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LysR-Type Transcriptional Regulator Contributes to *Pseudomonas cannabina* pv. *alisalensis* Virulence by Regulating Type Three Secretion System

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Abstract: *Pseudomonas cannabina* pv. *alisalensis* (*Pcal*) causes bacterial blight on cabbage. In a previous study, we screened for reduced virulence using Tn5 transposon mutants and identified a LysR-type transcriptional regulator (LTTR) as a potential virulence factor in *Pcal*. However, the role of LTTR in *Pcal* virulence has not been thoroughly investigated. In this study, we demonstrated that the *Pcal* NN14 mutant (with Tn5 insertion in the LTTR-encoding gene) showed reduced disease symptoms and bacterial populations in cabbage, indicating that LTTR contributes to *Pcal* virulence. RNA-seq analysis identified 39 LTTR-dependent genes. Genes associated with 13 of the type three secretion system (T3SS), two of flagellar apparatus, ABC transporters, and transcription factors were expressed at lower levels in the NN14 mutant compared to the wild type. Conversely, *tssH* and *hcp*, type six secretion system (T6SS)-related genes, showed higher expression in NN14. Furthermore, these differences in gene expression were observed in minimal medium, but not in nutrient-rich medium, suggesting that LTTR acts as a global regulator responsive to nutrient conditions. Additionally, LTTR activated the expression of T3SS-related genes during *Pcal* infection. We also demonstrated that NN14 showed a reduced ability to induce hypersensitive reaction (HR) cell death in non-host plants. Collectively, these results suggest that LTTR contributes to *Pcal* virulence by regulating T3SS in response to environmental changes.

Keywords: *Pseudomonas cannabina* pv. *alisalensis*; LysR-type transcriptional regulator; type three secretion system; RNA-seq; virulence; cabbage

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1. Introduction

Foliar plant-pathogenic *Pseudomonas* species cause diseases in a wide range of host plants. Generally, these pathogens colonize the epiphytic surface of host plants, enter through natural openings, mainly stomata, and then multiply in the intercellular apoplastic space [1]. In a successful disease cycle, plant-pathogenic *Pseudomonas* species utilize various virulence factors to suppress plant defense responses [1–4]. Studies on the model pathogen *P. syringae* pv. *tomato* (*Pst*) DC3000 have revealed key virulence factors, including the type three secretion system (T3SS) and the phytotoxin coronatine (COR). The T3SS is encoded by *hrp* and *hrc* genes, which are essential for eliciting a hypersensitive reaction (HR) in non-host plants and for pathogenesis in host plants [5]. Type three effectors (T3Es) contribute to suppressing host defenses and facilitating disease development [6–8]. *hrp* promoters are activated by the alternative sigma factor HrpL, a member of the extracytoplasmic factor

(ECF) family of sigma factors [9]. *hrp* genes are expressed at low levels in standard nutrient-rich medium, such as King's B (KB) or Luria–Bertani (LB) medium, but are induced in apoplast-mimicking minimal medium or in planta [10]. The phytotoxin coronatine (COR) is also a critical virulence factor in some *Pseudomonas* species [11–13]. COR is a non-host-specific polyketide toxin composed of coronafacic acid (CFA) and coronamic acid (CMA) [14,15]. The response regulator CorR positively regulates CFA and CMA operons in *Pst* DC3000 and is activated by HrpL [16]. COR stimulates jasmonic acid (JA) signaling and suppresses salicylic acid (SA)-dependent defense through antagonistic crosstalk [13].

Pseudomonas cannabina pv. *alisalensis* (*Pcal*) causes bacterial leaf spot and blight on Brassicaceae, including cabbage, broccoli, pak choi, Chinese cabbage, red cabbage, and green ball cabbage [17]. *Pcal* was formerly classified as *Pseudomonas syringae* pv. *maculicola* (*Psm*), but these species were reclassified based on bacteriological characteristics, genetic traits, and their ability to infect monocot plants such as oat (*Avena sativa*) and timothy (*Phleum pretense*) [11,17,18]. Currently, copper-based fungicides and antibiotics are the primary methods for controlling diseases caused by *Pcal* [17]. However, these conventional treatments target critical bacterial growth pathways, leading to the rapid emergence of resistant strains. Indeed, copper- and streptomycin-resistant strains of *Pcal* have already been identified [17]. As a result, neutralizing bacterial virulence factors has gained attention as an alternative approach for managing such bacterial diseases [19,20]. This strategy offers the advantage of exerting lower selective pressure compared to traditional copper fungicides and antibiotics [20]. Developing drugs that target virulence factors requires a thorough understanding of the virulence mechanisms of *Pcal* and their roles in disease progression.

Sakata et al. (2019) conducted a screening for reduced virulence using Tn5 transposon mutants to identify *Pcal* virulence factors [21]. Their study revealed that multiple virulence factors are required for successful infection, including T3SS, membrane transporters, transcriptional factors, and amino acid metabolism [21]. Among these mutants, the NN14 mutant (with Tn5 inserted in the LysR-type transcriptional factor gene) showed reduced virulence in cabbage. Therefore, the LysR-type transcriptional factor (LTTR) was identified as a *Pcal* virulence factor.

LTTRs are a well-characterized group of transcriptional regulators, highly conserved and ubiquitous in bacteria [22–24]. LTTRs are regarded as global transcriptional regulators, acting as dual-functional repressor–activators [25]. Structurally, LTTRs consist of an N-terminal DNA-binding domain and a C-terminal co-inducer-binding domain [19,25]. Co-inducers bound by LTTRs typically mediate a conformational change in the LTTR–DNA complex, leading to altered RNA polymerase affinity and the modulation of target gene expression. Thus, co-inducers are recognized as crucial for LTTR function [25].

