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Expression of the *Xanthomonas campestris* pv. vesicatoria *hrp* Gene Cluster, Which Determines Pathogenicity and Hypersensitivity on Pepper and Tomato, Is Plant Inducible

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The *hrp* gene cluster from *Xanthomonas campestris* pv. vesicatoria determines functions necessary not only for pathogenicity on the host plants pepper and tomato but also for the elicitation of the hypersensitive reaction on resistant host and nonhost plants. Transcriptional orientation and expression of the *hrp* loci were determined with *hrp*::Tn*3-gus* fusions. In addition, expression of the *hrp* loci was studied by RNA hybridization experiments. Expression of the *hrp* genes was not detectable after growth of the bacteria in complex medium or in minimal medium. However, high levels of induction of *hrp* gene expression were measured during growth of the bacteria in the plant. To search for a plant molecule responsible for this induction, we examined a variety of materials of plant origin for their ability to induce *hrp* gene expression. Filtrates from plant suspension cultures induced *hrp* genes to levels comparable to those induced in the plant. The inducing molecule(s) was found to be heat stable and hydrophilic and to have a molecular mass of less than 1,000 daltons.

The molecular mechanisms involved in plant-bacterium interactions during pathogenesis are complex and far from being understood. In the last few years, a number of bacterial genes that determine the outcome of the interaction between the bacterium and the plant have been identified and isolated. Most notable are two classes of genes required for basic compatibility: disease-specific (*dsp*) genes, which are associated with disease development in host plants but not with the induction of a hypersensitive response (HR) in nonhost plants (7, 27); and *hrp* genes, which are required for both the pathogenic interaction with host plants and the induction of the HR in resistant host and nonhost plants. *hrp* genes have been cloned from a number of different species of gram-negative phytopathogenic bacteria, e.g., *Erwinia amylovora*, *Pseudomonas solanacearum*, and pathovars of *Pseudomonas syringae* and of *Xanthomonas campestris* (for a review, see reference 35). Genetic analysis of *hrp* genes from these different organisms indicates that they determine basic pathogenicity functions necessary for any interaction with the plant. The elucidation of their biochemical function and their role in the plant-bacterium interaction is expected to lead to a molecular understanding of the mechanisms underlying bacterial plant diseases.

We have chosen the interaction between *X. campestris* pv. vesicatoria, the causal agent of bacterial spot disease, and its host plants, pepper (*Capsicum annuum* L.) and tomato (*Lycopersicum esculentum* Mill.), as a system for the analysis of *hrp* genes. After invasion of the plant via stomata or wounds, *X. campestris* pv. vesicatoria multiplies in the intercellular spaces of the leaf tissue, giving rise to disease symptoms (29). Depending on the susceptibility of the particular plant cultivar, two different types of reactions can be observed. In the susceptible plant, water-soaked lesions occur (compatible interaction). In the resistant plant, avirulent strains induce the HR and show only limited growth (incompatible interaction).

Recently, we described the identification in and isolation from *X. campestris* pv. vesicatoria of the *hrp* gene cluster, which spans a chromosomal region of about 25 kb. The genes are organized into at least six complementation groups, designated *hrpA* to *hrpF* (6). Transposon insertions into any of the six *hrp* loci eliminated both pathogenicity and the ability to induce the HR in resistant host and nonhost plants. Nonpathogenic mutants of *X. campestris* pv. vesicatoria were characterized by their inability to multiply significantly within leaves of pepper; the numbers of CFU recovered 10 days after inoculation were reduced 1×10^3- to 5×10^4-fold, compared with those of the wild-type strain. DNA sequences homologous to the *hrp* region and, in some cases, sharing functional homology, as assessed by complementation experiments, were also found in other pathovars of *X. campestris* (6).

As the interaction between the plant and the pathogen is a dynamic process involving signal exchange between the interacting organisms, we investigated the expression of the *hrp* loci in *X. campestris* pv. vesicatoria. Expression was studied at the RNA level and with gene fusions to the β-glucuronidase gene. Growth of the bacteria under different environmental conditions revealed that the *hrp* loci are activated during growth in the plant leaf but repressed in complex medium. Furthermore, we found that filtrates of pepper, tomato, and also tobacco cell suspension cultures contain a molecule(s) that induces *hrp* gene expression.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The bacterial strains and plasmids used are listed in Table 1. Strains of *Escherichia coli* were cultivated in Luria-Bertani medium (22). *Xanthomonas* strains were routinely grown at 28°C in NYG broth (33) or on NYG 1.5% agar. The minimal medium used was M9 medium (22) or Murashige-Skoog (MS) medium (24) supplemented with 2,4-dichlorophenoxyacetic acid (1 μg/ml); both media contained 2% sucrose as a carbohydrate source. Antibiotics were added to the media at the following final concentrations: kanamycin, 50 μg/ml; tetracycline, 10 μg/ml; and rifampin, 100 μg/ml.

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics[a]	Source or reference
Strains		
X. campestris pv. vesicatoria		
71-21	Pepper race 1; HR in ECW-30R; carries *avrBs3*	23
85-10	Tomato and pepper race 2; HR in ECW-10R; carries *avrBs1*	6
85-10::45, 85-10::311, and 85-10::321	Marker exchange mutants carrying Tn*3-gus*; Hrp^+	6
E. coli DH5α	F^- *recA* *ϕ80dlacZ* *ΔM15*	Bethesda Research Laboratories
Plasmids		
pLAFR3	Tc^r rlx^+ RK2 replicon	30
pRK2013	Km^r $TraRK2^+$ Mob^+ ColE1 replicon	11
pL6GUSC	pLAFR6 derivative containing promoterless β-glucuronidase gene; Tc^r	17
pXV2	pLAFR3 clone from *X. campestris* pv. vesicatoria 75-3 containing *hrp* genes	6
pXV9	pLAFR3 clone from *X. campestris* pv. vesicatoria 75-3 containing *hrp* genes	6
p311, pF312, pF314, pF316, pF318, and p321	pXV2::Tn*3-gus* derivatives	This study
pB2, pA9, pA14, pC17, pA22, pD29, pB35, pB40, pC44, p45, pC52, pD54, pD58, pB59, pE75, pB78, pB79, pB80, pB85, pC117, pD119, pB126, pD137, and pD140	pXV9::Tn*3-gus* derivatives	This study

[a] Tc^r, tetracycline resistant; Km^r, kanamycin resistant.

