

Regulatory Interactions Between Quorum-Sensing, Auxin, Cytokinin, and the Hrp Regulon in Relation to Gall Formation and Epiphytic Fitness of *Pantoea agglomerans* pv. *gypsophila*e

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Gall formation by *Pantoea agglomerans* pv. *gypsophila*e is controlled by *hrp/hrc* genes, phytohormones, and the quorum-sensing (QS) regulatory system. The interactions between these three components were investigated. Disruption of the QS genes *pagI* and *pagR* and deletion of both substantially reduced the transcription levels of the *hrp* regulatory genes *hrpXY*, *hrpS*, and *hrpL*, as determined by quantitative reverse-transcriptase polymerase chain reaction. Expression of *hrpL* in planta was inhibited by addition of 20 μ M or higher concentrations of the QS signal C₄-HSL. The *pagR* and *hrpL* mutants caused an equivalent reduction of 1.3 orders in bacterial multiplication on bean leaves, suggesting possible mediation of the QS effect on epiphytic fitness of *P. agglomerans* pv. *gypsophila*e by the *hrp* regulatory system. indole-3-acetic acid (IAA) and cytokinin significantly affected the expression of the QS and *hrp* regulatory genes. Transcription of *pagI*, *pagR*, *hrpL*, and *hrpS* in planta was substantially reduced in *iaaH* mutant (disrupted in IAA biosynthesis via the indole-3-acetamide pathway) and *etz* mutant (disrupted in cytokinin biosynthesis). In contrast, the *ipdC* mutant (disrupted in IAA biosynthesis via the indole-3-pyruvate pathway) substantially increased expression of *pagI*, *pagR*, *hrpL*, and *hrpS*. Results presented suggest the involvement of IAA and cytokinins in regulation of the QS system and *hrp* regulatory genes.

Pantoea agglomerans (previously *Erwinia herbicola*) has been transformed from a commensal and epiphytic bacterium associated with many plants into a host-specific tumorigenic pathogen, by acquiring a plasmid-borne pathogenicity island (PAI) (Barash and Manulis-Sasson 2007; Manulis and Barash 2003). Unique features of this PAI that are relevant to virulence include the presence of a functional *hrp/hrc* gene cluster encoding the type III secretion system (T3SS), a regulatory cascade controlling the Hrp regulon, genes encoding type III effectors, and a cluster of genes responsible for biosynthesis of indole-3-acetic acid (IAA) and cytokinin (CK) (Barash and Manulis-Sasson 2007). It has been demonstrated that gall formation by the two related pathogens, *P. agglomerans* pv. *gypsophila*e, pathogenic on gypsophila, and *P. agglomerans* pv. *betae*, pathogenic on gypsophila and beet, is Hrp dependent (i.e., mutations in the *hrp/hrc* gene cluster completely abolished

gall formation) (Mor et al. 2001; Nizan et al. 1997; Nizan-Koren et al. 2003). The regulatory cascade that activates the Hrp regulon in *P. agglomerans* pv. *gypsophila*e 824-1 (824-1) has been characterized (Nizan-Koren et al. 2003); it contains an *hrpXY* operon encoding a two-component system (i.e., *hrpS* encoding a transcriptional factor of the NtrC family and *hrpL* encoding an alternative sigma factor). The following signal transduction model was suggested: phosphorylated HrpY activates *hrpS*, HrpS activates *hrpL*, and HrpL activates genes containing the “*hrp* box” promoter. The *hrp* box is common to all the genes controlled by the Hrp regulon, which is essentially composed of genes encoding the T3SS and type III effectors (Alfano and Collmer 2004). Binding of HrpL protein to the *hrp* box promoter in *P. agglomerans* pv. *gypsophila*e and the precise relationship between its base composition and promoter activity have been described (Nissan et al. 2005).

The development of a plant tumor is characterized by increases in the number (hyperplasia) and size (hypertrophy) of cells, which are attributed to overproduction of IAA and CK (Morris 1986). It has been shown that gall-forming *P. agglomerans* strains possess two major metabolic pathways for IAA biosynthesis, the indole-3-acetamide (IAM) and the indole-3-pyruvate (IPyA) routes, in contrast to nonpathogenic strains, which carry only the IPyA pathway (Manulis et al. 1991). Inactivation of the plasmid-borne IAM pathway was achieved by mutagenesis of its genes encoding for tryptophan-2-monooxygenase (*iaaM*) or IAM hydrolase (*iaaH*) (Clark et al. 1993), whereas the chromosomal IPyA pathway was inactivated by mutagenesis of its key gene, *ipdC*, encoding for IPyA decarboxylase (Brandl and Lindow 1996; Manulis et al. 1998). The key gene for CK biosynthesis is isopentenyl transferase (*ipt*) (Morris 1986). A marker exchange mutant of the plasmid-borne *ipt* homolog (*etz*) was generated in *P. agglomerans* pv. *gypsophila*e and it prevented CK production (Lichter et al. 1995). Inactivation of either the IAM pathway or CK biosynthesis caused 40 to 50% reduction in gall size, whereas simultaneous inactivation of the two pathways for IAA and CK biosynthesis in *P. agglomerans* pv. *gypsophila*e also reduced gall size by approximately 50% but did not prevent gall formation (Manulis et al. 1998). It is noteworthy that a differential contribution of the IAM and IPyA pathways for IAA biosynthesis to pathogenicity and epiphytic fitness, respectively, was observed in *P. agglomerans* pv. *gypsophila*e (Manulis et al. 1998). Thus, inactivation of the IAM pathway substantially reduced gall formation in gypsophila cuttings but not epiphytic fitness on bean or gypsophila leaves, whereas inactivation of the IPyA

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pathway adversely affected epiphytic fitness but not pathogenicity. Taken together, it can be concluded that IAA and CK secreted by the pathogen may play a significant but secondary role in gall development, unlike *hrp/hrc* genes that perform a primary function (Barash and Manulis-Sasson 2007). Because both IAA and CK are required for stimulating hyperplasia and hypertrophy involved in gall formation (Morris 1986), it was hypothesized that type III effector proteins injected via the T3SS can manipulate the plant to produce the necessary IAA and CK for tumorigenesis (Barash and Manulis-Sasson 2007).

