Research Note

An *hrcU*-Homologous Gene Mutant of *Xanthomonas campestris* pv. *glycines* 8ra That Lost Pathogenicity on the Host Plant but Was Able to Elicit the Hypersensitive Response on Nonhosts

Changsik Oh,¹ Sunggi Heu,² Jae-Yong Yoo,¹ and Yongsup Cho¹

¹Department of Agricultural Biology, College of Agriculture and Life Sciences, Seoul National University, Suwon, Korea; ²Division of Plant Pathology, National Institute of Agricultural Science and Technology, Suwon, Korea

Accepted 6 April 1999.

Transposon mutagenesis was used to isolate nonpathogenic mutants of *Xanthomonas campestris* pv. *glycines* 8ra, which causes bacterial pustule disease in soybean. A 6.1-kb DNA region in which a mutation gave loss of pathogenicity was isolated and found to carry six open reading frames (ORFs). Four ORFs had homology with *hrcU*, *hrcV*, *hrcR*, and *hrcS* genes of *Ralstonia solanacearum* and *X. campestris* pv. *vesicatoria*. One nonpathogenic mutant, *X. campestris* pv. *glycines* H80, lost pathogenicity on soybean but was able to elicit the hypersensitive response (HR) on nonhost pepper and tomato plants. This mutant still multiplied as well as the wild type in the leaves or cotyledons of soybean. Although the DNA and amino acid sequences showed high homology with known *hrp* genes, the *hrcU*-homolog ORF is not required for HR induction on nonhost plants, pepper and tomato, or for the multiplication of bacteria in the host plant. This gene was only required for the pathogenic symptoms of *X. campestris* pv. *glycines* 8ra on soybean.

Additional keywords: Tn Ω Km, type III secretion systems, virulence factors.

The ability of bacteria to elicit pathogenicity in susceptible host plants and the hypersensitive response (HR) in nonhost plants is controlled by *hrp* genes (Alfano and Collmer 1996). In most cases, *hrp* genes are organized in a gene cluster spanning 23 to 40 kb of DNA (Bonas 1994; Alfano and Collmer 1997). *hrp* genes are divided into three groups according to function: (i) gene regulation; (ii) elicitor production; and (iii) type III secretion system. Sequence comparison of *hrp* genes that encode secretion systems established a subset of genes that are broadly conserved among animal and plant pathogens, and, therefore, they were recently renamed as *hrc* (hyper-

sensitive response and conserved; Bogdanove et al. 1996). *hrc* genes are considered to encode core components of the protein secretion pathway. In phytopathogenic bacteria, this system controls the secretion of several proteinaceous elicitors of the HR such as harpins (Wei et al. 1992; He et al. 1993; Arlat et al. 1994) and *avr* gene products (Leach and White 1996). Because of the functions of *hrp* genes mentioned above, *hrp* or *hrc* mutants lose pathogenicity on susceptible hosts and do not induce an HR on resistant host plants and/or nonhost plants.

Xanthomonas campestris pv. *glycines* 8ra causes bacterial pustule disease on susceptible soybean cultivars. Hwang et al. (1992) isolated two pathogenicity-related genes, ORF1 and ORF2, from *X. campestris* pv. *glycines* that are homologous to *hrpC3*, *hrpD1*, and *hrpD2* of *X. campestris* pv. *vesicatoria* (Bonas 1994). Besides these, no other *hrp* genes or pathogenicity genes have been reported from this pathogen. Here, we present the isolation and characterization of pathogenicity-related genes from *X. campestris* pv. *glycines* 8ra that have homology to known *hrp* genes.

To isolate the pathogenicity-related genes, *X. campestris* pv. *glycines* 8ra was mutagenized with Tn Ω Km (Felley et al. 1989) and tested on soybean cultivars Paldal and Pella for the loss of pathogenicity. One nonpathogenic mutant of *X. campestris* pv. *glycines* 8ra (of 2,000 tested) was isolated and named *X. campestris* pv. *glycines* H80. The growth rate of *X. campestris* pv. *glycines* H80 was not different from that of the wild-type strain in both nutrient broth and the host plant soybean cv. Paldal (Fig. 1). Though the mutant strain was not able to cause disease on the host plant, it was still able to cause the HR on the nonhost plants pepper (*Capsicum annuum* L. 'Chogwang') and tomato (*Lycopersicon esculentum* Mill. 'Seogwang'), as was the wild type (Fig. 2). Both mutant and wild-type strains were not able to induce the HR on the nonhost plant tobacco (*Nicotiana tabacum* cv. Samsun). The mutant could not be distinguished from the wild type with several different physiological and biochemical tests, and cellulolytic and proteolytic activities of the mutant were similar to those of the wild type. A Southern hybridization

Corresponding author: S. Heu; Current address: Division of Molecular Genetics, National Institute of Agricultural Science and Technology, Suwon, Korea; Telephone: 331-290-0341; Fax: 331-290-0392; E-mail: heu@niast.go.kr

Fig. 1. Growth patterns of *Xanthomonas campestris* pv. *glycines* 8ra and H80 in cotyledons of soybean. Cotyledons were inoculated with 10^4 CFU/ml of each bacterial strain. Multiplication assays were performed by grinding each cotyledon after measuring the weight in 1 ml of phosphate buffer (10 mM, pH7.5), followed by serial dilution and plating of the samples onto agar plates with appropriate antibiotics. The values reported are the means of three replicates. Error bars indicate the standard deviation.

experiment with Tn Ω Km as a probe showed that only a single copy of Tn Ω Km was inserted in the genomic DNA of the mutant (data not shown).

