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Expression of *Erwinia amylovora hrp* Genes in Response to Environmental Stimuli

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Seven *hrp* loci that are essential for the hypersensitive reaction elicited by *Erwinia amylovora* were transcriptionally fused with a derivative of transposon Tn*5*, containing the promoterless *Escherichia coli* β-glucuronidase reporter gene. The seven *hrp* fusions were used to monitor expression of the *hrp* loci in vitro and in planta. No significant expression was detected in rich medium for any of the fusions. However, five of them were expressed highly in planta and in inducing medium that contains mannitol, salts, and 5 mM $(NH_4)_2SO_4$. Expression of these five *hrp* loci is regulated by ammonium, nicotinic acid, complex-nitrogen sources, certain carbon sources, temperature, and pH. Under well-defined conditions, i.e., in inducing medium, no specific plant components were required for transcriptional activation of the *hrp* loci. The high levels of expression detected in vitro were comparable to those determined during the development of the hypersensitive reaction in tobacco. Differential levels of expression of the *hrp* loci occurred in host and nonhost plants. In pear, a host plant, expression of the *hrp* loci was delayed and greatly reduced compared with expression in tobacco leaves, a nonhost.

Erwinia amylovora causes the often devastating disease known as fire blight. The bacterium affects pear, apple, and several important ornamental plants (1). On nonhosts, e.g., tobacco, *E. amylovora* elicits a hypersensitive response (HR) (18, 19) that is characterized by rapid collapse of leaf tissue following infiltration of the intercellular spaces with suspensions containing ca. $\geq 10^7$ CFU/ml. Genetic analyses have enabled the identification of a multiple gene system of *E. amylovora* that functions in the elicitation of the HR in nonhost plants and pathogenicity in host plants (3, 5, 31, 32). A gene cluster, designated *hrp* (20), has been characterized by transposon mutagenesis and genetic complementation and has been shown to cause nonpathogenic bacteria, such as *Escherichia coli*, to elicit the HR (6, 8).

A most interesting question is how the bacteria trigger the HR. One hypothesis involves a compound or elicitor produced by bacteria that interacts with plant cells to initiate the HR (17). However, so far, all attempts to demonstrate the existence of an elicitor in culture filtrates that is active in a broad range of nonhost plants have failed. Little is known concerning the biochemical role that the products of *hrp* loci play in the plant-pathogen interaction, except that the predicted protein product of one *hrp* locus from *Pseudomonas syringae* pv. phaseolicola (11) and *E. amylovora* (28) share homologies with a highly conserved domain of several prokaryotic regulatory proteins (26). Some mutations in *hrp* genes of *P. syringae* pv. glycinea inhibit transcriptional induction of an avirulence gene (14). Thus, the study of the regulation of *hrp* genes is an important step in elucidating the molecular mechanism of HR elicitation and pathogenesis.

hrp genes of *E. amylovora* are repressed in rich media (6). Similar observations have been made with respect to *hrp* genes of some pathovars of *P. syringae* (14, 36). Studies utilizing metabolic inhibitors have suggested that active metabolism and de novo RNA and protein synthesis are required for induction of the HR in pepper (*Capsicum annuum* L.) by *Xanthomonas campestris* pv. vesicatoria (22). This requirement apparently reflects the need to overcome the lack of expression of *hrp* genes that occurs in rich media. Previous work in our laboratory indicated that the required period of de novo RNA or protein synthesis was reduced by incubating bacteria in a defined minimal medium (MM) (35). With *P. syringae* pv. glycinea, Huynh et al. (14) have shown that de novo transcription is not required for the HR induction when the bacteria are grown in a defined MM. Yucel et al. (36), using the K^+/H^+ exchange reaction (XR) assay, also demonstrated that incubating *P. syringae* in a nitrogen-deficient medium containing a metabolizable carbon source specifically abolished the sensitivity of the XR to transcriptional inhibitors. Thus, all of the *hrp* loci that have been studied so far are inhibited in rich media, and their expression is closely related to nutritional conditions (6, 13, 14, 24, 36). Nevertheless, our knowledge of *hrp* gene regulation in response to environmental stimuli is fragmentary; how expression of *hrp* genes in vitro is related to their function in planta is not known.

Here we report the transcriptional regulation of *hrp* genes of *E. amylovora* in response to environmental stimuli, both in vitro and in planta. We used Tn*5-gusA1*, a transposon that fuses target genes with the promoterless *gusA* gene of *E. coli* (27). The *gusA* gene product, β-glucuronidase (GUS), can be assayed easily and accurately by spectrophotometric, fluorometric, and histochemical methods (15, 16). Because *E. amylovora*, tobacco, and pear lack detectable GUS, *hrp*:: Tn*5-gusA1* fusions can be used to monitor expression of *E. amylovora hrp* genes both in vitro and in planta. On the basis of analysis of five fusions distributed throughout the *hrp* cluster, expression of *E. amylovora hrp* genes is transcriptionally regulated by ammonium, nicotinic acid, complex-nitrogen sources, certain carbon sources, pH, and temperature. Specific plant components are not required for transcriptional activation of *hrp* genes, and their high levels of expression in vitro are comparable to their levels of expression in plants. Interestingly, the levels of expression of *hrp* genes differ markedly in tobacco (nonhost) and pear (host) tissues.

