A NOVEL BACTERIAL LUXR-TYPE REGULATOR INVOLVED IN INTERKINGDOM SIGNALING BETWEEN PLANT GROWTH PROMOTING BACTERIA AND PLANTS

A Dissertation

by

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ABSTRACT

Difference with transcriptional regulators in bacterial quorum sensing systems containing a paired LuxR transcriptional regulator and a LuxI cognate signal synthase, solo LuxRs are not associated with signal synthase and have been shown to bind to exogenous signals. LuxR solos are common in plant-associated bacteria and increasingly recognized as playing important roles in plant-microbe interkingdom signaling. I identified the plant LuxR solo, PcsR2, in the plant growth-promoting species *Pseudomonas chlororaphis* 30-84 that was highly upregulated when bacteria were grown on plant roots or in root macerates. The encoding gene *pcsR2* is part of a chromosomal region containing 11 genes in three operons that is highly conserved among *P. chlororaphis* species and not other *P*.spp. Bioinformatic analysis suggests that the adjacent operons are involved in fatty acid desaturation, a function typically related to cell membrane fludity, and therefore, may be involved in bacterial adaptation to the plant niche.

Chapter 2 demonstrated that expression of *pcsR2* and genes encoding two fatty acid desaturases in adjacent operons were upregulated when this strain was grown on wheat roots and *pcsR2* responded specifically to root-derived substrates as compared to leaf-derived substrates. Moreover, a *pcsR2* mutant was impaired in the ability to utilize root nutrients and to colonize wheat roots and in bacterial phenazine production, a trait required for effective root colonization, biofilm production and biological control activity. I hypothesize that these changes are related to alterations in cell membrane fluidity, which may affect the activity of membrane bound transporters and regulators. Consistent with

this hypothesis, the activity of a two-component system with a membrane-located signal-sensor controlling the phenazine regulatory network was reduced in the *pcsR2* mutant, and expression of phenazine regulatory genes *in trans* restored phenazine production.

Chapter 3 demonstrates that mutation of *pcsR2* led to altered fatty acid production, and resulted in modified altered anisotropy consistent with alterations in cell membrane fluidity. Expression of two desaturases *in trans* partially demonstrated their role in these phenotypes. Mutation of *pcsR2* also affected stress tolerance. Preliminary evidences in Appendix suggest broader effects of *pcsR2* mutation on enzyme secretion, nutrient uptake and utilization, and plant immunity response, but require further study.

DEDICATION

To my parents, Wenhe Pan and Huawei Zhang, for all their love and support for my education in spite of poor condition

To my children, Denong Liu and Derong Liu, for the strength they provide me as a parent. May this dissertation show them that nothing is impossible if you have a dream and work hard towards it.

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Contributors

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NOMENCLATURE

2OHPCA	2-hydroxy-phenazine-1-carboxylic acid
2OHPZ	2-hydroxy-phenazine
AdoMet	S-adenosyl-methionine
AHL	*N*-Acyl homoserine lactone
ACP	Acyl carrier protein
Ap	Ampicillin
CDP-DAG	Cytidine diphosphate diacylglycerol
cFA	Cyclopropane fatty acid
CFU	Colony forming unit
Cyclohex	Cycloheximide
DAPG	2,4-diacetylphloroglucinol
DNA	Deoxyribonucleic acid
eDNA	extracellular DNA
EPS	Extracellular polysaccharide
Gm	Gentamicin
GFP	Green fluorescent protein
Ggt	*Gaeumannomyces graminis* var. *tritici*
HSAF	Heat-stable antifungal factor
HCN	Hydrogen cyanide
HTH	Helix-Turn-Helix
HIR	Herbivore-induced resistance

ISR	Induced systemic resistance
IAA	Indole-3-acetic acid
JA	Jasmonic acid
Km	Kanamycin
LB	Luria-Bertani
LM	Leaf macerate
LPS	Lipopolysaccharides
ML	Maximum likelihood
NCBI	National Center for Biotechnology Information
OHHL	N-(3-oxohexanoyl)-L-homoserine lactone
OD	Optical density
PAS	Per-ARNT-Sim
PBS	Phosphatebuffered saline
PC	Phosphatidylcholine
PCA	Phenazine-1-carboxylic acid
PCN	Phenazine-1-carboxamide
Pcs	Phosphatidylcholine synthase
PG	Phosphatidylglycerol
PE	Phosphatidylethanolamine
PmtA	Phospholipid N-methyltransferases
RFU	Relative fluorescence units
PGPR	Plant Growth-Promoting Rhizobacteria

pip	Proline iminopeptidase
PPMD	Pigment Production Medium-D
QS	Quorum sensing
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
RM	Root macerate
Rif	Rifampicin
ROS	Reactive oxygen species
SA	Salicylic acid
SAR	Systemic acquired resistance
TCST	Two-component signal transduction
Ti	Tumor inducing
UFA	Unsaturated fatty acids
VOC	Volatile organic compound
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

1.1. General Introduction

Prokaryotes and eukaryotes have cohabited and been co-exposed to each other's metabolites for billions of years (Gonzalez and Venturi, 2013). It has been postulated and confirmed that organisms from the different kingdoms have and continue to acquire mechanisms to sense and respond to signaling molecules from one another. This extensive cross-talk between prokaryotes and eukaryotes is termed interkingdom signaling (Shiner et al., 2005; Rumbaugh, 2007; Hughes and Sperandio, 2008) and plant-microbe interkingdom signaling is a rapidly emerging field (Gonzalez and Venturi, 2013; Kan et al., 2017).