Plasmids were introduced into *Xanthomonas* strains by conjugation with pRK2013 as a helper plasmid in triparental matings (8, 11).

Plant material and plant inoculations. Pepper cultivar ECW was used for these studies (23). The plants were kept in a growth chamber at 28°C (16 h of light) or 22°C (8 h of dark) and 80% relative humidity. Young, fully expanded leaves of 6-week-old pepper plants were inoculated with a bacterial suspension of 10^8 CFU/ml in 1 mM $MgCl_2$, unless otherwise stated. For the measurement of β-glucuronidase activity, small areas of the leaf were inoculated by use of a plastic syringe as previously described (31). For RNA isolation from bacteria, whole shoots were infiltrated under vacuum as described by Klement (16). Bacteria were recovered from infected leaves of pepper plants with 1 mM $MgCl_2$ by vacuum infiltration (16) and separated from the washing fluid by centrifugation. For the isolation of intercellular washing fluid from infected or uninfected leaves, the same method was used. After centrifugation, supernatants were filtered through 0.22-μm-pore-size nitrocellulose. The intercellular washing fluid was freeze-dried and dissolved in H_2O (one-tenth the original volume).

For the preparation of leaf extracts, fully expanded pepper leaves were harvested and frozen in liquid nitrogen. After removal of the main veins, the tissue was homogenized in 1 mM $MgCl_2$ (1 ml/g [fresh weight]) with or without polyvinylpyrrolidone (PVP 360; 0.1 g of PVP 360 per g [fresh weight]). The homogenate was separated by centrifugation for 30 min at 40,000 × *g*; the pellet was resuspended in 1 mM $MgCl_2$ (1 g/ml). For induction assays, the supernatant, the resuspended pellet, and whole homogenates of both preparations were tested, undiluted or at dilutions of 1/5, 1/10, and 1/100.

Plant cell cultures and isolation of conditioned medium. Callus suspension cell lines of *Nicotiana tabacum* cv. W38, pepper cv. ECW, and tomato cv. Money Maker were grown in MS medium (24) supplemented with 2% sucrose and 2,4-dichlorophenoxyacetic acid (1 μg/ml). Flasks (250 ml) containing 50 ml of suspension were incubated at 27°C on a shaker at 110 rpm for 7 days before 10% of the suspension was subcultured by dilution into fresh medium.

For obtaining tobacco-, pepper-, or tomato-conditioned medium, the cell-free filtrate of a 7-day-old suspension culture was filtered through 0.22-μm-pore-size nitrocellulose and stored at −80°C.

Biochemical treatments of TCM. The conditioned medium of tomato suspension cultures (TCM) was treated by various methods before bioassays were done. TCM was filtered through Centricon-3 columns (Amicon Division, W. R. Grace & Co., Beverly, Mass.) to remove molecules with a molecular mass of more than 3,000 daltons. The Centricon-3 filtrate was extracted twice with organic solvents as described previously (28). The extractions were performed at pHs ranging from 4 to 9. After extraction, the organic and aqueous phases were rotary evaporated and the remaining material was resuspended in water or 1 mM $MgCl_2$ before another rotary evaporation. Finally, the sediment was resuspended in water or 1 mM $MgCl_2$. For bioassays, different concentrations of the solution were used (undiluted and 10- and 100-fold diluted, as compared with the original concentration of TCM).

For gel filtration, a Bio-Gel P2 column (Pharmacia, Uppsala, Sweden) was used. Two milliliters of a 10-fold-concentrated Centricon-3 filtrate (after extraction with chloroform) was loaded onto the column (35-cm length; 2.6-cm diameter); 100 mM NaCl was used for elution, and 3-ml fractions were collected.

A Spectra/Por dialysis membrane with a molecular mass cutoff of 1 kDa was obtained from Spectrum (Los Angeles, Calif.).

For digestion of any proteins present in TCM after centrifugation through Centricon-3 and chloroform extraction, samples were digested with proteinase K (50 and 250 μg/ml) or with trypsin (100 and 500 μg/ml) for 3 h at pH 7 and 37°C. The proteases were removed by heat treatment of the samples, and the samples were filtered with Centricon-3. Undiluted samples were tested in bioassays. For digestion of any DNA present in TCM, a 4-ml sample was incubated with 23 U of DNase I for 5 h at 37°C.

Molecular genetic techniques. Standard molecular techniques were used (4, 21), unless otherwise stated.

RNA isolation. RNA from *X. campestris* pv. vesicatoria was purified by the hot phenol procedure as modified by Aiba et al. (1). Bacteria were grown in NYG medium overnight or for 3 days in planta. Cells were harvested by centrifugation and resuspended in 3 ml of 0.02 M sodium acetate (pH 5.5)–0.5% sodium dodecyl sulfate (SDS)–1 mM EDTA. After the addition of 3 ml of phenol (equilibrated in 0.02 M sodium acetate [pH 5.5]), the mixture was incubated at 60°C for 10 min with shaking. After centrifugation, the aqueous phase was extracted twice with phenol (60°C) and then twice with chloroform. The nucleic acids were precipitated by the addition of 0.1 volume of 3 M sodium acetate (pH 6.5) and 3 volumes of ethanol and collected by centrifugation. The pellet was dissolved in acetate-SDS-EDTA buffer. The ethanol precipitation was repeated twice and, finally, the RNA was resuspended in water.

