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The *hrp* pathogenicity island of *Pseudomonas syringae* pv. *tomato* DC3000 is induced by plant phenolic acids

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Plants produce a wide array of antimicrobial compounds, such as phenolic compounds, to combat microbial pathogens. The *hrp* PAI is one of the major virulence factors in the plant pathogen, *Pseudomonas syringae.* A major role of *hrp* PAI is to disable the plant defense system during bacterial invasion. We examined the influence of phenolic compounds on *hrp* PAI gene expression at low and high concentrations. There was approximately 2.5 times more *hrpA* and *hrpZ* mRNA in PtoDC3000 that was grown in minimal media (MM) supplemented with 10 μM of ortho-coumaric acid than in PtoDC3000 grown in MM alone. On the other hand, a significantly lower amount of *hrpA* mRNA was observed in bacteria grown in MM supplemented with a high concentration of phenolic compounds. To determine the regulation pathway for *hrp* PAI gene expression, we performed qRT-PCR using *gacS*, *gacA*, and *hrpS* deletion mutants.

Keywords: plant phenolic compounds, *Pseudomonas syringae*, plant-pathogen interaction, *hrp* PAI, qRT-PCR

Introduction

Pseudomonas syringae pv. *tomato* DC3000 (PtoDC3000) causes bacterial speck of tomato, an economically significant plant disease worldwide that is representative of numerous bacterial plant diseases for which effective controls are still needed (Buell *et al.*, 2003). PtoDC3000 causes bacterial specks on *Lycopersicon esculentum* (tomato) and *Arabidopsis thaliana* and induces hypersensitive responses (HRs), rapid defense-associated programmed death of those plant cells in contact with the pathogen, in many other non-host plant species (Alfano *et al.*, 2000). The ability of *P. syringae* to cause disease in its hosts and elicit HR in non-host plants is controlled by the *hrp* (hypersensitive response and pathogenicity) / *hrc* (hypersensitive response and conserved) genes located in a pathogenicity island (PAI), also known as *hrp* PAI. The virulence of *P. syringae* is also controlled by the *avr* (avirulence) and *hop* (Hrp-dependent outer protein) genes that encode effector proteins that are injected into plant cells (Alfano *et al.*, 2000). *Hrp* PAI is conserved among many gram-negative plant pathogenic bacteria and has been sequenced in *Pseudomonas*, *Erwinia*, *Xanthomonas*, and *Ralstonia* (Collmer *et al.*, 2000). The expression of PAI in most pathogenic bacteria is environmentally regulated, as is the *hrp* PAI in *P. syringae*. The *hrp* PAI is expressed at a very low level in nutrient-rich media, but is induced in plant apoplasts or when the pathogen is in close contact with host cells. Expression of *hrp* PAI is also induced in *hrp*-inducing minimal media (MM) that mimics plant apoplastic conditions (Galan and Collmer, 1999).

In *P. syringae*, *hrp* PAI is regulated by HrpL, an alternative sigma factor (Lindgren, 1997). The HrpL proteins of plant pathogenic bacteria have highly conserved amino acid sequences. Additionally, all of these HrpL proteins recognize a consensus sequence (GGAACC-N15/16-CCACNNA) called the "*hrp* box" in the promoters of the *hrp* operons and type III effectors (Fouts *et al.*, 2002). Induction of *hrpL* in *P. syringae* requires HrpS and HrpR proteins. The *hrpR* and *hrpS* genes are organized in an operon controlled by a promoter that is upstream of *hrpR*. The two proteins are highly homologous in sequence and form a heterodimer. The dimerization is believed to be crucial for the transcriptional activation of *hrpL* (Xiao *et al.*, 1994; Grimm *et al.*, 1995; Hutcheson *et al.*, 2001). *hrpS* alone is capable of activating the *hrpL* gene in *P. syringae* but only at a very low level. Maximal induction of *hrpL* requires both *hrpR* and *hrpS* (Bretz *et al.*, 2002). Both HrpR and HrpS contain an enhancer-binding domain and a motif that interacts with the σ54-RNA polymerase holoenzyme. Transcription of *hrpL* is consistently under the control of a σ54-dependent promoter in an alternate σ factor RpoN-dependent manner (Hendrickson *et al.*, 2000).

