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BACTERIAL AND PHYTOPLASMA DISEASES

Expression of *hrpG* and activation of response regulator HrpG are controlled by distinct signal cascades in *Ralstonia solanacearum*

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Abstract The transcriptional regulator HrpB activates the entire *hrp* regulon in the plant pathogen *Ralstonia solanacearum*. Through a complex multigene regulatory cascade PrhA–PrhR/PrhI–PrhJ–HrpG, expression of *hrpB* is induced in a *hrp*-inducing, nutrient-poor medium and in response to contact between the bacterium and plant cell. In this study, we analyzed the expression levels of these regulatory genes and *hrpB* using *lacZ* reporter strains grown in three different conditions: in a nutrient-rich or nutrient-poor medium and co-cultivated with *Arabidopsis thaliana* seedlings. We found that *prhA* and *prhIR* were expressed constitutively. Expression of *prhJ* and *hrpG* was PrhA-dependent in all three conditions. Despite the high level of *hrpG* expression in all cases, *hrpB* was induced only when the bacteria were co-cultivated with *A. thaliana* seedlings or grown in nutrient-poor medium. A mutation in the predicted phosphorylation site of *hrpG* greatly reduced the function of HrpG. From these results, we conclude that the *prhA*-dependent regulatory cascade controls the expression of *hrpG*, and a new cascade, which is induced by a signal from plant cells, activates HrpG by phosphorylation. Only when both signal cascades are effective is full expression of *hrpB* induced. We speculate that the metabolic status of the bacteria in the nutrient-poor medium also contributes to the second cascade.

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Introduction

The type III secretion system (TTSS) of Gram-negative plant and animal pathogens translocates effector proteins into host cells (Galán and Collmer 1999; Hueck 1998). In plant pathogenic bacteria, the TTSS is encoded by a cluster of approximately 20 *hrp* genes (Hueck 1998) that are organized into several operons (Arnold et al. 2003). TTSS-defective mutants of the soil-borne pathogen *Ralstonia solanacearum*, like other Gram-negative plant pathogens, are unable to cause disease on susceptible host plants or to elicit the hypersensitive reaction (HR) in resistant hosts. Mutant cells are unable to colonize the intercellular spaces of host plants (Hueck 1998; Kanda et al. 2003a).

TTSS construction in *R. solanacearum* is strongly influenced by the growth environment (Arlat et al. 1992; Genin et al. 1992; Mukaihara et al. 2004). The *hrp* regulon is repressed in nutrient-rich media. Concomitantly, a low-level expression of *hrpB*, which encodes an AraC-type transcriptional activator of the entire *hrp* regulon, is observed. Nutrient-poor conditions, which may mimic conditions in the intracellular spaces of plants, induce 20-fold expression of the *hrp* regulon. Plant signal(s) enhance the expression of the operons belonging to the *hrp* regulon 10- to 20-fold more than do nutrient-poor conditions (Marenda et al. 1998). The expression of *hrpB* is activated through a complex multigene regulatory cascade PrhA–PrhR/PrhI–PrhJ–HrpG (Fig. 1) initiated during bacterium-plant cell contact (Aldon et al. 2000; Brito et al. 1999, 2002; Cunnac et al. 2004). Plant cell signals are presumed

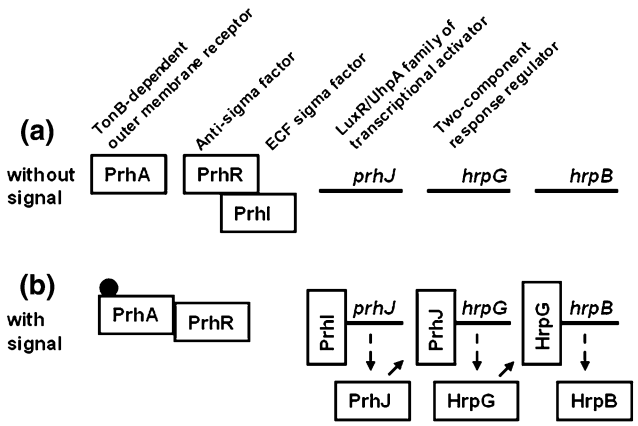

Fig. 1 Schematic diagram of a six-gene regulatory cascade. *Boxed letters* represent proteins. *Dashed lines* show transcription/translation. The function of each protein is shown on the *top*. **a** Without signal, **b** with unidentified signal (shown by a *small dot*)

to be recognized by PrhA, which is located on the outer membrane of the bacterial cell.