For radioactive labelling of the RNA, 15 μg of RNA was partially hydrolyzed for 5 min at 90°C in 0.1 M Tris-HCl (pH 9.5). The RNA was chilled on ice and incubated for 60 min at 37°C in kinase buffer (50 mM Tris-HCl [pH 9.5], 10 mM $MgCl_2$, 5 mM dithiothreitol) with 150 μCi of [γ-^{32}P]ATP (3,000 Ci/mmol) and T4 polynucleotide kinase. The reaction was stopped by the addition of EDTA and extraction with phenol-chloroform. Labelled RNA was separated from unincorporated [γ-^{32}P]ATP by chromatography on a small Sephadex G-50 column.

Assays of β-glucuronidase activity. For β-glucuronidase assays, the bacteria were grown in NYG, M9, or MS medium for 14 h, harvested by centrifugation, and resuspended in assay buffer (14). For assays of in planta-grown bacteria, pepper leaves were inoculated with 10^9 CFU/ml in 1 mM $MgCl_2$. Leaf discs (0.8-cm diameter) were taken 40 h postinoculation and macerated in 1 mM $MgCl_2$ each; aliquots were taken for the enzyme assays. The number of bacteria (CFU) per assay were calculated by plating appropriate dilutions on selective medium. β-Glucuronidase activity was determined in fluorometric assays with 4-methylumbelliferyl glucuronide as a substrate as described previously (14). One unit of β-glucuronidase was defined as nanomoles of 4-methylumbelliferone released per minute.

RESULTS

RNA expression studies. RNA hybridization experiments allowed analysis of the expression of the *hrp* gene cluster of *X. campestris* pv. vesicatoria (6). DNA of plasmids pXV2 and pXV9, which contain overlapping sequences and span the entire *hrp* gene cluster and several kilobases of flanking sequences (Fig. 1C), was digested with *Eco*RI. The DNA fragments were separated on an agarose gel and hybridized in a Southern blot experiment to radioactively labelled total RNA. The RNA was isolated from *X. campestris* pv. vesicatoria 71-21 after growth of the bacteria in complex medium (NYG) or in the susceptible pepper cultivar ECW. After growth in complex medium, no transcripts hybridizing to the DNA region containing *hrpA* to *hrpF* were observed (Fig. 1A). The hybridizing 2.2-kb *Eco*RI fragment (Fig. 1A, lane 1) present in pXV2 corresponds to the DNA region to the right of *hrpF* (Fig. 1C). In contrast, when RNA isolated from bacteria grown in pepper leaves for 3 days was used as a probe, a number of DNA fragments corresponding to different *hrp* loci hybridized: in pXV2, the 2.2-, 2.7-, 8.0-, 5.1-, and 4.0-kb *Eco*RI fragments (Fig. 1C). The latter three sequences are also present in pXV9 (Fig. 1B, lane 2). Hybridization of the 2.2-kb *Eco*RI fragment of pXV2 was enhanced 5- to 10-fold as compared with that in Fig. 1A; the role of this region in pathogenicity is uncertain, since a mutant carrying an insertion in this fragment was still pathogenic (6). The 31-kb fragment contains a portion of the 8-kb fragment plus the pLAFR3 vector; the 5.4-kb *Eco*RI fragment contains the leftmost 4.0-kb fragment from pXV2. In addition, in pXV9 the 4.5- and 2.0-kb *Eco*RI fragments and the leftmost 0.8-kb *Eco*RI fragment corresponding to the region not present in pXV2 hybridized. The small *Eco*RI fragments (0.5 and 0.8 kb) present in both plasmids (Fig. 1C) did not hybridize, indicating that this region is not expressed or is only weakly expressed. The weaker signal observed for the 31-kb *Eco*RI fragment of pXV9 may have been due to inefficient transfer. The 2.7-kb *Eco*RI fragment present only in pXV2 and containing part of the *hrpF* locus always showed weak hybridization to the RNA from in planta-grown bacteria. Differences in signal strength observed between hybridizing fragments do not necessarily reflect different transcription rates of the *hrp* genes or differences in RNA stability, since the hybridizing DNA fragments may represent more than one *hrp* locus (Fig. 1C). As an internal control, a DNA fragment containing the coding region of the avirulence gene *avrBs3* from *X. campestris* pv. vesicatoria 71-21, which is constitutively expressed (17), was used (Fig. 1A and B, lanes 3). The results were confirmed with different RNA preparations as probes for hybridization.

These results show that transcription of the *hrp* loci *hrpA* to *hrpF* is not detectable in bacteria grown in complex medium. The same result was obtained for bacteria grown in M9 minimal medium (data not shown). During growth of the bacteria in the plant, several *hrp* loci were induced.

***hrp* gene activation and orientation of *hrp*::*gus* fusions.** For a more quantitative analysis of the expression of single *hrp* loci, we determined the activity of gene fusions to the β-glucuronidase (*gusA*) gene under different growth conditions. Previously, a number of different Tn*3*-*gus* insertions were generated in pXV9 and pXV2 (Fig. 2B and C) (6, 26). Transposon Tn*3*-*gus* carries a promoterless gene for β-glucuronidase and allows transcriptional and translational fusions to target genes. The effects of the different Tn*3*-*gus* insertions on pathogenicity were reported previously (6). In the genomic mutants, obtained by marker exchange, all insertions, except those in strains 85-10::45, 85-10::311, and 85-10::321, were located in one of the six *hrp* loci (Fig. 2B and C), thereby inactivating *hrp* function. Since the chromosomal Tn*3*-*gus* insertion mutants were not able to grow in the plant (6), β-glucuronidase enzyme activity was determined for merodiploid *X. campestris* pv. vesicatoria 85-10 strains carrying Tn*3*-*gus* insertion derivatives of pXV2 and pXV9. These strains elicit the same phenotypic response on pepper plants as does wild-type strain 85-10.