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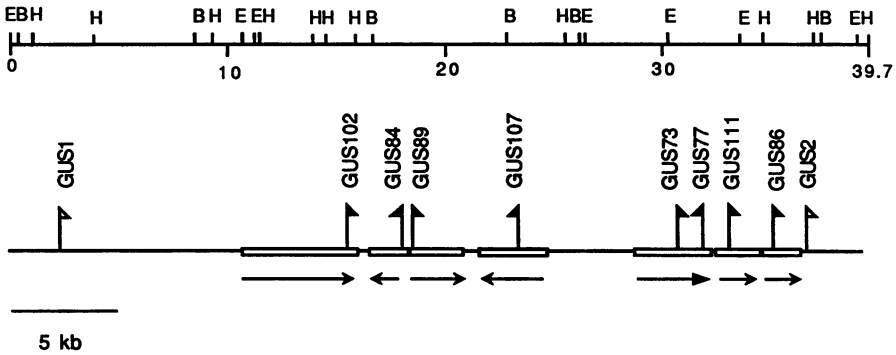

FIG. 1. Location of Tn*5-gusA1* insertions in the *hrp* gene cluster of *E. amylovora*. The restriction enzyme map of the *hrp* gene cluster is shown at the top, and the Tn*5-gusA1* insertions are shown below. The orientations of the insertions are indicated by the directions of the flags. Open flags indicate insertions that result in nonpathogenicity and reduced HR; closed flags indicated Hrp^- insertions. Open rectangles represent complementation groups in the *hrp* cluster; the arrows indicate transcriptional orientations. B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII.

MATERIALS AND METHODS

Construction of *hrp*::Tn*5-gusA1* insertions. *E. coli* CC118 harboring the cosmid pCPP430, which contains the entire cluster of *hrp* genes of *E. amylovora* Ea321 (8), was mutagenized with a Tn*5-gusA1* transposon on a lambda vector (27). Mutagenized cosmids were introduced into *E. coli* DH5α, and the resulting transconjugants were tested for elicitation of the HR in tobacco. Eight Hrp^- mutagenized cosmids were introduced into a Rif^r derivative of the wild-type strain Ea321 by triparental mating (10), by using the helper plasmid pRK600 (a gift from E. Signer). The eight Tn*5-gusA1* insertion mutants were created through marker exchange in a low-phosphate medium (4). Two HR-reduced insertions, GUS1 and GUS2, also were selected for marker-exchange mutagenesis. Expression of the *hrp* genes was monitored in tobacco leaf and immature pear fruit tissue and various media by monitoring GUS activity. The location of these eight insertions on the restriction endonuclease map of the *hrp* cluster was determined (Fig. 1).

Media and growth conditions. Three media were used for the GUS activity assay: Luria broth (LB) (21); MM that was modified from Miller's Minimal Medium (23) and contained, per liter, 3.51 g of K_2HPO_4, 1.5 g of KH_2PO_4, 1.0 g of $(NH_4)_2SO_4$, 0.25 g of L-asparagine, and 2.0 g of glucose (4); inducing medium (IM), modified from the XR assay buffer (2), containing 0.5 mM K_2SO_4, 0.5 mM $CaCl_2$, 0.5 mM MES (morpholineethanesulfonic acid), 175 mM mannitol, and 5 mM $(NH_4)_2SO_4$ and adjusted to pH 7.0. The log-phase culture grown in LB medium was inoculated into LB or MM and incubated at 24°C with shaking (200 rpm). The initial optical density at 620 nm of the inoculated culture was about 0.1. For inoculation of IM, the bacteria were concentrated and resuspended in IM to give an initial optical density at 620 nm of 0.4 (ca. 3×10^8 CFU/ml). The antibiotics used to maintain selection were kanamycin at 50 μg/ml and tetracycline at 10 μg/ml. The effects of carbon source, nitrogen source, and other compounds, e.g., nicotinic acid, on gene expression were determined in IM. The effect of temperature on *hrp* gene expression was determined at 18, 24, and 30°C in the three media described above.

GUS activity assay in vitro. GUS activity was assayed fluorometrically as described by Jefferson et al. (16). Log-phase cells (ca. 3×10^8 CFU) in 45 μl were mixed with an equal volume of 2× assay buffer (100 mM sodium phosphate buffer [pH 7.0], 0.2% Triton X-100, 20 mM β-mercaptoethanol, 2 mM 4-methylumbelliferyl glucuronide [Sigma M-9130]). The mixture was incubated at 37°C for 10 to 12 h, and the reaction was terminated by the addition of 10 μl of 2 M Na_2CO_3. The fluorescence of 4-methylumbelliferone (Sigma M-1381) was measured in 0.2 M Na_2CO_3 by excitation at 365 nm and emission at 455 nm with a spectrofluorometer (Model TKO 100; Hoefer Scientific Instruments). The fluorometer was calibrated with freshly prepared 4-methylumbelliferone standards in 0.2 M Na_2CO_3. The background fluorescence of wild-type *E. amylovora* lacking the Tn*5-gusA1* was subtracted from the readings of *hrp*::Tn*5-gusA1* strains. The corrected fluorescence readings were converted to picounits of GUS activity per CFU.

Assay of GUS activity in planta. (i) Tobacco cell suspensions. Suspension-cultured tobacco cells derived from *Nicotiana tabacum* L. var. Xanthi were maintained in Schenk-Hildebrandt medium as described by Atkinson et al. (2). Cells were transferred to fresh medium weekly. Tobacco cells from 50 ml of 3- to 5-day-old cultures were collected by filtration through Miracloth and washed once with two volumes of IM and adjusted to 125 mg (fresh weight) per 5 ml before use. The bacteria used were washed from Luria agar plates with IM and then inoculated into 5 ml of tobacco cell suspension at about 3×10^8 CFU/ml. The mixture was incubated at 24°C with shaking; samples for GUS activity assay were collected at intervals and assayed as described above.