Diverse microbes (bacteria, fungi, oomycetes) live in the nutrient-rich rhizosphere, a small-scale ecosystem described by Lorenz Hiltner in 1904(Hiltner, 1904), which consists of roots and the millimeters of surrounding soil that are influenced by plant root exudates (Bais et al., 2006; Hartmann et al., 2008). It is becoming increasingly clear that plants actively recruit a rhizosphere microbiome via purposeful exudation of carbon and nitrogen, and that plants benefit from the activities of plant growth promoting rhizosphere colonizing bacteria (PGPR) (Bakker et al., 2012; Berendsen et al., 2012; Pascale et al., 2020). In this specific ecological niche, it is hypothesized that plants and microbes efficiently communicate via chemically-mediated interkingdom signaling, shaping their evolution, fitness and contemporary ecology (Zhalnina et al., 2018). Research for sustainably improve plant yields has traditionally focused on plant breeding, however

increasingly efforts to manipulate plant-associated microbial communities to improve biotic and abiotic stress tolerance are being considered (Kumari et al., 2019). I hypothesize that at the heart of finding more effective ways to utilize PGPR is a better understanding of the mechanisms underlying how PGPR sense and respond to plant-derived signals to regulate the expression of traits involved in plant colonization and plant growth promotion.

To date, the most well characterized examples of interkingdom signaling between plants and microbes are those involved in the formation of intimate symbioses or pathogenesis between agrobacteria and host plants. For example, in response to the production of flavonoid signals by legumes, rhizobia produce signaling molecules known as Nod factors (lipochitooligosaccharides) required for rhizobial nodulation (Schultze and Kondorosi, 1998). Similarly, mycorrhizal fungi produce Myc factors (a mixture of sulphated and non-sulphated simple lipochitooligosaccharides) needed for mycorrhization in response to plant strigolactone signals (Parniske, 2008; Maillet et al., 2011). The interkingdom signaling between *Agrobacterium* and their host plants resulting in deoxyribonucleic acid (DNA) transfer is also well-characterized (Subramoni et al., 2014). Upon the perception of plant-derived phenolic compounds, the expression of virulence genes on Tumor-inducing (Ti) plasmid in *Agrobacterium* is induced and T-DNA is integrated into the plant nucleus. The expression of genes in T-DNA within the plant leads to the production of opines, which then activate genes for opine metabolism, as well as the TraR/TraI quorum-sensing (QS) system in *Agrobacterium* to further promote virulence and tumor inducing (Ti) plasmid conjugation. Moreover, increasingly new plant-derived

molecules (e.g. flavonoids, rosmarinic acid) are identified as signals for plant-microbe interactions continue to be discovered (Keshavan et al., 2005; Vandeputte et al., 2010; Corral-Lugo et al., 2016).

In addition to the interkingdom chemicals above, studies have demonstrated that signals used by bacteria to communicate among themselves to coordinate population level gene expression via quorum sensing, have also been co-opted into roles in plant-bacteria communication (described in detail below). For example, in Gram negative bacteria *N*-acyl homoserine lactones (AHLs) are the primary signal molecule used for quorum sensing regulation of bacterial traits involved in plant pathogen virulence, inhibition of plant pathogens, activation of induced systemic resistance (ISR) in plants, modulation of plant hormone and stress response, and plant growth promotion (Pierson and Pierson, 2007; Venturi and Fuqua, 2013; Pieterse et al., 2014). The prototypical AHL-based QS system consists of a LuxI-type AHL synthase that produces an AHL signal and a cognate LuxR-type transcriptional regulator that senses the signal and regulates the expression of target genes **(Figure 1.1A).** Once the intracellular concentration of the signal exceeds a specific threshold, the signal binds to the LuxR, which in turn binds to a relatively conserved DNA sequence known as the *lux* box in the promoter region of regulated genes, activating or repressing their expression (Waters and Bassler, 2005). Quorum sensing was first identified in *Vibrio fischeri*, where LuxR binds the signal and directly activates transcription of the *luxICDABE* operon, resulting in an exponential increase in the production of both the signal and bioluminescence (Nealson and Hastings, 1979; Engebrecht et al., 1983; Fuqua and Greenberg, 2002). Interestingly,

plant-derived AHLs or AHL-mimics can stimulate or repress QS-regulated responses in plant-associated bacteria (Corral-Lugo et al., 2016). These AHLs are produced by a variety of plants such as peas, rice, soybean, tomato and crown vetch, to name a few (Teplitski et al., 2000; Daniels et al., 2002; Teplitski et al., 2004).The production of AHL mimics by plants highlights the importance of LuxR-based signal-response regulators as contributing to the recognition of the chemical language involved in interkingdom communication (Loh et al., 2002).

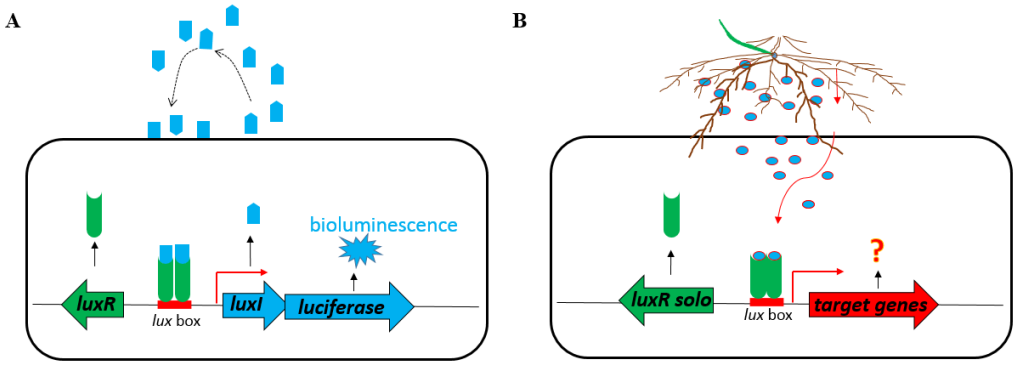

Figure 1.1 Comparion of prototypical LuxR/LuxI Quorum Sensing and the hypothetical function of LuxR solos in *P.chlororaphis*.
(A) The protypical LuxR/LuxI Quorum Sensing system in *Vibrio fischeri* which is involved in regulation of genes encoding luciferase. **(B)**The hypothetical function of LuxR solos in *P.chlororaphis*, which may regulate target genes involved with rhizosphere survial and growth promotion.

