Suppression of Defense Response in Plants by the *avrBs3/pthA* Gene Family of *Xanthomonas* spp.

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Effector genes of some plant-pathogenic bacteria, including some members of the *avrBs3/pthA* effector gene family from *Xanthomonas* spp., confer not only genotype-specific disease resistance but also pathogen aggressiveness or virulence. In addition, some effector gene products suppress induction of a nonspecific (or general) hypersensitive response (HR). To determine whether the *Xanthomonas avrBs3/pthA* gene family members *apl1*, *avrXa7*, or *avrXa10* also confer suppressor activity, we introduced constructs with each effector gene into *Pseudomonas fluorescens* 55 that expressed the entire *hrp* cluster from *P. syringae* pv. *syringae* in cosmid pHIR11. When inoculated to tobacco ‘Bright Yellow’, *P. fluorescens* (pHIR11) induces the HR and expression of four tobacco defense response genes: *HIN1*, *RbohB*, *PAL*, and *PR1*. When *P. fluorescens* double transformants that contained pHIR11 and constructs with *apl1*, *avrXa7*, or *avrXa10* were infiltrated into tobacco, the HR and expression of three defense response genes, *RbohB*, *PAL*, and *PR1*, were suppressed. The suppression of the HR and defense gene expression was more efficient in the transformants with the *apl1* and *avrXa7* than the transformant with *avrXa10*. Although expression of other defense genes was suppressed by the double transformants, *HIN1* expression was the same level as was observed after infiltration with *P. fluorescens* (pHIR11), suggesting that *HIN1* may not be involved directly in HR. Taken together, our data suggest that *avrXa7*, *avrXa10*, and *apl1*, when delivered to plant cells by the *P. syringae* pv. *syringae* *hrp* secretion system, can suppress nonhost HR and associated phenotypes.

From careful genetic analysis of the differential response of flax to the rust fungus *Melampsora lini*, Flor formulated the “gene-for-gene hypothesis” to explain cultivar-specific resistance to pathogens (Flor 1955; Keen 1990). According to this hypothesis, a single dominant avirulence (*avr*) gene of the pathogen induces a resistance response, which often includes an hypersensitive response (HR), only in plants that have a single corresponding resistance (*R*) gene; the presence of either the *avr* or *R* genes alone does not lead to induction of resistance. This hypothesis has proven to be true in many different combinations of pathogen races and plant cultivars (Baker et al. 1997; Keen 1990). The first *avr* gene was isolated from a bacterial pathogen, *Pseudomonas syringae* pv. *glycinea*, by testing HR development after the introduction of genomic library of an incompatible race into a compatible race

(Staskawicz et al. 1984). Many *avr* genes, now frequently referred to as effector genes, have been isolated not only from bacterial pathogens but also from fungal and viral pathogens (Baker et al. 1997; Bonas and Lahaye 2002; Lahaye and Bonas 2001; Leach et al. 2001). In addition, many *R* genes have been isolated from various plant hosts (Baker et al. 1997; Bonas and Lahaye 2002; Hammond-Kosack and Jones 1996).

Keen predicted that the mechanism for resistance induction in gene-for-gene interactions involved elicitor–receptor interactions, and suggested that the *avr* gene of the pathogen encodes a genotype-specific elicitor while the *R* gene of the resistant genotype encodes a corresponding receptor (Keen 1990). Recently, however, the requirement for third components in induction of genotype-specific incompatible defense responses was described (Martin et al. 2003; Shirasu and Schulze-Lefert 2003; Van der Biezen et al. 1998). For example, in *Arabidopsis*, a plant protein, RIN4, was found to be the target of the *P. syringae* AvrRpt2 protease, which is the product of *avrRpt2* (Axtell et al. 2003). RIN4 also binds to RPS2, the product of the *Arabidopsis* *R* gene *RPS2* (Axtell et al. 2003; Mudgett 2005). The incompatible (*avrRpt2*–*RPS2*) defense response is predicted to be induced when the RPS2 protein is disassociated from RIN4 due to the cleavage of RIN4 by AvrRpt2 (Axtell et al. 2003; Mudgett 2005). RPS5 protein is activated by phosphorylation of PBS1 (*R*) protein to induce incompatible (*avrPphB*–*PBS1*) defense after PBS1 is cleaved by the modified AvrPphB (*avr*) protein followed by its autophosphorylation (Mudgett 2005; Shao et al. 2003). These and other studies indicate that third components are critical, at least in some genotype-specific defense responses.

In addition to the genotype-specific effector proteins, several nonspecific (general or nonhost) effectors have been identified from the hypersensitive response and pathogenicity (*hrp*) gene clusters in plant-pathogenic bacteria (Alfano and Collmer 1997). These effectors are trafficked into plant cells via type III secretion system (TTSS) to induce HR in various nonhost plant species. However, the absence of HR after inoculation of bacteria known to secrete nonspecific effectors into susceptible host plants was a mystery. Recently, some genotype-specific effector gene products and *hrp* outer protein (*hop*) effectors of *Pseudomonas* were reported to suppress plant defense responses or the HR in susceptible host plants (Bretz and Hutcheson 2004; Mudgett 2005). For example, AvrPto of *P. syringae* pv. *tomato* suppresses plant-cell wall-based defense in the susceptible plant *Arabidopsis* (Hauck et al. 2003), while AvrPtoB from the same bacterium suppresses the HR and programmed cell death (PCD) in tomato (Abramovitch et al. 2003).