(ii) Tobacco leaves. Log-phase bacterial cultures were centrifuged and resuspended in the original volume of HR test buffer (5 mM potassium phosphate buffer, pH 6.5), and then 200-μl aliquots were infiltrated into each leaf panel at room temperature. Three discs (5 mm in diameter) were collected from each infiltrated panel at each sampling time, ground in 1 ml of HR assay buffer in a microcentrifuge tube, and assayed as described above. The number of bacterial cells present in the discs was determined by dilution plating.

(iii) Immature pear. Immature pear fruits were surface disinfested with 70% ethanol for 2 to 3 min; radial slices (0.5 to 0.8 cm thick) were cut and inoculated with 100 μl of bacterial suspension (3×10^8 CFU/ml). After incubation in a moist chamber at 25°C for 6, 12, and 24 h, three cylinders of pear tissue (about 0.5 g) were taken from each slice, ground in a centrifuge tube with 0.5 ml of HR assay buffer, and assayed as described above.

HR test in the presence of antibiotics. Bacterial suspensions, prepared as for the GUS activity assay in tobacco leaves, were infiltrated into each leaf panel. The bacterial

RNA polymerase inhibitor rifampin (200 μg/ml) or the protein translational inhibitor tetracycline (20 μg/ml) or chloramphenicol (50 μg/ml) was infiltrated into the same area at intervals after bacterial infiltration. The development of the HR was scored after incubation at room temperature for 18 h.

RESULTS

Mutagenesis of the *E. amylovora hrp* gene cluster with Tn*5-gusA1*. The *hrp* gene cluster of *E. amylovora* has been delineated by transposon mutagenesis and complementation analysis (6, 7). The present study focused on the regulation of *hrp* loci by creating *hrp-gus* operon fusions. For this purpose, the cosmid pCPP430, which contains the entire *hrp* cluster and bestows on *E. coli* the ability to elicit HR, was mutagenized by Tn*5-gusA1*. Eight Tn*5-gusA1* insertion mutants that failed to elicit the HR in tobacco were selected from 114 that were tested. The precise location of the insertions in the *hrp* cluster was determined by mapping with multiple restriction enzymes (Fig. 1). The leftmost insertion, GUS102, was mapped at 16.2 map units (1 map unit equals 1 kb; numbering begins at the leftmost *Eco*RI restriction site [Fig. 1]), and the rightmost insertion, GUS86, was mapped at 35.8 map units. The region covered by reporter transposon Tn*5-gusA1* insertions was about 20 kb, which was shown previously to be essential for HR elicitation and sufficient for XR elicitation (6). Eight of 10 HR^+ Tn*5-gusA1* insertions mapped to regions outside the *hrp* cluster located at the ends of the *E. amylovora* DNA cloned in pCPP430. According to an analysis of complementation groups of the *hrp* cluster with merodiploids and certain subclones (7), two of the eight Tn*5-gusA1* insertions, GUS73 and GUS77, are in the same complementation group. The other six insertions are located in individual complementation groups (Fig. 1). Subsequently, the eight Hrp^- marker-exchange haploids in a Rif^r derivative of the wild-type strain Ea321 were constructed. All the marker-exchange haploids containing the Tn*5-gusA1* transposon failed to elicit the HR in tobacco and to cause disease in pear (Hrp^-). The locations and orientations of the eight Tn*5-gusA1* insertions in the *hrp* gene cluster are shown in Fig. 1.

Expression of *E. amylovora hrp*::Tn*5-gusA1* insertions in vitro. Assay of GUS activity of eight *hrp*::Tn*5-gusA1* insertions indicated that *hrp* genes are not expressed in rich medium (LB). In several independent experiments, the GUS activity of these insertions (marker exchanged into *E. amylovora*) incubated in LB or MM was very low. However, five of the eight insertions, GUS84, GUS107, GUS73, GUS111, and GUS86, exhibited high levels of expression in IM (Fig. 2). Insertion GUS77 is in the same complementation group as GUS73 (7) but in the opposite orientation. Transcription of this unit is from left to right. Thus, insertion GUS77 is perhaps not in the correct orientation. The insertions GUS102 and GUS89 apparently are in separate complementation groups, but significant expression was not detected either in vitro or in planta. Recently, we have found that these two loci were negatively regulated by another *hrp* gene (34). Hence, data from the five transcriptional fusions are emphasized in this report.

Regulation of *hrp* genes in response to nitrogen and carbon sources. *hrp* genes are regulated in response to the concentration of NH_4^+ and other sources of nitrogen. Because *hrp* genes were repressed in rich medium and highly expressed in IM, we sought to identify the components responsible for repression. The addition of 50 mM $(NH_4)_2SO_4$ to IM reduced

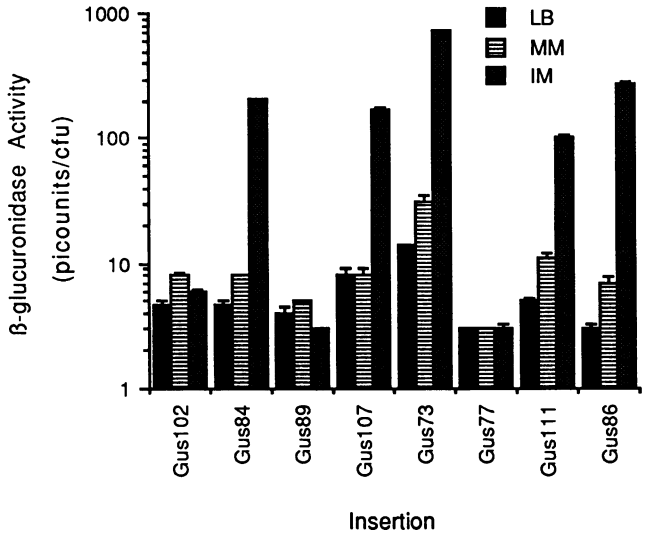

FIG. 2. GUS activity of eight *E. amylovora* Ea321 *hrp*::Tn*5-gusA1* mutants 6 h after incubation in three media. The bacteria were grown and assayed for GUS activity, as described in Materials and Methods. Error bars indicate one standard deviation calculated from triplicate samples.