LuxR solos comprise an interesting subgroup of LuxR regulators that lack a cognate signal synthase gene located with the gene encoding the LuxR, and recent observations suggest that LuxR solos are widespread among Gram negative bacteria and may comprise the majority of annotated *luxR* genes in sequenced genomes (Hudaiberdiev et al., 2015). LuxR solos having a typical AHL binding domain may respond to signals produced by an AHL QS system within the same cell or to an external signal, which is not necessarily an

AHL type molecule (Gonzalez and Venturi, 2013; Venturi and Fuqua, 2013). For example, the few well-characterized LuxR solos in plant-associated bacteria that have typical QS autoinducer-binding domains such as LesR, PipR, PsoR, NesR, XccR and OryR have one or two amino acid substitutions within the autoinducer-binding domain and do not respond to AHLs. Additionally, a number of atypical LuxR solos have been characterized that retain the Helix-Turn-Helix (HTH) DNA-binding at the C terminus, but have different types of signal receiver domains, indicating the potential for responding to an even broader array of signals (Brameyer et al., 2014; Hudaiberdiev et al., 2015).

My dissertation research focuses on unveiling the mechanisms underlying how plant growth-promoting rhizobacteria (PGPR) sense and respond to plant signals as well as regulate bacterial expression of traits important for plant growth promotion. The focal point will be on LuxR solos. My experimental system utilizes the well-characterized phenazine-producing PGPR *Pseudomonas chlororaphis* 30-84 (*P. chlororaphis* 30-84), which has been used as a biological control agent of take-all disease of wheat, caused by the fungus *Gaeumannomyces graminis* var. *tritici* (Ggt). Wheat is grown on more land area than any other commercial food crop and has been one of the leading sources of human food (Curtis et al., 2002). However, take-all disease attacks most varieties of wheat and is the most important soil-borne wheat root disease throughout the world (Freeman and Ward, 2004). Management of root diseases via biological control agents such as *P. chlororaphis* 30-84 represents an important biotechnology for suppressing these diseases while minimizing chemical inputs. The availability of a complete genomic sequence of *P. chlororaphis* 30-84 makes it possible to bioinformatically identify the potential LuxR

candidate genes and allow the creation of reporters to investigate the relationship between wheat signals and LuxR-mediated responses by *P. chlororaphis* 30-84 at the species level or in multi-trophic interactions at the community level. Molecular cloning and biochemical characterization of identified candidate genes hold significant promise to reveal novel components of interkingdom signaling and provide enriched genetic resources for manipulation of crops with broad-spectrum and durable resistance offered by PGPR.

The rationale for my research is that phenazine-producing bacteria such as *P. chlororaphis* 30-84 typically increase in take-all-suppressive soils especially those formed under dryland production (Mavrodi et al., 2012a; Mavrodi et al., 2012b; Parejko et al., 2012) and thus appear to be vital components of disease suppressive rhizosphere communities. Coordinated signal exchange and response between wheat and phenazine-producing bacteria during suppressive soil formation is likely to be crucial for the establishment, structure, and function of rhizosphere communities that result in collaborative defense against biotic and abiotic stresses in the environment.

The central hypothesis is that wheat secretes small molecules that serve as interkingdom signals to alter gene expression patterns in *P. chlororaphis* 30-84, and that this PGPR in turn senses and responds to the signals to provide a favorable environment for plant growth **(Figure 1.1B)**. **The overall objectives** of my research are to characterize the function of LuxR-type proteins in *P. chlororaphis* 30-84 in sensing and responding to interkingdom signals from wheat and based on this, to elucidate the molecular mechanisms influencing plant-microbe interactions **(Figure 1.1B)**. **The long-term goal** is

to improve our understanding of communication networks between wheat and phenazine-producing microbes, which may lead to more precise and effective application of plant-beneficial microbes for pathogen suppression and disease control. It may also help to develop novel management strategies for sustainable agriculture such as wheat breeding to utilize the phenazine-producing members of the rhizobiome more effectively.

1.2. Literature review

1.2.1. Root exudate and its function on plant-microbe interaction

Plants root continuously produce and secrete an enormous range of potentially valuable metabolites as root exudates into the rhizosphere. The majority of the well-studied interkingdom signals (e.g. AHL mimic, phenolic and flavonoids) have been identified from root exudates. Root exudates are comprised of a broad variety of metabolites (Dennis et al., 2010) and these are often divided into two classes. One is low-molecular weight compounds from plant primary metabolism (e.g., organic acid, sugars, and amino acids, nucleotides, fatty acids) and secondary metabolism (e.g., alkaloids, terpenoids, vitamins and phenolic), whereas the other is high-molecular weight compounds such as mucilage (polysaccharides) and proteins (Badri and Vivanco, 2009; Ahemad and Kibret, 2014). Roots release compounds via two main mechanisms. The first mechanism involves transport of root exudates across the cellular membrane and secretion into the surrounding rhizosphere. In the second mechanism, plant products are released from root border cells (or cells that function like root border cells), which separate from roots as they grow (Hawes et al., 2000; Vicré et al., 2005).

It has been estimated that plant roots release 20-40% of their photosynthetic fixed carbon as primary or secondary metabolites to the rhizosphere (Badri and Vivanco, 2009). The metabolic diversity in the qualitative and quantitative composition of plant exudates may be influenced at the level of plant species, cultivar, developmental stage, and/or by various environmental factors (Vacheron et al., 2013; Huang et al., 2014). These biologically active compounds come at a significant cost in terms of plant carbon and nitrogen, indicating their potential evolutionary significance for conditioning the rhizosphere. The desired outcome of this chemical language is the attraction and promotion of populations of plant-beneficial microbes, while deterring plant-detrimental microorganisms. It is becoming evident that plant exudates contribute to the establishment and structure of the community of organisms living in the rhizosphere (Leach et al., 2017; Zhalnina et al., 2018).

