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Harpin induces disease resistance in *Arabidopsis* through the systemic acquired resistance pathway mediated by salicylic acid and the *NIM1* gene

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Summary

Harpin, the product of the *hrpN* gene of *Erwinia amylovora*, elicits the hypersensitive response and disease resistance in many plants. Harpin and known inducers of systemic acquired resistance (SAR) were tested on five genotypes of *Arabidopsis thaliana* to assess the role of SAR in harpin-induced resistance. In wild-type plants, harpin elicited systemic resistance to *Peronospora parasitica* and *Pseudomonas syringae* pv. tomato, accompanied by induction of the SAR genes *PR-1* and *PR-2*. However, in experiments with transgenic *Arabidopsis* plants containing the *nahG* gene which prevents accumulation of salicylic acid (SA), harpin neither elicited resistance nor activated SAR gene expression. Harpin also failed to activate SAR when applied to *nim1* (non-inducible immunity) mutants, which are defective in responding to SA and regulation of SAR. In contrast, mutants compromised in responsiveness to methyl jasmonate and ethylene developed the same resistance as did wild-type plants. Thus, harpin elicits disease resistance through the *NIM1*-mediated SAR signal transduction pathway in an SA-dependent fashion. The site of action of harpin in the SAR regulatory pathway is upstream of SA.

Introduction

Harpin, the first bacterial hypersensitive response (HR)-elicitor characterized, is an acidic, heat-stable, glycine-rich, 44 kDa protein encoded by the *hrpN* gene (hypersensitive reaction and pathogenicity) of *Erwinia amylovora* (Beer *et al.*, 1991; Wei *et al.*, 1992). The bacterial pathogen causes fire blight disease of apple, pear and other members of the Rosaceae (van der Zwet and Beer, 1995). Harpin also induces resistance in a variety of plants against a broad array of pathogens (Qiu *et al.*, 1997; Wei and Beer, 1996). Subsequently, several similar Hrp proteins (harpins) have been characterized from different bacterial plant pathogens (Arlat *et al.*, 1994; Bauer *et al.*, 1995; Charkowski *et al.*, 1998; Cui *et al.*, 1996; He *et al.*, 1993; Kim and Beer, 1998; Preston *et al.*, 1995). Harpins elicit the HR, and some have been shown to elicit disease resistance (Bauer *et al.*, 1997; Strobel *et al.*, 1996; Wei and Beer, 1996; Wei *et al.*, 1998; S.V. Beer *et al.*, unpublished results).

Certain interactions between harpins and plants must occur for the induction of resistance (Hoyos *et al.*, 1996). In tobacco suspension cell cultures treated with HrpZ or HrpN protein, a variety of responses occur that lead to ion influxes across the membranes, alkalization of the growth medium, cell membrane depolarization and production of active oxygen species (Baker *et al.*, 1993; He *et al.*, 1994; Popham *et al.*, 1995; Wei *et al.*, 1992). Programmed cell death occurs in *Arabidopsis* suspension cultured cells in response to HrpZ (Desikan *et al.*, 1998). A potential role for phosphorylation in the regulation of these responses is suggested by observations that they are sensitive to K252a, an inhibitor of protein kinases (Baker *et al.*, 1993), and that tobacco leaves infiltrated with harpin or water accumulate a mitogen-activated protein kinase (MAPK) (Adám *et al.*, 1997). Together, these data suggest that application of harpin to plants initiates signal transduction events that lead to defence responses (see review by Boller and Felix, 1996).

Induced resistance in plants is regulated by complex signal transduction pathways that respond to infection by pathogens and specific abiotic inducers of resistance. One of the better understood signaling pathways in plant defence leads to the expression of systemic acquired resistance (SAR) (Delaney, 1997; Dorey *et al.*, 1997; Lawton *et al.*, 1995; Ryals *et al.*, 1996; Sticher *et al.*, 1997). Salicylic acid (SA) is an endogenous signaling molecule (Klessig and Malamy, 1994) which accumulates following pathogen exposure (Malamy *et al.*, 1990; Malamy *et al.*, 1996; Métraux *et al.*, 1990) and is required for induction of SAR. Application of SA induces resistance to the same spectrum of pathogens and activates the same set of genes as biological inducers of SAR (Kessmann *et al.*, 1994). Transgenic tobacco and *Arabidopsis* plants unable to accumulate SA, due to expression of an introduced bacterial gene encoding salicylate hydroxylase, are unable to express SAR and show other defects in resistance to pathogens (Delaney *et al.*, 1994; Gaffney *et al.*, 1993). Several functional synthetic analogs of SA, including 2,6-dichloroisonicotinic acid (INA) and benzo-1,2,3-thiadiazole-