LTTRs play important roles in various cellular pathways, including metabolism, oxidative stress response, antibiotic resistance, quorum sensing, cell motility, and virulence [25]. Specifically, some LTTRs have been identified as essential for the virulence of plant-pathogenic bacteria. In *Ralstonia solanacearum*, the causal agent of bacterial wilt, PhcA and PrhO are LTTRs associated with virulence [26–29]. PhcA is well characterized as a global regulator controlling diverse virulence-related genes, including those involved in plant cell wall degradation, motility, extracellular polysaccharide (EPS) synthesis, and T3SS [26–29]. PrhO positively regulates T3SS expression and entire T3Es, contributing to virulence in *R. solanasearum [29].* In *Xanthomonas oryzae* pv. *oryzae*, the causal agent of bacterial leaf blight of rice is GamR, an LTTR that acts as a positive regulator of two key *hrp* regulators and subsequent *hrp* genes [30]. Additionally, LTTRs have gained attention as drug targets due to their pivotal roles in regulating the expression of virulence factors [19]. Some LTTRs are already being utilized as drug targets for human diseases [19]. For instance, MvfR, an LTTR from *P. aeruginosa*, has emerged as a promising drug target to mitigate high levels of antibiotic resistance [31,32]. These examples suggest that targeting LTTRs could aid in developing novel treatment strategies for *Pcal*. However, the roles of LTTRs in

plant-pathogenic *Pseudomonas* species remain largely uncharacterized. Thus, we aimed to investigate the contribution of LTTRs to the virulence of *Pcal*.

This study aimed to clarify the role of LTTR in *Pcal* virulence and explore its potential as a target for novel treatment strategies. In this study, we conducted inoculation experiments on cabbage and demonstrated that LTTR contributes to *Pcal* virulence. RNA-seq analysis identified 39 LTTR-dependent genes, including those related to T3SS. RT-qPCR confirmed that T3SS-related genes, including *hrpL* and *avrPto*, along with *fliK*, *iaaL*, and *corR*, were up-regulated in wild-type (WT) *Pcal* in minimal medium, whereas this upregulation was weak or absent in NN14. In contrast, *tssH* and *hcp* were upregulated in NN14 in a minimal medium. These results suggest that LTTR acts as a global regulator responsive to nutrient conditions. Moreover, LTTR upregulates T3SS-related genes during infection. These results indicate that LTTR contributes to *Pcal* virulence by regulating T3SS.

2. Materials and Methods

2.1. Bacterial Strain and Growth

The pathogenic bacterial strain used in this study was *Pseudomonas cannabina* pv. *alisalensis* strain KB211 (*Pcal* KB211). The wild-type (WT) *Pcal* was cultured on King's B (KB) [33] agar medium at 28 °C. The NN14 mutant was cultured on a KB medium agar supplemented with kanamycin (10 μg/mL). Before bacterial inoculation, bacteria were scraped from the KB agar medium and suspended in sterile distilled water. The cell density of the bacterial suspensions were adjusted to an optical density at 600 nm (OD_{600}) using a Biowave CO8000 cell density meter (Funakoshi, Tokyo, Japan). An OD_{600} of 0.1 corresponds to 5×10^7 CFU/mL.

2.2. Bacterial In Vitro Growth Measurements

To evaluate bacterial growth in vitro, WT *Pcal* and NN14 were grown on KB broth at 28 °C with shaking. The bacterial suspensions were adjusted to an OD_{600} of 0.01 using fresh KB broth and immediately incubated at 28 °C with shaking. Bacterial growth was monitored 24 h and 48 h after incubation by measuring the OD_{600}. The assay was conducted in sterile polystyrene test tubes (Caplugs Evergreen, Buffalo, NY, USA), with three biological replicates for each strain.

2.3. Plant Materials

Cabbage (*Brassica oleracea* var. *capitate*) cv. Kinkei 201 (SAKATA SEED CORPORATION, Yokohama, Kanagawa, Japan) was used for *Pcal* virulence assays. Tobacco (*Nicotiana tabacum*) cv. Xanthi (Japan Tobacco Inc., Minato-ku, Tokyo, Japan) was used for the *Pcal* hypersensitive reaction (HR) cell death assay. Plants were grown from seeds at 23–25 °C under a light intensity of 200 $\mu Em^{-2}s^{-1}$ and a 16 h light/8 h dark photoperiod. For the inoculation assay, cabbage seedlings were used approximately two weeks after germination. Tobacco seedlings were used in the HR cell death assay approximately four weeks after germination.

2.4. Bacterial Inoculation and Population Measurements In Planta

For dip-inoculation, cabbage seedlings were submerged in bacterial suspensions (5×10^7 colony-forming units: CFU/mL) containing 0.025% Silwet L-77 (OSI Specialities, Danbury, CT, USA). The inoculated plants were transferred to a growth chamber with a 85–95% RH for the first 24 h, followed by 80–85% RH for the rest of the experimental period. For syringe-inoculation, bacterial suspensions (5×10^4 CFU/mL) were applied onto cabbage leaves using a 1 mL blunt syringe. After inoculation, the plants were maintained at 70–80% RH in growth chambers. Each treatment group contained at least three plants.

