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Sensitive and Pathovar-Specific Detection of *Xanthomonas campestris* pv. *glycines* by DNA Hybridization and Polymerase Chain Reaction Analysis

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Xanthomonas campestris pv. _glycines_ causes bacterial pustule disease on susceptible soybean leaves and produces a bacteriocin, named glycinecinA, against most xanthomonads including _Xanthomonas campestris_ pv. _vesicatoria_. One of the 5 isolated DNA regions responsible for bacteriocin production, a 1.7 kb DNA region for the glycinecinA gene, was used as a probe to detect the presence of the homolog DNA in other bacterial strains. Among 55 bacterial strains tested, only _X. campestris_ pv. _glycines_ showed the positive signal with glycinecinA DNA. Two oligomers, heu2 and heu4, derived from a glycinecinA DNA were used to carry out the polymerase chain reaction (PCR) analysis with chromosomal DNA from 55 different bacterial strains including 24 different strains of _X. campestris_ pv. _glycines_, 9 different pathovars of xanthomonads, and other 22 bacterial strains of different genus and species. By separation of the PCR products on agarose gel, a 0.86 kb DNA fragment was specifically detected when _X. campestris_ pv. _glycines_ was present in the amplification assay. The 0.86 kb fragment was not amplified when DNA from other bacteria was used for the assay. Southern analysis with glycinecinA DNA showed that the PCR signal was obtained with _X. cmapestirs_ pv. _glycines_ isolates from various geographic regions and soybean cultivars. Therefore, the 1.7 kb DNA region for the glycinecinA gene can be used for the pathovar-specific probe for the DNA hybridization and the primers heu2 and heu4 can be used for the pathovar-specific primers for the PCR analysis to detect _X. campestris_ pv. _glycines_.

Keywords : bacterial pustule, bacteriocin, glycinecinA.

Xanthomonas campestris pv. *glycines* causes bacterial pustule on susceptible soybean cultivars. This disease is characterized by small yellow-to-brown lesions with a raised pustule in the center. The lesions are restricted to the leaves and may merge to form large necrotic areas. The disease symptoms are due primarily to hypertrophy of host mesophyll cells, which can result in premature defoliation of infected plants. Yields are lowered because of reduced seed size, and under certain environmental conditions, serious economic losses can occur. A very high level of resistance to the bacterial pustule disease is conferred by a recessive gene designated *rxp*, originally found soybean cultivar CNS (Hwang and Lim, 1998). This resistance has endured for more than 30 years in spite of the fact that it is the only widely used source of resistance. However, once the field is infected, the control of this disease is very difficult and many of Korean soybean cultivars are susceptible to this disease. The only practical control for this disease is the use of disease-free seed and the eradication of infected plants from the field.

X. campestris pv. *glycines* produces the bacteriocin against the related pathogen (Fett et al., 1987). Bacteriocin production has been reported for several genera of plant-pathogenic bacteria: *Agrobacterium*, *Clavibacter*, *Erwinia*, and *Pseudomonas* (Vidaver, 1983). Bacteriocin production was first reported for *X. campestris* pv. *glycines* by Fett et al. (1987); however, none of *X. campestris* pv. *glycines* bacteriocins have been further characterized. Our previous data showed that the *X. campestris* pv. *glycines* 8ra produced the bacteriocin glycinecinA which had antimicrobial activity against most tested xanthomonads and more than one DNA region of *X. campestris* pv. *glycines* 8ra may correlate to the production of bacteriocin glycinecinA (Ahn and Cho, 1996; Woo et al., 1998). Among those, a DNA region responsible for the production of glycinecinA was cloned into the plasmid vector, pBluescriptSK+ and the 1.7 kb DNA region was sequenced in both strands of DNA. There is no known homology of glycinecinA gene to the DNA of other bacteria (Heu et al., 1999). The glycinecinA gene is a good candidate for a potential DNA hybridization probe and a potential PCR target for the detection of *X. campestris* pv. *glycines* since only *X. campestris* pv. *glycines* carries this bacteriocin gene.

Amplification of specific DNA sequences by means of the polymerase chain reactions (PCR) is very available for the detection of small numbers of the target organism (Audy et al., 1994; Frey et al., 1996; Hartung et al., 1993).