The translational of the *avrBs3/pthA* gene family in *Xanthomonas* spp. are transferred by the TTSS and some have been

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shown to be involved in virulence or disease aggressiveness in plants (Bai et al. 2000; Swarup et al. 1992; Yang et al. 1996, 2000). The *avrBs3/pthA* gene products share unique structural features; 15 to 30 repeats of 34 amino acids in their central portion, a leucine zipper-like motif, nuclear localization signals (NLSs), and acidic transcriptional activation domain in the C-terminus (Lahaye and Bonas 2001; Ponciano et al. 2003; Zhu et al. 1998). Because of their structural features, the AvrBs3/PthA proteins were predicted to be trafficked into the plant nucleus where they may regulate the expression of the genes required for development of either the genotype-specific HR or pathogenicity, depending on the combination of this gene family and plant species (Lahaye and Bonas 2001; Ponciano et al. 2003; Szurek et al. 2002).

Whether or not members of the *avrBs3/pthA* gene family function to suppress the nonhost HR is not known. To address this question, we expressed three representatives of the gene family, *avrXa7*, *avrXa10*, and *apl1*, from two *Xanthomonas* spp., *X. oryzae* pv. *oryzae* and *X. axonopodis* pv. *citri*, in the saprophyte, *P. fluorescens* 55, that harbors the cosmid clone pHIR11, which contains a full set of *hrp* genes from *P. syringae* pv. *syringae* 61 (Huang et al. 1988). The *hrp* gene cluster in pHIR11 encodes the components of the TTSS, harpin, and other nonspecific (general or broad) effectors (e.g., HopPsyA), as well as their regulatory proteins (Alfano et al. 1996; Alfano and Collmer 1997). *P. fluorescens* (pHIR11) elicits HR after infiltration into leaves of tobacco (Huang et al. 1988). However, when tobacco leaves are infiltrated with double transformants of *P. fluorescens* that carry pHIR11 and genes encoding some *Pseudomonas* TTSS effectors, elicitation of the HR is suppressed (Jamir et al. 2004). In this study, we use this well-characterized system to demonstrate suppressor activity for the effector genes from two *Xanthomonas* spp. (i.e., *avrXa7* and

avrXa10 from *X. oryzae* pv. *oryzae*, and *apl1* from *X. axonopodis* pv. *citri*).

RESULTS

Suppression of the HR in tobacco leaves by transformation with the *avrBs3/pthA* genes.

To determine whether the *avrBs3/pthA* gene family members *avrXa7*, *avrXa10*, and *apl1* encode suppressor functions, we transformed plasmids carrying each gene into *P. fluorescens* (pHIR11), the saprophyte *P. fluorescens* 55 which is expressing the *P. syringae* pv. *syringae* *hrp* gene cluster from cosmid pHIR11. Inoculation of tobacco leaves (*Nicotiana tabacum* L. cv. Bright Yellow) with 10^7 CFU/ml suspensions of *P. fluorescens* (pHIR11) or the double transformant, *P. fluorescens* (pHM1 + pHIR11), which contains the empty vector pHM1, caused a typical HR response (Fig. 1A-1). Induction of the HR by *P. fluorescens* (pHIR11) is dependent on a functional TTSS, because transformant *P. fluorescens* (pCPP2089), which is *P. fluorescens* carrying pHIR11 mutated at *hrcC* (Huang et al. 1991), did not induce HR (Fig. 1A-3). The *hrcC* gene encodes a member of a superfamily of outer membrane translocators that are components of the TTSS (Alfano and Collmer 1997); mutants in *hrcC* do not secrete various effectors, such as harpin and HopPsyA, to the extracellular fraction of bacterial cells (Alfano and Collmer 1997; Collmer et al.

Fig. 1. A, Hypersensitive response (HR) in tobacco leaves after inoculation with various *Pseudomonas fluorescens* 55 (*Pf*) transformants. *P. fluorescens* was transformed with pHIR11 from *P. syringae* pv. *syringae* and with the cloned avirulence (*avr*) genes. Plants were inoculated with *P. fluorescens* transformants (10^7 CFU/ml) with a needleless syringe. Photographs were taken 24 h after inoculation with *Pf*(pHM1 + pHIR11) (1); *Pf*(pCPP5024), pHIR11 mutated at *hrpZ* (2); *Pf*(pCPP2089), pHIR11 mutated at *hrcC* (3); *Pf*(pHap1 + pHIR11), *apl1* + pHIR11 (4); *Pf*(pH81 + pHIR11), *avrXa7* + pHIR11 (5); or *Pf*(pHa10 + pHIR11), *avrXa10* + pHIR11 (6). **B**, The *Pseudomonas* type III secretion system (TTSS) delivers AvrXa10 into rice cv. IRBB10 to elicit a genotype-specific HR response. Rice IRBB10, which contains the *Xa10* resistance gene, was inoculated with *Pf*(pHM1+pCPP5024) (1); *Pf*(pHa10+pCPP5024) (2); *Pf*(pCPP2089) (3); and *Xanthomonas oryzae* pv. *oryzae* PXO86 (4) at 10^7 CFU/ml with a needleless syringe. Photographs were taken 48 h after inoculation. **C**, *Apl1* suppresses the HrpZ-dependent HR. *Pf*(pHM1+pHIR11) (10^7 CFU/ml) was inoculated as challenge inoculum to tobacco at 2 h after a first inoculation with *Pf*(pHap1+pHIR11) (1), *Pf*(pCPP2089) (2), or *Pf*(pCPP5024) (3) at 10^7 CFU/ml. The area surrounded by the red line is the area of the leaf infiltrated with the initial inoculum, *Pf*(pHap1+pHIR11), *Pf*(pCPP2089), or *Pf*(pCPP5024), and the area surrounded by the blue line was the challenge infiltration, which was infiltrated with *Pf*(pHM1+pHIR11). Only the overlapping area infiltrated with *Pf*(pHap1+pHIR11) and *Pf*(pHM1+pHIR11) failed to show the HR. This suggests that *Pf*(pHap1+pHIR11) secretes *Apl1* into plant cells, and *Apl1* in plant cells suppresses the HrpZ-dependent HR induced by *Pf*(pHM1+pHIR11). **D**, A functional nuclear localization signal (NLS) domain is required for *Apl1* suppressor activity. An in-frame *apl1* mutant (*papl1-1Δ*) deleted in the second and third NLS domains was created in pBluescriptII by digestion of *apl1* with *BsmI* followed by religation. The double transformant *Pf*(*papl1-1Δ* + pHIR11) (2) showed an HR in tobacco, as did *Pf*(pHIR11) (3), whereas *Pf*(pNKBH1 + pHIR11) suppressed the HR (1). Thus, the plant nuclear localization of *Apl1* may be necessary for suppressor activity. Also, this result suggests that *Apl1* is delivered by the *Pseudomonas* TTSS into the plant cell.