To identify gene(s) responsible for the loss of pathogenicity, isolated total DNA of the mutant was digested with *Eco*RI and religated to produce the plasmid with the characteristics of Tn Ω Km. The ligated plasmid was transformed into *Escherichia coli* DH5 α and kanamycin-resistant colonies were selected. Selected kanamycin-resistant colonies contained a plasmid carrying a 6.1-kb DNA fragment from *X. campestris* pv. *glycines* 8ra flanked with Tn Ω Km and this plasmid was named pH80. In order to isolate wild-type genomic DNA corresponding to this selected region, 3,000 clones from a genomic library of *X. campestris* pv. *glycines* 8ra were screened by colony hybridization with pH80 as a probe. Two clones were selected and designated pCS1 and pCS2. Each had about 29 kb of genomic DNA inserted in the plasmid vector pLAFR3. Restriction enzyme analysis (Fig. 3A) and Southern hybridization experiments showed that the two clones were overlapped in the 6.1-kb *Eco*RI-*Eco*RI fragment of pCS2 and the 4.8-kb *Eco*RI-*Hind*III fragment of pCS1.

The 6.1-kb *Eco*RI-*Eco*RI fragment carrying the DNA region for the loss of pathogenicity of the mutant *X. campestris* pv. *glycines* H80 was subcloned into pBluescript II SK⁺ (Stratagene, La Jolla, CA) to create pCS2E2 and the nucleotide sequence of both strands in the entire fragment was determined (Figs. 3B and 4). Six open reading frames (ORFs) were found to extend from the left-hand *Eco*RI site to the right-hand *Eco*RI site (Fig. 3B). The ORFs were predicted to encompass nucleotides 124 to 1,197, 1,206 to 3,128, 3,151 to

Fig. 2. Responses of different plants to *Xanthomonas campestris* pv. *glycines* 8ra and H80. **A**, Disease symptoms on host plant, soybean (*Glycine max* L. 'Paldal'). Primary leaves of soybean seedling were inoculated with 10^8 to 5×10^8 CFU/ml of indicated bacteria and photographed 3 days after infiltration. Hypersensitive response (HR) on nonhost plants (**B**) pepper (cv. Chogwang) and (**C**) tomato (cv. Seogwang). Fully expanded leaves of these plants were inoculated with 10^8 to 5×10^8 CFU/ml of the indicated bacteria and photographed after 24 h. **A** and **B**: 1, *X. campestris* pv. *glycines* 8ra; 2, *X. campestris* pv. *glycines* H80; 3, *Pseudomonas syringae* pv. *syringae* Pss61; 4, 10 mM phosphate buffer; 5–7, *X. campestris* pv. *glycines* H80 (pCS2HBCEP). **C**: 1, *X. campestris* pv. *glycines* 8ra; 2, *X. campestris* pv. *glycines* H80; 3, *P. syringae* pv. *syringae* Pss61; 4, 10 mM phosphate buffer; 5, *X. campestris* pv. *glycines* NP1; 6–8, *X. campestris* pv. *glycines* H80 (pCS2HBCEP).

3,765, 3,895 to 4,809, 4,838 to 5,443, and 5,448 to 5,708, and were designated ORF4, ORF5, ORF6, ORF1, ORF2, and ORF3, respectively. It had been reported that the conserved DNA sequence known as a PIP-box (TTCGC-15N-TTCGC) was found upstream of most transcriptional units of the *hrp* gene cluster isolated from *X. campestris* pv. *vesicatoria* and *Ralstonia solanacearum* (Bonas 1994; Fenselau and Bonas 1995). Since PIP-boxes were found upstream of ORF1 and ORF4, these genes appear to form two different transcriptional units. The putative amino acid sequence and ribosome-binding sites of all genes are shown in Figure 4. *X. campestris* pv. *glycines* H80 was determined to have a 3.4-kb Tn Ω Km insertion between 842 and 843 bp, which is in the middle of ORF4. The DNA region including ORF1 and ORF2 was the same region reported to carry ORF1 and ORF2 by Hwang et al. (1992). However, the DNA sequencing results with both strands showed some nucleotide sequence differences from those of the reported ORF1 and ORF2. In our DNA sequence, with the DNAsis program (Hitachi Software, Yokohama, Japan), two putative translational starting codons, GTG and ATG, at the 3,895 and 4,003 bases, respectively, were found in the same frame of ORF1. If the putative PIP-box found in the 3,624-bp region is considered, the upstream GTG codon is more reasonable than the first ATG codon, which is reported as the putative translational start codon for ORF1 by Hwang et al. (1992). Also, the termination codon of ORF1 appears from our analysis to be at 4,809 instead of 4,541 nucleotides. Therefore, the predicted ORF1 extends from 543 to 915 nucleotides and encodes 304 amino acids. We also interpret our data to support changing the starting codon for ORF 2 from the previously reported ATG at 4,796 bp to a new ATG at 4,838 bp.

In order to determine if the nonpathogenic mutant phenotype could be complemented with this region, the subclone pCS2HBCP carrying the 9.5-kb DNA region from the *Hind*III site to the *Bgl*III site in the plasmid vector pCPP30 was constructed (Fig. 3; Deng et al. 1998) and transferred into *X.*

campestris pv. *glycines* H80. The transconjugant carrying the pCS2HBCP regained pathogenicity on soybean plants (Fig. 2) to the wild-type level.

The amino acid sequences of six deduced polypeptides were compared with sequences in GenBank by the BLAST program (Altschul et al. 1990) and the DNAsis program (Hitachi Software). Homologous regions were aligned with the CLUSTAL V program (EMBL Lab, Heidelberg, Germany). The similarities of these proteins with those previously reported are summarized in Table 1. The putative polypeptides of ORF4, ORF5, ORF6, ORF1, ORF2, and ORF3 were similar to HrpN (50.7% identity/77% similarity), HrpO (62% identity/74% similarity), HpaP (29.4% identity/57.4% similarity), HrpQ (31% identity/60% similarity), HrpT (81% identity/91% similarity), and HrpU (63% identity/73% similarity) of *R. solanacearum*, respectively. In the case of ORF5, the amino acid sequence had the highest identity (87%) and similarity (94%) with the amino acid sequence for the *hrpC2* gene isolated from *X. campestris* pv. *vesicatoria*. Since *hrpC2* is the only published data from *X. campestris* pv. *vesicatoria* in this region, it was not possible to compare the sequence of other ORFs with *hrp* genes isolated from *X. campestris* pv. *vesicatoria*. In general, these proteins were more similar to those of *R. solanacearum* and *X. campestris* pv. *vesicatoria* than to those of *P. syringae* pv. *syringae* and *E. amylovora* among phytopathogenic bacteria.