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An ideal situation for the application of PCR in many phytosanitary practices and disease epidemiology is presence of DNA sequences which are unique to a given pathogen (Bereswill et al., 1992; Leite et al., 1994; Pan et al., 1997; Schaad et al., 1995). A PCR technique that amplifies a segment of the genomic phaseolotoxin gene cluster by using a single pair of primers was recently developed. The method is specific for *P. syringae* pv. *phaseolicola* (Prosen et al., 1993).

Here we describe the identification of *X. campestris* pv. *glycines* by Southern hybridization with DNA region corresponding to the glycinecinA and confirm that the DNA region responsible for the production of glycinecinA is specific for *X. campestris* pv. *glycines*. PCR analysis using two oligomers, heu2 and heu4, from the DNA sequence of the glycinecinA can be used as a fast, very sensitive, and pathovar specific detection method for the bacterial pustule disease pathogen.

Materials and Methods

Bacterial strains. The *X. campestris* pv. *glycines* isolates and other bacterial species used for PCR analysis are listed in Table 1. Peptone sucrose agar (PSA) and Yeast-dextrose-$CaCO_3$ agar (YDC) were used as general plating media.

DNA manipulations. Total genomic DNA was isolated with phenol extraction and ethanol precipitation essentially as described by Sambrook et al. (1989). Plasmid miniprep was performed by standard procedures (Sambrook et al., 1989).

Hybridization analysis. Total genomic DNA digested with *Eco*RI and amplified DNA fragments were electrophoresed in 0.7% agarose gel by standard procedures (Sambrook et al., 1989). The DNA was then depurinated with 0.25 N HCl for 15 min and denatured in 0.25 N NaOH for 30 min. The denatured DNA was then transferred onto nylon membrane (Amersham Co.) using vacuum blotter (BioRad Co.). The transferred DNA was cross-linked on the membrane with UV for 3 min. The dried memebrane was prehybridized with hybridization solution [5X SSC, N-laurylsarcosine, 0.1% (w/v); SDS, 0.02% (w/v); Blocking reagent, 1% (w/v)] at 68°C for 1 hr. Hybridization was performed with fresh 10 ml hybridization solution containing denatured labelled DNA probe to the final concentration of 10 μl (10 ng/ml) for 100 cm^2 of membrane at 68°C for 16 hr.

DNA fragments used as probes were labelled with digoxigenins as described by the manufacturer (Boehringer Mannheim). The template DNA (2 μg) was denatured for 10 min at 100°C and quickly chilled on ice. The denatured DNA solution was mixed with 2 μl hexanucleotide mixture as a random primer, 2 μl dNTP mixture, and 1 μl Klenow fragment. The solution was incubated for 20 hr at 37°C. To stop the reaction, 2 μl of 0.2 M EDTA was added, and the labelled DNA was precipitated by adding 2.5 μl of 4 M LiCl and 75 μl prechilled (-20°C) 95% ethanol. The mixture was mixed well, incubated for 10 hr at -20°C, and centrifuged for 20 min at 14,000 rpm in a microcentrifuge. DNA pellet was washed with 50 μl of cold 70% ethanol, dried, and dissolved in 200 μl of TE buffer.

Hybridized membrane was washed twice with washing solution I (2X SSC, 0.1% SDS) at room temperature and followed by washing solution II (0.1X SSC, 0.1% SDS) twice at 68°C for 15 min. For the detection of hybridization, the membrane was rinsed with washing buffer [0.1 M maleic acid, pH 7.5; 0.15 M NaCl; 0.3 % Tween 20 (v/v)] for 5 min and incubated in 100 ml of buffer 2 [0.1 M maleic acid, pH 7.5; 0.15 M NaCl; 10% Blocking reagent (w/v)] for 30 min at room temperature. The membrane was incubated in 20 ml of newly prepared buffer 2 added with anti-digoxigenin-AP conjugate to the final concentration of 75 mU/ml, for 30 min at room temperature. The membrane was washed with 100 ml of washing buffer with gentle agitation for 15 min twice at room temperature, and the equilibration of the membrane was performed in 20 ml of buffer 3 (0.1 M Tris-Cl; 0.1 M NaCl; 50 mM $MgCl_2$, pH 9.5) for 5 min at room temperature. The substances, 45 μl NBT and 35 μl X-phosphate, were added to 10 ml of newly prepared buffer 3 and the membrane was immerged in the solution and incubated during the color development at room temperature in the dark. When desired spot or band intensities were achieved, the reaction is stopped by washing the membrane for 5 min with 50 ml of TE (pH 8.0) buffer.