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7-carbothioic acid S-methyl ester (BTH), faithfully mimic SA in activating SAR (Lawton *et al.*, 1996; Vernooij *et al.*, 1995; reviewed in Kessmann *et al.*, 1994). SAR induction is accompanied by the expression of a variety of genes, including those that encode pathogenesis-related (PR) proteins, which presumably play a role in the resistance phenotype (Alexander *et al.*, 1993; Beffa and Meins, 1996; Bol *et al.*, 1990; Sticher *et al.*, 1997; Ward *et al.*, 1991).

To elucidate the parts of the signal transduction pathway downstream of SA that lead to SAR, a variety of mutant screens have been used. These have involved assays for defective induction of pathogen resistance (Delaney *et al.*, 1995) and screens using SAR-gene-promoter fusions to reporter or selectable marker genes, coupled with treatment with SAR-inducing compounds like INA (Cao *et al.*, 1994; Shah *et al.*, 1997). In each case, mutations were discovered in the same gene, called *NIM1*, *NPR1* or *SAI1*, respectively, by each group (Cao *et al.*, 1994; Delaney *et al.*, 1995; Shah *et al.*, 1997). *nim1* mutants were shown to be unresponsive to SA, INA or BTH for induction of SAR (Delaney *et al.*, 1995; Lawton *et al.*, 1996) demonstrating that like SA, the synthetic compounds also act through a plant signaling pathway defined by the *NIM1/NPR1*. Because mutants at this locus retain the ability to accumulate SA, yet fail to respond to this compound, the *NIM1/NPR1* gene is believed to act between the site of action of SA and the induction of SAR-associated defence genes. The *NIM1/NPR1* gene was cloned in two laboratories (Cao *et al.*, 1997; Ryals *et al.*, 1997) and was shown to encode a protein that contains ankyrin repeats, motifs present in a variety of proteins and believed to mediate protein–protein interactions. The protein NPR1 or NIM1 is suggested to regulate PR gene expression by interacting with a transcription factor (Kim and Delaney, 1999; Zhang *et al.*, 1999).

Other induced resistance pathways exist that are independent of SAR (Niki *et al.*, 1998; Penninckx *et al.*, 1996; Vijayan *et al.*, 1998; Xie *et al.*, 1998). For example, certain growth promoting rhizobacteria can elicit a form of systemic resistance called induced systemic resistance (ISR) (Hoffland *et al.*, 1995; Liu *et al.*, 1995; van Loon *et al.*, 1998), which is distinct from SAR because it is not dependent upon SA accumulation and is not linked to the accumulation of SAR-associated gene products (Pieterse *et al.*, 1996; van Loon *et al.*, 1998). Furthermore, also unlike SAR, ISR appears to depend on signaling by jasmonic acid and ethylene, based on the inability to induce ISR in *Arabidopsis* mutants insensitive to these compounds (Pieterse *et al.*, 1998). Thus, at least two distinct pathways contribute to the suite of pathogen-induced resistance systems. These involve distinct signaling pathways that require either the accumulation of SA or the action of ethylene and jasmonic acid for SAR and ISR, respectively. Activation of SAR requires function of the *NIM1/NPR1* gene product. Curiously, however, although the induction of ISR occurs independently of SA, it is reported to depend upon action of the *NIM1/NPR1* gene product, as *npr1-2* mutants are unable to induce ISR (Pieterse *et al.*, 1998).

Induction of resistance by harpin could result from the activation of a variety of defence pathways. To better understand the mechanisms underlying harpin-induced disease resistance, we examined harpin-treated plants for accumulation of SAR-associated gene products, which would suggest that harpin functions through the SAR pathway. To assess the role of specific signaling pathways in harpin-induced resistance, we examined its effectiveness in several *Arabidopsis* genetic backgrounds, including SA-non-responsive *nim1-1*, jasmonate-insensitive *jar1-1* and ethylene-insensitive *etr1-1* and *etr1-3* mutants, and SA-non-accumulating NahG plants (Delaney *et al.*, 1994; Delaney *et al.*, 1995; Guzmán and Ecker, 1990; Schaller and Bleecker, 1995; Staswick *et al.*, 1992; Staswick *et al.*, 1998). Harpin was found to be an effective inducer of resistance to *Peronospora parasitica* and *Pseudomonas syringae* and caused induction of SAR genes in all genotypes except *nim1-1* and NahG. The present data indicate that harpin-induced resistance acts specifically through the SAR pathway, and does not depend upon *JAR1* or *ETR1* gene products.

