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Mutations in the Regulatory Gene *hrpG* of *Xanthomonas campestris* pv. vesicatoria Result in Constitutive Expression of All *hrp* Genes

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***hrpG* is a key regulatory gene for transcriptional activation of pathogenicity genes (*hrp*) of *Xanthomonas campestris* pv. vesicatoria. We identified three mutations in *hrpG* which render *hrp* gene expression constitutive in normally suppressing medium. The mutations in *hrpG* result in novel amino acid substitutions compared to mutations in related proteins, such as OmpR. In addition, mutated *hrpG* enhances the timing and intensity of plant reactions in infection assays.**

The interaction of the gram-negative bacterium *Xanthomonas campestris* pv. vesicatoria (hereafter *X. campestris*) with its host plants pepper and tomato is controlled by *hrp* (hypersensitive reaction and pathogenicity) genes (3), some of which are predicted to encode components of a type III protein secretion system (1, 5). *hrp* gene expression is suppressed in complex medium (NYG [5 g of peptone, 3 g of yeast extract, and 20 g of glycerol per liter]), but is induced in planta and in the synthetic XVM2 medium (16, 19) and is regulated by two genes, *hrpX* and *hrpG*. *hrpX* encodes a protein of the AraC family and activates transcription of the operons *hrpB* to *hrpF* (18). Expression of *hrpX* and *hrpA* depends on *hrpG*, which encodes a putative response regulator protein of two-component signal transduction systems (20). The predicted HrpG protein is most similar to proteins of a subclass containing *Escherichia coli* OmpR (4, 22) and *Agrobacterium tumefaciens* VirG (21). The *X. campestris hrpG* gene is expressed at a low level in NYG medium but requires *hrp* gene-inducing conditions for the activation of downstream genes (20). To achieve *hrp* gene expression in noninducing media, we performed a random mutagenesis of *hrpG* and selected for mutations rendering expression of all *hrp* genes constitutive in NYG medium.

First, a suitable reporter plasmid, pPCspec, was constructed by fusing a 320-bp fragment encompassing the *hrpG*-regulated *X. campestris hrpC* promoter to a promoterless *aadA* gene in pLAFR6 (2). The *aadA* gene, which confers resistance to spectinomycin, was amplified by PCR from the omega cassette (14) by using oligonucleotides 161 (5′ GGGGAGCTCAAACAAA GTTAAACATC 3′) and 162 (5′ GGGGAATTCATTATTTG CCGACTACC 3′) (restriction sites used for cloning are underlined). For mutagenesis of *hrpG*, plasmid pFG72, which carries *hrpG* on a 1.7-kb *Bam*HI-*Kpn*I fragment in the low-copy plasmid pUFR043 (gift from D. Gabriel, University of Florida, Gainesville), was transformed into the *E. coli* mutator strain Epicurian Coli XL1-Red (Stratagene, La Jolla, Calif.). Plasmid DNA was isolated at different time points and transformed into *E. coli* DH5α. Transformants were conjugated en masse with *X. campestris* 85-10 (11) carrying pPCspec and plated on NYG agar containing 400 μg of spectinomycin per ml. Eleven spectinomycin-resistant transconjugants, obtained at a frequency of 10^{-4}, were analyzed further. To test the effect of the mutated pFG72 derivatives on *hrp* promoter activity, they were first introduced into *X. campestris* 85-10 carrying pPC490, an *hrpC* promoter-*uidA* fusion in pL6GUSB (2). β-Glucuronidase (GUS) activities were determined after growth of bacteria in NYG and in *hrp* gene-inducing XVM2 medium. All 11 mutated *hrpG* plasmids (designated pFG72-1 to pFG72-11) activated the *hrpC* promoter in NYG. To determine the activity of all other known *hrp* promoters in presence of the mutated *hrpG* gene, pFG72-1 (*hrpG* herein referred to as *hrpG**) was conjugated into *X. campestris* strains carrying *hrp* promoter-*uidA* fusion plasmids pPA2, pPB1, pPD3, pPFI42, pPG1, and pPX2 (2, 6, 18–20), which express the *uidA* gene under the control of the *hrpA*, *hrpB*, *hrpD*, *hrpF*, *hrpG*, and *hrpX* promoters, respectively. *hrpE* promoter activity was determined by using pXV4::E525 (18). As shown in Fig. 1A, additional copies of the wild-type *hrpG* plasmid pFG72 resulted in somewhat higher GUS activities of bacteria grown in NYG medium compared to 85-10. However, in the presence of *hrpG**, all *hrp* promoters, except for the *hrpG* promoter, exhibited 20- to 1,000-fold higher activities in NYG compared to wild-type strain 85-10. In XVM2 medium, GUS activities in the presence of *hrpG** were in the same order of magnitude as those with additional copies of the wild-type gene and were 10-fold higher than those for strains containing only the genomic copy (Fig. 1B).

