Identification and Characterization of Nip, Necrosis-Inducing Virulence Protein of *Erwinia carotovora* subsp. *carotovora*

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Erwinia carotovora subsp. *carotovora* is a gram-negative bacterium that causes soft rot disease of many cultivated crops. When a collection of *E. carotovora* subsp. *carotovora* isolates was analyzed on a Southern blot using the harpin-encoding gene *hrpN* as probe, several harpinless isolates were found. Regulation of virulence determinants in one of these, strain SCC3193, has been characterized extensively. It is fully virulent on potato and in *Arabidopsis thaliana*. An RpoS (SigmaS) mutant of SCC3193, producing elevated levels of secreted proteins, was found to cause lesions resembling the hypersensitive response when infiltrated into tobacco leaf tissue. This phenotype was evident only when bacterial cells had been cultivated on solid minimal medium at low pH and temperature. The protein causing the cell death was purified and sequenced, and the corresponding gene was cloned. The deduced sequence of the necrosis-inducing protein (Nip) showed homology to necrosis- and ethylene-inducing elicitors of fungi and oomycetes. A mutant strain of *E. carotovora* subsp. *carotovora* lacking the *nip* gene showed reduced virulence in potato tuber assay but was unaffected in virulence in potato stem or on other tested host plants.

Erwinia carotovora is a phytopathogenic bacterium that causes soft rot of many crop plants all over the world. The species is divided into several subspecies, among which subsp. *carotovora* and subsp. *atroseptica* infect potato. *E. carotovora* subsp. *carotovora* is a broad-host-range pathogen that causes stem rot of potato plants and soft rot of harvested tubers and many other vegetables and fruit, whereas *E. carotovora* subsp. *atroseptica* causes mainly black leg of potato in the field (Pérombelon 2002). A third soft-rot pathogen, *E. chrysanthemi*, causes both kinds of symptoms on potato as well as rotting on many other cultivated crops. *E. carotovora* subsp. *carotovora* has been reclassified as *Pectobacterium carotovorum* subsp. *carotovorum* and *E. carotovora* subsp. *atroseptica* as *Pectobacterium carotovorum* subsp. *atrosepticum* (Hauben et al. 1998). Recently, *E. carotovora* subsp. *atroseptica* was suggested

to form a separate species, *Pectobacterium atrosepticum* sp. nov. (Gardan et al. 2003). The main virulence determinants of the soft rotting *Erwinia* spp. plant are cell wall-degrading enzymes, mainly pectinases and cellulases (Toth et al. 2003). Many of the corresponding genes have been cloned, and their secretion, regulation, and role in virulence studied in detail (Pérombelon 2002; Whitehead et al. 2002).

Some gram-negative bacteria in the genera *Erwinia*, *Pantoea*, *Pseudomonas*, *Ralstonia*, and *Xanthomonas* have been shown to possess a type III secretion machinery which they use to translocate virulence proteins (so-called effectors) into the host cell (He and Jin 2003). The genes coding for the components of the secretion system are called hypersensitive reaction and pathogenicity (*hrp*) genes due to their involvement in the outcome of the plant–pathogen interaction in both incompatible and compatible interactions. In incompatible hosts, type III secretion systems are needed for the secretion of avirulence (*avr*) proteins into the host cell, where they are recognized by a corresponding resistance gene product. This recognition leads to a hypersensitive response (HR): localized cell death caused by activation of the programmed cell death machinery of the host cells (Heath 2000). A second group of HR-inducing proteins secreted through the type III secretion system are harpins: small, acidic, glycine-rich, and heat-stable proteins (Büttner and Bonas 2003; Jin et al. 2003). When present in plant tissue, either as a result of infection or artificial infiltration into the intercellular space, harpins cause HR and induction of defense mechanisms (Bauer et al. 1995; Kariola et al. 2003).

The function of harpin during compatible interaction is not clear. Harpin of *Pseudomonas syringae*, HrpZ, has been shown to bind in plant protoplast membranes and form cation-conducting pores in lipid bilayers in vitro (Lee et al. 2001a,b) and *E. amylovora* harpin HrpN has been suggested to regulate ion channels of *Arabidopsis thaliana* suspension cells (El-Maarouf et al. 2001). Harpin-deficient mutants of *E. chrysanthemi* and *E. amylovora* were reduced in virulence on their susceptible hosts (Bauer et al. 1995; Wei et al. 1992). On the other hand, HrpZ of *Pseudomonas syringae* pv. *tomato* apparently is not needed for virulence on tomato, and ectopic expression of a harpin-like effector, PopA, of *Ralstonia solanacearum* during early infection even results in loss of virulence (Kanda et al. 2003).

Many fungal and oomycete pathogens produce an array of proteins and other elicitors able to induce HR and defense responses (Radman et al. 2003; Tyler 2002). Elicitins (conserved small sterol scavenging proteins with elicitor activity) are pro-

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duced by all *Phytophthora* spp. and by some *Pythium* spp. (Vauthrin et al. 1999). Cell walls of *Phytophthora sojae* and *Phytophthora megasperma* contain a 42-kDa transglutaminase, a glycoprotein that is able to induce the activation of defense-related genes in parsley (Brunner et al. 2002; Nürnberger et al. 1994). *Phytophthora parasitica* var. *nicotianae* produces an elicitor with a cellulose-binding domain and lectin-like characteristics (Gaulin et al. 2002; Mateos et al. 1997). Fungal xylanases are elicitors of defense responses in tobacco and tomato (Enkerli et al. 1999). When applied to tobacco or tomato leaves, these xylanases induce ethylene biosynthesis, production of phytoalexins, and pathogenesis-related (PR) proteins and cause cell death resembling the HR reaction. The xylanase of *Trichoderma viride* has been most extensively studied. It has been shown that the enzymatic activity of the xylanase is not necessary for its elicitor activity, and a specific binding site for xylanase protein on tobacco membrane has been characterized (Enkerli et al. 1999; Hanania and Avni 1997).

