Microbial Pathogenesis 142 (2020) 104091

Contents lists available at ScienceDirect

Microbial Pathogenesis

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

The cold shock family gene *cspD3* is involved in the pathogenicity of *Ralstonia solanacearum* CQPS-1 to tobacco

Ying Liu[a], Xi Tan[a], Haojin Cheng[a], Jie Gong[a], Yong Zhang[b], Daibin Wang[c,**], Wei Ding[a,*]

[a] College of Plant Protection, Southwest University, Chongqing, China
[b] College of Resources and Environment, Southwest University, Chongqing, China
[c] Chongqing Tobacco Science Research Institute, Chongqing, China

ARTICLE INFO

Keywords:
Cold shock proteins
cspD3
Ralstonia solanacearum
Swimming motility
Virulence

ABSTRACT

Cold shock proteins (Csps) are small and highly conserved proteins that have target RNA- and DNA-binding activities. Csps play roles in different cellular processes and show functional redundancy. *Ralstonia solanacearum*, the agent of bacterial wilt, has 4 or 5 Csps based on genome analysis. However, the functions of all Csps in *R. solanacearum* remain unclear. According to phylogenetic analysis, the Csps from *R. solanacearum* are clustered into a group with CspD from *E. coli*. Here, we studied the role of CspD3, which was closer to CspD of *E. coli* in the phylogenetic tree. A *cspD3* deletion strain was constructed to assess its effect on the phenotype of *R. solanacearum*, including growth, biofilm formation, motility, and virulence. The results showed that *cspD3* of *R. solanacearum* was not necessary for normal growth, cold-shock adaptation, or biofilm formation. However, deletion of *cspD3* in *R. solanacearum* CQPS-1 led to increased swimming motility, and the mean diameters of swimming haloes produced by the Δ*cspD3* mutant were 1.3-fold larger than those produced by wild-type strain and 1.2-fold larger than those produced by the complemented strain. More importantly, the virulence of the *cspD3* deletion mutant on susceptible tobacco plants was significantly attenuated compared to the wild-type strain. At 20 days after inoculation, the disease index of the Δ*cspD3* mutant was 2.27, which was reduced by 1.6-fold relative to the wild-type strain. To assess the molecular response influenced by *cspD3*, the expressions of the main motility-associated genes and virulence-associated genes including *flgM*, *fliA*, *pehS*, *pehR*, *hrpG*, *xpsR*, and *prhI* in *R. solanacearum* were measured. The results showed that the expressions of *hrpG*, *xpsR*, and *prhI* were significantly decreased in *cspD3* deletion mutant. Collectively, our findings showed that Csps are involved in the regulation of motility and virulence in *R. solanacearum*.

1. Introduction

When suffer a cold shock (rapid temperature drop), Organisms would product a series of cold-induced proteins (Cips) transiently; for example, the cold shock protein (Csp) family, RNA helicase csdA, exoribonucleases PNPase and RNaseR in *Escherichia coli* [1–3]. Csp family proteins (Csps) are small, highly conserved, and comprise the cold-shock domain (CSD, the RNA-binding domain). This CSD, which contains two nucleic acid-binding motifs (RNP1 and RNP2), has target RNA- and DNA-binding activities [4–6]. Csps could play roles in various cellular processes, including cellular growth, stress tolerance, and virulence [2,6,7].

CspA, the major cold-shock protein, was first described in *E. coli* [8]. To date, nine homologues of CspA have been identified in *E. coli*, named CspA to CspI. Among the nine Csps, five homologues (CspA, CspB, CspE, CspG and CspI) have been reported to be cold inducible [3,8–11]. It was reported that some Csps, such as CspE and CspC of *E. coli*, could be expressed during growth at normal temperatures [2,12]. The Csps in bacteria participate in different cellular processes and show functional redundancy. The nomenclature of certain Csps does not reflect their sequences in addition to their functions. For instance, CspD in *E. coli* plays an important role in nutrient stress adaptation, while it may respond to osmotic stress in *Listeria monocytogenes* [13,14]. Therefore, more functional experiments should be performed to identify the real role of Csps in different bacteria.

The gram-negative bacterium *Ralstonia solanacearum* is an important phytopathogen causing a devastating disease (bacterial wilt) in many plants [15–17]. The *R. solanacearum* species complex is widely distributed around the world and has a large number of hosts, which includes more than 50 plant families, including many important

* Corresponding author. No.2 Tiansheng Road, Beibei, Chongqing, China.
** Corresponding author. No.2 Tiansheng Road, Beibei, Chongqing, China.
E-mail addresses: 467572562@qq.com (D. Wang), dingw@swu.edu.cn (W. Ding).

https://doi.org/10.1016/j.micpath.2020.104091
Received 29 October 2019; Received in revised form 19 February 2020; Accepted 19 February 2020
Available online 20 February 2020
0882-4010/ © 2020 Published by Elsevier Ltd.

economic crops [17,18]. As a soil-borne pathogen, *R. solanacearum* survives and persists in various environmental conditions, including soil and water [19,20]. To adapt to different stressors, *R. solanacearum* has evolved a complicated regulatory network. The genome of the representative strain GMI1000 provides a molecular basis for understanding the characteristics of the species [21]. Due to the high variation among species, only one genome sequence cannot meet the needs for analysis. An increasing number of genome sequences are acquired for molecular analysis. To date, 121 genomes of *R. solanacearum* have been accessed in the National Center for Biotechnology Information (NCBI) database, providing strong support for analysing the molecular characteristics of this species. Based on the genome data and molecular biology research, the pathogenic networks of *R. solanacearum* are becoming clear, providing a basis for studying the molecular responses of certain genes or pathways [17].