1.2.2. Plant growth-promoting rhizobacteria

The term plant growth-promoting rhizobacteria (PGPR) was coined by Kloepper and Schroth in 1978 to describe rhizosphere bacteria that colonize the roots of plants and stimulate plant growth (Kloepper, 1978). Although a few researchers classify rhizobia and mycorrhizal fungi that form intimate symbioses with plants as PGPR, here my research focuses on plant-beneficial plant-symbiotic PGPR that colonize the rhizosphere, rhizoplane, and/or root cortex, but do not form specific symbiosis structures. A large number of bacterial taxa have been explored for their potential application as PGPR including, but not limited to Gram negative species: *Alcaligenes*, *Azospirillum* *Azotobacter*, *Chryseobacterium*, *Enterobacter*, *Klebsiella*, *Pseudomonas*,

Phyllobacterium , *Sphingomonas*, *Serratia* and Gram positive species: *Arthrobacter*, *Bacillus* and *Streptomyce* (Vejan et al., 2016).

Plant-beneficial PGPR has been shown to promote improvements in plant productivity and yield in a variety of important food and fiber crops (Zakry et al., 2012; Vejan et al., 2016; de Souza Vandenberghe et al., 2017; Kumari et al., 2019). PGPR may promote plant growth via direct effects on plant growth, development, and/or biotic and abiotic stress tolerance pathways, or indirectly via improvements in the rhizosphere environment via inhibition of pathogens or amelioration of stressful conditions (Saleem et al., 2007; Gupta and Dikshit, 2010; Etesami and Maheshwari, 2018; Yuan et al., 2020), although sometimes the distinction between direct and indirect effects may not be clear cut (Goswami et al., 2016). The bacterial services provided by PGPR have been reviewed extensively (Lugtenberg et al., 2001; Persello-Cartieaux et al., 2003; Lugtenberg and Kamilova, 2009; Backer et al., 2018; Kumari et al., 2019), but some aspects will be described further in the next sections.

1.2.2.1. Mechanisms of plant growth promotion

Mechanisms that directly promote plant growth can be demonstrated in the absence of other rhizosphere microbes (e.g. plant pathogen) (Mustafa et al., 2019). These modes of action include enhancement of nutrient availability or uptake and/or the production of phytohormones, volatile organic compounds (VOC) or other interkingdom signals that directly influence plant growth and development. PGPR providing these functions are typically labelled as biofertilizers and biostimulants.

For example, some plant-associated, non-symbiotic PGPRs are able to fix nitrogen via nitrogenase enzyme activity, providing additional nitrogen to the plant (Zakry et al., 2012; Ahemad and Kibret, 2014; Goswami et al., 2016). Multiple bacterial and cyanobacteria taxa have been shown to be capable of providing this service including: *Azoarcus*, *Azotobacter*, *Azospirillium*, *Bacillus*, *Burkholderia*, *Diazotrophicus*, *Enterobacte*r, *Gluconobacterium*, *Herbaspirillum*, *Paenibacillus*, *Pseudomonas* and *Anabaena* and *Nostoc*, respectively. Many PGPR also secrete organic acids (e.g. acetic, lactic, malic, succinic, tartaric, gluconic, 2-ketogluconic, oxalic and citric acids) and phosphatases (e.g. *Bacillus* and *Pseudomonas*) that convert organic forms of phosphorus to plant-available forms such as $HPO4^{2-}$ or H_2PO4^{-} by hydrolyzing the phosphoric esters (Rodríguez and Fraga, 1999; Sharma et al., 2013). Similarly, organic acids produced by PGPR solubilize potassium from rock (Han et al., 2006). PGPR also produce siderophores that chelate the insoluble iron in natural environments and transport it into the plant cells (Sarode et al., 2007). In addition to iron, bacterial siderophores can chelate other heavy metals, relieving stresses by metal toxicity (Rajkumar et al., 2010). Also, PGPR were reported to increase Zn uptake, and thus increase yield in crops (Ramesh et al., 2014).

Another direct mechanism used by PGPR for plant growth promotion involves the bacterial production of plant phytohormones. For example, most PGPR produce auxin/indole-3-acetic acid (IAA), which can augment endogenous auxin production in the roots. Typically this results in increased root surface and length, therefore plants have greater access to soil water and nutrients (Glick, 2012). Similarly cytokinin produced by bacteria and gibberellins produced by fungi have been shown to promote plant growth and

development (Nelson and Steber, 2016). For example, lettuce inoculated with *Bacillus subtilis* after 2 days led to approximately 10 times higher accumulation of cytokinin (e.g zeatin and its derivatives) in roots and shoots than controls, thereby increasing root and shoot weight nearly 30% after 8 days (Arkhipova et al., 2005). Tomato plants inoculated with the gibberellin-producing *Sphingomonas* sp. have a significant increase in various growth parameters such as shoot length, chlorophyll content, and shoot and root dry weights (Khan et al., 2014). In addition, 1-aminocyclopropane-1-carboxylate (ACC) deaminases produced by PGPR can degrade ACC, the immediate precursor of ethylene, releasing the inhibition of plant growth from ethylene under stress conditions (Nadeem et al., 2014). In addition to the phytohormone, PGPR may produce other signal molecules to improve plant growth and/or stress-tolerance. For example, VOCs (e.g. polyamines, 2,3-butanediol and acetoin) produced by *Bacillus* spp. result in significant growth promotion in *Arabidopsis* (Ryu et al., 2003; Zhou et al., 2016). It has been suggested that bacterial AHL production also may affect the expression of genes in host plants in a global manner, changing the accumulation of a variety of proteins such as auxin-responsive and flavonoid synthesis proteins (Mathesius et al., 2003).