The GacS/GacA two-component system plays a role in regulating *hrpRS* expression in *P. syringae* (Chatterjee *et al.*, 2003). GacS is a sensory histidine kinase, whereas GacA is a cognate response regulator. The GacS/GacA system is found in numerous bacteria, and this system serves as a master regulator of many bacterial phenotypes, including pathogenicity, production of toxins and antibiotics, quorum sensing, motility, production of exopolysaccharides, biofilm formation, and stress tolerance (Heeb and Haas, 2001). In PtoDC3000, the *gacA* mutation significantly attenuates the transcription of *hrpRS*, *rpoN*, and *hrpL* (Chatterjee *et al.*, 2003). Because *hrpL* is regulated by *hrpRS* and *rpoN*, the reduced *hrpL* expression in the *gacA* mutant likely results from the reduced expression of *hrpRS* and *rpoN*. The signal perceived

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by GacS and the mechanism by which GacA regulates the expression of *hrpRS* and *rpoN* are unknown. In addition to the GacS/GacA system, HrpA, the major component of the type III pilus, also affects the transcription of the *hrpRS* operon (Wei *et al.*, 2000; MacLean and Studholme, 2010).

Plants have evolved a number of inducible defense mechanisms against pathogen attack. Spread of resistance throughout plant tissues is referred to as systemic acquired resistance (SAR) (Durrant and Dong, 2004). SAR can be activated in many plant species by pathogens that cause necrosis, either as part of an HR or as a symptom of disease. The resistance conferred is long-lasting, sometimes for the lifetime of the plant, and effective against a broad spectrum of pathogens, including viruses, bacteria, fungi, and oomycetes. Molecularly, SAR requires the signal molecule salicylic acid (SA) and is associated with the accumulation of pathogenesis-related proteins that are thought to contribute to resistance (Ryals *et al.*, 1996; Sticher *et al.*, 1997). SA for SAR is synthesized via the shikimate-phenylpropanoid pathway. Phenylalanine is deaminated by phenylalanine ammonia lyase. Trans-cinnamic acid (TCA) is converted to ortho-coumaric acid (OCA) through ortho-hydroxylation. Para-coumaric acid (PCA) is an isomer of OCA. Finally, salicylic acid (SA) is produced by β-oxidation of OCA (Lee *et al.*, 1995).

Because a major role of phytopathogen *hrp* PAI is to neutralize the host defense system during bacterial invasion, it is possible that PtoDC3000 induces the expression of *hrp* PAI genes by recognizing certain phenolic compounds in plants (Ravirala *et al.*, 2007; Yang *et al.*, 2008). To identify plant compounds that induce the expression of *hrp* PAI genes, we examined the effect of OCA, PCA, TCA, and SA on *hrp* expression and on the *hrp* PAI regulatory pathway in PtoDC3000.

Materials and Methods

Bacterial strains, plasmids, media, and chemicals

All bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* and PtoDC3000 strains were grown at 37°C and 28°C, respectively. For induction of *hrp*/*hrc* genes, bacteria were grown overnight in Luria-Bertani medium. Cells were washed twice in 10 mM $MgCl_2$ and grown in *hrp*-inducing minimal medium (MM; 50 mM potassium phosphate, 7.6 mM $(NH_4)_2SO_4$, 1.7 mM $MgCl_2$, 1.7 mM NaCl, and 10 mM mannitol and fructose) that was previously adjusted to an optical density of 0.5–0.6 at 600 nm. Antibiotics (μg/ml) used were: ampicillin (Ap), 100 and kanamycin (Km), 20. OCA, PCA, TCA, and SA were purchased from Sigma-Aldrich.

Construction of mutants via *in vivo* marker exchange

DNA isolation and routine manipulations were performed using the standard methods previously described by Sambrook Russell (2001) or provided by the manufacturers of the reagents. To construct deletion mutants, the regions flanking each of the PtoDC3000 genes of interest were cloned into pGEM-T Easy or T-blunt (Table 1). Specific primer sets (Table 2) were designed based on the PtoDC3000 genome