E. carotovora RpoS (sigma-S or sigma-38) is an alternate sigma factor needed for the production of a negative regulator RsmA (Mukherjee et al. 1998). RsmA is an RNA-binding protein that promotes decay of harpin and enzyme transcripts (Liu et al. 1998). RpoS mutant does not express RsmA and, therefore, overproduced tissue-macerating enzymes and harpin (Andersson et al. 1999a; Mukherjee et al. 1998, 2000). Mutants of *E. carotovora* subsp. *carotovora* strain 71 overexpressing harpin caused HR when infiltrated into tobacco tissue (Cui et al. 1996). In this report, we have characterized HR-like response caused in tobacco tissue by an RpoS mutant of *E. carotovora* subsp. *carotovora* strain SCC3193. Although this strain did not appear to have a homologue of *Erwinia* harpin, it was able to elicit an HR-like cell death. We hypothesized that it might produce a functionally similar but nonhomologous harpin. Purification of the cell death-inducing factor led to the identification of necrosis-inducing virulence protein homologous to fungal and oomycete elicitors.

RESULTS

Not all *E. carotovora* subsp. *carotovora* strains have the *Erwinia* harpin gene *hrpN*.

Several *E. carotovora* strains belonging to subspecies *carotovora* and subsp. *atroseptica*, and *E. chrysanthemi* strain EC16, were studied by Southern analysis using a fragment of the *hrpN* gene of *E. carotovora* subsp. *carotovora* strain 71 as a probe (Fig. 1). Three *E. carotovora* subsp. *carotovora* isolates, all *E. carotovora* *atroseptica* isolates, and an *E. chrysanthemi* strain showed hybridizing bands suggesting that these strains have a gene homologous to *hrpN*. The other five *E. carotovora* subsp. *carotovora* strains did not show hybridization to the probe. This result suggests that not all *E. carotovora* subsp. *carotovora* isolates have a homologue of the *Erwinia* harpin gene or they have a homologue which is less conserved.

The *Erwinia* isolates were studied by polymerase chain reaction (PCR) using degenerate primers corresponding to the conserved regions of *Erwinia* harpin protein (AKEIGQFM and KPDDDGM). This PCR analysis revealed a 141-bp-long harpin-specific band amplified only from the strains that showed hybridization in the Southern blot (data not shown). This suggests that the other isolates do not carry a less-conserved form of *Erwinia* harpin but, rather, may lack the *hrpN* homologue completely.

Induction of HR-like response in tobacco leaf tissue by an *E. carotovora* RpoS mutant.

We wanted to find out whether the harpin-negative strains had a functionally similar protein to harpin. One of the *hrpN*-

negative strains, SCC3193, which originally was isolated from diseased potato stem, has been characterized at the molecular level. It is virulent on tobacco, in potato stem and tuber assays, and on *A. thaliana* (Norman-Setterblad et al. 2000; Pirhonen et al. 1991). Furthermore, an RpoS⁻ mutant of SCC3193, strain SCC8003, containing a chloramphenicol resistance marker (Cm^R) in the RpoS gene, has been shown to overproduce some secreted proteins in planta (Andersson et al. 1999a). When grown on solid minimal induction media (Huynh et al. 1989) at pH 5.7 and low temperature (15°C), SCC8003 cells were able to induce an HR-like response in tobacco tissue (Fig. 2A). No HR-like response could be seen when the cells had been grown on Luria-Bertani (LB) plates, on solid minimal induction media at normal growth temperature (28°C, Fig. 2A), or in liquid media in any conditions (data not shown). Wild-type strain SCC3193 did not cause induction of the HR-like response under any growth conditions (Fig. 2A.). These growing conditions—solid minimal induction media combined with low pH and low temperature—have been shown to induce type III-dependent secretion of *E. amylovora* HrpW, a protein showing partial homology to harpin (Gaudriault et al. 1998). Similar conditions in liquid media cause induction of type III secretion in many plant-pathogenic bacteria, possibly because these conditions mimic plant apoplastic fluid (Jin et al. 2003).

Purification and characterization of a plant cell death-inducing protein of *E. carotovora*.

Cells of the RpoS mutant strain SCC8003, grown under inducing conditions on minimal plates, were fractionated to find the cellular location of the cell death-inducing activity in the bacterial cells. Periplasmic proteins were collected after osmotic shock treatment and the intracellular activity was released by sonicating the cells. Filter-sterilized periplasmic fraction was effective in inducing cell death in tobacco leaf tissue (Fig. 2B). The inducing activity was sensitive to proteinase K treatment, suggesting that the inducer was a protein (data not shown).

The periplasmic proteins of SCC8003 were separated by cation exchange chromatography (Fig. 3A). Two of the fractions forming a peak at 12 min after the start of elution induced cell death in tobacco tissue. When these fractions were run on a sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel, they were found to contain several proteins (data not shown). Therefore, the active fractions were pooled and subjected to gel filtration (Fig. 3B). Two fractions, corresponding to a peak eluted out of the gel column with 1.5 ml of buffer, caused cell death in tobacco tissue. When these fractions were analyzed using SDS-PAGE, they were found to contain a single protein band with a size of approximately 27 kDa (data not shown). This protein band was cut out of the gel

Fig. 1. Southern analysis of *Erwinia* spp. with partial *hrpN* gene as probe. Bacterial species or subspecies: *Erwinia amylovora* (Ea), *E. chrysanthemi* (Ech), *E. carotovora* subsp. *carotovora* (Ecc), and *E. carotovora* subsp. *atroseptica* (Eca). Strains: 1, uncharacterised *E. amylovora* strain; 2, EC16; 3, 71; 4, SCC3193; 5 to 10, randomly chosen Ecc isolates (6, 8, 12, 17, 24, and 42, respectively); 11 to 15, randomly chosen Eca isolates (549, 612, 613, 622, 623, and 844, respectively).

and digested into peptides, which then were subjected to mass spectrometry to get amino acid sequences. Numerous peptide sequences were obtained. Six peptides were sequenced more than once, making it possible to determine the reliable parts of the peptides (Fig. 4).

Cloning of the *nip* gene coding for an *E. carotovora* subsp. *carotovora* homologue of fungal and oomycete necrosis-inducing elicitors.

Two of the peptide sequences (NFL/IDSSNTL/IHR and PAGYPEFTAASL/I) were utilized to design degenerate primers. Four primers were designed for each peptide, two partially overlapping forward primers and two partially overlapping reverse-oriented primers (Table 1). These primers were used in nested PCR reactions, taking into account two possible orientations of the peptides on the protein. Several bands could be seen in both of the final PCR reactions (data not shown). Of these, 16 were cloned and sequenced. One of the PCR fragments, approximately 500 bp in size, showed right primers in the ends as well as sequences corresponding to two of the other peptides internal to the ones used for cloning. The fragment contained a unique *Cla*I restriction site in the middle (Fig. 4).