Results

Harpin *induces expression of SAR genes*

In *Arabidopsis*, several PR genes, including *PR-1*, *PR-2* and *PR-5* are expressed co-ordinately with SAR (Ryals *et al.*, 1994; Uknes *et al.*, 1992). We monitored the expression of *PR-1* and *PR-2* in plants treated with the HrpN protein (harpin) from *Erwinia amylovora*. Harpin was obtained from a cell-free elicitor preparation (CFEP) made from cultured *Escherichia coli* cells containing a cloned *hrpN* gene. As a negative control a cell-free empty vector preparation (CFVP) was similarly prepared from *E. coli* cells that contain the vector without the *hrpN* insert. Plants sprayed with INA were used as a positive control for SAR in most experiments. Infiltration of harpin into the older lower leaves of *Arabidopsis* caused accumulation of *PR-1* transcripts in the untreated apices and the youngest leaves (Figure 1), showing systemic induction of the gene. Expression of *PR-1* occurred in a time-dependent manner, first being detected after 2 days, and increasing through 6 days after harpin application. Harpin-induced *PR-1* mRNA accumulation exceeded that mediated by treatment with 0.3 mM INA. No *PR-1* induction was observed in plants treated with the empty vector control extract (CFVP) (Figure 1).

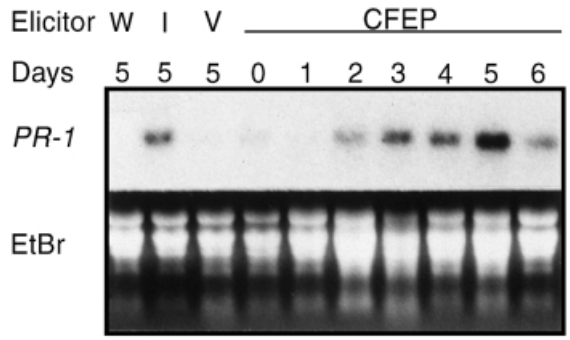

Figure 1. Harpin-induced systemic expression of the *PR-1* gene in Arabidopsis, ecotype Ws-O.
Three leaves of the plant were infiltrated with water (W), INA (I), empty vector preparation CFVP (V) and harpin-containing preparation CFEP. RNA was extracted from the apices and the three youngest leaves, and analyzed by Northern blot hybridization with a *PR-1* cDNA probe. Loading was monitored by staining the gel with ethidium bromide (EtBr). Days between treatment and RNA extraction are indicated.

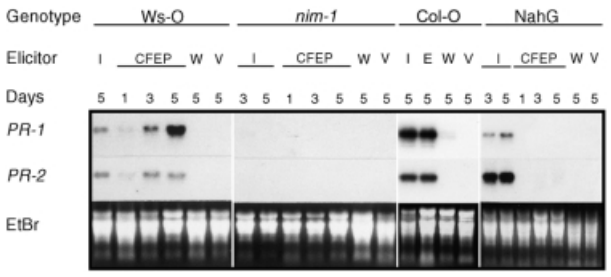

Figure 2. Requirements of SA accumulation and *NIM1* for harpin-induced SAR gene expression.
Plants were sprayed with water (W), INA (I), CFVP (V) or CFEP (E). After the RNA was isolated from the three youngest leaves, *PR-1* and *PR-2* RNA accumulation was analyzed by Northern blot hybridization with cDNA probes.

Gene induction in SAR-compromised genotypes

To test whether harpin-induced gene expression involves the SAR pathway, we assayed for *PR-1* and *PR-2* mRNA accumulation in salicylate hydroxylase (NahG)-expressing plants and in the *nim1-1* mutant (Figure 2). Both genotypes are unable to express SAR, which depends upon SA accumulation and signaling through the *NIM1* pathway (Delaney *et al.*, 1994; Delaney *et al.*, 1995; Gaffney *et al.*, 1993). Harpin and control solutions were sprayed onto wild-type, NahG and *nim1-1* plants; leaf tissues were collected for RNA analysis 1, 3 and 5 days after treatment. In wild-type (Col-O and Ws-O) plants, both *PR-1* and *PR-2* showed strong induction by harpin at days 3–5, while NahG and *nim1-1* plants showed no accumulation of these mRNAs at any timepoint assayed. Plants treated with the positive control, INA, showed induction of *PR-1* and *PR-2* in wild-type and NahG plants. INA is capable of inducing resistance in salicylate hydroxylase plants because it is not a substrate for this enzyme (Delaney *et al.*, 1994; Vernooij *et al.*, 1995). These data indicate that harpin-induced *PR-1* and *PR-2* expression requires a functional SAR signal transduction pathway.

