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Gene Involved in Transcriptional Activation of the *hrp* Regulatory Gene *hrpG* in *Xanthomonas oryzae* pv. oryzae

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A novel regulatory gene, *trh*, which is involved in *hrp* gene expression, is identified in the plant pathogen *Xanthomonas oryzae* pv. oryzae. In the *trh* mutant, expression of HrpG, which is a key regulator for *hrp* gene expression, is reduced both under the in vitro *hrp*-inducing condition and in planta.

Xanthomonas oryzae pv. oryzae is the causal agent of bacterial leaf blight of rice (23). Like other gram-negative phytopathogenic bacteria in the genera *Erwinia*, *Pseudomonas*, and *Ralstonia*, *Xanthomonas* spp. possess clustered hypersensitive response and pathogenicity (*hrp*) genes that play important roles for pathogenicity on host plants and for triggering a hypersensitive response on nonhost plants (1). The most conserved genes in the *hrp* cluster, called *hrc* (*hrp* conserved) genes (2), encode core components of a type III secretion system (TTSS) that delivers virulence factors from bacteria to host cells (6, 7, 20).

Expression of *hrp* genes is highly regulated and is generally suppressed in complex media but induced in planta and in certain nutrient-poor synthetic media (4, 21, 26, 33, 37). Although many *hrp*-regulatory genes have been isolated and a complicated regulatory system has been revealed in *Pseudomonas syringae*, *Erwinia* spp., and *Ralstonia solanacearum* (1, 3, 4, 5, 6, 12, 31, 32, 37, 38), only two *hrp*-regulatory genes, *hrpG and hrpX*, have been identified in xanthomonads so far (18, 34, 35). HrpG is predicted to be a response regulator, belonging to the OmpR family of a two-component regulatory system, although the corresponding kinase gene has not been identified (36). Phosphorylated HrpG is, therefore, predicted to regulate the expression of another *hrp*-regulatory gene, *hrpX*, the product of which belongs to the AraC regulator family, followed by transcriptional activation of other *hrp* genes (including TTSS structural genes) and genes encoding effector proteins secreted via a TTSS (34, 35).

To isolate and identify novel *hrp*-regulatory genes in *X. oryzae* pv. oryzae, we first conducted transposon mutagenesis using an EZ::TN transposome-mediated insertion system (Epicentre, Madison, WI) (27) on 74HrcQ::GUS, in which a promoterless β-glucuronidase (GUS) gene was inserted at +42 (+1 represents A of the initiation codon) in *hrcQ* (the first gene of the *hrpD* operon) in the genomic DNA of *X. oryzae* pv. oryzae strain T7174R (8, 29). Approximately 1,000 kanamycin-resistant clones were incubated in a *hrp*-inducing medium, XOM2 (26), at 28°C for 15 h, and GUS activity in each clone was measured (14, 26). In one clone, NRH867, GUS activity was significantly reduced compared to that of 74HrcQ::GUS, although the activity was not completely lost (Fig. 1). Growth of NRH867 in nutrient-rich NBY medium (28) and nutrient-poor XOM2 was similar to that of the parental strain 74HrcQ::GUS (data not shown).

Sequence analysis revealed that, in NRH867, the transposon was inserted at +310 in a putative transcriptional regulator gene (*trh* [transcriptional regulator for *hrp*]) (XOO0783 in the genomic database of *X. oryzae* pv. oryzae T7174 [17]). The coding sequence of *trh* is predicted to be 729 bp long (242 amino acids), and in motif analysis using ExPASy (http://www.expasy.org/prosite/), the product was predicted to be a member of the GntR regulator family with a helix-turn-helix motif in the N-terminal region of the protein (+31 to +50) (11). Transcriptional regulators of the family include activators, re-

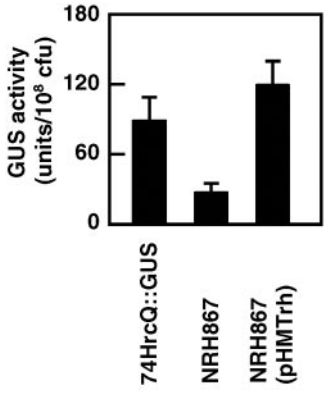

FIG. 1. Expression of *hrcQ*::*gus* in *trh* mutant NRH867. *X. oryzae* pv. oryzae strains were incubated in the *hrp*-inducing medium XOM2 for 15 h, and GUS activity per 1×10^8 CFU of bacteria was measured. Strain 74HrcQ::GUS contains a *hrcQ*::*gus* fusion gene in the genome, and NRH867, a derivative of 74HrcQ::GUS, has a transposon inserted in *trh*. Plasmid pHMTrh harbors a *trh* gene. Values are averages ± standard deviations ($n = 3$).