To study the expression of *hrp* genes in NYG medium, we tested bacteria from overnight cultures for β-glucuronidase activity. For in planta induction assays, bacteria were inoculated by injection into pepper leaves. During this study, it was found that a bacterial cell number of at least 10^6 CFU

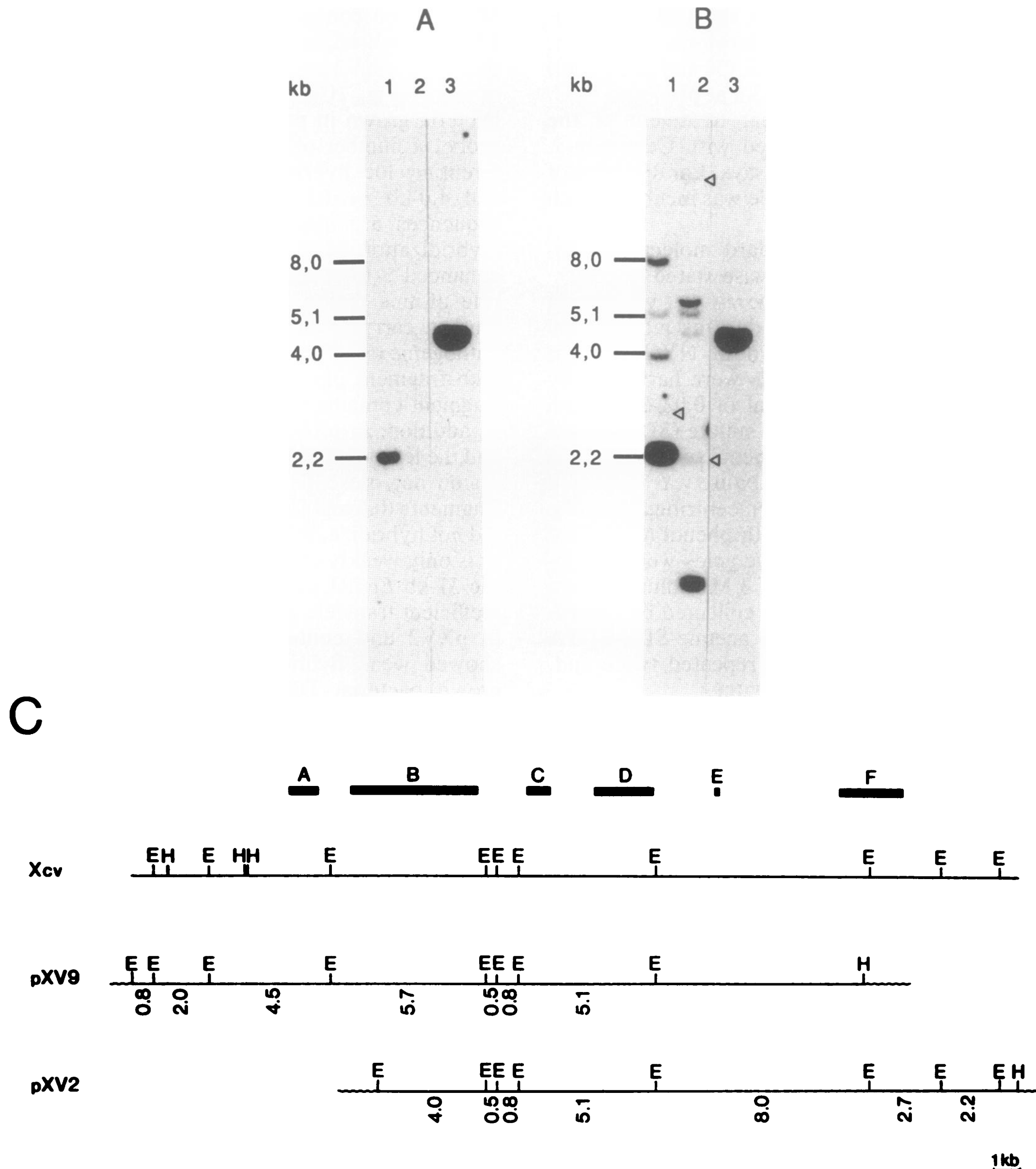

FIG. 1. (A and B) Southern blot of *Eco*RI-digested DNA of plasmids pXV2 and pXV9. Approximately 2 μg of DNA was separated on a 0.7% agarose gel. The blots were hybridized to radioactively labelled total RNA isolated from bacteria grown overnight in NYG medium (A) and from bacteria grown for 3 days in pepper cultivar ECW leaves (B). Lanes: 1, pXV2; 2, pXV9; 3, DNA of the internal *Bam*HI fragment of the coding region of *avrBs3* (17). The triangles mark the positions of weak bands. (C) Structure of the DNA region containing the *hrp* loci. The upper part represents the *hrp* region in *X. campestris* pv. vesicatoria (Xcv) which consists of six complementation groups. The *Eco*RI restriction maps of pXV9 and pXV2 are shown in the lower part; the sizes of the *Eco*RI fragments are indicated. The inserts of both clones are flanked by *Eco*RI (left side) and *Hin*dIII (right side) sites of the vector.

