glass filters (Maidstone, UK) preblocked with 5% BSA in binding buffer (Nürnberg et al., 1994). Subsequently, filters were subjected to γ-counting in a Wizard counter (Amersham Pharmacia). Nonspecific binding was determined in the presence of a 100-fold molar excess of unlabeled harpin_{PspH}. Binding of ¹²⁵I-harpin_{PspH} to 2.5 × 10⁶ tobacco protoplasts resuspended in 250 μL of Murashige and Skoog (1962) medium containing 0.4 M sucrose was performed as described above except that protoplasts were rinsed three times with 5 mL of ice-cold 0.24 M CaCl₂. Appropriate dilutions of ¹²⁵I-harpin_{PspH} with unlabeled harpin_{PspH} were used to reduce the costs and use of radioactivity.

Ion Flux Measurements and MAPK Activity Assays

Changes in ion concentrations (H⁺, K⁺, and Cl⁻) of elicited tobacco cells were determined as described (Nürnberg et al., 1994). Extracellular free Ca²⁺ concentration was calculated using MaxChelator software (version 2.5 for Windows; <http://www.stanford.edu/~cpatton/>) (Bers et al., 1994). Preparation of protein extracts from tobacco cells, in-gel protein kinase assays with myelin basic protein (MBP) embed-

ded in 10% polyacrylamide gels, and immune complex kinase activity assays using the antiserum Ab-p48N were performed as described (Zhang et al., 1998). For immunoblot analyses with the antiACTIVE mitogen-activated protein kinase (MAPK) antibody (Promega, Mannheim, Germany), tobacco protein extracts (30 μ g of protein) were separated on 10% SDS-polyacrylamide gels, proteins were transferred to nitrocellulose by semidry blotting, and membranes were incubated with the antiserum according to the supplier's instructions. A secondary goat anti-rabbit IgG antibody coupled to alkaline phosphatase was used to visualize immunoreactive proteins.

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