Internal bacterial populations were quantified at specific time points: 0, 1, 3, and 5 dpi. The photographs of the leaves were taken at 5 dpi. For dip-inoculated leaves, leaf samples were surface-sterilized using 10% H_2O_2 for three minutes, rinsed with sterile distilled water, and homogenized in sterile distilled water using a mortar and pestle. Bacterial populations

at 0 dpi were estimated using leaves collected 1 h post-inoculation (hpi) without surface sterilization. For syringe-inoculated leaves, bacterial growth in cabbage was assessed by harvesting leaf disks with a cork borer. The disks were homogenized in sterile distilled water. The diluted samples were plated onto solid KB medium plate. The number of bacterial colony-forming units (CFUs) was determined and expressed as CFU per gram or CFU per cm^2, based on the total weight of the leaf or the total leaf area, respectively. Each bacterial measurement was conducted in at least three independent experiments.

2.5. RNA Purification

To analyze the gene expression profiles of WT *Pcal* and NN14 genes under various nutrient conditions, bacteria were separately cultured under nutrient-rich and minimal nutrient environments. For nutrient-rich conditions, bacteria were grown in KB broth for 24 h, adjusted to 2×10^8 CFU/mL with fresh KB broth, and cultured for an additional 3 h. For minimal nutrient conditions, bacteria were grown in KB broth for 24 h, adjusted to 2×10^8 CFU/mL with fresh KB broth, cultured for 3 h, and subsequently incubated in mannitol–glutamate (MG) medium [34] for 30 min. Total RNA was extracted using ReliaPrep (Promega, Madison, WI, USA) following the manufacturer's protocol.

To assess gene expressions during infection, cabbage plants were syringe-inoculated with WT *Pcal* and NN14 at a concentration of 5×10^5 CFU/mL. Total RNA was extracted and purified from the infected leaves after 24 h using RNAiso Plus (Takara Bio, Kusatsu, Japan).

2.6. RNA-Seq Analysis

RNA-seq analysis was conducted as described in Sakata et al. (2023) [35]. Briefly, total RNAs isolated from WT *Pcal* and NN14 were processed to deplete rRNAs using the RiboMinus Transcriptome Isolation Kit for bacteria (Thermo Fisher Scientific Inc., Waltham, MA, USA). Libraries were then constructed using the Ion Total RNA-seq Kit v2 (Thermo Fisher Scientific Inc.) and Ion Xpress RNA-seq barcode (Thermo Fisher Scientific Inc.). RNA-seq templates were prepared using the Ion 540 Chef Kit on an Ion Chef system (Thermo Fisher Scientific Inc., Waltham, MA, USA). Analysis was performed using the CLC Genomics Workbench (Qiagen, Valencia, CA, USA), with the *P. syringae* pv. *maculicola* ES4326 genome (GeneBank accession number: CP047260) as a reference. Genome expression was quantified as RPKM values. The subsequent mathematical analyses were performed using R (version 4.3.2; R Foundation for Statistical Computing, Vienna, Austria). The expression ratio of NN14 against WT *Pcal* was calculated using the GLM method implemented in the edgeR package. One-way ANOVA was used to obtain the statistical data. To reduce the second type of error in statistical analysis (type II errors), genes were filtered at 0.1 of permissive FDR value. In addition, the effect size (eta-squared) was calculated using the etaSquared method implemented in the Isr package.

2.7. RT-qPCR

The DNase-treated RNA was reverse-transcribed using the ReverTra Ace qPCR RT Master Mix (TOYOBO, Osaka, Japan). The resulting cDNA was then subjected to RT-qPCR analysis with the primers specified in Supplementary Table S1 and THUNDERBIRD SYBR qPCR Mix (TOYOBO). The reactions were performed on a thermal cycler dice real time system (Takara Bio). To normalize gene expression levels, the *outer membrane porin F* (*oprF*) and *recombinase A* (*recA*) of *Pcal* KB211 were used as reference genes.

2.8. Hypersensitive Response Cell Death Assay

To assess the hypersensitive response (HR), tobacco leaves were syringe-infiltrated with WT *Pcal* and NN14 suspensions (5×10^7 CFU/mL and 1×10^8 CFU/mL) using a 1 mL blunt syringe. HR cell death symptoms were photographed at 48 hpi.

2.9. Hypertrophy-Inducing Activity Assay on Potato Tuber Tissue

Potato tuber disks were washed in tap water for 5 min and then rinsed with sterile distilled water several times. Inoculation was conducted by placing a toothpick on the WT and NN14 on a KB medium plate and then inserting it into the potato tuber disk. The disks were then incubated at 23 °C in darkness and photographed at 5 dpi.

2.10. Swimming Motility Assays

The swimming phenotypes of WT *Pcal* and NN14 were tested on 0.25% (*w*/*v*) MG medium plate. To inoculate the plates, fresh colonies grown on KB medium plate were inoculated by stabbing them into the center of the MG medium plate, which were then incubated at 23 °C. Swimming phenotypes were observed three days after incubation.

2.11. Statistical Analysis

All data are presented as the mean with standard error (SE). All statistical analyses were conducted using EZR (Saitama Medical Centre, Jichi Medical University, Saitama, Japan), a graphical user interface for R. Tukey's honestly significant difference (HSD) test or *t*-test was used to analyze gene expression profiles. Differences in $p < 0.05$ were considered statistically significant.