per assay was necessary for the accurate quantification of β-glucuronidase activity. To obtain this minimal number of bacteria per leaf disc, we used an inoculum of 10^9 CFU/ml; the resulting plant reaction was identical to the one that occurred after inoculation of bacteria at 10^8 CFU/ml. After growth of the bacteria in complex medium, no significant activity was recorded with any of the Tn*3-gus* insertions tested; enzyme activities were comparable to those encoded by a promoterless *gusA* gene (pL6GUSC; 0.01×10^{-10} U/CFU). However, after growth of the bacteria in planta, 12 of 30 insertion derivatives expressed β-glucuronidase at a significantly higher level than in complex medium (closed symbols in Fig. 2B and C; Table 2). The β-glucuronidase activities of the inactive insertions were, on average, 0.01×10^{-10} U/CFU for cells grown in complex medium and in the range of 0.1×10^{-10} to 0.7×10^{-10} U/CFU for cells grown in the plant. The reason for this difference in background activity is not clear. With the exception of *hrpE*, enzyme activity was found with at least one insertion in each *hrp* locus. As a positive control, we used strain 85-10 carrying an *avrBs3*::Tn*3-gus* fusion (17). The *avrBs3* gene was constitutively expressed, and β-glucuronidase activity was present

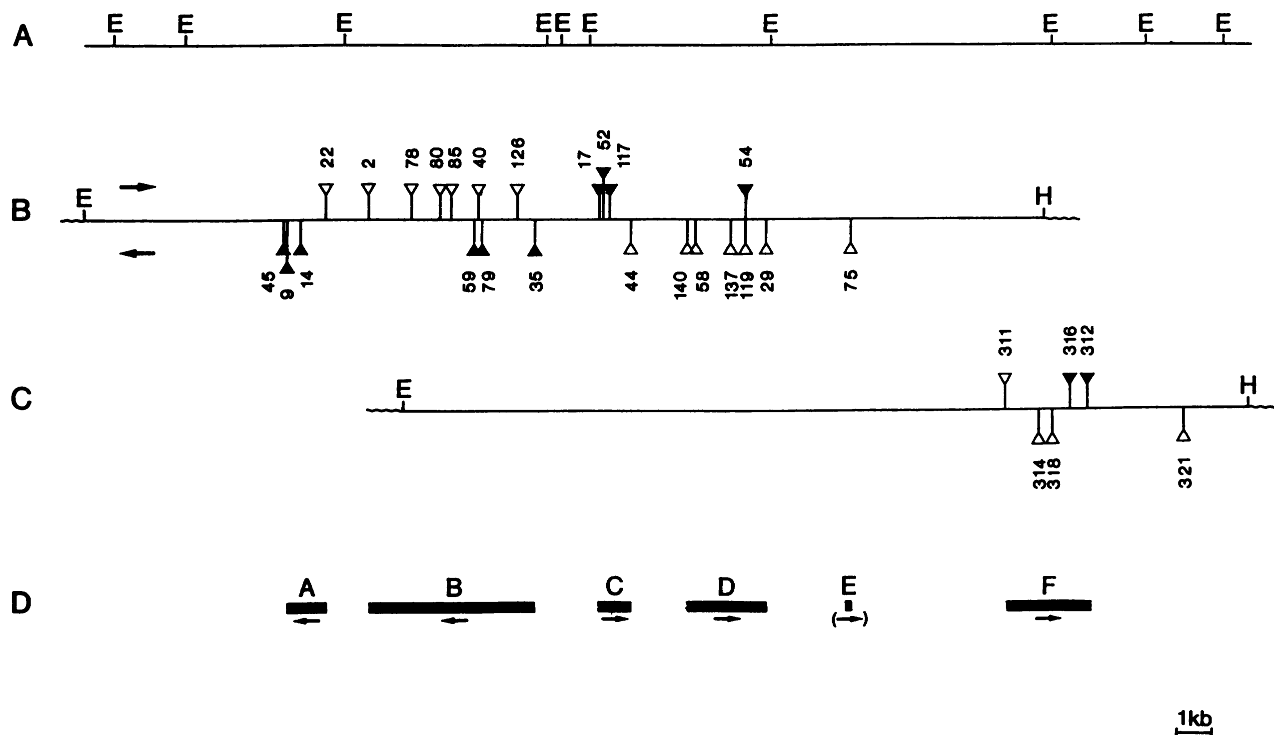

FIG. 2. Transposon insertions within pXV2 and pXV9 and analysis of transcription. (A) *Eco*RI (E) restriction map. (B and C) Sites of Tn*3-gus* insertions into the cosmid clones pXV9 and pXV2, respectively. Closed triangles represent insertions with β-glucuronidase activity and open triangles represent insertions without β-glucuronidase activity after growth of the bacteria in the plant. Tn*3-gus* insertions oriented from left to right with respect to the *gus* gene (see the arrows in panel B) are given above the line; insertions oriented from right to left with respect to the *gusA* gene are given below the line. (D) Transcriptional orientation of the *hrp* loci, deduced from the directions of plant-inducible Tn*3-gus* insertions.

at 10×10^{-10} U/CFU after growth of the cells in complex medium or in pepper leaves.

The absolute level of β-glucuronidase activity of inducible insertions differed not only between *hrp* loci but also between different insertions residing within the same locus (Table 2). This means that the specific activities do not reflect the rates of transcription of the different loci but rather properties of individual Tn*3-gus* insertions. The specific activities of the insertions induced during growth in the plant were in the range of 5×10^{-10} U/CFU (pC117) to 70×10^{-10} U/CFU, as for pF312. The results of these experiments suggest the presence of plant-inducible promoters that control transcription of the *hrp* loci.

To determine the direction of transcription of the different *hrp* loci, we determined the orientations of the insertions in plasmids pXV2 and pXV9 by restriction enzyme analysis and by Southern blot hybridization with the wild-type *hrp* region and a fragment from the *gusA* gene as probes. In cases in which several inducible Tn*3-gus* insertions were obtained for one particular *hrp* locus, these insertions were all in the same orientation with respect to the reporter gene (Fig. 2B and C). On the basis of these data, we deduced the transcriptional orientation of the *hrp* loci: *hrpA* and *hrpB* are transcribed from right to left, and *hrpC* to *hrpF* are transcribed from left to right (Fig. 2D). As the activity of the only insertion available in *hrpE*, pE75, is not inducible and the *gusA* gene is oriented from right to left, the orientation of transcription of *hrpE* is most likely from left to right. This orientation is also indicated by preliminary sequence data (10).