The putative ORF4 polypeptide was similar to YscU (34% identity/61% similarity) of *Yersinia* spp., SpaS (29% identity/60% similarity) of *Salmonella typhimurium*, and Spa40 (28% identity/56% similarity) of *Shigella flexneri*. Ysc proteins are involved in the translocation of Yop proteins or virulence factors in *Yersinia* spp. and Spa proteins are involved in the secretion of invasion antigens to the bacterial surface in *Salmonella* and *Shigella* spp. Putative proteins encoded by ORF5, ORF2, and ORF3 were similar to LcrD, YscR, and YscS in *Yersinia pestis* and InaV, SpaP, and SpaQ in *Salmonella typhimurium*, respectively. Putative ORF4, ORF1,

Fig. 3. A, Restriction enzyme maps of pH80, pCS1, pCS2, pCS2HBCP, and pCS2E2. Restriction enzyme site abbreviations: E, *Eco*RI; H, *Hind*III; B, *Bam*HI; V, *Eco*RV; P, *Pst*I; Bg, *Bgl*III; (v), an enzyme site on the vector. **B,** Open reading frames (ORFs) of pCS2E2. ORFs in pCS2E2 are indicated as boxes and arrow boxes. Horizontal arrows indicate transcriptional units.

EcoRI ----- PIP box -----
GAATTTCGCTGGCGAACAACCATTCGCGCTTCGCGCAGGCTACCCACACATTCGCGCTGTCAAGCGGATCGTTCCACCACAGCTACCCACAA 90
S/D ORF4 -->
TCGGGGGCGCAGCGATCGCGGAAGGAGGGCAATGTCTCGGAAGAAAAACCGAGAAGCCGACCGAGAAGAACTGCGGGATGCGCGCAGG 180
M S E E K T E K P T E K K L R D A R R
GACGGGGAAGTTCGGTTCAGCCCGGACGTCAGTGCAGCCGCGGTGCTTTTGGCGCGCTAATGGTCATGAAATCGGCCGCGGACTACTTC 270
D G E V P V S P D V I A A A V L F G A L M V M K S A G D Y F
TCCGACCACATGCGCGCATGTATGACGATCGGCTTCGATTTCGCGGAAAAATACACGCGATGCAACGGCAATAAACCGCGCAGCTCGGCCAT 360
S D H M R A L M T I G F D F P E N T R D A T A I N R A L G H
ATCGGTATCCAGGGATGTGTGTATGCTCCCTTGTGCTGGTGGCTGGCGTGGCAGGCGGTGCTTTCCAGACCCGCGCTG 450
I G I Q G L L L M L P L L V A C L V A G V A G G A F Q T G L
AATGCTCGCTCAAGCCGGTGGCGCCAAATTCGATTCCCTCAATCCCGCAACCGGGGTCAAGAACTCTTCTCGTTGCGAAGCCCTCATC 540
N A S L K P V A P K F D S L N P A T G V K K L F S L R S L I
AATCTACTGAAGTGTATCAAGGCCATCTTGATTTGGCGTGGTGTGTGGCGCGGTATCCGCATCTCATGCCGATGATCATGGCCCTT 630
N L L K L I K A I L I G V L W A G I R I L M P M I I G L
GCCTACCAGACACCACCTGACATTGCCAGATGCGCTGGCGGACGCTGGGGATGCTATTGCGATTGGGCGTGTGTGTGTCTGCTGCTG 720
A Y Q T P P D I A Q I A W R T L G M L F A L G V L L F V L V
GGCGCCGACACTGGAGCGTGCAGCACTGGCTGTTTCATCCGCGCAACGAATGAGCAAGGACGAGCAGAAGCGCGAAGTCAAGGAGAGC 810
G A A D W S V Q H W L F I R D K R M S K D E Q K R E V K E S

GAGGGCGACCCGAAATCAAGGGCAAGCGCAAGGAATTTGCCAAGGACATGGTGTTCGCGACCCGCGCAAGCGCTGCGCAAGGCCAAG 900
E G D P E I K G K R K E F A K D M V F G D P R E R V A K A K
GTGATGGTGGTCAACCCGACCATACCGCGTGGCACTGGCTACGAGCGATGACTTCGCGCTACCGCAGGTCTGTCGCAAGGCCGCTG 990
V M V V N P T H Y A V A L A Y E P D D F G L P Q V V A K G V
GACGACGCGCGCTGGAGCTGCGCGCTTCGCGCATAACAGGGCATTCGATCGTTCGCAATCCGCCACTGGCAGCTGCGCTGTATCAG 1080
D I A S T I K S I L L H A E A G H I I E S F G E L V V G G N L
GTTGAATTTGGGCGACGCGTGGCGCAACCACTGTTCGAAACGGTTGCGAGTGGTGTTCGCTGGGTGATGAACCTGGTTCGCGACCCAGC 1170
V E L G D A V P E P L F E T V A V V L R W V D E L G R D H S

