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BACTERIAL AND PHYTOPLASMA DISEASES

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Identification of *dspEF*, *hrpW*, and *hrpN* loci and characterization of the $hrpN_{Ep}$ gene in *Erwinia pyrifoliae*

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Abstract *Erwinia pyrifoliae*, the causal pathogen of shoot blight in the Asian pear tree (*Pyrus pyrifolia* cv. Singo), is host-specific and endemic to Korea. To identify the genes associated with the hypersensitive response (HR) and pathogenicity, a genomic library of *E. pyrifoliae* WT3 was constructed, and the cosmid clone *Escherichia coli* (pCEP33) was selected. Sequence analysis of 19.7-kb pCEP33 determined disease-specific (*dsp*) region homolog and approximately 40% of the *hrp* genes, which included *hrpW*, $hrpN_{Ep}$, *hrpV*, *hrpT*, *hrcC*, *hrpG*, *hrpF*, and partial *hrpE* homologs, with respect to the cluster of *Erwinia amylovora.* Additionally, two open reading frames, ORFD and ORFE, were found downstream of the *dspEF* region. The results of the sequence analysis showed that the pCEP33 did not contain any *hrp* regulatory genes or most of the genes encoding components of the Hrp protein secretion system. The $hrpN_{Ep}$ gene of *E. pyrifoliae* contained five intergenic nucleotide fragment insertions (INFIs) and produced the HR elicitor protein $harpin_{Ep}$, with a molecular mass of approximately 44 kDa. The purified $HrpN_{Ep}$ protein elicited faster and stronger HR when infiltrated into tobacco leaves than did $HrpN_{Ea}$ from *E. amylovora*. To observe the role of the *hrpL* gene in the expression of $HrpN_{Ep}$, the pEL2 containing *hrpL* was used to transform *E. coli* (pCEP33). Expression of $HrpN_{Ep}$ in *E. coli* (pCEP33 + pEPL2) was detected with an immunoblot using antiserum raised against $HrpN_{Ep}$, indicating a role of *hrpL* gene in enhancing the expression of $HrpN_{Ep}$.

Key words *Pyrus pyrifolia* · Phytopathogens · Enterobacteriaceae · Shoot blight · $Harpin_{Ep}$

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Introduction

Most Gram-negative phytopathogenic bacteria have *h*ypersensitive *r*esponse and *p*athogenicity (*hrp*) genes that are involved in the pathogenicity in host plants and induction of a hypersensitive response (HR) in nonhost plants (Bonas 1994). The *hrp* genes are generally localized in the chromosomal DNA of various phytopathogens and are organized in large 20- to 30-kb clusters (Kim and Alfano 2002; Kim and Beer 2000). Based on similarities in *hrp* gene organization and regulation, phytopathogenic bacteria are classified into two groups: group I (*Erwinia amylovora* and *Pseudomonas syringae*) and group II (*Ralstonia solanacearum* and species of *Xanthomonas*) (Alfano and Collmer 1997). The *hrp* genes of group I are regulated by the alternative sigma factors HrpL and HrpS (Frederick et al. 1993; Wei and Beer 1995; Xiao and Hutcheson 1994). In *E. amylovora*, two components, HrpX/HrpY, are additionally required to regulate *hrpL* and *hrpS* genes. In contrast, the *hrp* genes of group II are regulated by HrpX (*Xanthomonas* species) and HrpB (*R. solanacearum*) of the members of the AraC-type family of regulators (Wengelnik and Bonas 1996). There are nine highly conserved *hrp* genes in both plant and animal bacterial pathogens known as the *hrc* (*hrp* conserved) genes (Bogdanove et al. 1996), and they encode components of the type III secretion system (TTSS). The TTSS mediates translocation of effector proteins across the bacterial membrane and into the host. These effector proteins are often important for virulence and modulation of host defense responses (Galán and Collmer 1999).

Harpin is the first protein known to be secreted by the TTSS of bacterial phytopathogens. The harpin proteins $HrpN_{Ea}$ and HrpW of *E. amylovora* (Kim and Beer 1998; Wei et al. 1992) are HR elicitors, as are HrpN of *Pantoea agglomerans* pv. *gypsophiliae* (Mor et al. 2001), *P. stewartii* subsp. *stewartii* (Ahmad et al. 2001), *Pectobacterium carotovorum* (Mukherjee et al. 1997), and *P. chrysanthemi* (Bauer et al. 1995); HrpZ and HrpW of *P. syringae* pathovars (Charkowski et al. 1997, 1998; He et al. 1993); PopA of *R. solanacearum* (Arlat et al. 1994); and HpaG of *X. axonopodis* pv. *glycines* (Kim et al. 2003). In *E. amylovora*, $HrpN_{Ea}$ is a major HR elicitor in tobacco; an $hrpN_{Ea}$ mutant was nonpathogenic in pear plants and elicited no HR on tobacco (Wei et al. 1992). HrpW has also been reported as an HR elicitor in tobacco, but the *hrpW* mutant retained the wild-type ability to elicit HR in nonhosts other than tobacco and to cause disease in hosts (Kim and Beer 1998). DspEF of *E. amylovora* and WtsEF of *P. stewartii* subsp. *stewartii* also produced HR on tobacco leaves (Frederick et al. 1993; Kim and Beer 1998). However, *hrp* genes and HR elicitors have not currently been reported from *E. pyrifoliae*, a close pathogen of *E. amylovora*.

E. pyrifoliae causes a necrotic disease in Asian pear trees (*Pyrus pyrifolia* cv. Singo) and was first observed in 1995 in the pear orchards in Chuncheon, which is located in the northern part of Korea (Kim et al. 1999; Rhim et al. 1999; Shrestha et al. 2003). The disease symptoms in pear trees were similar to those caused by *E. amylovora*, causal agent of fire blight in apple, pear, and other rosaceous plants (Van der Zwet and Keil 1979). Based on the disease symptoms and microbiological characteristics, *E. pyrifoliae* has been categorized as a necrogen along with *E. amylovora* (Enterobacteriaceae). However, despite phenotypic features similar to those of *E. amylovora*, the low homology of total DNA-DNA relatedness, low sequence identity in 16S–23S rRNA intergenic transcribed spacer (ITS) region, and different plasmid profiles imply that *E. pyrifoliae* is genetically different from *E. amylovora* (Kim et al. 1999, 2001b; Rhim et al. 1999; Shrestha et al. 2003).

