Expression of Harpin_{Xoo} in Transgenic Tobacco Induces Pathogen Defense in the Absence of Hypersensitive Cell Death

Jian-Ling Peng, Zhi-Long Bao, Hai-Ying Ren, Jin-Sheng Wang, and Han-Song Dong

Key Laboratory of Monitoring and Management of Plant Diseases & Pests, Ministry of Agriculture of China, Department of Plant Pathology, Nanjing Agricultural University, Nanjing 210095 China.

Accepted for publication 16 April 2004.

ABSTRACT

Peng, J.-L., Bao, Z.-L., Ren, H.-Y., Wang, J.-S., and Dong, H.-S. 2004. Expression of harpin_{Xoo} in transgenic tobacco induces pathogen defense in the absence of hypersensitive cell death. *Phytopathology* 94:1048-1055.

Harpin_{Xoo}, encoded by the *hpaG_{Xoo}* gene of *Xanthomonas oryzae* pv. *oryzae*, is a member of the harpin group of proteins that induce pathogen resistance and hypersensitive cell death (HCD) in plants. We elaborated whether both processes are correlated in *hpaG_{Xoo}*-expressing tobacco (HARTOB) plants, which produced harpin_{Xoo} intracellularly. Resistance to fungal, bacterial, and viral pathogens increased in HARTOB, in correla-

tion with the expression of *hpaG_{Xoo}*, the gene *NPR1* that regulates several resistance pathways, and defense genes *GST1*, *Chia5*, *PR-1a*, and *PR-1b* that are mediated by different signals. However, reactive oxygen intermediate burst, the expression of HCD marker genes *hsr203* and *hin1*, and cell death did not occur spontaneously in HARTOB, though they did in untransformed and HARTOB plants treated exogenously with harpin_{Xoo}. Thus, the transgenic expression of harpin_{Xoo} confers nonspecific pathogen defense in the absence of HCD.

Additional keywords: *Alternaria alternata*, *Nicotiana tabacum*, *Ralstonia solanacearum*, *Tobacco mosaic virus*.

Harpins produced by several gram-negative plant-pathogenic bacteria constitute a peculiar group of proteins (28) that are acidic, glycine rich, protease sensitive, and heat stable, as characterized earlier for harpin_{Ea} (54), harpin_{Pss} (26), and harpin_{PspH} (49) encoded by the genes *hrpN*, *hrpZ_{Pss}*, and *hrpZ_{PspH}*, respectively, in *Erwinia amylovora*, *Pseudomonas syringae* pv. *syringae*, and *P. syringae* pv. *phaseolicola*. Harpin_{Xoo}, identified recently, is encoded by the 420-bp *hpaG_{Xoo}* gene in a Japanese strain of *Xanthomonas oryzae* pv. *oryzae* and contains 140 amino acids with a size of 15.6 kDa (55). *hpaG_{Xoo}* seems allelic to the *hpaG/hpaI* locus identified in a Philippines strain of the same bacterium (59), *X. axonopodis* pv. *glycines* (29), and *X. campestris* pv. *vesicatoria* (38), and is not homologous with other harpin genes (55). In amino acid sequence, harpin_{Xoo} is 97.2% identical to the deduced HpaI protein (55). A difference is that harpin_{Xoo} contains three (55) while HpaI contains four (59) internal repeats of the GGG(N)GG motif. Whereas *Escherichia coli* transformed with *hpaG_{Xoo}* produces active harpin_{Xoo} (35,55) and the HpaG protein from *X. axonopodis* pv. *glycines* elicits hypersensitive cell death (HCD) in several plants (29), whether *hpaI* and *X. campestris* pv. *vesicatoria* *hpaG* can encode active proteins has not been determined (38). Moreover, *hpaG_{Xoo}* and harpin_{Xoo} are smaller than many other harpin genes and harpins. For example, harpin_{Ea} and *hrpN* are 42 kDa and 1,300 bp (54), and *hrpZ_{Pss}* is 712 bp (26). Even so, harpin_{Xoo} induces HCD and pathogen resistance (35,55) similarly to other harpins (18,33,34,56).

