Ectopic Expression of *Ralstonia solanacearum* Effector Protein PopA Early in Invasion Results in Loss of Virulence

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Submitted 30 September 2002. Accepted 17 January 2003.

Ralstonia solanacearum OE1-1 (OE1-1) is pathogenic to tobacco. The type III-secreted effector protein *popA* of OE1-1 showed 97.6% identity to *popA* of *R. solanacearum* GMI1000, which is not pathogenic to tobacco. Reverse transcription-polymerase chain reaction analysis showed that *popA* in OE1-1 was expressed at 3 h after inoculation (HAI), but not before, in infiltrated-tobacco leaves. Pathogenicity analysis using a *popABC* operon-deleted mutant of OE1-1 (ΔABC) showed that *popABC* is not directly involved in the pathogenicity of OE1-1. When Papa, which constitutively expresses *popA*, was infiltrated into tobacco leaves, *popA* was expressed by 0.5 HAI. Papa could no longer multiply or spread in tobacco leaves and was no longer virulent. Moreover, the hypersensitive response (HR) and expression of HR-related genes were not induced in Papa-infiltrated leaves. Papa was also avirulent in a tobacco root-dipping inoculation assay. These results suggest that the expression of *popA* in Papa immediately after invasion triggers the suppression of bacterial proliferation and movement, resulting in loss of virulence. However, Papa retained its virulence when directly inoculated into xylem vessels. This result suggests that tobacco plants can recognize PopA when it is expressed early in disease development, and respond with an effective defense in the intercellular spaces.

Additional keyword: bacterial wilt

Bacterial wilt caused by *Ralstonia solanacearum* (Yabuuchi et al. 1995) is one of the most devastating bacterial plant diseases in the tropics, subtropics, and warm temperature regions worldwide (Hayward 1991). *R. solanacearum* GMI1000 (GMI1000), which is nonpathogenic to tobacco plants and pathogenic to tomato plants, elicits a hypersensitive response (HR) when infiltrated into tobacco leaves (Boucher et al. 1985). GMI1000 possesses *hrp* (hypersensitive response and pathogenicity) genes, which confer the bacterium's ability to elicit the hypersensitive response (HR) in tobacco leaves or to be pathogenic to tomato plants (Boucher et al. 1987; Arlat et al. 1992; Van Gijsegem et al. 1995). Several putative *hrp*-

encoded proteins of this bacterial pathogen share homology with proteins from the animal pathogens of the species *Yersinia*, *Salmonella*, and *Shigella* (Galan and Collmer, 1999). The proteins are assumed to be structural constituents of the type III secretion machinery, which translocates effector proteins out of the cell (Van Gijsegem et al. 1995; Wei et al. 1992; He et al. 1993; Arlat et al. 1994; Gaudriault et al. 1997; Bogdanove et al. 1998; Mudgett and Staskawicz 1998).

In most gram negative plant-pathogenic bacteria, one or two genes in each *hrp* cluster encode a heat-stable glycine-rich extracellular protein, termed harpin, that can elicit the HR when applied externally to nonhost plant cells (Alfano and Collmer, 1997). The first harpin was isolated from *Erwinia amylovora* (Wei et al. 1992). In *E. amylovora*, HrpN_{Ea} is important for the elicitation of the HR in tobacco and pathogenicity to apples and pears. In nonpectolytic *E. chrysanthemi* strains, HrpN_{Ech} required for the HR elicitation in tobacco, contributes in a minor way to infectivity on witloof chicory (Bauer et al. 1994, 1995). In *Pseudomonas syringae* pv. *syringae* B728A harpin affects virulence on beans only when the bacteria are inoculated onto the surface of leaves (Loniello et al. 1995). However, the general role of harpins in pathogenesis is still uncertain.

In GMI1000, the harpin PopA contains abundant glycine and alanine but no cysteine, and the protein purified from in vitro cultures of GMI1000 can induce an HR-like response in tobacco (Arlat et al. 1994). The induction of PopA accumulation in transgenic tobacco plants, which express *popA* from GMI1000 under control of the *hsr203J* promoter, induces the HR and systemic acquired resistance (Belbahri et al. 2001). These pieces of evidence suggest that PopA functions as an elicitor for the HR induction in tobacco plants.

PopA is secreted via the Hrp machinery, and is cotranscribed in an operon with *popB* and *popC*. PopB has functional nuclear localization signals, and PopC has leucine-rich repeat (Gueneron et al. 2000; Van Gijsegem et al. 2000). PopB and PopC are also secreted through the Hrp machinery. PopA is involved in the avirulent activity to a Petunia line (Arlat et al. 1994). On the other hand, both a *popA* mutant and a *popABC* mutant of GMI1000 retain full virulence on tomato plants and ability to elicit the hypersensitive response in tobacco plants, suggesting that the *popABC* operon is not required for GMI1000 to provoke disease and induce HR (Arlat et al. 1994; Gueneron et al. 2000). Therefore, there is no evidence that *popA* alone controls the pathogenicity of the bacteria and the bacterial host range.

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The nucleotide and amino acid sequence data of *popA*, *popB*, and *popC* of *Ralstonia solanacearum* OE1-1 can be found at DDBJ/EMBL/GenBank as accession number AB100882.