A quorum-sensing (QS) regulatory system has recently been characterized in *P. agglomerans* pv. *gypsophilae* 834-1 (Chalupowicz et al. 2008): *P. agglomerans* pv. *gypsophilae* produces *N*-butanoyl-L-homoserine lactone (C₄-HSL) as a major and *N*-hexanoyl-L-homoserine lactone (C₆-HSL) as a minor QS signal. Homologs of *luxI* and *luxR* regulatory genes, *pagI* (encoding C₄-HSL synthase) and *pagR* (encoding the transcriptional regulator), were characterized and shown to be convergently transcribed. The deduced PagI (23.8 kDa) and PagR (26.9 kDa) as well as the QS signals showed striking similarity to those in *Serratia* sp. ATCC 39006 (Thomson et al. 2000). The

presence of the QS signals and the regulatory genes in pathogenic as well as in nonpathogenic strains of *P. agglomerans*, which lack the pPATH plasmid, suggested that they do not reside on the pathogenicity plasmid and are quite likely chromosomal (Chalupowicz et al. 2008). Disruption of either *pagR* or *pagI* or both genes simultaneously in strain 824-1 reduced gall size in gypsophila cuttings by 50 to 55% when plants were inoculated with 10⁶ CFU/ml. Higher reduction in gall size (70 to 90%) was achieved by overexpression of *pagI* or addition of exogenous C₄-HSL (Chalupowicz et al. 2008).

The reduction of gall development in QS mutants could be attributed to a positive effect of this global regulatory system on the Hrp regulon. Alternatively, it could be caused by a negative effect of QS on biosynthesis of IAA and CK that leads to reduction in gall size. It is noteworthy that a reduction of approximately 50% was reported for *pagI* or *pagR* mutants (Chalupowicz et al. 2008), which is similar to that obtained by inactivation of IAA and CK biosynthesis pathways (Manulis et al. 1998). Initial support for the first premise was obtained by monitoring the expression of the *hrp/hrc* regulatory gene *hrpL* and the T3SS effector *pthG* in *pagI* mutant background using quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) under in vitro conditions (Chalupowicz et al. 2008). The possibility that the observed reduction in gall size resulted from suppression of IAA or CK biosynthesis through inactivation of the QS system was tested in the present study and was ruled out because QS mutations did not affect the expression of *iaaH*, *ipdC*, and *etz*. These preliminary results prompted us to further investigate the regulatory interrelationships among biosynthesis of IAA and CK, QS, and activation of the Hrp regulon under in planta conditions. The present study was undertaken to i) demonstrate the effect of the QS system on expression of the Hrp regulon in planta and ii) elucidate the interactions between biosynthesis of IAA and CK, the QS regulatory system, and expression of the Hrp regulon.

Table 1. Relevant primers used for quantitative reverse-transcriptase polymerase chain reaction analysis

Name	Sequence (5'→3')	Expressed gene
gyrBRTFow	GGTGAAAGTGCCGGATCCTAA	<i>gyrB</i>
gyrBRTRev	TGCTGCTCAACCGCTGTTT	...
hrpLRTFow	GGTACAGATGACCTGGCTGGAA	<i>hrpL</i>
hrpLRTRev	CCAAAAACCCACGTCTCTGG	...
hrpSRTFow	ATCCAGTTACAGCCTCTC	<i>hrpS</i>
hrpSRTRev	CTAATCCCAACACAAATCG	...
hrpXRTFow	TGCCATCTACCTGATTGAC	<i>hrpX</i>
hrpXRTRev	CCGTTCTGCTCTCTTACC	...
hrpYRTFow	TGAGCGAAGCGGGAAATG	<i>hrpY</i>
hrpYRTRev	GGTATTGCGAGTGAATCTGG	...
RFow	TACGCCGAAGTTGCGGTCAT	<i>pagR</i>
RRev	ACCGCATGTGTGGCGTT	...
IFow	ACAGCCGGCTAATCTCACAACA	<i>pagI</i>
IRev	TGGGCATGCATAACGGGAAGTT	...
iaaFow	TTTCCGGATCCAGGCATTGT	<i>iaaH</i>
iaaRev	AATAAAGCCCGCCAGCCATTCT	...
etzFow	GAGAGGTCTGGCTGATGGG	<i>etz</i>
etzRev	CTTCTGGCAAGGATAGCACTC	...
rsmAFow	TCGTCGAGTTGGTGAACCCTCAT	<i>rsmA</i>
rsmARev	ATCTCTTCGCGATGCACAGACT	...

RESULTS

The *hrp/hrc* regulatory genes *hrpXY*, *hrpS*, and *hrpL* are controlled by the QS system in planta.

It has been previously reported that disruption of *pagI* in 824-1 significantly reduced the transcript level of the regulatory gene *hrpL* in cultures grown on minimal A medium (Chalupowicz et al. 2008). To further corroborate this observation, the expressions of the other Hrp regulatory genes, namely,

Table 2. Bacterial strains and plasmids used in this study

Strains, plasmids	Relevant characteristics ^a	Reference or source
Strains		
<i>Escherichia coli</i>		
DH5α	<i>lacZ</i> ΔM15 Δ (<i>lacZYA-argF</i>) U169 <i>gyrA96</i>	Invitrogen ^b
<i>Pantoea agglomerans</i> pv. <i>gypsophilae</i>		
824-1	Pathogenic strain with spontaneous Rif ^r , gypsophila pathovar	Manulis et al. 1991
MxI	Rif ^r , Spec ^r , 824-1 marker exchange mutant with Ω cartridge in <i>pagI</i> (AHL ⁻)	Chalupowicz et al. 2008
MxR	Rif ^r , Km ^r , 824-1 marker exchange mutant with kanamycin cassette in <i>pagR</i>	Chalupowicz et al. 2008
MxAIR	Rif ^r , Km ^r , 824-1 marker exchange with double deletion in <i>pagI</i> and <i>pagR</i>	Chalupowicz et al. 2008
MxL	Rif ^r , Spec ^r , 824-1 marker exchange mutant of Tn3-Spice in <i>hrpL</i>	Nizan-Koren et al. 2003
MxJ	Rif ^r , Spec ^r , 824-1 marker exchange mutant of Tn3-Spice in <i>hrpJ</i>	Nizan-Koren et al. 2003
MxIaaH	Rif ^r , Km ^r , 824-1 marker exchange mutant in <i>iaaH</i> (<i>Ehg824-1MX54</i>)	Manulis et al. 1998
MxEtz	Rif ^r , Km ^r , 824-1 marker exchange mutant in <i>etz</i> (<i>Ehg824-1MX37</i>)	Manulis et al. 1998
MxIpdc	Rif ^r , Spec ^r , 824-1 marker exchange mutant in <i>ipdC</i> (<i>Ehg824-1MX119</i>)	Manulis et al. 1998
PD713	Pathogenic strain on gypsophila	Manulis et al. 1991
Plasmids		
pWM1029	Km ^r , Spec ^r , broad-host-range plasmid for green fluorescent protein expression	Cooley et al. 2003
pAKC891	Spec ^r , 3.0-kb <i>EcoRI-SacI</i> fragment containing <i>rsmA_{rhg}</i> in pCL1921	Ma et al. 2001
pCLrsmA	Spec ^r , 3.0-kb <i>SacI</i> fragment containing <i>rsmA_{pag}</i> in pCL1920	This study

^a Rif^r, Spec^r, and Km^r indicate resistance to rifampicin, spectinomycin, and kanamycin, respectively.