Table 1. **Bacterial strains and plasmids used in this study**

Strain or plasmid	Relevant characteristics[a]	Source or reference
Strains		
P. syringae pv. *tomato* DC3000 (PtoDC3000)	Plant pathogen, wild-type strain, Rif^r	ATCC BAA-871
BL712	PtoDC3000 Δ*gacS* (0.5 kb deletion)	This study
BL723	Tc^r, BL712 containing pBL315	This study
BL710	PtoDC3000 Δ*gacA* (0.5 kb deletion)	This study
BL724	Tc^r, BL710 containing pBL316	This study
BL442	PtoDC3000 Δ*hrpS* (0.8 kb deletion)	This study
BL725	Tc^r, BL442 containing pBL317	This study
E. coli		
Top10	Transformation host for cloning vector	Invitrogen
DH5α	Transformation host for cloning vector	TaKaRa
S17-1 λ_{pir}	S17-1 derivative, RK2 *tra* regulon, *pir*, host for *pir* dependent plasmids	Simon *et al.* (1983)
Plasmids		
pGEM-T Easy	Ap^r, Cloning vector	Promega
T-blunt	Ap^r, Km^r, Cloning vector	Solgent
pLO1	Km^r, SacB, Suicide vector	Lenz *et al.* (1994)
pRK415	Tc^r, Broad-host-range cloning vector	Cha *et al.* (2008)
pBL146	Ap^r, Derivative of pGEM-T Easy containing the *gacS* deletion mutation	This study
pBL164	Km^r, Derivative of pLO1 containing the *gacS* deletion mutation	This study
pBL315	Tc^r, Derivative of pRK415 containing the wild-type *gacS* gene	This study
pBL305	Ap^r, Derivative of pGEM-T Easy containing the *gacA* deletion mutation	This study
pBL307	Km^r, Derivative of pLO1 containing the *gacA* deletion mutation	This study
pBL316	Tc^r, Derivative of pRK415 containing the wild-type *gacA* gene	This study
pBL148	Ap^r, Derivative of pGEM-T Easy containing the *hrpS* deletion mutation	This study
pBL165	Km^r, Derivative of pLO1 containing the *hrpS* deletion mutation	This study
pBL317	Tc^r, Derivative of pRK415 containing the wild-type *hrpS* gene	This study

[a] Ap^r, ampicillin resistance; Km^r, kanamycin resistance, Rif^r, rifampicin resistance, Tc^r, tetracycline resistance

Table 2. Primers used in this study

Name	Oligonucleotide sequence (5′→3′)*, Forward	Oligonucleotide sequence (5′→3′)*, Reverse
Oligonucleotides used for construction of gene deletion mutations and complementation assays		
gacS-L	(*Xba*I) TATCTAGAGACGAAACGCCACAGAAAAT	(*Bam*HI) TAGGATCCGTGAAATAGCCACCCAGCAT
gacS-R	(*Bam*HI) TAGGATCCAAACCCTGCTCAAACAGGAC	(*Sac*I) TAGAGCTCGGGCTGATCGGTAGTTGAGA
gacA-L	(*Xba*I) TCTAGATTCCGGGCATAGTTCAAAAC	(*Bam*HI) CATGGATCCCGAACAAGGTCATGGTCATC
gacA-R	(*Bam*HI) CATGGATCCCGAACAAGGTCATGGTCATC	(*Eco*RI) GAATTCACACCTCAGGCTCCACAAGA
hrpS-L	(*Xba*I) TATCTAGATATCGGTAGGGTCGTCTTGG	(*Bam*HI) TAGGATCCTCCTCGTCCAGATCATCCTC
hrpS-R	(*Bam*HI) TAGGATCCTACGCTCTATCGACGCATCA	(*Sac*I) TAGAGCTCCATGAACTGGGCGACTTTTT
Oligonucleotides used for qRT-PCR		
qRT-16S	CAGCTCGTGTCGTGAGATGT	CACCGGCAGTCTCCTTAGAG
qRT-*gacA*	TGTGAAGAAGACCCGTTTCC	AATCTGCGGGCTGATGTAAC
qRT-*hrpS*	CAGTTTCGTCGCGATCTGTA	AAACGTGTGAACAGGGGAAC
qRT-*rpoN*	ACATCGAACAAAAGCCCAAC	GTATCGACCGGCAGTTCATT
qRT-*hrpL*	CTCCAGTGCGTGTTTCTTGA	AGCTTTCCTGATACGGCTGA
qRT-*hrpA*	GGTTGATGCACAAGCCAAG	ACGTCCATGGTCTGCTTCTT
qRT-*hrpZ*	ACAAGGCCCAGTTCCCTACT	TTGACCAATGACGTCGAGAG

* Underlines indicate the restriction enzyme sites for the enzymes indicated in the primer names

sequence (GenBank ID: AE016853.1). We used the previously described *in vivo* marker exchange to replace the chromosomal copies of *gacS, gacA, hrpS* in *Pto*DC3000 with the altered pBL164, pBL307, and pBL165 copies without leaving any remaining integrated plasmid sequences (Cha *et al.*, 2008, 2012; Yang *et al.*, 2011). All final mutant strains and clones derived from PCR fragments were verified by PCR amplification and DNA sequencing.