R. solanacearum OE1-1 (OE1-1) is pathogenic on tobacco plants (Date et al. 1993; Hikichi et al. 1999). Previous studies on the behavior of OE1-1 in susceptible and resistant cultivars of tomato suggest that vigorous growth of the bacteria in the roots and collars followed by growth in the upper stems are necessary for wilt symptom development (Hikichi et al. 1999; Nakazawa-Nasu et al. 1999). *hrp* mutants of OE1-1, which lack a functional type III secretion machinery, lose their ability to colonize and multiply in host plants immediately after invasion and cannot induce host responses or provoke disease (Kanda et al. in press). However, no type III effectors involved in the bacterial growth and pathogenicity have yet been reported.

In this paper, we analyze the kinetics of *popA* expression and its involvement in the proliferation and movement of OE1-1 in tobacco plants. Our results suggest that *R. solanacearum* down-regulates expression of *popA* early in host invasion. Strains constitutively expressing *popA* can no longer cause disease unless they are inoculated directly into host xylem vessels.

RESULTS

popABC operon of OE1-1.

Based on the nucleotide sequence data of GMI1000, a 5.5-kb DNA fragment containing the *popABC* operon was polymerase chain reaction (PCR)-amplified and the DNA fragment was cloned into pUC118 to create pPop. The nucleotide sequences of *popA*, *popB*, and *popC* were determined from this plasmid.

OE1-1 *popA* consisted of 1,017 nucleotides, with 97.6% identity to *popA* of GMI1000. The putative consensus promoter sequence of HrpB-regulated genes, PIP-box (plant-inducible-promoter, TTTCG-N16-TTTCG, Salanoubat et al. 2002) was detected upstream of *popA* at the same position as in GMI1000. PopA of OE1-1 showed high identity (95.0%) to PopA of GMI1000 in the amino acid sequence but it had deletions of residues 24 to 25, 186 and 193 to 195 compared to PopA of GMI1000.

PopB and PopC of OE1-1 showed high identities (98.2 and 98.9%, respectively) to those of GMI1000 in the deduced amino acid sequences. Bipartite nuclear localization signals and leucine-rich repeat sequences were detected in PopB and PopC of OE1-1, respectively, similarly as those of GMI1000.

Analysis of bacterial gene expression in infiltrated tobacco leaves.

To obtain the precise kinetics of bacterial gene expression in plants, tobacco leaves infiltrated with bacteria were used for expression analysis of *popA* and *hrpY*. Total RNA was extracted from tobacco leaves infiltrated with OE1-1 at 1, 3, and 6 h after infiltration (HAI) and analyzed by reverse transcription-polymerase chain reaction (RT-PCR).

Expression of *popA* of OE1-1 was detected at 3 HAI while that of *hrpY* was detected at 1 HAI (Fig. 1). A more precise

Fig. 1. Reverse transcription-polymerase chain reaction analysis of *popA* and *hrpY* of *Ralstonia solanacearum* strains infecting tobacco leaves. Total RNA was isolated from tobacco leaves infiltrated with *R. solanacearum* OE1-1 at 1, 3, and 5 h after inoculation.

kinetic analysis of *popA* expression in OE1-1 infiltrated into tobacco leaves at 0.5, 1, 2, 3, and 5 HAI showed that *popA* was not expressed until 3 HAI (Fig. 2B). In tobacco leaves infiltrated with water, no PCR products specific for *popA* or *hrpY* were detected (data not shown).

To analyze the function of *popABC* in the pathogenicity of OE1-1, the *popABC* operon, *popB* and *popC*, or *popC* of OE1-1 were replaced with the tetracycline resistance gene using marker exchange to produce strains ΔABC , ΔBC , and ΔC , respectively. Analysis using genomic PCR and Southern hybridization confirmed the correct replacement of the *popABC* operon, *popB* and *popC*, or *popC* with the tetracycline resistance gene (data not shown). In peptone yeast (PY) medium and in Boucher's medium, ΔABC , ΔBC and ΔC grew in a similar manner to OE1-1 (data not shown).

Tobacco plants inoculated with ΔABC , ΔBC or ΔC by all inoculation methods wilted rapidly in a manner identical to the plants inoculated with wild type strain OE1-1, suggesting that the *popABC* operon is not essential for the pathogenicity (Table 1).

To determine if *popBC* has an effect on the expression of *popA*, total RNA was extracted from tobacco leaves infiltrated with ΔBC or ΔC at 0.5, 1, 2, 3, and 5 HAI and analyzed by RT-PCR. In this *popBC* background, expression of *popA* was detected at 3 HAI just as in wild type strain OE1-1 (Fig. 2B), indicating that deletion of *popB* and *popC* did not influence the expression of *popA* in ΔBC and ΔC .

When PCR amplified, using each RNA sample, from OE1-1 and any transformants-infiltrated tobacco leaves as a template, without cDNA synthesis reaction, any bands were not observed.

A *popA*-expressing transformant of ΔABC .

To investigate the effects of constitutive expression of *popA* and *popABC* on bacterial virulence, strain ΔABC was transformed with plasmids pPapa and pPABC (Table 2), which contain *popA* open reading frame (ORF) and ORFs of *popABC* from OE1-1 fused to a constitutively active promoter from *Burkholderia glumae* (Hikichi et al. 1999), to create Papa and

Fig. 2. Reverse transcription-polymerase chain reaction analysis of *popA* of *Ralstonia solanacearum* A, OE1-1 and Papa at 0, 1, 3, and 5 h after incubation in Boucher's medium and B, OE1-1, ΔBC , ΔC , and Papa at 0.5, 1, 2, 3, and 5 h after infiltration into tobacco leaves.