A LysR-type transcriptional activator PhcA was originally identified as a positive regulator of the production of a major virulence factor, extracellular polysaccharide (EPS) (Brumbley et al. 1993). In addition to EPS, PhcA coordinates several virulence factors, positively regulating β-1,4-endoglucanase and pectin methylesterase and negatively regulating polygalacturonases and motility (Schell 2000). The levels of active PhcA are sensitive to the density of the bacterial cells (Clough et al. 1997). PhcA activity starts to increase at a cell density of 10^7 CFU/ml and reaches its maximum at 5×10^8 CFU/ml. Recently, PhcA was demonstrated to negatively regulate TTSS expression (Genin et al. 2005). A *phcA* mutation dramatically increases *hrpB* and *hrp* regulon expression even in nutrient-rich medium.

HrpG, a two-component response regulator, is the key component of the regulatory cascade and directly controls *hrpB* expression (Brito et al. 1999). HrpG has been shown to integrate three major signals: physical contact with the plant host (Aldon et al. 2000), the bacterial metabolic status related to growth conditions (Brito et al. 1999), and a PhcA-dependent quorum sensing signal (Genin et al. 2005). How these three signals interact with each other in different environments to control HrpG activity has not yet been completely elucidated. To address this question, we investigated the expression levels of five genes (*prhA*, *prhI*, *prhR*, *prhJ*, and *hrpG*) in the complex multigene regulatory cascade (Fig. 1) in different growth conditions. We found that all five genes were expressed at high levels in all culture conditions tested. We also found that phosphorylation of HrpG, activated by plant signal(s), was necessary for the induction of *hrpB* expression.

Materials and methods

Bacterial strains and culture conditions

Escherichia coli and *R. solanacearum* strains used are listed in Table 1. *E. coli* was grown in LB medium at 37°C, and *R. solanacearum* was grown at 28°C in rich B medium (Boucher et al. 1985), hydroponic plant culture medium with 2% sucrose (Fujiwara et al. 1992), or was co-cultivated with *A. thaliana*. Seedlings of *A. thaliana* cv. Landsberg grown aseptically for 10 days on a plant agar medium (Fujiwara et al. 1992) were mixed with *R. solanacearum* cells in liquid plant medium with 2% sucrose and incubated at 25°C with rotation and a 16-h day/8-h night cycle.

Reporter strain construction

Plasmid ppop containing *popA*, *popB*, and *popC* (Kanda et al. 2003b) was digested with *Eco*RI and *Sph*I and cloned into pK18mobsacB (Schäfer et al. 1994) to construct ppop2. A 6.3-kb *Bam*HI-digested promoterless *lacZYA* fragment from pUC–lacZYA (Mukaihara et al. 2004) was inserted into ppop2 to generate ppop3. The *lacZYA* fragment was inserted into position 321 of the 1017-bp *popA* gene. This plasmid was used to construct a *popA–lacZYA* fusion strain in wild-type strain OE1-1 of *R. solanacearum* (Kanda et al. 2003a) by homologous recombination.

Table 1 Strains used in this study

Strain	Genotype or relative characteristics	References
Escherichia coli		
DH12S	Host strain for DNA manipulation	Invitrogen
S17-1	Host strain for plasmid mobilization	Simon et al. (1983)
Ralstonia solanacearum		
OE1-1	Wild type	Kanda et al. (2003a)
RK5046	OE1-1 *hrpB–lacZYA*	This study
RK5050	OE1-1 *popA–lacZYA*	This study
RK5051	RK5050 Δ*hrpB*	This study
RK5120	OE1-1 *hrpG–lacZYA*	This study
RK5124	OE1-1 *prhJ–lacZYA*	This study
RK5130	OE1-1 *prhIR–lacZYA*	This study
RK5134	OE1-1 *prhA–lacZYA*	This study
RK5198	RK5046 Δ*hrpG*	This study
RK5218	RK5130 Δ*prhA*	This study
RK5220	RK5120 Δ*prhA*	This study
RK5224	RK5124 Δ*prhA*	This study
RK5240	OE1-1 D51N mutation in *hrpG*	This study
RK5243	RK5046 D51N mutation in *hrpG*	This study

A *hrpB* gene fragment was amplified from chromosomal DNA of OE1-1 by PCR with primers hrpBA1 and hrpBB1 (Table 2) and cloned into pBluescript KS(+) to construct phrpB11. The *lacZYA* fragment was inserted into phrpB11 digested with *Bgl*II to make phrpB16. The *Bam*HI–*Hin*dIII fragment of phrpB16 was subcloned into pK18mobsacB to generate phrpB18 for *hrpB–lacZYA* fusion strain construction. The *lacZYA* was inserted into position 878 of the 1434-bp *hrpB* gene.