An interesting question concerns relationships between HCD and resistance induced by harpins. Harpin_{Ea} (18,39) and harpin_{Pss} (48) stimulate systemic acquired resistance (SAR). HCD and the accumulation of reactive oxygen intermediates (ROIs), which mediate the development of HCD (1,39), also occur in leaves infiltrated (39) and suspension-cultured cells treated with either of

the harpins (7,56). Nevertheless, spraying of plants with harpin_{Ea} or harpin_{Pss} induces pathogen resistance without visible cell death (18,48). In *Arabidopsis* spp. treated with harpin_{Ea}, cell death is not required for SAR, though both processes occur simultaneously (39). In transgenic tobacco plants expressing *hrpZ_{PspH}* driven by the *Potato virus X*-activated promoter, HCD is induced by infection with the virus (49). Transgenic apple (4) and potato (36) plants expressing *hrpN* increase resistance to *E. amylovora* and *Phytophthora infestans*, respectively. However, whether HCD occurs in *hrpN*-expressing plants and whether resistance develops in *hrpZ_{PspH}*-expressing plants have not been determined. Thus, assay of both HCD and resistance in the same system is essential for elaborating relationships between both processes in transgenic plants. Here, we present evidence that *hpaG_{Xoo}*-expressing tobacco plants develop nonspecific resistance to pathogens without accompanying HCD.

MATERIALS AND METHODS

Plants and microbes. Tobacco (*Nicotiana tabacum*) cv. Xanthi and its transgenic lines were grown in the greenhouse for 38 days before use, except when specified elsewhere. *Tobacco mosaic virus* (TMV) was maintained in an aqueous solution of 18 mg ml⁻¹ at 4°C. *Alternaria alternata* was maintained on potato dextrose agar medium at 4°C, and multiplied on the same medium before use (17). *Ralstonia solanacearum* was maintained in distilled water under 25°C. *Agrobacterium tumefaciens* strain EHA105, harboring the binary vector pBI121 (27,32), was used in construction of the transformation unit. *Escherichia coli* strains BL21 (DE3) and BL21 (DE3) PHRF4, containing the prokaryotic expression vector pET30(a) and pET30(a)::*hpaG_{Xoo}* (55), respectively, were used to prepare inactive control proteins and harpin_{Xoo}. They were lyophilized and maintained at -80°C. Bacteria were multiplied before use as described (17,23,30).

Transgenic plant generation. A full-length *hpaG_{Xoo}* gene was excised from pET30(a) and inserted into pBI121 between the CaMV 35S promoter and the β -glucuronidase gene *uidA* by

Corresponding author: H.-S. Dong; E-mail address: hsdong@njau.edu.cn

digestion with restriction enzymes *Xba*I and *Bam*HI and ligation with T₄ DNA ligase (9). The resulting pBI-hpaG construct (Fig. 1A) was sequenced to verify correct orientation of genes and transferred into *A. tumefaciens* EHA105 by electroporation. Tobacco was transformed with pBI-hpaG or pBI121 by soaking leaf disks with the appropriate *A. tumefaciens* suspensions. Tissue culture and plant regeneration were done as described (9). Seed from the T₁ and T₂ generations were screened on an MS agar medium (23) in the presence of kanamycin at 150 µg ml⁻¹ (27,32). After genomic integration and expression of *hpaG*_{X₀₀} and production of harpin_{X₀₀} in transgenic plants were determined, the *hpaG*_{X₀₀}-expressing tobacco (HARTOB) plants were compared with untransformed plants in height and weight of the entire plant, weight and size of leaves, and flowering time. Plants containing the vector pBI121 only were similarly selected and designated as Vec lines.

Nucleic acid manipulation. Standard protocols (9,45) were used. RNA and genomic DNA were isolated from leaves. Polymerase chain reaction (PCR) and DNA blot analyses were conducted for integration of *hpaG*_{X₀₀} into tobacco genomes. Expression of the transgene and defense-related genes were studied by reverse transcriptional (RT)-PCR or RNA gel blot analyses. The *hpaG*_{X₀₀} cDNA used as probe in DNA and RNA blot analyses were produced by PCR from the *E. coli* strain BL21 (DE3) PHRF4. Sequences of PCR and RT-PCR products were confirmed as described (39). Various nucleic acids were fractionated by electrophoresis in agarose gels and visualized by staining with ethidium bromide, using DL2000 markers (Takara Biotech, Co., Dalian, China) to indicate size. In Southern blot hybridization, 3 µg of genomic DNA was digested with *Eco*RI prior to electrophoresis and, after that, blotted to Nylon membranes. Three replicates of blots were hybridized with a digoxigenin-labeled *hpaG*_{X₀₀} probe (45). PCR was conducted for 25 cycles using 3 µg of genomic DNA as template and specific primers, producing an intact *hpaG*_{X₀₀} sequence. In Northern blot hybridization, 20 µg of RNA was resolved by electrophoresis, blotted to Nylon membranes, and probed with P³²-dCTP-labeled *hpaG*_{X₀₀} cDNA (8, 18,39). For RT-PCR, reaction conditions were optimized for each gene (39). RNA (2 µg) was treated with RNase-free DNase and used as template to synthesize the first-strand cDNA. An equal volume of cDNA was amplified for 25 to 26 cycles using specific primers (Table 1). The *EF1α* gene was used as a standard and amplified with specific primers, resulting in a 495-bp product (39). Products were quantified in gels by a scanner attached to an Image System (SX-100; Shanghai Sixing Biol. Tech. Co., Shanghai, China).