ABC, respectively. Papa and ABC grew in a similar manner to OE1-1 in potato sucrose (PS) medium (Wakimoto et al. 1968) and in Boucher's medium (data not shown).

Tobacco plants inoculated with OE1-1 by root dipping or stem dipping started to wilt 5 days after inoculation (DAI) and 3 DAI, respectively (Table 1). All the inoculated plants wilted at 10 and 7 DAI, respectively. When OE1-1 was infiltrated into tobacco leaves, the leaves started to wilt at 5 DAI, and then the plants wilted at 10 DAI.

Neither Papa nor ABC were virulent when inoculated into tobacco plants using root dipping or when infiltrated into tobacco leaves (Table 1). However, Papa and ABC retained their virulence to tobacco plants when inoculated using the stem dipping method, suggesting that only the transformants inoculated directly into xylem vessels could induce wilting. Because these results indicate that ABC showed the same pathogenicity to tobacco plants as Papa, only Papa was used in the following studies.

Though OE1-1 and Δ ABC showed the same pathogenicity to tomatoes, eggplants, and sweet peppers, Papa, inoculated through the roots, lost its virulence to these *Solanum* spp. plants but it retained its virulence when inoculated through the cut stems (data not shown).

RT-PCR analysis showed that *popA* was constitutively expressed in Papa incubated in Boucher's medium (Fig. 2A), and was expressed in tobacco leaves infiltrated with Papa at 0.5 HAI (Fig. 2B). Expression of *hrpY* of Papa was detected immediately after invasion in a similar manner to that of OE1-1 (data not shown).

The population of wild type strain OE1-1 in infiltrated tobacco leaves increased to 5×10^8 CFU/cm² at 5 DAI. On the other hand, the population of Papa in the infiltrated region of the tobacco leaves drastically decreased from 3×10^6 CFU/cm² at 2 DAI to 5×10^4 CFU/cm² until 5 DAI, suggesting that the proliferation of *popA*-constitutively expressing strain Papa is inhibited in the intercellular spaces (Fig. 3).

In the plate-printing assay (Fig. 4), though OE1-1 and Δ ABC were detected at any area at 14 DAI, Papa was never detected beyond the bacteria-infiltrated area, suggesting that Papa lacked the ability to move into the periphery through the intercellular spaces in the infiltrated tobacco.

Expression of plant HR and induced resistance-related genes in tobacco leaves infiltrated with the bacteria.

PopA purified from GMI1000 cultures functions as an HR elicitor (Arlat et al. 1994). The HR-related tobacco genes, *hsr203J* and *hin1*, are specifically expressed in tobacco leaves inoculated with *R. solanacearum* 8107 (8107), which like GMI1000, induces HR and systemic acquired resistance in infiltrated tobacco leaves and is nonpathogenic to tobacco (Kiba et al. In press). To determine whether these HR-related host genes might play a role in suppression of bacterial growth and movement, accumulation of the mRNAs of *hsr203J* and *hin1* mRNAs in Papa-infiltrated tobacco leaves was analyzed by northern hybridization.

In all bacteria-infiltrated tobacco leaves, weak signals for mRNAs of *hsr203J* were first detected at 3 HAI. In tobacco leaves infiltrated with the tobacco nonpathogen 8107, the accumulation reached a maximum at 6 HAI (Fig. 5). On the other hand, *hsr203J* was only weakly expressed in tobacco leaves infiltrated with tobacco pathogen OE1-1 and constitutive expression of PopA in OE1-1 mutant strain Papa.

In 8107-infiltrated tobacco leaves, mRNAs of *hin1* were first detected at 3 HAI, and mRNA signal intensity reached a maximum at 6 HAI. In OE1-1-infiltrated leaves, a weak signal for *hin1* was first detected at 3 HAI. Signal intensity increased gradually, reached a maximum at 9 HAI, and then drastically decreased (Fig. 5). On the other hand, in Papa-infiltrated leaves, weak signals for *hin1* were detected at 3 to 9 HAI, and a strong signal was detected at 12 HAI (Fig. 5), suggesting that kinetics of *hin1* expression in Papa-infiltrated leaves differs from that in OE1-1-infiltrated leaves.

DISCUSSION

In GMI1000, transcription units 1, 2, 3, and 4 of the *hrp* gene clusters are induced in the Boucher's medium or in coculture with plant cells through the *hrpB* regulatory gene (Aldon et al. 2000, Arlat et al. 1992, Genin et al. 1992, Brito et al. 1999, Marenda et al. 1998, Van Gijsegem et al., 1995). The PIP-box has been detected in the upstream regions of each transcript unit in GMI1000 (Salanoubat et al., 2002). The box was also detected in the upstream regions of *popA* and *hrpY* in

Table 1. Virulence of *R. solanacearum* to tobacco plants by different inoculation methods