The PCR fragment corresponding to the purified protein was used as a probe in a Southern blot on *Cla*I-cut chromosomal DNA of *E. carotovora* subsp. *carotovora* strain SCC3193. The probe hybridized to two fragments, 4 and 4.5 kb in size (data not shown). *Cla*I-cut chromosomal DNA, 3 to 5 kb in size, was purified from agarose gel and cloned into pBluescriptII plasmid cut with the *Cla*I enzyme. The transformants were screened by colony hybridization using the PCR fragment as a probe. Plasmid clones corresponding to both of the *Cla*I fragments were isolated and sequenced from the *Cla*I ends to get the remaining sequence of the gene (Fig. 4).

The sequence of the gene corresponding to the purified cell death-inducing protein was used to search databases with BLAST to find homologous proteins (Table 2). The best homology was to a deduced protein encoded by open reading frame (ORF) 9 of a hemolysin-encoding region of *Vibrio pommerensis*, an atypical marine *Vibrio* sp. isolated from the Baltic Sea (Jores et al. 2003). The *E. carotovora* subsp. *carotovora* protein also was homologous to necrosis- and ethylene-inducing protein elicitors found in many oomycete and fungal pathogens of plants. These protein elicitors have been found to induce plant defense systems and cause cell death in many dicots (Fellbrich et al. 2002; Keates et al. 2003). Several fungal and oomycete pathogens seem to have more than one elicitor-encoding gene (Table 2). Homologous proteins also can be found in some non-pathogenic organisms (Table 2). The *E. carotovora* homologue

Fig. 2. A, Induction of cell death in tobacco tissue by *Erwinia carotovora* subsp. *carotovora* RpoS mutant. Strains: 1 to 4, RpoS mutant SCC8003; 5 to 8, wild-type strain SCC3193; 9, buffer control. Growth conditions: 1 and 5, LB at 28°C; 2 and 6, minimal induction medium at 28°C; 3 and 7, LB at 15°C; 4 and 8, minimal induction medium at 15°C. **B**, Induction of cell death by partially purified necrosis-inducing protein (Nip) of *E. carotovora* subsp. *carotovora*. Samples: 1, periplasmic proteins of wild-type strain SCC3193 grown in inducing conditions; 2, periplasmic proteins of RpoS mutant SCC8003 grown in inducing conditions; 3, buffer control; 4, periplasmic proteins of isopropyl- β -D-thiogalactoside (IPTG)-induced *Escherichia coli* carrying pBluescript (empty vector); 5, periplasmic proteins of IPTG-induced *E. coli* carrying pNip (*nip* gene under lac promoter in pBluescript). **C**, Complementation of the necrosis-negative phenotype of the Nip⁻ RpoS⁻ double mutant with the cloned *nip* gene. Strains: 1, RpoS⁻ mutant 8002; 2, Nip⁻ RpoS⁻ double mutant SCC3200; 3, buffer control; 4, Nip⁻ RpoS⁻ double mutant SCC3200 carrying pBluescript; 5, Nip⁻ RpoS⁻ double mutant SCC3200 carrying pNip. Leaves were photographed 2 days after infiltration.

was named Nip (necrosis-inducing protein). The Nip protein showed no homology or resemblance to harpins.

When part of the *nip* coding sequence was used as a probe in a Southern blot, all the analyzed *E. carotovora* isolates and *E. chrysanthemi*, but not *E. amylovora*, were seen to possess a homologue (Fig. 5). Homologues of the *E. carotovora* subsp. *carotovora* *nip* gene can be found using a TBLASTN search in the genomic DNA sequence of *E. carotovora* subsp. *atroseptica* 1043 but not in genomic databases covering other plant-pathogenic bacteria. The deduced protein sequence of *E. carotovora* subsp. *atroseptica* Nip showed 90% identity and 93% similarity to *E. carotovora* subsp. *carotovora* Nip protein, whereas the corresponding *E. chrysanthemi* protein was 71% identical and 84% similar to *E. carotovora* subsp. *carotovora* Nip. All the *Erwinia* homologues appear to have a typical Sec-dependent signal sequence when analyzed by SignalP program (Nielsen et al. 1997).

To verify that Nip was the plant cell death-inducing protein in the periplasm of the *E. carotovora* subsp. *carotovora* RpoS mutant, the protein was produced in *Escherichia coli* and used in plant assays. The *nip* gene was cloned into pBluescript vector with its own ribosome-binding site and translation start and stop codons. Expression of the gene was controlled by the lac promoter of the vector. The construct was transduced into *E. coli* TOP10F' cells and expression of the protein induced by IPTG (isopropyl- β -D-thiogalactoside). Periplasmic proteins from the transductant were prepared and injected into tobacco tissue. This showed that the periplasmic fractions isolated from an *Erwinia carotovora* subsp. *carotovora* RpoS mutant and *Escherichia coli* cells harboring the cloned gene caused identical necrosis in tobacco tissue (Fig. 2B).

Nip is responsible for the necrosis-inducing phenotype of the RpoS mutant.

We wanted to study whether Nip was the only inducer produced by the *E. carotovora* subsp. *carotovora* RpoS mutant, or whether the strain overexpressed several inducers. A pBluescript construct was created, where almost the whole *nip* gene, between restriction sites *NheI* and *EcoRI* (Fig. 4), was replaced with a chloramphenicol marker. This construct was transduced into strain SCC8002 containing a *Km^r* marker in its *rpoS* gene (Andersson et al. 1999a). SCC8002 is phenotypically similar to the RpoS strain SCC8003. Marker-exchange mutants lacking the *Ap^r* marker but carrying both the *Km^r* and *Cm^r* markers in the chromosome were screened, and the *nip* mutation was verified by Southern blotting (data not shown). Induction of necrosis in tobacco tissue by the Nip⁻ RpoS⁻ double mutant (SCC3100) was studied. The double mutant had lost its ability to cause necrosis in tobacco tissue, which suggests that Nip is the only necrosis-inducing protein overexpressed in the RpoS mutant strains of *E. carotovora* subsp. *carotovora*. The necrosis-negative phenotype of the Nip⁻ RpoS⁻ double mutant could be complemented by the plasmid construct producing the Nip protein (Fig. 2C).

