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BACTERIAL AND PHYTOPLASMA DISEASES

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Type III secretion machinery-deficient mutants of *Ralstonia solanacearum* lose their ability to colonize resulting in loss of pathogenicity

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Abstract *Ralstonia solanacearum* OE1-1 (OE1-1) induced necrotic lesions in an infiltrated area of tobacco leaves 72 h after infiltration, and the leaves had wilted at 5 days. Here we report phenotypes of the OE1-1 mutant deleted *hrpB* or *hrpY* with respect to colonization and proliferation in infiltrated tobacco leaves and the induction of host responses immediately after invasion. An *hrpB*-deleted mutant and an *hrpY*-deleted mutant grew similar to the parent strain, OE1-1, in vitro. When infiltrated into tobacco leaves, the mutants lost their ability to induce necrotic lesions and provoke the disease. Populations of the mutants in the infiltrated area were retained equally after infiltration, and the mutants were not detected in any other region. Transcripts of *hsr*203J and *hin*1, which are marker genes of plant–microbe interactions and were detected 8 h after infiltration of OE1-1 in the infiltrated area of tobacco leaves, were not detected in the mutant-infiltrated tobacco leaves. These results suggest that the *hrp* mutants, which are deficient in type III secretion machinery, lose their ability to colonize and multiply in host plants immediately after invasion, resulting in a loss of their ability to induce host responses and the subsequent provocation of disease.

Key words *Ralstonia solanacearum* · Type III secretion machinery-deficient mutants · Parasitic fitness

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The nucleotide sequence data reported are available in the DDBJ/EMBL/GenBank databases under the accession number AB094669

Introduction

Ralstonia solanacearum is a soil-born phytopathogenic bacterium and vascular pathogen responsible for the bacterial wilt disease that causes severe loss of many important crops in tropical, subtropical, and warm temperate regions of the world (Hayward 1991). The bacteria generally invade plant vascular tissues from wounded roots or natural openings from which secondary roots subsequently emerge (Hayward 1991; Kelman and Sequeira 1965). Bacterial colonization in the stems results in browning of the xylem, foliar epinasty, and a lethal, generalized wilt.

In several gram-negative phytopathogenic bacteria including *R. solanacearum*, the *hrp* genes have been identified as essential determinants for disease development on compatible hosts and elicitation of the hypersensitive response (HR) on resistant plants (Alfano and Collmer 1997; Genin et al. 1992; Van Gijsegem et al. 1995). The *hrp* genes encode the type III secretion pathway, and nine of the *hrp* genes have been renamed *hrc* (HR and conserved) to indicate that they encode conserved components that are also present in the type III secretion machinery of the animal pathogens *Yersinia*, *Shigella*, and *Salmonella* (Bogdanove et al. 1996).

Ralstonia solanacearum GMI1000 (GMI1000) is nonpathogenic to tobacco and elicits the HR in infiltrated tobacco leaves (Boucher et al. 1985). In GMI1000, the *hrp* gene clusters comprise more than 20 genes organized in at least seven transcriptional units. Transcription units 1, 2, 3, and 4 are induced in minimal media or in co-culture with plant cells through HrpB protein (Aldon et al. 2000; Arlat et al. 1992; Boucher et al. 1985, 1987; Brito et al. 1999; Genin et al. 1992; Marenda et al. 1998). The *hrp* genes are also involved in the biogenesis of *hrp* pili, which are mainly composed of HrpY protein (Van Gijsegem et al. 1995, 2000). It is thought that *hrp* pili comprise the type III secretion machinery through which the type III effectors would be translocated into the plant cells. Therefore, it is thought that the *hrpB* mutant and the *hrpY* mutant of GMI1000 lose their ability to construct the type III secretion machinery, resulting in loss of induction of the HR in tobacco leaves

and provocation of disease in susceptible plants (Van gijsegem et al. 2000; Vasse et al. 2000). Vasse et al. (2000) reported that *hrp* mutants of GMI1000 show reduced colonization and multiplication in the vascular system of tomato roots 18 days after inoculation. However, they did not mention the ability of the mutants to colonize and multiply or the host responses in the intercellular spaces immediately after invasion. It has remained unclear how the type III effectors affect bacterial colonization in host plants or the responses of the host plants, such as the expression of specific genes immediately after invasion, because the bacterial behavior and host response in the host plant root inoculated with the bacteria are not always observed synchronously.