Protein analysis. Soluble proteins were isolated from leaves as described (3) with several modifications. The protease inhibitor phenylmethylsulfonyl fluoride (PMSF), which protects harpins from destruction by proteases (54), was applied at 0.1 mmol liter⁻¹ to the protein extraction solution. Supernatants from centrifuging (25,000 × g, 25 min, 4°C) leaf homogenates were heated in a boiling water bath for 10 min, cooled to room temperature, and centrifuged similarly. The second supernatants were supplemented with PMSF and maintained overnight at -4°C. After centrifuging, the third supernatants were lyophilized and maintained at -80°C. The protein preparations were tested for bioactivity in aqueous solutions and fractioned in ammonium sulfate. Segments precipitated by the salt between 29 and 60% (wt/vol) were dissolved in ethylene diamine teraacetic acid at 0.2 mmol liter⁻¹, dialyzed against water, and purified by anion HiPre Q XL exchange chromatography (AKTA Explore 100) (3). Purified proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins also were isolated from intercellular spaces of leaves, as described (13), in the presence of PMSF, lyophilized, and maintained as above. Partial aqueous solutions of the protein preparations were heated in a boiling water bath for 10 min, while another part remained untreated;

both were tested for bioactivity and resolved by a native PAGE. Bioactivity of the two types of plant proteins was evaluated, in comparison with harpin_{X₀₀} and inactive proteins (18) from *E. coli* strains and PHRF4 and BL21 (DE3), based on the occurrence of a hyperactive response (HR) in tobacco leaves infiltrated with each of protein preparations at 10 µg ml⁻¹, a concentration sufficient for the response (39).

Histochemistry. The fifth and sixth leaves were infiltrated with a harpin_{X₀₀} solution (10 µg ml⁻¹) or water as a control because the inactive proteins from BL21 (DE3) did not cause any response (39). Both responses were investigated 12 h posttreatment. ROI burst was observed based on H₂O₂ accumulation after staining leaves with diaminobenzidine (DAB) (1). Excised leaves were transferred to DAB aqueous solution (pH 3.8) at 1 mg ml⁻¹ and maintained at room temperature for 8 h, followed by clearing with 95% ethanol until tissues were judged clear. Micro-HR was monitored based on observing dead cells after staining leaves with lactophenol trypan blue (1,39,51). Briefly, 1.5-cm squares of tobacco leaves were placed in lactophenol trypan blue solution, composed of 10 ml of lactic acid (85% aqueous), 10 ml of water-saturated phenol, 10 ml of glycerol (98%), 10 ml of distilled water, and 15 mg of trypan blue. The dye solution was infiltrated into the leaf intercellular spaces with the aid of a vacuum pump and a bell jar. The infiltrated tissues then were heated in a boiling water bath for 5 to 10 min and incubated at room temperature for 6 to 8 h. Stained leaf tissues were cleared in chloral hydrate solution (2.5 g ml⁻¹) overnight or until tissues were judged clear. Cleared leaves for ROI burst or cell death were observed under a microscope. To determine the possibility that both responses spontaneously occur in HARTOB, every third leaf on the plants was investigated similarly at a 10-day intervals until flowering.

Disease and resistance scoring. Plants were inoculated with *R. solanacearum* and with TMV or *A. alternata* 48 and 38 days, respectively, after transplanting plants to the soil. Inoculation was performed for *R. solanacearum* by infiltrating seedling tops with 20-µl bacterial suspensions (10⁷ CFU ml⁻¹), for TMV (1 µg ml⁻¹ of solution) by rubbing leaves using a finger in the presence of abrasive diatomaceous earth, and for *A. alternata* by dropping 25 µl of a conidial suspension (10⁶ spores ml⁻¹) on each of six sites of leaf surfaces (17,18,30). Inoculated plants were grown in the greenhouse and investigated for infection (17) 7 days later. For plants inoculated with TMV or *A. alternata*, number and size of lesions on leaves were determined. Meanwhile, size of necrosis on stems caused by *R. solanacearum* was scored. Disease severity (DS) was expressed as number of lesions per leaf for TMV, mean size of lesions for *A. alternata*, and mean size of necrosis for *R. solanacearum*. Correspondingly, resistance was given as percent decrease of DS in HARTOB plants, relative to that in untransformed or Vec plants, quantified using the formula 100 × (mean DS in control plants – mean DS in HARTOB)/mean DS in the control.