DNA sequencing of *hrpG** in pFG72-1 revealed a single G-to-A mutation at position 130 of the *hrpG* coding sequence, leading to an E44K exchange in the translation product. Since this mutation destroys a *Sac*I restriction site, the remaining 10 pFG72 derivatives were tested for presence or absence of this site. While eight of them had lost the *Sac*I site and probably carry the same mutation as pFG72-1, the *Sac*I site was present in two plasmids, pFG72-2 and pFG72-3. Sequence analysis revealed a G-to-A exchange at position 595 of *hrpG* in pFG72-2, leading to a D199N substitution, and an A-to-G mutation at position 581 in pFG72-3, resulting in an H194R exchange. All three *hrpG* mutations had identical effects on the activity of the *hrpG*-dependent promoters (data not shown).

Only a few known mutations in OmpR-type transcriptional activators render the protein constitutively active. These mu-

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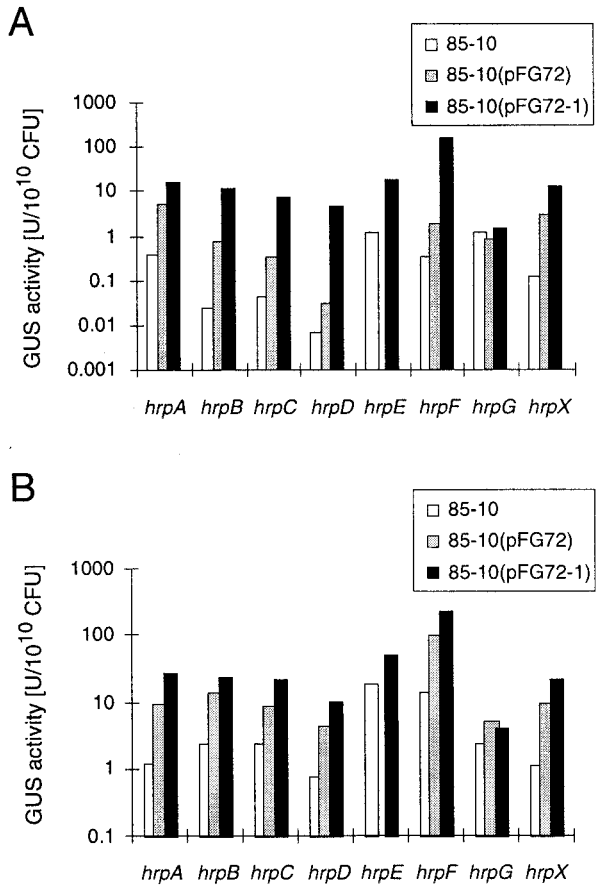