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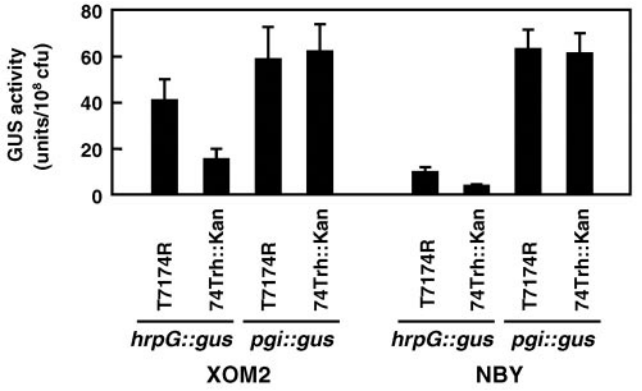

FIG. 2. Effects of mutation in *trh* on expression of *hrpG*. A plasmid harboring *hrpG*::*gus* fusion genes inserted into the broad-host-range vector pHM1 (13) was introduced into T7174R and 74Trh::Kan. Each transformant was incubated in XOM2 and NBY for 15 h, and GUS activity per 10^8 CFU was measured. As a control, a plasmid harboring a *gus*-fused phosphoglucose isomerase gene (*pgi*), expression of which is independent of *hrp* (27), was used. Values are averages ± standard deviations ($n = 3$).

pressors, and molecules that both activate and repress a wide range of bacterial operons (19). The reduced GUS activity in NBH867 was complemented by introduction of plasmid pHMTrh, which harbors a ~900-bp PvuII-SphI fragment containing a *trh* gene inserted into the broad-host-range vector pHM1 (13) (Fig. 1). Higher GUS activity was observed in the transformant NRH867 (pHMTrh) than in 74HrcQ::GUS, which is probably due to overexpression of *trh* from multiple copies of the gene by introduction of the plasmid.

We cloned a ~7.9-kb SacI-NotI fragment containing a *trh* gene inserted with a kanamycin resistance gene from the genomic DNA of NRH867 in pBluescript II SK(+) (Stratagene, La Jolla, CA) and generated the *trh* mutant 74Trh::Kan from the wild-type strain T7174R by marker exchange mutagenesis using the plasmid. Then expression of *hrcU*, *hrpXo*, and *hrpG* in 74Trh::Kan was investigated using plasmids harboring each *hrp* gene fused with a promoterless *gus* gene. GUS activity in all of the transformants, even in that transformed with the plasmid harboring the *hrp*-regulatory gene *hrpG* fused with *gus*, was reduced, but not completely lost, compared to that in those derived from T7174R after a 15-h incubation in XOM2 (Fig. 2) (data not shown). The expression level of a phosphoglucose isomerase gene (*pgi*) in 74Trh::Kan, which is independent of a *hrp*-regulatory system (27) and is used as a control, was similar to that in T7174R.

To investigate the involvement of Trh in transcription of *hrpG*, real-time PCR was conducted with total RNA extracted from bacterial cells cultured in XOM2 for 15 h using a QuantiTect SYBR green real-time PCR kit (QIAGEN, Valencia, CA). The time course of the amplification of the PCR products was measured by the iCycler iQ real-time PCR System (Bio-Rad, Richmond, VA). As shown in Table 1, transcription of *hrpG* in 74Trh::Kan was reduced compared to that in T7174R.

