Physiological and Molecular Plant Pathology 103 (2018) 137–142

Contents lists available at ScienceDirect

Physiological and Molecular Plant Pathology

journal homepage: www.elsevier.com/locate/pmpp

Characterization of response regulator GacA involved in phaseolotoxin production, hypersensitive response and cellular processes in *Pseudomonas syringae* pv. *actinidiae* A18

Lixin Zhang*, Yanru Shi, Zhiran Wu, Genjia Tan**

Department of Plant Pathology, College of Plant Protection, Anhui Agricultural University, Hefei, 230036, China

ARTICLE INFO

Keywords:
Pseudomonas syringae pv. *actinidiae*
Phaseolotoxin
GacA
Motility
HR
Biofilm

ABSTRACT

Pseudomonas syringae pv. *actinidiae* is the phytopathogen responsible for kiwifruit canker, one of the most important kiwifruit bacterial diseases worldwide. In this work, two phaseolotoxin-related genes (*gacA* and *glmS*) were cloned and identified through transposon mutagenesis in conjunction with *Escherichia coli* toxin bioassay, which resulted in completely abolishment in the production of phaseolotoxin in *P. syringae* pv. *actinidiae* A18. Further study showed that motility ability, exopolysaccharides production and biofilm formation were reduced in the *gacA*-defective mutant. Moreover, a diminished hypersensitive response in tobacco was observed with the *gacA*-defective mutant when compared to wild-type strain after infiltration. Our experimental results represented a first view that GacA might play a key role in controlling phaseolotoxin biosynthesis, hypersensitive response and cellular processes in this bacterium.

1. Introduction

The bacterium *Pseudomonas syringae* pv. *actinidiae*, the causal agent of canker in kiwifruit (*Actinidia* spp.) vines, is responsible for severe outbreaks of bacterial canker of kiwifruit currently occurring around the world including China [1,2]. The disease consists of die-back on young canes, frequently accompanied by rust red exudates from trunks and canes, and dark-brown spots with chlorotic haloes on leaves [3–5]. *P. syringae* pathogens are known to be spread long distance by seed and vegetative material [6–8]. Recent findings demonstrated that pollen, equipment and people are responsible for transmission of *P. syringae* pv. *actinidiae* [9], whilst atmosphere also could be a possible mode for remote movement of this bacterium [8]. It is crucial to rapidly and accurately diagnose *P. syringae* pv. *actinidiae* populations on infected orchards for management strategies [9,10], and thus more polymerase chain reaction (PCR) assays have been developed and validated for detecting *P. syringae* pv. *actinidiae* from cultured bacteria or symptomatic kiwifruit tissues [11–15]. However, little is known about molecular basis of this bacterium in kiwifruit plants, although the complete genomes of several strains of *P. syringae* pv. *actinidiae* have been sequenced [8] and some genes involved in the virulence process were predicted by genomic analysis [2].

Bacterial two-component systems act as critical roles in the regulation of numerous cellular processes [16,17]. In the *P. syringae* group, the GacS/GacA two-component system has proven to be necessary for quorum sensing signals, phytotoxins, antibiotics, extracellular proteases, lesion formation and disease development [18,19]. Since bacteria are often challenged by exposure to various forms of environmental stress, each specific Gac system controls a different set of phenotypes within various species or strains to enable bacteria to adapt to changing environments [19,20]. Therefore, it is important to demonstrate the roles of the Gac system and interactions with different components of the network in various bacteria [21].

Phaseolotoxin is a host-non-specific tripeptide phytotoxin that is produced by *P. syringae* pv. *phaseolicola* and *P. syringae* pv. *actinidiae* pathogens. It functions as virulence factors of the pathogens, and induces chlorosis on leaves of several plant species by reversible inhibition of ornithine carbamoyltransferase [22–24]. *P. syringae* pv. *actinidiae* A18 is a pathogenic strain isolated from canker cane samples collected from diseased kiwifruit tree (cv. Hongyang) in Anhui Province of China. Its 16 S rRNA and 16s-23s ITS sequences (GenBank Accession Nos. MF944254 and MF944255) showed that it is very similar (99% identity) to *P. syringae* pv. *actinidiae* strain ICMP9617 (GenBank accession No. CM002753).