In this study, we used *R. solanacearum* OE1-1 (OE1-1), which is pathogenic to tobacco and induces necrotic lesions in infiltrated areas of tobacco leaves 72 h after bacterial infiltration (Hikichi et al. 1999). We directly infiltrated *R. solanacearum* into tobacco leaves. We used this method because bacterial colonization and proliferation in the infiltrated leaves can be observed synchronously and evaluated immediately after invasion.

Using this infiltration system, we first analyzed the phenotype and population of *hrpB*-deleted and *hrpY*-deleted mutants of OE1-1 in infiltrated tobacco leaves. Second, expression of the early inducible genes in plant–microbe interactions, *hsr*203J (Pontier et al. 1994, 1998, 1999; Tronchet et al. 2001) and *hin*1 (Gopalan et al. 1996; Kiba et al. 2003; Pontier et al. 1999), were analyzed in the mutant-infiltrated tobacco leaves. These results indicate that the type III-deficient mutants of OE1-1 lost their ability to colonize in tobacco leaves and to induce the host response, resulting in loss of the ability to provoke the disease. These results also suggest that the effectors, translocated into host cells through the type III secretion machinery, are involved in the ability of the bacteria to colonize immediately after invasion.

Materials and methods

Bacterial strains, plasmids, and culture conditions

The bacterial strains and plasmids used in this study are listed Table 1. *R. solanacearum* isolates were grown in PS medium (Wakimoto et al. 1968) in a 200-rpm shaker at 30°C. Antibiotics were added at standard concentrations as required. Boucher's minimal medium (Boucher et al. 1985) supplemented with glutamate, 20 mM at final concentration, and PS medium were used to measure bacterial growth. Cultures at 150 ml in 500-ml flasks, with their initial optical density at 600 nm (OD_{600}) fixed at 0.001 with the spectrophotometer (Ultrospec1000; Amersham Bioscience, Piscataway NJ, USA), were incubated in a 200-rpm shaker at 30°C; bacterial growth was measured spectrophotometrically as culture OD_{600} (Hikichi et al. 1999).

Chemicals and enzymes

Growth medium components were purchased from Nakarai (Kyoto, Japan), and the electrophoresis chemicals and nylon transfer membranes were from Bio-Rad (Richmond, VA, USA). DNA restriction and modification enzymes were from Takara (Kyoto, Japan). All other chemicals were from Sigma (St. Louis, MO, USA). Oligonucleotides were purchased from Hokkaido System Science (Sapporo, Japan).

DNA manipulations

Isolation of plasmid and chromosomal DNA, cloning, subcloning, Southern hybridization, and the polymerase chain reaction (PCR) were performed in accordance with

Table 1. Bacterial strains and plasmids

Designation	Relevant characteristics	References or sources
Escherichia coli		
DH5α	*F-endAI relA ϕ80 lacZΔM15 hsdR17 supE44 thi-I recAI gyrA96*	Takara
BL2(DE3)	*hsdS gal* (*λcI ts857 ind1 Sam7* UV5-T7 gene1)	Novagen, Inc
Ralstonia solanacearum		
OE1-1	Wild-type, pathogenic to tobacco, eggplant isolate	Hikichi
ΔhrpB	OE1-1 *hrpB*::Km^R	This study
ΔhrpY	OE1-1 *hrpY*::Km^R	This study
Plasmid		
pUC118	Ap^R	Takara
pBluescript	Ap^R	Stratagene
pET15b	Ap^R	Novagen, Inc
pUCD3101pro	Km^R	Hikichi
pUCK191	Containing kanamycin resistance gene, Km^R	From Dr. Tsuge
pB1	0.9-kbp *Eco*RI and *Kpn*I fragment containing *hrpC* in pUC118, Ap^R	This study
pB1-2	1.8-kbp *Bam*HI and *Kpn*I fragment containing *hrpA* in pB1. Ap^R	This study
phrpBKm	1.4-kbp *Kpn*I fragment containing kanamycin resistance gene in pB1-2 Ap^R, Km^R	This study
pY1	1.3-kbp *Xba*I and *Bam*HI fragment containing *hrpW* and *hrpX* in pUC118, Ap^R	This study
pY1-2	1.5-kbp *Bam*HI and *Kpn*I containing *hrp*G and *prhJ* in pY1. Ap^R	This study
phrpYKm	1.4-kbp *Bam*HI fragment containing kanamycin resistance gene in pY1-2, Ap^R, Km^R	This study
pEThrpY	0.26-kbp *Nde*I and *Bam*HI containing *hrpY* in pET15b, Ap^R	This study