The Tn*3-gus* derivative p45 was also plant inducible (Table 2). Previous studies revealed that marker exchange mutant 85-10::45 was Hrp$^+$ and that this insertion mapped to the left of the *hrpA* locus (6). Since the *gusA* gene in p45 is in the same transcriptional orientation as *hrpA*, the insertion may be within the transcription unit of the *hrpA* locus.

***hrp* gene activity under different growth conditions.** As transcription of the *hrp* loci was induced after inoculation of the bacteria into the plant, we tried to mimic conditions within the intercellular space of leaves by using minimal media. We determined β-glucuronidase activity after 14 h of growth of bacteria in M9 medium and in MS plant tissue culture medium. For each *hrp* locus, one representative, inducible insertion was tested in *X. campestris* pv. vesicatoria 85-10 merodiploids. Strain 85-10(pE75) was included as a negative control. The level of β-glucuronidase activity in bacteria grown in the plant was several orders of magnitude higher than that in bacteria grown in M9 or MS medium (Table 3). Strain 85-10(pE75) showed β-glucuronidase activity after growth in M9 medium comparable to that of strains with inducible *gusA* fusions. Therefore, we consider the slightly higher activities observed for all strains tested in minimal media (Table 3) to be background.

To isolate the putative *hrp*-inducing factor(s) from the plant, we analyzed various plant materials for their ability to induce *hrp* gene expression. Strain 85-10(pF312), with a *hrpF-gusA* fusion, was chosen as the test strain. This strain gave the highest levels of β-glucuronidase activity when grown in the plant (Table 2). No expression was detected when the bacteria were grown in intercellular washing fluids

TABLE 2. β-Glucuronidase activities of *hrp*::Tn*3-gus* fusions in *X. campestris* pv. vesicatoria 85-10 after growth in pepper cultivar ECW

Tn*3-gus* insertion derivative	Locus	β-Glucuronidase activity (10^{-10}U/CFU)[a]	Inducibility[b]
pA9	*hrpA*	8.8	+
pA14	*hrpA*	12.7	+
pA22	*hrpA*	0.5	−
pB2	*hrpB*	0.7	−
pB78	*hrpB*	ND[c]	ND
pB85	*hrpB*	0.1	−
pB80	*hrpB*	ND	ND
pB59	*hrpB*	9.7	+
pB40	*hrpB*	0.1	−
pB79	*hrpB*	13.9	+
pB126	*hrpB*	0.0	−
pB35	*hrpB*	22.9	+
pC17	*hrpC*	7.0	+
pC52	*hrpC*	7.9	+
pC117	*hrpC*	4.3	+
pC44	*hrpC*	0.1	−
pD140	*hrpD*	0.1	−
pD58	*hrpD*	0.6	−
pD137	*hrpD*	0.1	−
pD119	*hrpD*	0.1	−
pD54	*hrpD*	10.7	+
pD29	*hrpD*	0.3	−
pE75	*hrpE*	0.2	−
pF314	*hrpF*	0.4	−
pF318	*hrpF*	0.1	−
pF316	*hrpF*	6.7	+
pF312	*hrpF*	70.0	+
p45	*hrp*$^+$	11.0	+
p311	*hrp*$^+$	0.1	−
p321	*hrp*$^+$	0.1	−
pXV2	Wild-type clone	0.06	−
pXV9	Wild-type clone	0.01	−

[a] Calculated as described in Materials and Methods. Values are averages of three independent experiments with triplicate samples each. Samples were taken 40 h postinoculation.

[b] β-Glucuronidase activities above 10^{-10} were considered to be plant inducible (+); those below this threshold were considered to be not plant inducible (−).

[c] ND, not determined.

recovered from pepper leaves or in pepper leaf extracts containing whole homogenates or soluble or insoluble leaf material (see Materials and Methods). We also tested the intercellular washing fluid of pepper cultivar ECW 3 days after infection with *X. campestris* pv. vesicatoria wild-type cells. However, the filtrate of this fluid was only able to cause a slight induction of expression of the *gusA* gene in the test strain, and this induction could not reliably be reproduced.

The *hrp* genes were, however, efficiently induced by a factor(s) in the filtrate recovered from TCM (Table 3). Initial experiments, carried out with strain 85-10(pF312), were extended to include insertions in *hrp* loci *hrpA*, *hrpB*, *hrpC*, and *hrpD*, as summarized in Table 3. The levels of β-glucuronidase activity recorded were comparable to those obtained after growth of the bacteria in pepper leaves. Only strain 85-10(pB35) showed significantly higher induction in the plant than in TCM. Maximum β-glucuronidase activity in the plant was measured 40 h postinoculation. In the in vitro experiments, comparable levels of activity were obtained after an induction period of 14 h.

It should be mentioned that besides the attempt to identify conditions for *hrp* gene induction, we tested whether the bacteria were able to grow in the respective medium. The generation times of the bacteria were 3 h in complex medium and intercellular washing fluid and 6 to 8 h in minimal medium, results which were expected. Previous studies had shown that genomic marker exchange mutants carrying Tn*3-gus* in one of the *hrp* loci failed to grow in the plant (6). The question was whether the *gusA* gene in these mutants could be induced in vitro to the levels observed for the respective merodiploid strains. The genomic marker exchange mutants were able to grow in vitro; however, when TCM was tested for the induction of β-glucuronidase, the activity was very low for insertions in *hrpA*, *hrpB*, *hrpC*, and *hrpD*. Only mutant strain *X. campestris* pv. vesicatoria 85-10::hrpF312 could be induced in TCM to levels comparable to those shown in Table 3 for pF312 in TCM.