Statistical analysis. Assays were conducted three times, each involving 15 plants, except if specified in figure captions. For all quantitative determinations, data were analyzed by *t* test at *P* ≤ 0.05 using the Microsoft Analysis Tool. For differences in disease severity, each HARTOB line was compared with untransformed or Vec plants; lines 6, 7, and 15 were subjected to multiple comparisons as well.

RESULTS

Expression of the *hpaG*_{X₀₀} gene in tobacco. Following transformation of tobacco with the *hpaG*_{X₀₀}-containing vector (Fig. 1A) and screening of T₁ and T₂ seed, 38 possible *hpaG*_{X₀₀}-containing lines were obtained. Transformation with the vector pBI121 only and similar screening produced five Vec lines. The *hpaG*_{X₀₀} gene was analyzed by PCR, which produced a sequence 100% identical to that deposited in GenBank based on Blast

analyses. The *hpaG_{X00}* sequence was detected in transformed callus, regenerated seedlings, and the 38 *hpaG_{X00}*-transgenic plants at various stages, but was not detected in untransformed and Vec plants (Fig. 1B, intermediate; and data not shown). Southern blot hybridization confirmed the result, and additionally revealed that two copies of the transgene were present in transgenic plants except for line 30, which had one copy (Fig. 1B, bottom).

To determine whether the *hpaG_{X00}* insert was transcribed, an RT-PCR protocol was applied to detect accumulation of *hpaG_{X00}*

Fig. 1. Generation of transgenic tobacco plants. **A**, The *hpaG_{X00}*-transformation unit pBI-Hrp constructed in the vector pBI121. Labels on bottom include the kanamycin-resistant gene *NPT II*, the 35S promoter, and the full-length (420-bp) *hpaG_{X00}*, and those on top are restriction sites X (*Xba*I), Sm (*Sma*I), Sa (*Sac*I), and E (*Eco*RI). **B**, Polymerase chain reaction and Southern blot hybridization (bottom) analyses of *hpaG_{X00}* from the genome of *T₂* plants of *hpaG_{X00}*-transgenic lines coded on top. Markers (M) are 2,000, 1,000, 750, and 500 bp. **C**, Morphological comparison between untransformed plants and *T₂* plants of *hpaG_{X00}*-expressing tobacco (HARTOB) and the Vec line 1 generated from transformation with pBI121. Five experiments each involving 15 plants gave similar results.

mRNA in 20 *hpaG_{X00}*-containing transgenic plants, using the *EF1 α* gene as a standard (Fig. 2A, left), because it is highly conserved and constitutively expressed in eukaryotes (2,22). The sequences of RT-PCR products showed an identity of 100% for *hpaG_{X00}* to that deposited in GenBank and 94 to 98% for *EF1 α* to published sequences cloned from different organisms (39). Replicates of RT-PCR performance indicated that *hpaG_{X00}* was expressed in both *T₁* and *T₂* plants of the 20 *hpaG_{X00}*-transgenic lines tested (Fig. 2A, top gel panel; and data not shown); levels of the gene expression varied in HARTOB lines (Fig. 2A, left; and data not shown). We further assayed three lines to compare results of RT-PCR with northern blot hybridization. Both methods re-

Fig. 2. Levels of *hpaG_{X00}* expression in *hpaG_{X00}*-expressing tobacco (HARTOB) plants. Three HARTOB lines and the Vec line 1 in the *T₂* generation were tested. **A**, Comparison of reverse transcriptional-polymerase chain reaction (RT-PCR) (left) and Northern blot hybridization. In RT-PCR analysis, *EF1 α* (495 bp) was used as a standard gene. **B**, Levels of *hpaG_{X00}* expression. After electrophoresis, RT-PCR products were quantified in a gel documentation system. Gene expression levels were given as relative units by defining that in Vec plants as 1.