Harpin-induced resistance to Peronospora parasitica

To determine if harpin can induce disease resistance in *Arabidopsis*, the growth of the oomycete pathogen *P. parasitica* in harpin-treated plants was determined. Three lower leaves of 20-day-old wild-type Col-O and Ws-O seedlings were infiltrated with CFEP or CFVP. Five days later, the plants were inoculated with *P. parasitica* strains Noco2 and Emwa, which are virulent on *Arabidopsis* ecotypes Col-O and Ws-O, respectively. The development of infection was observed macroscopically and by staining leaves with lactophenol-trypan blue and microscopic examination. The data in Figure 3 clearly show the effectiveness of harpin-induced resistance against the oomycete pathogen. Control plants treated with CFVP or water were obviously infected and supported growth of large numbers of conidiospores. In contrast, plants treated with harpin were less infected, as indicated by few conidiospores growing on the leaves. Thus, the severity of infection, based on the numbers of conidiospores per leaf, was remarkably reduced by treatment with harpin, suggesting that harpin induces systemic resistance to the oomycete in two ecotypes of *Arabidopsis*.

Resistance also developed following spray application of harpin to leaves of 14-day-old wild-type *Arabidopsis* seedlings (Figure 4). Substantial pathogen growth was observed in leaves of plants treated with CFVP. In contrast, only a few conidiophores grew on the leaf surfaces and a few oospores and hyphae were produced within the leaf tissues of harpin-treated plants.

Harpin-induced resistance to Pseudomonas syringae *pv. tomato DC3000*

We then assayed for specificity of harpin-induced resistance in *Arabidopsis*. We found that harpin elicited resistance against *P. syringae* pv. tomato DC3000 (Figure 5a,b). Bacterial growth was reduced in the untreated upper leaves after infiltration of harpin into the lower three leaves of the plant. At each timepoint, the bacterial population in harpin-treated plants was less than that in control plants. Thus, disease resistance elicited by harpin occurs systemically in leaves not directly treated with harpin. When harpin was applied by spraying, bacterial multiplication was also reduced. The bacterial population increased in 4 days approximately 5000-fold and 3000-fold in CFVP-treated plants of ecotypes Col-O and Ws-O, respectively, while in harpin-treated plants an approximate 400-fold increase was observed in the two ecotypes during the

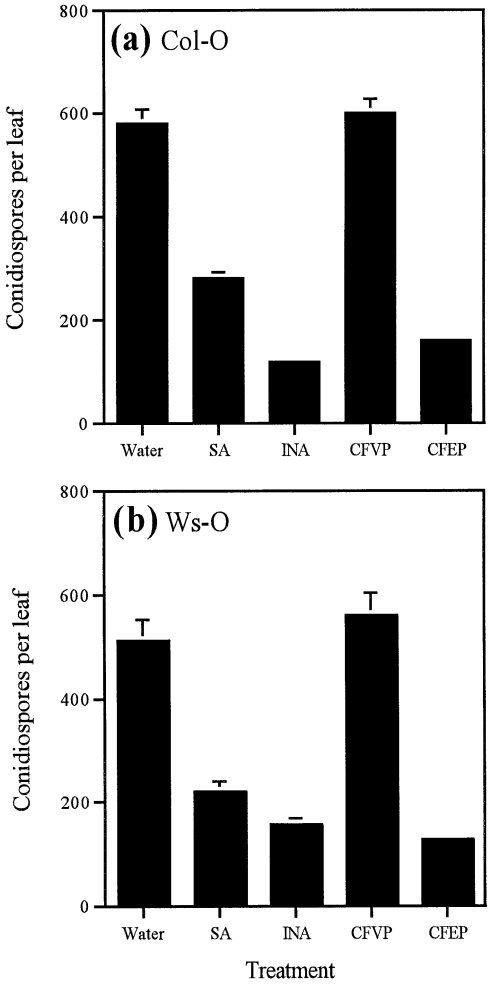

Figure 3. The effect of several elicitors on infection by *P. parasitica* in wild-type Arabidopsis.
Three lower leaves of the plants were infiltrated with the indicated elicitor or control solutions. After 5 days, plants were inoculated with the oomycete pathogen. Five days after inoculation, upper leaves were detached and washed in distilled water to obtain a spore suspension which was counted using a haemocytometer. The numbers of conidiospores per leaf are shown as the average of five samples with SD.