Nip is needed for virulence in potato tubers.

The necrosis-inducing proteins of fungal and oomycete pathogens have been characterized as elicitors of cell death and defense reactions in plants. In one of these pathogens, *Fusarium oxysporum* f. sp. *erythroxyli* infecting *Erythroxylum coca*, the elicitor-encoding chromosomal gene has been inactivated by gene replacement (Bailey et al. 2002). The lack of the elicitor or its overproduction did not affect the virulence of the pathogen. We wanted to see if whether Nip was needed for virulence of *Erwinia carotovora* subsp. *carotovora*. Therefore, the *nip* mutation was transduced into wild-type strain SCC3193 by selecting the *Cm^r* phenotype, and the virulence of the Nip⁻ single

mutant SCC3200 was compared with the corresponding wild-type strain SCC3193 in virulence tests on several plants. Under the experimental conditions which were used, virulence of the *E. carotovora* subsp. *carotovora* Nip⁻ mutant was found to be unimpaired on sterile tobacco seedlings and on lettuce, eggplant, cauliflower, broccoli, celery, and sweet potato, and on potato stems (data not shown).

Ability of the Nip⁻ mutant strain SCC3200 and the wild-type strain SCC3193 to cause rotting of potato tubers was studied using potato cv. Bintje. Each potato tuber was inoculated with the wild-type strain, the Nip⁻ mutant, and a negative control consisting of a noninoculated wound. Results showed that, although individual potato tubers varied substantially in their susceptibility to infection caused by both of the bacterial strains, the Nip mutant caused consistently less rotting (Fig. 6). In all the tubers, the weight of the rot caused by the mutant was less than half (average 17 ± 14 % standard deviation) of the rotting caused by the wild-type strain in the same tuber. Similarly, the virulence of the mutant cells harboring either the empty vector or the Nip clone was compared in potato tubers. The defect in the virulence of the *nip* mutant could be complemented by the

Fig. 3. Purification of the cell death-inducing protein of *Erwinia carotovora* subsp. *carotovora*. **A**, Cation exchange chromatography of periplasmic fraction isolated from RpoS mutant SCC8003. **B**, Gel filtration of the active fraction from the cation exchange column. Peaks containing cell death-inducing activity are marked with arrows. The protein present in the peak from gel filtration chromatography was sequenced with mass spectrometry.

plasmid-encoded Nip protein, suggesting that it is the mutation in the *nip* gene and not its downstream effect that causes the reduced virulence in tuber tests (Fig. 6).

DISCUSSION

Type III secretion systems are widely distributed in animal- and plant-pathogenic bacteria and in symbionts (Galán and

Collmer 1999). The presence of the type III cluster in an uncharacterized bacterial strain has been suggested to be a general indicator of bacterial virulence (Stuber et al. 2003). In two soft rot *Erwinia* spp., *E. carotovora* and *E. chrysanthemi*, type III secretion machinery has been characterized and found to contribute to virulence or ability of the pathogen to grow in planta (Rantakari et al. 2001; Yang et al. 2002). Similar to previous results, harpin, one of the effectors secreted

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1 - TTTTGTGACTATTGATAGTGTGGTTTAAAGGACTACCATTATTCAAGGAGTTAAT - 60
      NheI
61 - ATGCTTTTAGGAAAAAGAGCGCTAGCCTTCTCAATACTGTTCACTACGTTTCTTCA - 120
    - M L L G K K R A L A F S I L F T T F S S
121 - CATGCAGATACTTTCCAAAATTAAACCAATCCTTGCCATCCGGAATTGATGCGCGGGCG - 180
    - H A D T F P K L N Q S L E S G I D A R A
181 - ATTGCACCGGTATTTGATTTTGTGCTGCTTTCCAGCGCCGAGTGAGCCGT - 240
    - I A P V F D F D S D G C F P S A G V S R
241 - AGCGGTGTGCAAAACGGCGGCTGAAACCATCGGGCAATATAACAGGTGGTGCCGATGG - 300
    - S G V Q N G G L K P S G N I T G G C R W
301 - AGTAACCTTCTTGACTCGTCAAATACGCTGCACCGGTACGCTGTGTTAATTCTAGTGGT - 360
    - S N F L D S S N T L H R Y A C V N S S G
361 - GCTCGCTATTGTGGTAGTTTCTATTCACTCTATTTTTAAAGATCAGATACTGAATGGA - 420
    - A R Y C G S F Y S L Y F L K D Q I L N G
421 - GTAAATTCAGGCCACCGCCATGATTGGGAACATGTTGCAATCTGGACTAAAAATGGTGTG - 480
    - V N S G H R H D W E H V A I W T K N G V
481 - GTAACGCATGGTAGCTACAGTGCGCATGGAAAATTGACGACTAAAGATGCGGCGAGCATC - 540
    - V T H G S Y S A H G K L T T K D A A S I
      ClaI
541 - GATAACAGGATGGTCATCTGAAATTTGTTTATCACAAGATGGGGCATTAAACCATGCC - 600
    - D K Q D G H L K F V Y H K D G A L T H A
601 - TTTTCGTTTCTCTAAAACGAATGAAAATGCGGAAAATCCGTATAAAAAATTTGTAACGCCA - 660
    - F R F S K T N E N A E N P Y K K F V T P
661 - GACATCATCAGTTGGTACACCATGTTTGGTGATGGCATTAAATAACAGGAATGCGAAAC - 720
    - D I I S W Y T M F G D G I N N Q E L R N
721 - CGATTAAATGCATTCGACTATGGGTCGGCCTCGATTCTCTCAAGGACAACAATTTTCTG - 780
    - R L N A F D Y G S A S I P L K D N N F L
      EcoRI
781 - ACTAACCTAAATAATGGTCGTCGGCAGGCTATCCAGATTCACGGCTGCCAGTCTAACG - 840
    - T N L N N G R P A G Y P E F T A A S L T
841 - ACATCGAAATAA - 852
    - T S K *

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Fig. 4. Sequenced peptides shown on the deduced protein sequence coded for by the *nip* gene of *Erwinia carotovora* subsp. *carotovora* strain SCC3193. Peptides, which were identified with mass spectrometry, are marked with boxes on the protein sequence, and the ones used for designing degenerate primers for cloning are highlighted. Signal sequence is marked with bold letters, the conserved heptamer GHRHDWE is underlined, and the stop codon is marked with an asterisk. Restriction sites, which we used for cloning and constructing deletion derivative of the gene, are marked with bold and the name of the enzyme has been marked above the nucleotide sequence.