standard procedutes (Ausbel et al. 1994; Sambrook et al. 1989). The nucleotide sequence of *hrpY* from *R. solanacearum* OE1-1 was determined with a DNA sequencer (model 373; Perkin-Elmer, Foster City, CA, USA) according to the manufacturer's instructions. *R. solanacearum* strains were transformed by electroporation with a Gene-pulser (Bio-Rad) at a capacitance of 25 microfarads and an electric field of 12.5 kV/cm. Mid-log phase cells were made competent for electroporation by washing four times in 10% glycerol. Competent cells were stored at −80°C. Marker exchange mutagenesis of *R. solanacearum* OE1-1 with the plasmids was conducted by electroporation.

Deletion of *hrpB* gene

A 4.3-kbp DNA fragment containing *hrpC* was PCR-amplified with the primers RI-B1 (5′-GGAATTC ACCACGACACACTGAGTTGC-3′) with an added *Eco*RI site (underlined) and Kpn-B1 (5′-GGGGTACCTC AACAAAGCTBCGCCTTG-3′) with an added *Kpn*I site (underlined), designed based on the DNA sequence of GMI1000, from the genome DNA of OE1-1. The *Eco*RI- and *Kpn*I-digested 900-bp fragment was ligated into pUC118, and pB1 was created. A 1.8-kbp DNA fragment containing *hrpA* was PCR-amplified with the primers Kpn-B2 (5′-GGGGTACCATGCATCTGACTCGACGA-3′) with an added *Kpn*I site (underlined) and Bam-B2 (5′-CGGGATCCGAAAAAGCCTCCGTGCATGC-3′) with an added *Bam*HI site (underlined), designed based on the DNA sequence of GMI1000. The *Kpn*I and *Bam*HI-digested 1.8-kbp fragment was ligated into pB1, and pB1-2 was created. A 1.4-kbp *Kpn*I-digested fragemnt containing the kanamycin resistance gene from pUCK191 (kindly donated by Dr. Tsuge) was ligated into the *Kpn*I site of pB1-2, and phrpBKm was created. The resulting plasmid, phrpBKm, was exchanged into the wild-type strain OE1-1 chromosome by homologous recombination, and kanamycin-resistant transformants were screened. Deletion of the *hrpB* gene in chromosomes of selected colonies was certified by Southern hybridization using the 1.4-kbp fragment containing the kanamycin resistance gene.

Deletion of *hrpY* gene

A 1.3-kbp fragment containing *hrpW* and *hrpX* was PCR-amplified with the primers XbaY-3 (5′-GCTCTAGAGT CCGTCCCATCAACGT CTC-3′) with an added *Xba*I site (underlined) and BamY-4 (CGGGATCCGATTCGGC TCAGCGAACGTG-3′) with an added *Bam*HI site (underlined), designed based on the DNA sequence of GMI1000 from the genome DNA of OE1-1. The *Eco*RI- and *Kpn*I-digested 1.3-kbp fragment was ligated into pUC118, and pY1 was created. A 1.5-kbp DNA fragemnt containing *hrpG* and *prhJ* was PCR-amplified with the primers Kpn-Y2 (5′-GGGGTACCAGAGTAAGAA GGGCGCGCAG-3′) with an added *Kpn*I site (underlined) and Bam-Y1 (5′-CGGGATCCGTTCCACAACGGCG TGCATC-3′) with an added *Bam*HI site (underlined), designed based on the DNA sequence of GMI1000. The *Kpn*I- and *Bam*HI-digested 1.5-kbp fragment was ligated into pY1, and pY1-2 was created. The 1.4-kbp *Kpn*I-digested fragment containing the kanamycin resistance-gene from pUCK191 was ligated into the *Kpn*I site of pY1-2, and phrpYKm was created. The resulting plasmid, phrpYKm, was exchanged into the wild-type strain OE1-1 chromosome by homologous recombination, and kanamycin-resistant transformants were screened. Deletion of the *hrpY* gene in chromosomes of selected colonies was certified by Southern hybridization using the 1.4-kbp fragment containing the kanamycin resistance gene as the probe.