Although many studies have analysed Csps in other organisms, such as *E. coli*, and *Bacillus subtilis*, there is limited research on the Csps of *R. solanacearum*. Previous studies have reported that Csps are involved in the regulation of virulence, invasiveness, and motility in pathogens [7,22]. It is uncertainly whether Csps play roles in regulating the cellular progression of *R. solanacearum*. The annotation information of Csps in *R. solanacearum* species complex could be easily obtained based on the genome data (www.ncbi.nlm.nih.gov/genome). In this study, we compared Csps of *R. solanacearum* strain CQPS-1 with the Csps from *E. coli* and the representative *R. solanacearum* strain GMI1000. And then, one of *csp* genes (*cspD3*) was chosen for further study according to the result of phylogenetic analysis. To analyse the function, we constructed a *cspD3* deletion strain, characterized the phenotypes including motility, biofilm formation, and virulence and compared the phenotypes with the wild-type strain. The present study will contribute to a better understanding of the role of Csps in *R. solanacearum* strains.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Bacterial strains and plasmids used in this study are described in Table 1. *R. solanacearum* strains were grown at 30 °C in BG medium, B liquid medium, or MM medium [23]. *E. coli* strains were grown at 37 °C in Luria-Bertani (LB) medium. When necessary, antibiotics were added into the media at the following final concentrations: ampicillin (Amp, 100 mg L^{-1}), kanamycin (Km, 50 mg L^{-1}), polymyxin B (PB, 50 mg L^{-1}). Growth was detected by measuring the optical density (OD) at 600 nm. The bacterial growth curve was monitored according to Yang et al. [24].

Table 1
Bacterial strains and plasmids used in this study.

Strains and plasmids	Relevant characteristics[a]	Reference or source
E. coli		
DH5α	Strain used in cloning procedures	[25]
S17-1	Strain used for plasmid mobilization	[26]
R. solanacearum		
CQPS-1	Wild-type, biovar 3, phylotype I, isolated from tobacco	[27]
CQ101	CQPS-1 △*cspD3*	This work
CQ102	CQPS-1 △*cspD3*::*cspD3*^{WT}	This work
Plasmids		
pK18mobsacB	Sucrose counter-selection allelic exchange vector; Km^r	[28]
pUC18-mini-Tn7T-Gm	Mini-Tn7 delivery plasmid; Amp^r; Gm^r	[29]
pTNS2	Helper plasmid encoding the site-specific TnsABC + D transposition pathway; Ap^r	[29]

[a] Amp^r, ampicillin resistance; Km^r, kanamycyn resistance; Gm^r, gentamicin resistance.

2.2. Sequence analysis of Csps in R. solanacearum

The Csps of *R. solanacearum* strains were analysed according to the genome data. The accession numbers of *R. solanacearum* GMI1000 in GenBank were AL646052 and AL646053 (chromosome and megaplasmid, respectively), and the accession numbers of CQPS-1 were CP016914 and CP016915. The amino acid sequences were aligned using Clustal X [30]. The phylogenetic tree was generated using neighbor-joining (NJ) with 1,000 bootstrap resamplings in MEGA version 7 [31]. The sequence of *cspD3* was amplified with the primer pair cspD3-F/cspD3-R and verified by the Beijing Genomics Institute (BGI, Beijing, China).

2.3. Deletion of cspD3 genes

Mutants with gene deletions were generated based on homologous recombination by pK18mobsacB as described by Zhang et al. [32]. Briefly, two flanking fragments of *cspD3* (670 bp and 551 bp) were amplified from *R. solanacearum* CQPS-1 genomic DNA by using primer pairs p510A1BamHI/p510B1C and p510A2C/p510B2HindIII, respectively. p510B1C and p510A2C are fully complemented. A second round of PCR was performed to generate the DNA fragment (approximately 1.2 kb) without the open-reading frame of *cspD3* by using primers p510A1BamHI and p510B2HindIII. The generated DNA fragment was cloned into *Bam*HI-*Hin*dIII-digested pK18mobsacB to obtain pK18p510. After the sequence was validated, the plasmid pK18p510 was delivered from *E. coli* strain S17-1 into the *R. solanacearum* strain CQPS-1 by conjugative transfer. A Deletion mutant was generated through consecutive homologous recombination events. The successful *cspD3* mutant was verified by PCR and Sanger sequence with primers p510A1BamHI and p510B2HindIII. The primers used in this study are listed in Table S1 (see Supporting Information).

2.4. Complementation analyses

In the present study, complementation analysis was performed with a pUC18-mini-Tn7T-Gm-based site-specific chromosomal integration system as previously described [29,33]. Briefly, a DNA fragment containing the coding sequence of the *cspD3* gene and its upstream region of approximately 600 bp, empirically harbouring its native promoter, was PCR amplified from the genomic DNA of CQPS-1 with primers p510C1BamHI and p510C2HindIII and finally sub-cloned into pUC18-mini-Tn7T-Gm to generate the complementary plasmid pUCp510C. After validating the sequence, a cope of the *cspD3* gene was integrated into the chromosome of the *cspD3* mutant at a single attTn7 site (25 bp downstream of *glmS*), and complementary strains were generated, which were confirmed by colony PCR with the primers glmsdown and Tn7R [32].

2.5. Biofilm assay

The biofilm formation assay of *R. solanacearum* was performed as previously described [24]. Briefly, 200 μL of B liquid medium supplemented with 1/200 (v/v) overnight-cultured bacterial suspension was inoculated in 96-well polystyrene microtiter plates, and then incubated without shaking at 30 °C for 24 h. Biofilms were stained with crystal violet (Solarbio, Beijing, China), dissolved in 95% ethanol and quantified by measuring the absorbance at 530 nm (A_{530}). The assay was performed three times.

2.6. Swimming motility test

A swimming motility test was performed as previously reported with some modifications [34]. Strains were grown in MM medium

supplemented with glucose at a final concentration of 20 mM final concentration for 1 day at 30 °C and then diluted to 1.10^8 CFU ml^{-1}. The bacterial suspension was stab-inoculated into MM medium semi agar (0.3%) plates supplemented with 20 mM glucose. The diameter of the swimming halo was measured after 48 h and 72 h of incubation at 30 °C. The experiment was repeated twice, with ten plates for each strain per replicate.