A 2-kb DNA fragment containing both *hrpG* and *prhJ* was amplified with primers prhRA1 and hpaBB1 and cloned into pUC119 to generate pUChrpG2. The *Bam*HI–*Hin*dIII fragment of pUChrpG2 was recloned into pK18mobsacB to make phrpG10. The *lacZYA* fragment was inserted into phrpG10 digested with *Sal*I or *Sal*I/*Xho*I to make phrpG2 for *hrpG–lacZYA* fusion strain construction and pprhJ2 for *prhJ–lacZYA* construction. The *lacZYA* fragment was inserted into position 451 of the 741-bp *hrpG* gene and position 226 of the 525-bp *prhJ* gene.

A 2-kb DNA fragment containing *prhI* and *prhR* was amplified with primers prhIA1 and prhRB1 and cloned into pGEM-3Zf(+) (Promega, Madison, WI, USA) to generate pprhIR2. The *Kpn*I-digested *lacZYA* fragment was inserted into pprhIR2 to make pprhIR4. The *Eco*RI–*Sph*I fragment of pprhIR4 was cloned into pK18mobsacB to generate pprhIR5 for *prhIR–lacZYA* fusion strain construction. The *lacZYA* fragment was inserted at 6 bp upstream from the start codon of the 510-bp *prhI* gene.

A 2.8-kb DNA fragment containing *prhA* was amplified with primers prhORFxb and popAB10 and cloned into pK18mobsacB to generate pprhA1. After digestion with *Sph*I, the linearized plasmid was blunt-ended with T4 DNA polymerase. The *Sma*I-digested *lacZYA* fragment was inserted into the linearized, blunt-ended plasmid to make pprhA2 for *prhA–lacZYA* fusion strain construction. The *lacZYA* fragment was inserted into position 477 of the 2313-bp *prhA* gene.

All the pK18mobsacB-based plasmids were transferred to wild-type OE1-1 through *E. coli* S17-1 (Simon et al. 1983), and the *lacZYA* reporter strains were constructed by homologous recombination.

Construction of deletion strains

The 2.8-kb *Eco*RI–*Hin*dIII fragment of pUC118–hrpAC containing *hrpA* and *hrpC* (Kanda et al. 2003a) was recloned into the same sites of pK18mobsacB to construct phpAC53 for *hrpB* deletion.

Two DNA fragments flanking the *hrpG*-coding sequence were amplified with primers prhJA1 and hrpGA3 for the 3′-end region and hrpYA2 and hrpGB3 for the 5′-end region. Both 0.7- and 1.2-kb fragments were mixed and subjected to a second PCR with primers prhJA1 and hrpYA2. The resulting 1.8-kb fragment was cloned into TA vector p3T (MOBiTec, Goettingen, Germany) and recloned into pK18mobsacB to generate pK18dhrpG4.

Table 2 Primers used in this study

Primer	Sequence (5′–3′)[a]	Enzyme
hrpBA1	CTCGGATCCGCGGGCTGGTTCGACTGA	*Bam*HI
hrpBB1	CTCAAGCTTGGCTCAGCGCCAGATGGT	*Hin*dIII
prhRA1	GCATGATCGAGGTGCAGC	
hpaBB1	CCCAAGCTTCGCTACTGGCGGGACAAC	*Hin*dIII
prhIA1	CCGGAATTCAGCAGCAGGTTCAGTGCG	*Eco*RI
prhRB1	CGCGTCGACTCAGAGCCGGCTGATGCG	*Sal*I
prhORFxb	GCTCTAGATGCCTGCGCTTTCCTCCAC	*Xba*I
popAB10	CCCAAGCTTAGTGAATAACCTTTGAGGGC	*Hin*dIII
prhJA1	ATGAACGCCCGGGTTCAG	
hrpGA3	GGGGAACGCCAAAGCGGTCAATTAAGCAAACG	
hrpYA2	CTCGGATCCGATCGGTTCCACAACGGC	*Bam*HI
hrpGB3	TGACCGCTTTGGCGTTCCCCGTTTCATG	
prhAA6	GAATTCGGTTTCTCGCCTGCAGCGGTG	*Eco*RI
prhAA7	GGATCCGTCCGAGGCCGCCCGGCCTC	*Bam*HI
prhAB3	GGATCCCGTGCGGCGGACTCCCGGGGT	*Bam*HI
prhAB4	CTCAAGCTTCACGGACTGGCCCGATTG	*Hin*dIII
hrpGDN1	CTCCTGGGCATTGATCATCAGCAG	
hrpGDN2	CTGCTGATGATCAATGCCCAGCAG	
M13-47	CGCCAGGGTTTTCCCAGTCACGAC	
RV-M	GAGCGGATAACAATTTCACACAGG	