Overexpression and isolation of HrpY protein

A 260-bp DNA fragment containing *hrpY* ORF was PCR-amplified with the primers, NdeI-hrpY (5′-GGAATTC CATATGGCAGGCGTTCCGAAAC-3′) with an added *Nde*I site (underlined) and Bam-hrpY (5′-CGG GATCCTTAGCTGATCAGGTCCTTGGC-3′) with an added *Bam*HI site (underlined), designed based on the DNA sequence of GMI1000 from the genome DNA of OE1-1. The *Nde*I and *Bam*HI-digested 249-bp fragment was ligated into T7 expression vector pET15b (Novagen, Madison, WI, USA) to create pEThrpY. The construct was transformed into *Escherichia coli* overexpression strain BL21 (DE3), and protein expression was induced with 2 mM isopropyl-β-D-thiogalactopyranoside (IPTG). The recombinant HrpY protein containing His-tag was purified from sonicated bacterial cells using MagExtractor –His-tag– (Toyobo, Osaka, Japan) and then treated with thrombin (Novagen). These procedures were done according to the manufacturer's instructions. Proteins were separated by sodium dodecyl sulfate-polyacrylamide electrophoresis (SDS-PAGE) on 15% gels and stained with coomassie brilliant blue (Ausbel et al. 1994; Sambrook et al. 1989). Purified recombinant HrpY protein (0.6 μg) at a concentration of 15 μg/ml and mixtures with the bacteria were infiltrated into a tobacco leaf.

Detection of mRNA of *hsr*203J and *hin*1

Total RNA was isolated from five pieces of 1 cm^2 leaf punches in the bacterial infiltrated area of tobacco leaves in accordance with standard procedures (Ausbel et al. 1994; Sambrook et al. 1989). Total RNA (10 μg) was separated by 1.5% agarose gel under the denatured condition and blotted onto Hybond N+ membrane. The blot was hybridized with DIG-labeled RNA probe of *hsr*203J and *hin*1, which are isolated from genomic DNA of *Nicotiana tabacum* cv. SamsunNN and cloned into pBluescript II SK+ (Kiba et al. 2003). Hybridization was performed at 68°C for 16 h in ULTRAhyb (Ambion, Austin, TX, USA). The blots was washed in 2 × SSPE with 0.1% SDS at 68°C and 0.1 × SSPE with 0.1% SDS at 68°C. Hybridization was performed using the DIG RNA labeling and detection kit (Roche,

Mannheim, German) according to the manufacturer's instructions.

Pathogenicity assays

Tobacco (*N. tabacum* cv. Bright Yellow) plants were grown in pots containing a mixture of vermiculite and peat moss (3:1) in a growth room at 25°C under 10,000 lux for 16 h/day by watering with five times-diluted Hoagland's solution (Hikichi et al. 1999). Fully expanded leaves of 8-week-old tobacco plants were infiltrated with 40-μl of the bacterial solutions at 10^8 cfu/ml using a 1-ml syringe (Termo, Tokyo, Japan). Leaves were rated on a 0–2 disease index scale: 0, no wilting; 1, wilting in infiltrated leaves; 2, wilting in whole plants. Each assay was repeated in five successive trials.

Eight-week-old tobacco plants inoculated with the bacteria were grown in the water culture pots (Yamato Water Culture Pot No. 1; Yamato Plastic, Tokyo, Japan) with the five times-diluted Hoagland's solution in a growth room at 25°C under 20,000 lux for 16 h/day. Tobacco roots were dipped in the bacterial solutions at 10^8 cfu/ml, described elsewhere (Kanda et al. 2003). Plants were rated on a 0–4 disease index scale: 0, no wilting; 1, 1%–25% wilting; 2, 26%–50% wilting; 3, 51%–75% wilting; and 4, 76%–100% wilted or dead. Each assay was repeated in five successive trials.

To measure the growth of *R. solanacearum* strains, five pieces of 1 cm^2 leaf punches in the bacterial infiltrated area of tobacco leaves were ground, diluted, and plated on Hara-Ono plates (Hara and Ono 1983) to quantify the bacteria; infiltration and planting were done five times. We treated 20 plants with each strain for each trial, yielding 100 plants for each strain.

Results

Phenotype of the *hrpB*-deleted mutant and the *hrpY*-deleted mutant

The *hrpB*-deleted mutant (ΔhrpB) and *hrpY*-deleted mutant (ΔhrpY) retained their ability to grow in the minimum medium and the rich medium (PS medium), similar to the parent, OE1-1 (Fig. 1). In tobacco leaves infiltrated with OE1-1, necrotic lesions appeared in the infiltrated area 72 h after infiltration (Fig. 2), the leaves wilted on day 5 after infiltration, and the tobacco plants wilted heavily 10 days after infiltration (Fig. 3A). On the other hand, no symptoms appeared in the tobacco leaves infiltrated with ΔhrpB or ΔhrpY (Fig. 2), and the leaves and the plants had not wilted at all 14 days after infiltration (Fig. 3A).