ORF5 -->
GACGGCGATGGAGCTCTGCCATGCTAGGAGATCGCGTGCAGGCGACAAGGTATTTTCGCTTACAGCGCGAAGTCGCCATCGCCGCTTGG 1260
D G D G A L P C * M R A T R Y F A Y S G E V A I A A L V
TGGTGGCGGTTCATCGGGCTGATGATCCTGCCGCTGCCAACGCCGCTGATCGATACGCTGCTGGGCATCAACATCACCTTGAGCGTGGTGC 1350
V A V I G L M I L P L P T P L I D T L L G I N I T L S V V L
TGTTGATGGTCACGATGATGTGCGCGACTCGATCTCGCTCTCGTCTTTCCTTCCCTGCTGCTGTTCACACACTGCTGCGGTTATCCT 1440
L M V T M Y V P D S I S L S S F P S L L L F T T L L R L S L
TGAATATCGCTCGACGAAGTCGATTTCTGCTGCACGCGGAGGCGGGCCATATTCGAGAGCTTTGGCGAGTTGGTGGTTCGCGCGCAATC 1530
N I A S T I K S I L L H A E A G H I I E S F G E L V V G G N L
TGGTGGTAGGCTGCTGCTTTTCITGATCATCACCGTGCAGTTCATGTCGTCGCAAGGGCTCCGAACGCGTTGCCGAGGTGCGGTG 1620
V V G L V V F L I I T T V Q F I V I A K G S E R V A E V G A
CACGCTTCACGTTGGACGCAATGCCCGTAAGCAGATGAGTATCGATGCGCGACCTGCGCGCGCGCAACCTGACCCGAGATGAAGCAGGCC 1710
R F T L D A M P G K Q M S I D A D L R G G N L T A D E A R R
GCAACCGCGCGCGCTGCCATGGAAAGCCAACTGCACGCGCGCATGGACGCGGCCATGAAATTCGTCGCAAGGGTGTGCGATCGCGCGCC 1800
K R A R L P M E S Q L H G G M D G A M K F V K G D A I A A L
TGGTAATTACCATGGTCAACATCTGCGCGGCATCGTGGTGGCGGTGACGTACACGCGATGACCGCGGCGCAAGCGGCCAACCCTTTG 1890
V I T M V N I L A G I V V G C T T Y H G M T A G E A A N R F A
CGATCTGCTGCGTGGGATGCCATGGTGTGCGAGATTGCTGCTGATATCGGTGGCTGCGCGCGCTCATGATCACCCGCGTGGCCA 1980
I L A S V G D A M V S Q I A S L L I S V A A G V M I T R V A N
ACGAGAACGAGACAAGGCTCAGCTCGCTCGGGCTCGATATCGGCGCTCAGCTCACCAGCAATGCGCGCGCCCTGATGGCAGCGAGCGTGC 2070
E N E T L S S L G L D I G R Q L T S N A R A L M A S V L
TGCTGGCTGCTTTGCGTTCGTCCTCGATTCCCGCGCGTGTGTTCTGCTGCTGGCAGCGCGCTGCGCGCGCGGCTATACGATCT 2160
L A C F A F V P G P A V L F L L L A A A V G A G G Y T I W F
GGCGCAAGCAACGGATATCAGCGGCACCGATCAGCGCAAACTGCTTCGAGAGCCGTAAAGGCGCCAAAGGCGAGGCGCGCACATTC 2250
R K Q R D I S G T D G R K L P S T S R K G A K G E A P H I R
GCAAAAACGCCCTGATTTCGCTCACCCCTGTCGATGCTGCTGTCGCGCAGCTGGCCAATCTGCTCGACCCCGCGCGCTTGTATCAGG 2340
K N A P D F A S P L S M R L S P Q L A N L L D P A R L D Q A
CGATCGAAAGCGAGCGCGCAAGTTGGTCGAGTTGCTGGGCTTTGCCGGAATCGGCATATGGCAGAGCGAGTCCCTGCGAGGACA 2430
I E S R R K L V E L L G L F P F P G I G I W Q S E S L Q D M
TGCAATACGAGGTGTTGATTACAGATGTCCCGGAGACACGCGCGCAATTTGGGGGACACCGACGACATGCAAGCGCGCTGGCGCGCCAGG 2520
Q Y E V L I H D V P E T R A E L G D T D D M Q A A L A R Q A
CCATCGCACCCCTGCACGCGCGCACGCACTGTTTCGTCGGCATCCAGGAACCCAGTGGATGCTGGAGCAGGTGGCTGTGGACTATCCCG 2610
I A P L H A R T H L F V G I Q E T Q W M L E Q V A V D Y P G
GGCTGGTTGCGGAGGTCAACAAGCGCATGCCCGCACACGCATTCGCGATGTGTTCGCGCGCTGCTGGAAGAAAGGATTCGCGTGGCA 2700
L V A E V K A M P A Q R I A D V L R R L L L E E R I P V R N
ATATCAAGAGCATCTGGAGAGTCTGGTGGTATGGGTCCGAAGGAAAAGGACCTTCTGATGTTGACCGAATATGTGCGCTGCGATCTCA 2790
I K S I L E S L V V W G P K E K D L L M L T E Y V R C D L S
GCCGTATCTTGGCATACCGCAACCGCAGGCGCGGCGAGTTCGCTGCGGTGATGCTGACCATGCTGTGGAACAACTGATCAGGCAAT 2880
R Y L A H T A T A G T G Q L P A V M L D H A V E Q L I R Q S
CCATTCGCGCGACCGCGCGCGCAATTTCTGCGCCCTTCGCGCGGAGCAGGCGAACCAGCTTGTGCAACAGGTGGAACGCATCTGGGCG 2970
I R A T A A G N F L A L P P E Q A N Q L V E Q V E R I V G D

(continued on next page)

Fig. 4. Nucleotide sequence of 6,125-bp *EcoRI-EcoRI* fragment of pCS2. Dduced amino acid sequence is shown by single-letter codes below. Potential ribosome-binding sites (S/D) preceding six open reading frames (ORFs) are underlined; horizontal arrows indicate orientation of transcription. Vertical arrowhead indicates Tn Ω Km insertion into ORF4. PIP-box (TTCGC-15N -TTCGC) is located upstream of ORF1 and ORF4 and underlined with double line.

ORF2, and ORF3 proteins had some homology with HrcU, HrcQ, HrcR, and HrcS involved in the type III secretion pathway in *Rhizobium fredii*. Putative ORF4, ORF5, ORF1, ORF2, and ORF3 proteins also had some homology with the proteins involved in the flagellar biosynthesis, which are FlhB, FlhA, FliY, FliP, and FliQ of *Bacillus subtilis*, respectively (Table 1).