^b Invitrogen, San Diego, CA, U.S.A.

hrpXY and *hrpS*, as well as *hrpL*, were measured by qRT-PCR in planta. The PCR primers employed for amplification of each gene are given in Table 1. Gypsophila cuttings were dip inoculated with cell suspensions (10^6 cells/ml) of 824-1 and its QS mutant strains MxI, MxR, and Mx Δ IR (Table 2). After incubation for 16 h at 25°C, the total RNA was extracted from the inoculated plants and the levels of mRNA were determined as described below. Inactivation of *pagI* and *pagR* and simultaneous deletion of the two genes (*pagI/R*) reduced the transcript levels of *hrpXY* by 3.6-, 2.2-, and 3.3-fold, respectively; those of *hrpS* by 1.6-, 4.5-, and 5.3-fold, respectively; and those of *hrpL* by 5.5-, 9.0-, and 25-fold, respectively, compared with those in the wild-type strain 824-1 (Fig. 1). These results indicate that each member of the Hrp regulatory system is under the control of QS and that both functional *pagI* and functional *pagR* are required for activating the cascade of these regulatory genes.

High concentrations of C₄-HSL were previously shown to reduce gall development (Chalupowicz et al. 2008); therefore, we further investigated whether expression of *hrpL* was affected by addition of C₄-HSL. Gypsophila cuttings were infected by cell suspension (10^6 cells/ml) of the wild-type strain 824-1 in the absence and presence of C₄-HSL at 2, 20, and 200 μ M, and total RNA was extracted and evaluated for *hrpL* expression by qRT-PCR, as described above. The results (Fig. 2) revealed that the transcript level of *hrpL* was slightly higher after addition of 2 μ M C₄-HSL than that produced by 824-1. However, substantial reductions of two- and fourfold were observed in response to 20 and 200 μ M of C₄-HSL, respectively, compared with the wild-type control strain.

Colonization of *P. agglomerans* pv. *gypsophila* in planta is dependent on functional QS system and inhibited by high levels of C₄-HSL.

The effect of the QS system on colonization of gypsophila cuttings by *P. agglomerans* pv. *gypsophila* was assessed during early stages of infection. The multiplication of the QS mutants *pagI*⁻ (MxI) and *pagI/R*⁻ (Mx Δ IR) was compared with that of the wild-type strain (824-1) and of an *hrpJ*⁻ (MxJ) mutant deficient in functional T3SS (Nizan-Koren et al. 2003).

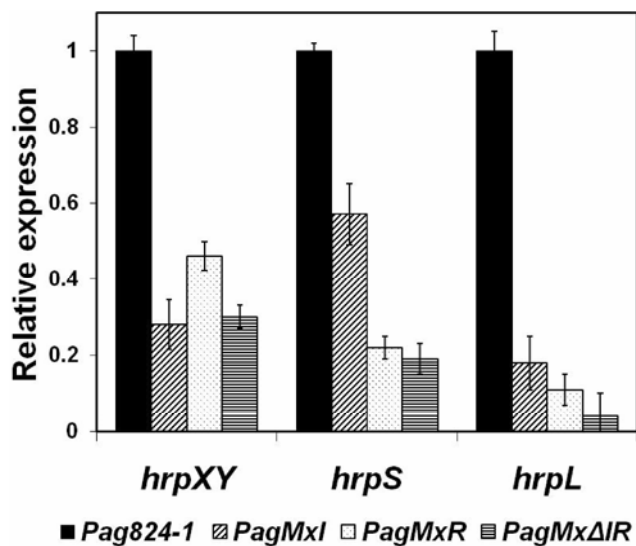


Fig. 1. Transcript levels of *hrpXY*, *hrpS* and *hrpL* in the wild-type *Pantoea agglomerans* pv. *gypsophila* 824-1 (*Pag824-1*) and quorum-sensing mutants (*PagMxI*, *PagMxR*, and *PagMx Δ IR*) in planta. Template cDNAs were generated from total RNA extracts of infected gypsophila cuttings 16 h after inoculation. Gene expression was normalized with *gyrB* used as an endogenous reference and *Pag824-1* as the control. Results represent three different experiments with standard deviation.

Gypsophila cuttings were inoculated with 10^6 cells/ml and the bacterial population was monitored after 1, 2, 3, and 4 days as described below. The populations of 824-1 and *hrpJ* mutant in gypsophila cuttings increased by approximately two orders of magnitude during the initial 24 h, compared with increases by only one order of magnitude in the populations of *pagI* and *Pag Δ IR* mutants (Fig. 3A). After 2, 3, and 4 days, the populations of the two QS mutants as well as that of *hrpJ* mutant remained similarly smaller (by 2.0 to 2.67 orders) than that of the wild type (Fig. 3A). These results could imply that the QS system might affect growth earlier than the Hrp system, and during the period in which the increased multiplication appeared to be Hrp independent (Valinsky et al. 2002). Addition of 200 μ M C₄-HSL to 824-1 inhibited growth by approximately one order after 4 days compared with the wild type without addition of C₄-HSL, which correlates with the effect of excessive signal on *hrpL* expression (Fig. 2).

To further support the above result, gypsophila cuttings were inoculated with 824-1 and with MxI and Mx Δ IR mutants into which a plasmid for green fluorescent protein (GFP) expression (pWM1029) was introduced, as described below. Confocal images of longitudinal sections taken 6 days after inoculation with GFP-tagged 824-1 revealed the presence of abundant bacterial aggregates colonizing the xylem and adjacent parenchymal cells, in contrast to the two QS mutants, which exhibited only scattered bacterial aggregates (Fig. 3B).

Effects of *pagI* and *pagR* on epiphytic fitness.