2.7. Pathogenicity test

The pathogenicity of the strains was tested on eight-week-old tobacco plants (cv. Yunyan 87, a susceptible cultivar, Yuxi Zhong Yan Tobacco Seed Co., Ltd., China) by soil drenching. The overnight bacterial culture in B medium was suspended in sterile deionized water to 10^8 cfu/ml (OD_{600nm} = 0.1). Each plant was inoculated by pouring 10 mL of suspension. The disease index was monitored every two days for 20 d. The experiment was repeated twice for each tested strain.

2.8. RNA extraction, cDNA synthesis, and quantitative real-time PCR

The strains were incubated in MM medium to comparable cell densities (OD_{600nm} ~ 0.5). The bacterial cells were collected after centrifugation and frozen in liquid nitrogen. Total RNA was extracted with TRNzol reagent (Tiangen Biotech Co., Ltd., Beijing, China). Then RNA samples were reverse transcribed with the iScript™ cDNA Synthesis Kit (BIO-RAD, USA). Quantitative real-time PCR (qRT-PCR) was used to analyse the relative expression of genes as previously described [35]. The primers are shown in Table S1, and *serC* was used as a housekeeping gene [35]. Seven genes were investigated including the flagella regulon genes *flgM* and *fliA*, two-component system genes *pehS* and *pehR*, OmpR family response regulator gene *hrpG*, and virulence-associated genes *xpsR* and *prhI* [17,36].

3. Results

3.1. Sequence analysis of Csps in R. solanacearum CQPS-1

Based on the annotation of genome data, the representative *R. solanacearum* strain GMI1000 had 5 Csps (CspA, CspC, CspD, CspD2, and CspD3), but there were only 4 Csps in strain CQPS-1, including CspC, CspD, CspD2, and CspD3. From the phylogenetic analysis of all Csps from *R. solanacearum* and *E. coli*, all Csps from *R. solanacearum* were clustered into a group with CspD from *E. coli*. Moreover, CspD3 from *R. solanacearum* was closer to CspD of *E. coli*, although the bootstrap value was low, only 30% (Fig. 1A). In this study, we concentrated on the role of CspD3 in *R. solanacearum* strain CQPS-1. The conserved nucleic acid-binding motifs (RNP1 and RNP2) were found in CspD3 of *R. solanacearum* (Fig. 1B) based on the sequence of CspD from *E. coli* [37]. Compared with CspD of *E. coli*, CspD3 of *R. solanacearum* had a mutated RNP2 (Fig. 1B).

3.2. R. solanacearum cspD3 is not necessary for normal growth and cold-shock adaptation

To evaluate the role of *cspD3* in different biological processes of *R. solanacearum*, a deletion mutant derivative was generated from strain CQPS-1. We first investigated the phenotypic alterations of the strains, such as the shape and growth. The colony morphologies of the wild-type, mutant, and complemented strains on BG agar plates containing tetrazolium chloride (TTC) are shown in Fig. 2A. The colonies of mutant strains were highly fluidal, irregularly shaped, and cream coloured, similar to those of the wild-type and complemented strain. After that, the growth curves of these strains were evaluated, showing that the loss of the *cspD3* gene had no significant impact on growth at 30 °C (Fig. 2B).

To investigate whether the *cspD3* gene of *R. solanacearum* plays a role in adapting to low temperature, and the growth status was tested when the strains were shifted from 30 °C to 15 °C in the exponential phase. As shown in Fig. 2C and 16 h after the transition from 30 °C to 15 °C, the OD_{600} values of the wild-type, mutant, and complemented strains were 0.67, 0.59, and 0.66, respectively, and 24 h after, the OD_{600} values were 0.89, 0.84, and 0.81, respectively. The Δ*cspD3* mutant showed no difference compared with the wild-type strain, CQPS-1, and the complemented strain when the strains were exposed to cold stress. This result suggested that the *cspD3* gene had no important function in the adaptation of *R. solanacearum* to cold shock.

3.3. Deletion of cspD3 had no influence on biofilm formation

To evaluate whether the *cspD3* gene contributes to normal biofilm formation, a 96-well polystyrene microtiter plate assay was performed. Biofilms were stained and quantified after strains were incubated without shaking in B liquid medium at 30 °C for 24 h. It was found that the Δ*cspD3* mutant produced similar biofilms as the wild-type strain CQPS-1, and the complemented strain (Fig. 2D).

3.4. Deletion of cspD3 enhances the swimming motility of R. solanacearum

The swimming motilities of the wild-type, Δ*cspD3* mutant and complemented strains were compared in this study (Fig. 3). At 72 h after inoculation in MM medium semi agar, the mean swimming haloes produced by the Δ*cspD3* mutant, wild-type CQPS-1 and the complemented strains were 46.8 ± 0.23 mm, 39.4 ± 0.43 mm, and 36.2 ± 1.84 mm, respectively. The results showed that the Δ*cspD3* mutant had a significantly larger mean diameter after 72 h of inoculation compared to the wild-type and complemented strains (Fig. 3B, one-way analysis of variance and Tukey test, p-value < 0.05).

3.5. Virulence of the cspD3 deletion mutant is decreased on tobacco plants

To assess the role of *cspD3* in *R. solanacearum* pathogenicity, an inoculation assay was performed on susceptible tobacco plants with the Δ*cspD3* mutant, complemented and wild-type strains. As shown in Fig. 4, the tobacco plants inoculated with the wild-type strain began to show wilt symptoms on the 4th day after inoculation, while the plants inoculated with the Δ*cspD3* mutant wilted on the 6th day after inoculation. The disease index of the Δ*cspD3* mutant on the 20th day after inoculation was 1.6-fold reduced compared with the wild-type strain. After the strain was complemented with *cspD3*, the virulence was restored to wild-type levels. This result indicated that the virulence of the *cspD3* deletion mutant was decreased in susceptible tobacco plants compared to the wild type strain.