In this work, Tn Ω Km was used to generate a nonpathogenic mutant, *X. campestris* pv. *glycines* H80. This mutant showed no physiological or phenotypic differences from the wild-type strain except in terms of pathogenicity. The mutant strain H80 was not able to cause disease on its host plant soybean. This phenotype is very similar to those of *pth* (pathogenicity) mu-

Fig. 4. (continued from preceding page)

```

ACCAGGCGCAGCATCCGCTGGCGGTGGTCGCATCGATGGACGTACGCCGCTATGTGCGCCGATGATCGAAGCAGCGCTGACGTGGCTGC 3060
  Q A Q H P L A V V A S M D V R R Y V R R M I E A R L T W L Q
AGGTTTATTCGTCCAGGAATGGGTTCGAGGTGCAATTACAGCGCTCGGAGGGTGGTGGTGTGATGCGCAACCCCGCTCCGTCAT 3150
  V Y S F Q E L G S E V Q L Q P I G R V V V *
ORF6 -->
GTGCGCATCCCTGCGGCTCAGCGGCGCACTGCAACGGCGCGGCTCTGCCACGCGCGGCTCGGCGCAGCGCTCCAGTCTCTGCAA 3240
  M R I L P V S G A L Q R P A A P A T P A R S A Q R S S F L Q
CTGCGCCAGCGACTGCGCAGCGCGCAGCTCGCGCTTCCCTGCGATGCTGTTGCCGCGCAGTGCGACGAGGATCGACCGGAGCCCGATGCC 3330
  L R Q R L R S A Q L A L P C M L L P P Q C D E D R P E P D A
GAGGACAGGTTACCGAAGCCATGACGGCGCGCCGCAACAGGACCGCGGATTACCTGTCGATGGCAGCAATACCAAGAGCCACTC 3420
  E D R F T E A H D G A P A T T D P A L P V D G T K Y Q E P L
CAAGGCACCGCAATGGCGCGTGGGCGACATATCGCCACCGAGTGGATACGCACGAGCGCGCCAGATGGCGATCGACACATCGCG 3510
  Q G T D N G A V G R H I A T E W I R T Q R A Q M A I D H I A
CTGCGGGTGGCGGAGTCTGCAACGCCACGCGAGTGGCGAGCGAGGAGCTGGGAAGCCTGGCTGGATATCGATCAAGGGCTCGTCGCA 3600
  L R V A E F C N A T P V R S A G S W E A W L D I D Q G L V A
CAGACGAGTGTGTTTTCGCGCTTTCGCGGACGAGTATCGCTTCGCTTCAATACAGTTCGCGGAGCGCGCGGAGGTACTTTGGTGC 3690
  Q T T L F L R L S P D Q L S L R F N T S S P D A R E V L A
GGAAGGCGCTGAGGCGTGCATGAACTCCACGCTGAGTAGCAGCTTCCAGATCAGCATCGAGGTTGTCTAACGCGGCAGTGGCGAC 3780
  G R Q R L E A A L N S T L S S T L Q I S I E V V *
AGAAAGCCATTTGGAGGAATCCATCTGTCTAACCGAGCAGAGCCAGGCTCCACCGGCACGTGCACTCTCTCAGCGTTGACGCGTTGCG 3870
ORF1 -->
CCCCGAGCGCGCACAGCTGAGCAGGGTGTTCGGCGATCCACGCGCGCATGCCAATGCGGCTTTGCGACGCACTGCCGCTCGCATCCACCC 3960
  M F G D P R A A C Q C G F A T H C R R I P P
GATGAGGCGGCGCCCTGCGCCTGCAGTTGGACACCGGCCAGATGGATGTGCGGATTGCCGCGCGCAGCGGCTGGCGCTGCTACTGAAC 4050
  D E A A R L R L Q L D T G Q M D V R I A A R D G L A L L L N
GAGGACGACCACGCACTCCGCGTTTCGATCGCGGGGATATTACTGGCCGATCGCCTCGCGGCTCTTTCGCGCGTGGGCTGGCGCTGCC 4140
  E D D H A L R V S I A G I L L A D R L G A L A P L G L G A A
GAGGTCACTCGCTTCGAGCGCGATGACAGAGCCAGACGACTGCCACGGCATCGGCATCACACTGGGCGATCTCGATGCGATTCGCTGACG 4230
  E V I A F E R D A E P D D C H G I G I T L G D L D A I A L T
GCAAGCGCCCCCTGCTGGCCACGCTGCGGACCGCAGTTGCCGCTTGGCGGCGCGCGCCACTGCCAGTCTGGCTGGCCGCACTGCGC 4320
  A S A P L L A T L R T A V A A L A A P A P L P V W L A A L R
GTCAACACGCGCTGCGCATCGGCGAGCGCACCGCATCGGCGCGCTGTGTCAGTGCCTGCGGCTGGCGATGTTGCTGCTGCTGCAAGC 4410
  V N T R L R I G E R T A S A A L L Q S L R P G D V L L H C T
GCCTCGGCTGCGGCCACCGCGCGAGGTGCTTGGGGTATCGCGGGGCGCGGTGTTGCGCGCTCCGGTACGCGTGAACCTGCAACAG 4500
  A S A A A T S G E V L W G I A G A V L R A P V R L N L Q Q
ATGATTTTGGAGGCCACCCCAACATGCAGCAGATACGTTTGAAGCCGAAGTCGCACAGTCCGCCAGCAACGTTGCGGAACGGAACG 4590
  M I L E A A T P T M Q H D T F E P E V A Q S A S N V A E L E L
CCGGTGCAGCTGGAAGTGGACCAACTTTCGCTGTCTATCGACGTTGTGCGGTTTGCAGCCCGGGCAGATCTGGAGTTTTCGCTGCGCG 4680
  P V Q L E V D Q L A L S L S T L S G L Q P G Q I L E L S V P
GTGGATCAAGCCGACATCCGCTGGTGGTTTACGGCCAGACCATCGGCATCGGCAGGTTAGTGACAGTGGGCGAACATCTTGGCGTGCAG 4770
  V D Q A L M A M S L V V Y G Q T I G I G R L V T V G E H L V Q
ORF2 -->
ATCCTCAGCATGTGCGAGAGCAGCATGCAGATGCCTGAGCTGGCTCGCTGCTGCTGGTGGTTCATCATGTGGGCTGCTGCCCTTCGC 4860
  I L S M S E S T H A D A * M L G L L P F A
AGCGATGGTGGTACCTCCTACCAAGATCGTGGTGGTGTGCGCTGCTGCGCAATGCGATCGGGGTACAGCAGGTGCCGCCGAACAT 4950
  A M V V T S Y T K I V V V L G L L R N A I G V Q Q V P P N M
GGTCTCTCAATGGCGTTGCGTTGCTGGTGTGCTGCTTGTGATGGCACCGGTGGGTATGGAGGCGTTCAAGGCCCGCGCAGAACTACGG 5040
  V L N G V A L L V S C F V M A P V G M E A F K A A A Q N Y G
TGCCGGGTCCGATAACAGCCGTGTGGTGGTCTGCTCGACGCTGCCGGGAGCCGTTTCGGCAATTCCTCTCAAACACACACCGGAACG 5130
  A G S D N S R V V V L L D A C R E P F R Q F L L K H T R E R
CGAAAGGCCCTTTTCATGCGTTTCAGCACAACAGATCTGGCCCAAGGACAAGGCCGCCACGCTCAAGTCGGACGACCTGCTTGTCTTGGC 5220
  E K A F F M R S A Q Q I W P K D K A A T L K S D D L L V L A
GCCGCGCTTCACGCTCAGCGAATTGACCGAAGCCTTCGCGATCGGCTTTCTGTTGATCTGGTGTATATCGTCATCGACCTGGTGGGC 5310
  P A F T L S E L T E A F R I G F L L Y L V F I V I D L V V A
CAACGCCCTGATGGCGATGGGCTTTTCGAGGTCACCCGACCAATGTGGCGATCCCATCAAGCTGCTGCTGTTTGTGCAATGGATGG 5400
  N A L M A M S L Q V T P T N V A I P F K L L L F V A M D G
ORF3 -->
CTGGTCAATGCTGATTTCATGGCCTGGTCTGAGCTATCGGTGATGCCATGGACCACGACGATCTAGTGGATTTACCTCCGAAGCGTTGC 5490
  W S M L I H G L V L S Y R * M D H D D L V R F T S E A L L
TGCTCTGCTCAAGGTCTCTTTCGCGGTGGTGGTGTGCTGCGCTGGCGGGCTGCTGATCGCCTTCATCCAGGCGGTGATGTCGCTGC 5580
  L C L K V S L P V V G V A A L A G L L I A F I Q A V M S L Q
AGGACGCACTGATTTCTGCTGCGCTCAAGCTGGTGGTGGTGGCGCCATTCGCGTGACGCGCGCGTGGGCGCGTGGCGCATCATGC 5670
  D A S I S F A L K L V V V V A A I A V T A P W G A S A I M Q
AGTTCGGCCAGGCATGATGACGCGCGCTTCCATGATCCGTCGATTCTCCCGGCACCTTGGCGCCCTCGGTTTCGACCGAGCATGG 5760
  F G Q A L M Q A A F P *
TACCTCGCATCACCATGCGAAGTCCGAGGCACTGGCGCAGCTTCGCGCTACCGACGCTGCAACACACATACCGCGCTTCGCTCTCGCGC 5850
  ACCGCGAGGCGACGACGCGCGCTCCGTTTCGCTGGACGGCCAGGAAGATGAGCTCGATGCCACCGAGCAGGAAGAGAAGCAAGCCAAAGC 5940
  GAGTGTCATCTGCGCGGGCGGGTGAGCGTGGCGCTGCTGCGGCCAAGGGCGTGAGCAGGGCCAGGGCGATCAGCACGGCAGTGGCGC 6030
  CGACGCGCATGCCAGCATCCGACAGACGAGACCGTGGCAAGCTGGCGCGCCACGCGCTTTGAGCGACGGCGTGCATGCACGCATCGACG 6120
  AATTC
EcoRI