PCR primers. Two oligonucleotides from the borders of ORF1 of the glycinecinA genes inserted in the plasmid pSGEB1 with the sequence 5'-GACCGAAATGTATTCTTGGG-3' (heu2) and 5'-CATTGCGACTAGCAAGG-3' (heu4) were used for amplification by PCR (Fig. 1). All primers were synthesized by Bioneer Co. (Chungbuk, Korea) and used according to company specifications.

PCR amplification and analysis of PCR products. Reaction mixture were assembled on ice and started in a preheated (95°C) PTC-100 programmable Thermal Cycler (Model 60, MJ Research, Inc., Watertown, MA). Each reaction mixture in 25 μl contained 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.3), 0.2 mM each of dATP, dTTP, dGTP, and dCTP, 1 μM each primer, 1.25 units of native Taq DNA polymerase (Perkin Elmer, Foster City, CA), and 25 ng of genomic DNA. Genomic DNA from all listed bacterial strains was tested for specificity of heu2 and heu4. PCR was performed at 95°C for 2 min, 35 cycles of 94°C for 1.5 min, 52°C for 1.5 min, and 72°C for 1.5 min, and final extension at 72°C for 10 min. The amplified DNA fragments were electrophoresed in 1.0% agarose gels in Trisborate-EDTA buffer and visualized with ultraviolet light following ethidium bromide staining.

Results and Discussion

Specificity of the DNA probe. Previous studies (Heu et al., 1999) had showed that the 1.7 kb DNA fragment which is responsible for the production of bacteriocin, glycinecinA, was subcloned into pBluescriptSK+ to make pSGEB1 (Fig. 1). pSGEB1 carries 2 open reading frames (ORFs) and both of them are required for the production of bacteriocin, glycinecinA. Since this 1.7 kb DNA fragment

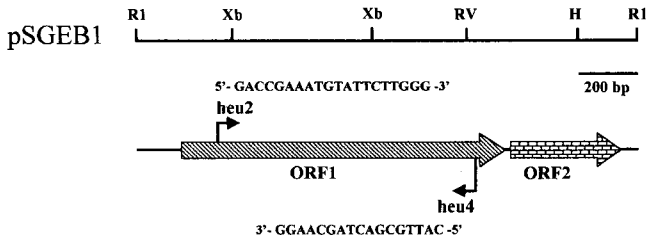

Fig. 1. Schematic diagram of the 1.7 kb DNA fragment which is responsible for the production of bacteriocin glycinecinA. The plasmid vector for the 1.7 kb DNA insert is pBluescriptSK+. The scale (in Kb) and the segments contained in the pSGEB1 are depicted above the physical map of the fragment. Arrows pointing to the right and left indicate the location and orientation of the heu2 and heu4 primers, whose sequence is also given. Two arrow boxes indicate the putative open reading frames for the glycinecinA gene. R1, Xb, RV, and H stand for *Eco*RI, *Xba*I, *Eco*RV, and *Hin*dIII, respectively.

was enough to produce glycinecinA in *E. coli*, this DNA fragment was used to search the presence of homolog DNA in other plant pathogenic or nonpathogenic bacteria. First of all, total genomic DNA from 12 bacterial strains including four *X. campestris* pv. *glycines* strains, *X. campestris* pv. *vesicatoria, X. axonopodis* pv. *citri, X. oryzae* pv. *oryzae*, *X. axonopodis* pv. *aurentifolia*, *X. campestris* pv. *campestris*, *X. arboricola* pv. *pruni, X. axonopodis* pv. *dieffenbachiae,* and *P. syringae* pv. *glycinea* were isolated for southern hybridization and the 1.7 kb insert DNA of pSGEB1 was used as a probe. Among those of 12 bacterial strains, total genomic DNA of four *X. campestris* pv. *glycines* were only hybridized with this probe (Fig. 2). It means that this probe is a pathovar-specific probe. To prove