To complement the deletion mutants (BL712, BL710, and BL442), each of the respective genes was PCR-amplified from genomic DNA using the primer sets listed in Table 2 and cloned into the broad-host plasmid pRK415. The constructs were then introduced by mating into the mutants. Complementation tests were conducted as phenotype experiments.

RNA isolation and quantitative real-time RT-PCR

Total RNA isolation and quantitative real-time RT-PCR (qRT-PCR) were conducted as described previously (Cha *et al.*, 2012). Briefly, Total RNA from the bacteria was isolated by using the SV total RNA isolation system (Promega). Genomic DNA was eliminated by treatment with gDNA Eraser (TaKaRa) for 2 min at 42°C. RNA from cells induced *in vitro* was quantified using a nanodrop spectrophotometer. cDNA was synthesized using a PrimeScript™ RT reagent Kit (TaKaRa) with random hexamers and 0.5 μg of RNA. qRT-PCR was carried out using SYBR Primix Ex *Taq*™ (TaKaRa) with a StepOne Real-Time PCR System (Applied Biosystems), as previously described (Cha *et al.*, 2012). Specific primers (Table 2) that amplify the mean products of approximately 200 bp were used to detect the 16S rRNA, *gacA*, *hrpS*, *rpoN*, *hrpL*, *hrpA*, and *hrpZ* transcripts. The standard curves used to quantify relative cDNA levels were made from 5-fold serial dilution of the genes of interest and were included in every assay. The following PCR amplification program was used: 30 min at 95°C and 40 cycles of 5 sec at 95°C and 34 sec at 60°C. All data were analyzed using StepOne analysis software version 2.1 (Applied Biosystems). The fold induction of mRNA was determined from the threshold cycle values (Ct), which were normalized first for 16S rRNA gene expression (endogenous control; evidences invariant expre-

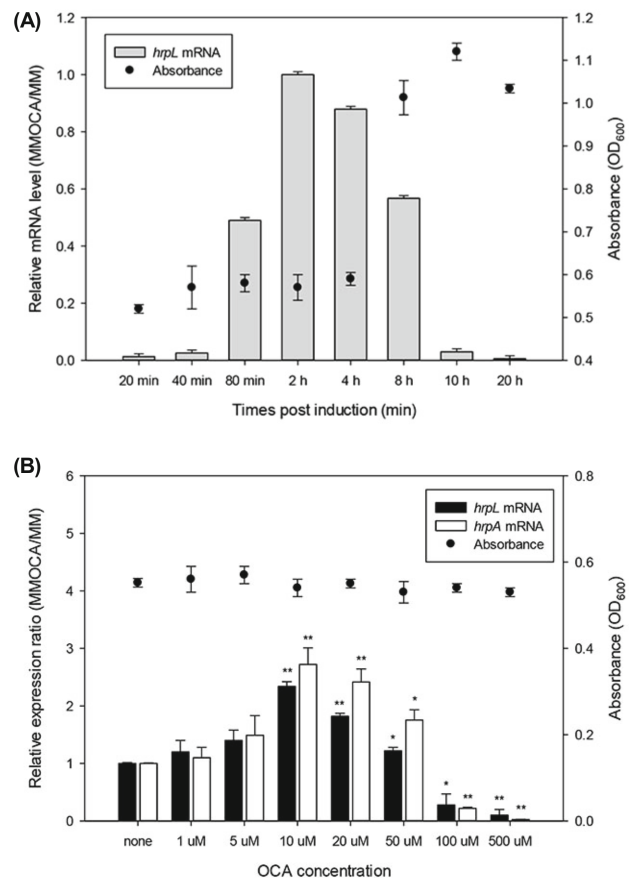

Fig. 1. The relative mRNA levels of *hrp* genes of *Pseudomonas syringae* pv. *tomato* DC3000 in minimal medium (MM) supplemented with ortho-coumaric acid (OCA) compared with those in MM without OCA. (A) The relative mRNA levels of *hrpL* at different induction times. PtoDC-3000 was grown for 20 min, 40 min, 80 min, 2 h, 4 h, 8 h, 10 h, and 20 h in MM. (B) The relative mRNA level of *hrpL* and *hrpA* at different OCA concentrations. PtoDC3000 was grown for 2 h in MM. The growth of PtoDC3000 in MM supplemented with different concentrations of OCA was recorded. The data are expressed as the average of three replicates ± SD. Asterisks indicate a significant difference from the none OCA (*P < 0.05, **P < 0.01).