TABLE 1. Accumulation of *hpa1* and *hrpG* transcripts in the *trh* mutant of *X. oryzae* pv. oryzae[a]

Strain	Relative expression value[b]		
	gyrB	*hpa1*	*hrpG*
T7174R	104.1 ± 23.1	24,863.7 ± 5,509.1	193.6 ± 62.1
74Trh::Kan	144.4 ± 33.1	3,895.7 ± 1,709.6	29.1 ± 16.1

[a] Total RNA (10 ng) extracted from each bacterial strain after incubation in XOM2 was used as a template. The primers were designed with Beacon Designer software, version 2.0 (Bio-Rad), as follows: for *gyrB*, the sense primer was 5′-GGCGAGCACAATGGCATT-3′ and the antisense primer was 5′-CCATCC TTCTGCGGGATGT-3′; for *hpa1*, the sense primer was 5′-AAGCCAGGACA CAACGTTCG-3′ and the antisense primer was 5′-GAAGCAGGGCCGAGAT GAG-3′; and for *hrpG*, the sense primer was 5′-AGGCACTGACCCACTTT C-3′ and the antisense primer was 5′-ATCGGAAGCACCACTCTC-3′.

[b] The threshold cycle (C_T) was determined using iCycler iQ real-time detection system software, version 3.0 (Bio-Rad), and the relative expression value was calculated as $1/2^{C_T} \times 10^8$. Values are averages ± standard deviations for three independent experiments.

TABLE 2. Accumulation of *trh* and *hpa1* transcripts under *hrp*-inducing (XOM2) and non-*hrp*-inducing (NBY) conditions[a]

Medium	Relative expression value[b]		
	gyrB	*trh*	*hpa1*
XOM2	104.1 ± 23.1	4.7 ± 0.6	24,863.7 ± 5,509.1
NBY	113.7 ± 67.1	5.4 ± 0.6	73.3 ± 28.2

[a] Total RNA (10 ng) extracted from each bacterial strain after incubation in XOM2 was used as a template. The primers used for *trh* were 5′-AACAACTT GAAGCCGAAGG-3′ (sense primer) and 5′-CCACGTACTGCATGAAAC C-3′ (antisense primer). Primers for detection of *hpa1* and *gyrB* transcripts are given in Table 1, footnote *a*.

[b] The threshold cycle (C_T) was determined, and the relative expression value was calculated as $1/2^{C_T} \times 10^8$. Values are averages ± standard deviations for three independent experiments.

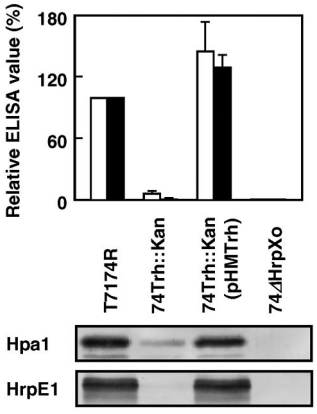

FIG. 3. Accumulation of Hpa1 and HrpE1 in the culture supernatant and in bacterial cells. (Top) For quantification of Hpa1 and HrpE1 secreted in the culture supernatant, an ELISA was conducted. The values shown are ELISA values (ΔA_{405}/h) relative to those from T7174R and are averages ± standard deviations for five independent experiments. White and black bars, Hpa1 and HrpE1, respectively. (Bottom) For detection of Hpa1 and HrpE1 in the bacterial cells, Western blot analysis was conducted using 5 μg bacterial proteins per lane. For each assay, polyclonal antibodies against Hpa1 and HrpE1 (unpublished data) were used as the primary antibody and an alkaline phosphatase-conjugated goat anti-rabbit antibody (Bio-Rad) was used as the secondary antibody. Reactions were visualized using *p*-nitrophenyl phosphate (1 mg/ml) for ELISA and nitroblue tetrazolium (337.5 μg/ml) and 5-bromo-4-chloro-3-indolyl phosphate (175 μg/ml) for the immunoblot assay. Strain 74ΔHrpXo is a *hrpXo*-deficient mutant described previously (25).

TABLE 3. Decrease of *hpa1* expression in the *trh*-defective mutant during growth in rice leaves and pathogenicity of the mutants

Strain[a]	Bioluminescence (cpm)[b,c]	No. of bacteria (10^7 CFU)[c,d]	Lesion length (mm)[c,e]
74Hpa1::Lux	4,802.8 ± 1,521.3	4.7 ± 3.2	40.0 ± 7.8
74Hpa1::Lux/Trh::Kan	910.0 ± 434.0	4.0 ± 2.2	37.8 ± 9.2

[a] Each bacterium was used to inoculate the susceptible rice cultivar IR24.
[b] Bioluminescence from 1-cm-long leaf sections, including the inoculation site, was measured 3 days after inoculation, and photons per minute are shown.
[c] Values are averages ± standard deviations for five inoculated leaves.
[d] Bacterial numbers from 1-cm-long leaf sections, including the inoculation site, were measured by plating on the medium 3 days after inoculation.
[e] Measured 2 weeks after inoculation.