[a] Underlined sequence corresponds to restriction enzyme (right column) site

Two DNA fragments flanking the *prhA*-coding sequence were amplified with primers prhAA6 and prhAB3 for the 5′-end region and primers prhAB4 and prhAA7 for the 3′-end region. Both PCR products were TA-cloned to make p3TprhA6.3 and p3TprhA7.1, respectively. The *Eco*RI–*Bam*HI fragment of p3TprhA6.3 and the *Bam*HI–*Hin*dIII fragment of p3TprhA7.1 were cloned into pK18mobsacB pre-digested with *Eco*RI–*Hin*dIII by three-way ligation to generate pprhA3.

All pK18mobsacB-based plasmids were used to construct deletion mutants using homologous recombination. The entire gene region of *hrpB*, *hrpG*, or *prhA* was deleted.

Construction of strains with a point mutation in the *hrpG* gene

Both the 5′ and 3′ halves of *hrpG* were amplified with primers RV-M and hrpGDN1 or primers M13-47 and hrpGDN2, respectively, using pUChrpG2 as a template. The two PCR products were mixed and subjected to a second PCR with primers M13-47 and RV-M. The final product was digested with *Sal*I and *Hin*dIII and cloned into pK18mobsacB to make phrpG10DN, which was used for homologous recombination.

Measurement of β-galactosidase activity

The β-galactosidase assay was performed as described by Miller (1992), with some modifications. When the cell density was low, *R. solanacearum* cells were collected by centrifugation and concentrated in distilled water. Values are expressed as the means of at least three independent experiments.

Virulence assays

The virulence of *R. solanacearum* strains was measured using an infiltration method as described by Kanda et al. (2003a). Fully expanded leaves of 4-week-old tobacco plants (*Nicotiana benthamiana*) were infiltrated with 50 µl of the bacterial suspension at 10^8 cells/ml using a 1-ml syringe without needle. Each assay was repeated in three successive trials.

Results

hrp regulon induction

The *hrp* regulon contains at least six operons in the *hrp* locus, and five of these six operons are controlled by HrpB in *R. solanacearum* (Cunnac et al. 2004). A *popA* operon, which has three genes, *popA*, *popB*, and *popC*, belongs to the *hrp* regulon (Arlat et al. 1994). We constructed a *popA–lacZYA* reporter strain RK5050 and used it to monitor *hrp* regulon expression in this study because *popABC* is not involved in the pathogenicity of *R. solanacearum* (Arlat et al. 1994; Kanda et al. 2003b). As reported previously (Cunnac et al. 2004; Van Gijsegem et al. 1995), the *popA* operon was not expressed in nutrient-rich B medium (Table 3). We used a plant medium with 2% sucrose (sucrose medium) as the nutrient-poor culture condition, instead of the *hrp*-inducing minimal medium with glutamate (MMG; Boucher et al. 1985) because the expression level of the *popA* operon was higher in sucrose medium than in MMG (data not shown). The expression level of the *popA* operon in nutrient-poor medium was 10 times higher than that in nutrient-rich medium (Table 3). When RK5050 was mixed with aseptically grown *A. thaliana* seedlings and incubated in sucrose medium, *popA* expression was 10 times higher than in the nutrient-poor condition (Table 3). Co-cultivation of RK5050 cells with seedlings of the host plant tomato *Solanum lycopersicum* cv. Moneymaker induced less *popA* expression than did co-cultivation with *A. thaliana* (data not shown). The induction was completely abolished in the *hrpB* mutant RK5051, indicating that the *hrp* regulon induction in nutrient-poor medium and when co-cultivated with *A. thaliana* seedlings were HrpB-dependent. We therefore opted to use the B medium, sucrose medium, and co-cultivation with *A. thaliana* seedlings as the three culture conditions for *hrp* regulon expression for this study.