2000). Furthermore, expression of a heat-stable, nonspecific harpin elicitor encoded by the *hrpZ* gene is critical for HR induction, because *P. fluorescens* (pCPP5024), which is *P. fluorescens* carrying pHIR11 mutated at *hrpZ* (Alfano et al. 1996), did not elicit the HR (Fig. 1A-2). No HR was observed in tobacco after infiltration with *P. fluorescens* (pHIR11) cotransformed with plasmids pHap1, containing *apl1* (a *pthA* homolog) from *X. axonopodis* pv. *citri* (Ishihara et al. 2004; Swarup et al. 1992; Tsuyumu et al. 2001); pH81, containing *avrXa7* from *X. oryzae* pv. *oryzae* (Hopkins et al. 1992; Ishihara et al. 2004); or pHa10, containing *avrXa10* from *X. oryzae* pv. *oryzae* (Hopkins et al. 1992; Ishihara et al. 2004) (Fig. 1A-4, -5, and -6, respectively). The vector harboring the *avr* genes did not influence suppressor activity because cotransformants of *P. fluorescens* (pHIR11) that contained *apl1* and *avrXa10* on derivatives of vector pBluescript II (pNKBH1 and pCH4, respectively) also did not induce the HR (data not shown). We confirmed that *P. fluorescens* transformants maintained these plasmid constructs for up to 10 days after inoculation into tobacco leaves (data not shown).

To confirm that *P. fluorescens* containing the *hrp* cluster could deliver an *avrBs3/pthA*-gene-family effector protein, we introduced *P. fluorescens* (pHa10 + pCPP5024), which carries *avrXa10* and the *hrp* gene cluster mutated at *hrpZ*, into rice cv. IRBB10, which carries *Xa10*, the resistance gene that corresponds to *avrXa10*. *P. fluorescens* (pHa10 + pCPP5024) elicited an HR in IRBB10 (Fig. 1B-2) that is similar in appearance to the race-specific HR elicited by *X. oryzae* pv. *oryzae* PXO86, which contains *avrXa10* (Fig. 1B-4). No HR was observed after infiltration of rice leaves with *P. fluorescens* (pHM1 + pCPP5024) and *P. fluorescens* (pCPP2089), both with no *avrXa10* effector gene (Fig. 1B-1 and -3). These results demonstrate that AvrXa10, the product of an *avrBs3/pthA* gene, can be delivered into plants by the *Pseudomonas* TTSS.

Supplemental data suggesting that the AvrBs3/PthA proteins are delivered to the plant cell by the TTSS was obtained using a challenge inoculation test (Fig. 1C). In this experiment, *P. fluorescens* (pHM1+pHIR11) was infiltrated into tobacco leaves as a challenge inoculum 2 h after pre-inoculation with *P. fluorescens*

(*avrBs3/pthA*+pHIR11). The HR was not observed in areas of the leaf where the infiltrations overlapped, suggesting that the suppression of the HR occurred from within the plant cells after delivery of the AvrBs3/PthA proteins. Additionally, we observed that *P. fluorescens* transformant with *apl1* gene mutated in NLS domain could not suppress HR (Fig. 1D-2).

In planta viability of *P. fluorescens* transformants.

When *P. fluorescens* (pHIR11) at 10^7 CFU/ml was inoculated into the intercellular space of the leaves of *N. tabacum* cv. Bright Yellow, the in planta population remained constant at 10^6 CFU/cm² for 6 h after inoculation (hai) and then decreased thereafter to 10^3 CFU/cm² by 24 hai (Fig. 2). On the other hand, double transformants containing *apl1* or *avrXa10*, *P. fluorescens* (pNKBH1 + pHIR11) or *P. fluorescens* (pCH4 + pHIR11), respectively, maintained populations of 10^6 CFU/cm² even after 24 hai (Fig. 2). This data is consistent with the suppression of the HR in tobacco by the *avrBs3/pthA* gene effectors.