GUS activity by 80%. At 150 mM $(NH_4)_2SO_4$, the expression level was similar to that in rich medium (Fig. 3). Similar effects on expression occurred when NH_4Cl was used instead of $(NH_4)_2SO_4$. However, the same concentrations of NaCl and Na_2SO_4 had little effect (data not shown). Therefore, high concentrations of NH_4^+ appear to repress gene expression.

To ascertain whether gene expression is regulated by other nitrogen sources, 5 mM urea, 0.5% Casamino Acids, 5 mM glutamic acid, glycine, leucine, tryptophan, threonine, asparagine, and histidine, representative of different nitrogen metabolic pathways, were added individually to IM with 5 mM $(NH_4)_2SO_4$. Bacteria grew as well in IM with 0.5% Casamino Acids as they did in LB medium, but *hrp* gene expression was completely repressed. The growth rate of the bacteria in IM was low and was not affected by any of the other supplements, but certain supplements to IM lowered

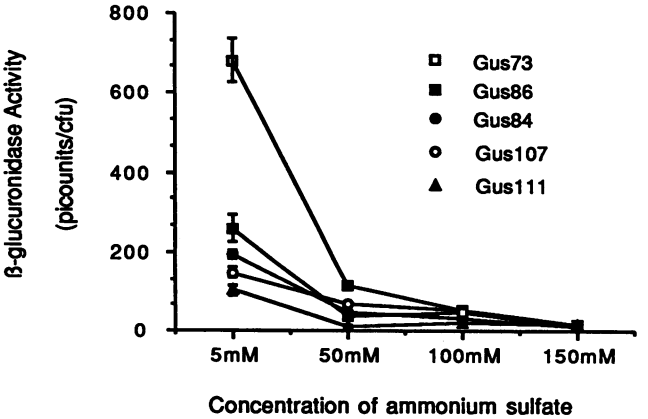

FIG. 3. Effect of NH_4^+ concentration on expression of *E. amylovora hrp* genes. Bacteria were incubated in IM containing 5 to 150 mM $(NH_4)_2SO_4$ and sampled at 6 h. Error bars indicate one standard deviation calculated from triplicate samples.

TABLE 1. Effect of different amino acids and urea on expression of *E. amylovora hrp* genes

Nitrogen source[a]	Mean activity ± SD (pU/CFU) of GUS from:				
	GUS84	GUS107	GUS73	GUS111	GUS86
Urea	156 ± 5.0	177 ± 5.0	405 ± 9.0	153 ± 5.0	53 ± 4.7
Glutamic acid	183 ± 4.0	283 ± 36.0	421 ± 19.0	259 ± 2.8	253 ± 2.7
Glycine	79 ± 6.0	104 ± 1.3	181 ± 18.0	78 ± 7.6	139 ± 4.2
Leucine	102 ± 2.5	107 ± 3.0	187 ± 13.0	82 ± 4.0	145 ± 5.0
Tryptophan	96 ± 6.4	106 ± 4.0	229 ± 28.0	72 ± 3.6	131 ± 4.0
Threonine	149 ± 8.0	133 ± 3.0	304 ± 14.0	125 ± 4.0	197 ± 4.0
Asparagine	42 ± 5.0	55 ± 3.3	45 ± 1.0	26 ± 11.0	92 ± 3.8
Histidine	15 ± 0.8	30 ± 3.3	20 ± 1.7	31 ± 4.0	16 ± 1.7
Casamino Acids	2 ± 0.5	19 ± 1.9	5 ± 0.6	4 ± 0.6	2 ± 0.3
IM [5 mM $(NH_4)_2SO_4$]	175 ± 4.0	155 ± 3.0	720 ± 56.0	114 ± 3.1	199 ± 6.1

[a] Each nitrogen source at a concentration of 5 mM was added to IM, which contained 5 mM $(NH_4)_2SO_4$; Casamino Acids were added at 0.5%.

expression levels. In particular, histidine and asparagine markedly repressed *hrp* gene expression (Table 1). These results suggest that the *hrp* genes of *E. amylovora* are expressed under conditions of nitrogen deficiency. However, it is not known why only histidine and asparagine, of the seven amino acids tested, significantly inhibited *hrp* gene expression.

GUS activity of the five transcriptional *hrp*::Tn*5-gusA1* fusions also was determined in IM lacking 175 mM mannitol but containing individual carbon sources at 20 mM (mannitol, fructose, glycerol, sucrose, glucose, or maltose). Maltose (20 mM) essentially eliminated *hrp* gene expression in all fusions except GUS86, but expression was restored by the addition of 20 mM mannitol. Glucose strongly repressed fusions GUS73, GUS84, and GUS111; fusions GUS107 and GUS86 were somewhat inhibited by glucose. The other carbon sources tested had little or no effect on expression (Table 2).

Inhibition of *hrp* genes by other components. Nicotinic acid, pH, and temperature also regulate *hrp* genes. Acidic conditions favor expression of *hrp* genes. The expression levels of the five fusions were three times higher in MM at pH 5.5 than those at pH 7.0 (data not shown). When the pH of IM was adjusted to 7.0, no significant reduction resulted from the addition of compounds such as amino acids and nicotinic acid. However, during incubation of bacteria in IM, the pH decreased to about 5.5, which favors *hrp* gene expression. Nicotinic acid (2 mM) either alone or combined with NH_4^+ or amino acids inhibited *hrp* gene expression (Table 3). For instance, *hrp* genes were expressed at high levels in IM [with 5 mM $(NH_4)_2SO_4$ or when the $(NH_4)_2SO_4$ was replaced by 5 mM glutamic acid] but were repressed by the addition of 2 mM nicotinic acid. In addition, HR development was effectively inhibited by periodic infiltration, following bacterial infiltration, of 2 mM nicotinic acid in buffer into tobacco leaves. Periodic infiltration of the same buffer lacking nicotinic acid did not affect HR development (data not shown).