It is now well established that the QS system regulates epiphytic fitness in *Pseudomonas syringae* (Quinones et al. 2005). Previous (Chalupowicz et al. 2008) and present results suggest that the QS system in 824-1 governs the Hrp regulon. The following experiment was intended to determine whether the QS affected epiphytic fitness of *Pantoea agglomerans* pv. *gypsophila* and whether this effect could be attributed to its interaction with the Hrp regulon. To test this hypothesis, we investigated the effects of mutants disrupted in the QS system (i.e., *pagI*⁻ and *pagR*⁻) and in the T3SS system (*hrpL*⁻) on epiphytic fitness of 824-1. Bean leaves were inoculated by dipping them in bacterial suspension (10^6 cells/ml) and the plants were subjected to moist followed by dry conditions, as described below. During the first 48 h, under wet conditions, the bacterial populations of the wild-type strain and the mutants increased by approximately two orders of magnitude (Fig. 4). However, after the 48 h of dry conditions, the populations of *pagR*⁻ and the

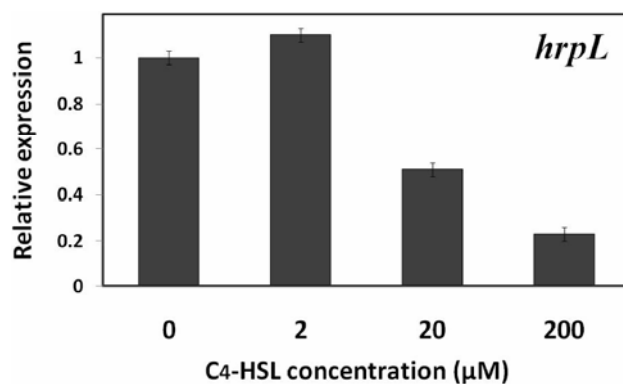
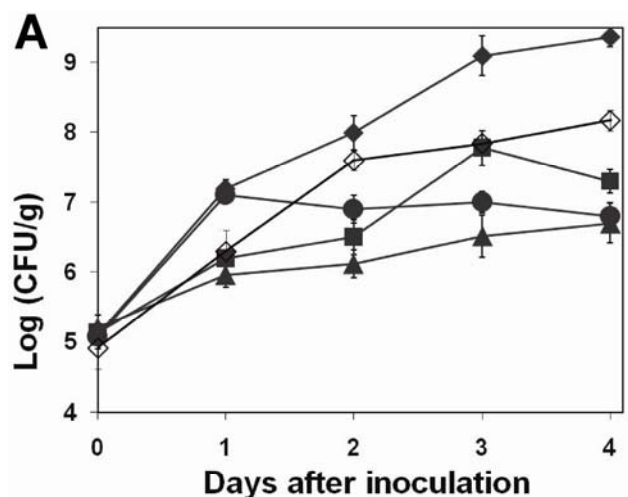


Fig. 2. Expression of *hrpL* in response to exogenous C₄-HSL. The transcript levels of *hrpL* were examined in *Pantoea agglomerans* pv. *gypsophila* 824-1 (*Pag824-1*) 16 h after inoculation of gypsophila cuttings, and in the presence of 2, 20, and 200 μ M C₄-HSL. Relative levels of *hrpL* were normalized with *gyrB* used as an internal reference and *Pag824-1* without addition of C₄-HSL as control. Results represent three different experiments with standard deviation.

hrpL⁻ mutants showed similar reductions of 1.3 orders of magnitude, whereas mutation in *pagI* caused only a slight decrease, of 0.7 orders. Taken together, these data could suggest that T3SS effectors might contribute to the effect of the QS on epiphytic fitness.

Disruption of biosynthetic pathways for IAA and CK affects the transcription levels of QS and Hrp regulatory genes.

The interrelationships among biosynthesis of IAA and CK, the QS regulatory system, and activation of the Hrp regulon were studied in planta. As indicated above, two routes for IAA biosynthesis are present in *P. agglomerans* pv. *gypsophila*: the pPATH-borne IAM pathway and the chromosomal IPyA pathway. Mutants in *iaaH* and *ipdC* inactivated the IAM and IPyA routes, respectively, whereas a mutant in *etz* disrupted the biosynthesis of CK (Manulis et al. 1998). Initially, we used a qRT-PCR with primers described in Table 1 to examine the



◆ Pag824-1 ■ PagMxI ▲ PagMxΔIR
● PagMxJ ◇ 200μM C4-HSL

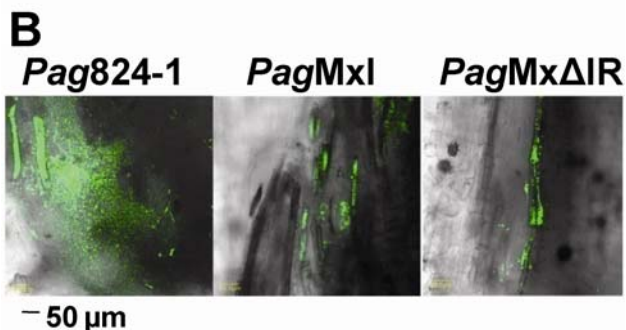


Fig. 3. Effects of quorum-sensing (QS) system on multiplication of *Pantoea agglomerans* pv. *gypsophila* in planta. **A**, Multiplication of the QS and *hrpJ* mutants compared with the wild type (with and without addition of C₄-HSL) in gypsophila cuttings. Cuttings were dipped in the various bacterial suspensions (10⁶ cells/ml) for 45 min and colonization was determined after 1, 2, 3, and 4 days. *P. agglomerans* pv. *gypsophila* 824-1 (*Pag824-1*), wild type; *PagMxI*, a marker exchange mutant in *pagI*; *PagMxΔIR*, a deleted *pagI/R* mutant; *PagMxJ*, a marker exchange mutant in *hrpJ* and addition of 200 μM C₄-HSL during inoculation with *Pag824-1*. Results presented are averages of three independent experiments with five replicates for each time point. Bars indicate standard deviations. **B**, Longitudinal section of infected gypsophila cuttings visualized 6 days after inoculation by confocal fluorescent microscopy. Gypsophila cuttings were inoculated by dipping in a solution (10⁶ cells/ml) of *Pag824-1*, *PagMxI*, and *PagMxΔIR* harboring the plasmid pWM1029. The images are representative of three independent experiments (Scale bars, 50 μm).

possibility that *pagI* or *pagR* mutants affected the transcription of *iaaH*, *ipdC*, and *etz*, but negative results were obtained (not shown). In contrast, when the effects of *iaaH*, *ipdC*, and *etz* mutants on the transcriptional levels of *pagI*, *pagR*, *hrpL*, and *hrpS* were tested, significant changes were obtained (Fig. 5). Transcription of *pagI* was reduced by factors of 5.9 and 2.8 in *iaaH* and *etz* mutants, respectively, compared with 824-1 (wild type) but increased 2.8-fold in *ipdC* mutant; transcription of *pagR* decreased by factors of 3.3 and 2.9 in *iaaH* and *etz* mutants, respectively, but increased 3.4-fold in *ipdC* mutant; transcription of *hrpL* was reduced by factors of 3.6 and 3.8 in *iaaH* and *etz* mutants, respectively, but increased 3.1-fold in *ipdC* mutant; transcription of *hrpS* was reduced by factors of 9.0 and 5.6 in *iaaH* and *etz* mutants, respectively, but increased 3.8-fold in *ipdC* mutant.