The double transformants containing *apl1* and *avrXa10* with the mutated *hrcC*, *P. fluorescens* (pNKBH1 + pCPP2089) or *P. fluorescens* (pCH4 + pCPP2089), respectively, also maintained levels of 10^6 CFU/cm² even after 24 hai (Fig. 2). The maintenance of populations at these levels also was observed after inoculation with *P. fluorescens* (pCPP2089) and *P. fluorescens* alone (data not shown). Taken together, these data suggest that the loss of viability of *P. fluorescens* (pHIR11) was dependent on TTSS effectors, and that the AvrBs3/PthA proteins may protect bacterial populations in planta by suppressing the defense responses elicited by the presence of pHIR11.

Fig. 2. Populations of *Pseudomonas fluorescens* (Pf) transformants over time after inoculation of tobacco leaves. Suspensions (10^7 CFU/ml) of *P. fluorescens* transformants were infiltrated into tobacco leaves as described in the materials and methods. At 6-h intervals, the inoculated area (approximately 1 cm²) was excised and homogenized in mortar with pestle containing 1 ml of distilled water. Numbers of in planta bacterial cells (log CFU/cm²) were determined by serial dilution plate assay on YP agar (1% tryptone and 0.5% yeast extract, pH 6.8) containing appropriate antibiotics. Pf(pHIR11) (●); Pf(pCH4+pHIR11), *avrXa10*+pHIR11 (■); Pf(pNKBH1+pHIR11), *apl1*+pHIR11 (▲); Pf(pCH4+pCPP2089), *avrXa10*+pHIR11 mutated at *hrcC* (□); and Pf(pNKBH1+pCPP2089), *apl1*+pHIR11 mutated at *hrcC* (△).

Fig. 3. **A**, Immunoblot analysis of cellular and extracellular harpin from *Pseudomonas fluorescens* 55 (Pf) transformants expressing the *hrp* cluster alone, Pf(pHIR11), or in combination with the *avrXa10* gene, Pf(pCH4+pHIR11). Cellular (lane 1) and extracellular (lane 2) harpin from Pf(pHIR11) and cellular (lane 3) and extracellular (lane 4) harpin from Pf(pCH4+pHIR11) were detected after separation and blotting using anti-harpin antibodies and an antirabbit second antibody conjugated with alkaline phosphatase (AP). **B**, Sodium dodecyl sulfate polyacrylamide gel electrophoresis profile of extracellular proteins from Pf(pHM1+pHIR11), Pf(pHap1+pHIR11), or Pf(pCPP2089). The band confirmed by time-of-flight mass spectrometry to be HopPsyA (indicated by arrows) was detected in the supernatant of Pf(pHM1+pHIR11) (lane 1) and Pf(pHap1+pHIR11) (lane 2) but not of Pf(pCPP2089) (lane 3).

Secretion of HrpZ and HopPsyA in vitro.

Western blot analysis using the antibody raised against HrpZ revealed that transformants *P. fluorescens* (pHIR11), *P. fluorescens* (pCH4 + pHIR11) (Fig. 3A), and *P. fluorescens* (pNKBH1 + pHIR11) (data not shown) secreted HrpZ to the culture medium with equal efficiency. Strains containing the *hrcC* mutant *P. fluorescens* (pCPP2089) and the *hrcZ* mutant *P. fluorescens* (pCPP5024) did not secrete HrpZ, in either the presence or absence of an *avrBs3/pthA* gene (data not shown). Detection of a known periplasmic protein, alkaline phosphatase, in only the cellular fraction (data not shown) indicated that HrpZ was secreted efficiently via TTSS from *P. fluorescens*. Furthermore, Apl1 protein was detected in the culture medium of *P. fluorescens* (pHap11 + pHIR11), but no alkaline phosphatase was detected by using immunoblotting and matrix-assisted laser desorption-ionization time-of-flight mass spectrometry (TOF-MS) (data not shown).

A 42-kDa band, which is the expected size of HopPsyA, was observed in the concentrated supernatant of *P. fluorescens* (pHM1+pHIR11) and *P. fluorescens* (pHap11+pHIR11), but not of *P. fluorescens* (pCPP2089) when these strains were grown in *hrp*-derepressing media (Fig. 3B). This band was cut and treated with trypsin to produce its specific peptide fragments. TOF-MS analysis (gi|30231122; mass, 41,161 Da; score, 121; expect, 4.6e-07) confirmed that the peptide fragments are from HopPsyA. Thus, the secretion of HopPsyA is TTSS dependent and the mechanism by which the *avrBs3/pthA* gene products suppress the HR does not involve interference of effector secretion via the TTSS.

Oxidative burst in tobacco suspension culture.

When tobacco cell suspension cultures are treated with various elicitors, a biphasic oxidative burst (OXB) is observed at an early stage of the defense response; the second peak of OXB has been reported to be associated with HR elicitation (Glazener et al. 1996; Hammond-Kosack and Jones 1996). We detected the OXB after addition of the *P. fluorescens* single and double transformants to tobacco cell suspension cultures using luminol-dependent chemiluminescence (LDC) (Glazener et al. 1996). This assay is based on light production due to the oxidation of luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) in response to reactive oxygen species (ROS). We observed clear biphasic LDC peaks (10 and 180 min after inoculation) when suspensions of *P. fluorescens* (pHIR11) and *P.*

fluorescens (pHM1+pHIR11) were added to tobacco BY-2 suspension cultures (Fig. 4). When the transformant containing the *hrcC* mutation, *P. fluorescens* (pCPP2089), was inoculated, the second peak was not observed (Fig. 4). These data are consistent with previous findings (i.e., the TTSS effector genes carried by pHIR11 are responsible for the induction of second peak). The double transformants, *P. fluorescens* (pCH4 + pHIR11) with *avrXa10* (Fig. 4) and *P. fluorescens* (pNKBH1 + pHIR11) with *apl1* (data not shown), also did not induce the second peak of LDC. These results suggest that the AvrBs3/PthA proteins suppress the second phase of ROS production, which is the phase associated with the HR.