3. Results

3.1. The Importance of LTTR in Pseudomonas cannabina pv. alisalensis Virulence

We first measured the in vitro growth of WT and NN14 *Pseudomonas cannabina* pv. *alisalensis* (*Pcal*) to determine potential multiplication ability. No significant growth differences were observed between WT *Pcal* and NN14 at 24 h and 48 h post-incubation in KB medium (Supplementary Figure S1). These results demonstrate that there are no significant differences in the growth capabilities of these strains under optimized nutrient-rich conditions.

To investigate the role of LTTR in *Pcal* virulence, we conducted bacterial inoculation experiments. Cabbage plants were dip-inoculated with either WT *Pcal* or NN14. Leaves inoculated with WT *Pcal* exhibited chlorosis, whereas those inoculated with NN14 showed milder symptoms (Figure 1A). Additionally, bacterial populations of NN14 were significantly reduced compared to those of WT *Pcal* (Figure 1B). To further assess whether NN14 is impaired in apoplastic growth and virulence, we performed syringe infiltration to bypass stomatal defenses. Leaves inoculated with NN14 showed no symptoms and had reduced bacterial populations compared to those inoculated with WT *Pcal* (Figure 1C,D). Together, these results indicate that LTTR contributes to *Pcal* virulence in cabbage.

3.2. Gene Expression Profiles of WT and NN14 Pseudomonas cannabina pv. alisalensis

To compare gene expression profiles between WT *Pcal* and NN14, both strains were cultured, and their transcriptional expression levels were analyzed using RNA-seq. This analysis revealed 39 LTTR-dependent genes exhibiting at least a twofold change in expression with false discovery rate (FDR) values of less than 1% (Table 1). In NN14, several genes associated with the T3SS, flagellar apparatus, ABC transporters, transcription factors including *corR,* and *iaaL* showed reduced expression compared to WT *Pcal* (Table 1). Specifically, the expression of several T3SS-related genes was downregulated in NN14 relative to WT *Pcal*, including seven genes related to T3SS machinery, two genes to type three helper proteins, and three genes encoding for T3Es (Table 1). Additionally, *hrpL*, which encodes an alternate RNA polymerase sigma factor required for T3SS gene expression, was suppressed in NN14 (Table 1). Conversely, genes related to the type six secretion system (T6SS) were upregulated in NN14 compared to WT (Table 1).

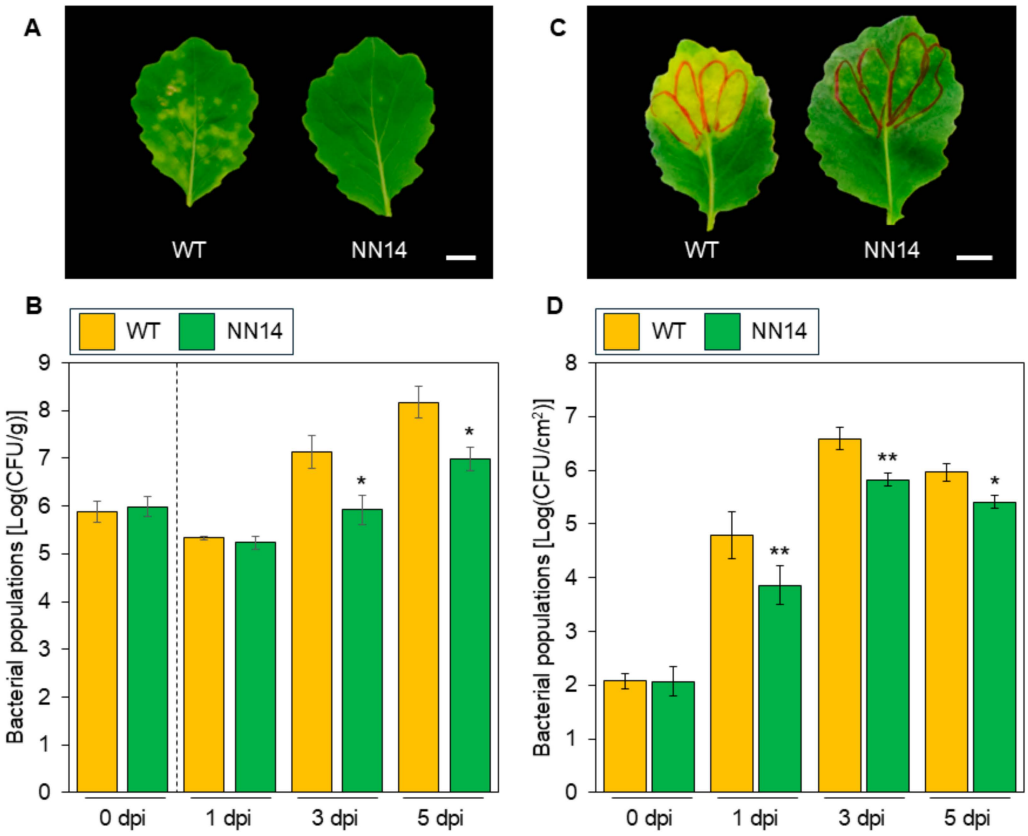

Figure 1. Disease phenotypes and bacterial populations of wild-type (WT) and NN14 *Pseudomonas cannabina* pv. *alisalensis* KB211 in cabbage after dip- and syringe-inoculation. (**A**) Representative images of cabbage leaves dip-inoculated with WT and NN14 bacterial suspensions (5×10^7 CFU/mL) containing 0.025% Silwet L-77. (**B**) Internal bacterial populations in dip-inoculated leaves were quantified at 0, 1, 3, and 5 days post-inoculation (dpi). (**C**) Disease symptoms in syringe-inoculated cabbage with WT and NN14 (5×10^4 CFU/mL). (**D**) Internal bacterial populations in syringe-inoculated leaves were quantified at 0, 1, 3, and 5 dpi. The leaves were photographed at 5 dpi. The scale bar represents 1 cm. Asterisks indicate a significant difference from WT in a *t*-test (* $p < 0.05$, ** $p < 0.01$).