Mechanisms by which PGPR promote plant growth and development indirectly result from improvements in the rhizosphere environment via inhibition of pathogens, or amelioration of stressful conditions. PGPR involved in disease control are typically labeled biocontrol agents and these agents or the microbial products they produce are regulated as biopesticides. Common PGPR taxa involved in biological control include *Bacillus*, *Burkholderia*, *Enterobacter*, *Herbaspirillum*, *Ochrobactrum*, *Pseudomonas*,

Serratia, *Staphylococcus*, and *Stenotrophomonas* (Mustafa et al., 2019). Common antagonistic metabolites produced by PGPR include antibiotics, hydrolytic enzymes and bacteriocins (Beneduzi et al., 2012) and common classes of antibiotics include phenazines, pyoluteorin, pyrrolnitrin, cyclic lipopeptides, 2,4-diacetylphloroglucinol (DAPG), hydrogen cyanide (HCN) and rhamnolipid, which were selected for their ability to inhibit plant pathogens and suppress plant diseases (Haas and Keel, 2003) Common targets of biological control include diseases caused by *Pythium*, *Rhzoctonia*, and *Fusarium* (Haas and Défago, 2005; Lugtenberg and Kamilova, 2009; Tariq et al., 2017). Other antagonistic compounds released from PGPR include hydrolytic enzymes such as proteases, lipases, glucanases, chitinases and cellulases, which typically degrade pathogen cell walls and thus inhibit the growth and activity of phytopathogens (Neeraja et al., 2010). In addition, some PGPR such as *Bacillus cereus*, *Bacillus subtilis*, *Serratia marcescens*, *Enterobacter cloacae*, *Pseudomonas pyogenes*,and *Pseudomonas chlororaphis* produce bacteriocins like cerein, subtilin, marcescins, cloacins, pyocins, and tailocins, respectively, that inhibit closely related competitors (Cascales et al., 2007; Subramanian and Smith, 2015; Dorosky et al., 2017).

In addition to producing antibiotic compounds, PGPR can protect plants against pathogens by triggering induced systemic resistance (ISR) in plants. In the 1990s, it was discovered that host plants inoculated with PGPR such as *Pseudomonas* or *Serratia* acquired systemic disease resistance (reduction in severity or incidence of disease) to the subsequent pathogen attack and this PGPR induced defense was termed ISR (Kloepper et al., 1992; van Loon et al., 1998). Further studies revealed that ISR is often regulated by a

network of interconnected jasmonic acid/ethylene (JA/ET) signaling pathways, which share some signaling components with systemic acquired resistance (SAR) and herbivore-induced resistance (HIR)(Pieterse et al., 2014). The known elicitors triggering ISR include lipopolysaccharide (LPS), salicylic acid (SA), siderophore (e.g. pyoverdin), antibiotics (e.g. DAPG and pyocyanin), VOC (e.g 2R,3R-butanediol and acetoin), chitin, flagella, cyclic lipopeptide, biosurfactants, β-glucans, the antifungal factor DAPG, and AHLs (Lugtenberg and Kamilova, 2009; Glick, 2012; Pieterse et al., 2014).

PGPR have also been shown to promote plant growth under environmental stresses relying on similar signaling pathways (Goswami et al., 2016; Ahkami et al., 2017; Etesami and Maheshwari, 2018) or via the production of extracellular matrix, which may serve as a natural humectant that may improve water availability in the root rhizosphere through increased water retention (Chang et al., 2007). Certainly, PGPR may use one or more of these mechanisms to promote plant growth in the rhizosphere and/or to cope with biotic and abiotic stresses.

1.2.2.2. Importance of microbial response to plant signals

Essential for the effective delivery of services provided by PGPR to the host plant, PGPR must be able to sense and respond to the host plant. This includes the ability of PGPR to effectively find and colonize plants (Chin-A-Woeng et al., 2000). Successful colonization is a multi-genic process and a variety of bacterial traits and specific genes have been identified that contribute to this process (Benizri et al., 2001; Lugtenberg et al., 2001; Persello-Cartieaux et al., 2003; Lugtenberg and Kamilova, 2009; Compant et al., 2010). For example, chemotactic motility (e.g. swimming, swarming, gliding and

twitching) driven by flagellar, pili or fimbria is a prerequisite for PGPR to migrate to the rhizosphere and root (de Weert et al., 2002). Some plant-produced chemo-attractants for PGPR include L-leucine and dicarboxylic acids, L-malic acid, L-malate, citric acid, and fusaric acid (de Weert et al., 2002; de Weert et al., 2004; Rudrappa et al., 2008). After arriving in the rhizosphere, PGPR must be able to propagate and adapt to the new environment. These traits correspond to the formation of biofilms and the production of secondary metabolites and are often regulated by quorum sensing. In addition, other traits include the ability to utilize root exudates as nutrients and alter the outer membrane or cell surface components to adapt to the rhizosphere environment and/or escape host defenses (Compant et al., 2010). Interestingly, a recent analysis of genes in *Pseudomonas simiae* required for colonization of *Arabidopsis thaliana* roots by sequence-driven saturation mutagenesis revealed a broad array of genetic determinants of plant root colonization genes purported to have roles on motility, carbon metabolism, cell wall biosynthesis, amino acid transport and metabolism, and other unknown functions (Cole et al., 2017). Previous work has also shown that root colonization of bacteria is affected by a variety of other environment factors like soil texture, chemistry, and structure affecting water infiltration and nutrient availability (Liddell and Parke, 1989; Ownley et al., 1991). Thus, a thorough understanding of the mechanisms by which microbes sense and respond to their environment is of general importance to utilize the beneficial function of PGPR efficiently and reliably.