same period (Figure 5a,b). This reduction is similar to that caused by synthetic inducers of SAR (Uknes *et al.*, 1992).

Harpin-induced disease resistance requires SA accumulation and NIM1 function

Unlike wild-type plants, harpin treatment of NahG and *nim1-1* plants failed to induce resistance to *P. parasitica* (Figure 4) and *P. syringae* pv. tomato DC3000 (Figure 5c,d). These results are consistent with the failure of harpin to induce SAR gene expression in these SAR-defective genotypes. Because harpin fails to induce resistance in these SAR-disabled genotypes, it is not likely to be directly antimicrobial, but rather to act through induction of an endogenous plant defence pathway requiring SA and defined by the *NIM1* gene. Harpin, SA and INA produced similar levels of resistance to *P. parasitica* and *P. syringae* pv. tomato DC3000 (Figures 3 and 5). All three compounds also failed to induce resistance in *nim1* mutants, highlighting the central role of the *NIM1* gene in defence signaling.

Harpin induces resistance in jasmonate and ethylene response mutants

To determine whether harpin-induced resistance requires signaling by jasmonate or ethylene, we tested the response to harpin in the methyl jasmonate insensitive mutant *jar1-1* (Staswick *et al.*, 1992), ethylene insensitive mutants *etr1-1* and *etr1-3* (formerly *ein1-1*) (Guzmán and Ecker, 1990; Schaller and Bleecker, 1995), and isogenic wild-type Col-O plants. Plants were sprayed with harpin, subsequently inoculated with *P. parasitica*, and disease development was monitored. Macroscopic and microscopic observations showed that a similar level of resistance developed in wild-type and three mutant lines following application of harpin (Figure 6). All CFVP-treated plants supported vigorous growth of *P. parasitica* within leaf tissues. In contrast, harpin-treated plants were nearly free of infection. Seven days after inoculation, 70–90% of the CFVP-treated mutant and wild-type plants were infected, compared to 5–10% of harpin-treated mutant and wild-type plants infected. Thus, jasmonate and ethylene signaling systems do not appear to affect the function of harpin in inducing resistance to the oomycete pathogen.

Discussion

The aim of this study was to determine the mode of action through which the HrpN protein (harpin) of *E. amylovora* elicits disease resistance in *Arabidopsis*. We examined wild-type *Arabidopsis* (Col-O and Ws-O), two SAR defective genotypes (NahG and *nim1-1*), a jasmonate-insensitive mutant (*jar1-1*), and two ethylene-insensitive mutants (*etr1-1* and *etr1-3*) for their responsiveness to harpin and established chemical elicitors of SAR. Both phenotypic and molecular data support our conclusion that harpin-induced resistance in *Arabidopsis* functions through activation of SAR that requires accumulation of SA and regulation by the *NIM1/NPR1* gene product. The demonstration of alternative resistance signaling pathways mediated by jasmonic acid and ethylene indicates the possibility for multiple actions of an inducer in triggering resistance signal transduction. Because methyl jasmonate and ethylene-insensitive mutants developed resistance following the application of harpin, the involvement of jasmonic acid and ethylene signaling mechanisms in the