Table 3 *popA* expression in *Ralstonia solanacearum* in different culture conditions

Strain	Genotype	β-Galactosidase activity[a]		
		Rich medium	Poor medium	Co-cultivation[b]
RK5050	*popA–lacZYA*	13 (0.5)	133 (3)	1240 (132)
RK5051	*popA–lacZYA* Δ*hrpB*	0.2 (0)	0.4 (0)	11 (0.8)

[a] Cells were grown to an OD600 of about 0.1, treated with SDS–chloroform and assayed. Mean values of at least six measurements are in Miller units with standard errors in parentheses

[b] Cells were incubated with two or three seedlings of *Arabidopsis thaliana* in plant medium at 25°C with 16-h day/8-h night

Expression of genes in a complex regulatory cascade under different culture conditions

A six-gene regulatory cascade has been proposed to regulate *hrpB* expression (Fig. 1) (Brito et al. 2002). We constructed five reporter strains to monitor the expression of these genes. None of the reporter strains were pathogenic to the host plant (Fig. 2, data not shown). Expression of *prhA* was constitutive (Marenda et al. 1998; unpublished result); hence, we used *prhA* as a reference gene. Gene expression levels were normalized against the expression level of *prhA* in RK5134 (Table 4). No difference was observed in the relative expression levels of *prhIR* (RK5130) in the three culture conditions (Table 4). The relative expression levels of *prhJ* (RK5124) and *hrpG* (RK5120) were slightly lower in nutrient-poor medium than in nutrient-rich medium or the co-cultivation with *A. thaliana* seedlings (Table 4). The expression level of *hrpB* (RK5046) was 10 times higher in nutrient-poor medium than in nutrient-rich medium, as demonstrated previously (Arlat et al. 1992; Genin et al. 1992), and was increased even more in the co-cultivation with *A. thaliana* seedlings, as demonstrated previously (Marenda et al. 1998).

Effect of PrhA on the expression of genes in the regulatory cascade

Expression of *prhJ* and *hrpG* is reduced in the *prhA* mutant background during co-cultivation with *Arabidopsis* cell suspensions (Brito et al. 1999). We re-examined the influence of PrhA on the expression of the regulatory cascade genes in three different culture conditions. The expression of *prhIR* in the Δ*prhA* background was the same as in the wild type in all three conditions (Table 4, RK5218 vs. RK5130). This result was expected because PrhR is thought to interact with PrhA (Brito et al. 2002), which is not supposed to regulate transcription of *prhR*. The expression levels of both *prhJ* and *hrpG* were reduced in the Δ*prhA* background in all three conditions, although the extent of reduction differed between culture conditions and genes (Table 4, RK5224 vs. RK5124 and RK5220 vs. RK5120). From these results, we conclude that PrhA is necessary for full expression of *prhJ* and *hrpG* regardless of the culture condition in which *R. solanacearum* cells grow.

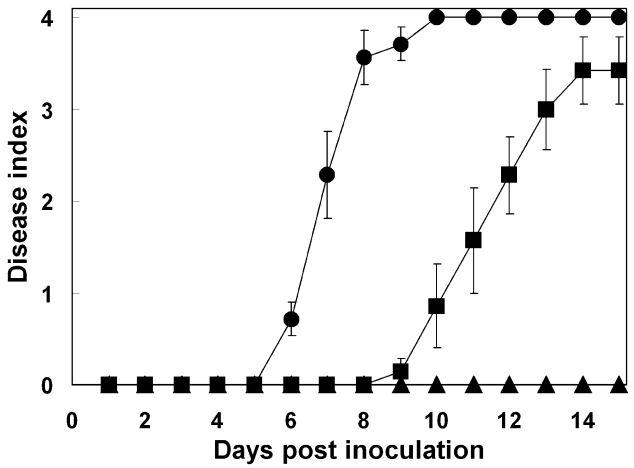

Fig. 2 Virulence of *hrpGD51N* mutant on tobacco. Tobacco (*Nicotiana benthamiana*) plants, 4 weeks old, were inoculated by leaf infiltration with 50 µl of a 10^8 cells/ml suspension and monitored daily for disease development. Plants were rated on a disease index scale as follows: 0 (no wilting), 1 (1–25% wilting), 2 (>25–50% wilting), 3 (>50–75% wilting), and 4 (>75–100% wilting or dead). Wild type OE1-1 (*circles*), *hrpG* mutant RK5120 (*triangles*), and *hrpGD51N* mutant RK5240 (*squares*). Points are the average of three separate experiments containing at least nine plants. *Bars* indicate standard errors