Recently, a Japanese *Erwinia* sp. that causes bacterial shoot blight of pear was reported to be more closely related to *E. pyrifoliae* than to the typical *E. amylovora* (Beer et al. 1996; Jock et al. 2003a, 2003b; Kim et al. 2001a; McGhee et al. 2002). In addition, a recent comparative study using amplified fragment length polymorphism (AFLP) and *groEL* sequence analysis showed that *Erwinia* pathogen of pear in Japan is closely related to *E. pyrifoliae* and is distinct from *E. amylovora* (Maxson-Stein et al. 2003; Mizuno et al. 2000). However, the plasmid numbers, restriction sites in the plasmids, and other molecular features of Japanese pear pathogen were distinct from those of *E. pyrifoliae* (Kim et al. 2001a; Maxson-Stein et al. 2003; Mizuno et al. 2000).

In Korea, major pear orchards are located in southern areas and only a few in northern areas. The shoot blight disease caused by *E. pyrifoliae* has appeared only in the north including Chuncheon but in no other area of Korea or in any other country (Shrestha et al. 2003). Additionally, *E. pyrifoliae* has been shown to have a narrow host range, causing disease only in Asian and some European pear trees but not in apple or in any other plants (Kim et al. 2001b). Thus, it is essential to identify the genes associated with the pathogenicity of this host-specific pathogen, *E. pyrifoliae*. In this study, *dspEF*, *hrpW*, and *hrpN* loci were characterized along with partial genes of *hrpC* locus using the representative strain WT3, which was collected during the first outbreak in the region Jichonri, Chuncheon, Korea in 1995 (Shrestha et al. 2003).

Materials and methods

Bacterial strains, plasmids, and culture media

The bacterial strains and plasmids used in this study are listed in Table 1. The strains of *E. pyrifoliae* and *E. amylovora* were routinely grown on mannitol glutamic yeast (MGY) agar plates (Keane et al. 1970) and maintained in nutrient broth (NA) containing 20% glycerol at −70°C (Shrestha et al. 2003). The *Escherichia coli* strains were grown at 37°C on Luria-Bertani (LB) agar plates or in LB broth. All media were amended with appropriate antibiotics (ampicillin 50 μg/ml, chloramphenicol 20 μg/ml, tetracycline 50 μg/ml) for the culture of recombinant clones.

Construction of genomic libraries

To construct the genomic library, total genomic DNA was isolated from pathogenic *E. pyrifoliae* strain WT3 (KCCM 10283) by the cesium chloride gradient method as described by Sambrook and Russell (2001). The DNA was partially digested with restriction enzyme *Sau*3AI, treated with calf intestine alkaline phosphatase, and 20- to 40-kb fragments fractionated using a 5%–30% linear sucrose gradient method, as described by Staskawicz et al. (1987). The cosmid vector pLAFR3 was isolated from *E. coli* HB101 using the cesium chloride gradient method, digested with *Bam*HI, and treated with calf intestine alkaline phosphatase. Constructed vector arms and inserts were ligated and subsequently packaged in vitro with a DNA packaging kit (Boehringer Mannheim, Detroit, MI, USA). *E. coli* HB101 cells were infected with the packaging mix, and transducts were selected on LB agar plates supplemented with tetracycline 50 μg/ml. Two thousands clones were picked with a sterile toothpick and transferred onto new LB agar plates containing tetracycline. Two replicates for each plate were made and stored at 4°C.

Screening of cosmid pCEP33

To select the cosmid clone that caused HR on nonhost tobacco, 2000 colonies from the genomic library were screened with an HR assay. Each clone for total protein extraction was added to 50 ml of LB broth supplemented with tetracycline 50 μg/ml and cultured at 37°C for overnight. The cells (2×10^8 CFU/ml) were centrifuged at 3000 *g*

Table 1. List of bacterial strains used in the present study

Strain/plasmid	Description	Source/reference
Erwinia pyrifoliae		
WT3 (KCCM 10283)	Isolated from *Pyrus pyrifolia* cv. Singo, Jichonri, Chuncheon, Korea, 1995	Shrestha et al. (2003)
Ep1(DSM 12162)	Isolated from *P. pyrifolia*, Korea, 1996	Kim W-S et al. (1999)
Ep4 (DSM 12394)	*P. pyrifolia*, Korea, 1997	Kim W-S et al. (1999)
Ep8 (DSM 12393)	*P. pyrifolia*, Korea, 1995	Kim W-S et al. (1999)
Ep16^T (DSM 12163)	*P. pyrifolia*, Korea, 1996	Kim W-S et al. (1999)
E. amylovora		
ATCC 15580^T	Type strain isolated from *P. communis*, UK	ATCC
LMG 1877	Isolated from *Cydonia oblonga*, Denmark	LMG
LMG 1946	Isolated from *P. communis* cv. Durondeau, Belgium	LMG
LMG 2068	Isolated from *Rubus idaeus*, USA	LMG
Escherichia coli		
DH5α	F$^-$, ϕ80d*lacZ*ΔM15, Δ (l*acZYA-argF*),U169, *deoR*, *recA*1 *endA*1, *hsdR*17(rk$^-$mk$^+$), *phoA*, *supE*44	Takara
HB101	*supE*44, Δ(mcrC-mrr), *recA*13, *ara*-14 *proA*2, *lacY*1, *galK*2, *rspL*20, *xyl*-5, *mtl*-1, *leuB*6, *thi*-1	Takara
BL21(DE3) pLysS	F$^-$, *omp*T, *hsd*S$_B$ ($r_B^-r_B^-$), *dcm*, *gal*, (DE3), pLysS, Cm^r	Promega
Vectors/clones		
pET-15b	Ap^r, Cm^r, T7 *lac* promoter	Novagen
pGEM-T easy	Ap^r	Promega
pHCE IA	Ap^r, HCE promoter	Takara
pLAFR3	Tc^r, Mob	Staskawicz et al. (1987)
pUC19	Ap^r	Gibco/BRL
pCEP33[a]	A cosmid of 19.7 kb, derived from genomic DNA of *E. pyrifoliae* WT3, cloned into pLAFR3	This work
pEPN2	*hrpN*$_{Ep}$ gene from cosmid pCEP33, cloned into *Nde*I-*Bam*HI restriction sites of pET-15b vector, transformed into BL21 (DE3) pLysS	This work
pEAN2	*hrpN*$_{Ea}$ gene of *E. amylovora*, cloned into *Nde*I-*Bam*HI restriction sites of pET-15b vector, transformed into BL21 (DE3) pLysS	This work
pEPL2	*hrpL* gene of WT3, cloned into *Nde*I-*Bam*HI restriction sites of pHCE IA vector, transformed into DH5α	This work

Superscript T, type strain; ATCC, American Type Culture Collection; DSM, Deutsche Sammlung von Mikroorganism; KCCM, Korean Culture Center of Microorganisms, LMG, Laboratorium voor Microbiologie; Ap, ampicillin; Cm, chloramphenicol; Tc, tetracycline
[a]The cosmid used for characterization of *hrp* genes by full-sequence analysis

for 10 min and sonicated in 2-[*N*-morpholino]ethanesulfonic acid (MES) buffer pH 5.5 containing 1 mM phenylmethylsulfonyl fluoride (PMSF) as described by Wei et al. (1992). The sonicated cells were centrifuged at 9000 *g* for 10 min at 4°C. Crude extracts were infiltrated into tobacco leaves (*Nicotiana tabacum* cv. Burley), which were grown in artificial soil in a greenhouse. After infiltration the plants were grown at 25° ± 3°C for 1–2 days, after which necrotic lesions were observed. One cosmid, pCEP33, elicited HR on tobacco when used to transform *E. coli* HB101. Thus, cosmid pCEP33 was selected for full sequence analysis.