Since the *hrp* genes of *X. campestris* pv. vesicatoria not only are involved in the growth of the bacteria in susceptible pepper and tomato plants but also are required for the induction of the nonhost HR in tobacco, we subsequently analyzed the filtrates of both pepper and tobacco cell suspension cultures. Interestingly, not only tomato- and pepper-conditioned medium but also tobacco-conditioned medium induced *hrp* gene expression, as monitored by the β-glucuronidase activity of the Tn*3-gus* insertion in pF312. The levels of activity were essentially in the same range for culture filtrates obtained from the three different plant species; however, induction by TCM was two- to threefold higher (data not shown). Culture filtrates of cell suspensions from plant species other than tomato, pepper, and tobacco were not tested. As MS medium alone does not induce *hrp* gene expression (Table 3), we hypothesized that an inducing factor(s) must be released from plant cells grown in this medium.

TABLE 3. Expression of *hrp*::Tn*3-gus* fusions in *X. campestris* pv. vesicatoria 85-10 under different growth conditions

Locus	Plasmid	β-Glucuronidase activity (10^{-10} U/CFU) in[a]:				
		NYG	MS	M9	Pepper	TCM
hrpA	pA14	0.080	0.240	0.170	13	7
hrpB	pB35	0.003	0.020	0.004	23	3
hrpC	pC52	0.004	0.036	0.008	8	6
hrpD	pD54	0.004	0.035	0.006	11	7
hrpF	pF312	0.005	0.054	0.053	70	100
hrpE	pE75	0.002	ND[b]	0.07	0.2	0.03

[a] Calculated as described in Materials and Methods. Values are averages of three independent experiments with duplicate samples each.

[b] ND, not determined.

Properties of the inducing factor(s). To identify the factor(s) inducing *hrp* gene activity, we chose TCM as a source. TCM was treated in different ways to determine the physical and chemical properties of the inducer (for details, see Materials and Methods). *hrp* gene induction was monitored by incubation of test strain 85-10(pF312) in the respective media for 14 h and the measurement of β-glucuronidase activity. To determine the minimal concentration of TCM necessary for *hrp* gene induction, we measured β-glucuronidase activity after induction with different concentrations of TCM diluted in water. The dose-response curve (Fig. 3) showed a sigmoidal shape. TCM could be diluted 10-fold and still contain 75% of the inducing activity. Combustion (2 h at 800°C) destroyed the inducing activity, indicating that the factor(s) is organic. Boiling of TCM (20 min at 100°C) and

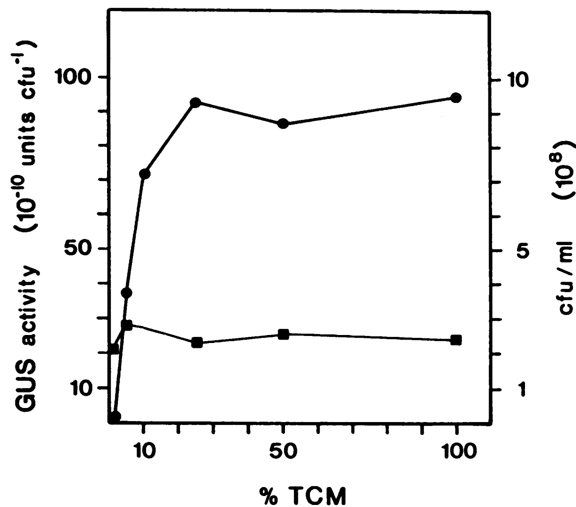

FIG. 3. Dose-response curve showing the β-glucuronidase (GUS) activity of strain 85-10(pF312) after growth for 14 h in different concentrations of TCM (●). TCM was diluted with water. Cell numbers per sample were determined by plating appropriate dilutions on selective agar medium (■).

repeated freezing-thawing had no effect. As the inducing activity was not removed by extraction with chloroform, butanol, or ethyl acetate, we assume that the factor(s) is hydrophilic. However, activity was lost after extraction of TCM with phenol. To determine whether the active factor(s) is charged, we loaded TCM onto a mixed-bed resin and a DEAE-Sephacel (anion-exchange) column. In both cases, no inducing activity was present in the flowthrough. Dialysis and fractionation of TCM on a Bio-Gel P2 column showed that the active molecules were smaller than 1 kDa. Digestion of a Centricon-3 filtrate of TCM with proteinase K, trypsin, or DNase I did not lower the activity. The inducer(s) was not precipitated after the addition of methanol, ethanol, or acetone to a final concentration of 80%. In summary, these treatments showed that the inducing factor(s) is small, organic, hydrophilic, and heat stable.