```

tants of *X. campestris* pv. *malvacearum* (Chakrabarty et al. 1997) and *dsp* (disease specific) mutants of *Erwinia amylovora* (Bogdanove et al. 1998; Gaudriault et al. 1997). However, the DNA sequence of the mutated region of *X. campestris* pv. *glycines* 8ra showed a high degree of homology with known *hrc* genes. The 6.1-kb DNA region carrying the mutation consists of six ORFs, and these ORFs had a homology with *hrp* or *hrc* genes isolated from other phytopathogenic bacteria. The organization of isolated ORFs in the cluster looks very similar to that of transcriptional units III and IV of the *hrp* gene cluster isolated from *R. solanacearum* (Fig. 5). Sequence data showed that the ORF4 has over 50% homology with HrcU of *R. solanacearum*. HrcU is involved in the *hrp*-dependent type III secretion systems (Kim et al. 1997; Lidell and Hutcheson 1994). All known *hrcU* gene mutants have lost pathogenicity on the host plant and were not able to induce the HR on nonhost plants. However, despite the homology with known *hrcU* gene, the ORF4 mutant of *X. campestris* pv. *glycines* 8ra lost pathogenicity on the host plant soybean, but was still able to induce the HR on nonhost plants, pepper and tomato (Jones and Fett 1985; Schulte and Bonas 1992). It is known that polar mutation is predominant in Tn Ω Km insertions (Felley et al. 1989). Since ORF4, ORF5, and ORF6 are in one transcriptional unit, it is likely that ORF4, ORF5, and ORF6 genes are all untranscribed due to the insertion of Tn Ω Km in ORF4. This suggests that some genes in this cluster, though they have good homology with known *hrp* and *hrc* genes, are involved just in pathogenicity but not in HR induc-

tion on nonhost plants. In support of this, the phenotype of another mutant, NP1, of *X. campestris* pv. *glycines* 8ra that has mutation(s) in ORF1 and/or ORF2 was very similar to those of H80. NP1 was not able to cause disease on its host plant (Hwang et al. 1992). When we tested NP1 on tomato and pepper, it did induce the HR on both plants (Fig. 2).