3.6. Deletion of cspD3 decreased the expression of virulence-associated genes of R. solanacearum

Previous experiments showed that deletion of *cspD3* could influence the swimming motility and virulence of *R. solanacearum*. To assess the molecular response influenced by *cspD3*, we measured the expression of the main motility-associated genes and virulence-associated genes in *R. solanacearum*. The results indicated that deletion of *cspD3* affected the expression of *pehS*, *hrpG*, *xpsR*, and *prhI* (Fig. 5). The expression of *pehS* in the *cspD3* deletion mutant was much higher than that in the wild-type strain and complemented strain. The expression of *hrpG*, and *xpsR* was much lower in the *cspD3* deletion mutant. The expression of *prhI* in the *cspD3* deletion mutant was not significantly lower than that in the wild-type strain but was significantly lower than that in the complemented strain. Although the *flgM* was overexpressed in the wild-type strain, there were no significant differences compared with the wild-type strain and the complemented strain.

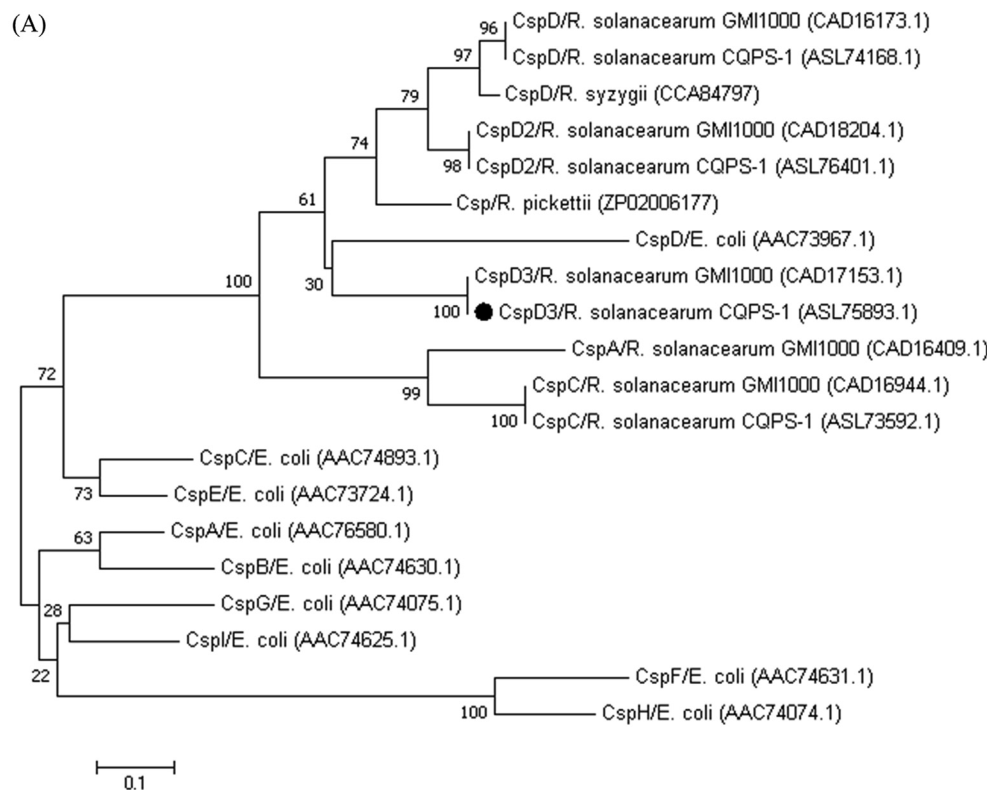

Fig. 1. Amino acid sequence analysis of Csps in *R. solanacearum* CQPS-1. (A) Evolutionary relationships of the CspDs from *R. solanacearum* CQPS-1 with Csps from other bacterial species by using the neighbor-joining method. Values at the branches indicate the percentage of bootstrap support for 1000 resamplings. Black circle indicates CspD3 from *R. solanacearum* CQPS-1. (B) Amino acids sequence comparison between *R. solanacearum* CspD3 and *E. coli* CspD. RNA-binding motifs RNP1 and RNP2 are labeled as red boxes. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

4. Discussion

Csps belonging to a family of nucleic acid-binding proteins are related to a series of physiological functions including growth and stress adaptation in some organisms [1,2,38]. *R. solanacearum*, a species of Betaproteobacteria, is an important phytopathogen causing huge economic losses [15]. According to the genome data, there are 5 Csps in *R. solanacearum* strain GMI1000, and 4 Csps in strain CQPS-1. Until now, the molecular functions of all Csps in *R. solanacearum* are not known. It is known that CspD3 of *R. solanacearum* was phylogenetically closer to the CspD of *E. coli* from the phylogenetic analysis, (Fig. 1). Based on this information, this study explored the function of the *cspD3* genes in *R. solanacearum*. The results suggested that *cspD3* does not play an important role in cold-shock adaptation. However, it is involved in regulating the virulence and swimming motility of *R. solanacearum* CQPS-1.

CspD in different bacteria is involved in various cellular progresses. Previous study reported that CspD of *E. coli* was induced by stationary-phase growth and nutrient starvation [14], and involved in biofilm formation and persister cell development [39,40]. In *B. subtilis*, CspD is cold-induced [41]. In Listeria monocytogenes, CspD is involved in osmotic stress adaptation [13]. The results of this study suggested that *cspD3* of *R. solanacearum* was not necessary for normal growth, biofilm formation, or cold-shock adaptation.