Accumulation of the *hpa1* transcript, which is expressed in a *hrp*-dependent manner, also decreased in 74Trh::Kan. On the other hand, accumulation of the DNA gyrase subunit B (*gyrB*) transcript, which is used for a reference, was not reduced in the mutant in comparison to that in the wild-type strain. These results indicate that Trh directly or indirectly activates *hrpG* transcription, followed by increased expression of other *hrp* genes.

Interestingly, expression of *hrpG* increased after incubation in XOM2 (*hrp* inducing) even without *trh* compared with incubation in NBY (not *hrp* inducing), although expression of *hrpG* was higher in the presence of *trh* (Fig. 2). And after incubation in NBY, *hrpG* expression was lower but up-regulated by Trh. These results suggest that at least two factors are involved in transcriptional activation of *hrpG*. One is mediated by Trh, which functions both under the nutrient-rich non-*hrp*-inducing condition (NBY) and under the nutrient-poor *hrp*-inducing condition (XOM2). The other is mediated by an unknown factor(s), which functions only under *hrp*-inducing conditions. In *R. solanacearum*, four genes, *prhA*, *prhI*, *prhR*, and *prhJ*, have been identified as regulatory genes involved in *hrpG* expression (4, 5, 16). The regulatory cascade in which these genes are involved is reported to function specifically during plant-bacterium interactions. Although there have been no reports of genes corresponding to *prh* genes in xanthomonads, a regulator(s) other than Trh controlling *hrpG* expression may be present, which specifically functions under *hrp*-inducing conditions, including during plant-bacterium interactions.

Expression of *trh* under different growth conditions was examined by a real-time PCR method using total RNA extracted from T7174R incubated in XOM2 or NBY. Although accumulation of the *hpa1* transcript seemed to be much lower after incubation in NBY than in XOM2, the amount of the specifically amplified fragment for *trh*, as well as that for *gyrB*, did not significantly differ between the two growth conditions (Table 2), suggesting constitutive expression of the *trh* gene.

We also investigated the expression and secretion in 74Trh::Kan of HrpE1 and Hpa1, which have been reported to be major components of the *hrp* pilus and a harpin-like protein, respectively, and to be secreted to the culture supernatant under the *hrp*-inducing condition (9, 10, 30). We incubated 74Trh::Kan under the *hrp*-inducing condition, and bacterial proteins in 1 ml culture were separated into intracellular and extracellular fractions by centrifugation at 10,000 × *g* for 5 min. One hundred microliters of supernatant, with bacteria completely removed by filtration, was used for an enzyme-linked immunosorbent assay (ELISA). Proteins in bacterial cells were extracted with 300 μl of B-PER bacterial protein extraction reagent (Pierce, Rockford, IL), and the concentrations were measured with a protein assay kit (Bio-Rad) using bovine serum albumin as a reference. The samples (5 μg) were added to 150 μl Laemmli buffer (15) and used for sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis, followed by an immunoblot assay to detect Hpa1 and HrpE1, respectively. Accumulation of these proteins in the extracellular fraction from the *trh* mutant was greatly reduced compared with that from the wild type, likely because of less expression of their own genes and of the genes encoding components of the type III secretion apparatus (Fig. 3). The lower level of secretion of the protein was restored by introduction of pHMTrh. We also confirmed lower levels of accumulation of Hpa1 and HrpE1 in the intracellular fraction of 74Trh::Kan and complementation by introduction of plasmid pHMTrh (Fig. 3).

Finally, we investigated the involvement of *trh* in the expression of *hrp* genes in rice leaves by using strain 74Hpa1::Lux and the *trh* mutant 74Hpa1::Lux/Trh::Kan, in which a fragment containing a promoterless *lux* operon derived from pUCD623 (22) was inserted at the EcoRI site located downstream of the *hpa1* promoter. Expression of the gene is regulated by HrpG and is specifically expressed under the *hrp*-inducing condition (9). The virulence of *hpa1* mutants is lower, but they are still pathogenic on rice (9, 39). The strains were diluted in water (optical density at 600 nm [OD_{600}], 0.3; approximately 1×10^8 CFU/ml) and inoculated by the clipping method (25) onto flag leaves and the next leaves of rice (*Oryza sativa* L. cv. IR24) grown in a greenhouse at 28 ± 5°C. Expression of *hpa1* was examined by measuring bioluminescence using a video-intensified microscope camera and analyzed by ARGUS-100 (Hamamatsu Photonics, Hamamatsu, Japan) (24). During infection, accumulation of bioluminescence from rice leaves inoculated with 74Hpa1::Lux/Trh::Kan was lower than that from leaves inoculated with 74Hpa1::Lux. At 3 days after inoculation, the level of