Involvement of HrpG phosphorylation in *hrpB* induction and virulence

Although *hrpG* was highly expressed in all three culture conditions, the induction level of *hrpB* differed significantly depending on the culture conditions. We speculated that HrpG must be modified posttranslationally in nutrient-poor medium and with co-cultivation with *A. thaliana* seedlings. Because HrpG is annotated as a response regulator of two-component signal transduction systems, HrpG could be phosphorylated posttranslationally. A conserved phosphorylation site D51 was identified on the basis of the amino acid alignment of several response regulators (Brito et al. 1999). We mutated the D51 residue in HrpG to Asn and examined the influence on *hrpB* expression. While the *hrpG* null mutation in RK5198 reduced *hrpB* expression to the basal level, the expression of *hrpB* greatly decreased but was not completely abolished in *hrpGD51N* mutant RK5243 when co-cultivation with *A. thaliana* seedlings or in nutrient-poor medium (Table 5). The expression of *popA* decreased to the same extent as *hrpB* in the *hrpGD51N* mutant (data not shown).

Leaves of *N. benthamiana* plants were inoculated with wild-type OE1-1, *hrpGD51N* mutant RK5240, and *hrpG* null mutant RK5120. No disease symptoms were observed in RK5120-infiltrated plants (Fig. 2). Necrotic lesions were observed at 2 days post-inoculation (dpi) on the leaves infiltrated with OE1-1, and the plants wilted within 10 days (Fig. 2). On the other hand, RK5240-infiltrated leaves had necrotic lesions at 3–4 dpi; the plants started to wilt at 9 dpi and wilted much more slowly than OE1-1-infiltrated plants (Fig. 2), indicating that the mutation of the phosphorylation site in *hrpG* severely reduces the virulence of *R. solanacearum*.

Table 4 Expression of genes involved in *hrp* gene regulatory cascade in *Ralstonia solanacearum* grown in different culture conditions

Strain	Genotype	β-Galactosidase activity					
		Rich medium		Poor medium		Co-cultivation	
		Actual[a]	Relative[b]	Actual[a]	Relative[b]	Actual[a]	Relative[b]
RK5046	*hrpB–lacZYA*	70 (2)	0.09	278 (44)	0.9	630 (87)	1.4
RK5120	*hrpG–lacZYA*	1172 (56)	1.5	314 (26)	1.0	736 (45)	1.7
RK5124	*prhJ–lacZYA*	933 (80)	1.2	249 (27)	0.9	686 (74)	1.5
RK5130	*prhIR–lacZYA*	1009 (48)	1.3	388 (21)	1.3	474 (63)	1.1
RK5134	*prhA–lacZYA*	768 (17)	1	289 (20)	1	445 (14)	1
RK5220	*hrpG–lacZYA ΔprhA*	178 (22)	0.2	173 (10)	0.6	158 (14)	0.3
RK5224	*prhJ–lacZYA ΔprhA*	423 (51)	0.6	61 (5)	0.2	71 (17)	0.2
RK5218	*prhIR–lacZYA ΔprhA*	1697 (241)	2.2	470 (62)	1.6	353 (34)	0.8

[a] Cells were grown to an OD600 of about 0.1, treated with SDS–chloroform and assayed. Mean values of at least six measurements are in Miller units with standard errors in parentheses

[b] Relative values were calculated to normalize levels of gene expression. Expression level of *prhA* was used as reference in each culture condition

Table 5 *hrpB* expression in wild-type *Ralstonia solanacearum* and *hrpG* mutants with a point mutation in the putative phosphorylation site

Strain	*hrpG*	β-Galactosidase activity[a]	
		Poor medium	Co-cultivation
RK5046	Wild type	193 (2)	630 (87)
RK5198	Deletion	47 (6)	44 (4)
RK5243	D51 N	83 (3)	110 (22)

[a] Cells were grown to an OD600 of about 0.1, treated with SDS–chloroform and assayed. Mean values of at least six measurements are in Miller units with standard errors in parentheses