Table 1. Primers used in this study

Primer location ^a	Name of the primer	Sequence 5'→3' ^c
<i>hrpN</i> forward	MP30	gciaagaratiggicarttyatg
<i>hrpN</i> reverse	MP31	caticrctcrttciggyt
Nip peptide FL/IDSSNTL/IHR ^b	FORW1	aaytyhtigaywsiaayac
	FORW2	gaywsiaayacihicay
	REV3	ckrtgiadigtrttiswswrtc
	REV4	tgidiadtrttiswswrtciadr
Nip peptide PAGYPEFTAASL ^b	FORW5	ccigciggitayccigartty
	FORW6	yccigarttyacigcigenws
	REV7	adiswigcigcigtraaytcn
	REV8	gcigtraaytciggrtance
<i>nip</i> promoter	EXP1	ggtaccgcagcctaataatattcggttc
<i>nip</i> 3' end	EXP2	gagctctatttcgatgctgtagactgg

^a Nip = necrosis-inducing protein.

^b Mass spectrometric analysis did not separate isoleucine and leucine.

^c d = a/g/t, h = a/c/t, i = inosine, k = g/t, r = a/g, s = c/g, w = a/t, y = c/t.

through the type III secretion machinery of soft-rotting *Erwinia* spp. and related bacteria, has been shown to be needed for full virulence in *E. chrysanthemi* (Bauer et al. 1995). Therefore, it was surprising to find out that approximately half of the *E. carotovora* subsp. *carotovora* strains seem to lack the *hrpN* gene. One of the *hrpN*-negative strains, SCC3193, which originally was isolated from diseased potato stems, has been characterized at the molecular level. It is virulent on tobacco, in potato stem and tuber assays, and on *A. thaliana* (Norman-Zetterblad et al. 2000; Pirhonen et al. 1991). We hypothesized that it may produce a harpin which is not homologous to the harpin encoded by the *hrpN* gene but is functionally similar.

When RpoS mutant strains of a harpinless SCC3193 were grown in conditions known to induce type III secretion, the bacterial cells were able to induce an HR or HR-like response in tobacco tissue. The response of the plant tissue resembled the HR caused in tobacco tissue by harpins, suggesting that a harpin-like protein was produced by the overexpressing RpoS strain. This inducing activity was found to be caused by a protein present in the periplasm of the plate-grown cells. The

Fig. 5. Southern analysis of *Erwinia* spp. with *nip* gene as probe. Bacterial species or subspecies: *E. amylovora* (Ea), *E. chrysanthemi* (Ech), *E. carotovora* subsp. *carotovora* (Ecc), and *E. carotovora* subsp. *atroseptica* (Eca). Strains: 1, uncharacterised *E. amylovora* strain; 2, EC16; 3, 71; 4, SCC3193; 5 to 10, randomly chosen Ecc isolates (6, 8, 12, 17, 24, and 42, respectively); 11 to 15, randomly chosen Eca isolates (549, 612, 613, 622, 623, and 844, respectively).

inducing protein was purified and peptide sequences were obtained by mass spectrometry. The deduced sequence of the *E. carotovora* subsp. *carotovora* necrosis-inducing protein (Nip) did not resemble any harpin but showed homology to a deduced protein encoded by an uncharacterized ORF in hemolysin-encoding region of a marine *Vibrio* sp. and necrosis- and ethylene-inducing protein elicitors produced by plant pathogens in the genera *Phytophthora*, *Pythium*, and *Fusarium*. The homologue of *Phytophthora sojae* was shown to be expressed during the transition from biotrophy to necrotrophy and was suggested to be involved in killing of the host tissue in the

Fig. 6. Virulence of necrosis-inducing protein (Nip) mutant is reduced in potato tuber assay. Strains: Wt, SCC3193; Nip⁻ mutant SCC3200; Nip⁻ mutant SCC3200 carrying pBluescript; Nip⁻ mutant SCC3200 carrying pNip (cloned gene under lac promoter in pBluescript). Results are presented as weight of macerated tissues 3 days after inoculation. Columns represent mean values ($\pm$ standard deviation) of eight potato plants inoculated with either SCC3193 and SCC3200 or SCC3200 carrying pBluescript and SCC3200 carrying pNip. Assay was repeated three times with similar results.

Table 2. Identification of homologues to *Erwinia carotovora* subsp. *carotovora* necrosis-inducing protein (Nip) in databases by BLASTP and TBLASTN search

Organism	Name of the protein ^a	Gene bank ID	Homology identity/similarity (%) ^b	E value
Bacteria				
<i>E. carotovora</i> subsp. <i>atroseptica</i>	Necrosis-inducing protein	50122010	90/93	e-134
<i>E. chrysanthemi</i>	...	NA	71/84	e-114
<i>Vibrio pommerensis</i>	Hypothetical protein	14331088	56/71	7e-96
<i>Streptomyces coelicolor</i> A3(2)	Putative secreted protein	14331088	38/54	2e-36
<i>Bacillus halodurans</i>	Necrosis- and ethylene-inducing protein	15612958	27/39	9e-10
Eukaryotes				
<i>Phytophthora sojae</i>	Necrosis-inducing-like protein	21327103	34/45	9e-16
	Necrosis-inducing-like protein	21327104	30/41	5e-13
	Necrosis-inducing-like peptide	12698672	27/39	1e-09
<i>P. parasitica</i>	Necrosis-inducing protein NPP1	13346474	31/43	9e-15
<i>P. infestans</i>	Necrosis-inducing protein NPP1	13430412	31/42	3e-14
<i>Pythium aphanidermatum</i>	25-kDa protein elicitor PaNIE	5834379	32/41	3e-11
<i>Neurospora crassa</i> OR74A	Hypothetical protein	21327103	36/50	3e-22
<i>Magnaporthe grisea</i> 70-15	Hypothetical protein	38101840	34/52	6e-25
	Hypothetical protein	38102735	32/45	1e-17
	Hypothetical protein	38108306	31/44	4e-14
	Hypothetical protein	38099500	29/42	1e-11
<i>Aspergillus fumigatus</i>	...	NA	26/39	6e-15
	...	NA	26/41	5e-06
<i>A. nidulans</i> FGSC A4	Hypothetical protein	49092022	27/42	2e-10
	Hypothetical protein	49091774	27/39	7e-09
<i>Fusarium oxysporum</i> f. sp. <i>erythroxyli</i>	Necrosis- and ethylene-inducing peptide Nep1	2697132	28/42	6e-07
<i>Gibberella zeae</i> PH-1 (<i>F. gramineae</i>)	Hypothetical protein	46115104	33/46	8e-21
	Hypothetical protein	46126819	32/43	1e-17
	Hypothetical protein	46139957	30/47	4e-15
	Hypothetical protein	46123279	28/41	6e-07
<i>Verticillium dahliae</i>	Necrosis- and ethylene-inducing peptide	42742373	27/42	4e-11