When inoculated with OE1-1 by root-dipping, tobacco plants wilted heavily 7 days after inoculation (Fig. 3B). In contrast, when inoculated into tobacco plants by root dipping, neither ΔhrpB nor ΔhrpY grew in the roots or moved to any other sites of the plants (data not shown); moreover, the plants had not wilted at all 14 days after inoculation (Fig. 3B).

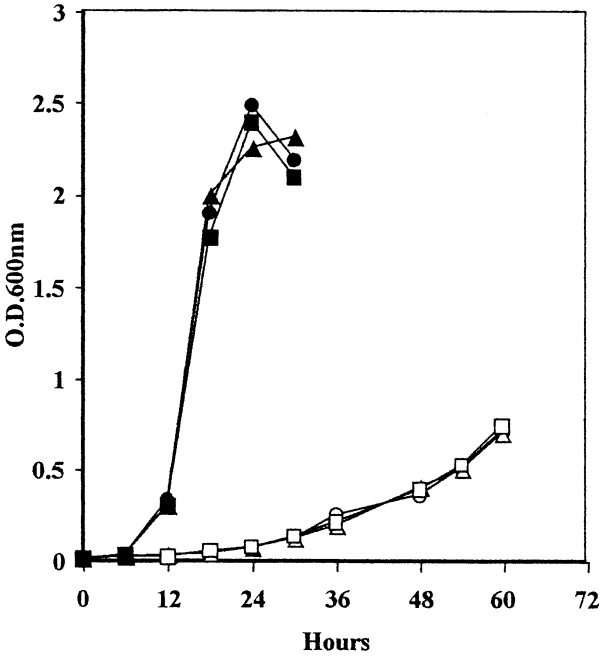

Fig. 1. Growth of *Ralstonia solanacearum* wild-type and *hrp* mutants on different medium. Wild-type OE1-1 (*closed circles*), *hrpB*-deleted mutant (*closed triangles*), and *hrpY*-deleted mutant (*closed squares*) grown in PS medium. OE1-1 (*open circles*), *hrpB*-deleted mutant (*open triangles*), and *hrpY*-deleted mutant (*open squares*) are grown in Boucher's minimal medium. Results shown are the means of three replicated experiments; within each experiment, every strain was replicated three times

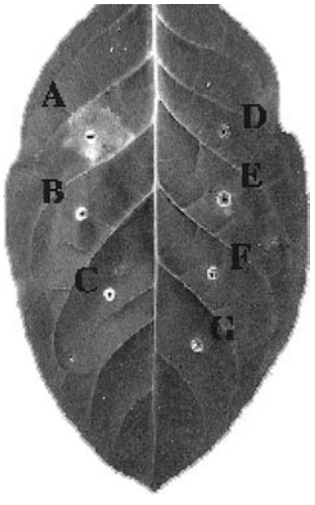

Fig. 2. Symptoms on tobacco leaves infiltrated with the recombinant HrpY protein or the mixtures of the recombinant HrpY protein with the *hrp* mutants 72 hr after infiltration. The 40-μl solution of *R. solanacearum* OE1-1 (*A*), *hrpB*-deleted mutant (*B*), and *hrpY*-deleted mutant (*C*) at 10^8 cfu/ml were infiltrated into a full expanded leaf of 8-week-old tobacco plants. Recombinant HrpY protein (0.6 μg) at the concentration of 15 μg/ml (*D*), mixtures with the *hrpB*-deleted mutant (*E*) or the *hrpY*-deleted mutant (*F*), and buffer (*G*) were infiltrated into the tobacco leaf