Recombinant DNA techniques

Standard techniques for DNA manipulation, such as plasmid DNA preparation, ligation, competent cell preparation, and transformation were followed as described by Sambrook and Russell (2001). Preparation of the plasmid DNA (Wizard Minipreps; Promega, Madison, WI, USA) and recovery of DNA fragments from agarose gel (Geneclean II Kit; Bio101, Rutherford, CA, USA) were performed as described in the manufacturers' manuals. The restriction enzymes, dNTPs, *Taq* polymerase, T4 DNA ligase, and DNA marker used in this study were supplied by Promega and Takara (Ohtsu, Japan).

Sequencing of cosmid pCEP33 and alignments

To determine the genes associated with HR in cosmid pCEP33, full nucleotide sequences were analyzed. Cosmid pCEP33 was digested with restriction enzyme *Hin*dIII at 37°C for 1–4 h. Six of the fragments derived after digestion were subcloned into plasmid vector pUC19 and used to transform *E. coli* DH5α. The subcloned DNA was sequenced by labeled primer sequencing using universal M13 forward and reverse primers and subsequently sequenced by primer walking. The sequencing was done at Macrogen in Korea using an automated ABI 3730 xl DNA Analyzer (Applied Biosystems, Foster City, CA, USA) and repeated for each subclone to minimize sequence errors. The homology search was performed using BLAST version 2.0, at the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/BLAST). Similarly, the software package, DNASTAR (Windows 3.88, Madison, WI, USA) was used for alignments of the nucleotides and amino acids in this study. GenBank data were used for alignment of each *hrp* gene.

PCR amplification of *hrpN*$_{Ep}$ gene

To analyze whether intergenic nucleotide fragment insertions (INFIs) are present only in strains of *E. pyrifoliae* or also exist in *E. amylovora* strains, primers hrpNEPF1 (5′-GTGGTTTAACGGGGCTGCTG-3′) and hrpNEPR1 (5′-AGCTTAAGCCGCGCCCAG-3′) were designed from the *hrpN*$_{Ep}$ gene. The chromosomal DNA (50 ng) of five strains of *E. pyrifoliae* (WT3, Ep1, Ep4, Ep8, Ep16) and four strains of *E. amylovora* (ATCC 15580, LMG 1877, LMG 1946, LMG 2068) were amplified using these primers (Table 1). The polymerase chain reactions (PCRs) were performed in a final volume of 25 µl containing 75 mM Tris-HCl (pH 9.0), 2 mM $MgCl_2$, 50 mM KCl, 20 mM $(NH_4)_2SO_4$, a 200 µM concentration of each deoxynucleotide triphosphate (dNTPs), 20 pmol of each primer, 1 µl dimethylsulfoxide (DMSO), and 5 units of *Taq* polymerase (Biotools; B&M Labs, S.A., Madrid, Spain). Initial denaturation was carried out at 94°C for 1 min followed by 30 cycles consisting of denaturation at 94°C for 15 s, annealing at 64°C for 15 s, extension at 72°C for 1 min, and an additional final extension at 72°C for 7 min. The PCR products were analyzed by agarose gel electrophoresis using 1× TAE (40 mM Tris-acetate, 1 mM EDTA) buffer and visualized on 0.7% of agarose gel after staining with ethidium bromide and photographed with a direct screen instant camera, Polaroide DS-34 (Polaroid, Cambridge, MA, USA).

Expression of *hrpN*$_{Ep}$ and *hrpN*$_{Ea}$ genes in *E. coli*

The HR elicitor gene, which was identified in cosmid pCEP33, was designated as *hrpN*$_{Ep}$ to distinguish it from *hrpN*$_{Ea}$ of *E. amylovora*. The *hrpN*$_{Ep}$ and *hrpN*$_{Ea}$ genes were amplified from 50 ng of cosmid DNA and chromosomal DNA of *E. coli* (pCEP33) and *E. amylovora*, respectively, by PCR using primers hrpNF (*Nde*I) 5′-GGAA TTCCATATGAGTCTGAATACAAGT-3′and hrpNR (*Bam*HI) 5′-CGGGATCCCATGCGACAGCTTAAG-3′. The PCRs were performed in a final volume of 25 µl as noted for amplification of the *hrpN*$_{Ep}$ gene. Initial denaturation was carried out at 94°C for 1 min followed by 35 cycles consisting of denaturation at 94°C for 15 s, annealing at 58°C for 30 s, extension at 72°C for 1 min, and an additional final extension at 72°C for 7 min. The 1287 bp of *hrpN*$_{Ep}$ and the 1212 bp of *hrpN*$_{Ea}$ genes amplified by PCR were then cloned into the expression vector pET-15b to transform *E. coli* BL21 (DE3) pLysS. The plasmid containing the *hrpN*$_{Ep}$ gene was designated pEPN2 and the plasmid pEAN2 for the *hrpN*$_{Ea}$ gene (Table 1). Each clone with pEPN2 and pEAN2 was added to 50 ml LB broth supplemented with ampicillin 50 µg/ml and cultured at 37°C; then 0.5 mM of isopropyl-D-thiogalactopyranoside (IPTG) (Sigma-Aldrich, St. Louis, MO, USA) was added when the cell reached 0.4 OD_{600}. The cells were centrifuged at 3000 *g* for 10 min and sonicated in MES buffer (pH 5.5) containing 1 mM PMSF, as described before for screening cosmid clones by HR. The sonicated cells were boiled for 10 min and centrifuged at 9000 *g* for 10 min at 4°C.