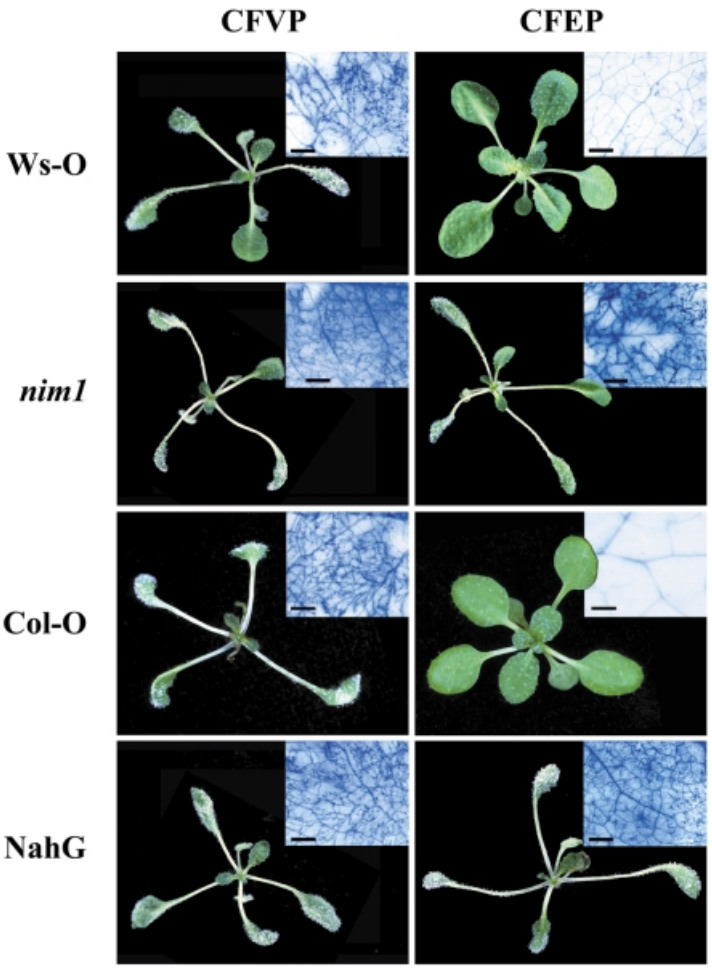

Figure 4. Growth of *P. parasitica* on and in the leaves of wild-type and SAR-disabled lines of Arabidopsis.
Harpin (CFEP) or control solution (CFVP) was sprayed on the plants. Five days later plants were inoculated with *P. parasitica* and photographed 5 days after inoculation. Inserts show the *in planta* growth of the oomycete. To observe the *in planta* growth of the pathogen, the first true leaf (Col-O treated with CFEP) or the upper leaves were stained with trypan blue 5 days post-inoculation and photographed under the microscope. Leaf vascular tissues are stained pale blue; oomycete hyphae and oospores stain more darkly. Bar = 800 μm.

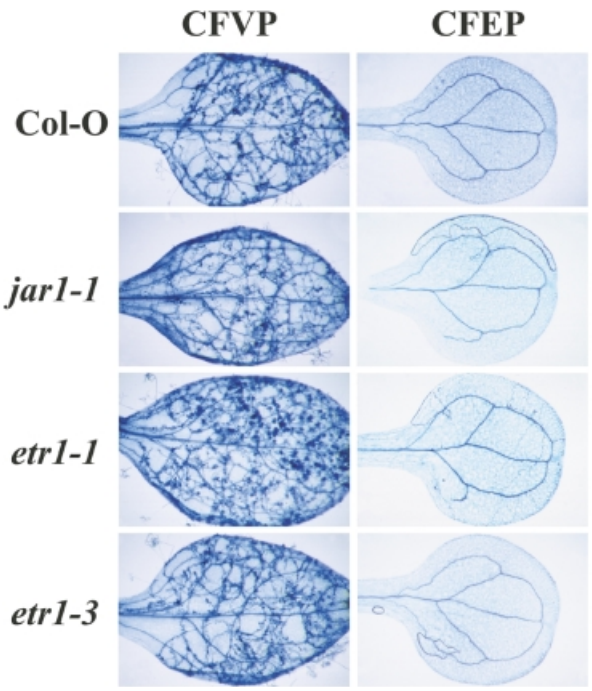

Figure 6. Harpin-induced resistance to *P. parasitica* in Arabidopsis mutants impaired in sensitivity to methyl jasmonate and ethylene.
Methyl jasmonate insensitive mutant (*jar1-1*), ethylene insensitive mutants (*etr1-1* and *etr1-3*), and wild-type plants were treated with CFVP or CFEP, challenged with *P. parasitica* and stained to visualize oomycete structures.