The nomenclature of the Csp family is based on the first reported Csp (named CspA), which is related to cold shock. This nomenclature may be misleading and limited because Csps in different processes of organisms play diverse roles not only in cold-shock adaptation [42]. The Csps in eubacteria could be classified into five clades and 12 subclades based on the phylogenetic analysis of the 89 Csps [42]. The Csps of *R. solanacearum* were clustered into one clade (clade III), together with the CspDs from Gammaproteobacteria. However, the relationship between the function and the phylogenetic clade needs to be further verified.

The data shown in this study suggested that the swimming motility of *R. solanacearum* was enhanced after deleting *cspD3* (Fig. 3B). Swimming motility, which is mediated by flagella, together with twitching motility, which is mediated by the type IV pilus system, is required for the pathogen to colonize plant roots during the early stage of infection [43–45]. The increased swimming motility may provide a better chance for *R. solanacearum* strains moving towards the plant rhizosphere and invading the tissue of roots. However, the virulence of the Δ*cspD3* mutant decreased based on the pathogenicity data (Fig. 4). Another research reported by Perrier previously showed that the deletion of *efpR* (a gene involved in adapting to the host) caused increased mobility and decreased virulence [34]. Although swimming motility is a virulence trait of *R. solanacearum*, its increase does not result in the increase of pathogenicity directly.

Based on the increasing knowledge about the molecular characterization of *R. solanacearum*, the virulence network has been comprehensively studied previously [17,36]. Phenotype results demonstrated that deletion of *cspD3* could influence swimming motility. Swimming motility is mediated by the PehSR two-component system and flagella [43,46]. qRT-PCR results showed that deletion of *cspD3* influences the expression of *pehS*, but not the flagella regulon genes *flgM* and *fliA*, which means that *cspD3* could affect the two-component system PehSR to control motility. HrpG is a key regulatory node, that plays very important roles in host-bacterial interactions [19]. Additionally, XpsR could enhance the expression of extracellular polysaccharide (EPS)-related genes [17]. The expression levels of both *hrpG* and *xpsR* were decreased in the *cspD3* deletion mutant (Fig. 5). It can be speculated that CspD3 in *R. solanacearum* is involved in the positive regulation of

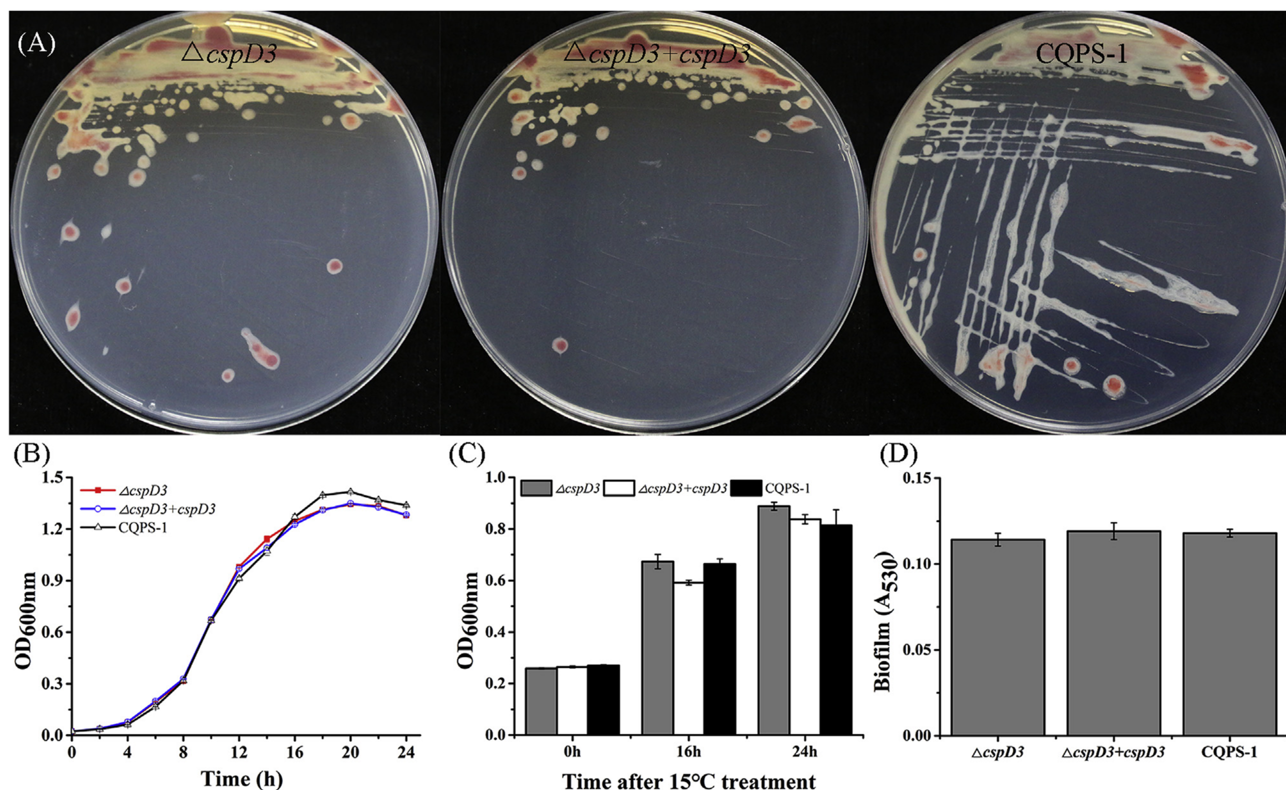

Fig. 2. Phenotypes, growth, and biofilm formation of the *cspD3* deletion mutant, complemented and wild-type strains. (A) Colony morphology of strains on BG agar plates containing tetrazolium chloride. (B) The growth curves of strains under normal growth conditions. Strains were cultured at 30 °C in B liquid medium and cell density was detected by measuring the OD_{600}. (C) The growth of strains at different time points after temperature downshifting. The strains were initially grown in B medium at 30 °C for approximately 8 h and then moved to a lower temperature (15 °C) to administer cold shock. (D) Biofilm formation of strains after incubation in B medium for 24 h in 96-well plates. Error bars indicate the standard error.