The mutant H80 was able to multiply as well as the wild type both in liquid culture and in the host plant soybean. Most *hrp* gene mutants cannot multiply in host plants. However, this *hrcU*-homologous gene mutant, *X. campestris* pv. *glycines* H80, was able to grow as well as wild type in the host plant, but it cannot induce disease in that host plant. This suggests that these *hrc/hrp*-homologous genes in *X. campestris* pv. *glycines* 8ra may be required for secretion of virulence factors but not for the induction of HR in nonhost plants. In the animal-pathogenic bacteria, virulence factors such as Yop (*Yersinia* outermembrane proteins) of *Yersinia* spp. and Ipa (invasion plasmid antigens) of *Shigella flexneri* are secreted through the type III secretion pathway. However, in the phytopathogenic bacteria, no virulence factors that are secreted through the *hrp*-dependent type III secretion system are reported. Cloned genes had a homology with *hrcU*, *hrcV*, *hrcQ*, *hrcR*, and *hrcS*, which are major components of *hrp*-dependent type III secretion systems. Therefore, in *X. campestris* pv. *glycines* 8ra, these genes may be components of a secretion system and this secretion system may be involved in the secretion of some virulence factors, like the animal-pathogenic bacteria, without affecting bacterial multiplication

Table 1. Homologs of putative polypeptides of *Xanthomonas campestris* pv. *glycines* 8ra *hrp*-homologous genes

Category and species	Similarity of protein to <i>X. campestris</i> pv. <i>glycines</i> 8ra protein					
	ORF4	ORF5	ORF6	ORF1	ORF2	ORF3
Pathogenic determinants						
<i>X. campestris</i> pv. <i>vesicatoria</i>		HrpC2 (87/94)				
<i>Ralstonia solanacearum</i>	HrpN (50.7/77) ^a	HrpO (62/74)	HpaP (29.4/57.4)	HrpQ (31/60)	HrpT (78/89.5)	HrpU (63/73)
<i>Pseudomonas syringae</i> pv. <i>syringae</i>	HrpY (33/63)	HrpI (33/53)			HrpW (44/69)	HrpO (26/64)
<i>Erwinia amylovora</i>	HrcU (30/57)	HrcV (37/59)			HrcR (40/68)	HrcS (34/70)
<i>Yersinia enterocolitica</i>	YscU (34/61)	LcrD (43/65)				
<i>Y. pestis</i>		LcrD (42/62)		YscQ (25/56)	YscR (50/73.6)	YscS (36/66)
<i>Salmonella typhimurium</i>	SpaS (29/60)	InaV (43/66)			SpaP (39/69)	SpaQ (29/62)
<i>S. flexneri</i>	Spa40 (28/56)	MxiA (43/69)			Spa24 (41/70)	Spa9 (30/60)
Flagellar proteins						
<i>B. subtilis</i>	FlhB (34/62.5)	FlhA (38/58)		FliY (18.7/54.6)	FliP (42/68)	FliQ (28/59)
<i>Y. enterocolitica</i>	FlhB (31.6/60.5)	FlhA (40/65)				
<i>S. typhimurium</i>	FlhB (34/62.5)	FliH (39/63)			FliP (48/70)	
<i>Escherichia coli</i>		FlhA (39/64)			FliP (49/75)	FliQ (24/65)
<i>R. fredii</i>	HrcU (28/54.6)			HrcQ (24/55.6)	HrcR (49/72)	HrcS (30/69)

^a Identity/similarity values are expressed in percentages as estimated by the CLUSTAL V program (EMBL Lab, Heidelberg, Germany).

Fig. 5. Genetic organization of *hrp*-homologous genes from *Xanthomonas campestris* pv. *glycines* 8ra and *hrp* gene cluster of *Ralstonia solanacearum* and *X. campestris* pv. *vesicatoria*. Transcription units (horizontal arrows) and relative positions of the genes are presented. Genes with related encoded proteins are represented by the same shape. Maps were drawn according to Van Gijsegem et al. (1995) for *R. solanacearum* and Fenseleau et al. (1992) for *X. campestris* pv. *vesicatoria*. Boxes are not drawn to scale.

(Cornelis 1994). In *X. campestris* pv. *glycines*, virulence factors such as auxin and cytokinin, toxin, EPS (exopolysaccharide), protease, and cellulase are reported (Fett and Dunn 1987; Paxton 1972). No differences were observed for the production of EPS and proteolytic and cellulolytic activities between wild-type *X. campestris* pv. *glycines* 8ra and mutant *X. campestris* pv. *glycines* H80. Whether or not the secretion of other virulence factors such as auxin, cytokinin, and toxin is different between wild type and mutant, we don't know yet. Those possibilities are under investigation.

ACKNOWLEDGMENTS

We thank I. Hwang for the bacterial strain *X. campestris* pv. *glycines* NP1 he provided as well as his fruitful comments, and S. H. Choi for technical assistance. We also thank S. Hutcheson for the preparation of this manuscript and the sincere discussion for this paper. This work was supported in part by NON DIRECTED RESEARCH FUND, Korea Research Foundation.