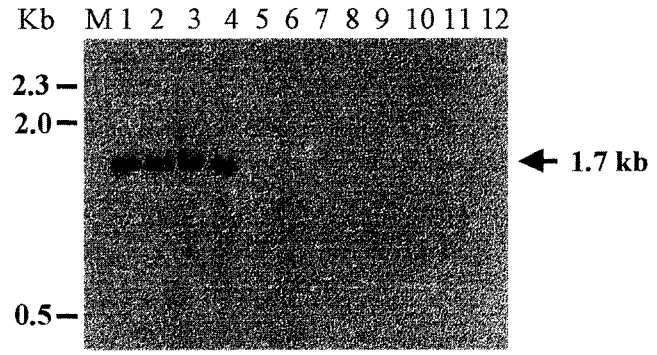

Fig. 2. Southern hybridization analysis of total genomic DNA of several different bacteria. DNA was digested with *Eco*RI, fractionated on a 0.7 % agarose gel, transferred to nylon membrane, and probed with Dig-labeled pSGEB1. Bacterial strains tested are; 1, *X. campestris* pv. *glycines* 8ra; 2, OCSF; 3, OCSG; 4, OCSH; 5, *X. campestris* pv. *vesicatoria* 824; 6, *X. axonopodis* pv. *carotae* ATCC10547; 7, *X. oryzae* pv. *oryzae* KXO169; 8, *X. axonopodis* pv. *aurentifolii* NCPPB3654; 9, *X. campestris* pv. *campestris* XCCK9501; 10, *X. arboricola* pv. *pruni* XPK9502; 11, *X. axonopodis* pv. *dieffenbachiae* 192; and 12, *P. syringae* pv. *glycinea* race 4.

Table 1. Bacterial strains used for Southern hybridization and PCR

Bacterial species	Strain
Xanthomonas campestris pv. *glycines*	8ra, ocsF, ocsG, ocsH, LMG7403(=NCPPB1124) LMG7404(=NCPPB1717) 980, 1017, 1018, 1032, 1034, 1037 1038, 1040, 1041, 1045, 1046, 1048, 1036, 1148, 1149, 1151, 1185, 1189
X. axonopodis pv. *aurentifolia*	NCPPB3654
X. campestris pv. *campestris*	XCCK9501
X. campestirs pv. *citri*	875
X. axonopodis pv. *carotae*	ATCC10547
X. axonopodis pv. *dieffenbachiae*	192
X. arboricola pv. *pruni*	XPK9502
X. campestris pv. *sesami*	914
X. campestris pv. *vesicatoria*	824(race 1), 833(race 3)
X. oryzae pv. *oryzae*	KXO169
Acidovorax avenae pv. cattlyae	2290
Agrobacterium tumefaciens	NT1
Burkholderia glumae	947
Clavibacter michiganensis subsp. michiganensis	1611
Erwinia amylovora	542
Erwinia carotovora subsp. carotovora	1940
E. carotovora subsp. atroseptica	498
E. chrysanthemi	1837
E. trachiphila	282
Escherichia coli	DH5a
Pseudomonas aeruginosa	
P. syringae pv. actinidiae	1714
P. syringae pv. chichorii	905
P. syringae pv. corrugata	758
P. syringae pv. glycinea	race 4
P. syringae pv. marginalis	499
P. syringae pv. syringae	Pss61
P. syringae pv. lachrymans	308
P. syringae pv. tomato	
P. viridiflava	460
Ralstonia solanacearum	2063(race1), 2029(race3)

specificity of this probe at the pathovar level, the number of bacterial strains was increased to 55 (Table 1). Twenty more *X. campestris* pv. *glycines* strains from various regions of the world were included and 3 more xanthomonads and 21 more bacterial strains other than xanthomonads were added. None of total genomic DNA isolated from those bacteria except *X. campestris* pv. *glycines* did hybridize with the probe. Of the 55 strains tested, the 24 strains of *X. campestris* pv. *glycines* all gave a positive hybridization signal. All *X. campestris* pv. *glycines* strains naturally isolated from various regions and host plants in Korea carried a 1.7 kb DNA fragment like *X. campestris* pv. *glycines* 8ra from USA, LMG7403 from Zambia and