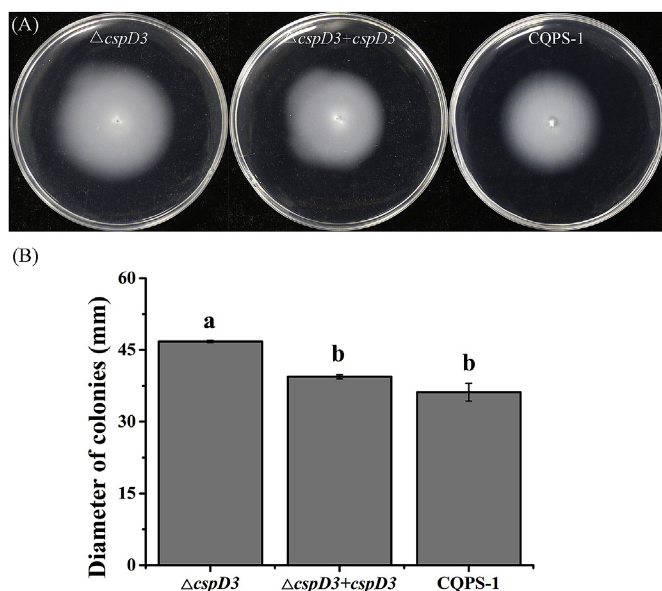

Fig. 3. Swimming motility of the *cspD3* deletion mutant, complemented and wild-type strains. (A) The pictures of swimming halos observed after incubation at 30 °C after 72 h. (B) The diameter of halos measured at 72 h. Error bars indicate the standard error. Different letters indicate significant difference as determined by ANOVA and Tukey's HSD (lowercase difference p-value < 0.05).

the virulence network.

It has been observed that *R. solanacearum* strains are spreading from the lowlands to the highlands and to cold areas in China recently [47]. *R. solanacearum* strains could survive at relatively low temperatures for a long time [48]. Exploring the cold-shock response mechanism of *R. solanacearum* would provide more evidence for studying the adaptation to low temperature. In the future, the function of the other 3 Csps genes should be studied to better understand the molecular strategy for adapting to the environment, especially cold-shock adaptation.

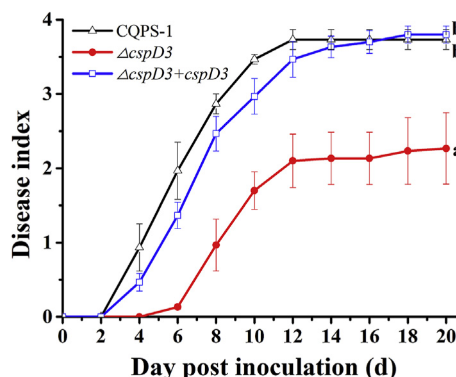

Fig. 4. Virulence of the *cspD3* deletion mutant and complemented strains vs the wild-type strain on tobacco plants. Disease progression was recorded every two days for 20 d by using a 0 to 4 disease index. Error bars indicate the standard error. Different letters indicate significant difference as determined by ANOVA and Tukey HSD (lowercase difference p-value < 0.05).

5. Conclusion

In conclusion, our study provides evidence that CspD3 might act as a positive regulator in the virulence regulation network of *R.*

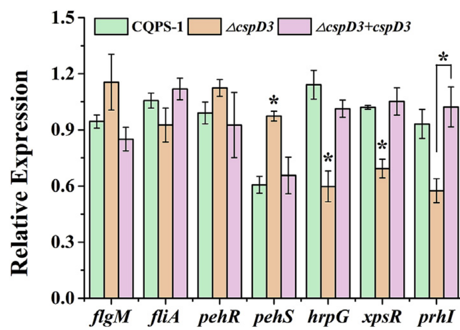

Fig. 5. Relative expression of the flagella regulon and virulence genes. The relative expression of seven genes including the flagella regulon genes *flgM* and *fliA*, two-component system genes *pehS* and *pehR*, OmpR family response regulator gene *hrpG*, and virulence-associated genes *xpsR* and *prhI*. *SerC* was used as a control gene to normalize the expression of the target genes. Error bars indicate the standard error. Asterisks indicate significant differences as determined by ANOVA and Tukey's HSD (p-value < 0.05).

solanacearum. More evidence should be provided to explore the molecular mechanism by which CspD3 regulates the virulence of *R. solanacearum* in the future. Furthermore, it is also necessary to evaluate the function of other Csps in *R. solanacearum*.

Author contributions

Conceived and designed the experiments: WD, and YL. Performed the experiments: YL, HC, XT, JG, and YZ. Analysed the data: YL. Wrote and revise the paper: YL, YZ, DW, and WD.

Statement

We declare that there are no potential competing interests. All authors have read and approved this version of the article, and due care has been taken to ensure the integrity of the work. We certify that the submission is original work and is not under review at any other publication.

Declaration of competing interest

The authors declare that they have no competing interests.

Acknowledgments

The research was funded by the Major Project of the China National Tobacco Corporation [110201601025(LS-05)] and [110201901042(LS-05)], and the Key Project from China National Tobacco Corporation Sichuan Branch (SCYC201908 and SCYC202010).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.micpath.2020.104091.