DISCUSSION

In this study, we investigated the mode of expression of the *hrp* genes from *X. campestris* pv. vesicatoria. This *hrp* gene cluster has recently been isolated and consists of at least six complementation groups, designated *hrpA* to *hrpF* (6). Hybridization experiments showed that RNA transcripts corresponding to the *hrp* region were detectable in *X. campestris* pv. vesicatoria after growth of the bacteria in the plant. No expression was observed after growth of the cells in complex medium, or expression was below the detection limits of the conditions used (Fig. 1). The plant inducibility of genes in the *hrp* cluster was confirmed with gene fusions to the β-glucuronidase (*gusA*) gene (Table 2 and Fig. 2). The finding that the levels of expression of inducible insertions within one *hrp* locus were not identical may have been due to an effect of sequences at the site of the Tn*3-gus* insertion on the transcription of the *gusA* gene. Also, translational fusions could influence enzyme activity. Preliminary experiments with *hrp*::Tn*3-gus* fusions revealed the induction of β-glucuronidase activity after inoculation into tobacco leaves, suggesting that *hrp* genes not only are induced in the pepper plant, the natural host, but also may be induced in the nonhost plant tobacco as well. This suggestion would imply that inducing conditions for *hrp* genes in a certain pathovar are not plant species specific and that the host range of the pathogen is controlled by a different mechanism, e.g., avirulence genes, as has been suggested before (18, 34). When bacteria were grown in minimal medium M9 or MS, no *hrp* expression was detected. Thus, the *hrp* genes from *X. campestris* pv. vesicatoria isolated and characterized to date exhibit a pattern of regulation similar to that of *hrp* genes from other phytopathogenic bacteria, e.g., from *X. campestris* pv. campestris (15) and *P. syringae* pv. phaseolicola (9, 19, 25). In these cases, the *hrp* genes, like those from *X. campestris* pv. vesicatoria, are suppressed in complex medium and inducible in the host plant. For *P. solanacearum* and *E. amylovora*, preliminary data indicate the induction of *hrp* genes in tomato root exudate and tobacco-conditioned medium (3) and in the nonhost plant tobacco (5), respectively. A striking difference between the *hrp* gene clusters from *X. campestris* pv. vesicatoria and the other phytopathogens studied to date is the lack of expression observed in minimal medium. This finding indicates that, in *X. campestris* pv. vesicatoria, the regulation of the *hrp* genes is not simply a matter of catabolite repression. Although the levels of reporter gene activities measured cannot be directly compared between the different systems under study, it is obvious that the expression of the *hrp* loci in *X. campestris* pv. vesicatoria and *P. syringae* pv. phaseolicola (9, 25) is inducible by a factor of several hundredfold to thousandfold under growth conditions in planta compared with in vitro. The potentially complex regulation of *hrp* gene clusters is apparent from the work of Fellay et al. with *P. syringae* pv. phaseolicola. In a preliminary report (9), they stated that osmolarity, medium composition, and also a plant signal were all found to contribute in regulating the expression of *hrp* genes. The involvement of a plant factor was inferred from the high levels of expression of *hrpL* and *hrpS* from *P. syringae* pv. phaseolicola in the plant, whereas the other *hrp* loci were induced in minimal medium.

In addition, we have demonstrated that a heat-stable, hydrophilic plant factor(s) with a low molecular weight induces the expression of *hrp* genes in *X. campestris* pv. vesicatoria. The nature of this factor(s) is not known yet. On the basis of the characterization of its properties, it could be a carbohydrate with a charged side chain or a small peptide which would be protease insensitive. A small heat-stable plant factor(s) was also postulated by Arlat et al. (3) to induce *hrp* genes in *P. solanacearum*. Unexpectedly, the inducer(s) was not recovered from intercellular washing fluids of pepper leaves, but such extracts may poorly reflect the microenvironment within the plant leaf. In addition, the invading bacteria may contribute to the release of the plant factor(s), e.g., by secretion of degradative enzymes. The ability of conditioned cell suspension medium, also reported by others (3), to induce *hrp* gene expression may be due to a combination of the presence of the inducing factor(s) and the provision of balanced nutritional conditions, i.e., the absence of suppressors, which may optimize *hrp* gene expression. This idea is supported by the observation that dilution of TCM with complex medium completely suppresses *hrp* gene induction (13). The basal medium of the tomato suspension cultures, MS, tested with or without 2,4-dichlorophenoxyacetic acid, did not induce the expression of *hrp* genes. It should be noted that although both the plant and conditioned medium from cell suspension lines of different plant species clearly induced *hrp* gene expression, the *hrpB* locus was induced to a lower level in TCM. Whether *hrpB*

needs a different factor that is absent from or suppressed in TCM is not clear.

The *hrp* loci from *X. campestris* pv. vesicatoria were induced in planta and also, to various levels, in TCM, whereas expression was suppressed in complex and minimal media. Therefore, expression of the different *hrp* loci might be regulated by the same mechanism. We predict that the *hrp* promoter regions share common sequence elements required for this regulation. The induction of gene expression could be controlled by either the inactivation of a repressor or the activation of a positively regulating molecule. In addition, there might be autoregulation of the transcription of the *hrp* loci because the expression in TCM of all *hrp* loci, except for *hrpF*, seemed to be dependent on an intact copy of the respective locus in the cell. This result was also observed for the expression of *hrpL* and *hrpS* in *P. syringae* pv. phaseolicola (9). Plant-induced genes necessary for the interaction with the plant have previously been described for other bacteria, most notably *Agrobacterium tumefaciens* and *Rhizobium* spp. The expression of *vir* genes in *A. tumefaciens* (28) and *nod* genes in *Rhizobium* spp. (20) is specifically regulated by phenolic compounds released from the plant. Studies on the mechanism of *vir* gene regulation in *A. tumefaciens* led to the identification of a two-component regulatory system homologous to systems evolved in other prokaryotes for sensing and adapting to changes in the environment (2). It is conceivable that *hrp* genes are regulated in a similar way by being activated only when the bacteria meet the proper environmental conditions. To date, there is genetic evidence in *P. syringae* pv. phaseolicola (9, 19) for a regulatory *hrp* locus (*hrpS*), the sequence of which shares homology with the sequences of two-component regulatory proteins (12). A locus positively regulating the synthesis of extracellular enzymes and whose sequence is homologous to those of two-component regulatory proteins has recently been identified in *X. campestris* pv. campestris (32). Mutants showed reduced pathogenicity; the effect of this locus on other pathogenicity functions, e.g., *hrp*, remains to be determined.

Whether the plant signal for *hrp* induction in *X. campestris* pv. vesicatoria is a secondary metabolite, as in *Agrobacterium*- or *Rhizobium*-plant interactions, or a primary metabolite is not clear. Since the bacteria grow within the plant, one could imagine that the factor(s) might have a dual function in being a signal for *hrp* gene induction as well as a nutritional factor. Further analysis of the functions encoded by *hrp* genes from *X. campestris* pv. vesicatoria and the role of plant factors in regulating gene expression should allow the biochemical basis underlying the basic pathogenicity of the bacterial spot pathogen to be determined.

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