Discussion

HrpB is the factor that directly regulates the *hrp* regulon. When *hrpB* is constitutively expressed from a strong promoter, the *hrp* regulon is induced even in nutrient-rich medium (Tamura et al. 2005). HrpG is the upstream regulator of HrpB in the complex regulatory cascade. In this study, we clearly demonstrated that the aspartate residue at the predicted phosphorylation site of HrpG (D51) was important for HrpG function. HrpG belongs to a family of response regulators of two-component signal transduction systems (Salanoubat et al. 2002). We speculate that a new signal cascade activates the HrpG protein by phosphorylation. HrpG proteins in *Xanthomonas* and *R. solanacearum* share more than 40% amino acid identity. The importance of HrpG phosphorylation has been suggested for *X. campestris* pv. *vesicatoria* (Wengelnik et al. 1996, 1999). We have measured the expression levels of *prhA*, *prhIR*, *prhJ*, and *hrpG* and monitored *hrpB* expression in different culture conditions. Taking these results together, we can summarize the patterns of signal integration by HrpG in different culture conditions (Fig. 3). On the basis of this model, two distinct signal cascades are involved in HrpG activation and, in turn, the induction of *hrpB* expression. The first cascade is the well-known, complex regulatory cascade composed of PrhA–PrhR/PrhI–PrhJ–HrpG (Brito et al. 2002). Signals are recognized by PrhA and finally trigger induction of *hrpG* expression. This cascade controls *hrpG* transcription. The newly demonstrated second cascade starts from an unidentified receptor, goes through a two-component signal transduction pathway, and finally activates HrpG. The second cascade is involved in posttranslational modification of HrpG, presumably by phosphorylation. While we have no answer to the question of why *R. solanacearum* uses such a complex system, both cascades are necessary for the formation of active HrpG and *hrpB* induction.

Both *prhA* and *prhIR* were constitutively expressed in all conditions. In the nutrient-rich medium, where *R. solanacearum* cells have no physical contact with plant cells, *prhJ* and *hrpG* were expressed in a PrhA-dependent manner via the multigene regulatory cascade (Fig. 3a). On the other hand, the second cascade was not active because there were no plant signals. As a result, HrpG remained inactive, and *hrpB* expression was not induced. In the nutrient-poor medium, the expression of *prhJ* and *hrpG* seemed to be at a basal level (Table 4). Even though the expression level of *hrpG* was slightly lower in the nutrient-poor medium than in the nutrient-rich medium, HrpG could induce *hrpB* expression at a much higher level because HrpG was activated through the new signal cascade (Fig. 3b). The metabolic signal in the nutrient-poor medium could be sensed by the new signal cascade and activate HrpG. When the bacteria was co-cultivated with

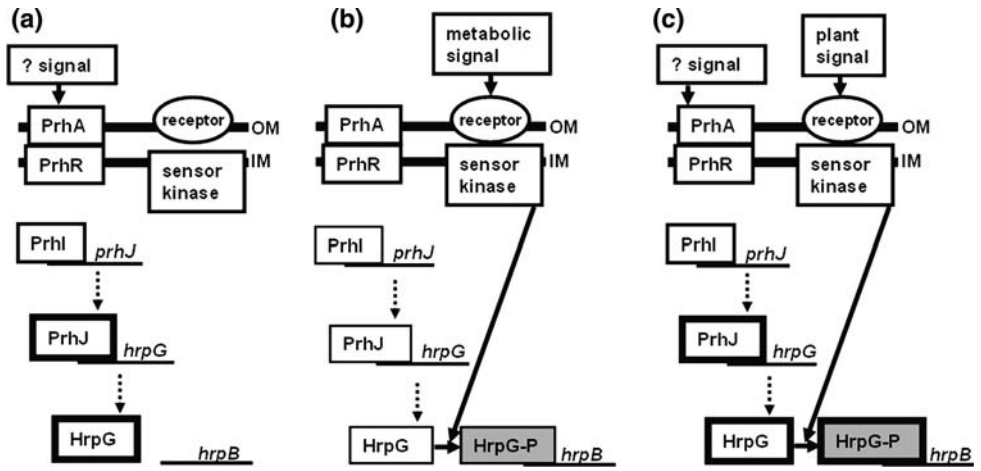

Fig. 3 Proposed models for the two signal cascades for HrpG activation in *Ralstonia solanacearum* cells cultivated in **a** nutrient-rich medium, **b** nutrient-poor medium, or **c** co-cultivated with *A. thaliana* seedlings. The *left half* represents the complex multigene regulatory cascade, and the *right half* shows the new cascade including the two-component system. *OM* and *IM* represent outer and inner membranes. Gene names are given on the *thin lines*. *Dashed lines* show transcription/translation. *Boxed letters* represent proteins. *Thick boxed lines* indicate a higher expression level. *Shaded boxes* show the phosphorylated HrpG

A. thaliana seedlings, *prhJ* and *hrpG* were expressed through the first cascade. Expressed HrpG was fully activated by the second cascade and induced a higher level of *hrpB* expression (Fig. 3c).