ssion under different growth conditions) and then for the Ct of the wild-type strain. The relative expression ratios were calculated using a mathematical model, which included an efficiency correction for the RT-PCR efficiency of the individual transcripts (Pfaffl, 2001). The data are expressed as the average of triplicate samples.

Statistical analysis

All experiments were performed at least in triplicate and all data are presented as mean ± the standard deviation (SD). Significant differences among the groups were determined using the unpaired two-tailed Student's *t*-test calculated in SigmaPlot for Windows version 12.5. A value of $P<0.05$ was accepted as an indication of statistical significance.

Results and Discussion

Relative *hrpL* mRNA expression at different induction times

To determine the appropriate induction time of PtoDC3000 *hrp* PAI genes, the mRNA level of PtoDC3000 was examined at various induction times. We selected an alternative sigma factor *hrpL* that is required for the expression of genes encoding effectors and structural components, as well as type III secretion system (TTSS), to represent the *hrp* PAI genes in our analyses. The level of *hrpL* mRNA increased in bacteria 2 h after transfer to MM. Peak expression of *hrpL* mRNA occurred 2 h after induction and mRNA levels decreased after that time (Fig. 1A). We therefore examined the mRNA levels of *hrp* PAI genes in PtoDC3000 that was grown in MM and MM supplemented with phenolic compounds 2 h after induction.

Change of *hrpL* mRNA expression level after exposure to OCA

We used qRT-PCR to examine the effects of OCA on the expression of *hrp* PAI genes. The expression of *hrpL* and *hrpA* (encoding a structural protein of the TTSS pilus) were examined in bacteria grown in MM and MM supplemented with OCA at concentrations of 1, 5, 10, 20, 50, 100, and 500 μM. More *hrpL* and *hrpA* mRNA were observed in PtoDC-3000 grown in MM supplemented with low concentrations of OCA (10 μM, 20 μM, and 50 μM) than in PtoDC3000 grown in MM alone (Fig. 1B). However, PtoDC3000 supplemented with high concentrations of OCA (100 μM and 500 μM) contained less *hrpL* and *hrpA* mRNA than PtoDC-3000 grown in MM alone. No obvious inhibition of bacterial growth was observed when OCA was added to MM supplemented with up to 500 μM. Therefore, the examinations of the influence of phenolic compounds on TTSS gene expression are divided into two groups; low concentration and high concentration.

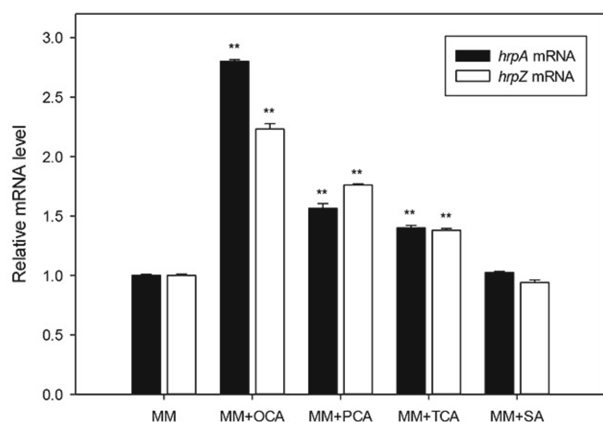

Fig. 2. The relative mRNA levels of *hrpA* and *hrpZ* of PtoDC3000 in minimal medium (MM) and MM supplemented with ortho-coumaric acid (OCA), para-coumaric acid (PCA), trans-cinnamic acid (TCA), and salicylic acid (SA) at a concentration of 10 μM. PtoDC3000 was grown for 2 h in MM supplemented with phenolic compounds. The data are expressed as the average of three replicates ± SD. Asterisks indicate a significant difference from the mock control (*$P < 0.05$, **$P < 0.01$).