Purification of N-terminal His-tagged $HrpN_{Ep}$ and $HrpN_{Ea}$ proteins

To purify N-terminal His-tagged $HrpN_{Ep}$ and $HrpN_{Ea}$, *E. coli* strains with pEPN2 and pEAN2, respectively, were grown in LB broth and overexpressed following IPTG induction. The buffers were prepared as described in the handbook for high-level expression and purification of 6× His-tagged proteins (Qiagen Strasse, Hilden, Germany) and adjusted to pH 8.0 using NaOH. The cells were harvested by centrifugation and resuspended in 0.5 ml lysis buffer pH 8.0 (50 mM NaH_2PO_4, 300 mM NaCl, and 10 mM imidazole), sonicated, and again centrifuged. The supernatants were loaded into Ni-NTA agarose (Qiagen Strasse) and washed with washing buffer pH 8.0 (50 mM NaH_2PO_4, 300 mM NaCl, and 20 mM imidazole). His-tagged proteins were then eluted using elution buffer pH 8.0 (50 mM NaH_2PO_4, 300 mM NaCl, and 250 mM imidazole) as described in the handbook of the manufacturer. The eluted proteins were dialyzed with distilled water to remove the imidazole. The purified proteins, $HrpN_{Ep}$ and $HrpN_{Ea}$, were separated on a 12% resolving polyacrylamide gel with a 5% stacking gel. The molecular weights of both the $HrpN_{Ep}$ and $HrpN_{Ea}$ were determined by the image analyzer program for Windows, UVDocMw version 9.03 (UVItec, Cambridge, UK), using sodium dodecyl sulfate-polyacrylamide gel electrophoreses (SDS-PAGE) molecular weight standards, and broad-range markers (Bio-Rad Laboratories, Hercules, CA, USA).

Comparative HR assay by $HrpN_{Ep}$ and $HrpN_{Ea}$

For comparative HR assays, purified $HrpN_{Ep}$ and $HrpN_{Ea}$ were quantified with a densitometer (Bio-Rad Laboratories) and diluted in MES buffer to 5, 10, 20, and 30 µg/ml for infiltration into tobacco leaves. After infiltration the tobacco plants were grown in the greenhouse, and the HR was observed at 6-h intervals for 48 h. Three replicates were conducted, and the experiment was repeated four times.

Transformation of the *hrpL* homolog in *E. coli* (pCEP33)

To observe the role of the *hrpL* gene in HR induction in *E. coli* (pCEP33), chromosomal DNA (50 ng) of *E. pyrifoliae* WT3 was amplified using primers hrpLF (*Nde*I) 5′-GGAATTCCATATGACAGAAATTCACCTGCAGTC-3′ and hrpLR (*Bam*HI) 5′-CGGGATCCGCGCATTA TCCGTCTATCCAAC-3′. Initial denaturation was carried out at 95°C for 3 min followed by 28 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 30 s, extension at 72°C for 15 s, and an additional final extension at 72°C for 7 min.

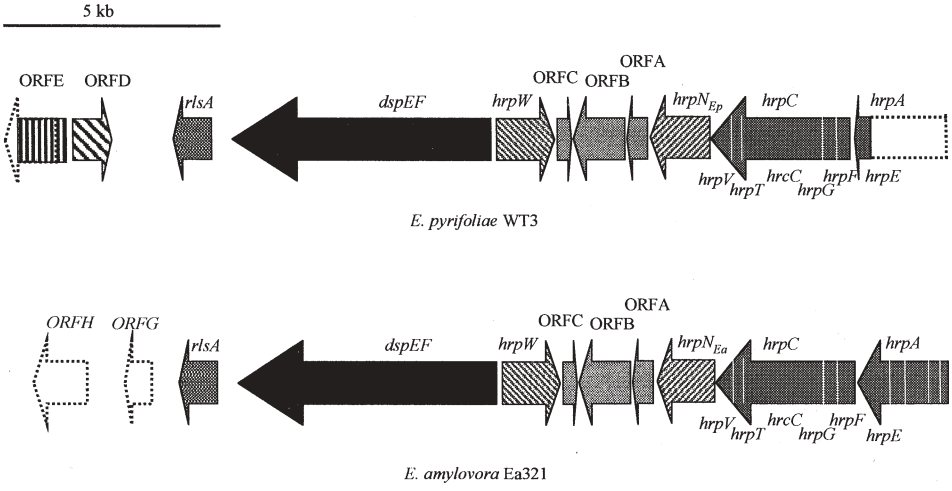

Fig. 1. Comparison of *hrp* genes and their organization in *Erwinia pyrifoliae and E. amylovora*. *Arrows* indicate the orientation of transcription for each gene. *ORFD* and *ORFE* are designated for two newly predicted open reading frames (ORFs) in *E. pyrifoliae*, which are located downstream of the *dspEF* and *rlsA* genes. The *open* and *dotted lines* indicate the predicted structure for the *hrpA* operon, including the partial *hrpE* gene

The amplified *hrpL* gene was cloned into pHCE IA expression vector and transformed into *E. coli* DH5α. The resulting clone, pEPL2, was then transformed into *E. coli* HB101 (pCEP33) by preparing competent cells using the calcium chloride method as described by Sambrook and Russell (2001). The *E. coli* cells containing both pCEP33 and pEPL2 were screened on LB agar supplemented with both ampicillin and tetracycline (50 μg/ml each).

Detection of $HrpN_{Ep}$ by immunoblotting

The purified $HrpN_{Ep}$ (1 mg/ml) was injected into a rabbit, and polyclonal antibodies were raised as described by Harlow and Lane (1999). Injections were given three times, at 2- to 3-week intervals, in complete Freund's adjuvant (Sigma-Aldrich). The antiserum was collected after 8 weeks, and immunoglobulins containing the anti-$HrpN_{Ep}$ antibodies were precipitated with ammonium sulfate. The antibody was preserved at −70°C.

The total proteins from cells of *E. pyrifoliae* WT3, *E. coli* (pCEP33), *E. coli* (pCEP33 + pEPL2), and *E. coli* (pEPN2) were prepared as described earlier for the isolation of total proteins for screening HR-inducible cosmid clones. The total proteins were run through SDS-PAGE using 12% acrylamide and transferred onto a 0.2-μm nitrocellulose membrane (Bio-Rad Laboratories) in a Mini Trans-Blot Electrophoretic Transfer cell (Bio-Rad Laboratories) as described by Sambrook and Russell (2001). The membrane was washed three times with 2% blocking solution and incubated with antibodies raised against $HrpN_{Ep}$; the membrane was again washed with TBS-Tween buffer (10 mM Tris-HCl, pH 8.0; 150 mM NaCl; 0.05% Tween 20 solution) and incubated with anti-rabbit antibodies conjugated with alkaline phosphatase (Sigma-Aldrich). Finally, the membrane was colorized with 5-bromo-4-chloro-3-indoyl phosphate (BCIP)/nitroblue tetrazolium (NBT) tablet (0.15 mg/ml BCIP, 0.30 mg/ml NBT, 100 mM Tris buffer, and 5 mM $MgCl_2$, pH9.5) (Sigma Fast BCIP/NBT; Sigma-Aldrich).