FIG. 1. Activity of *X. campestris hrp* promoters in the presence of wild-type *hrpG* and *hrpG**. Wild-type strain 85-10 carrying plasmid-borne transcriptional *hrp* promoter-*uidA* fusions and no additional *hrpG*, plasmid-borne *hrpG* (pFG72), and *hrpG** (pFG72-1), respectively, were grown for 16 h in NYG (A) and XVM2 (B). For technical reasons, *hrpE* promoter activity was determined with *hrpG** expressed in pDSK600. GUS assays were performed as described previously (16). Specific GUS activities are the average of two experiments and are graphically displayed with a logarithmic scale (1 U = 1 nmol of 4-methylumbelliferyl-β-D-glucuronide released per bacterium per min).

tations have all been mapped to the N-terminal domain of a given protein (Table 1 and Fig. 2). For example, in the *Agrobacterium* irG protein, N54E or I106L substitutions (N80 or I132 in Fig. 2) resulted in constitutive *vir* gene expression (12). A D55E substitution in the *E. coli* OmpR protein rendered it active independent of the sensor kinase EnvZ (8). Whether the E44K mutation in the N-terminal domain of HrpG mimics the predicted phosphorylation of the D60 residue in HrpG (20) is a matter of speculation. Interestingly, the other two mutations in *X. campestris* HrpG identified here, H194R and D199N, are located in the C-terminal domain of the protein. In contrast to HrpG, substitutions at corresponding positions in *E. coli* OmpR (E193K and E198K) and PhoB (D192G) led to loss of function (9, 13, 15). It has been shown for OmpR that the region from amino acid 192 to amino acid 199 forms a loop exposed at the protein surface, which has been proposed to control RNA polymerase activity by direct interaction with its α-subunit (10). The H194R and D199N substitutions in HrpG might, therefore, lead to transcriptional activation due to alteration of contact with RNA polymerase.

Because *hrpG* is a key regulator for the interaction of *X. campestris* with plants, we wondered whether the three mutations in *hrpG* alter the plant reaction after infection. To avoid interference of mutated *hrpG* with the wild-type gene, a 950-bp *Sac*I fragment spanning most of the *hrpG* coding region was deleted. The mutation was introduced into the chromosome of *X. campestris* wild-type strain 85-10 using suicide plasmid pOK1 (7), resulting in mutant 85ΔG. Transconjugants of 85ΔG harboring pFG72, pFG72-1, pFG72-2, or pFG72-3 were inoculated into leaves of susceptible (ECW) and resistant (ECW-10R) pepper plants (11). Symptoms caused by wild-type and mutated *hrpG* bacteria in ECW were indistinguishable. On pepper ECW-10R, strain 85-10 induces the hypersensitive reaction (HR), a rapid, localized cell death reaction, approximately 8 to 10 h after inoculation. Bacteria expressing *hrpG** induced a complete, confluent collapse (earliest macroscopically visible reaction) on ECW-10R as soon as 4 h after inoculation (Fig. 3A). No difference was observed between *hrpG** and mutated *hrpG* present in pFG72-2 and pFG72-3. The minimal concentration of *hrpG** bacteria needed to induce the HR on ECW-10R was two- to fourfold lower than those for 85-10 and 85ΔG(pFG72). Thus, *hrpG** enhances the HR induction with respect to both time and efficiency. We then tested whether a stronger HR was also observed in the nonhost plant tobacco, which is resistant to *X. campestris*. Different

TABLE 1. Amino acid substitutions in HrpG and selected homologous proteins and their phenotypic effect

Protein	Mutation	Phenotype[a]	Effect	Reference
HrpG	E44K	+	*hrp* gene expression in complex medium	This study
OmpR	R42H	−	Defective for transcription activation of *ompC*	13
OmpR	R42C	−	Defective for transcription activation of *ompC*	13
OmpR	L43R	+	Increased curli expression	17
OmpR	D55E	+	Sensor kinase-independent activity	8
VirG	N54D	+	*vir* gene expression in the absence of the inducer	12
VirG	I106L	+	*vir* gene expression in the absence of the inducer	12
HrpG	H194R	+	*hrp* gene expression in complex medium	This study
HrpG	D199N	+	*hrp* gene expression in complex medium	This study
OmpR	E193K	−	Defective for transcription activation of *ompC* and *ompF*	15
OmpR	A196V	−	Defective for transcription activation of *ompC*	13
OmpR	E198K	−	Defective for transcription activation of *ompC*	13
PhoB	V190M	−	Defective for interaction with RNA polymerase	9
PhoB	D192G	−	Defective for interaction with RNA polymerase	9
PhoB	D192N	−	Defective for transcription activation of *phoA*	9

[a] + and − refer to constitutively active and loss-of-function phenotypes, respectively.