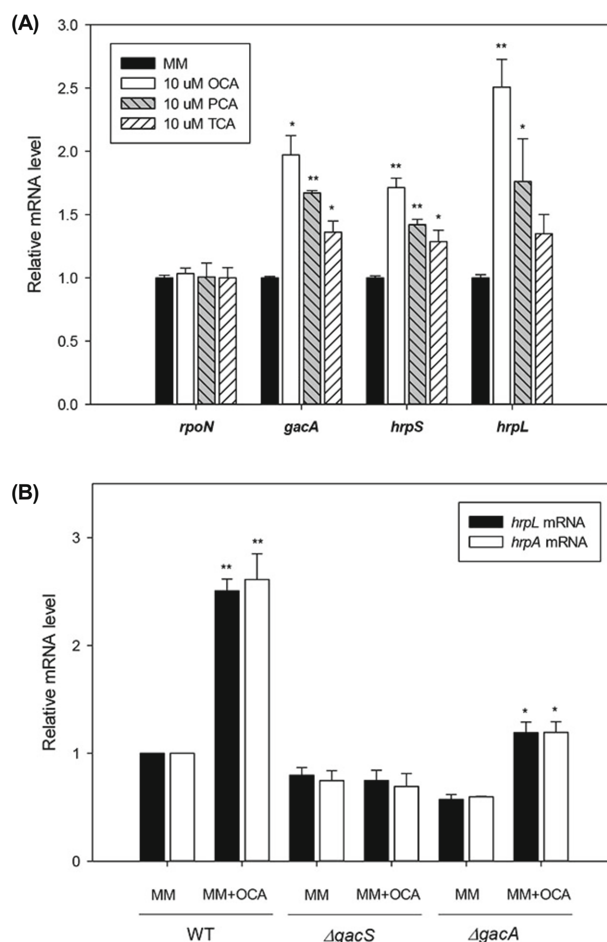

Fig. 3. (A) The relative mRNA levels of TTSS regulator genes of PtoDC-3000 in minimal medium (MM) and MM supplemented with ortho-coumaric acid (OCA), para-coumaric acid (PCA), and trans-cinnamic acid (TCA) at a concentration of 10 μM. (B) The relative mRNA levels of *hrpL* and *hrpA* of WT, Δ*gacS* mutant, and Δ*gacA* mutant in MM and MM supplemented with OCA at a concentration of 10 μM. PtoDC3000 was grown for 2 h in MM supplemented with phenolic compounds. The data are expressed as the average of three replicates ± SD. Asterisks indicate a significant difference from the mock control (*$P < 0.05$, **$P < 0.01$).

Effect of phenolic compounds at low concentration

Because low concentration OCA induced the expression of *hrpA*, we further investigated the effect of other phenolic compounds on the expression of an additional TTSS gene, *hrpZ* (TTSS harpin gene). More *hrpA* and *hrpZ* mRNA was observed in PtoDC3000 grown in MM supplemented with OCA, PCA or TCA at concentrations of 10 μM than in PtoDC3000 grown in MM alone (Fig. 2). *hrpA* and *hrpZ* mRNA did not increase after exposure of PtoDC3000 to SA. The concentration of these phenolic compounds in healthy leaves is reported to be approximately 0.5 μM, and the phenolic compounds accumulate to 10 μM after exposure to the phytopathogen (Montesano *et al.*, 2005). Approximately 2.5-fold higher *hrpA* and *hrpZ* mRNA levels was observed in PtoDC3000 grown in MM supplemented with 10 μM of OCA (the level of OCA in plants after exposure to the phytopathogen) in comparison to bacterial cells grown in MM alone (Fig. 2). This result indicates that a higher expression of TTSS may be induced in the bacterial cells for a defensive response against the plant responses because of the accumulated level of the phenolic compound in hosts caused by bacterial infection. Therefore, physiologically relevant concentrations of phenolic acid can induce TTSS gene expression.

Because we observed an induction of *hrpA* and *hrpZ* expression in PtoDC3000, we investigated the regulatory mechanism of the TTSS pathway that is affected by these compounds. mRNA levels of PtoDC3000 *gacA*, *hrpS*, *rpoN*, and *hrpL* were measured between bacterial cells grown in MM and MM supplemented with 10 μM OCA, PCA, or TCA. PtoDC3000 cultures grown in MM with 10 μM OCA, PCA, or TCA produced more *gacA*, *hrpS*, and *hrpL* mRNA than those grown in MM alone (Fig. 3A). However, similar *rpoN* mRNA levels were found between bacterial cells grown in MM and those grown in MM supplemented with phenolic compounds. This result suggests that *hrpA* and *hrpZ* are induced by the GacA-HrpRS-HrpL pathway and not the GacA-RpoN-HrpL pathway.