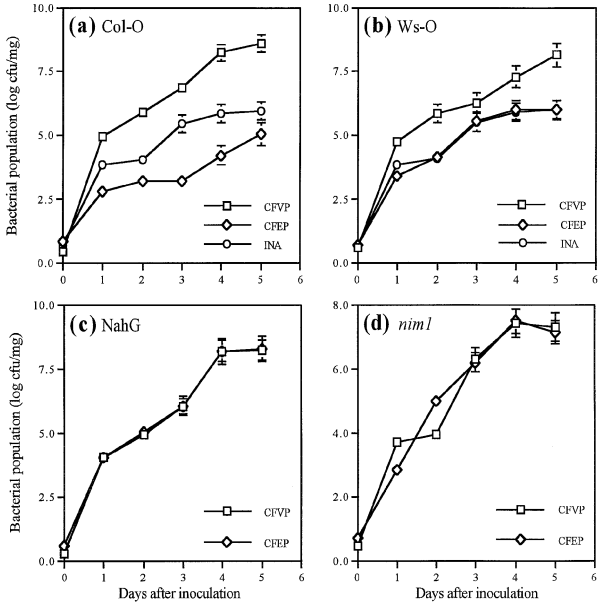

Figure 5. Populations of *P. syringae* pv. tomato DC3000 in wild-type and SAR-impaired genotypes of Arabidopsis.
Plants were treated by spraying whole plants with CFEP, INA or CFVP, and inoculated by spraying bacterial suspension 5 days after the application of elicitors. Bacteria were recovered from leaf tissues on the indicated day by homogenizing surface-sterilized leaves. For every timepoint five samples were included; data shown are mean colony forming units (cfu) per mg of leaf tissues as determined by plate counts.

action of harpin for pathogen resistance seems unlikely, and further supports the conclusion that harpin acts through the SAR pathway.

An important understanding from this study is that harpin induces resistance by a signaling process that begins upstream of SA, leads to activation of PR genes, and is regulated by *NIM1* gene product. This distinguishes harpin from other elicitors. First, SA dependence and PR gene activation distinguishes harpin-induced resistance from ISR, which is neither dependent on SA nor associated with PR gene expression (Pieterse *et al.*, 1996; Pieterse *et al.*, 1998). Second, SA-dependence also distinguishes harpin from INA and BTH that act downstream of SA (Lawton *et al.*, 1996; Vernooij *et al.*, 1995). Finally, harpin-induced resistance to *P. parasitica* isolate Noco2 is different from the constitutive, *NPR1*-independent and defensin gene (*PDF1.2*) expression-associated resistance to the same isolate in *Arabidopsis* mutant *cpr5* that expresses both *NPR1*-dependent and *NPR1*-independent resistance (Bowling *et al.*, 1994; Bowling *et al.*, 1997). Otherwise, because harpin induces resistance to bacteria, it may also activate antibacterial genes that have not been defined and are suggested to require regulation by *NPR1/NIM1* (Bowling *et al.*, 1997; Clarke *et al.*, 1998).

This study presents a preliminary understanding of a harpin-triggered signal transduction process. The SA-dependent and *NIM1/NPR1*-mediated signal transduction pathway may be only one signaling pathway used by harpin for resistance induction, although we were able to rule out dependence on the JAR1 and ETR1 signaling pathways. In addition to resistance against pathogens (Qiu *et al.*, 1997; Wei and Beer, 1996; Wei *et al.*, 1998; this work), several other beneficial effects occur in plants treated with harpin, including the enhancement of plant growth (H. Dong and S.V. Beer, unpublished results; Qiu *et al.*, 1997; Wei *et al.*, 1998) and the repellency of insects (Zitter and Beer, 1998). The mechanisms that underlie these diverse beneficial effects of harpin are not known. Nevertheless, previous data suggest the involvement of reactive oxygen intermediates (for its implication see Alvarez *et al.*, 1998; Dangl *et al.*, 1996; Jabs *et al.*, 1996) and programmed cell death, calcium ion channels and protein kinase cascades in interactions of Hrp proteins with plants (Adám *et al.*, 1997; Baker *et al.*, 1993; Dong *et al.*, 1999; He *et al.*, 1994; Popham *et al.*, 1995; Wei *et al.*, 1992). These may together account for the pleiotropic effects of harpins in plants. Further explorations of the relationships between signaling pathways that affect the several beneficial effects of harpin are underway.