References

[1] S. Phadtare, Recent developments in bacterial cold-shock response, Curr. Issues Mol. Biol. 6 (2004) 125–136.
[2] R. Keto-Timonen, N. Hietala, E. Palonen, A. Hakakorpi, M. Lindstrm, H. Korkeala, Cold shock proteins: a minireview with special emphasis on csp-family of *enteropathogenic Yersinia*, Front. Microbiol. 7 (2016) 1151.
[3] K. Nakashima, K. Kanamaru, T. Mizuno, K. Horikoshi, A novel member of the cspA family of genes that is induced by cold shock in *Escherichia coli*, J. Bacteriol. 178 (1996) 2994–2997.
[4] D. Landsman, Rnp-1, an rna-binding motif is conserved in the DNA-binding cold shock domain, Nucleic Acids Res. 20 (1992) 2861–2864.
[5] K. Schroder, P. Graumann, A. Schnuchel, T.A. Holak, M.A. Marahiel, Mutational analysis of the putative nucleic acid-binding surface of the cold-shock domain, cspb, revealed an essential role of aromatic and basic residues in binding of single-stranded-DNA containing the Y-box motif, Mol. Microbiol. 16 (1995) 699–708.
[6] V. Chaikam, D.T. Karlson, Comparison of structure, function and regulation of plant cold shock domain proteins to bacterial and animal cold shock domain proteins, Bmb Rep 43 (2010) 1–8.
[7] A.K. Eshwar, C. Guldimann, A. Oevermann, T. Tasara, Cold-shock domain family proteins (csps) are involved in regulation of virulence, cellular aggregation, and flagella-based motility in *Listeria monocytogenes*, Front. Cell. Infect. Microbiol. 7 (2017) 453.
[8] J. Goldstein, N.S. Pollitt, M. Inouye, Major cold shock protein of *Escherichia coli*, Proc. Natl. Acad. Sci. U.S.A. 87 (1990) 283–287.
[9] S.J. Lee, A.G. Xie, W.N. Jiang, J.P. Etchegaray, P.G. Jones, M. Inouye, Family of the major cold-shock protein, cspa (Cs7.4), of *Escherichia coli*, whose members show a high sequence similarity with the eukaryotic Y-box binding-proteins, Mol. Microbiol. 11 (1994) 833–839.
[10] N. Wang, K. Yamanaka, M. Inouye, CspI, the ninth member of the CspA family of *Escherichia coli*, is induced upon cold shock, J. Bacteriol. 181 (1999) 1603–1609.
[11] J.P. Etchegaray, M. Inouye, CspA, CspB, and CspG, major cold shock proteins of *Escherichia coli*, are induced at low temperature under conditions that completely block protein synthesis, J. Bacteriol. 181 (1999) 1827–1830.
[12] T.R. Czapski, N. Trun, Expression of csp genes in *E. coli* K-12 in defined rich and defined minimal media during normal growth, and after cold-shock, Gene 547 (2014) 91–97.
[13] B. Schmid, J. Klumpp, E. Raimann, M.J. Loessner, R. Stephan, T. Tasara, Role of cold shock proteins in growth of *Listeria monocytogenes* under cold and osmotic stress conditions, Appl. Environ. Microbiol. 75 (2009) 1621–1627.
[14] K. Yamanaka, M. Inouye, Growth-phase-dependent expression of *cspD*, encoding a member of the CspA family in *Escherichia coli*, J. Bacteriol. 179 (1997) 5126–5130.
[15] J. Mansfield, S. Genin, S. Magori, V. Citovsky, M. Sriariyanum, P. Ronald, M. Dow, V. Verdier, S.V. Beer, M.A. Machado, I. Toth, G. Salmond, G.D. Foster, Top 10 plant pathogenic bacteria in molecular plant pathology, Mol. Plant Pathol. 13 (2012) 614–629.
[16] A.C. Hayward, Characteristics of *Pseudomonas solanacearum*, J. Appl. Bacteriol. 27 (1964) 265–277.
[17] S. Genin, T.P. Denny, Pathogenomics of the *Ralstonia solanacearum* species complex, Annu. Rev. Phytopathol. 50 (2012) 67–89.
[18] S. Genin, C. Boucher, *Ralstonia solanacearum*: secrets of a major pathogen unveiled by analysis of its genome, Mol. Plant Pathol. 3 (2002) 111–118.
[19] S. Genin, Molecular traits controlling host range and adaptation to plants in *Ralstonia solanacearum*, New Phytol. 187 (2010) 920–928.
[20] B. Álvarez, E.G. Biosca, M.M. López, On the life of *Ralstonia solanacearum*, a destructive bacterial plant pathogen, Curr. Res. Technol. Educ. Top. Appl. Microbiol. Microb. Biotechnol. 1 (2010) 267–279.
[21] M. Salanoubat, S. Genin, F. Artiguenave, J. Gouzy, S. Mangenot, M. Arlat, A. Billault, P. Brottier, J. Camus, L. Cattolico, Genome sequence of the plant pathogen *Ralstonia solanacearum*, Nature 415 (2002) 497–502.
[22] C. Loepfe, E. Raimann, R. Stephan, T. Tasara, Reduced host cell invasiveness and oxidative stress tolerance in double and triple *csp* gene family deletion mutants of *Listeria monocytogenes*, Foodb. Pathog. Dis. 7 (2010) 775–783.
[23] C.A. Boucher, P.A. Barberis, D.A. Demery, Transposon mutagenesis of *Pseudomonas solanacearum*: isolation of Tn5-induced avirulent mutants, Microbiology 131 (1985) 2449–2457.
[24] L. Yang, W. Ding, Y.Q. Xu, D.S. Wu, S.L. Li, J.N. Chen, B. Guo, New insights into the antibacterial activity of hydroxycoumarins against *Ralstonia solanacearum*, Molecules 21 (2016) 468.
[25] D. Hanahan, Studies on transformation of *Escherichia coli* with plasmids, J. Mol. Biol. 166 (1983) 557–580.
[26] R. Simon, U. Priefer, A. Pühler, A broad host range mobilization system for in vivo genetic engineering: transposon mutagenesis in gram negative bacteria, Nat. Biotechnol. 1 (1983) 784–791.
[27] Y. Liu, Y.M. Tang, X.Y. Qin, L. Yang, G.F. Jiang, S.L. Li, W. Ding, Genome sequencing of *Ralstonia solanacearum* CQPS-1, a phylotype I strain collected from a highland area with continuous cropping of tobacco, Front. Microbiol. 8 (2017) 974.
[28] A. Schafer, A. Tauch, W. Jager, J. Kalinowski, G. Thierbach, A. Puhler, Small mobilizable multi-purpose cloning vectors derived from the *Escherichia coli* plasmids pK18 and pK19: selection of defined deletions in the chromosome of *Corynebacterium glutamicum*, Gene 145 (1994) 69–73.
[29] K.H. Choi, J.B. Gaynor, K.G. White, C. Lopez, C.M. Bosio, R.R. Karkhoff-Schweizer, H.P. Schweizer, A Tn7-based broad-range bacterial cloning and expression system, Nat. Methods 2 (2005) 443–448.
[30] F. Jeanmougin, J.D. Thompson, M. Gouy, D.G. Higgins, T.J. Gibson, Multiple sequence alignment with Clustal x, Trends Biochem. Sci. 23 (1998) 403–405.
[31] S. Kumar, G. Stecher, K. Tamura, MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets, Mol. Biol. Evol. 33 (2016) 1870–1874.
[32] Y. Zhang, F. Luo, D.S. Wu, Y. Hikichi, A. Kiba, Y. Igarashi, W. Ding, K. Ohnishi, PrhN, a putative marR family transcriptional regulator, is involved in positive regulation of type III secretion system and full virulence of *Ralstonia solanacearum*, Front. Microbiol. 6 (2015) 357.
[33] Y. Zhang, J. Li, W.Q. Zhang, H.L. Shi, F. Luo, Y. Hikichi, X.J. Shi, K. Ohnishi, A putative LysR-type transcriptional regulator PrhO positively regulates the type III secretion system and contributes to the virulence of *Ralstonia solanacearum*, Mol. Plant Pathol. 19 (2018) 1808–1819.
[34] A. Perrier, R. Peyraud, D. Rengel, X. Barlet, E. Lucasson, J. Gouzy, N. Peeters, S. Genin, A. Guidot, Enhanced in planta fitness through adaptive mutations in EfpR, a dual regulator of virulence and metabolic functions in the plant pathogen