1.2.2.3. Pseudomonas PGPR

Pseudomonas spp. are widespread and account for a sizable portion of the microbe

in diverse environments due to their utilization of a broad variety of organic compounds and production of versatile secondary metabolites (Loper et al., 2012). Many *Pseudomonas* strains isolated from plant roots or rhizospheres have been utilized as PGPR especially for biological control and many of these were isolated from naturally disease suppressive soils (Weller and Cook, 1983; Vincent et al., 1991; Schroth and Hancock, 1982; Weller et al., 2002). Common taxa of *Pseudomonas* PGPR include *P. fluorescent, P. chlororaphis, P. protegens, and P. brassicacearum,* and as much as one half of the genes may be shared across these genomes (Loper et al., 2012).

The majority of *Pseudomonas* biological control PGPR produce antibiotics, which contribute to the suppression of a variety of soil-borne diseases. For example, pyoluteorin and oomyxin A produced by *P. fluorescens* Pf-5 and *P. fluorescens* Hv 37, respectively, were correlated with suppression of damping off disease caused by *Pythium ultimum* (Howell and Stipanovic, 1980; Howie and Suslow, 1991); cyanide produced by *P. fluorescens* CHA0 was shown to be involved in suppression of black root rot caused by *Thielaviopsis basicola* (Voisard et al., 1989); 2,4-diacetylphloroglucinol (DAPG) produced *P. fluorescens* CHA0 and *P. fluorescens* Q2-87 contributed to suppression of take-all disease caused by Ggt (Keel et al., 1991; Vincent et al., 1991); and phenazine production by *P. fluorescens* 2-79 and *P. chlororaphis* 30-84 also contributed to the suppression of take-all disease(Ownley et al., 1992; Pierson and Thomashow, 1992).

P. chlororaphis 30-84 and phenazine production

P. chlororaphis 30-84, the focus of this study, is a well-characterized PGPR strain isolated from the roots of wheat grown in a take-all disease suppressive soil(Pierson and

Thomashow, 1992; Pierson et al., 2013). Production of phenazines by *P. chlororaphis* 30-84 was shown to be the primary mechanism of disease suppression (Pierson and Weller, 1994; Pierson and Pierson, 1996). Phenazine production by *P. chlororaphis* 30-84 is necessary for effective inhibition of the take-all pathogen Ggt (Pierson and Thomashow, 1992), persistence of *P. chlororaphis* 30-84 in the wheat rhizosphere (Mazzola et al., 1992), biofilm formation (Maddula et al., 2006), and wheat seedling drought tolerance (Mahmoudi et al., 2019).

Phenazines comprise a large group of nitrogen-containing, heterocyclic secondary metabolites naturally produced by a diversity of prokaryotes: mainly *Pseudomonas* and a few other bacterial taxa including *Brevibacterium, Burkholderia, Erwinia, Methanosarcina, Mycobacterium, Nocardia, Sorangium, Streptomyces, Pectobacterium, Pelagiobacter,* and *Vibrio* (Turner and Messenger, 1986; Pierson and Pierson, 1996; Mavrodi et al., 2006; Mentel et al., 2009; Mavrodi et al., 2010; Pierson and Pierson, 2010). Phenazines differ from one another in the type of functional groups attached to the core ring structure and due to the sharing of electrons across the ring structure exhibit a variety of brilliant colors. Phenazines are the products of the shikimic acid pathway and chorismic acid is the branch point where phenazine and aromatic acid biosynthesis are diverted. Elegant work demonstrated that phenazine production in bacteria is catalyzed by a conserved operon *PhzABCDEFG*, which encodes the enzymes for the biosynthesis of the core phenazine, phenazine carboxylic acid (PCA), and many bacteria produce several phenazines from PCA via the activity of terminal-modifying enzymes (Mavrodi et al., 2001). For example, PhzH in *P. chlororaphis* PCL1391 converts PCA into phenazine-1-

carboxamide (PCN)(Chin-A-Woeng et al., 2001); PhzO in *P. chlororaphis* 30-84 converts PCA into 2-hydroxy-phenazine-1-carboxylic acid (2OHPCA) and 2-hydroxy-phenazine (2OHPZ) (Delaney et al., 2001); PhzM, PhzS, and both in *P. aeruginosa* PAO1 converts PCA into 5-methyl-phenazine-1-carboxylic acid (5MPCA), 1-hydroxy-phenazine (1OHPZ) and pyocyanin (PYO), respectively (Mavrodi et al., 2001). Different phenazines inhibit a different spectrum of bacteria, fungi, and even higher plants and animals (Toohey et al., 1965; Yu et al., 2018a). The antibiotic action of phenazines relates to the production of reactive oxygen species (ROS) which may decouple oxidative phosphorylation in the host organism (Baron et al., 1989; Mavrodi et al., 2006; Das and Manefield, 2012). In addition to pathogenic inhibition, phenazines have been shown to play roles inROS generation, electron shuttling, and iron chelation in other *Pseudomonas* species (Pierson and Pierson, 2010; Wang et al., 2010; Wang et al., 2011; Recinos et al., 2012; Das et al., 2015). Studies also have shown that the production of phenazines has broad transcriptomic consequences (Dietrich et al., 2006; Wang et al., 2016). For example, comparative transcriptomic analysis of wild type and phenazine biosynthetic mutants in *P. chlororaphis* 30-84 revealed that a total of 802 genes were differentially expressed in the phenazine-producing and non-producing derivatives (Wang et al., 2016).

1.2.2.4. Phenazine biosynthesis and regulation in P. chlororaphis 30-84

P. chlororaphis 30-84 produces PCAas well as 2-hydroxy-PCA (2OHPCA), and a small amount of 2-hydroxy-phenazine (2OHPZ) (Pierson and Thomashow, 1992). The operon for phenazine biosynthesis in *P. chlororaphis* 30-84 was originally characterized as *PhzXYZABCDE* and the modifying enzyme *phzO* converts PCA into 2OHPCA and

2OHPZ, which contributes to the bright orange color of *P. chlororaphis* 30-84 (Pierson and Thomashow, 1992; Pierson et al., 1995; Delaney et al., 2001; Mavrodi et al., 2006). Previous studies demonstrated that phenazine biosynthesis in *P. chlororaphis* 30-84 is regulated by a complex network of regulatory genes organized in a hierarchical manner (Pierson et al., 1998b; Whistler and Pierson III, 2003; Pierson and Pierson, 2010; Wang et al., 2012; Wang et al., 2013; Yu et al., 2018b). At the heart of this network is control by the LuxR-type transcriptional regulator (PhzR), part of the PhzR/PhzI quorum sensing (QS) system that directly regulates phenazine biosynthesis (Pierson et al., 1994; Wood and Pierson, 1996). A second set of QS system CsaR/CsaI, indirectly influences phenazine production (Zhang and Pierson, 2001). QS gene regulation is discussed further in the next section.