Table 1. The differential gene expression between WT *Pcal* and NN14.

Category	Locus Tag	Gene	Function	Log_2 Fold Changes	FDR Values
Type three secretion system	PMA4326_RS06565	*hrpL*	Sigma-70 family RNA polymerase sigma factor	−4.25	4.9×10^{-22}
	PMA4326_RS06450	*hrpB*	Type III secretion system inner rod subunit SctI	−5.15	5.9×10^{-17}
	PMA4326_RS06455	*hrcJ*	Type III secretion inner membrane ring lipoprotein SctJ	−4.01	2.6×10^{-9}
	PMA4326_RS06570	*hrpK*	Type III helper protein HrpK	−4.88	6.9×10^{-9}
	PMA4326_RS06520	*hrcR*	Type III secretion system export apparatus protein SctR	−4.23	7.5×10^{-7}
	PMA4326_RS06480	*hrcC*	Type III secretion system outer membrane ring subunit SctC	−3.66	5.1×10^{-5}

Table 1. *Cont.*

Category	Locus Tag	Gene	Function	Log_2 Fold Changes	FDR Values
	PMA4326_RS06465	*hrpE*	Type III secretion system stator protein SctL	−4.01	1.5×10^{-3}
	PMA4326_RS06555	*hrcV*	Type III secretion system export apparatus subunit SctV	−3.65	5.1×10^{-3}
	PMA4326_RS19805	*hopAk1*	Type III helper protein HopAK1	−3.07	6.2×10^{-3}
	PMA4326_RS06525	*hrcQ*	Type III secretion system protein	−4.53	5.6×10^{-3}
	PMA4326_RS24740	*hopAA1−2*	Type III effector HopAA1-2	−2.17	5.1×10^{-4}
	PMA4326_RS23615	*hopI1*	Type III effector HopI1	−2.63	1.4×10^{-5}
	PMA4326_RS03310	*avrPto*	Type III effector avrPto	−2.17	2.6×10^{-8}
Type six secretion system	PMA4326_RS26840	*hcp*	Type VI secretion system tube protein Hcp	2.16	9.1×10^{-11}
	PMA4326_RS26870	*tssH*	Type VI secretion system ATPase TssH	2.08	3.4×10^{-3}
Flagellar	PMA4326_RS06545	*fliI*	FliI/YscN family ATPase	−3.78	1.5×10^{-3}
	PMA4326_RS06535	*fliK*	Flagellar hook-length control protein FliK	−4.30	3.0×10^{-3}
Transporter	PMA4326_RS30565		MFS transporter	−1.46	3.2×10^{-8}
	PMA4326_RS20255	*aatP*	amino acid ABC transporter ATP-binding protein	−2.03	1.1×10^{-3}
Transcription factor	PMA4326_RS12490	*lttR*	LysR family transcriptional regulator	3.71	1.4×10^{-6}
	PMA4326_RS24665	*corR*	Response regulator transcription factor	−4.23	1.7×10^{-8}
Others	PMA4326_RS25720	*iaaL*	AMP-binding protein	−3.17	4.2×10^{-6}
	PMA4326_RS02410		Restriction endonuclease	−5.68	1.0×10^{-20}
	PMA4326_RS03605		Amidinotransferase	−4.12	6.1×10^{-5}
	PMA4326_RS03305		AAA family ATPase	−1.60	7.5×10^{-7}
	PMA4326_RS20160		Argininosuccinate synthase	2.00	8.2×10^{-4}
	PMA4326_RS26995		Acyl-CoA dehydrogenase	−3.04	6.3×10^{-3}
Hypothetical protein	PMA4326_RS19340		Hypothetical protein	−3.83	1.9×10^{-14}
	PMA4326_RS06460		Hypothetical protein	−3.76	5.1×10^{-5}
	PMA4326_RS06540		Hypothetical protein	−3.41	6.5×10^{-4}
	PMA4326_RS06575		Hypothetical protein	−2.52	1.2×10^{-3}
	PMA4326_RS26765		Hypothetical protein	2.06	2.8×10^{-3}
	PMA4326_RS15740		Hypothetical protein	−3.06	3.4×10^{-3}

We next confirmed the gene expression profiles in rich and minimal media using RT-qPCR. *hrpL* and *avrPto* were significantly upregulated in only WT *Pcal* in minimal medium (Figure 2A,B). The *hrcC* gene also tended to be upregulated in WT *Pcal* compared to NN14 in minimal medium (Figure 2C). In minimal medium, the *fliK*, *iaaL*, and *corR* genes exhibited differential expression patterns between WT *Pcal* and NN14. The *fliK* gene was upregulated in both strains, though the increase in NN14 was significantly less pronounced compared to WT *Pcal* (Figure 2D). The *iaaL* gene was upregulated exclusively in WT *Pcal* (Figure 2E). Furthermore, the *corR* gene showed a significant increase in expression only in WT *Pcal* (Figure 2F). On the other hand, *tssH* was upregulated in NN14 in the minimal medium

(Figure 2G). The expression of *hcp* in NN14 was upregulated in minimal medium compared to WT *Pcal*, although this increase was not statistically significant (Figure 2H). These results revealed that WT *Pcal* and NN14 show different gene expression patterns in a minimal medium, suggesting that LTTR is involved in regulating gene expression under specific environmental conditions. Since flagellin-related and COR-related gene expressions were down-regulated in NN14, we also investigated swimming motility and COR production assays. The swimming ability of WT *Pcal* and NN14 was comparable (Supplementary Figure S2). We also investigated COR production using a hypertrophy-inducing activity test on potato tuber tissues [36]. Potato tuber tissues inoculated with WT *Pcal* and NN14 showed a hypertrophy response (Supplementary Figure S3), indicating that NN14 was not impaired in COR production.