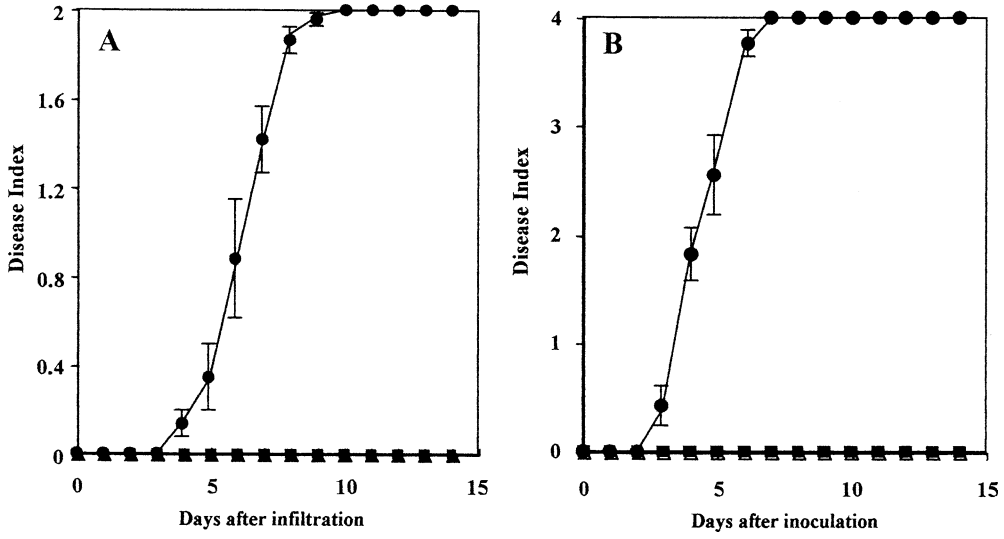

Fig. 3. Pathogenicity of *hrp* mutants of *R. solanacearum*. Full expanded leaves of 8-week-old tobacco plants were infiltrated with the 40-μl solution of *R. solanacearum* OE1-1 (*closed circles*), *hrpB*-deleted mutant (*closed triangles*), and *hrpY*-deleted mutant (*closed squares*) at 10^8 cfu/ml using a 1-ml syringe (**A**). Roots of 8-week-old tobacco plants were dipped in the solution of OE1-1 (*closed circles*), *hrpB*-deleted mutant (*closed triangles*), and *hrpY*-deleted mutant (*closed squares*) at 10^8 cfu/ml (**B**). Points shown are means of five experiments, each containing 20 plants per treatment. *Error bars*, standard deviation of the mean

Population of *hrp* mutants infiltrated into tobacco leaves

The population of OE1-1 in the infiltrated area of tobacco leaves increased by an order of three at 2 days after infiltration and then decreased drastically (Fig. 4). The bacterial population of ΔhrpB or ΔhrpY did not change much, remaining at 10^6–10^7 cfu/cm^2 for 10 days after infiltration.

Using the tissue printing immunoassay with the antibody against OE1-1 (Nakazawa-Nasu et al. 1999), the bacteria were detected 3 and 5 days after infiltration at the periphery of the OE1-1-infiltrated area and the petiole of the bacteria-infiltrated leaves, respectively. No signal was detected in the periphery of the mutant-infiltrated area or the petiole of the mutant-infiltrated leaves until 10 days after infiltration (data not shown).

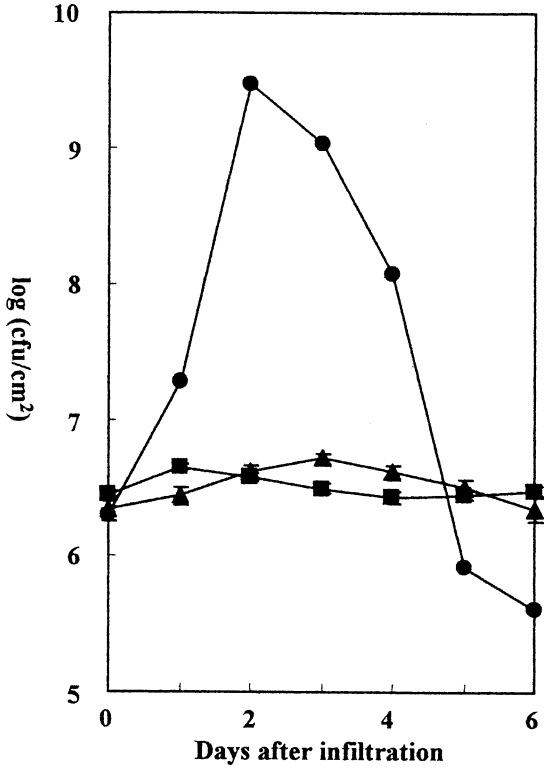

Fig. 4. Growth of *R. solanacearum* OE1-1 and the *hrp* mutants in the infiltrated area of tobacco leaves. *Closed circles*, *R. solanacearum* OE1-1; *closed triangles*, *hrpB*-deleted mutant; *closed squares*, *hrpY*-deleted mutant. Similar results were obtained in three independent trials. *Error bars*, standard deviation of the mean