Low temperature is more favorable for expression of *hrp* genes of *E. amylovora* than high temperature. Highest expression occurred at 18°C of the three temperatures tested (Table 4). GUS activity was two- to threefold greater at 18°C than at 24°C in either LB or MM. When the bacteria were

TABLE 2. Effect of different carbon sources on expression of *E. amylovora hrp* genes

Carbon source[a]	Mean activity ± SD (pU/CFU) of GUS from:				
	GUS84	GUS107	GUS73	GUS111	GUS86
Mannitol	102 ± 11.0	86 ± 9.2	260 ± 33.2	108 ± 4.3	135 ± 11.5
Mannitol + M[b]	102 ± 6.7	142 ± 6.2	356 ± 35.7	123 ± 1.5	181 ± 9.9
Fructose	92 ± 9.0	102 ± 11.8	177 ± 18.5	120 ± 7.8	90 ± 14.2
Fructose + M	106 ± 4.0	153 ± 5.7	292 ± 36.5	120 ± 11.9	145 ± 5.7
Glycerol	100 ± 6.2	99 ± 4.9	138 ± 11.5	105 ± 3.4	115 ± 5.7
Glycerol + M	105 ± 6.7	196 ± 13.9	269 ± 30.2	111 ± 5.5	191 ± 10.0
Sucrose	104 ± 7.1	63 ± 11.0	163 ± 4.5	106 ± 6.6	91 ± 4.0
Sucrose + M	103 ± 4.7	75 ± 5.4	453 ± 31.0	109 ± 5.3	64 ± 5.3
Glucose	33 ± 1.3	81 ± 15.2	19 ± 2.3	40 ± 2.4	156 ± 7.1
Glucose + M	92 ± 10.0	72 ± 14.7	34 ± 5.0	45 ± 10.9	100 ± 17.4
Maltose	27 ± 1.4	14 ± 1.7	5 ± 0.6	3 ± 0.5	81 ± 5.7
Maltose + M	103 ± 6.4	128 ± 6.4	368 ± 39.2	120 ± 16.0	149 ± 6.9
IM (M)	207 ± 2.5	170 ± 3.6	682 ± 16.0	103 ± 2.6	269 ± 8.7

[a] Each carbon source at a concentration of 20 mM was added to IM minus 175 mM mannitol.
[b] M, mannitol at 175 mM.

TABLE 3. Effect of nicotinic acid on expression of *E. amylovora hrp* genes

Nitrogen source[a]	Mean activity ± SD (pU/CFU) of GUS from:				
	GUS84	GUS107	GUS73	GUS111	GUS86
NA	5 ± 0.6	6 ± 0.6	17 ± 0.8	6 ± 0.5	4 ± 0.5
$(NH_4)_2SO_4$	175 ± 4.0	155 ± 3.0	720 ± 56.0	114 ± 3.1	199 ± 6.1
$(NH_4)_2SO_4$ + NA	7 ± 0.5	10 ± 0.9	15 ± 0.4	6 ± 1.2	4 ± 0.6
Glutamic acid	183 ± 4.0	107 ± 3.0	421 ± 19.0	82 ± 4.0	253 ± 28.0
Glutamic acid + NA	13 ± 0.5	14 ± 0.9	22 ± 1.3	6 ± 0.8	5 ± 0.7
Leucine	102 ± 2.5	107 ± 3.0	187 ± 13.0	82 ± 4.0	145 ± 5.0
Leucine + NA	3 ± 0.8	8 ± 0.9	16 ± 1.2	5 ± 1.2	3 ± 0.5

[a] Individual nitrogen sources were added to IM without $(NH_4)_2SO_4$ at a concentration of 5 mM, except for nicotinic acid (NA), which was added at a concentration of 2 mM.

incubated in IM, there was no significant effect of temperature on the *hrp* gene expression, except for fusion GUS107.

Expression of *hrp* genes in planta. (i) *hrp* expression in a nonhost plant, tobacco. Two approaches were taken to determine whether plant components provide additional expression of *hrp* genes. First, bacteria were incubated with cultured tobacco cells suspended in IM. Levels of expression of *hrp* loci during coincubation with tobacco cell suspensions were the same as those in IM (Fig. 4). As in the in vitro assay, no GUS activity was detected for the insertions GUS102, GUS89, and GUS77 in tobacco cell suspensions. However, the other five transcriptional fusions were highly expressed under both conditions. The time course of gene activation in tobacco cell suspensions was also similar to that in IM (Fig. 5). GUS activity increased beginning 2 h after incubation, reached the maximum after 6 h, and then decreased with further incubation. Secondly, the eight Tn*5-gusA1* insertions infiltrated in whole tobacco leaves confirmed that the expression of *hrp* genes was similar to that in IM and in tobacco cell suspensions. Only those fusions that were expressed in IM or in tobacco cell suspensions were expressed in whole tobacco leaves (Fig. 4). Thus, transcriptional expression of the *hrp* loci is not increased in the environment of plant tissue culture or in the whole plant.