Suppression of induction of phenylalanine ammonia-lyase.

Phenylalanine ammonia-lyase (PAL) is a key enzyme in the phenylpropanoid pathway, which leads to the production of many phytoalexins or reactive compounds during HR development after treatment with various elicitors (Hammond-Kosack and Jones 1996; Latunde-Dada and Lucas 2001). When *P. fluorescens* (pHIR11) was added to a tobacco BY-2 suspension culture, two peaks of PAL activity were observed at 10 and 60 min after inoculation (Fig. 5). However, when *P. fluorescens* (pCPP2089) or *P. fluorescens* (pCH4 + pHIR11) with *avrXa10* were added, the second peak was very low (Fig. 5). Inoculation with the *apl1*-containing double transformant, *P. fluorescens* (pNKBH1 + pHIR11), resulted in an even lower second peak (data not shown). These data suggest that suppression of the defense cascade by the tested AvrBs3/PthA proteins occurs upstream of PAL induction.

Expression of defense genes in tobacco.

Upon inoculation to tobacco leaves, some elicitors, such as harpin, induce the expression of many defense genes, such as pathogenicity-related (PR) genes (Bent 1996; Fritig et al. 1998; Hammond-Kosack and Jones 1996). We examined the expression of several tobacco defense response genes (*HIN1*, *RbohB*, *PAL*, and *PR1*) by reverse-transcription polymerase chain reaction (RT-PCR) using a known constitutive gene (*EF1 α* , tobacco elongation factor in transcription) as the control. *HIN1* commonly is used as a marker gene for the HR because it is expressed highly, rapidly, and specifically in response to HR-inducing bacteria and elicitors (Goplan et al. 1996; Kiba et al. 2003; Lee et al. 2001). *RbohB* is a core protein of NADPH oxidase, the enzyme responsible for the OXB,

Fig. 4. Chemiluminescence from the oxidative burst (OXB) of tobacco BY-2 suspension culture. PAL activity was assayed by monitoring formation of cinnamic acid. *Pf*(pHIR11) (●), *Pf*(pCH4+pHIR11) (■), *Pf*(pCPP2089) (○), *Pf*(pCH4+pCPP2089) (□), and buffer control (△). LDC = luminol-dependent chemiluminescence; data are the means $\pm$ standard error of three independent samples.

Fig. 5. Phenylalanine ammonia lyase (PAL) activity in tobacco BY-2 suspension culture cells after cocultivation with *Pseudomonas fluorescens* (*Pf*) transformants. The OXB was assayed by luminol-dependent chemiluminescence (LDC) as described in the experimental procedures. *Pf*(pHIR11) (●), *Pf*(pCH4+pHIR11) (■), *Pf*(pCPP2089) (○), *Pf*(pCH4+pCPP2089) (□), and buffer control (△). PAL = phenylalanine ammonia lyase; data are the means $\pm$ standard error of five independent samples.

and its expression is induced by several elicitors in tobacco (Torres et al. 2002; Yoshioka et al. 2003). *PAL* and *PR1* are well-known plant-inducible defense genes (Hammond-Kosack and Jones 1996; Kiba et al. 2003; Latunde-Dada and Lucas 2001; Sasabe et al. 2000). The genes on cosmid pHIR11 cause induction of all four defense genes as early as 4 hai, as evidenced by infiltration of tobacco with *P. fluorescens* (pHM1 + pHIR11), which contains the empty vector pHM1 (Fig. 6). When *P. fluorescens* (pHM1 + pCPP2089) (the *hrcC* mutation) (data not shown) or *P. fluorescens* (pHap11 + pCPP2089) (Fig. 6) were infiltrated into tobacco, the four defense genes were not induced, suggesting that the expression of these defense genes is dependent on the presence of functional pHIR11 in *P. fluorescens*. When the double transformants containing *apl1*, *P. fluorescens* (pHap11 + pHIR11) or *avrXa7*, *P. fluorescens* (pH81 + pHIR11) were infiltrated into tobacco leaves, *RbohB*, *PAL*, and *PR1* gene expression were not detected at 4 (Fig. 6) or 6 hai (data not shown). However, the expression of the *HIN1* gene was detected at both 4 (Fig. 6) and 6 hai (data not shown). On the other hand, the double transformant containing *avrXa10*, *P. fluorescens* (pHa10 + pHIR11) did not induce expression of *RbohB* and *PR1* but did induce expression of the *HIN1* and *PAL* genes at 4 hai (Fig. 6). Although *PAL* was induced when *avrXa10* was present in *P. fluorescens*, the expression was lower than induction by *P. fluorescens* (pHM1 + pHIR11) (Fig. 6). Thus, the AvrBs3/PthA proteins suppressed the expression of *RbohB*, *PR1*, and *PAL* genes but not the *HIN1* gene. Although the *HIN1* gene is considered a marker of the HR (Goplan et al. 1996; Kiba et al. 2003; Lee et al. 2001), even if it was induced in tobacco inoculated with *P. fluorescens* that carried any of the *avrBs3/pthA* genes in combination with the *hrp* cluster (pHIR11), no HR was observed. Therefore, the suppression observed by the AvrBs3/PthA proteins may occur downstream of *HIN1* induction.