In addition to QS, phenazine biosynthesis is regulated by two, two component signal transduction (TCST) systems, GacS/GacA and RpeA/RpeB. TCST systems are widely distributed among bacterial taxa and account for a strikingly high proportion of the bacteria genome (2-3%) (Stover et al., 2000; Paulsen et al., 2005). TCST systems are normally comprised of a membrane-bound histidine kinase as a sensor for environment stimuli and a transcriptional response regulator. The GacS/GacA system was originally identified as global activator of cyanide in *Pseudomonas fluorescens* (Laville et al., 1992) and has been identified in a wide range of Gram-negative bacteria to control production of secondary metabolites, extracellular enzymes involved in plant-microbes interactions (Heeb and Haas, 2001). In *P. chlororaphis* 30-84, GacS/GacA regulate phenazine production post-translationally via the regulation of small non-coding RNA (Wang et al.,

2013) and mutations of either GacS (the signal sensor) or GacA (cytoplasmic response regulator) lead to deficiency in the production of phenazine as well as loss of protease, HCN, and the AHL signal (Chancey et al., 1999). Spontaneous mutants contribute to biofilm context and the survival of wild-type *P. chlororaphis* 30-84 in the wheat rhizosphere (Clancey et al., 2002; Driscoll et al., 2011). In the RpeA/RpeB system RpeA (repressor of pheanizne expression) is a membrane bound histidine kinase that deactivates RpeB (the cytoplasmic response regulator), which positively regulates phenazine production (Wang et al., 2012). RpeA/RpeB act upstream of other phenazine regulatory genes including the transcriptional regulator Pip (phenazine inducing protein) and PhzR (Whistler and Pierson III, 2003; Girard et al., 2006; Wang et al., 2012; Wang et al., 2013). Phenazine biosynthesis is also affected by mutations in the promotor region, and other genes that affect primary metabolism also affect phenazine biosynthesis (Yu et al., 2017; Yu et al., 2018b).

1.2.3. LuxR family regulators

1.2.3.1. LuxI/LuxR QS system

Bacteria have a delicate capacity to sense and detect a variety of alterations in the environment, and in response to such stimuli, bacteria initiate signal transduction pathways accordingly. This ability enables bacterial adaption and survival in complex and dynamic environments (Mukherjee and Bassler, 2019). QS is a well-known cell-cell signal transduction mechanism enabling microbes to coordinate group- behavior (such as production of secondary metabolites and biofilm, conjugation, and motility) via the production of small diffusible molecular signals in a cell density manner (Miller and

Bassler, 2001). QS systems have been identified in many Gram-positive and Gram-negative taxa and many species have more than one QS system (Waters and Bassler, 2005).

The paradigm QS system is comprised of two components LuxI/LuxR and was first characterized for control of bioluminescence in the marine symbiont *Vibrio fischer* (Nealson and Hastings, 1979; Fuqua et al., 1996). The two adjacent, divergently transcribed genes *luxI* and *luxR* encode an N-Acyl Homoserine Lactone (AHL) synthase, in this case, N-(3-oxohexanoyl)-L-homoserine lactone (OHHL), and a cytoplasmic transcriptional regulator, respectively (Eberhard et al., 1981; Engebrecht and Silverman, 1984). The substrates catalyzed by LuxI are produced from the cellular precursors S-adenosyl-methionine (AdoMet) and an acyl-Acyl Carrier Protein (acyl-ACP), an intermediate from the fatty acid biosynthesis pathway (Moré et al., 1996; Schaefer et al., 1996). The signal is freely diffusible through the cellular membrane and at low cell densities, signals are present at a low concentration, whereas at high cell densities they accumulate and then passively diffuse back into the bacterial cell. When the signal accumulates intracellularly to a threshold concentration, it binds to a LuxR protein and transforms the protein into dimers or multimers. The LuxR complex in turn binds to a 20-basepair palindromic DNA region (*lux* box) in the promoter of the adjacent *luxCDEFG* operon encoding bioluminescence and activates transcription exponentially (Engebrecht and Silverman, 1984). Most LuxR homologs bind DNA as dimers or multimers (Loh et al., 2002).

Since the discovery QS in *Vibrio fischer*, a wealth of LuxI/LuxR homologs have been investigated in a variety of microbes, controlling a broad range of microbial traits

involved in host-microbe pathogenic interaction (Pierson et al., 1998b). The prototypes include the LasR/LasI and RhlR/RhlI QS systems in the opportunistic human pathogen *P. aeruginosa,* which regulate the production of the phenazine pyocyanin in a hierarchical manner. In this system the LuxI homologs, LasI and RhlI catalyze the synthesis of two different AHLs, N-(3-oxododecanoyl)-homoserine lactone and N-(butyryl)-homoserine lactone, respectively (Schuster and Greenberg, 2006; Jimenez et al., 2012). Perhaps the most well studied QS system involved in microbe-plant interactions is TraR/TraI, regulating the Ti plasmid transfer genes in the plant pathogen *Agrobacterium tumefaciens.* In this system the TraI AHL synthase produces *N*-3-(oxooctonoyl)-L-homoserine lactone (3-oxo-C8-HSL), which interacts with TraR to activate transcription of the Ti plasmid-encoded *tra* genes as well as *traI*. Thus under high cell density this leads to autoinduction of the QS system (Fuqua and Winans, 1994). The QS systems ExpI/ExpR, CarI/CarR in the plant pathogen *Erwinia carotovora* causing soft-rot disease also are involved in regulation of genes important for virulence, including the production of celluloses and pectinases, enzymes involved in the degradation of plant cell walls, and carbapenem antibiotics, respectively (Andersson et al., 2000). Similarly, EsaI/EsaR in *Pantoea stewartii*, the causal agent of corn Stewart's wilt and leaf blight, regulate the production of capsular extracellular polysaccharide (EPS), a virulence factor, in response to the production of the AHL signal N-(3-oxohexanoyl)-L-homoserine lactone (von Bodman and Farrand, 1995).