^a NA = not available, refers to unannotated genomic sequence from where the homologous protein sequence has been identified with TBLASTN search.

^b Homology to *E. carotovora* subsp. *carotovora* Nip.

necrotrophic phase of the life cycle of the fungus (Qutob et al. 2002). Homologous proteins can be found by BLAST searches from the genomic sequences of *Magnaporthe grisea*, two *Aspergillus* spp., and *Gibberella zeae*, all of them having more than one homologue. Homologous sequences also exist in nonpathogenic organisms *Streptomyces coelicolor*, *Neurospora crassa*, and *Bacillus halodurans*, suggesting that the function of the protein is not restricted to pathogenesis. It was shown by Qutob and associates (2002) that only the homologous proteins of some of the plant pathogens caused cell death in plants. The presence of the Nips in soft-rotting *Erwinia* spp. and in fungal and oomycete pathogens of plants suggests that the interactions of these pathogens with their hosts share similar features. The common feature may be a necrotrophic lifestyle in at least one phase of the life cycle.

A 24-kDa necrosis- and ethylene-inducing protein has been purified from culture filtrates of *Fusarium oxysporum* f. sp. *erythroxyl* (Bailey et al. 1997). It causes cell death resembling HR in plants even when applied as foliar spray (Jennings et al. 2000). Similar proteins have been characterized from *Pythium aphanidermatum* and several *Phytophthora* spp., indicating the presence of a novel family of elicitor proteins (Veit et al. 2001). These proteins have been shown to cause induction of plant defense mechanisms, alteration of host plasma membranes and chloroplast structure, and induction of a wide array of stress-related genes (Fellbrich et al. 2000, 2002; Keates et al. 2003). The homologue in *Pythium aphanidermatum*, PaNie, was shown to cause DNA fragmentation in carrot protoplasts and induction of PR1 protein in *Arabidopsis thaliana*, suggesting that these elicitors cause induction of programmed cell death in plant tissue. However, no resistance to pathogens after elicitor treatment has been reported. It is unclear at the moment whether the cell death induced by these elicitors is an HR or necrosis.

The function of the fungal and oomycete necrosis- and ethylene-inducing proteins, apart from the induction of plant defense mechanisms and cell death, is unknown. The presence of a homologue in a hemolysin-encoding region in *Vibrio* spp. suggests that it may be needed for attachment or have some other activity on cell membranes of the host, activities which also are associated with harpins. Overexpression or lack of the protein did not affect the virulence of *F. oxysporum* f. sp. *erythroxyl* on its host plant, *Erythroxylum coca* (Bailey et al. 2002). A mutant strain deleted for most of the *Erwinia carotovora* subsp. *carotovora* nip gene seemed similarly fully virulent on axenic tobacco plants and on several plants representing a wide variety of possible host plants of *E. carotovora* subsp. *carotovora*. It also, apparently, was fully virulent on potato stems; whereas, in potato tubers, the mutant was severely reduced in virulence. It is not known if the virulence phenotype and the necrosis seen in tobacco leaf tissue are caused by the same function of the protein. Nip may be a membrane-damaging toxin or may have an enzymatic activity which is needed during growth of *E. carotovora* subsp. *carotovora* in potato tuber, whereas cell death in tobacco may be caused by recognition of a pathogen-associated molecular pattern (PAMP) by receptors of the innate immunity system (Nürnberg and Brunner 2002). The elicitation activity of *Trichoderma* xylanase has been shown to be independent of its enzymatic activity and reside in an exposed pentapeptide (Rotblat et al. 2002). Even in bacterial flagellin and oomycete 42-kDa transglutaminase, small internal peptides are responsible for the elicitation activity (Brunner et al. 2002; Felix et al. 1999). The necrosis-inducing proteins are not very conserved except for seven amino acids (GHRHDWE) in the middle of the gene, a sequence possibly representing a PAMP. However, all large deletions in *Phytophthora* protein NPP1 reduced the necrosis-inducing ac-

tivity (Fellbrich et al. 2002), arguing that the necrosis-inducing proteins have some sort of activity, and it is this activity rather than the recognition of the PAMP which is needed for the production of necrosis in leaf tissue.

The *E. carotovora* subsp. *carotovora* Nip protein seem to be produced in conditions known to induce type III secretion. Because all the *Erwinia* Nip homologues contain a Sec-dependent signal sequence, they are most unlikely to be secreted through a type III secretion system. These results suggest that growth on minimal medium combined with low pH and low temperature probably induces the production of a wide array of virulence proteins, which may be secreted out of the bacterial cell by various secretion systems.

MATERIALS AND METHODS

Strains, plasmids, and basic techniques.