Nucleotide sequence accession number

Full nucleotide sequences of cosmid pCEP33 of *E. pyrifoliae* have been deposited in the GenBank database under accession no. AY530755. The nucleotide sequence of the *hrpL* gene has been deposited under accession no. AY532654.

Results

Sequence analysis of cosmid pCEP33

Among 2000 colonies screened from the genomic library of *E. pyrifoliae* WT3, one cosmid pCEP33 caused HR on tobacco when used to transform *E. coli*. The full sequence of cosmid pCEP33 was analyzed and determined to be 19.7 kb; it consisted of approximately 40% of the *hrp* genes of the *hrp* cluster in *E. amylovora*. Cosmid pCEP33 contained *rlsA*, *dspEF*, *hrpW*, *hrpN$_{Ep}$*, *hrpV*, *hrpT*, *hrcC*, *hrpG*, *hrpF*, and the partial *hrpE* homologs along with open reading frames ORFA, ORFB, and ORFC previously described in *E. amylovora* (Fig. 1). The *rlsA* gene of *E. pyrifoliae* had 93.5% nucleotide sequence similarity to that of *E. amylovora* (Zhang and Geider 1999). The homology percentages of the each *hrp* gene identified in the cosmid pCEP33 with those of other phytopathogens are given in Table 2. A comparison of the *hrp* genes of *E. pyrifoliae* with those of phytopathogenic bacteria showed the highest homologies with those of *E. amylovora* and then with those of *Pantoea agglomerans*. Comparatively low homologies were obtained with soft rot erwinias, such as *Pectobacterium carotovorum* and *P. chrysanthemi*. The major difference between *E. pyrifoliae* and *E. amylovora* was observed in their HR elicitor genes *hrpN$_{Ep}$* and *hrpN$_{Ea}$*, respectively. The *hrpN$_{Ep}$* gene was identified in a 8.4-kb *Hin*dIII-*Hin*dIII fragment, whereas the *hrpN$_{Ea}$* gene was located in a 1.3-kb *Hin*dIII-*Hin*dIII fragment (Wei et al. 1992). The *hrpN$_{Ep}$* gene was 1287 bp in size, and five intergenic nucleotide fragment insertions (INFIs) were observed when alignment was

Table 2. Comparison of *hrp* genes sequences of *Erwinia pyrifoliae* with corresponding genes of various Gram-negative phytopathogenic bacteria

Gene[a]	Percentage identity[b]							
	Erwinina amylovora		*Pantoea stewartii* pv. *stewartii*		*Pectobacterium chrysanthemi*		*Pectobacterium carotovorum*	
	Nucleotides	Amino acids	Nucleotides	Amino acids	Nucleotides	Amino acids	Nucleotides	Amino acids
hrpL	92.5	95.1	64.7	72.7	50.3	53.0	49.1	51.1
$hrpN_{Ep}$	83.3	86.1	52.3	59.0	44.5	41.1	49.1	47.6
hrpV	90.3	89.7	53.3	57.3	41.6	37.6	25.0	21.6
hrpT	95.9	96.9	57.4	64.6	48.4	45.2	46.4	46.9
hrcC	93.0	96.2	69.5	80.9	61.4	65.3	61.5	67.1
hrpG	93.3	93.1	60.5	56.6	36.3	31.9	33.3	27.7
hrpF	95.6	94.7	76.0	81.3	59.6	58.7	60.9	58.7
hrpW	83.8	82.6	–	–	–	–	–	–
dspE	89.6	89.9	53.9[c]	57.1[c]	–	–	–	–
dspF	91.4	90.7	57.6[d]	57.9[d]	–	–	–	–
ORFA	81.2	78.8	–	–	–	–	–	–
ORFB	83.6	80.1	–	–	–	–	–	–
ORFC	83.8	81.1	–	–	–	–	–	–

The nucleotide sequences of *hrp* genes of *E. pyrifoliae* in this study have been deposited in the GenBank database under accession nos. AY530755 and AY532654

–, sequences that were not available in the GenBank database; ORF, open reading frame

[a] Sequences of the *hrp* genes were obtained from GenBank accession nos. U97504, Y13831, AF083619, AF083620, M92994, and U56662 for *E. amylovora*; AF282857 for *Pantoea stewartii* subsp. *stewartii*; AF501263 for *Pectobacterium chrysanthemi*; and AY293288 for *Pectobacterium carotovorum* subsp. *carotovorum*

[b] Percentage identity was analyzed using Microsoft program MegaAlign (DNASTAR, Madison, WI, USA)

[c] *dspE* gene of *E. pyrifoliae* was homologous to *wtsE* gene of *P. stewartii* subsp. *stewartii*

[d] *dspF* gene of *E. pyrifoliae* was homologous to *wtsF* gene of *P. stewartii* subsp. *stewartii*

performed with the 1212 bp $hrpN_{Ea}$ gene of *E. amylovora* using the software package MegaAlign (DNASTAR, Madison, WI, USA).

In addition to the *hrp* genes, two open reading frames (ORFs) were identified downstream of a region containing genes homologous to *dspEF* and *rlsA*; they were designated ORFD and ORFE, as shown in the physical map (Fig. 1). The BLAST search analysis performed for ORFD indicated 50% homology to the transcriptional regulator protein (LysR) of *P. aeruginosa* PA01 and *P. syringae* pv. *tomato* strain DC3000. Another partial ORFE, which was opposite in its transcriptional orientation to ORFD, showed 32%–35% homology to a number of proteins, such as the putative oxidoreductase of *Streptomyces coelicolor* involved in NAD binding, and siderophore and secondary metabolite biosynthesis, transport, and catabolism. In our sequence analysis for the *hrpL* gene of *E. pyrifoliae* WT3, we found 100% and 92.5% homologies to those previously reported for *E. pyrifoliae* Ep4 and *E. amylovora*, respectively. Meanwhile, the PCR of chromosomal DNAs using primers hrpNEPF1 and hrpNEPR1 showed amplification of a ~1.1-kb fragment only from $hrpN_{Ep}$ gene of different strains of *E. pyrifoliae* but not from *E. amylovora* strains (Fig. 2). The amplified fragments were confirmed as a part of $hrpN_{Ep}$ by sequencing.