TABLE 1. Relevant characteristics of *hpaG_{X00}*, defense, and hypersensitive cell death-associated genes determined in this study

Gene	GenBank accession no.	Primers	Size (bp) of RT-PCR product ^a
<i>hpaG_{X00}</i>	AF02197, AY139029	5'-GCGGATTGTGTTATCGATTTC-3' 5'-TATTACTGCATTGATGCGCTTCC-3'	420 (same for PCR)
<i>NPR1</i>	U76707	5'-TACTCTCTATCAGAGGCACTTATTGGACGT-3' 5'-CCATAGTGGCTTGTGTCGATCATGA-3'	565
<i>Chia5</i>	X77110	5'-TCTCATGTTTCTCTTCTCCGG-3' 5'-CAAAGTAACCTAGCAATCCTCTACC-3'	1,015
<i>PR-1a</i>	X05959	5'-GGCGTTCTCTTTTACAATTGCCTTCAT-3' 5'-AACGGACTTTCGCCTCTATAATTACCTG-3'	495
<i>PR-1b</i>	X66942	5'-ATGGGATACTCCACAACAT-3' 5'-TTACTAGACATCAGTTGGGAAG-3'	537
<i>hsr203</i>	X77136	5'-TGTAATACACTGTCTACACGC-3' 5'-GATAAAAGCTATGTCCCACTCC-3'	618
<i>hin1</i>	Y07563	5'-GAACGGAGCCTATTATGGCCCTTCC-3' 5'-CATGTATATCAATGAACACTAAACGCCGG-3'	867
<i>GST1</i>	D10524, D90500	5'-GCGATCAAAGTCCATGGTAG-3' 5'-TTATTTTTCGACGTTCTCCA-3'	639

^a RT-PCR = reverse transcriptional-polymerase chain reaction.

vealed a similar pattern in the transgene expression in lines 15, 6, and 7 (Fig. 2A), which were representative of HARTOB plants in *hpaG_{X00}* expression levels ranging from low and intermediate to high (Fig. 2B, right). Finally, the *hpaG_{X00}*-expressing tobacco (HARTOB) and Vec plants were compared with untransformed plants for several botanical traits. Based on height and weight of the entire plant, weight and size of leaves, and flowering time, seven HARTOB lines (lines 2, 4, 6, 7, 16, 21, and 32) and the Vec line 1 that evidently resembled untransformed plants (Fig. 1C; and data not shown) were selected for the further study.

Production of harpin_{X00} in HARTOB plants. To determine the production of harpin_{X00} in HARTOB plants, we compared SDS-PAGE gel patterns and bioactivity of soluble proteins from leaf tissues of untransformed plants, Vec, and HARTOB lines. We found that only proteins from HARTOB plants were active; ex-

tents of the HR they induced in tobacco leaves were comparable with that induced by harpin_{X00} prepared regularly from the recombinant *E. coli* strain (Fig. 3A). Inversely, proteins isolated from untransformed or Vec plants did not cause the HR (Fig. 3A). A

Fig. 3. Production of harpin_{X00} in *hpaG_{X00}*-expressing tobacco (HARTOB) plants. T₂ plants of HARTOB lines 6, 7, and 15 were observed; data from line 7 are shown here. Untransformed plants (Utp) and the Vec line 1 in the T₂ generation were tested as controls. **A**, Bioactivity assays of proteins from leaf tissues, compared with water and preparations of inactive proteins (BL21) and harpin_{X00} (PHRF) from *Escherichia coli*. **B**, Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (PAGE) patterns of the above preparations. Lane 1, purified PHRF; lane 2, BL21 after partial purification that removed heat-sensitive proteins; lane 3, purified harpin_{X00} from HARTOB; lane 4, HARTOB proteins after partial purification; lane 5, Vec protein preparations after partial purification. Arrows indicate harpin_{X00}. **C**, Native PAGE patterns and bioactivity assays of proteins from intercellular spaces of leaves, compared with those from *E. coli*. Codes: 1 and 2, unheated and heated proteins from Vec; 3 and 4, unheated and heated proteins from HARTOB; 5 and 6, harpin_{X00} from *E. coli* and leaf tissues of HARTOB.