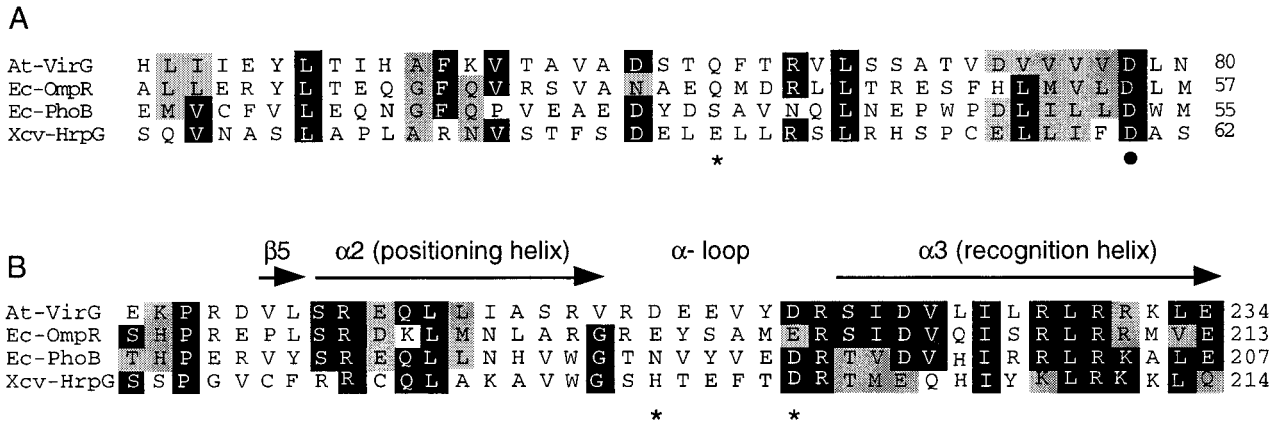

FIG. 2. Protein sequence alignment of *X. campestris* HrpG and selected homologs. (A) Only the relevant region in the N terminus of proteins is shown. Highly conserved amino acids are boxed in black. At, *A. tumefaciens*; Ec, *E. coli*; Xcv, *X. campestris* pv. vesicatoria. (B) Only the relevant part of the C-terminal region of proteins is shown. Structural and functional elements are indicated above the sequence. The positions of β-sheets, recognition helices, and α-loops are based on structural data for OmpR (10). The asterisks mark the positions of three independent substitutions in HrpG; the dot indicates the position of the conserved aspartate residue, which is predicted to be phosphorylated (10). Database accession numbers: *A. tumefaciens* VirG, SWISS-PROT P06664; *E. coli* OmpR, SWISS-PROT P03025; *E. coli* PhoB, SWISS-PROT P08402; *X. campestris* pv. vesicatoria HrpG, GENBANK U57625.

densities of bacterial suspensions were inoculated into leaves of *Nicotiana tabaci* cv. Xanthi plants (Fig. 3B). Inoculation of the *hrpG** strain induced a confluent HR 1 day after inoculation. In contrast, strains 85ΔG(pFG72) and 85-10 induced only a weak chlorotic and necrotic reaction 2 to 3 days after infection.