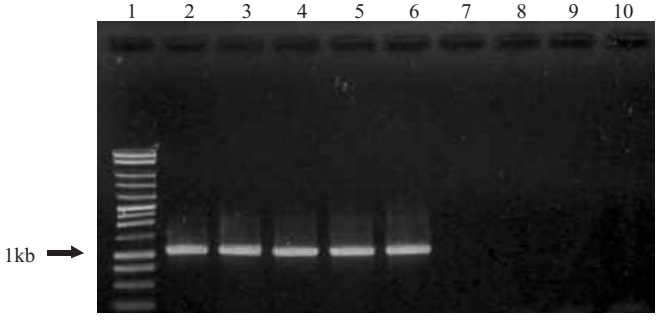

Fig. 2. Amplification of chromosomal DNA (50 ng) of various strains of *Erwinia pyrifoliae* and *E. amylovora* using primers hrpNEPF1 and hrpNEPR1. Polymerase chain reaction (PCR) products were visualized on 0.7% agarose gel after staining with ethidium bromide. Lanes: *1*, 1-kb ladder (Promega); *2–6*, *E. pyrifoliae* WT3, Ep1, Ep4, Ep4, Ep8, and Ep16, respectively; *7–10*, *E. amylovora* ATCC 15580, LMG 1877, LMG 1946, and LMG 2068, respectively. The *arrow* indicates approximately 1.1-kb amplified fragments only from the $hrpN_{Ep}$ gene of *E. pyrifoliae*

Comparative HR assay of $HrpN_{Ep}$ and $HrpN_{Ea}$

The clone pEPN2, in which the $hrpN_{Ep}$ gene was expressed under the T7 promoter, produced the HR-inducing protein $harpin_{Ep}$. The $HrpN_{Ep}$ contained 429 amino acid residues without cysteine. The molecular mass of $HrpN_{Ep}$ was 44 kDa; and it was acidic and rich in glycines, with 85.9% amino acid sequence identity to $HrpN_{Ea}$ of *E. amylovora* (Fig. 3). In a comparative HR assay using 5–30 μg/ml of the purified HR elicitor proteins prepared from the clones transformed with pEPN2 and pEAN2, $HrpN_{Ep}$ HR occurred more rapidly than with $HrpN_{Ea}$ (Table 3). Because infiltration of $HrpN_{Ep}$ (18 h) and $HrpN_{Ea}$ (36 h) (5 μg/ml) gave distinguishable HR activation, the HR assays were not performed below this concentration. This experiment was confirmed by repeating it four times, with three replications each time.

Table 3. HR plant assay performed for $HrpN_{Ep}$ and $HrpN_{Ea}$ of *Erwinia pyrifoliae* and *E. amylovora*, respectively, at various concentrations

Concentration (μg/ml)	HR at various times									
	14 h		18 h		24 h		36 h		48 h	
	$HrpN^{a}_{Ep}$	$HrpN^{b}_{Ea}$	$HrpN_{Ep}$	$HrpN_{Ea}$	$HrpN_{Ep}$	$HrpN_{Ea}$	$HrpN_{Ep}$	$HrpN_{Ea}$	$HrpN_{Ep}$	$HrpN_{Ea}$
5	–	–	+	–	+	–	+	+	++	+
10	+	–	+	+	++	+	++	+	++	+
20	+	+	++	++	+++	++	++++	++++	++++	++++
30	++	++	+++	+++	++++	+++	++++	++++	++++	++++
MES buffer	–	–	–	–	–	–	–	–	–	–

+, 25% collapse of tissues of tobacco leaves by HR; ++, 50% collapse of tissues of tobacco leaves by HR; +++, 75% collapse of tissues of tobacco leaves by HR; ++++, >75% collapse of tissues of tobacco leaves by HR; –, negative reaction, no HR symptoms

[a] Purified HR elicitor protein of *E. pyrifoliae*, expressed in *Escherichia coli* BL21 (DE3) pLysS under the T7 promoter of the pET-15b vector

[b] Purified HR elicitor protein of *E. amylovora*, expressed in *E. coli* BL21 (DE3) pLysS under the T7 promoter of the pET-15b vector

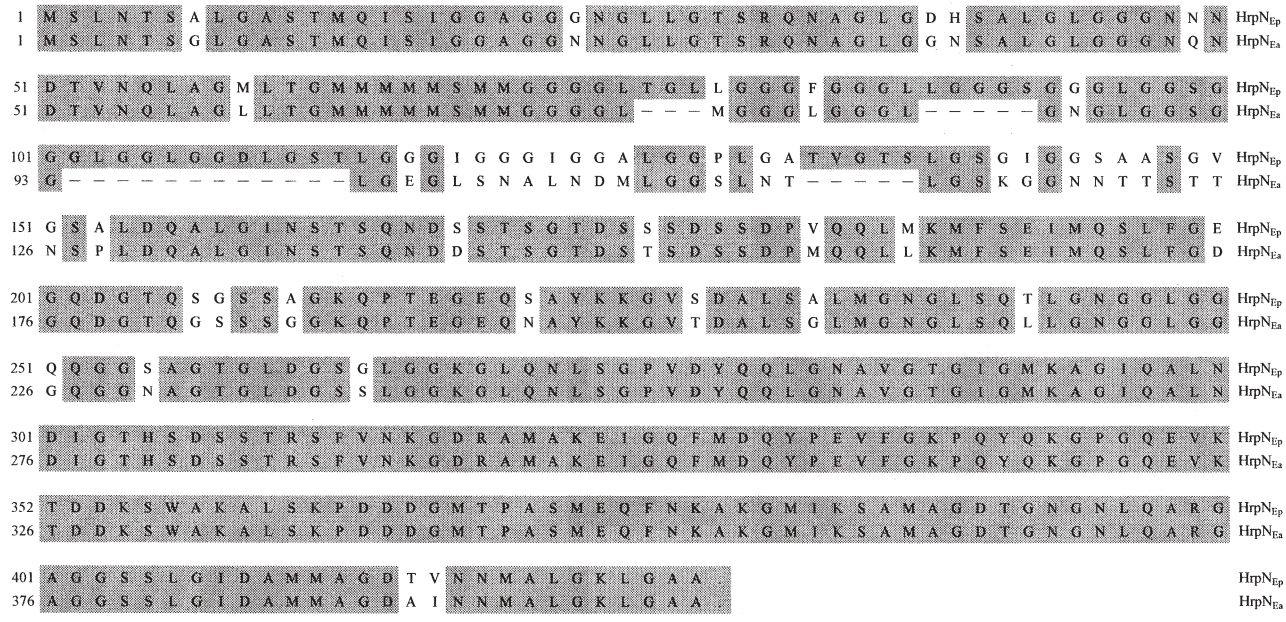

Fig. 3. Alignment of deduced amino acid sequences of $HrpN_{Ep}$ of *Erwinia pyrifoliae* WT3 and $HrpN_{Ea}$ of *E. amylovora* Ea321. $HrpN_{Ep}$ has 429 amino acids and a molecular mass of 44 kDa; it is glycine-rich but lacks cysteine. Identical amino acids are shaded. *Gaps* represent insertions in the deduced amino acids of $HrpN_{Ep}$ in *E. pyrifoliae* that do not exist in $HrpN_{Ea}$ of *E. amylovora*