Alternaria alternata

Tobacco mosaic virus

Ralstonia solanacearum

Fig. 4. Responses of three *hpaG_{X00}*-expressing tobacco (HARTOB) lines to pathogens. Plants inoculated with *Ralstonia solanacearum* and *Alternaria alternata* or Tobacco mosaic virus were photographed 15 and 7 days after inoculation, respectively. Mean reduction in disease severity of HARTOB plants, relative to that of the Vec line 1, is presented in red. Similar results were obtained from five sets of experiments, investigating a total of 75 plants of each genotype.

purified protein from the HARTOB line 7 was abundant in total proteins and had the same size as harpin_{Xoo} from *E. coli* (Fig. 3B). Similar results were obtained for lines 6 and 15 (data not shown). These results suggest that active harpin_{Xoo} is present in tissues of HARTOB plants. To determine the possibility that harpin_{Xoo} is present intercellularly in HARTOB plants, we compared gel patterns and bioactivity of proteins from intercellular spaces and tissues of leaves. Protein preparations from leaf tissues still contained a protein that co-migrated in gel with harpin_{Xoo} from *E. coli* following the extraction of leaf intercellular proteins, which did not contain such a component (Fig. 3B). Although several proteins were detected in leaf intercellular spaces, none of them co-migrated with harpin_{Xoo} on gel (Fig. 3C, left). These proteins no longer appeared after heating and centrifuge, a procedure that removes heat-sensitive proteins (54). Moreover, proteins from leaf intercellular spaces of both HARTOB and Vec plants did not cause the HR in tobacco when tested in comparison with harpin_{Xoo} from *E. coli* and leaf tissues of HARTOB (Fig. 3C, right). These results suggest that harpin_{Xoo} is not present intercellularly in HARTOB plants.

Pathogen resistance in HARTOB plants. We investigated infection of the seven HARTOB lines (Figs. 4 and 5) by TMV, *R. solanacearum*, and *A. alternata* and evaluated resistance to the pathogens. Size of brown spot lesions on leaves caused by *A. alternata*, numbers of lesions on leaves caused by TMV, and stripe necrosis on stems caused by *R. solanacearum* were markedly reduced in HARTOB plants compared with the controls (Fig. 4). For example, stripe necrosis developed progressively and expanded in 10 days to basal stems from inoculation sites in un-

transformed and Vec plants. In contrast, symptoms were restricted around inoculation sites or expanded slightly in HARTOB plants during the same period. *T* tests indicated that differences in disease severity were significant between each of the HARTOB lines tested and untransformed or Vec plants. Multiple comparisons suggested that severities in diseases caused by the three pathogens all were significantly different among lines 6, 7, and 15. We regarded the significant decrease in disease severity as an indicator of resistance developed in HARTOB. On this basis, levels of resistance to the fungal, bacterial, and viral pathogens in the seven lines ranged correspondingly from 21.6 to 52.0, 16.6 to 70.0, and 29.9 to 84.3% (Fig. 5). Lines 15, 6, and 7 showed the lowest, moderate, and greatest resistance, respectively, against the three pathogens (Fig. 5). Comparing Figures 2B and 5, levels of *hpaG*_{Xoo} mRNA accumulation clearly were consistent with levels of resistance in the three lines.

Expression of defense-related genes in HARTOB plants. Previous studies have shown that defense response genes like *GST1*, *Chia5*, *PR-1a*, and *PR-1b* are involved in distinct defense pathways (5,6,10–12,20,43) under modulation by the regulator NPR1 (37,46). Particularly, some of these genes are activated by exogenous application of harpins to plants during the induction of pathogen resistance (18,39,48). To determine possible activation of molecular defense response in HARTOB plants, we performed an RT-PCR protocol to monitor mRNA accumulation of these genes in lines 6, 7, and 15 compared with the control plants. Sequences produced by RT-PCR were confirmed. Expression patterns of the five genes varied in HARTOB and control plants (Fig. 6A). *NPR1* and *PR-1b* had low constitutive expression in control plants. However, their expression was enhanced strikingly in HARTOB. All of the other genes were expressed in HARTOB but not control plants. Moreover, the five genes were expressed at the lowest, intermediate, and greatest levels in lines 7, 6, and 15, respectively (Fig. 6B). Comparing these results with those in Figures 2B and 5B, it is clear that levels of the defense-related gene expression are consistent with levels of *hpaG*_{Xoo} expression and resistance in the HARTOB plants.

ROI burst and HCD induced by the application of harpin_{Xoo}. We investigated ROI burst and expression of HCD-marker genes *hin1* (7,25,52) and *hsr203* (41,42), which are essential for the HCD pathway (1,16,24) and micro-HR (1,39) in leaves of untransformed tobacco plants following infiltration with the protein. First, no expression of *hin1* and *hsr203* was detected in the control, but both genes were induced greatly by harpin_{Xoo} (Fig. 7A). The genes started to accumulate transcripts by 3 h after treatment and reached an optimal accumulation by 5 to 7 h. Second, ROI burst was induced by the application of harpin_{Xoo} in 12 h, based on leaf staining with DAB. Little staining was evident in the control, whereas the staining was extensive over harpin_{Xoo}-infiltrating areas on leaves (Fig. 8A). Finally, micro-HR was observed in harpin_{Xoo}-treated leaves within 12 h (Fig. 8B). Thus, ROI bursts and the HCD-marker genes express concomitantly with cell death in the plants responding to harpin_{Xoo}.