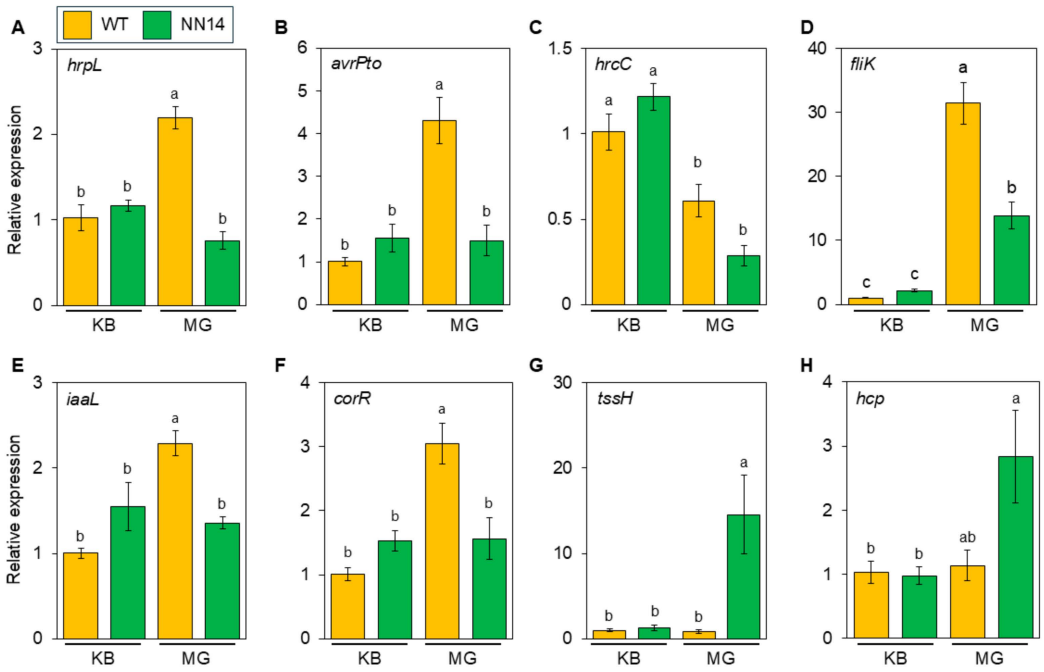

Figure 2. Gene expression profiles in wild-type (WT) and NN14 *Pseudomonas cannabina* pv. *alisalensis* KB211 under nutrient-rich and nutrient-poor conditions. The expression profiles of type three secretion system-related genes (including *hrpL* (**A**), *avrPto* (**B**), and *hrcC* (**C**)), *fliK* (**D**), *iaaL* (**E**), *corR* (**F**), type six secretion system-related genes *tssH* (**G**), and *hcp* (**H**) were analyzed using a real-time quantitative reverse transcription-polymerase chain reaction (RT-qPCR) with gene-specific primer sets (Table S1). Bacteria were cultured in nutrient-rich KB medium or transferred to nutrient-poor MG medium for 30 min before RNA extraction. Expression was normalized using *oprF* and *recA*. Vertical bars represent the standard error of at least three biological replicates. Different letters indicate a significant difference among treatments based on Tukey's HSD test ($p < 0.05$).

We next investigated the gene expression profiles during *Pcal* infection. *hrpL* and *avrPto* were highly expressed in WT *Pcal* compared to NN14 (Figure 3A,B). In contrast, no significant difference was observed in the expression levels of *hrcC*, *iaaL*, *corR*, and *hcp* between WT *Pcal* and NN14 (Figure 3C–F). These results indicate that LTTR plays a crucial role in regulating T3SS expression during *Pcal* infection.