Expression of *hsr*203J and *hin*1 mRNA

To investigate the responses of tobacco leaves infiltrated with *hrp* mutants, expression of *hsr*203J and *hin*1, which are marker genes for interactions between the bacteria and tobacco plants (Kiba et al. 2003), was observed by Northern hybridization analysis. In the area infiltrated with OE1-1, mRNAs of *hsr*203J and *hin*1 were detected 8h after infiltration (Fig. 5). In contrast, no signal was detected in the area infiltrated with either ΔhrpB or ΔhrpY until 24h after infiltration, similar to those infiltrated with water (data not shown).

Function of HrpY protein as an effector

To elucidate whether HrpY protein functions directly as an elicitor between the bacteria and host plants, similar to flagellin, *hrpY* was first PCR-amplified from the genomic DNA of OE1-1. The ORF of *hrpY* from OE1-1 consisted of 249 nucleotides and showed 99.5% identity with *hrpY* from GMI1000. The 23rd nucleotide of the ORF of OE1-1 was replaced from C to A, resulting in replacing the eighth residue of the deduced amino acid of HrpY protein of OE1-1 from Asn to Thr.

When the recombinant HrpY protein was infiltrated into tobacco leaves, no symptoms were seen in the leaves (Fig. 2). In tobacco leaves infiltrated with a mixture of ΔhrpB or ΔhrpY and recombinant HrpY protein, the bacterial population remained the same 0–10 days after infiltration (data

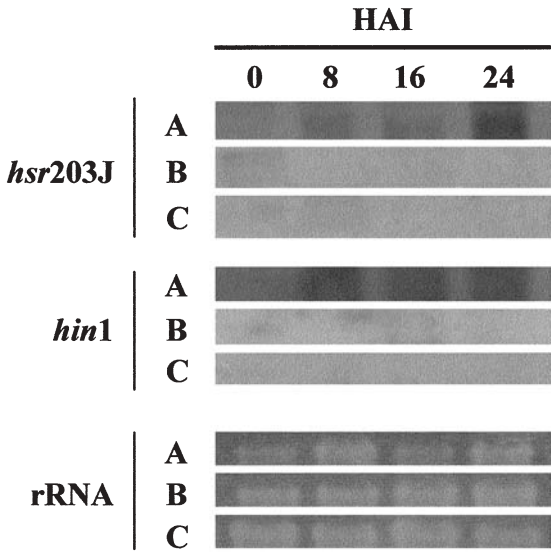

Fig. 5. Time course of *hsr*203J and *hin*1 mRNA expression in infiltrated area of tobacco leaves infiltrated with *R. solanacearum* OE1-1 (**A**), *hrpB*-deleted mutant (**B**) and *hrpY*-deleted mutant (**C**). Total RNA (10μg) was hybridized with gene sequences indicated on the left. *HAI*, hours after infiltration

not shown), and there were no symptoms, similar to leaves infiltrated with ΔhrpB or ΔhrpY (Fig. 2).

Discussion

Based on results from microscopic observations, Vasse et al. (2000) suggested that *hrp* mutants, such as *hrpB* mutant and *hrcV* mutant, of GMI1000 show reduced infection, colonization, and multiplication ability in the vascular system of tomato roots. The mutants also induce a defense reaction similar to a vascular HR at one protoxylem pole of invaded tomato plants. Vasse et al. noted that the reduced bacterial multiplication of the *hrp* mutants could be either the cause or the consequence of their apparent inability to overcome the plant response. However, Vasse et al. did not mention colonization or proliferation of the mutants in intercellular spaces immediately after invasion.