LITERATURE CITED

- Alfano, J. R., and Collmer, A. 1996. Bacterial pathogens in plants: Life up against the wall. *Plant Cell* 8:1683-1698.
- Alfano, J. R., and Collmer, A. 1997. The type III (Hrp) secretion pathway of plant pathogenic bacteria: Trafficking Harpins, Avr proteins, and death. *J. Bacteriol.* 179:5655-5662.
- Altschul, S. F., Fish, W., Miller, W., Myers, E. W., and Lipman, D. J. 1990. Basic local alignment search tool. *J. Mol. Biol.* 215:403-410.
- Arlat, M., Van Gijsegem, F., Huet, J. C., Penollet, J. C., and Boucher, C. A. 1994. PopA1, a protein which induces a hypersensitivity-like response on specific *Petunia* genotype, is secreted via the Hrp pathway of *Pseudomonas solanacearum*. *EMBO J.* 13:543-553.
- Bogdanove, A. J., Beer, S. V., Bonas, U., Boucher, C. A., Collmer, A., Coplin, D. L., Cornelis, G. R., Huang, H., Hutcheson, S. W., Panopoulos, N. J., and Gijsegem, F. V. 1996. Microcorrespondence; unified nomenclature for broadly conserved *hrp* genes of phytopathogenic bacteria. *Mol. Microbiol.* 20:681-683.
- Bogdanove, A. J., Kim, J. F., Wei, Z., Kolchinsky, P., Charkowski, A. O., Conlin A. K., Collmer, A., and Beer, A. 1998. Homology and functional similarity of an *hrp*-linked pathogenicity locus, *dspEF*, of *Erwinia amylovora* and the *avirulence* locus *avrE* of *Pseudomonas syringae* pathovar *tomato*. *Proc. Natl. Acad. Sci. USA* 95:1325-1330.
- Bonas, U. 1994. *hrp* genes of phytopathogenic bacteria. *Curr. Top. Microbiol. Immunol.* 192:79-98.
- Chakrabarty, P. K., Duan, Y. P., and Gabriel, D. W. 1997. Cloning and characterization of a member of the *Xanthomonas avr/pth* gene family that evades all commercially utilized cotton *R* genes in the United States. *Phytopathology* 87:1160-1167.
- Cornelis, G. R. 1994. *Yersinia* pathogenicity factors. *Curr. Top. Microbiol. Immunol.* 192:243-263.
- Deng, W., Preston, G., Collmer, A., Chang, C., and Huang, H. 1998. Characterization of the *hrpC* and *hrpRS* operons of *Pseudomonas syringae* pathovar *syringae*, tomato, and glycinea and analysis of the ability of *hrpF*, *hrpG*, *hrcC*, *hrpT*, and *hrpV* mutants to elicit the hypersensitive response and disease in plants. *J. Bacteriol.* 180:4523-4531.
- Felley, R., Krisch, H. M., Prentki, P., and Frey, J. 1989. Omegon-Km: A transposable element designed for in vivo insertional mutagenesis and cloning of genes in Gram-negative bacteria. *Gene* 76:215-226.
- Fenselau, S., Balbo, I., and Bonas, U. 1992. Determinants of pathogenicity in *Xanthomonas campestris* pv. *vesicatoria* are related to proteins involved in secretion in bacterial pathogens of animals. *Mol. Plant-Microbe Interact.* 5:390-396.
- Fenselau, S., and Bonas, U. 1995. Sequence and expression analysis of the *hrpB* pathogenicity operon of *Xanthomonas campestris* pv. *vesicatoria* which encodes eight proteins with similarity to components of the Hrp, Ysc, Spa, and Fli secretion systems. *Mol. Plant-Microbe Interact.* 8:845-854.
- Fett, W. F., and Dunn, M. F. 1987. Auxin production by plant-pathogenic pseudomonads and xanthomonads. *Appl. Environ. Microbiol.* 53:1839-1845.
- Gaudriault, S., Malandrin, L., Paulin, J. P., and Barny, M. A. 1997. DspA, an essential pathogenicity factor of *Erwinia amylovora* showing homology with AvrE of *Pseudomonas syringae*, is secreted via the Hrp secretion pathway in a DspB-dependent way. *Mol. Microbiol.* 26:1057-1069.
- He, S. Y., Huang, H. C., and Collmer, A. 1993. *Pseudomonas syringae* pv. *syringae* HarpinPss: A protein that is secreted via the Hrp pathway and elicits the hypersensitive response in plants. *Cell* 73:1255-1266.
- Hwang, I., Lim, S. M., and Shaw, P. D. 1992. Cloning and characterization of pathogenicity genes from *Xanthomonas campestris* pv. *glycines*. *J. Bacteriol.* 174:1923-1931.
- Jones, S. B., and Fett, W. F. 1985. Fate of *Xanthomonas campestris* infiltrated into soybean leaves: An ultrastructural study. *Phytopathology* 75:733-741.
- Kim, J. F., Wei, Z., and Beer, S. V. 1997. The *hrpA* and *hrpC* operons of *Erwinia amylovora* encode components of a type III pathway that secretes harpin. *J. Bacteriol.* 179:1690-1697.
- Leach, J., and White, F. F. 1996. Bacterial avirulence genes. *Annu. Rev. Phytopathol.* 34:153-179.
- Lidell, M. C., and Hutcheson, S. W. 1994. Characterization of the *hrpJ* and *hrpU* operons of *Pseudomonas syringae* pv. *syringae* Pss61: Similarity with components of enteric bacteria involved in flagellar biogenesis and demonstration of their role in Harpin_{pss} secretion. *Mol. Plant-Microbe Interact.* 7:488-497.
- Parsot, C. 1994. *Shigella flexneri*: Genetics of entry and intercellular dissemination in epithelial cells. *Curr. Top. Microbiol. Immunol.* 192:217-241.
- Paxton, J. D. 1972. Toxin production by *Xanthomonas phaseoli* var. *sojensis*. Page 377 in: *Phytotoxins in Plant Diseases*, Proceeding of the NATO Advanced Study Institute, Pugnuchiuso, Italy, June 1970. R. K. S. Wood, A. Ballio, and A. Graniti, eds. Academic Press, London.
- Schulte, R., and Bonas, U. 1992. Expression of the *Xanthomonas campestris* pv. *vesicatoria* *hrp* gene cluster, which determines pathogenicity and hypersensitivity on pepper and tomato, is plant inducible. *J. Bacteriol.* 174:815-823.
- Srivastava, S. L., and Bias, B. S. 1986. Involvement of pectolytic and cellulolytic enzymes in bacterial leaf pustule of soybean. *Farm Sci. J.* 1:82-84.
- Van Gijsegem, F., Gough, C., Zischek, C., Niqueux, E., Arlat, M., Genin, S., Barberis, P., German, S., Castello, P., and Boucher, C. 1995. The *hrp* gene locus of *Pseudomonas solanacearum*, which controls the production of a type III secretion system, encodes eight proteins related to components of the bacterial flagellar biogenesis complex. *Mol. Microbiol.* 15:1095-1114.
- Wei, Z., Laby, R. J., Zumoff, C. H., Bauer, D. W., He, S. Y., Collmer, A., and Beer, S. V. 1992. Harpin, elicitor of the hypersensitive response produced by the plant pathogen *Erwinia amylovora*. *Science* 257:85-88.