Inoculation methods ^a	Bacteria	Disease Index				
		2 ^b	4	6	8	10
Root dipping	OE1-1	0	0	38.4	76.3	100
	Δ ABC	0	0	39.1	79.4	100
	Δ BC	0	0	36.4	82.5	100
	Δ C	0	0	40.5	75.2	100
	Papa	0	0	0	0	0
	ABC	0	0	0	0	0
Leaf infiltration	OE1-1	0	0	19.1	52.9	100
	Δ ABC	0	0	20.1	59.3	100
	Δ BC	0	0	18.3	53.5	100
	Δ C	0	0	25.1	60.0	100
	Papa	0	0	0	0	100
	ABC	0	0	0	0	0
Stem dipping	OE1-1	0	41.6	91.7	100	0
	Δ ABC	0	37.9	89.1	100	100
	Δ BC	0	42.9	79.8	100	100
	Δ C	0	38.1	96.1	100	100
	Papa	0	43.0	95.0	100	100
	ABC	0	39.0	94.0	100	100

^a Eight-week-old tobacco plants were inoculated by dipping the roots of the plants in the bacterial suspension at 10^8 CFU/ml for 20 min (Root dipping), infiltration of the bacterial suspension at 10^8 CFU/ml in an approximately 50- μ l volume into the tobacco leaves using a disposable syringe (Leaf infiltration) or dipping the cut stems in the bacterial suspension at 10^8 CFU/ml for 20 min (Stem dipping). Plants were rated daily on a 0 to 4 disease index. Each point represents the mean disease index of 60 plants combined from five separate experiments.

^b Days after inoculation of the bacteria.

OE1-1. Moreover, in a *hrpB*-deleted mutant of OE1-1 cultured in the Boucher's medium, *popA* was not expressed and the mutant lost its parasitic fitness in host plants and its pathogenicity (Kanda et al. In press), suggesting that expression of *hrp* genes and *pop* operon in OE1-1 are likely also to be regulated by HrpB as in GMI1000. However, RT-PCR analysis showed that the timing of the *popA* expression in OE1-1 infiltrated into tobacco leaves differed from that of *hrpY*, suggesting that one or more other regulators in addition to HrpB could control expression of *popA* in OE1-1.

The induction of *hrpB* expression is activated by a two-component response regulator, HrpG, in response to nutrient conditions and metabolic signals (Brito et al. 1999). Expression of *hrpG* is sensed by a surface signaling mechanism composed by PrhA-PrhR-PrhI activated after physical bacterium-plant cell contact (Brito et al. 2002). Moreover, accumulated 3-OH palmitic acid methyl ester, which controls expression of the Phc regulon in response to cell density of the bacteria, represses expression of *hrp* genes (Schell, 2000), and type III-secreted proteins of a *phcA*-mutant of OE1-1 were secreted when incubated in the rich medium (K. Ohnishi, unpublished data). Therefore, expression of *hrp* regulon may be elaborately regulated since it may be affected not only by each other but also by other factors such as nutrient conditions and metabolic signals.

Gueneron et al (2000) have suggested a possibility that some of the *popB* sequence deleted in a *popB* mutant of GMI1000 cultured in Boucher's medium might play a role in transcription of *popA*. The nucleotide sequence of *popB* of OE1-1 showed high identity (99.2%) to that of GMI1000, nonetheless RT-PCR analysis showed that deletion of *popBC* did not affect expression of *popA*. Moreover, in the Boucher's medium, though expression of *hrpY* was detected immediately after incubation in OE1-1 cultured (data not shown), expression of *popA* was not detected until 3 h after incubation. Gueneron et al (2000) have also reported that the level of induction of the *popABC* operon is significantly lower in the presence of plant cells than in the Boucher's medium, whereas the level of transcription of *hrp* units 2, 3, and 4 is similar or slightly higher in the presence of plant cells than in the Boucher's medium. Constitutive expression of *popA* in Papa was not affected by deletion of *popBC* and did not affect expression of *hrpY*. Therefore,

expression of *popA* may be regulated by not only HrpB but also nutrient conditions or metabolic signals, which may be transduced in OE1-1 infected into host plants and incubated in Boucher's medium.

Infiltration of OE1-1 induces necrotic lesion of tobacco leaves at 60 HAI. The necrotic lesion differed from necrosis induced at 24 HAI by infiltration of 8107 nonpathogenic to tobacco (Kanda et al. In press). Induction of the necrotic lesion depends on secretion of effectors through *hrp* secretion machinery, similarly as pathogenicity of the bacteria (Kanda et al. In press). Infiltration of Papa induced the necrotic lesion at 60 HAI (data not shown), and Papa showed virulence when inoculated through cut stem, suggesting that constitutive expression of *popA* in Papa will not affect delivery of effectors involving in induction of necrotic lesion and virulence. Moreover, RT-PCR analysis showed that pattern of *hrpY* expression in Papa in vitro and in planta showed similarly as that of OE1-1. These results suggest that constitutive expression of *popA* in Papa does not affect expression of other *hrp* genes or prevent delivery of other essential effectors, but is directly involved in loss of virulence of Papa inoculated through the roots and infiltrated into leaves.

RT-PCR analysis of *hrpY* pilin gene expression suggests that the type III secretion pilus might be constructed immediately after bacterial invasion and that PopA expressed in Papa during 3 HAI is likely secreted into tobacco cells through the system, in the same way that *P. syringae* pv. *glycinea* delivers AvrB within a few minutes of contact with plant cells (Huynh et al. 1989). Proliferation of Papa in vitro showed that constitutively expression of *popA* did not influence bacterial growth. Therefore, PopA protein expressed in Papa immediately after invasion influences the induction of a resistance response as an effector in the infiltrated spaces, and suppresses the bacterial growth and movement in the intercellular spaces, resulting in regulation of the bacterial virulence.