DISCUSSION

The expression of *avr* genes, including the *avrBs3/pthA* gene family members tested in this study, in their host plants causes cell death if the host contains the corresponding *R* gene (Kim et al. 2002; Van den Ackerveken et al. 1996). Thus, the Avr proteins function as elicitors for a genotype-specific HR with the

aid of the *R* gene in a plant cell. However many *avr* genes, especially those in the β -*avr* group (Jackson et al. 1999), are required for virulence in susceptible hosts (Bai et al. 2000; Jackson et al. 1999; Kearney and Staskawicz 1990; Kjemtrup et al. 2000; Lahaye and Bonas 2001; Marois et al. 2002; Ponciano et al. 2003; Swarup et al. 1992; Tsiamis et al. 2000; White et al. 2000; Yang et al. 1996, 2005). Thus, the idea is emerging that *avr* gene function in virulence may involve the suppression of basal or nonspecific defense responses, including the HR, in susceptible or nonhost plants (Abramovitch et al. 2003; Hauck et al. 2003; Jackson et al. 1999; Tsiamis et al. 2000).

In this study, we show that products of three *avrBs3/pthA* gene family members suppress several components of nonspecific plant defense responses in tobacco, including i) the nonspecific HR (Fig. 1), ii) the expression of several plant defense genes (Fig. 6), iii) the OXB (Fig. 4), and iv) the second induced peak of activity of a key defense enzyme, *PAL* (Fig. 5). We also have observed that these effector genes suppress lignification associated with defense responses (*unpublished data*). In addition to tobacco, we demonstrated that the three effector genes used in this study also suppress these defense responses in other plants, such as rice and Japanese orange (*unpublished data*). Thus, suppression of defense responses may be one mechanism for plant-pathogenic bacteria to escape inhibitions imposed by the HR and other plant defense responses elicited by their nonspecific elicitors such as harpin or HopPsyA.

When *P. fluorescens* contained combinations of *avrBs3/pthA* and the cosmid pHIR11, it did not induce the expression of several defense genes in tobacco (Fig. 6). Unlike other *avr* genes shown to suppress the HR, these AvrBs3/PthA proteins are trafficked into plant nuclei (Suzrek et al. 2002; Yang et al. 2000). Thus, one possibility is that their function in disease resistance or aggressiveness may result from their interactions with plant components that lead to altered plant gene expression (Marois et al. 2002; Yang et al. 2000; Zhu et al. 1998, 1999). Our data do not address a mechanism of activity for these proteins. However, based on our data, any action to suppress would be upstream of the induction of *RbohB*, *PR1*, and *PAL* in the cascade leading to the HR. Furthermore, because the double transformants containing these *avrBs3/pthA* genes induced *HIN1* gene expression in spite of their failure to induce the HR, either the AvrBs3/PthA proteins must also suppress genes downstream of the *HIN1*, or *HIN1* is not directly involved in the HR cascade.

It is interesting that the double transformant *P. fluorescens* (*avrXa10* + pHIR11) caused a low level of *PAL* gene induction, although reduced compared with controls, whereas the double transformant expressing *avrXa7* and *apl1* did not induce *PAL* gene expression. This difference in activity may be linked to differences in the strength of contribution of each gene to pathogenicity or disease aggressiveness. In fact, the *avrXa7* gene is reported to contribute to disease aggressiveness in rice missing the *Xa7* gene, whereas the *avrXa10* gene does not function in aggressiveness (Bai et al. 2000; Ishihara et al. 2004; Ponciano et al. 2003; White et al. 2000).

Our work demonstrates that three members of the highly related *avrBs3/pthA* gene family found in *Xanthomonas* spp. can be transferred from a saprophyte, *P. fluorescens*, by means of the *P. syringae* pv. *syringae* TTSS, and can suppress the induction of nonspecific defense responses induced by *P. syringae* pv. *syringae* elicitors in tobacco. These results are intriguing due to the unique structure of the *Xanthomonas* effector gene products and the fact that, unlike other effector proteins, most AvrBs3/PthA proteins require translocation into the nucleus for function, at least in their host plants. Our future work will be directed at determining the mechanisms by which this gene family causes HR suppression.

Fig. 6. Reverse-transcription polymerase chain reaction (RT-PCR) analysis of expression pattern of defense genes. Tobacco leaves were inoculated with each *Pseudomonas fluorescens* (Pf) transformant, and total RNA was isolated at 4 h after inoculation. *HIN1*, *RbohB*, *PAL*, and *PR1* genes were tested as inducible defense genes, and the *EF1* gene was used as a constitutively expressed control gene. The entire experiment was repeated three times.

MATERIALS AND METHODS

Culture of bacterial cells.