HR elicitation and detection of $HrpN_{Ep}$ in *E. coli* (pCEP33) and *E. coli* (pCEP33 + pEPL2)

The sonicated, boiled protein of *E. coli* (pCEP33) caused HR when infiltrated into tobacco leaves. The HR elicited by *E. coli* (pCEP33) was similar to that produced by cell suspensions of *E. pyrifoliae* WT3 and was observed 18 h after infiltration (Fig. 4). However, infiltration of living cell suspensions of *E. coli* (pCEP33) did not cause HR on tobacco leaves (Fig. 4). The Western blot analysis using antiserum raised against $HrpN_{Ep}$ detected no expression of the HR elicitor $HrpN_{Ep}$ from the sonicated, boiled protein of *E. coli* (pCEP33) (Fig. 5). However, $HrpN_{Ep}$ was detected in sonicated, boiled protein of *E. coli* (pCEP33 + pEPL2) by Western blot analysis using $HrpN_{Ep}$ as antiserum (Fig. 5) in our effort to understand the role of the *hrpL* homolog in HR elicitation. In addition, HR was produced after infiltration with living cells suspension of *E. coli* (pCEP33 + pEPL2) and sonicated, boiled protein (Fig. 4).

Discussion

Molecular characterization of the full *dspEF*, *hrpW*, and *hrpN* loci along with a partial *hrpC* locus presented in this article showed that the *hrp* genes of *E. pyrifoliae* WT3 were similar to those of *E. amylovora* (Fig. 1), although their total genomic backgrounds were clearly different (Hacker and Kaper 2000; Kim et al. 1999; Shrestha et al. 2003). This result supports the hypothesis that the *hrp* genes of *E. pyrifoliae* and *E. amylovora* might have been acquired during evolution by horizontal transfer. For further characterization of the *hrp* genes of *E. pyrifoliae*, their sequences

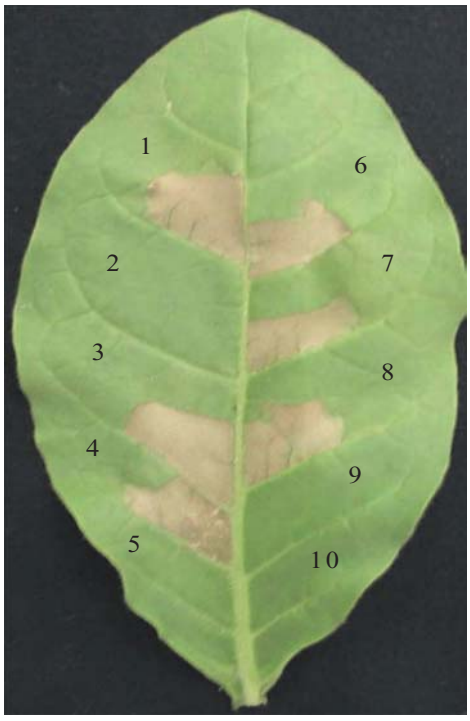

Fig. 4. Elicitation of the hypersensitive response (HR) in tobacco leaf after infiltration with sonicated, boiled protein isolated from *Escherichia coli* (pCEP33). Living cells suspensions were the infiltrates in panels: *1*, *Erwinia pyrifoliae* WT3 (2×10^8 CFU/ml); *2*, *E. coli* (pCEP33) (2×10^8 CFU/ml); *3*, *E. coli* (pCEP33 + pEPL2) (2×10^8 CFU/ml). The same concentration of cells (2×10^8 CFU/ml) was used for protein preparation for each sample. Sonicated, boiled proteins were used in panels *4*, $HrpN_{Ep}$ (40 µg/ml) isolated from *E. coli* (pEPN2); *5*, *E. coli* (pLAFR3); *6*, *E. pyrifoliae* WT3; *7*, *E. coli* (pCEP33); *8*, *E. coli* (pCEP33 + pEPL2); *9*, *E. coli* (pEPL2); and *10*, MES buffer. The leaf was photographed 24 h after infiltration

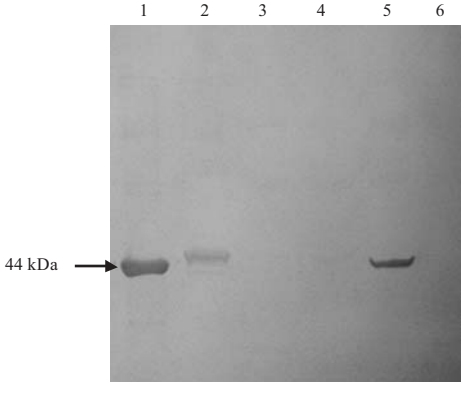

Fig. 5. Detection of the expression of $HrpN_{Ep}$ from sonicated and boiled proteins of *Escherichia coli* (pCEP33 + pEPL2) by Western blot using antiserum raised against purified $HrpN_{Ep}$. Lanes: *1*, purified $HrpN_{Ep}$ from *E. coli* (pEPN2) (2 µg); *2*, *Erwinia pyrifoliae* WT3; *3*, *E. coli* (pCEP33); *4*, *E. coli* (pLAFR3); *5*, *E. coli* (pCEP33 + pEPL2); *6*, *E. coli* (pEPL2). *Arrow* indicates 44-kDa $HrpN_{Ep}$

were analyzed and categorized into three groups based on the degree of their homology with those of *E. amylovora* (Table 2). Group I included the *hrpV*, *hrpT*, *hrpG*, *hrcC*, *hrpF*, and *hrpE* genes, which showed higher homologies to those of *E. amylovora.* These genes are involved in the secretion of type III secretion proteins in *E. amylovora* (Kim et al. 1997). Group II included the *dspEF*, *hrpW*, and $hrpN_{Ep}$ genes of *E. pyrifoliae*, which are related to disease specificity or avirulence and HR in *E. amylovora* (Bogdanove et al. 1998; Gaudriault et al. 1997; Kim and Beer 1998; Wei et al. 1992) and had relatively low homologies to those of *E. amylovora*. Group III included ORFD and ORFE, which had no sequence homology to the *hrp* genes of any other phytopathogenic bacteria. These two ORFs of *E. pyrifoliae* showed no homology even to the ORFG and ORFH of *E. amylovora*, which are located in similar regions just downstream of the *dspEF* gene. Thus, we were led to presume that these two ORFs of *E. pyrifoliae* may be related to functions other than pathogenicity.