R. solanacearum generally invades from wounded roots or natural openings from which secondary roots subsequently emerge, and then proliferates in the intercellular spaces of the inner cortex and vascular parenchyma before invasion into xylem vessels (Hayward, 1991; Seile et al. 1997; Vasse et al. 1995). Pathogenicity analysis of Papa inoculated through the roots showed that a resistance response was induced in the

Table 2. Bacterial strains and plasmids

Designation	Relevant Characteristics	Reference or Source
<i>E. coli</i> DH5 α	F-endAI, relA, ϕ 80, lacZAM15, hsdR17, supE44, thi-I, recAI, <i>gyrA</i> 96	Hanahan, 1983
<i>Ralstonia solanacearum</i> OE1-1	wild-type, race 1, biovar 1,	Date et al. 1993
8107	wild-type, race 1, biovar 1,	this study
Δ ABC	OE1-1 Δ popABC::Tc ^r	this study
Δ BC	OE1-1 Δ popBC::Tc ^r	this study
Δ C	OE1-1 Δ popC::Tc ^r	this study
Papa	A transformant of Δ ABC with pPapa, Km ^r	this study
ABC	A transformant of Δ ABC with pPABC, Km ^r	this study
Plasmids		
pUC118	Ap ^r	Vieira and Messing, 1987
pMT5056	Ap ^r , Tc ^r , pBebd2 derivative	Tsuda et al. 1995
pUCDpro3101	Km ^r , pUCD3101 derivative	Hikichi et al. 1999
pPOP	5.5-kb fragment containing <i>popABC</i> operon in pUC118, Ap ^r	this study
pHA	1.03-kb <i>KpnI</i> and <i>EcoRI</i> fragment containing <i>hrpA</i> in pUC118, Ap ^r	this study
pABC	1.0-kb <i>KpnI</i> and <i>EcoRI</i> fragment containing <i>prhA</i> in pHA, Ap ^r	this study
pBC	2.09-kb <i>KpnI</i> and <i>EcoRI</i> fragment containing <i>popA</i> in pHA, Ap ^r	this study
pC	2.66-kb <i>KpnI</i> and <i>EcoRI</i> fragment containing <i>popB</i> in pHA, Ap ^r	this study
pABC-tet	pABC with the 1.4-kb tetracycline resistance gene in <i>KpnI</i> site of pABC, Ap ^r , Tc ^r	this study
pBC-tet	pBC with the 1.4-kb tetracycline resistance gene in <i>KpnI</i> site of pABC, Ap ^r , Tc ^r	this study
pC-tet	pC with the 1.4-kb tetracycline resistance gene in <i>KpnI</i> site of pABC, Ap ^r , Tc ^r	this study
pPapa	1.0-kb fragment containing <i>popA</i> in pUCDpro3101, Km ^r	this study
pPABC	4.7-kb fragment containing <i>popABC</i> in pUCDpro3101, Km ^r	this study

intercellular spaces of the roots similarly as that in the leaves. In addition, virulence in Papa inoculated into the xylem vessels suggests that PopA expressed in the bacteria existing in xylem vessels might not function as an effector for resistance induction or that defense responses induced by early PopA expression are not effective in the xylem.

hsr203J is specifically activated during the early steps of incompatible plant-pathogen interactions and activation of *hsr203J* is correlated with programmed cell death occurring in response to diverse pathogens (Pontier et al. 1994, 1998, 1999). *hsr203J* may be a signaling component or a detoxification effector (Tronchet et al. 2001). Expression of *hin1* is activated rapidly by harpins from *E. amylovora* and *P. syringae*. *Hin1* protein is homologous to the NDR1 protein required in the *Arabidopsis* spp. to mediate disease resistance induction by different *R* genes specific for the pathogen (Gopalan et al. 1996). These evidences indicate that the expression of these genes is involved in the HR induction. Though PopA possesses HR-elicitor activity when infiltrated into tobacco leaves (Arlat et al. 1994), the induction of necrosis by the HR in tobacco leaves infiltrated with Papa was not observed (data not shown). Moreover, the expression patterns of *hsr203J* and *hin1* in Papa-infiltrated tobacco leaves suggest that PopA expressed during 3 HAI would not function as a strong elicitor for the HR.

Kinetics of *hin1* expression in Papa-infiltrated leaves differed from that in OE1-1-infiltrated leaves. In tobacco, salicylic acid (SA) is a critical signaling molecule in the pathway leading to local and systemic disease resistance as well as the expression of acidic types of pathogenesis-related (PR) genes and the *hsr203J* (Guo et al. 2000, Ohashi and Ohshima 1992, Pontier et al. 1994). In addition, recent studies have demonstrated that jasmonic acid (JA) and ethylene also mediate resistance to certain pathogens, wounding responses, and expression of basic types of PR genes (Memelink et al. 1990, Vijayan et al. 1998, Xu et al. 1994). In 8107-infiltrated leaves, expression of *hsr203J* is regulated by the SA-dependent signaling pathway, whereas the *hin1* are regulated by the SA-independent pathway (Kiba et al. In press). SA- and JA-mediated pathways including gene expression are affected by JA and SA, respectively (Pena-Cortes et al. 1993, Sano et al. 1994, Doares et al. 1995, Niki et al. 1998, Thomma et al. 2000). Therefore, delay of expression of *hin1* in Papa-infiltrated tobacco leaves might be involved in a change in valance between SA- and JA-mediated pathways, resulting in induction of a resistance response different from the HR to suppress proliferation and movement of Papa in the intercellular spaces.