(ii) *hrp* expression in a host, immature pear. Immature pear fruits were inoculated with the eight strains containing Tn*5-gusA1* insertions. No specific host plant-inducible locus was found, on the basis of GUS activity following incubation for 24 h. The *hrp* loci expressed in immature pear fruits were the same as those in tobacco or IM (Fig. 6). However, expression of the *hrp* genes in pear was delayed and at a much lower level than in tobacco (Fig. 5 and 6). In tobacco, 3 h after infiltration, *hrp* genes were induced; after 6 h, expression was greatest, and after 10 h, GUS activity had decreased (Fig. 5). The time of *hrp* gene expression detected for the Tn*5-gusA1* fusions correlated with the appearance of visible cell collapse of tissues infiltrated with the wild-type strain, Ea321. Cell collapse usually occurred 8 to 10 h after infiltration of Ea321, and the number of bacterial cells recovered from tobacco leaves 5 h after inoculation were unchanged. At 10 h, only ca. 1% of the initial inoculum was recovered. However, the populations recovered from Tn*5-gusA1* insertion mutants were similar to those of the initial inoculum, even 12 h after infiltration (data not shown).

In pear fruits, during the first 12 h, no significant expression of *hrp* loci was detected. After 24 h, fusions GUS84, GUS73, GUS111, and GUS86 were expressed at 10 to 20% of their maximum activity in tobacco leaves. Also, after 24 h, ooze was apparent in pears inoculated with the wild-type strain and the population of wild-type bacteria had increased at least 100-fold. GUS activity detected from fusion GUS107 did not differ significantly between pear and tobacco (Fig. 4

TABLE 4. Effect of temperature on expression of *E. amylovora hrp* genes

Medium	Fusion	Mean activity ± SD (pU/CFU) of GUS at:		
		18°C	24°C	30°C
LB	GUS107	24 ± 2.1	9 ± 0.9	2 ± 0.5
	GUS73	31 ± 1.6	12 ± 0.7	5 ± 0.6
	GUS86	7 ± 0.8	2 ± 0.7	1 ± 0.7
MM	GUS107	62 ± 3.0	24 ± 0.8	19 ± 1.8
	GUS73	84 ± 2.4	31 ± 1.0	31 ± 2.0
	GUS86	36 ± 1.3	14 ± 1.0	10 ± 0.9
IM	GUS107	315 ± 7.4	188 ± 15.8	114 ± 5.3
	GUS73	418 ± 6.0	378 ± 8.0	371 ± 12.8
	GUS86	108 ± 5.7	93 ± 9.9	91 ± 4.5

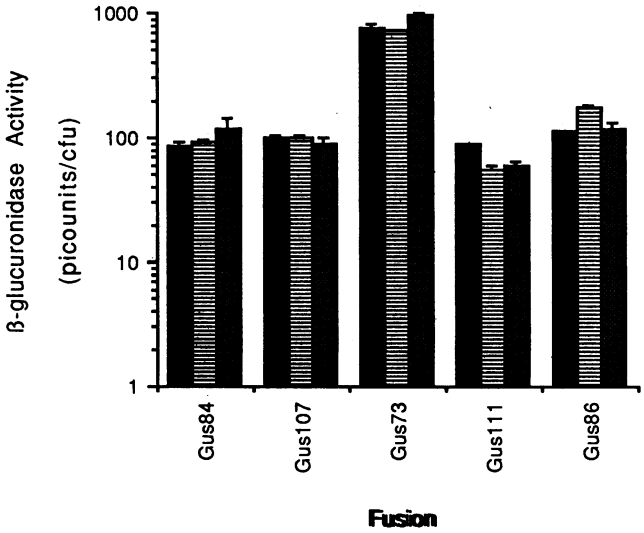

FIG. 4. GUS activity of *hrp*::Tn*5-gusA1* fusions after incubation for 6 h in IM, tobacco cell suspensions, and tobacco leaves. Error bars indicate one standard deviation calculated from triplicate samples. ■, tobacco cell suspension; ▤, IM; ▨, tobacco leaf.

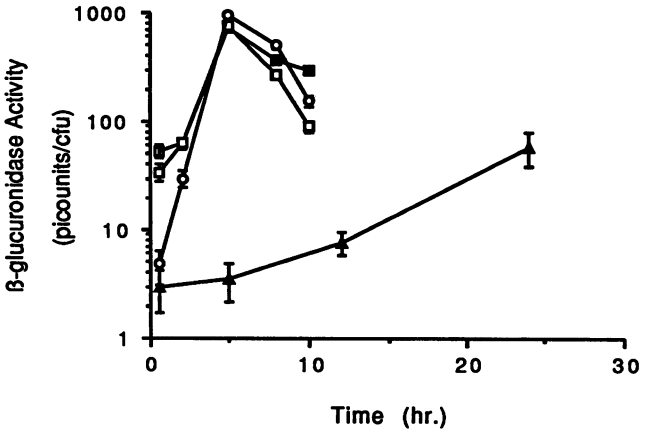

FIG. 5. Expression of fusion GUS73 over time in IM, tobacco cell suspension, tobacco leaf, and immature pear fruit. Four other fusions, GUS84, GUS107, GUS111 and GUS86, showed similar temporal expression. Error bars indicate one standard deviation calculated from triplicate samples. ○, tobacco leaf; ■, IM; □, tobacco cell suspension; ▲, pear fruit.

and 6). This fusion is situated in a positive regulatory locus, whose predicted protein product (28) is homologous to a highly conserved domain of several prokaryotic regulatory proteins, including HrpS of *P. syringae* pv. phaseolicola (11, 26).