The possibility that these mutants could be complemented by either IAA or CK was tested. Addition of 100 μM IAA to the *iaaH* and *ipdC* mutants restored the expression of *pagI*, *pagR*, *hrpL*, and *hrpS* to wild-type levels; namely, it increased the expression of these genes in the *iaaH* mutant and decreased expression of them in the *ipdC* mutant, respectively. Similarly, addition of 20 μM 6-benzylamionpurine (BA) to the *etz* mutant increased the expression of *pagI*, *pagR*, *hrpL*, and *hrpS* to wild-type levels (results not shown). The differential effects of the two IAA biosynthetic pathways on the transcription of the QS and Hrp regulatory genes were surprising because they have an identical end product. Moreover, to the best of our knowledge, CK have not previously been reported to affect transcription of bacterial genes. These two intriguing findings are addressed below (Discussion).

The post-transcriptional regulator RsmA has been reported to play a central role in the interaction between the QS systems and virulence (Mole et al. 2007). The possibility that QS affects the expression level of *rsmA* in 824-1 was tested in *pagI* and *pagR* mutants by using PCR primers of *rsmA* listed in Table 1. No effect of either of the two mutants on *rsmA* could be detected (results not shown).

DISCUSSION

The function of genes encoding T3SS and type III effectors is critical for gall formation by *P. agglomerans* pv. *gypsophila* and *P. agglomerans* pv. *betae* (Barash and Manulis-Sasson

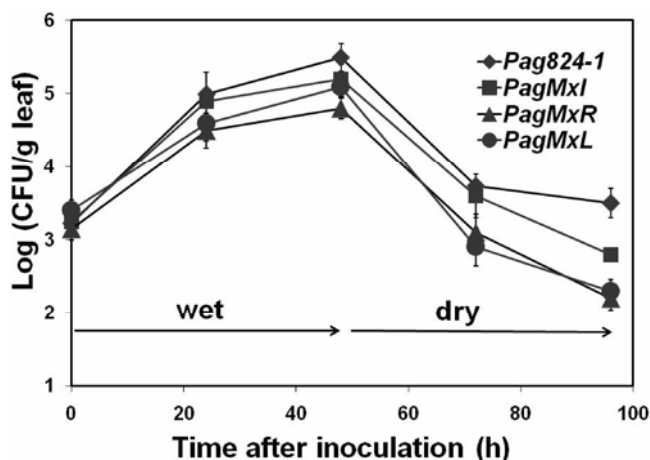


Fig. 4. Population size of *Pantoea agglomerans* pv. *gypsophila* 824-1 (*Pag824-1*), *PagMxI* (*pagI*⁻), *PagMxR* (*pagR*⁻), and *PagMxL* (*hrpL*⁻) on bean leaves. The leaves were kept moist for the first 48 h after inoculation and then kept dry for the rest of the experiment. The vertical bars represent the standard error of the mean log₁₀-transformed bacterial population size per gram of leaf.

2007). A linear cascade of regulatory genes (*hrpXY*, *hrpS*, and *hrpL*) that activates the Hrp regulon via the *hrp* box promoter regulates these genes in *P. agglomerans* pv. *gypsophila* (Nizan-Koren et al. 2003). We have previously reported that expression of the regulatory gene *hrpL* and the type III effector *pthG* were significantly reduced in a *P. agglomerans* pv. *gypsophila* mutant deficient in C_4 -HSL production when grown

on minimal medium (Chalupowicz et al. 2008). In the present study, we further corroborate the role of the QS system in controlling the Hrp regulon by studying its effects on *hrpXY* and *hrpS*, in addition to *hrpL*, in planta at an inoculum level of 10^6 CFU/ml. The results presented unequivocally demonstrate that both *pagI* and *pagR* are required for full activation of the Hrp regulon (Fig. 1). Information concerning the effect of the QS

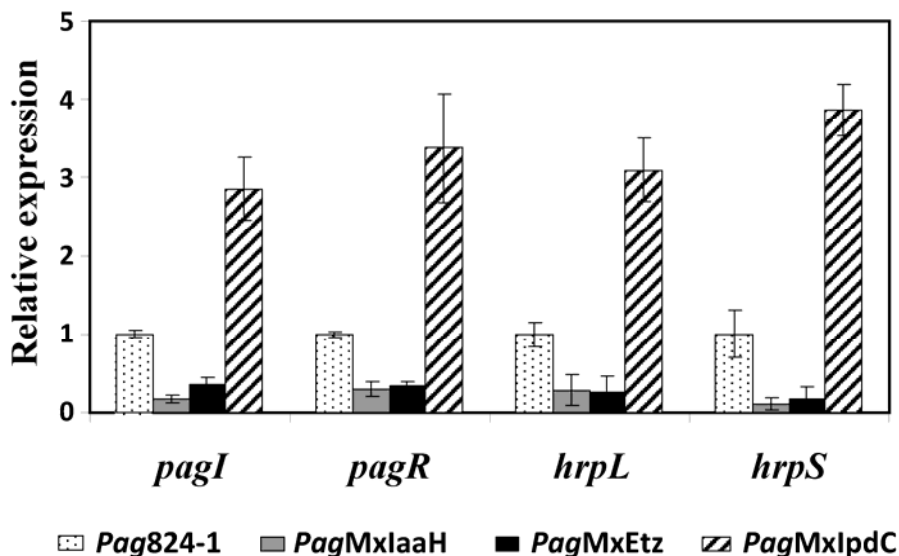


Fig. 5. Effects of mutations in indole-3-acetic acid (IAA) and cytokinin (CK) biosynthetic genes on *pagI*, *pagR*, *hrpL*, and *hrpS* transcript levels as determined by quantitative reverse-transcriptase polymerase chain reaction. *Pantoea agglomerans* pv. *gypsophila* MxIaaH (*PagMxIaaH*), *PagMxEtz*, and *PagMxIpdC* are marker exchange mutants in *iaaH*, *etz*, and *ipdC*, respectively. cDNA samples were generated from total RNA of *Pag824-1* and the various mutants after growth for 16 h in gypsophila cuttings. Experiments were repeated three times with three replicates for each strain.