In the evolution war between plants and pathogens, since the hosts have developed the ability to recognize effectors (Nimchuk et al. 2001), *AvrPphF* in *P. syringae* pv. *phaseolicola* masks the presence of an unknown *avr* gene (Tsiamis et al. 2000). Although the selection pressure on the pathogen without any virulence function of the *Avr* factor will result in selection for loss or mutated versions of the *Avr* gene, *Avr* factors with a virulence role will be maintained in the pathogen population (Van der Hooft et al. 2002). Papa lost its virulence not only to tobacco plants but also to other *Solanum* spp. plants. Since *popA* is already known to be an avirulence gene to specific *Petunia* genotypes (Arlat et al. 1994) and might possess unknown virulence functions to other plants, the bacteria might regulate expression of *popA* but not diminish the expression of *popA* nor delete *popA* to infect *Solanum* spp. plants. Otherwise, its expression might be delayed to allow time for other bacterial factors to be delivered into plant cells. These additional factors might prevent the plants from sensing PopA and other virulence factors.

Genomic analysis reveals that 14 ORFs coding for products related to *Avr* determinants and 51 candidates for the type III

secretion-dependent effectors are distributed on the chromosome and megaplasmid of GMI1000 (Salanoubat et al. 2002). Single and multiple frameshifts or insertion sequence insertions occur in putative virulence gene homologues. The emergence of a plant resistance gene that recognizes a virulence gene would abolish the value of the corresponding virulence gene. Salanoubat et al. (2002) have demonstrated that deactivating gene mechanisms may be the result of an evolutionary elimination of genes that have become liabilities for the pathogen. The results in this study suggested that deletion of *popABC* genes did not affect pathogenicity of OE1-1. However, expression of *popA* immediately after invasion resulted in suppression of the bacterial colonization and proliferation. Moreover, expression of *popA* was suppressed immediately after bacterial invasion into host plants. Taken together, to infect into host plants and cause the disease, *R. solanacearum* not only eliminates and deactivates genes that show a disadvantage for bacterial colonization and proliferation, but also control the expression of genes such as *popA*.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions.

The bacterial strains and plasmids are listed in Table 2. *R. solanacearum* strains were routinely grown in PS medium (Wakimoto et al. 1968) at 30°C. *Escherichia coli* strains were grown in LM medium (Hanahan 1983) at 37°C. The following antibiotics were used in selective media in the amounts indicated: 50 µg of ampicillin per ml; 50 µg of kanamycin per ml; and 50 µg of tetracycline per ml. The populations of *R. solanacearum* OE1-1 and Papa in planta were assayed in three independent experiments using Hara-Ono plates (Hara and Ono 1983) and Hara-Ono plates containing kanamycin, respectively.

Plant assays.

Tobacco plants (*Nicotiana tabacum* cv. Bright Yellow) were grown in pots containing a mixture of vermiculite/peat moss

Fig. 3. Population dynamics of Papa and OE1-1 in infiltrated tobacco leaves. The graph shows the average number of bacteria per square centimeter. Closed circles = OE1-1; closed squares = Papa.

Fig. 4. Cut sites of tobacco plants in plate-printing assay. Tobacco leaves that infiltrated OE1-1, Δ ABC, or Papa were detached and then cut into three pieces with razor blades at 14 days after infiltration. Stems of the tobacco plants were also cut into three pieces. Dashed lines indicate cut sites. Bacteria were infiltrated into a hatched region.

(3:1) in a growth room at 25°C under 10,000 lux for 16 h per day, and watered with five times-diluted Hoagland's solution (Hikichi et al. 1999). Eight-week-old test plants were inoculated at a bacterial concentration of 10^8 CFU/ml, and then the plants were grown in water culture pots (Yamato Water Culture Pot No.1, Yamato Plastic Co. Ltd., Tokyo) with five times-diluted Hoagland's solution in a growth room at 25°C under 10,000 lux for 16 h per day (Hikichi et al. 1999).

Virulence assays.

The tobacco plants were inoculated with the bacteria using three types of inoculation methods, root-dipping, leaf-infiltration, and stem-dipping. For plant inoculations, the bacteria were resuspended at an optimal density at 600 nm (OD_{600}) of 0.1 (1.0×10^8 CFU/ml). For inoculation by root-dipping, the roots of tobacco plants were dipped in the bacterial solution at 10^8 CFU/ml for 20 min and then washed in running water. For infiltration, the bacterial solutions at 10^8 CFU/ml in an approximately 50- μ l volume, measured according to indicators in syringes, were infiltrated into tobacco leaves using a disposable syringe, and for stem-dipping the stems of tobacco plants, with roots and collars removed, were dipped in the bacterial solution at 10^8 CFU/ml for 20 min and then washed in running water. Tobacco plants, inoculated by roots-dipping and stem dipping, were grown, as described in plant assays.