Our research focused mainly on the $hrpN_{Ep}$ gene of group II, because it is the only gene that differed in size from its homologous $hrpN_{Ea}$, whereas the rest of the *hrp* genes identified were similar in size to their corresponding genes in *E. amylovora*. The size difference between the $hrpN_{Ep}$ and $hrpN_{Ea}$ of *E. pyrifoliae* and *E. amylovora*, respectively, was due to the INFIs, which produced four notable fragments insertions at the amino acids level of $HrpN_{Ep}$ (Fig. 3).

To understand whether the INFIs play a role in the HR response of $HrpN_{Ep}$ different from that of $HrpN_{Ea}$, a comparative HR assay was performed. HR was faster and stronger with $HrpN_{Ep}$ than with $HrpN_{Ea}$, suggesting that the INFIs have a synergic effect in elicitating an HR response on tobacco leaves (Table 3).

Hao and Wei (2001) reported that fragments $HrpN_{Ea}$4370 (spanning amino acids 43–70 in $HrpN_{Ea}$) and $HrpN_{Ea}$ 140176 (spanning amino acids 140–176 in $HrpN_{Ea}$) are two domains responsible for full HR activity. These domains are located in the N-terminal region of $HrpN_{Ea}$, and each domain contains an α-helical unit and an acidic unit. In this study, two domains were found in the N-terminal region of $HrpN_{Ep}$ that were similar to those of $HrpN_{Ea}$. The INFIs were found between the two HR domains of $HrpN_{Ep}$. Deletion analysis of $HrpN_{Ep}$ showed that the N-terminal region is important for HR elicitation because the C-terminal region (spanning amino acids 190–428 in $HrpN_{Ep}$) did not cause HR in tobacco (data not shown). Therefore, we also speculated that the INFIs combined with the α-helical and acidic units caused the three-dimensional structure of $HrpN_{Ep}$ that differed from $HrpN_{Ea}$ and thereby enhanced the HR activity of $HrpN_{Ep}$. A detailed domain study of $HrpN_{Ep}$ by deletion and mutation analyses is ongoing to understand the exact role of INFIs in the $hrpN_{Ep}$ gene. Because no fragment was amplified from any of the studied strains of *E. amylovora* by PCR using a primer corresponding to INFIs (Fig. 2), this result indicated that only *E. pyrifoliae* contained the INFIs in the $hrpN_{Ep}$ gene and suggested that it evolved independently from the $hrpN_{Ea}$ gene.

In *E. amylovora*, mutations in the *hrp* regulatory genes abolished the organism's pathogenicity to the host plant and its ability to elicit HR in a nonhost plant (Kim et al. 1997;

Wei and Beer 1993; Wei et al. 2000). However, *E. coli* (pCEP33) produced HR consistently without the regulatory *hrpL*, *hrpXY*, and *hrpS* genes when tobacco leaves were infiltrated with sonicated, boiled protein. This result is the first time HR was induced in the absence of regulatory genes and a full set of components of the type III secretion apparatus when compared to that of *E. amylovora* and other phytopathogenic bacteria. Despite the occurrence of HR, the expression of $HrpN_{Ep}$ could not be detected in *E. coli* (pCEP33) by Western blot analysis, even from a 20-fold concentration of the protein. It is possible that $HrpN_{Ep}$ is expressed at a basal level without a regulatory gene, and that this level is sufficient for HR elicitation (Fig. 4) but not for detection by Western blot analysis (Fig. 5). Another reason may be the presence of other HR elicitors, DspEF and HrpW, which also could be expressed minimally as $HrpN_{Ep}$ in *E. coli* (pCEP33) and produce HR when infiltrated into tobacco. To understand the role of the *hrpL* gene in the expression of $HrpN_{Ep}$, *E. coli* (pCEP33) was transformed with a plasmid pEPL2 containing an *hrpL* homolog of *E. pyrifoliae*. Clear detection of $HrpN_{Ep}$ by Western blotting without concentrating the protein isolated from *E. coli* (pCEP33 + pEPL2) indicated that HrpL enhances the expression of $HrpN_{Ep}$. Meanwhile, HR was produced after infiltration with living cell suspensions of *E. coli* (pCEP33 + pEPL2), although this clone did not have the complete genes required for secretion (Fig. 1). The *hrpL* gene does not seem to be involved in secretion of the $hrpN_{Ep}$ gene. Instead, HrpL may be overexpressed in the expression vector pHCE IA, and the transcription of HR elicitors such as $HrpN_{Ep}$, HrpW, and DspEF may be enhanced. The elicitors then could have leaked from self-lysed cells of *E. coli* (pCEP33 + pEPL2), inducing HR in tobacco (Fig. 4). Mutation analysis is underway to help us understand the HR caused by *E. coli* (pCEP33).

Conclusions

The $hrpN_{Ep}$ of *E. pyrifoliae* and $hrpN_{Ea}$ of *E. amylovora* may have evolved independently, although their structures and functions are similar to each other. In addition to the *hrp* genes, such independent evolution is thought to have occurred with other characteristics of *E. pyrifoliae*. Our previous study on the effects of temperature on the growth of these two pathogens showed that *E. pyrifoliae* is more cold-tolerant than *E. amylovora* (Shrestha et al. 2001; Shrestha et al. 2005). Regarding the epidemic survey of shoot blight disease, *E. pyrifoliae* has so far been found only in the north, which is much colder than the south, the major pear cultivation area in Korea (Shrestha et al. 2003). In another of our preliminary studies, *E. pyrifoliae* was not transmitted by insects (data not shown), although this point requires further detailed study. Therefore, these characteristics of *E. pyrifoliae* might explain its restriction to northern areas, suggesting that geographical evolution might have been occurred even within a single country.

Meanwhile, three plasmids (pEP36, pEJ30, pEA29) of *E. pyrifoliae,* Japanese *Erwinia* sp., and *E. amylovora*, respectively, were sequenced recently (Jock et al. 2003a; Maxson-Stein et al. 2003). The comparative sequence analysis of these plasmids showed that the Japanese *Erwinia* sp. is more closely related to *E. pyrifoliae* than to *E. amylovora*. The close relationship of these two pathogens could be due to their similar geographic regions (i.e., Korea and Japan). It is presumed that Korean *E. pyrifoliae* and Japanese *Erwinia* are not only phylogenetically related, their pathogenicity may also be similar. To our knowledge, Japan, as Korea, does not use bactericides or resistant host cultivars to control shoot blight disease, so no direct selection pressure is exerted on the pathogen. Therefore, analysis of the *hrp* genes of Korean *E. pyrifoliae* and Japanese *Erwinia* sp. can indicate how *E. pyrifoliae* and *E. amylovora* are evolving independently.

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