We also investigated the effect of low concentration phenolic compounds on TTSS regulatory pathway. To investigate whether OCA induces TTSS through the GacS/A pathway, we compared the mRNA levels of *hrpL* and *hrpA* in the wild-type bacterium, *ΔgacS* mutant, and *ΔgacA* mutant

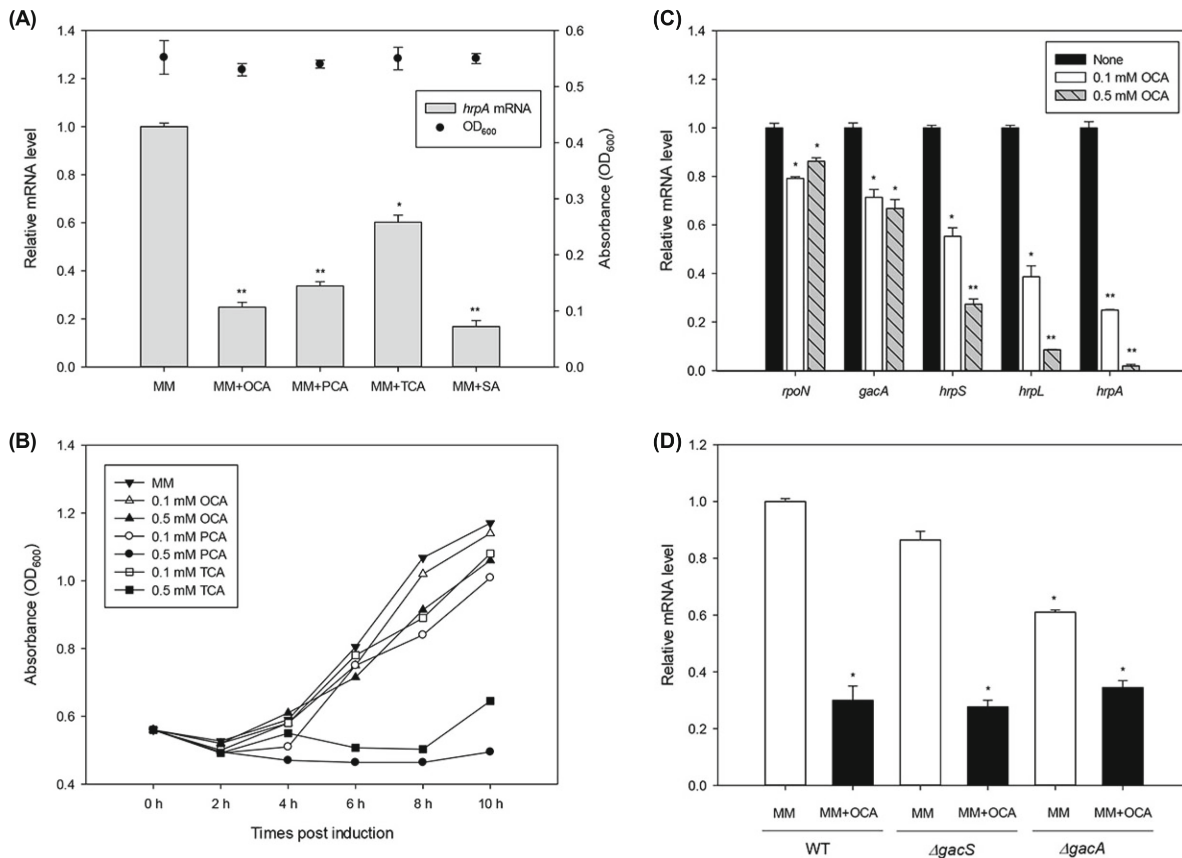

Fig. 4. **(A) The relative mRNA levels of *hrpA* of PtoDC3000 in minimal medium (MM) and MM supplemented with ortho-coumaric acid (OCA), para-coumaric acid (PCA), or trans-cinnamic acid (TCA) at a concentration of 100 μM.** The growth of PtoDC3000 in MM and MM supplemented with OCA, PCA, TCA, or SA was recorded. (B) The growth of PtoDC3000 in MM and MM supplemented with OCA, PCA ,or TCA at a concentration of 100 or 500 μM. PtoDC3000 was cultured in MM containing 100 or 500 μM of OCA, PCA, or TCA at 28°C with shaking. During culture, the optical density at 600 nm was measured at the time points indicated in the graph. (C) The relative mRNA levels of TTSS regulator genes of PtoDC3000 in MM and MM supplemented with 100 and 500 μM OCA. (D) The relative mRNA levels of *hrpA* of WT, *ΔgacS* mutant, and *ΔgacA* mutant in MM and MM supplemented with OCA at a concentration of 100 μM. PtoDC3000, *ΔgacS* mutant, and *ΔgacA* mutant were grown for 2 h in MM supplemented with phenolic compounds. The data are expressed as the average of three replicates ± SD. Asterisks indicate a significant difference from the mock control (*$P < 0.05$, **$P < 0.01$).

grown in MM with or without OCA. The wild-type and *ΔgacA* mutant grown in MM supplemented with OCA had higher mRNA levels than those grown in MM alone. However, similar *hrpL* and *hrpA* mRNA levels were observed in *ΔgacS* mutants grown in MM and those grown in MM supplemented with OCA (Fig. 3B). These results suggest that OCA induces the TTSS gene expression through recognizing GacS and increasing the expression of *gacA* mRNA. Because little is known about the nature of the signals perceived by GacS or the regulation of GacA, the effect of plant phenolic acids on PtoDC3000 TTSS system discovered in this study gives an important meaning in pathogenesis of this strain. However, the increased expression of TTSS genes induced by low concentration OCA in PtoDC3000 is not necessarily caused by GacA. With the complexity of the TTSS regulatory system revealed in PtoDC3000, we can't rule out the possibility of other unknown regulators affected by OCA, not GacA.

Effect of phenolic compounds at high concentration