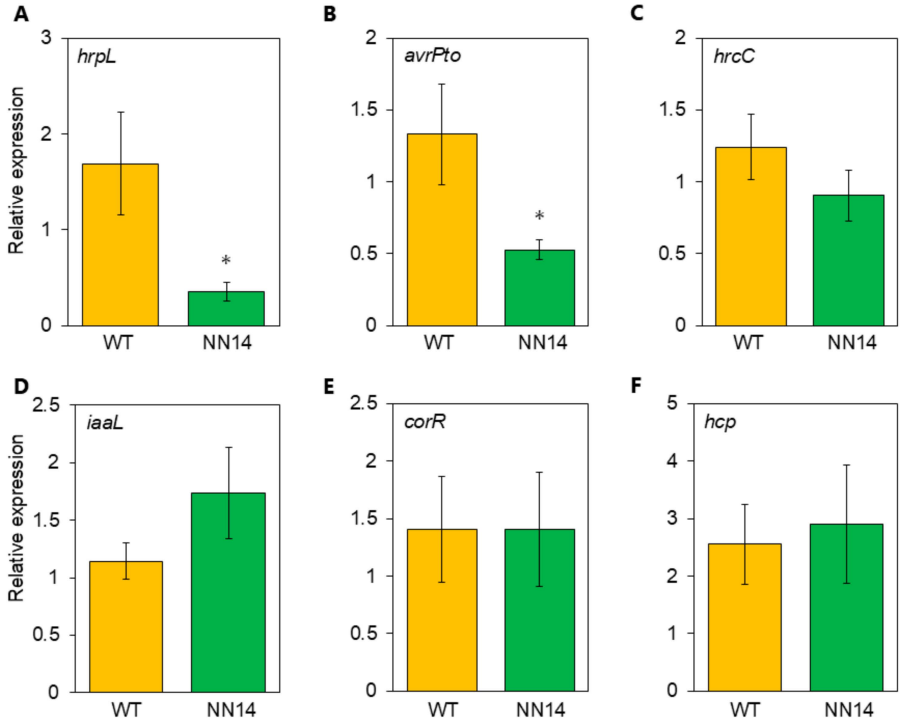

Figure 3. Gene expression profiles in wild-type (WT) and NN14 *Pseudomonas cannabina* pv. *alisalensis* KB211 in planta. The expression profiles of type three secretion system-related genes (including *hrpL* (**A**), *avrPto* (**B**), and *hrcC* (**C**)), *iaaL* (**D**), *corR* (**E**), and *hcp* (**F**) were analyzed using a real-time quantitative reverse transcription-polymerase chain reaction (RT-qPCR) with gene-specific primer sets (Table S1). Cabbage leaves were syringe-inoculated with a bacterial suspension of WT and NN14 (5×10^5 CFU/mL). Total RNAs were collected at 24 h post-inoculation (hpi). Expression was normalized using *oprF* and *recA*. Vertical bars represent the standard error of at least three biological replicates. Asterisks indicate a significant difference from WT in a *t*-test (* $p < 0.05$).

3.3. Hypersensitive Response in Tobacco Inoculated with Pseudomonas cannabina pv. alisalensis WT and NN14

WT *Pcal* elicits a hypersensitive response (HR) cell death in tobacco leaves [35], and some T3Es are responsible for HR elicitation [37]. We therefore assessed whether HR induction in tobacco leaves was altered in NN14. When the bacterial suspensions were adjusted to 5×10^7 CFU/mL, HR cell death was induced by WT *Pcal*, but not by NN14 (Figure 4A). Conversely, at 1×10^8 CFU/mL, NN14 exhibited identical HR cell death development when compared to WT (Figure 4B). These results indicate that the ability of NN14 to cause HR is reduced but not completely lost.

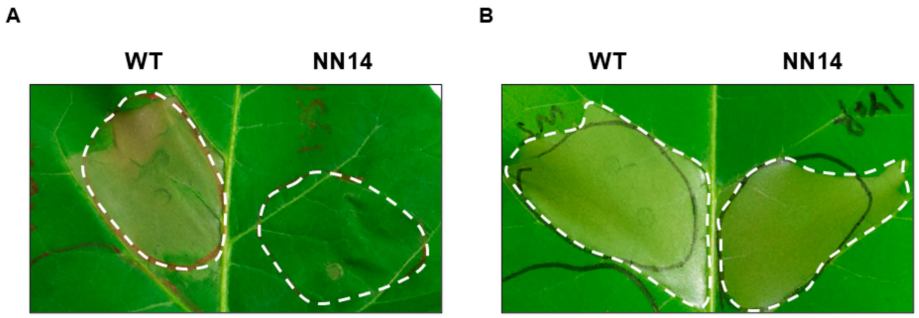

Figure 4. Hypersensitive response cell death elicited by wild-type (WT) and NN14 *Pseudomonas cannabina* pv. *alisalensis* KB211 in tobacco leaves. Tobacco leaves were syringe-inoculated with bacterial suspensions at 5×10^7 CFU/mL (**A**) and 1×10^8 CFU/mL (**B**). The inoculated area was outlined with white dotted lines. Photos were taken at 48 hpi.

4. Discussion

LTTR, a transcriptional regulator, has been identified as a potential virulence factor in *Pcal* [35]. While LTTRs are a well-characterized group of transcriptional regulators, their specific role in plant-pathogenic *Pseudomonas* species has not been extensively investigated. Thus, we aimed to elucidate the role of LTTR in *Pcal* virulence. We demonstrated that the NN14 strain (LTTR mutant) exhibited reduced disease symptoms and bacterial populations on cabbage, indicating that LTTR contributes to *Pcal* virulence. Through RNA-seq analysis, we revealed that multiple genes, including those related to the T3SS, showed significantly altered expression levels in NN14. Notably, the gene expression differences between the WT and NN14 mutant were observed only under minimal medium conditions and not in nutrient-rich medium. These results suggest that LTTR functions as a global regulator, enabling *Pcal* to sense and respond to varying nutrient conditions. Additionally, T3SS-related genes in NN14 showed lower expression compared to WT during infection. Therefore, our results suggest that the upregulation of T3SS-related genes by LTTR is a crucial factor contributing to *Pcal* virulence.

Disease symptoms and bacterial populations on cabbage inoculated with NN14 were significantly reduced compared to those inoculated with WT *Pcal* after dip-inoculation (Figure 1A,B). Furthermore, NN14 exhibited significant reductions in disease symptoms and bacterial populations compared to WT following syringe-inoculation, which bypasses stomatal-based defense mechanisms (Figure 1C,D). These results indicate that LTTR contributes to *Pcal* virulence and multiplication even after the bacteria have entered the plant tissue. Similarly, LTTRs are crucial for the virulence of *Ralstonia solanacearum* [27,29]. Previous studies have shown that the inactivation of *phcA*, one of the LTTRs, results in reduced virulence [27]. Additionally, the deletion of *prhO*, another LTTR, leads to significantly impaired virulence [29]. Collectively, these results suggest that LTTRs play a vital role in the virulence of several bacterial pathogens.