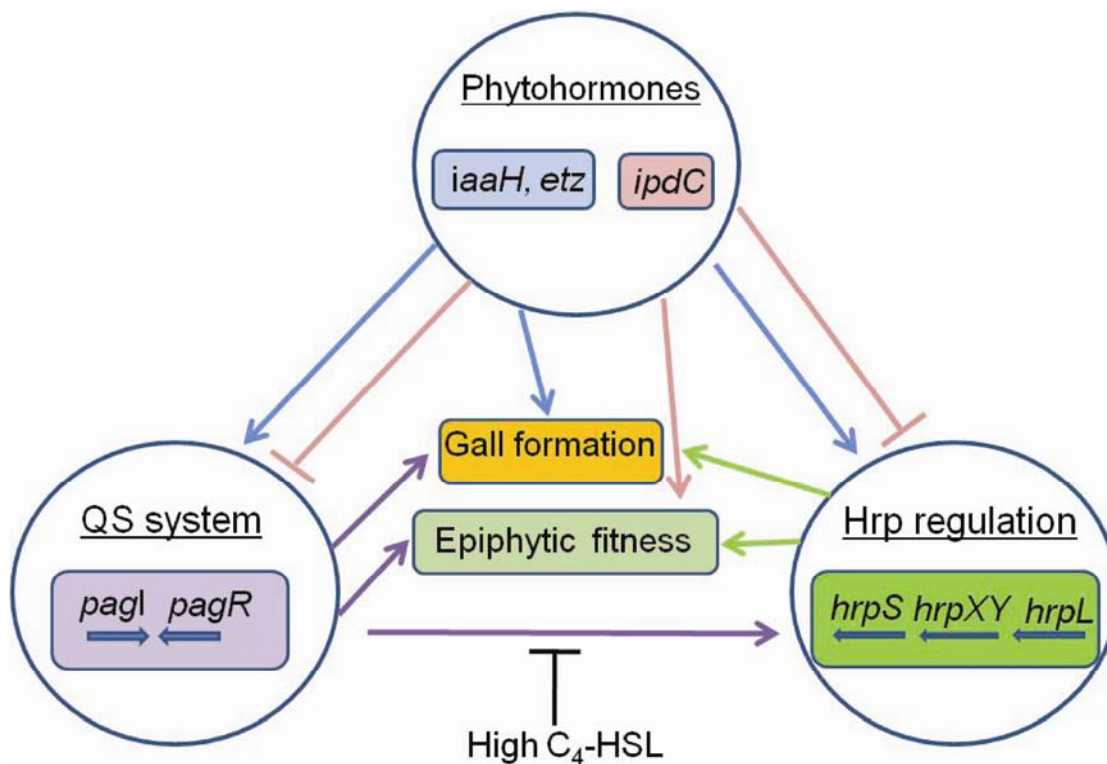


Fig. 6. Relationships among the phytohormones, quorum-sensing (QS) system, and Hrp regulatory genes of *Pantoea agglomerans* pv. *gypsophila* 824-1 and their effects on gall formation and epiphytic fitness. The various components are denoted by circles. Genes and phenotypes are represented by rectangles. Arrows indicate upregulation and barred arrows indicate downregulation. The colors of the arrows correspond to the respective genes. At low inoculum level (10^6 cells/ml), the biosynthetic pathways for indole-3-acetic acid (IAA) (*iaaH*) and cytokinin (*etz*) positively regulated the expression of both the QS (*pagI*, *pagR*) and the Hrp regulatory (*hrpS*, *hrpXY*, *hrpL*) genes. The IAA pathway (*ipdC*) negatively regulated the transcription of the Hrp regulon and QS system. The QS system induced expression of the Hrp regulatory genes. High C_4 -HSL concentrations negatively affected the Hrp regulon. Gall formation and epiphytic fitness were affected by all three systems.

system on the Hrp regulon is very limited. In the case of *Pectobacterium carotovorum* subsp. *carotovorum*, cell-to-cell communication systems are responsible for regulating *hrpL* and *hrpN*, which are related to the T3SS system, as well as major virulence factors of this pathogen; namely, cell-wall-degrading enzymes (Barnard et al. 2007). In the presence of low concentrations of C₄-HSL, the *pagR* either directly activates the transcription of *hrpXY*, *hrpS*, and *hrpL* or, alternatively, its effect is mediated by other as-yet-unknown genes. Some support for the direct activation hypothesis could be inferred from the presence of putative *lux* boxes in the promoters of these genes. The minimal consensus sequence for *lux* box according to Whiteley and Greenberg (2001) is [NNCT-(N₁₂)-AGNN]. Sequences that could meet this consensus in the promoter regions of *hrpXY* [AACT-CTGTAGATCTGC-AGCC], *hrpS* [TCCT-TACTCGTGGTTC-AGCA], and *hrpL* [AGCT-GACAGTAT CAGG-AGTG] were identified. Whether these sequences can bind *pagR* and thereby activate transcription remains to be determined by gel shift experiments. It is also noteworthy that a sequential regulation is responsible for the activation of *hrpL* (Nizan-Koren et al. 2003). Thus, either activation of *hrpXY* expression, in itself, is sufficient for the upregulation of *hrpS* and *hrpL* or activation of *hrpS* stimulates expression of *hrpL*. It is not yet known whether the QS system directly controls each of the three genes or whether only the upstream genes of the cascade are being activated. Another point of interest is that the transcription of *hrpL* by *pagR* could be substantially overridden by addition of excess exogenous C₄-HSL (Fig. 2). This finding correlates with a previous observation that gall size on gypsophila cuttings can be reduced by up to 90% by exposure to exogenous C₄-HSL (Chalupowicz et al. 2008). It is possible to hypothesize that the presence of a high concentration of AHL could interfere with the binding of the transcription activator *pagR* to the DNA as reported by Tsai and Winans for the YenI/yenR system in *Yersinia enterocolitica* (Von Bodman et al. 2008). Another possibility is that the premature activation of the *hrp* regulatory cascade by a high concentration of the signal caused its suppression by the plant (Chalupowicz et al. 2008).