TABLE 4. Lesion lengths on rice leaves inoculated with dilution series of 74Trh::Km

Strain	Lesion length (mm)[a] on rice leaves inoculated with the following dilution of a bacterial suspension[b]:					
	×1	×5	$\times5^2$	$\times5^3$	$\times5^4$	$\times5^5$
T7174R	76.83 ± 16.49	56.33 ± 20.26	54.50 ± 5.54	47.83 ± 9.28	31.00 ± 12.88	23.76 ± 16.06
74Trh::Km	62.67 ± 15.08	54.83 ± 23.40	47.17 ± 16.62	40.67 ± 16.01	31.50 ± 19.60	22.50 ± 8.41

[a] Measured 2 weeks after inoculation. Values are averages ± standard deviations for six inoculated leaves.
[b] A bacterial suspension concentrated to an OD_{600} of 0.3 was diluted 5, 5^2, 5^3, 5^4, and 5^5 times and used to inoculate the rice cultivar IR24.

bioluminescence from the *trh* mutant strain was ca. five times lower than that from the *trh*$^+$ strain (Table 3). Simultaneously, the bacterial numbers in 1-cm-long leaf sections including the inoculation site were measured by plating on the medium. Unexpectedly, there was no significant difference in the increase in the number of bacteria between the two strains. Moreover, leaves infected with each strain showed disease symptoms from 6 days after inoculation, and there was no significant difference in lesion lengths between the two strains by 2 weeks after inoculation.

For further comparison of virulence between two strains with or without *trh*, dilution series of a bacterial suspension (1/5, $1/5^2$, $1/5^3$, $1/5^4$, and $1/5^5$; original OD_{600} of suspension, 0.3) of T7174R and 74Trh::Kan were prepared and inoculated onto rice leaves, and lesion lengths were measured 2 weeks after inoculation. With decreasing bacterial concentrations, the appearance of disease symptoms was delayed and lesion lengths became shorter in leaves inoculated with each strain. There were no significant differences in lesion formation between the two strains (Table 4). These results suggest that expression of *hrp* genes (at least *hpa1*) in the *trh* mutant is decreased (but not completely lost) not only under the in vitro *hrp*-inducing condition but also during bacterial growth in rice leaves and that even reduced expression of *hrp* genes and reduced secretion of TTSS effectors due to a mutation in *trh* may be sufficient for bacterial growth in host plants and the development of disease symptoms, although the possibility that expression of *hrp* genes other than *hpa1* does not decrease, unlikely in the case of XOM2, cannot be excluded. In *R. solanacearum*, mutations in *prh* genes do not necessarily result in loss of pathogenicity, suggesting that there are multiple cascades to induce expression and activation of HrpG (4, 5, 16). In *X. oryzae* pv. oryzae, expression of *hrp* genes may be induced by an unknown cascade(s) to a level sufficient for pathogenicity on rice even without Trh.

In this work we showed that a putative transcriptional regulatory gene, *trh*, is involved in expression of a *hrp*-regulatory gene, *hrpG*, in *X. oryzae* pv. oryzae, although whether the regulation is direct or indirect remains unknown. The *trh* gene is located far from both the *hrp* gene cluster and *hrp*-regulatory genes *hrpG* and *hrpXo*, although *prh* genes are located adjacent to the *hrp* gene cluster in *R. solanacearum*. Moreover, *trh* is located near the gene cluster for the type II secretion system (XOO0771 to XOO0781), which implies the possibility of involvement of *trh* in expression of genes other than *hrp*, especially genes for construction of the type II secretion system. However, we found no difference between the wild type and the *trh* mutant in the extracellular activity of cellulase, which is secreted via the type II secretion system, under either *hrp*-inducing or non-*hrp*-inducing conditions (data not shown). To clarify direct or indirect transcriptional activation of *hrpG* by Trh and involvement of Trh in expression of genes other than *hrpG*, further investigation is required.

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