In our study using leaf infiltration, which resulted in synchronous proliferation of the bacteria and the host response, OE1-1 proliferated vigorously in the intercellular spaces of tobacco leaves and induced necrotic lesions, with the tobacco leaves wilting heavily. Retention of a population of *hrp* mutants in the intercellular spaces might result from loss of their ability to grow in the intercellular spaces and induce a host response. A type III-secreted effector protein, PopA protein, purified from the culture supernatant of GMI1000, is able to induce the HR in infiltrated tobacco leaves (Arlat et al. 1994). Pathogenicity analysis of *popA*-deficient mutants of GMI1000 (Arlat et al. 1994), OE1-1 (Kanda et al. 2003), and 8107 (Kiba et al. 2003) indicated that PopA protein is not directly involved in the pathogenicity of *R. solanacearum*. However, OE1-1 suppresses *popA* expression, allowing escape from the defense response of host plants immediately after invasion of the intercellular spaces (Kanda et al. 2003). Such evidence suggests that interactions between the bacteria and host plants immediately after invasion might be involved in colonization by the bacteria in the intercellular spaces, and OE1-1 might secrete effectors necessary for multiplication of the bacteria in the intercellular spaces via the type III secretion system. The *hrp* mutants cannot colonize the intercellular spaces, as the mutants might not secrete the effectors.

The *hsr*203J gene is specifically activated during the early steps of incompatible plant–pathogen interactions, and its activation is correlated with programmed cell death, which occurs in response to diverse pathogens (Pontier et al. 1994, 1998, 1999). The *Hsr*203J may be a signaling component or a detoxification effector (Tronchet et al. 2001). The Hin1 protein is homologous to the NDR1 protein required in *Arabidopsis* to mediate disease resistance induction by a different R gene specific for the pathogen (Gopalan et al. 1996). Kiba et al. (2003) suggested that the timing of *hsr*203J and *hin*1 expression may be a marker for identifying the compatibility or incompatibility between bacteria and tobacco plants. Expression of the genes is not induced in tobacco leaves infiltrated with type III secretion machinery-deficient mutants, suggesting that the infiltrated tobacco leaves might not be able to recognize the mutants as pathogens.

Felix et al. (1999) demonstrated that flagellin (the main component of the bacterial flagellum) of *Pseudomonas syringae* pv. *tabaci* is a general elicitor of resistance induction in some plants, and plants have a highly sensitive and selective perception system for flagellin. Che et al. (2000) demonstrated the resistance response in cultured rice cells induced by flagellin in the incompatible strain of *Pseudomonas avenae* but not in flagellin of the compatible strain; and the resistance responses in cultured rice cells are induced by the monomer-type flagellin. Hrp-dependent type III secretion machinery is believed to be similar to the secretion apparatus of flagellin in the flagella, and it may be involved in the biogenesis of pili that are mainly composed of the HrpY protein (Van Gijjjsegem et al. 1995, 2000). HrpY protein is also thought to travel through the apparatus to be added to the pili at their tips. The function of HrpY protein, except for *hrp* pili formation, has remained unclear. Results from this study using recombinant HrpY protein suggest that HrpY protein is not an effector involved in the induction of the necrotic lesion or in provocation of the disease. HrpY protein is the main component of *hrp* pili, and expression of *hrpY* depends on *hrp* regulation conducted by *hrpB*. These results suggest that ΔhrpY loses its ability to construct the entire type III secretion machinery, similar to ΔhrpB, resulting in its losing ability to induce necrotic lesions and provoke the disease.

Although the population of OE1-1 in the infiltrated tobacco leaves increased vigorously at 2 days after infiltration and then decreased drastically, the bacterial population of *hrp* mutants did not change much. In addition, the *hrp* mutants did not induce a host response (e.g., with a necrotic

legion or expression of specific genes in the bacteria–host interactions). These results suggest that the type III secretion machinery-deficient mutants lose their ability to determine compatibility between the bacteria and host, which results from loss of their colonization in tobacco plants immediately after invasion. Alfano et al. (2000) demonstrated that the pathogenicity island of *P. syringae* has a tripartite mosaic structure. The *hrp/hrc* gene cluster is conserved and is flanked by a unique exchangeable effector locus and a conserved effector locus. Deletion of these loci reduces bacterial growth and abolishes bacterial pathogenicity, suggesting that these loci contribute to parasitic fitness and pathogenicity in plants. Rantaskari et al. (2001) suggested that soft-rot bacteria *Erwinia carotovora* subsp. *carotovora* also has *hrp* clusters, and the type III secretion machinery during early phyllosphere colonization of the bacteria plays a role in enabling bacteria to multiply faster at early stages of infection. Our results in this study suggest that the type III secretion machinery-deficient mutants of OE1-1 lost their ability to colonize in the tobacco leaves and induce the subsequent signaling pathway of the tobacco plants. The mutants may lose their ability to be parasitic in tobacco leaves. Taken together, OE1-1 may produce effectors translocated through the type III secretion machinery into host plants immediately after invasion, and the effectors might be involved in the parasitic fitness of OE1-1.

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