HR elicitation in the absence of de novo RNA and protein synthesis. Previous studies have indicated that for bacteria grown in a complete medium, a minimum 3-h period of induction is required in plant tissue, during which de novo RNA and protein synthesis occur (35). Since *hrp* genes are activated in IM, we determined whether bacteria grown in IM are able to elicit the HR in the absence of de novo RNA or protein synthesis. Infiltrated suspensions of the wild-type strain Ea321 grown in LB or MM with rifampin, tetracycline, or chloramphenicol did not elicit the HR. Infiltration of antibiotic after a 3-h induction period in tobacco leaf did not affect HR development. Thus, de novo RNA and protein synthesis are necessary for elicitation of HR when the bacteria are grown in LB or MM, in which *hrp* genes are repressed. However, when the bacteria were incubated in IM for 5 to 6 h, they elicited the HR in the presence of rifampin; further induction in tobacco plant was not required (Fig. 7). In the presence of the translational inhibitors tetracycline and chloramphenicol, even though the bacteria were incubated in IM, they elicited the HR only after an induction period of 0.5 h in the plant (Fig. 7).

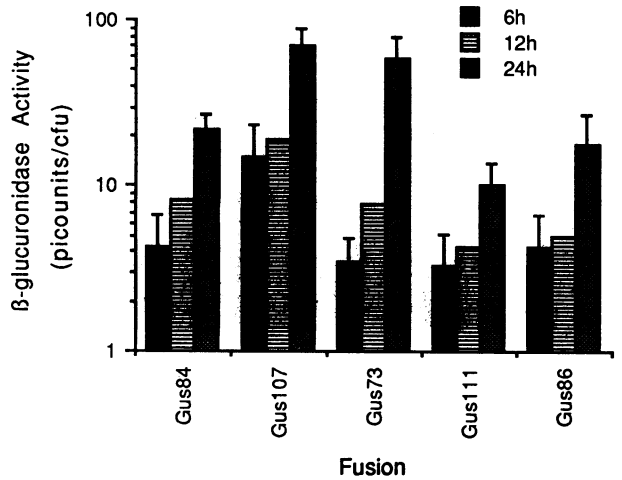

FIG. 6. Expression of five *hrp*::Tn*5-gusA1* fusions in pear. About 0.5 g of pear tissue was collected after incubation for 6, 12, or 24 h. Error bars indicate one standard deviation calculated from triplicate samples.

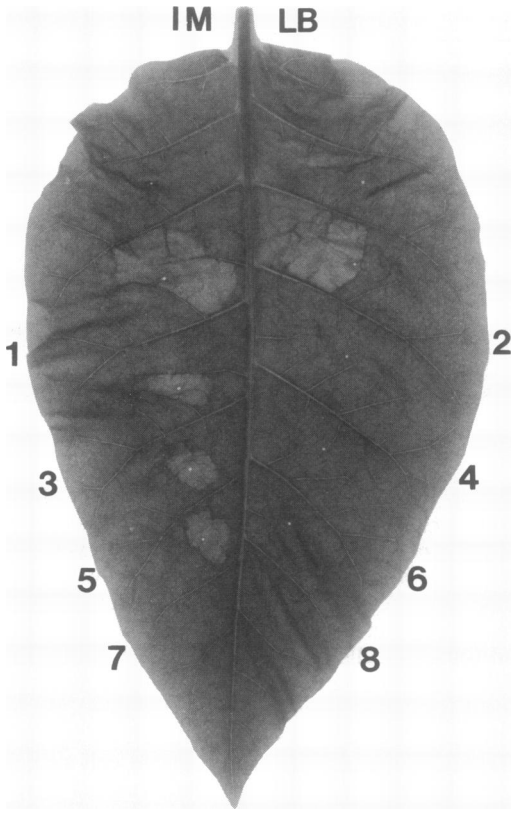

FIG. 7. Ability of *E. amylovora* Ea321 to induce the HR in the presence of antibiotic. Bacteria incubated in IM (samples 1, 3, 5, and 7) or LB (samples 2, 4, 6, and 8) were pelleted and resuspended at 4×10^8 CFU/ml in 5 mM potassium phosphate buffer (pH 6.5) and infiltrated into separate leaf panels. Samples 3 and 4 were treated with rifampin (200 μg/ml) immediately prior to infiltration. Tetracycline (20 μg/ml) for samples 5 and 6 and chloramphenicol (50 μg/ml) for samples 7 and 8 were infiltrated into the areas in which the bacteria had been infiltrated 0.5 h earlier. The leaf was detached for photography after 18 h of incubation at room temperature.

DISCUSSION

Previous characterization of the *hrp* gene cluster of *E. amylovora* has indicated that it spans about 40 kb of DNA, but only about 22.5 kb of the region is needed for elicitation of XR (6). Mutant GUS102, created in this study, is the leftmost one that failed to elicit the HR and XR. The mutants with insertions located to the left of the complementation group that includes GUS102 showed the phenotype of reduced HR and nonpathogenicity to pear, which is similar to *dsp* mutants described by Barny et al. (3) in *E. amylovora* CFBP1430. Insertion GUS86 is situated at the right border of the region required for the XR. (The insertion GUS2 to the right of GUS86 showed reduced HR and nonpathogenicity to pear.) Because the eight Hrp$^-$ Tn*5-gusA1* insertions in-

cluded in this study covered the essential region of the *hrp* cluster, our expression studies represent the regulation of the *E. amylovora hrp* gene cluster in response to environmental stimuli. Eight Tn*5-gusA1* insertions were located in seven different complementation groups. Only five *hrp* loci that fused with Tn*5-gusA1* were expressed under the defined conditions used in this study; no expression was detected for two *hrp* loci in which insertions GUS102 and GUS89 were located either in vitro or in planta. In an effort to explore the relationships of the *hrp* genes, a number of double marker-exchange mutants were recently constructed. GUS102 and GUS89 were highly expressed when additional insertions were present in the leftmost 3.8-kb *Eco*RI fragment of the cluster (34). Apparently, these two *hrp* loci are negatively regulated, and their transcriptional orientation is consistent with that of reporter transposon insertions. How these two *hrp* loci are regulated and the roles that they play in HR elicitation are unknown.