In this work, the random Tn5 mutagenesis of the *P. syringae* pv. *actinidiae* A18 genome was combined with *Escherichia coli* toxin

* Corresponding author. Department of Plant Pathology, College of Plant Protection, Anhui Agricultural University, Hefei, 230036, China.
** Corresponding author. Department of Plant Pathology, College of Plant Protection, Anhui Agricultural University, Hefei, 230036, China.
E-mail addresses: lxzhang@ahau.edu.cn (L. Zhang), tgj63@163.com (G. Tan).

https://doi.org/10.1016/j.pmpp.2018.07.001
Received 6 May 2018; Received in revised form 4 July 2018; Accepted 5 July 2018
Available online 05 July 2018

Table 1
Bacterial strains and plasmids used in this study.

Strains and plasmids	Relevant characteristics[a]	Reference/Source
Strains		
E. coli DH5α	*F⁻ λ⁻ ϕ80dLacZΔM15Δ(lacZYA-argF)U169 recA1 endA1 hsdR17* ($r_k^-m_k^-$) *supE44 thi*-1 gyrA *relA1*	Takara, China
E. coli DH1		Takara, China
P. syringae pv. *actinidiae*		
A18	Rif^r, the pathogen of bacterial canker of kiwifruit, wild-type isolated from the diseased kiwifruit plants in China	Lab collection
A1843	Rif^r, Km^r, Tn5 mutant defective in *gacA*	This study
A18Δ*gacA*	Rif^r, Km^r,Cm^r; *gacA* deletion mutant strain	This study
A18Δ*gacA*-C	Rif^r, Km^r, Tc^r, A18Δ*gacA* complemented with pLgacA	This study
Plasmids		
pMD19-T	Amp^r, cloning vector	Takara, China
pLAFR3	Tc^r, broad host range cloning vector	[28]
pRK2013	Km^r, used as mobilizing plasmid in triparental crosses	[29]
pK18mobsacB	Km^r, Small mobilizable vector, sucrose-sensitive (*sacB*)	[30]
PMDgacA	A 834 bp fragment of *gacA* from strain A18 subcloned into pMD19-T	This study
PK18gacA	Km^r, Cm^r, deleted *gacA* fragment cloned into pK18*mobsacB*	This study
pLgacA	Tc^r, pLAFR3 derivative carrying a fragment encoding the *gacA* gene, utilize to complement	This study

[a] Amp^r, Cm^r, Km^r, Rif^r and Tc^r = ampicillin, chloromycetin, kanamycin, rifampicin, and tetracycline resistant, respectively.

bioassay [25] to screen deficient/altered phenotypes of phaseolotoxin production. We present our findings on the characterization of Tn5 mutants impaired in *gacA* and *glms* genes, which established a direct regulatory influence at the phaseolotoxin production level in the strain A18. Moreover, the roles of GacA in the regulation of motility, extracellular exoproducts, biofilm formation and hypersensitive response were investigated.

2. Materials and methods

2.1. Strains, plasmids and growth conditions

The bacterial strains and plasmids used in this work are listed in Table 1. *E. coli* strains were cultivated on LB medium (10 g/L of tryptone, 5 g/L of yeast extract, 10 g/L of NaCl, final pH of 7) at 37 °C. *P. syringae.* pv. *actinidiae* A18 and mutant derivatives were grown on LB medium, King's B agar (KBA) [26] or M9 medium [27] at 18 or 28 °C. For phaseolotoxin production, *P. syringae* pv. *actinidiae* was grown in M9 medium at 18 °C for 48 h. When appropriate, antibiotics were used at the following concentrations: ampicillin (Amp), 50 μg/mL; kanamycin (Km), 50 μg/mL; chloromycetin (Cm), 34 μg/mL; rifampicin (Rif), 50 μg/mL; and tetracycline (Tc), 20 μg/mL.