Ralstonia solanacearum, PLoS Pathog. 12 (2016) 1006044.

[35] L. Yang, J.N. Chen, Y. Liu, S.T. Zhang, S.L. Li, W. Ding, Validation of reference genes for quantitative gene expression analysis in *Ralstonia pseudosolanacearum* CQPS-1 under environment stress, J. Microbiol. Methods 148 (2018) 104–109.

[36] N. Peeters, A. Guidot, F. Vailleau, M. Valls, Ralstonia solanacearum, a widespread bacterial plant pathogen in the post-genomic era, Mol. Plant Pathol. 14 (2013) 651–662.

[37] K. Yamanaka, L. Fang, M. Inouye, The CspA family in *Escherichia coli*: multiple gene duplication for stress adaptation, Mol. Microbiol. 27 (1998) 247–255.

[38] M.H.W. Weber, M.A. Marahiel, Bacterial cold shock responses, Sci. Prog. 86 (2003) 9–75.

[39] Y. Kim, T.K. Wood, Toxins Hha and CspD and small RNA regulator Hfq are involved in persister cell formation through MqsR in *Escherichia coli*, Biochem. Bioph. Res. Co. 391 (2010) 209–213.

[40] Y. Kim, X.X. Wang, X.S. Zhang, S. Grigoriu, R. Page, W. Peti, T.K. Wood, *Escherichia coli* toxin/antitoxin pair MqsR/MqsA regulate toxin CspD, Environ. Microbiol. 12 (2010) 1105–1121.

[41] M.H.W. Weber, M.A. Marahiel, Coping with the cold: the cold shock response in the Gram-positive soil bacterium *Bacillus subtilis*, Philos. Trans. R. Soc. Lond. Biol. Sci. 357 (2002) 895–906.

[42] T. Yu, R. Keto-Timonen, X.J. Jiang, J.P. Virtanen, H. Korkeala, Insights into the phylogeny and evolution of cold shock proteins: from enteropathogenic *Yersinia* and *Escherichia coli* to eubacteria, Int. J. Mol. Sci. 20 (2019) 4059.

[43] J. Tans-Kersten, H.Y. Huang, C. Allen, *Ralstonia solanacearum* needs motility for invasive virulence on tomato, J. Bacteriol. 183 (2001) 3597–3605.

[44] J. Tans-Kersten, D. Brown, C. Allen, Swimming motility, a virulence trait of *Ralstonia solanacearum*, is regulated by FlhDC and the plant host environment, Mol. Plant Microbe Interact. 17 (2004) 686–695.

[45] H.L. Liu, Y.W. Kang, S. Genin, M.A. Schell, T.P. Denny, Twitching motility of *Ralstonia solanacearum* requires a type IV pilus system, Microbiol-Sgm 147 (2001) 3215–3229.

[46] C. Allen, J. Gay, L. SimonBuela, A regulatory locus, pehSR, controls polygalacturonase production and other virulence functions in *Ralstonia solanacearum*, Mol. Plant Microbe Interact. 10 (1997) 1054–1064.

[47] G.F. Jiang, Z. Wei, J. Xu, H.L. Chen, Y. Zhang, X.M. She, A.P. Macho, W. Ding, B.S. Liao, Bacterial wilt in China: history, current status, and future perspectives, Front. Plant Sci. 8 (2017) 1549.

[48] A. Milling, F. Meng, T.P. Denny, C. Allen, Interactions with hosts at cool temperatures, not cold tolerance, explain the unique epidemiology of *Ralstonia solanacearum* race 3 biovar 2, Phytopathology 99 (2009) 1127–1134.