Bacteria and plasmids used in this study were listed in Table 1. *P. fluorescens* (strain 55) was cultured at 27°C in YP (1% tryptone and 0.5% yeast extract, pH 6.8) or LB (1% tryptone, 0.5% yeast extract, and 1% NaCl, pH 7.0) media. Where appropriate, these media were supplemented with antibiotics at the following concentrations: nalidixic acid (Nx), 60 µg/ml; tetracycline (Tc), 50 µg/ml; kanamycin (Km), 50 µg/ml; and ampicillin (Ap), 200 µg/ml (100 µg/ml for *Escherichia coli*).

Culture of tobacco cells.

Tobacco Bright Yellow (BY-2) suspension cultures were grown at 27°C with aeration at 130 rpm in modified Murashige and Skoog (MS) medium (Wako, Osaka, Japan) supplemented with sucrose (30 g/liter), thiamine (1 mg/liter), myo-inositol (100 mg/liter), KH₂PO₄ (200 mg/liter), and 2,4-dichlorophenoxyacetic acid (0.2 mg/liter) (Sasabe et al. 2000). The cell suspension (10 ml) was transferred into 100 ml of fresh medium every 7 days.

Inoculation test.

Tobacco *N. tabacum* L. cv. Bright Yellow plants were grown and inoculated as described (Huang et al. 1988). Rice cv. IRBB10 was cultivated in a growth chamber and inoculated with a needleless syringe as described (Ishihara et al. 2004). An overnight culture of *P. fluorescens* transformants grown in YP broth with appropriate antibiotics was centrifuged and resuspended in sterile distilled water. The HR development was tested by infiltration of the bacterial suspension (10⁷ CFU/ml) with a needleless syringe into the intercellular space of the tobacco seedling leaves.

For challenge inoculations (Fig. 1C), tobacco Bright Yellow leaf panels were infiltrated with *P. fluorescens* (pHap11+pHIR11), *P. fluorescens* (pCPP2089), or *P. fluorescens* (pCPP5024) at 10⁷ CFU/ml. After 2 h, suspensions of *P. fluorescens* (pHM1+pHIR11) (10⁷ CFU/ml) were infiltrated into the panels such that part of the area infiltrated overlapped with the first inoculum.

Immunoblot analysis.

Overnight cultures of *P. fluorescens* transformants grown at 27°C in YP broth were centrifuged and resuspended in *hrp* de-

repressing medium at an optical density at 600 nm (OD₆₀₀) of 0.4, and then grown at 27°C to an OD₆₀₀ of 0.8 as described (Alfano et al. 1996). The cultures were centrifuged and the pellets resuspended in sterile distilled water to collect the cellular fraction. The supernatant was concentrated 30-fold by ultrafiltration (centricon-10; Amicon, Tokyo) to collect the extracellular fraction. Each sample was adjusted to the equivalent of 6 µg of protein, and then boiled in 1× sample buffer (0.125 M Tris-Cl, pH 6.8, 4% sodium dodecyl sulfate [SDS], 20% glycerol, and 10% 2-mercaptoethanol) for 3 min. The proteins were separated by 12% SDS-polyacrylamide gel electrophoresis, and transferred to a polyvinylidene difluoride membrane (Nihon Genetics Co., Ltd, Tokyo). Harpin (HrpZ of *P. syringae* pv. *syringae*) was detected by Western blotting using the antibody raised against the epitopic peptide of HrpZ in rabbit (prepared by Tanpaku-Seisei-Kogyo Co., Gunma, Japan), using 4-nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl-pposphate solution (Roche, Tokyo) and antirabbit immunoglobulin G antibody conjugated with alkaline phosphatase (Sigma, Tokyo) as described (Sambrook et al. 1989).

TOF-MS analysis.

For TOF-MS analysis, supernatant of *P. fluorescens* transformants cultured in *hrp* de-repressing medium were prepared as for immunoblot analysis and concentrated 300-fold by trichloroacetic acid/acetone. The proteins of each sample were separated in 12% SDS-polyacrylamide gels by electrophoresis. The 42-kDa band suspected to be HopPsyA was extracted from the gel and treated with trypsin to collect the peptide fragments as described by the manufacturer (Bruker Daltonics Japan, Yokohama, Japan). This mixture of peptide fragments was analyzed by Bruker Daltonics autoflex and the result was analyzed by Matrix Science Mascot Search.

Detection of OXB.

The degree of oxidative burst was determined by assaying the amount of hydrogen peroxide with LDC (Glazener et al. 1996). Four-day-old tobacco suspension cultures in 100 ml of MS medium were collected on filter paper (Whatman Japan, Tokyo) and washed with distilled water. The washed cells were resuspended in 100 ml of MES buffer (0.5 mM MES, 0.5 mM CaCl₂, 0.5 mM K₂SO₄, and 175 mM mannitol, pH 5.8) and incubated with shaking at 130 rpm at 27°C. After 30 min of incubation, the tobacco cells were collected on filter paper, and