Experimental procedures

Plant growth and pathogen maintenance

Arabidopsis thaliana ecotypes Columbia (Col-O) and Wassilewskija (Ws-O) were used in all experiments. NahG transgenics (Delaney *et al.*, 1995; Gaffney *et al.*, 1993) and *nim1-1* mutant plants (Delaney *et al.*, 1995) were previously produced from the Col-O and Ws-O ecotypes. Methyl jasmonate response mutants *jar1-1* and ethylene response mutants *etr1-1* and *etr1-3* were derived from Col-O (Guzmàn and Ecker, 1990; Schaller and Bleecker, 1995; Staswick *et al.*, 1992), and their seeds (accession numbers CS8072, CS237 and CS3037) were provided by the Arabidopsis Biological Resource Center at the Ohio State University (Columbus, OH, USA). All the genotypes were grown in greenhouse soil mix at 21°C and 14 h light per day for vegetative growth, at 18°C and 12 h day length for infection by *Peronospora parasitica*, and at 24°C and 14 h day length for infection by *Pseudomonas syringae* pv. tomato DC3000 (Koncz *et al.*, 1992).

Peronospora parasitica strain Emwa and Noco2 were maintained by weekly culture on *Arabidopsis* ecotypes Ws-O and Col-O. Conidial suspensions were made from infected leaves and inoculated as described previously (Uknes *et al.*, 1992). *Pseudomonas syringae* pv. tomato DC3000 was cultured on L-Agar medium (Gerhardt *et al.*, 1981) prior to inoculation of plants.

Preparation of elicitors and treatment of plants

INA (2,6-dichloroisonicotinic acid) was kindly provided as 25% wettable powder by Dr Kay Lawton (Novartis Crop Protection, Inc., Research Triangle Park, North Carolina, USA). INA was used at 0.3 mM in water except when otherwise noted. Salicylic acid (SA) was used at 0.3 or 0.5 mM in water as described. Harpin was

prepared as a cell-free elicitor preparation (CFEP) from *E. coli* strain DH5-α harboring plasmid pCPP2139, which contains the *hrpN* of *E. amylovora* (Wei *et al.*, 1992) in the expression vector pCPP50 (Bauer *et al.*, 1997). The cell-free empty vector preparation (CFVP) was similarly made, except that the DH5-α strain contained only the vector, pCPP50. The HR-eliciting activity of CFEP and CFVP was determined by infiltrating opposite leaf panels of Xanthi NN tobacco leaves with dilutions of both preparations. The undiluted CFVP did not elicit HR, and was used as a negative control for harpin-containing CFEP. The concentration of harpin in CFEP was determined by HPLC by Eden Bioscience Corporation (Bothell, WA, USA). Inducing compounds, elicitor preparations and controls were applied by spraying the plants to run-off with an atomizer (Devilbiss no. 15) 14 days after sowing except when otherwise noted. Five days later, plants were inoculated using an atomizer with a *P. parasitica* conidial suspension containing 5×10^4 conidiospores per ml, or with a suspension of *P. syringae* pv. tomato DC3000 at 5×10^8 cfu per ml of water. Ecotype Col-O and genotypes derived from it (NahG, *jar1-1*, *etr1-1* and *etr1-3*) were inoculated with the virulent *P. parasitica* isolate Noco2; Ws-O and *nim1* were inoculated with the virulent isolate Emwa. Inoculated plants were maintained under the conditions described above for 5 days before infection was assessed. Each induction–inoculation combination included six pots, and each pot contained 15–25 seedlings.

Evaluation of infection

Infection by *P. parasitica* was judged based on the presence of conidiophores on the leaf surfaces (Koncz *et al.*, 1992). Conidiospores on leaves were estimated by counting spores in leaf washes using a haemocytometer under the microscope and expressed as conidiospores per leaf. Oomycete growth in leaves was examined using an Olympus BX60 microscope following staining with lactophenol trypan blue and clearing with chloral hydrate (Uknes *et al.*, 1992). To monitor *in planta* bacterial multiplication, leaves of inoculated plants were detached at designated times, sterilized with 70% ethanol and homogenized in sterile water; bacteria were recovered from the resulting homogenates by culturing on L-agar medium (Gerhardt *et al.*, 1981).

RNA blot analyses

RNA was prepared from experimental and control plants using the RNeasy Plant Mini Kit (Qiagen, Chatsworth, CA, USA), size-fractionated by agarose gel electrophoresis (Clark, 1997), and transferred to Immobilo-N transfer membrane (Millipore). Replicate blots were hybridized to ^{32}P[dCTP]-labeled Arabidopsis SAR gene cDNA probes *PR-1* and *PR-2* as described previously (Church and Gilbert, 1984). Loadings were standardized by calculating the total RNA (4 μg per lane) of samples and verified by ethidium bromide (EtBr) staining of gels.

Acknowledgements

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