Absence of spontaneous ROI burst and HCD in HARTOB plants. We found that ROI burst and HCD did not occur spontaneously in HARTOB plants. In leaves of these plants treated with water, *hin1* and *hsr203* were not expressed (Fig. 7B), hydrogen peroxide did not accumulate (Fig. 8A), and little cell death was evident (Fig. 8B). Further, results from monitoring plants at a 10-day interval until flowering suggested that none of these responses occurred in the plants throughout development. Then, we determined that HARTOB plants still had the potential to respond to harpin_{Xoo}. First, the accumulation of *hin1* and *hsr203* mRNA was conspicuous in HARTOB plants by 7 h after treatment with harpin_{Xoo}, whereas little expression of both genes had been detected in these plants as tested immediately prior to the treatment (Fig. 7C). Second, hydrogen peroxide clearly accumulated in harpin_{Xoo}-infiltrated leaves of the plants (Fig. 8A).

Fig. 5. Levels of pathogen resistance in seven *hpaG*_{Xoo}-expressing tobacco (HARTOB) lines. Resistance was given as percent decrease in disease severity, relative to that in the Vec line 1.

Finally, micro-HR evidently developed in the plant leaves in 12 h after infiltration with harpin_{X₀₀} (Fig. 8B).

DISCUSSION

We have generated HARTOB plants and shown that they express the *hpaG_{X₀₀}* gene concomitantly with resistance to selected pathogens representing plant-pathogen relationships and plant defenses. First, unlike biotrophic pathogens, such as *Peronospora* oomycetes, *Alternaria alternata* typically is necrotrophic and similar to TMV in causing necrosis in hosts. Unlike *P. syringae* that usually infects leaves, *R. solanacearum* usually infects plant roots and then spreads throughout the plant. In *Arabidopsis* spp., resistance to biotrophic pathogens and some leaf-infecting bacteria is induced via the salicylic acid (SA)-mediated SAR pathway (15,20,44), whereas induced resistance to necrotrophic pathogens is mediated by jasmonic acid (JA) or ethylene (50). Both pathways can intersect under the modulation by the NPR1 protein

(6,46), which regulates transcription of relevant defense genes (37,40,57). Second, resistance to *P. syringae* in *Arabidopsis cpr* (constitutive expressor of pathogenesis-related [PR] genes) mutants is both NPR1 dependent and NPR1 independent (5,10,11), indicating that the SAR pathway branches at NPR1 for bacterial resistance. Finally, harpins induce resistance to TMV in tobacco (48), to *P. parasitica* and *P. syringae* in *Arabidopsis* spp. (18,39) by activating the SAR pathway, and to insects in both plants via ethylene signaling (19). How harpins confer resistance to *R. solanacearum* is unclear, though harpin-induced resistance was described first with the pathogen (53). The correlation of NPR1 expression with resistance to the three pathogens predicts that NPR1 is critical for nonspecific defense in HARTOB plants.

Defense genes tested here are involved in plant basal defense pathways mediated by SA, JA, or ethylene (12). SA mediates transcription of *Chia5*, *PR-1a*, and other genes encoding acidic PR proteins (21,44), whereas JA or ethylene mediates *PR-1b* and other basic PR genes (50). *Arabidopsis* GST genes encoding glutathione S-transferases are one of three groups of SA-independent defense genes induced by *P. parasitica* (43). Roles of GST homologs (QB842845 and QB843150) in tobacco resistance are unclear. Harpins can stimulate the accumulation of SA and ethylene (19,58) and the expression of acidic and basic PR genes in *Arabidopsis* spp. and tobacco (18,39). We found that pathogen resistance developed in correlation with the expression of these genes

Fig. 6. Expression of defense-related genes in *hpaG_{X₀₀}*-expressing tobacco (HARTOB). HARTOB lines 6, 7, and 15 were compared with untransformed (Utp) and Vec plants for transcripts of five defense-related genes determined by reverse transcriptional-polymerase chain reaction, using *EF1α* (495 bp) as a standard. **A**, Expression patterns. **B**, Expression levels.