It is well established that the *hrp/hrc* gene cluster and type III effectors are necessary for growth of gram-negative phytopathogenic bacteria in planta (Alfano and Collmer 2004; Lindgren 1997). The results presented in Figure 3A imply that under QS conditions (i.e., initial inoculum up to 10⁶ CFU/ml) the QS system regulates the bacterial growth rate in planta, most likely through control of the Hrp regulon. This result is in accordance with the demonstration that disruption of the QS genes suppressed expression of the Hrp regulatory genes (Fig. 1) and gall development (Chalupowicz et al. 2008). Interestingly, the colonization by the bacteria in the xylem and adjacent parenchymal cells in gypsophila cuttings appeared in aggregates, which might indicate formation of a biofilm (Koutsoudis et al. 2006). In contrast to the growth of plant-pathogenic bacteria within the plant, which is strongly affected by the action of Hrp genes, bacterial colonization of leaf surfaces employs a range of strategies for growth and survival (Lindow and Brandl 2003). These include, for example, locally increasing the nutrient concentration (Brandl and Lindow 1998), and exopolysaccharide (EPS) production and motility (Quinones et al. 2005). Moreover, bacterial cells form aggregates that endow them with much-enhanced ability to survive periodic desiccation stress and to increase their epiphytic fitness (Monier and Lindow 2003). These colonization strategies are generally controlled by QS and additional global regulatory systems (Quinones et al. 2005; von Bodman et al. 2003). The contribution of *hrp/hrc* genes to epiphytic fitness of *Pseudomonas syringae* pv. *syringae* has been reported previously (Hirano et al. 1999). The results presented in Figure 4 show that the *Pantoea agglomerans* pv.

gypsophila mutant disrupted in *hrpL* exhibited a similar reduction in epiphytic fitness to that of *P. agglomerans* pv. *gypsophila* disrupted in *pagR*, whereas the reduction by *pagI* mutant was lower. These results suggest that, in addition to the above-mentioned factors, *hrp/hrc* genes of *P. agglomerans* pv. *gypsophila* contribute to epiphytic fitness of pathogenic bacteria, presumably by internal growth in tissues under the leaf surface.

A most exciting finding of the present study is the regulatory effects of the biosynthetic pathways for IAA and CK on the Hrp regulon and QS system (Fig. 5). Both mutants, in *iaaH* and in *etz*, that disrupted the IAM pathways for biosynthesis of IAA and CK, respectively, caused substantial reductions in the expression of the Hrp regulatory genes *hrpL* and *hrpS* and the QS genes *pagI* and *pagR* compared with the levels in the wild-type control. In contrast, a mutant in *ipdC* which disrupted the IPyA pathway for IAA caused a substantial increase in the transcription of all these genes (Fig. 5). Auxins and CK are both major classes of plant growth regulators known to be involved in a variety of processes at the whole-plant and cellular levels (Buchanan et al. 2000). The role of IAA as a signaling molecule in bacteria has also been previously demonstrated (Spaepen et al. 2007). It has already been reported that, similarly to our results, disruption of the IAM pathway in *E. chrysanthemi* caused reduced expression levels of genes encoding for Hrp and T3SS effectors such as *hrpL*, *hrpA*, and *dspE*, and also of genes encoding endopectate lyases (Yang et al. 2007). However, to the best of our knowledge, there have been no reports on the involvement of CK in regulation of HSL-mediated QS and Hrp regulatory genes. Moreover, the complementation of the *etz* mutant by addition of BA strongly suggests that CK may act as signaling molecules in bacteria.

Because the disrupted IAM and IPyA pathways reduced and induced, respectively, expression of the Hrp regulon and QS genes (Fig. 5), it appears that the end product of the IAM pathway may act as a positive signal and that of the IPyA pathway as a negative signal for the Hrp regulon and QS system. The contrasting signaling by the product of these two pathways is difficult to reconcile and is not understood because they both suppose to synthesize IAA. However, it is noteworthy that whereas IAA is the only end product of the IAM hydrolase (*iaaH*), the IPyA decarboxylase (*ipdC*) forms indole-3-acetaldehyde, which can be either oxidized to IAA or reduced to indole-3-ethanol, which might be further conjugated or metabolized (Manulis et al. 1991). Whether a different end product from IAA is formed, which could account for the observed difference in signaling of the two pathways, can only be speculated; further studies are required to resolve the above-mentioned questions. It is noteworthy that no effect of *ipdC* on the QS system and the Hrp regulon has been reported previously. Interestingly, IAA and some synthetic auxins have been reported as signals that upregulate *ipdC* expression in *Azospirillum brasilense* (Broek et al. 1999, 2005).

The interactions among IAA and CK, the QS system and the Hrp regulatory genes of *824-1*, and their effects on gall formation and epiphytic fitness as presented in this study are summarized in Figure 6. It should be pointed out that the QS is one component of a global regulatory system that includes additional genes such as *RsmA*, *rsmB*, *GacA*, and *GacS* (Brencic and Winans 2005; Lapouge et al. 2008) that have not been addressed in the present study. However, the finding that the *pagI* and *pagR* mutants do not exert any effect on transcription of *rsmA* in *P. agglomerans* pv. *gypsophila* could imply that, unlike *Pectobacterium carotovorum* subsp. *carotovorum* (Mole et al. 2007) and similarly to *Pseudomonas aeruginosa* (Burrowes et al. 2006; Pessi et al. 2001), the QS system does not exert its positive control of the Hrp regulatory genes through *RsmA*.

Regardless of the mechanisms involved in signaling of the QS and Hrp regulon by IAA and CK, the present results suggest that production of these phytohormones by *Pantoea agglomerans* pv. *gypsophilae* may contribute to optimal expression of the T3SS secretion system and of type III effectors by the pathogen, in addition to their contribution to gall induction.

MATERIALS AND METHODS

Bacterial strains and growth conditions.

The bacterial strains used in the present study are listed in Table 2. Wild-type and mutant strains of *P. agglomerans* pv. *gypsophilae* were grown in Luria-Bertani (LB) broth or agar at 28°C, whereas *Escherichia coli* strains were cultured on the same medium at 37°C. Antibiotics were used at the following concentrations: kanamycin at 50 µg/ml, spectinomycin at 50 µg/ml, and rifampicin at 150 µg/ml. All strains were maintained as glycerol stocks at -80°C (Sambrook et al. 1989).