LMG7404 from Zimbabwe (data not shown). The other 31 strains, representing closely and distantly related bacteria, were all negative in the tests. The pathovar identity of these strains was examined by inoculation tests to assay HR on nonhost plant tomato and pustule disease on host soybean plant. In support with the Southern hybridization result, all 24 strains of *X. campestris* pv. *glycines* strains showed positive signal with probe were able to produce clear inhibition zone on the seed bacteria, *X. campestris* pv. *vesicatoria*. Since it was found in all of the *X. campestris* pv. *glycines* strains investigated, it can be used as a pathovar-specific marker for this bacterial species. DNA hybridization had been used to identify human pathogens, symbiotic pathogens, or plant pathogens. With a specific probe, this method was found to be a reliable way to identify *X. campestris* pv. *glycines* among other plant pathogenic bacteria.

Specificity of the amplification reaction. Since the 1.7 kb insert DNA of pSGEB1 was hybridized only to total genomic DNA of *X. campestris* pv. *glycines*, the two primers were designed from the DNA sequence of ORF1 between two ORFs of glycinecinA to test the possibility of the pathovar-specific amplification of the glycinecinA gene using PCR analysis (Fig. 1). One primer, heu2, was derived from the 134 bp down stream of the putative translational start codon for ORF1 and another primer, heu4, was derived from the 63 upstream of the putative termination codon for ORF1. A 860-bp product was amplified with a primer pair, heu2/heu4, from *X. campestris* pv. *glycines* (Fig. 3). Of the 55 strains tested, the 24 strains of *X. campestris* pv. *glycines* isolated from various soybean cultivars and geographic regions all gave a 860 bp product (data not shown). Similarly, strains of other *Xanthomonas* spp. and other genera of bacteria listed in Table 1 did not yield PCR products. Since the 860-bp fragment was by far predominant PCR product verified by DNA sequencing, no attempt was made to further optimize the specificity of amplification with the heu2 and heu4 primers.

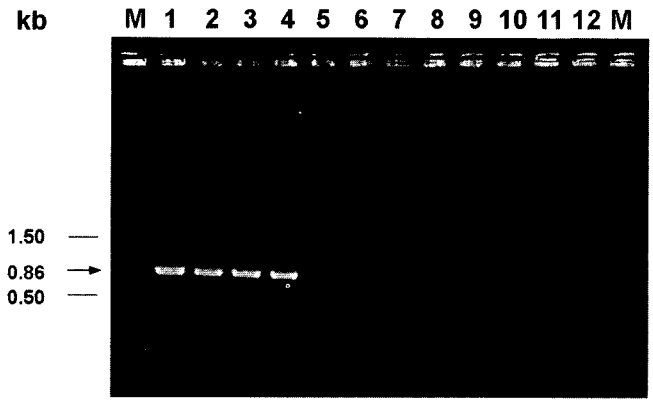

Fig. 3. Ethidium-bromide stained gel of PCR-amplification products directed by heu2 and heu4 primers. Template DNAs (25 ng) were from; 1, *X. campestris* pv. *glycines* 8ra; 2, OCSF; 3, OCSG; 4, OCSH; 5, *X. campestris* pv. *vesicatoria* 824; 6, *X. axonopodis* pv. *carotae* ATCC10547; 7, *X. oryzae* pv. *oryzae* KXO169; 8, *X. axonopodis* pv. *aurentifolii* NCPPB3654; 9, *X. campestris* pv. *campestris* XCCK9501; 10, *X. arboricola* pv. *pruni* XPK9502; 11, *X. axonopodis* pv. *dieffenbachiae* 192; and 12, *P. syringae* pv. *glycinea* race 4.

The specificity for the detection of *X. campestris* pv. *glycines* by the method described here is due in large to the fact that the DNA region serving as a probe for hybridization and a PCR target is absent from other pathogens, saprophytes, or epiphytes. This 1.7-kb DNA fragment does not hybridize to DNA from a large number of bacterial strains, which include representatives of nonpathogenic bacteria as well as plant pathogenic bacteria. In addition to the pathovar-specific DNA probe, a primer pair heu2/heu4 derived from this DNA probe can be a pathovar-specific primer to detect amplified pathovar-specific DNA fragment for *X. campestris* pv. *glycines* using PCR. This can be a good tool to detect *X. campestris* pv. *glycines* from the samples such as soybean leaves and seeds that have a complex population of bacteria.

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