Fig. 7. Expression of hypersensitive cell death-marker genes *hin1* and *hsr203* in different plants. **A**, Results from untransformed plants sprayed with water or harpin_{X₀₀}. **B**, Results from *hpaG_{X₀₀}*-expressing tobacco (HARTOB), Vec line 1, and untransformed (Utp) plants without treatment. **C**, Results from HARTOB plants sprayed with harpin_{X₀₀}. Gene expression was determined by reverse transcriptional-polymerase chain reaction, using *EF1α* (495 bp) as a standard, at indicated times or 7 h posttreatment. M = markers. HARTOB lines are numbered on tops in **B** and **C**.

in HARTOB lines, particularly line 7, which is most resistant to the three pathogens and expresses the transgene and defense-related genes at the greatest extent. These results imply that distinct defense pathways are activated in HARTOB plants.

Cellular localizations of harpins greatly affect subsequent activation of HCD and defense pathways. First, HCD often is initiated in concert with cellular sites of initial action by a particular harpin. Although ROI burst and HCD are induced in HARTOB and untransformed plants by the application of harpin_{Xoo}, both events do not occur spontaneously in the transgenic plants. One possible reason is that harpin_{Xoo} is localized in the plant cell, because a sequence encoding a signal peptide that directs intercellular localization of proteins (13) was not involved in the *hpaG*_{Xoo}-transformation unit. HCD occurred in transgenic tobacco plants expressing *hrpZ*_{PspH} only when the gene was fused with such a sequence (49). In *Arabidopsis* spp., harpin_{Ea} induces

HCD while altering functions of mitochondria (39,56) and promoting cytochrome C release from the organelle into the cytosol (31), which is essential for downstream HCD signaling (12). Nevertheless, the presence of harpin_{Pss} in the cytoplasm and chloroplast of transgenic tobacco plants is required for ROI burst and HCD, which are dependent on NADPH-oxidases localized on the plasma membrane (14). Second, harpin_{Ea} and harpin_{Xoo} induce resistance when acting both intercellularly and intracellularly (39; this study). Transgenic tobacco and tomato plants expressing harpin_{Ea} were resistant to *P. infestans* (36) and *P. nicotianae* (H.-S. Dong and S. V. Beer, unpublished data), no matter whether *hrpN* was fused with a signal peptide-encoding sequence. Finally, plants may rely on distinct receptors to recognize harpins for different effects. In tobacco, binding of harpin_{PspH} to cell membranes is correlated with defense gene expression, which, however, is abolished by a kinase inhibitor (33), indicating that a membrane-bound kinase receptor is required. Harpin_{PspH} also can make pores in artificial membranes and cause Ca²⁺-channel responses (34). If similar responses occur during infection of plants, the pores may help release nutrients from cells to support bacterial growth and provide virulence effectors that function in cells (28,47) with an entry to the cell. In this case, ion-gated receptors may recognize specific harpins. It is of great interest to elaborate signaling mechanisms underlying different actions by harpins.

In conclusion, we have demonstrated clearly that nonspecific defense to pathogens develops without accompanying ROI burst and HCD in HARTOB plants. Our results should stimulate further studies of whether HCD and resistance pathways work separately in response to harpins and how harpins perform different effects according as their recognition by plants.

ACKNOWLEDGMENTS

The Century-Across Talent Award (Jiaokehan-2002-48), the Natural Science Foundation (3008001 and 30370969), and the 863 Plan (2002AA245011) of China and the High-Technique Plan of Jiangsu Province (BG2001307) supported this study. We thank S. V. Beer and H. S. Aldwinckle (Cornell University, NY) for useful discussion on the study, M. J. Davis (University of Florida) for critical comments on the earlier version of this article and kind instruction for revision, two anonymous reviewers for suggestions important to the subject, and D.-H. Liu (Chinese Academy of Agricultural Science, Beijing) for gifts of the pBI121 vector and *A. tumefaciens* strain EHA105.

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Fig. 8. Reactive oxygen intermediate (ROI) burst and cell death in leaves of untransformed (Utp) and *hpaG*_{Xoo}-expressing tobacco (HARTOB) (Htb) plants. HARTOB lines 6, 7, and 15 were tested; data from line 7 are shown here. Leaves were stained at 12 h after infiltration with harpin_{Xoo} or water and observed under a microscope. Scale bar = 0.25 mm. **A**, Views of diamino-benzidine-stained leaves. Tissues with ROI burst were stained orange. **B**, Views of trypan blue-stained leaves. Micro-hypersensitive response were shown as areas stained blue (left panel). On the right panel, projecting granular lipoglands are not related to any particular treatment.

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