Plants were coded and inspected for wilting symptoms daily for 14 days. Plants were rated on a zero-to-four disease index scale: 0 = no wilting; 1 = 1 to 25% wilting; 2 = 26 to 50% wilting; 3 = 51 to 75% wilting; and 4 = 76 to 100% wilting or dead. Each assay was repeated in five successive trials. Within each trial, we treated 12 plants with each strain, yielding 60 plants for each strain.

Plate-printing assay was performed as follows (Fig. 4). At 14 DAI with OE1-1, Δ ABC and Papa, the bacteria-infiltrated leaves were detached and then cut into three pieces with the razor blades. Stems of the tobacco plants were also cut into three pieces. Cut site of each piece was placed on the Hara-Ono medium, and the medium was incubated at 30°C for 3 days.

Bacterial population in tobacco plants.

The Papa or OE1-1-infiltrated area (1 cm²) in tobacco leaves was excised from five of each set of plants at 1, 2, 3, 4, and 5 DAI and ground using a mortar and pestle. The original solution and 10-fold serial dilutions of it were dispensed in 0.1 ml of samples and spread onto three plates of the selective agar media of Hara and Ono for OE1-1, or media containing kanamycin at 50 μ g/ml for Papa. The numbers of colonies were

counted after 2 days of incubation at 30°C to estimate the population.

General DNA and RNA manipulations.

Isolation of genomic DNA, plasmid DNA manipulations, PCR, and Southern blot analyses were performed using standard techniques (Sambrook et al. 1989). *R. solanacearum* was transformed by electroporation (Allen et al. 1991). Double-stranded DNA sequencing templates were prepared with GenElute Plasmid Miniprep Kits (SIGMA Chemical Co., St. Louis, MO U.S.A.). Sequences were determined using an Automated DNA Sequencer Model 373 (Applied Biosystems, Tokyo). DNA sequence data were analyzed using the DNASIS-Mac software (Hitachi Software Engineering, Yokohama, Japan).

Isolation of the *popABC* operon.

A 5.5-kb DNA fragment was PCR-amplified using the following primers based on the nucleotide sequence of GM1000: 5'-GCTCTAGAGCAACACCGTATCGAGCAGC-3' (named Xba-propopAhrpA) with an added *Xba*I site (underlined) and 5'-CCCAAGCTTGATCAACGAAGGCGACAGC-3' (named Hind-hrpApopA) with an added *Hind*III site (underlined), and *Ex Taq* DNA polymerase (Takara, Ohtsu, Japan). An *Xba*I and *Hind*III-digested fragment was ligated into the *Xba*I and *Hind*III sites of pUC118 (Vieira and Messing, 1987) to create pPop.

A *popABC* operon-deleted mutant.

A 1,030-bp fragment (named HA fragment) was PCR-amplified from the genomic DNA of OE1-1 with the following primers: 5'-GGGGTACCGCATTCGGCAATGAAGAAG C-3' (named Kpn2-1) with an added *Kpn*I site (underlined) and 5'-CGGGATCCAATACGTC GGCAACAGCGGC-3' (named Bam1-2) with an added *Bam*HI site (underlined). A *Kpn*I and *Bam*HI-digested HA fragment was ligated into the *Kpn*I and *Bam*HI sites of pUC118 to create pHA. A 1,032-bp fragment (named ABC fragment) was PCR-amplified from the genomic DNA of OE1-1 with 5'-CGGAATTCTACAACGGCG CGACCATGC-3' (named Eco3-1) with an added *Eco*RI site (underlined) and 5'-GGGGTACCAACCTTTGAGGGCAGG GGGT-3' (named Kpn4) with an added *Kpn*I site (underlined). A 2,092-bp fragment (named BC fragment) was PCR-amplified from the genomic DNA of OE1-1 with Eco3-1 and 5'-GGGGTACCGAAAGCGGACAGCCGTTGCC-3' (named Kpn8-1) with an added *Kpn*I site (underlined). A 2,656-bp fragment (named C fragment) was PCR-amplified from the genomic DNA of OE1-1 with Eco3-1 and 5'-GGGGTACCG ACGATCAGTCGTCGTCGTC-3' (named Kpn6-1) with an added *Kpn*I site (underlined). The ABC fragment, BC fragment, and C fragment were digested with *Kpn*I and *Eco*RI, and the fragments were ligated into the *Kpn*I and *Eco*RI sites of pHA to create pABC, pBC, and pC, respectively. The *Kpn*I-digested fragment containing the tetracycline-resistance gene from pMT5056 (Tsuda et al. 1995) was ligated into the *Kpn*I sites of pABC, pBC, and pC to create pABC-tet, pBC-tet, and pC-tet, respectively. Δ ABC, Δ BC, and Δ C were created using the pABC-tet, pBC-tet, and pC-tet, respectively, by the marker-exchange. Each plasmid was electroporated into OE1-1 and then tetracycline-resistant transformants were isolated. PCR-amplification using genomic DNA of the transformants as templates and pop3 (5'-TCGAAGACGGCCAGATCACC-3') and pop5 (5'-TGTTCTGGCGCAGCAAGCAG-3') as primers was performed. When used genomic DNA of OE1-1 as a template, a 5.6-kb DNA fragment was detected. Transformants with pABC-tet, pBC-tet, and pC-tet in which 2.3-, 3.3-, and 3.8-kb DNA fragments were detected, respectively, were

Fig. 5. Northern blot hybridization analysis of *hsr203J* and *hin1* in tobacco leaves infiltrated with *Ralstonia solanacearum* strains.

selected. DNA sequences of these fragments were analyzed using pop3 and pop5 as primers, as described above and Δ ABC, Δ BC and Δ C were selected.