Our RNA-seq analysis identified 39 LTTR-dependent genes in minimal medium (Table 1). Many T3SS-related genes exhibited lower expression in NN14 compared to WT (Table 1). Additionally, several other genes associated with the flagellar apparatus, ABC transporters, transcriptional factors such as *corR,* and *iaaL* were less expressed in NN14. Conversely, T6SS-related genes, including *tssH* and *hcp*, showed higher expression in NN14 (Table 1). These results indicate that LTTR modulates multiple *Pcal* virulence factors. Many LTTRs are considered global transcriptional regulators, acting as either activators or repressors of single or operonic genes [25,38,39]. Modrzejewska et al. (2021) revealed that BsrA in *Pseudomonas aeruginosa* PAO1161 directly regulates 35 genes and indirectly influences many others [19,40]. Additionally, PhcA in *R. solanacearum* GMI1000 affects approximately 30% of the genes in its genome either directly or indirectly [41]. Collectively, LTTRs function as activators or repressors for a wide range of genes, controlling various cellular processes, including virulence.

T3SS-related gene expressions were up-regulated in minimal medium in WT *Pcal* compared to NN14 (Figure 2A,B). Conversely, there was no significant difference in gene expression patterns between the WT and NN14 in nutrient-rich medium (Figure 2A,B). Previous research has highlighted the relationship between the T3SS and environmental conditions in *P. syringae*, showing that T3SS is activated under nutrient-deficient, low-osmotic, and acidic conditions (e.g., minimal medium) but suppressed under high-pH, high-osmolarity, and nutrient-rich conditions (e.g., KB medium) [4]. Moreover, Rashid et al. (2016) showed that GamE, one of the putative LTTRs, positively regulates T3SS via two key *hrp* regulators in *Xanthomonas oryzae* pv. *oryzae* in nutrient-poor synthetic medium [30]. Nutrient-poor conditions mimic the condition of leaf surfaces and intercellular apoplastic space. Indeed, T3SS-related gene expressions in NN14 showed significantly lower expression compared to those in WT in planta (Figure 3). Previous studies have shown that the regulation of T3SS-related genes by LTTRs in planta and their contribution to virulence. For example, the *R. solanacearum* Δ*prhO* mutant exhibited significantly reduced T3SS expression and impaired virulence in tomato and tobacco plants [29]. Perrier et al. (2018) revealed that

T3SS-related genes are induced by PhcA in planta [41]. Together, LTTR regulates bacterial virulence factors, including T3SS, by responding to nutrient conditions, leading to bacterial pathogen virulence.

Our RNA-seq and RT-qPCR analyses in minimal medium revealed that LTTR regulates various genes, including *fliK* and *corR*, in addition to T3SS-related genes. However, the LTTR mutation did not affect swimming ability (Supplementary Figure S2) or COR production (Supplementary Figure S3). Additionally, there was no significant difference in the expression levels of these genes in planta (Figure 3). These results suggest that the significantly reduced T3SS expression in planta is likely the primary cause of the reduced virulence observed in the NN14 mutant. Fan et al. (2020) reported that CrgA, another LTTR, represses cell motility in *R. solanacearum*, thereby influencing disease development in host plants [42]. These results indicate functional diversity and differential contributions to virulence among various LTTRs. Further investigations under different environmental conditions and exploration of other phenotypes are required to more precisely elucidate the function of LTTR.

Targeting bacterial virulence factors is a promising strategy to combat drug resistance. Recent studies have highlighted LTTRs as potential drug targets, as several LTTRs play essential roles in regulating the expression of virulence factors [19]. In *Pcal*, LTTRs control the T3SS and contribute to bacterial virulence, making them attractive candidates for drug development. Typically, ligand or co-inducer binding to the C-terminal domain of LTTRs facilitates RNA polymerase recruitment, initiating transcription [25,43]. Thus, one potential strategy is to develop antagonists that block LTTR ligands, thereby inhibiting LTTR function [43]. For example, ligand analogs targeting MvfR, an LTTR from *P. aeruginosa*, have already been synthesized [44,45]. Additionally, Mandal et al. (2016) identified ribavirin as an inhibitor that binds to AphB, an LTTR from *Vibrio cholerae*, at its ligand/co-inducer-binding pocket [43]. The computational and screening approaches used in these studies could be applied to develop inhibitors targeting LTTRs in *Pcal*, offering a promising avenue for future research.

5. Conclusions

In conclusion, our findings suggest that LTTR functions as a global regulator, responding to nutrient availability and facilitating the adaptation of *Pcal* to changing environments. Notably, mutations in LTTR genes resulted in reduced expression of T3SS-related genes in planta, indicating that LTTR plays a crucial role in promoting the expression of these genes during infection, thereby contributing to *Pcal* virulence. Further research is required to elucidate the complex regulatory mechanisms of LTTR, with the ultimate aim of developing novel drugs that inhibit its function.

Supplementary Materials: The following supporting information can be downloaded at the following website: https://www.mdpi.com/article/10.3390/bacteria3040033/s1, Figure S1: Bacterial populations in vitro; Figure S2: Swimming motility assay; Figure S3: Coronatine quantification; Table S1: Primer sets used in this study.

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