DNA manipulation.

Isolation of plasmid DNA from *E. coli* and other DNA manipulations were performed according to standard procedures (Ausubel et al. 1995; Sambrook et al. 1989) or as recommended by the supplier. Synthetic oligonucleotides (Table 1) were synthesized according to the manufacturer's specifications by Sigma-Aldrich (Rehovot, Israel). The *E. coli* and *P. agglomerans* strains were transformed by electroporation with a Gene Pulser apparatus (Bio-Rad Laboratories, Hercules, CA, U.S.A.) according to manufacturer's instructions.

To clone the *rsmA* gene, a fosmid library of *824-1* was screened via colony hybridization with a labeled probe from the plasmid pAKC891 containing the *rsmA* gene from *P. agglomerans* pv. *gypsophilae* PD713 (Ma et al. 2001). Positive clones were then digested with *SacI* and analyzed by Southern blot hybridization, using the insert of pAKC891 as a probe. The *rsmA* from *P. agglomerans* pv. *gypsophilae* was localized in a 3-kb DNA fragment, which was cloned into the *SacI* site of pCL1920 to produce pCL*rsmA*. The cloned *rsmA* with its promoter was sequenced and submitted to the GenBank as accession no. FJ465141.

Plant inoculation.

Cuttings of *Gypsophila paniculata* var. Golan (Danziger Ltd., Bet Dagan, Israel) were inoculated according to Lichter and associates (1995). After removal of an approximately 2-mm section from the bottom of the stem, the cuttings (10 for each treatment) were inoculated by dipping into a bacterial suspension of 10⁶ cells/ml for 16 h at 25°C prior to RNA extraction, or were placed in trays filled with vermiculite for visualization by confocal microscopy 6 days after inoculation. The greenhouse temperature was maintained at 22 to 25°C and high humidity was maintained by computer-controlled mist sprinklers (every 20 min for 10 s). Bacterial colonization in *gypsophila* cuttings was carried out as follows: at various time intervals, five cuttings per treatment were collected and a 10-mm segment was excised from the bottom of each cutting and washed thoroughly with sterile distilled water. Each stem segment was then macerated in 1 ml of 10 mM phosphate buffer (pH 7.0) and serial 10-fold dilutions were plated on LB agar medium containing appropriate antibiotics. The CFU per gram fresh weight was counted and the average of three independent experiments was calculated. Assays with addition of exogenous C₄-HSL (Sigma-Aldrich), IAA (Sigma-Aldrich), and BA (Sigma-Aldrich) were performed by dipping *gypsophila* cuttings into a bacterial suspension of 10⁶ cells/ml in the presence of 2, 20, or 200 µM C₄-HSL, 100 µM IAA, or 20 µM BA.

Epiphytic fitness test.

Determination of epiphytic fitness was carried out essentially as in previous studies (Lindow 1993; Quinones et al. 2005). Common bean seedlings (*Phaseolus vulgaris* cv. Sigma) were grown for 2 weeks on a greenhouse bench and the plants were inoculated by immersing the leaves in 1 liter of bacterial suspension in a sterile container. The bacterial suspension was prepared from cells grown on LB selective agar plates incubated overnight at 28°C, and were suspended in sterile 10 mM potassium phosphate buffer (pH 7.0) that was diluted with 1 mM potassium phosphate buffer to a final concentration of 10⁶ cells/ml. Pots, each containing 10 plants, were immersed in a suspension of a given bacterial strain for 1 min. Immediately after inoculation, the pots were covered with plastic bags and held for 48 h at 26 to 28°C, so that high relative humidity was maintained. Ten individual leaves from treated plants were harvested at various times and placed individually in large test tubes containing 20 ml of washing buffer (100 mM potassium phosphate buffer, pH 7.0, containing 0.1% bacto-peptone). The test tubes were then sonicated in an ultrasonic bath for 7 min and vortexed briefly for 15 s, and 10-fold serial dilutions were plated onto LB selective agar plates. The number of colonies was calculated and the epiphytic bacterial sizes were log-transformed before calculating mean population sizes for a given strain.

Expression analysis by qRT-PCR.

For RNA isolation, wild-type and mutant strains of *Pantoea agglomerans* pv. *gypsophilae* were grown on minimal A medium plates for 16 h at 28°C. The cells were then scraped from the plates, washed once with sterile water, and suspended in sterile water to an optical density at 600 nm of 0.5. RNA was extracted from inoculated cuttings as described above for plant inoculation. MasterPure RNA purification kit (Epicentre Biotechnologies, Madison, WI, U.S.A.) was used to isolate total RNA. Reverse transcription was carried out from 1 µg of total RNA pretreated with the Turbo DNA-free (Ambion Inc., Austin, TX, U.S.A.) using the Verso cDNA kit (Thermo Scientific, Epsom, U.K.) with random hexamer primers. The resulting cDNAs were subjected to PCR amplification with SYBR green PCR master mix (Applied Biosystems, Warrington, U.K.). The specific primers used in this study were designed by using the AlleleID program (Premier BioSoft International, Palo Alto, CA, U.S.A.) according to the sequence of the corresponding genes. All primers were stored at a concentration of 10 µM, and 0.3 µl of each forward and reverse primer were used per 15-µl reaction. The primer pairs as well as the degenerate primers for the internal normalization control gene, *gyrB*, are presented in Table 1. The expected size of the amplicons was approximately 85 bp. Real-time detection was performed with a Rotor-Gene 3000 device (Corbett Research, Sydney, Australia) and the results were analyzed with the Rotor-Gene 6 software. The endogenous control was the *gyrB* gene and the calibrator was the wild-type *824-1*. A mixture of all cDNAs used in the various treatments was employed as a template for calibration curves designed for each pair of primers. Relative quantification was based on cycle threshold (Ct) ($\Delta\Delta C_T$). The ΔC_T value of the sample was determined by subtracting the average Ct value of the target gene from the average Ct value of the endogenous control gene. The $\Delta\Delta C_T$ value was then calculated by subtracting the ΔC_T value of the sample from that of the wild type from the same batch. Each experiment was conducted three times and statistical analysis was performed with the Excel software (Microsoft, Seattle).

Confocal images.

Images of gall sections inoculated with fluorescent bacteria (*P. agglomerans* pv. *gypsophilae* strains harboring the plasmid

pWM1029) were examined under a confocal laser-scanning microscope (model CLSM FluoView 500a; Olympus, Tokyo).

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