Construction of pPapa and pPABC.

A 1.0-kb DNA fragment containing a *popA* ORF was PCR-amplified from OE1-1 with the following primers: 5'-GGA AGATCTATGTCAGTCGGAACATC-3' (named popA-Bgl) with an added *Bgl* II site (underlined) and 5'-GCC AAGCTTACATCGGCTGCGTCGAGG-3' (named popA-Hind) with an added *Hind*III site (underlined). The *Bgl*II and *Hind*III-digested DNA fragment was ligated into the *Bam*HI and *Hind*III sites of pUCD3101pro containing the constitutively active promoter from *Burkholderia glumae* (Hikichi et al. 1999) to create pPapa.

A 4.7-kb DNA fragment containing ORFs of the *popABC* operon was PCR-amplified from OE1-1 with popA-Bgl and 5'-GCTCTAGATCAACGCTGCCTCGACGGACT-3' (named xba-popC) with an added *Xba*I site (underlined). The *Bgl*II and *Xba*I-digested DNA fragment was ligated into the *Bam*HI and *Xba*I sites of pUCD3101pro to create pPABC.

pPapa and pPABC were electroporated into Δ ABC to create Papa and ABC, respectively.

RT-PCR of *popA* and *hrpY*.

The bacteria-infiltrated area in tobacco leaves (0.6 g) was homogenized in RNA extraction buffer (100 mM glycine pH 9.5, 10 mM EDTA, 0.1 M NaCl, 1% [wt/vol] sodium dodecyl sulfate (SDS), 0.1% [wt/vol] bentonite), extracted with phenol/chloroform/isoamyl alcohol (25:24:1) twice, and then extracted with chloroform/isoamyl alcohol (24:1). The aqueous phase was mixed with one-third volume of 10 M LiCl and incubated at -20°C for 2 h. After centrifugation at $17,500 \times g$ for 30 min, the pellet was washed with 2M lithium solution (2 M LiCl, 50 mM EDTA), and then the pellet was dissolved in Tris-EDTA (TE) buffer. Phenol extraction and ethanol precipitation were performed to recover the RNA. DNase I (Ambion, Texas, U.S.A) was treated to remove the genomic DNA containing RNA preparation prior to RT-PCR.

The bacteria at OD₆₀₀ of 0.8 were incubated in Boucher's medium (Boucher et al. 1985) at 30°C for appropriate time, and RNA was extracted with High Pure RNA Isolation Kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's protocols.

cDNA fragments of *popA* and *hrpY* were synthesized from total RNA (6 μg) using Reverse Transcriptase RAV-2 (Vieira and Messing, 1987) with the primers 5'-GTTGGCACCG TTGACATCG C-3' (named SQ-) and 5'-CGGGGATCCTT AGCTGATCAGGTCCTTGGC-3' (named hrpY-Bam), respectively. PCR was then carried out using 5'-CTGGGTG AAGCTGCTGAAGGC-3' (named SQ+) and SQ- for the

amplification of a 320-bp DNA fragment specific to *popA*, and 5'-GGAATTCATATGGCAGGCGTTCCGAAC-3' (named Nde-hrpY) and hrpY-Bam for the amplification of a 250-bp DNA fragment specific to *hrpY*.

Northern analysis of *hsr203J* and *hin1*.

Total RNA (10 μg) was separated in a 1.5% agarose gel under denaturing conditions and blotted onto Hybond N+ membrane (Amersham Pharmacia Biotech, Buckinghamshire, U.K.) and then cross-linked to the blots by UV illumination at $1200 \times 100 \mu\text{J}/\text{cm}^2$ using a Funa-UV-Crosslinker (Funakoshi, Tokyo). The membranes were prehybridized for at least 1 h in Ultra-Hyb (Ambion, Austin, TX U.S.A.) at 68°C . The membranes were hybridized at 68°C for 16 h with DIG-labeled RNA probes (Roche Molecular Biochemicals, Indianapolis, IN U.S.A.) of *hin1*-SNN (Kiba et al. in press) and *hsr203J*-BY (Kiba et al. in press) according to the manufacturer's manual. After washing in $2 \times$ SSPE with 0.1% SDS at 68°C and $0.1 \times$ SSPE with 0.1% SDS at 68°C , the membranes were immersed in a blocking solution (Roche Molecular Biochemicals) for 60 min and then incubated with anti-digoxigenin-AP Fab fragments (10^4 dilution). Ig-bound RNA was visualized with CDP-Star according to the manufacturer's protocols (Roche Molecular Biochemicals). Chemifluorescence was detected by exposing the membrane to X-ray film for 5 to 30 min.

ACKNOWLEDGMENTS

We would like to thank Dr. C. Allen for critical reading of the manuscript. This work was supported by Grants-in Aid for Scientific Research on priority area (A) from the Ministry of Education, Science, Sports and Culture of Japan.

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