Table 1. Bacteria and plasmids used in this study

Strains or plasmids	Relevant characteristics ^a	Reference or source
<i>Escherichia coli</i> DH5α	F [−] , <i>endA1</i> , <i>hsdR17</i> (<i>rk</i> + <i>mk</i> −), <i>supE44</i> , <i>thi</i> −1, <i>recA1</i> , <i>gyrA96</i> , <i>relA1</i> , <i>φ80dlacZAM15A</i> (<i>lacZYA</i> - <i>argF</i>) <i>U169</i> , Nx ^r	BRL Co.
<i>Pseudomonas fluorescens</i> 55	Nx ^r	Huang et al. 1988
<i>Xanthomonas oryzae</i> pv. <i>oryzae</i> PXO86	Philippine race 2 strain, carrying <i>avrXa10</i>	Hopkins et al. 1992
Plasmids		
pHIR11	Broad-host range vector, pLAFR3 (Staskawicz et al. 1987) derivative carrying <i>P. syringae</i> pv. <i>syringae</i> 61 <i>hrp/hrc</i> cluster, Tc ^r	Huang et al. 1988
pCPP2089	pHIR11 [<i>hrcC::TnpA</i>], Tc ^r Km ^r	Huang et al. 1991
pCPP5024	pHIR11 [<i>hrpZ::Km</i>], Tc ^r Km ^r	Alfano et al. 1996
pHM1	Broad-host range cosmid derivative of pRI40, IncW, <i>parA</i> , Sp ^r Sm ^r	R. Innes, Indiana University
pHap11	pHM1 derivative carrying <i>aplI</i> with endogenous promoter, Sp ^r Sm ^r	Ishihara et al. 2004
pH81	pHM1 derivative carrying <i>avrXa7</i> with endogenous promoter, Sp ^r Sm ^r	Ishihara et al. 2004
pHa10	pHM1 derivative carrying <i>avrXa10</i> with endogenous promoter, Sp ^r Sm ^r	Ishihara et al. 2004
pCH4	pBluescript KS+ (Stratagene) derivative carrying <i>avrXa10</i> with endogenous promoter, Ap ^r	Zhu et al. 1999
pNKBH1	pBluescriptII SK− (Stratagene) derivative carrying <i>aplI</i> with endogenous promoter, Ap ^r	This laboratory
<i>papI</i> −1Δ	pBluescriptII containing an in-frame deletion in <i>aplI</i> (lacks second and third NLS domains) created by digestion of <i>aplI</i> with <i>BsmI</i> followed by religation	This laboratory

^a Resistant to nalidixic acid (Nx^r), tetracycline (Tc^r), kanamycin (Km^r), spectinomycin (Sp^r), streptomycin (Sm^r), and ampicillin (Ap^r); NLS = nuclear localization signal.

0.15 g of the wet cells were placed in a stainless steel dish and resuspended in 3 ml of fresh MES buffer. Luminol (5-amino-2,3-dihydro-1,4-phthalazinedione, 20 µl) (Wako, Osaka, Japan) stock solution (8.8 mg in 1 ml of 1 N NaOH and 9 ml of MES buffer, pH 7.0, stored at 4°C) was added to the tobacco suspension culture and left for 2 to 3 h at room temperature in the dark until oxidative generation was stabilized (called aging) before chemiluminescence measurement. After this aging process, *P. fluorescens* transformants at approximately 10⁶ CFU/ml were added. Chemiluminescence was read every 1 min for a duration of 1 s by a chemiluminescence detector (CLD-100, Tohoku Electronic Industrial Co., Ltd., Sendai, Japan).

Assay of PAL activity.

After transferring 4-day-old cultures of tobacco suspension cells into fresh modified MS medium, a suspension of *P. fluorescens* transformants (approximately 10⁶ CFU/ml) was added. At intervals, the tobacco cells and bacteria were collected by centrifugation at 5,500 rpm for 10 min at 25°C. The pellet was resuspended in 1.5 ml of extraction buffer (0.1 M potassium phosphate, pH 8.8, containing 1.4 mM 2-mercaptoethanol) and ground in the presence of quartz in a mortar with a pestle at 4°C. The homogenate was centrifuged at 9,000 rpm at 4°C for 10 min. The supernatant was collected and used for the PAL assay as described (Latunde-Dada and Lucas 2001). After 1 h of incubation at 40°C, the crude extract (166 µl) was added into 833 µl of a 2% (wt/vol) solution of L-phenylalanine in 50 mM Tris-HCl, pH 8.8, the formation of cinnamic acid was determined spectrophotometrically at 290 nm. Protein concentration was determined by the method of Bradford (Sambrook et al. 1989). PAL activity is expressed as relative value.

RT-PCR analysis.

After inoculation, tobacco (*N. tabacum* L. cv. Bright Yellow) leaves were collected and ground in liquid nitrogen, and total RNA in the leaves was isolated by an RNeasy plant minikit (Qiagen, Tokyo). Total RNA were used for reverse transcription by Omniscript RT kit (Qiagen). Synthesized cDNA was used for PCR. Primer combinations for amplification of genes were: *EF1α*, 5'tcacatcaacattgtggtcattggc3' and 5'ttgatctggtcaagagcctcaag3'; *HIN1*, 5'aatttgacggagcctattatggcctcc3' and 5'acgcaagctgtagtattctgtgaccctg3'; *RbohB*, 5'tttctctgaggttgccagccaccactaa3' and 5'gccttcattgttgacaatgtcttaaca3'; *PAL*, 5'aggaactgatagttatggtgttacaactgg3' and 5'gccttccttaggctgcaactcgaataatcc3'; and *PRI*, 5'ttggatgccataacacagctcgtgcaga3' and 5'ctagacatccaacgaacgagttacg3'. We optimized the cycle number by analyzing the samples after different cycle numbers (for example, 17, 20, 25, and 30 cycles). Semiquantitative RT-PCR was done in the exponential phase by the optimized cycle number for each of the samples (*EF* and *RbohB*: 27 cycles, *HIN1*, *PAL*, and *PRI*: 23 cycles; Fig. 6).

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