To reexamine the effect of *hrpG** on susceptible plants in a bacterial genetic background resembling the natural situation, the wild-type copy was replaced by *hrpG** in strains 85-10 and 82-8 (11) with pOK1 (7) to generate 85* and 82*. Chromosomal *hrpG** accelerated the HR induction, as described above for *hrpG** expressed from a plasmid. However, there was a clear difference in timing and intensity of disease symptoms on the susceptible plant, ECW. Water-soaked lesions, typical early disease symptoms, appeared much earlier (49 h) and were stronger than with the wild-type strains (65 to 72 h). These effects were not due to increased bacterial growth in the presence of *hrpG**, as determined by bacterial growth curves in planta (data not shown).

In conclusion, we identified three mutations in the key *hrp* regulatory gene *hrpG* rendering expression of downstream genes constitutive. The mutations residing in the C-terminal domain of HrpG are particularly interesting, because similar mutations in homologous proteins lead to inactivation. The

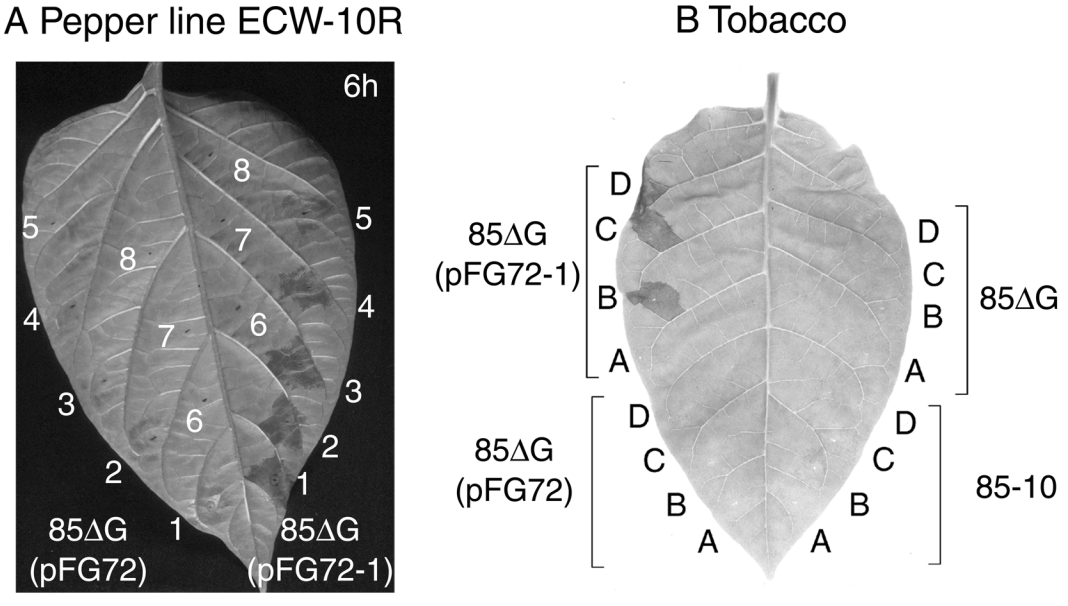

FIG. 3. Effect of *hrpG** on HR induction on resistant plants. (A) HR induction on pepper line ECW-10R after inoculation with 85ΔG(pFG72) and 85ΔG(pFG72-1). Bacteria were grown overnight on NYG agar and resuspended in H_2O. Serial twofold dilutions of bacterial suspensions, from 5×10^8 to 4×10^6 CFU/ml (no. 1 to 8), were infiltrated into the intercellular space of a fully expanded leaf of an 8-week-old plant. The photograph of the underside of the leaf was taken 6 h after inoculation. (B) Reaction of *Nicotiana tabacum* cv. Xanthi after inoculation with different *X. campestris* strains. Strains 85-10, 85ΔG, 85ΔG(pFG72), and 85ΔG(pFG72-1) were inoculated at bacterial densities of 2×10^7 (A), 2×10^8 (B), 8×10^8 (C), and 2×10^9 (D) CFU/ml. The photograph was taken 24 h after inoculation.

amplifying effect of mutated *hrpG* on both disease symptoms and cell death is intriguing and might be indicative of more efficient Hrp type III protein delivery into the plant tissue.

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