E. carotovora subsp. *carotovora* wild-type strain SCC3193 (Pirhonen et al. 1988), its RpoS mutants SCC8002 (Andersson et al. 1999a) and SCC8003 (Andersson et al. 1999b), *E. carotovora* subsp. *carotovora* strain 71 (Zink et al. 1984), and *E. chrysanthemi* strain EC16 (Chatterjee and Starr 1977) have been described earlier. Uncharacterized *E. amylovora* strain was provided by S. W. Hutcheson (University of Maryland, College Park, U.S.A.) and the randomly chosen *E. carotovora* subsp. *carotovora* isolates (strains 6, 8, 12, 17, 24, and 42) and *E. carotovora* *atroseptica* isolates (strains 549, 612, 613, 622, 623, and 844) by P. Persson (Swedish University of Agricultural Sciences, Uppsala, Sweden). Competent cells of *Escherichia coli* strain TOP10 F' were purchased from Invitrogen (Invitrogen AB, Lidingö, Sweden). Transduction of chromosomal markers and plasmids into SCC3193 and its derivatives was done with T4GT7 as previously described (Pirhonen et al. 1991). pBluescript II KS plasmid was obtained from Stratagene (La Jolla, CA, U.S.A.). Plasmid DNA was prepared by using QIAPrep Spin Kit and chromosomal DNA with DNeasy Kit of Qiagen (Hilden, Germany). Potato tubers, cv. Bintje, were obtained from MTT Agrifood Research Finland, and the other vegetables were bought from the local supermarket.

Growth conditions.

Erwinia carotovora subsp. *carotovora* and *E. carotovora* *atroseptica* strains were grown at 28°C and *Escherichia coli* at 37°C on LB medium supplemented with appropriate antibiotics (Ap, 150 µg ml⁻¹; Km, 25 µg ml⁻¹; and Cm, µg ml⁻¹) when needed. For the induction of Nip production, *Erwinia carotovora* subsp. *carotovora* cells were grown for 3 days at 15°C on solid minimal induction medium (Huynh et al. 1989) supplemented with 10 mM mannitol and 0.5% sucrose. *Nicotiana tabacum* cv. Samsun plants used in HR assays were grown in greenhouses at approximately 22°C. Plants were used before flowering. At 1 to 2 days before the HR assays, plants were transferred to constant light. Axenic tobacco plants used for virulence tests were grown on Murashige-Skoog medium in a growth cabinet at 22°C with a 16-h light regime. Potato plants, cv. Bintje, used in the stem virulence assay were grown in the greenhouse until they were approximately 50 cm high.

Leaf-infiltration assay of bacterial cells and periplasmic proteins.

Bacterial strains carrying RpoS mutations were grown to pure cultures on fresh LB plates overnight. Cells were harvested from the plates, spread onto minimal induction plates, and grown at 15°C for 4 days. Approximately 50 mg of the bacterial cells of each strain were collected from the plate and resuspended into 1 ml of 5 mM MES (morpholineethanesulfonic acid) buffer, pH 6.5. This bacterial suspension was injected into tobacco leaf

tissue with a 1-ml syringe. The control cultures were grown for 4 days on minimal plates or 2 days on LB plates before harvesting the cells for the leaf infiltration assay.

Periplasmic proteins of *E. carotovora* subsp. *carotovora* and *Escherichia coli* were collected with modified osmotic shock procedure as described previously (Pirhonen et al. 1991). Periplasmic fractions were filter-sterilized to remove residual cells, and a protease inhibitor cocktail (Complete EDTA free; Roche, Mannheim, Germany) was added. The suspension was diluted 10 times into MES buffer and used immediately for HR assay. When needed, protein preparations were stored in -20°C as aliquots, and more protease inhibitor was added immediately after melting the samples for use. Care was taken that all the solutions that were injected into tobacco tissue were at room temperature.

Protein purification, digestion, and mass spectrometry.

Periplasmic fractions of SCC8003 containing cell death-inducing activity were applied to a cation exchange column in a fast performance liquid chromatography system and washed with 50 mM sodium acetate buffer, pH 5.2. After washing, a 0 to 0.3 M NaCl gradient in 50 mM sodium acetate buffer was added to the column, during which fractions were collected and the protein amounts detected at 280 nm. Fractions were tested immediately for ability to cause HR-like response in tobacco leaf. The two positive fractions were pooled and subjected to gel filtration using 20 mM sodium acetate buffer, pH 5.2, with 150 mM NaCl. Fractions were eluted and tested for cell death-inducing activity in tobacco and run on a 12% PAGE gel. Two active fractions, corresponding to a peak eluted out of the column when 1.6 ml of buffer had run out, showed only one band in silver-stained gel (Shevchenko et al. 1996). This band immediately was cut out of the gel and subjected to tryptic cleavage in gel (Wilm et al. 1996). Mass spectrometric analysis of the peptides was performed in a Micro-mass Q-TOF electrospray instrument (Manchester, U.K.). After the initial peptide scanning, several peptides were selected for further analysis by fragmentation. Biolyx Peptide sequencing software was used for the final analysis of the fragmentation pattern.

PCR.

Nested PCR with degenerate primers was used to clone part of the *nip* gene, assuming that the peptides used to design the primers could be in either orientation. First, it was assumed that peptide 1 (FL/IDSSNTL/IHR) was upstream of peptide 2 (PAGYPEFTAASL/I) in the protein. Chromosomal DNA of the strain SCC3193 (1 μl) was used as a template together with the outermost primers FORW1 (AAYTTYHTIGAYWSIWSIA AYAC) for peptide 1 and REV7 (ADISWIGCIGCIGTRAAYT CN) for peptide 2 in the first reaction. The PCR reaction mixes contained 1 $\times$ buffer and 20 pmol of each primer, 2 mM each nucleotide, 3 mM MgCl_2 , 2.5 μl of fresh dimethyl sulfoxide, and 2.5 units of *Taq* polymerase in 50 μl of reaction mix. The reaction was run in gradient PCR apparatus with program containing 10 min at 97°C ; 35 cycles with 1 min at 95°C , 2 min at 40 to 60°C (at 2°C intervals), and 1.5 min at 72°C ; followed by a final 10 min at 72°C . The PCR reactions were pooled and 5 μl of this mix was used as a template together with corresponding overlapping nested primers FORW2 (GAY WSIWSIAAYACIHTICAY) and REV8 (GCIGTRAAYTCIGG RTANCC) in gradient PCR with the same program as before.