To investigate the effect of high concentrations of phenolic compounds on TTSS gene expression, we compared the level of *hrpA* mRNA in PtoDC3000 grown in MM and that grown in MM supplemented with 100 μM OCA, PCA, TCA, or SA. A significantly lower level of *hrpA* mRNA was observed in bacteria grown in MM supplemented with a high concentration phenolic compounds than in those grown in MM alone (Fig. 4A). To determine whether this reduction of *hrpA* mRNA levels was caused by the inhibition of bacterial growth, we observed the bacterial growth in MM and MM supplemented with OCA, PCA or TCA at 2 h intervals between 0 and 10 h after induction (Fig. 4B). There was no obvious inhibition of bacterial growth in MM supplemented with ≤ 500 μM. However, there was no increase in the absorbance of PtoDC3000 grown in MM supplemented with 500 μM PCA or TCA over the 10 h observational period. These observations suggest that high concentrations of OCA reduce TTSS gene expression, while having little effect on bacterial growth.

Because we observed a reduction of the expression of *hrpA* in PtoDC3000 grown in MM supplemented with high concentrations of OCA, we investigated the regulatory mechanism through which high concentrations of phenolic compounds affected the TTSS pathway. mRNA levels of *gacA*, *hrpS*, *rpoN*, and *hrpL* of PtoDC3000 were observed between bacterial cells grown in MM with or without OCA at concentrations of 100 and 500 μM. The level of ptoDC3000 *gacA* and *hrpS* mRNA decreased in a dose-dependent manner in MM supplemented with high concentrations of OCA (Fig. 4C). However, PtoDC3000 *rpoN* mRNA level was not directly influenced by high concentrations of OCA. Finally, we observed a significantly lower amount of *hrpL* and *hrpA* mRNA in PtoDC3000 grown in MM supplemented with OCA than in PtoDC3000 grown in MM alone.

To investigate whether OCA induces TTSS through the GacS/A pathway, we compared the level of *hrpA* mRNA level in the wild-type bacterium, *ΔgacS* mutant, and *ΔgacA* mutant grown in MM and MM supplemented with 100 μM OCA (Fig. 4D). There were lower levels of *hrpA* mRNA in wild-type bacteria, *ΔgacS* mutant bacteria, and *ΔgacA* mutant bacteria grown in MM supplemented with a high concentration of OCA than in bacteria grown in MM alone. These results suggest that although exposure to high concentrations of OCA reduces the TTSS expression by the GacA-HrpRS-HrpL pathway, this regulation is not completely dependent upon activation of GacS/A.

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