Assay of GUS activity showed that *hrp* genes are inhibited in complete medium, such as LB. This is consistent with previous results of mRNA analysis and HR studies in our laboratory (35) and also HR and XR analyses done in other laboratories with other pathogens (14, 36). Examination of the effects of several nitrogen and carbon sources showed that the *hrp* genes of *E. amylovora* are regulated by the concentrations of ammonium ion, Casamino Acids, histidine, asparagine, nicotinic acid, certain carbon sources, pH, and temperature. In the *E. amylovora*-tobacco system, when high concentrations of ammonium sulfate or ammonium chloride were added, *hrp* genes were repressed to levels similar to those in LB. The ammonium ion clearly is the important component, as sodium chloride and sodium sulfate had no significant effect. These results contrast with those of Mindrinos et al. (24), who presented evidence for repression of *hrp* genes of *P. syringae* pv. phaseolicola by high osmotic concentration. No significant difference was found in gene expression between LB and MM. Thus, three components of MM—glucose, nicotinic acid, and asparagine—seem to be responsible for repression of *hrp* genes.

It is surprising that only histidine and asparagine, of the seven individual amino acids tested, greatly inhibited gene expression. No relationship between growth rate and gene activity was observed when the fusions were grown in IM supplemented with the different amino acids. Thus, the biochemical mechanism of gene repression by histidine and asparagine is unknown. It is interesting that repression of *E. amylovora hrp* genes by 2 mM of nicotinic acid in vitro is correlated with the function of *hrp* genes in planta. Starr and Mandel (30) reported that nicotinic acid is required for the growth of *E. amylovora* in glucose-salts-basal medium and that nicotinamide (an amide of nicotinic acid) also can be used directly as a nitrogen source. Perhaps, nicotinic acid affects the nitrogen status of *E. amylovora*, which in turn affects *hrp* gene expression. Nicotinamide is an essential component of pyrimidine nucleotides that function as coenzymes for many oxidoreductases. The study of the mechanisms by which nicotinic acid represses *hrp* genes of *E. amylovora* may help elucidate the function of *hrp* genes.

Only the *hrp* loci expressed in IM were highly expressed in tobacco; no solely plant-inducible gene was found. Gene expression, monitored by GUS assay, was closely related to development of the HR in tobacco. Only cells incubated in IM, which induces *hrp* gene expression in the wild-type strain Ea321, are able to elicit HR in the presence of the transcriptional inhibitor rifampin. This suggests that no specific plant components are required for transcriptional activation of *E. amylovora hrp* genes and that incubation in IM, which activates the five *hrp* loci, also permits transcription of all bacterial genes required for elicitation of the HR. These results are consistent with those of Huynh et al. (14), who found that *hrp* genes of *P. syringae* pv. glycinea are transcribed in a defined MM at pH 5.5. Similarly, Yucel et al. (36) found that transcriptional activation of *hrp* genes during coculture with tobacco cells in XR assays is not dependent on plant cells but can be achieved by incubating the bacteria in nitrogen-deficient medium. These results and the finding that some of *hrp* genes are functionally conserved in nonpathogenic bacteria, such as *E. coli* (33), suggest that *hrp* gene products act at a basic metabolic level.

Nutritional status is important for transcription of *E. amylovora hrp* genes, rather than the presence of plant-derived compounds, which serve to turn on the symbiosis and virulence genes of *Rhizobium* (25) and *Agrobacterium* (29) species, respectively. Although we found no evidence that plant-derived compounds are required in transcriptional activation of *E. amylovora hrp* genes, we cannot rule out that possibility. A specific plant signal may be required for translation or protein processing, because translational inhibitors prevented HR elicitation and a 0.5-h induction stage in plant leaves was required.

Genetic analysis of *E. amylovora* and other plant pathogenic bacteria has shown that mutants that do not elicit the HR in nonhost plants also are nonpathogenic to host plants (3, 5, 9, 12, 20, 31, 32). Thus, the *hrp* gene cluster is required for both HR elicitation and pathogenicity. How the same set of genes can function in both compatibility and incompatibility is unclear. Interestingly, our studies showed that *E. amylovora hrp* genes are regulated differently in host and nonhost plants, in terms of both time and the level of expression. In the nonhost plant tobacco, the *hrp* genes were quickly turned on and maximally expressed 5 to 6 h after infiltration. The time of maximal *hrp* gene expression in tobacco leaves was correlated with the initial appearance of visible symptoms of cell collapse in the infiltrated area caused by the wild-type strain. Symptoms of cell collapse usually were clearly evident 8 to 10 h after infiltration; by then, the bacterial numbers of the wild-type strain had decreased to less than 1% of the initial concentration. Thus, under stressed conditions in the nonhost plant, once the *hrp* genes were expressed maximally, the bacteria began to die. This suggests that quick and high expression of the *hrp* genes in the nonhost plant not only causes a quick defense reaction of the plant (HR) but also sacrifices the bacteria; this reaction leads to the incompatible interaction. In contrast, in the host plant pear, only low GUS activity was detected during the first 24 h after inoculation; by this time, bacterial cell numbers had increased at least 100-fold and disease development was clearly underway. These results suggest that the different environments of nonhost plant (tobacco leaf) and host plant (immature pear fruit) affect *hrp* gene expression. Thus, differential *hrp* gene regulation between host and nonhost plants may be one of the mechanisms that determine whether an organism causes disease or elicits the HR in a particular plant-microbe interaction. Further study is required to elucidate the molecular mechanism that controls differential *hrp* gene expression in host and nonhost plants.

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