R. solanacearum cells sense several signals to induce *hrpB* expression and the *hrp* regulon. A nondiffusible signal present in the plant cell wall was identified as the factor that induced a *hrpB–gfp* reporter gene (Aldon et al. 2000). Although this signal is thought to be recognized by PrhA, the signal could instead be used by the second cascade (Fig. 3c) because the complex regulatory cascade composed of PrhA–PrhR/PrhI–PrhJ–HrpG was activated in the absence of plant signals (Table 4), and the activation of both cascades by signal recognition is needed for *hrpB* induction. PrhA belongs to the TonB-dependent siderophore-receptor proteins, which are located in the outer membrane (Marenda et al. 1998). Siderophore-receptor proteins bind specific iron-siderophore complexes and transport iron into the periplasm. Although PrhA is not related to iron transport (Marenda et al. 1998), PrhA might bind compounds that resemble siderophores, such as nonribosomal peptides (Neilands 1995).

HrpG integrates the PhcA-dependent quorum sensing signal (Genin et al. 2005). Recently, we reported that PhcA binds to the promoter region of *prhIR* and represses *prhIR* expression (Yoshimochi et al. 2009). At high cell density, active PhcA represses *prhIR* expression, and *hrpG* expression is dramatically reduced because the complex signal cascade is not working. We observed that at high cell density *hrpB* expression was reduced even in the co-cultivation with *A. thaliana* seedlings (unpublished data). In this situation, the first cascade may be inactive while the newly demonstrated second cascade is active.

The *hrpG* null mutant completely lost pathogenicity (Fig. 2) (Brito et al. 1999). In contrast, although the *hrpGD51N* mutant had reduced pathogenicity on tobacco, it was still able to cause disease. Concomitantly, HrpGD51N could induce *hrpB* expression, although at a low level. HrpG is a member of the OmpR/PhoB subfamily of response regulator transcription factors with a C-terminal winged helix-turn-helix DNA-binding motif (Toro-Roman et al. 2005); members of this subfamily are known to bind as tandem dimers to DNA direct repeat recognition sequences. The OmpR/PhoB family has a common dimerization mechanism mediated by the α4–β5–α5 interface (Toro-Roman et al. 2005). Phosphorylation induces a conformational change in the α4–β5–α5 face that in turn promotes dimerization. It is well known that dimerization allows or increases DNA binding to the promoter recognition element (Simonovic and Volz 2001). The α4–β5–α5 interface contains 11 well-conserved amino acid residues (Toro-Roman et al. 2005). HrpG has three residue substitutions, which could affect its interactions at the dimer interface. Nonphosphorylated HrpG might form dimers at low efficiency and bind to the *hrpB* promoter, resulting in weak expression of *hrpB*. The *hrpB* expression of RK5046 (wild type *hrpG*) grown in nutrient-rich medium (Table 4) was similar to that of RK5243 (*hrpGD51N*) grown with *A. thaliana* seedlings (Table 5). In both conditions, *hrpG* is expressed, but HrpG might not be phosphorylated (Fig. 3a, c). When *hrpG* was supplied in trans from a multicopy plasmid in RK5198 (Δ*hrpG*), *hrpB* expression was highly

induced even in nutrient-rich medium (unpublished data), also in support of the idea that nonphosphorylated HrpG can bind to the *hrpB* promoter. HrpG is known to regulate the expression of several virulence genes other than *hrpB* (Valls et al. 2006). Nonphosphorylated HrpG could activate some of these virulence genes.

More than 40 sensor kinases of two-component systems have been annotated in *R. solanacearum* GMI1000 (Cunnac et al. 2004). HrpG is an orphan, and no cognate kinase genes have been identified. By using a yeast two-hybrid system, HrpG of *X. axonopodis* pv. *citri* was found to interact with a single prey derived from the XAC3683 gene that encodes a composite sensor-histidine kinase/response regulator (Alegria et al. 2004). We have identified a homologous gene rsp1676 encoding a composite two-component regulatory protein on the megaplasmid of *R. solanacearum* GMI1000 (Salanoubat et al. 2002). XAC3683 shows 32% identity in amino acids to RSp1676. This composite kinase could be a candidate for the cognate kinase that phosphorylates HrpG. We are planning to test phosphotransfer activity from RSp1676 to HrpG to confirm that RSp1676 could be a counterpart of HrpG.

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