2.2. Tn5 mutagenesis and transposon-insertion mapping

A random transposon library of *P. syringae* pv. *actinidiae* A18 was generated using the EZ-Tn5™ < R6K*gori*/KAN-2 > Tnp Transposome™ Kit (Epicentre, Madison, WI, USA.) according to the manufacturer's instructions. Electroporation were carried out in a Bio-Rad Gene Pulser Xcell™ Electroporation System in accordance with the manufacturer's recommendations. The transformants were subsequently isolated by plating serial dilutions of the LB medium containing Rif and Km. Mutants were screened for altered phaseolotoxin production relative to wild-type by the *E. coli* toxin bioassay [25]. Individual colonies of putative mutants from *P. syringae* pv. *actinidiae* A18 were confirmed by PCR and Southern blotting. Six mutants completely abolishing phaseolotoxin production was selected for further analysis. The inserted region was identified by thermal asymmetric interlaced PCR (TAIL-PCR) as described by Liu and Huang [31]. Sequencing, BLAST analysis, and alignment with the corresponding sequence from the genome of *P. syringae* pv. *actinidiae* ICMP9617 (GenBank Accession No. CM002753) were performed.

2.3. Construction of the gacA knock-out mutant and complementation

To verify the role of *gacA* in *P. syringae* pv. *actinidiae* A18, deletion of the *gacA* region was carried out by homologous recombination according to the strategy of PCR-based fusions [32]. Approximately 400 bp of the upstream and downstream regions of *gacA* were amplified using primers gacA1U_up_fw, gacA1D_up_rev, and gacA2U_down_fw, gacA2D_down_rev (Table S1), respectively. The Cm resistance gene *cat* was amplified from plasmid pACYC184 [33] with Primers Chl-F and Chl-R (Table S1). All PCR fragments generated for vector construction were cloned into plasmid PMD19-T, transformed into *E. coli* DH5α, and validated through sequencing performed by Invitrogen (Shanghai). The three amplicons were fused into an about 1.7 kb fragment, in which the *cat* gene is located between the other two amplicons. The fusion PCR product was then cloned into PK18*mobsacB* [30] to generate the suicide plasmids, named PK18gacA (Table 1).

Subsequently the PK18gacA was verified by sequencing and mobilized into *P. syringae* pv. *actinidiae* A18 via tri-parental mating [34] using pRK2013 helper plasmid as described by Figurski et al. [29]. The transconjugants were selected by plating on LB plates with Km and Rif, followed by counterselection on LB plates containing Rif and sucrose to identify double cross-over recombinants. The sucrose-resistant but Km-sensitive transconjugants were checked by PCR using primers gacA2-F and gacA2-R (Table S1), and confirmed by sequencing. One of the confirmed mutants (A18Δ*gacA*) was used for further study.

For mutant complementation, the DNA fragment containing the *gacA*-coding region was amplified from genomic DNA of strain A18 by PCR using the primers gacA1-F/gacA1-R (Table S1), and cloned into the PMD19-T vector. After verification by sequencing, the insert was excised and ligated into the broad host range vector pLAFR3 [28]. The obtained recombinant plasmid was transferred into the mutant strain A18Δ*gacA* by tri-parental mating, producing complementary strain A18Δ*gacA*-C. Moreover, studies of growth of these strains were carried out in liquid KBA medium, LB medium and M9 medium, respectively, to determine whether the mutants were able to grow the same as their parent strains [35].

2.4. Phaseolotoxin assays

Phaseolotoxin production by *P. syringae* pv. *actinidiae* A18 and derived mutants was assayed by the *E. coli* growth inhibition assay as described previously [25,36] with slight modification. Strains were grown at 18 °C in M9 medium for 48 h, then 150 mL of supernatants from these cultures were vaccum concentrated at −50 °C to a volume of 5 mL as crude toxin. 100 μL of crude toxin was spotted to a paper disk (0.6 cm diameter) and evaporated to dryness. A lawn of *E. coli* DH1 was plated on LB agar medium and the paper discs containing supernatant from the tested cultures were placed on this plate. Then plates were incubated at 37 °C and examined for zones of inhibition of growth of *E.*