Because the order of the peptides on the protein was not known, it was taken into account that peptide 2 could be before peptide 1 in the protein. Therefore, a second set of primers, FORW5 (CCIGCIGGITAYCCIGARTTY) and REV3 (CKRTGIADIGTRTTISWISWRTC), corresponding to this lat-

ter order of peptides, was used together with the chromosomal DNA as template with the same program as before, followed by a second PCR using corresponding nested primers FORW6 (YCCIGARTTYACIGCIGCNWS) and REV4 (TTGIADIGTR TTISWISWRTCIAADR) with a 5- μl aliquot of the previous reaction as template. All visible PCR products seen in the gels were cloned by using TOPO TA cloning kit of Invitrogen (Invitrogen AB) and sequenced.

The plasmid pNip containing the *nip* gene under lac promoter of pBluescript was constructed by using PCR primers EXP1 (GGTACCGCAGGCTAAATATATTCGTGTTC) for the 5' end of the *nip* gene and EXP2 (GAGCTCTTATTTTCGATGTCGTTAGACTGG) for the 3' end of the gene. The PCR reaction contained 1 $\times$ buffer, 10 pmol of each primer, 2 mM each nucleotide, 2 mM MgCl_2 , and 2 U of *Pfu* DNA polymerase (Promega Corp., Madison, WI, U.S.A.) in a 50- μl reaction. The PCR was run with the following program: 10 min at 97°C ; 30 cycles with 1 min at 95°C , 1 min at 55°C , and 1.5 min at 72°C ; followed by a final 10 min at 72°C . The PCR fragment was cloned into TOPO TA cloning vector and sequenced before transferring it into pBluescript.

Marker-exchange mutagenesis.

Two 4-kb *Cla*I fragments, containing either upstream or downstream sequences and halves of the *nip* gene in pBluescript, were utilized to construct a deletion derivative of the *nip* gene. The clone containing the upstream sequences and the 5' half of the *nip* gene was cut with two restriction enzymes: *Nhe*I, present at the start of the *nip* gene and *Hind*III, present in the vector. This restriction removed almost the whole 5' end of the *nip* gene. The linearized plasmid was blunted with Klenow and ligated to similarly blunted *Bam*HI fragment of pHP45 Ω -Cm (Fellay et al. 1987). The ligation was plated on LB plates supplemented with Cm to select for the clones which carried the Cm^r gene. From this clone, a large fragment, containing the upstream chromosomal sequence and the Cm^r gene, was cut out of the construct with *Sac*I, present 3.5 kb upstream of the *nip* gene in the chromosomal DNA, and *Eco*RI, present in the vector. A second clone, containing the 3' end of the *nip* gene and the downstream chromosomal DNA, was cut with *Eco*RI and *Sac*I, which deleted almost all the sequence coding for the 3' end of the *nip* gene. This plasmid was ligated with the fragment containing the upstream sequences and Cm^r marker. The final construct contained approximately 3.5 kb upstream DNA followed by 28 nucleotides of the *nip* gene Cm^r marker, 32 last nucleotides of the *nip* gene, and 4 kb of downstream chromosomal DNA. The construct was verified by sequencing the joints between the chromosomal DNA and the insert. This clone was transferred into SCC8003 and used for marker exchange mutagenesis.

For marker exchange mutagenesis the SCC8003 strain, carrying the in vitro mutagenized *nip* gene in a plasmid, was grown in LB medium with Cm at 28°C for 48 h followed by incubation in room temperature for 2 weeks. Bacterial cells were then diluted 1/100 in LB medium and grown overnight at 28°C , then plated to single colonies on LB plates supplemented with Cm. Single colonies were screened for Ap^r . Two Cm^r Ap^s strains were characterized by Southern for the presence of the Cm^R marker and for the absence of the *Cla*I site in the *nip* gene.

Southern blot analysis.

Chromosomal DNA, 2 μg of each bacterial strain, was cut with *Hind*III and *Xho*I, run in 1% agarose gel, and blotted onto Nylon membrane (Hybond-N; Amersham International, Buckinghamshire, U.K.). The presence of the *E. carotovora* *hrpN* gene in bacterial strains was studied by using part of the cod-

ing sequence (bases 751 to 891) of the *hrpN* homologue of *E. carotovora* subsp. *carotovora* strain 71 as probe. The hybridization was done at 55°C by using α -³²P-labeled DNA as probe, and the same temperature was used when washing the filter twice for 30 min in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)-0.1% SDS before exposing it on film. Essentially the same conditions were used in hybridizations with *nip* gene as a probe. When the *Nip*⁻ marker-exchange mutant was verified with Southern by using the *nip* coding sequence and *Cm*^r marker as probes, the hybridizations and washings were performed at 65°C.

Virulence assays.

SCC3193 and its mutant derivatives were grown overnight on LB plates supplemented with appropriate antibiotics when needed. Potato tubers and other vegetables were rinsed with tap water and dried, then stabbed to a depth of 1 cm with a sterile pipette tip. Visible amount of bacterial culture was transferred from the plate with a sterile toothpick into the wound, which then was sealed with sterile vaseline. Each potato tuber or vegetable was inoculated with the wild-type strain, the *Nip*⁻ mutant SCC3200, and a negative control consisting of a non-inoculated wound. The inoculated vegetables were placed on wet paper tissues on a tray, sprayed with sterile water, and kept in the dark at room temperature. Results were scored after 3 to 4 days by cutting the vegetables in halves and weighting the macerated tissue removed from the sites of inoculation. Each vegetable was inoculated in several replicas and the tests were repeated two to five times.

Potato stems of greenhouse-grown potato plants were inoculated with visible amount of fresh plate-grown cells, 10 cm from the top, after which the plants were sealed in plastic bags and kept at room temperature in dim light. Results were scored 3 to 4 days later. At least five stems were inoculated with each strain and the tests were repeated four times. Axenic tobacco plants were sprayed with sterile water, wounded on three leaves, and inoculated by pipetting approximately 10 µl of overnight liquid culture, containing 2×10^7 bacteria, on each wound. The inoculated plants were kept in plastic growth chambers in dim light at room temperature, and the symptoms were followed daily.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

The Wellcome Trust Sanger Institute *Erwinia carotovora* blast server:
www.sanger.ac.uk/cgi-bin/blast/submitblast/e_carotovora
 The Institute for Genomic Research *Erwinia chrysanthemi* search engine:
tigrblast.tigr.org/ufmg/index.cgi?database=e_chrysanthemi|seq