QS also has been shown to be important for the regulation of the symbiosis trait between host and microbes. For example, RhiR/RhiI in the large symbiotic plasmid

pRL1JI as well as CinI/CinR in chromosome of *Rhizobium leguminosarum* regulate genes associated with root nodulation and survival (Daniels et al., 2002). As mentioned above, in the plant growth promoting rhizobacteria (PGPR) *P. chlororaphis* 30-84 the PhzR/PhzI system has been shown to regulate the production of phenazines, important for rhizosphere competence and pathogen inhibition.

Although QS is recognized as a mechanism used by bacteria to self-regulate bacteria traits, demonstrations of positive and negative cross-communication among bacteria in mixed populations, and between plants and microbes via the production of signal mimics or signal quenching mechanisms revealed that QS is a common intercellular language among bacteria and even between hosts and microbes. For example, using QS reporters it was shown that wheat root-associated bacteria can produce signals that positively or negatively affect PhzR/PhzI regulation of phenazine production in *P. chlororaphis 3*0-84 (Pierson et al., 1998a; Strauss, 1999; Morello et al., 2004). AHL mimics (e.g. furanones) produced by eukaryotes (e.g. algae and plants) were also shown to interfere with bacteria QS (Teplitski et al., 2000; Manefield et al., 2001). The production of AHL mimics by plants highlights the importance of LuxR-based signal-response regulators as contributing to the recognition of the chemical language involved in interkingdom communication (Loh et al., 2002)

1.2.3.2. LuxR solos

Although LuxR transcriptional regulators were first characterized as components of QS systems, it is now recognized that a majority of the annotated *luxR*-type genes do not have a *luxI* gene in proximity on the bacterial chromosome (Hudaiberdiev et al., 2015).

This type of unpaired LuxR is referred to as a LuxR solo (or orphan LuxR) (Fuqua, 2006; Subramoni and Venturi, 2009a). Similar to the LuxRs of two-component QS systems, typical LuxR solo regulators consist of about 250 amino acids and have an autoinducer-binding domain at the N-terminus and a conserved HTH DNA-binding motif at the C-terminus. As reported previously, LuxR-type proteins exhibit low identities (18–25%), however, in most QS LuxR-type proteins nine amino acid residues involved in autoinducer-binding and DNA-binding are highly conserved (Gonzalez and Venturi, 2013). Although not universally accepted, the definition of LuxR solo has been expanded to include atypical LuxR solos, e.g. LuxR-type receptors that do not have a classic autoinducer domain (Brachmann et al., 2013; Brameyer et al., 2015b). Atypical LuxR solos characterized to date retain the HTH DNA-binding at the C-terminus, but have either Per-ARNT-Sim (PAS) signal-sensing domains, REC (receiver) signal receiver domains, or unidentified domains in place of the autoinducer-binding domain at the N-terminus (Wang et al., 2006; de Bruijn and Raaijmakers, 2009; Brameyer et al., 2014). In the present study, in keeping with the broad definition of LuxR solos, typical and atypical solo LuxR-family homologs will be included in our discussion of LuxR solos regardless of the domains at the N-terminus. LuxR solos are believed to expand the regulatory repertoire of the prototypical QS systems, wherein the LuxR solos or the expression of their encoding genes respond to exogenously produced AHLs, signal-mimics, or as yet unidentified interkingdom signals (Teplitski et al., 2000; Subramoni and Venturi, 2009b; Martínez et al., 2015; Coutinho et al., 2018). It is believed that these widespread LuxR solos may mediate novel interkingdom communication, such as between symbiotic-living bacteria

and their hosts (Subramoni and Venturi, 2009a; Gonzalez and Venturi, 2013; Brameyer et al., 2015a).

To date, several LuxR solos in plant-associated bacteria are known to play roles in plant pathogenesis or symbiosis, and some of these LuxR solos or the expression of their encoding genes respond to plant signals. The genes encoding these LuxR solos are usually located on the bacterial chromosome in close proximity to a proline iminopeptidase (*pip*) gene (Gonzalez and Venturi, 2013), which releases an N-terminal residue from a peptide, typically a proline. The promoter region of *pip* in all these strains contains a 20 bp inverted repeat sequence (*lux* box). XccR in the plant pathogen *Xanthomonas campestris* pv. *campestris* was the first LuxR solo reported to respond to unknown plant signals and both XccR and Pip are important for plant virulence (Zhang et al., 2007). Likewise, in other pathogenic bacteria, LuxR solos such as OryR in *Xanthomonas oryzae* pv. *oryzae* (Ferluga et al., 2007; Ferluga and Venturi, 2009; González et al., 2013), XagR in *Xanthomonas axonopodis* pv. *glycines(Chatnaparat et al., 2012),* XocR in *Xanthomonas oryzae* pv. *oryzicola* (Xu et al., 2015), and PsaR2 in *P. syringae* pv. *actinidiae* (Patel et al., 2014) respond to plant signals and are involved in bacterial pathogenicity, mobility, or host colonization. The genes encoding the LuxR solo NesR in the symbiotic plant-beneficial bacteria *Sinorhizobium meliloti* (Patankar and González, 2009) and PipR in the cottonwood endophyte *Pseudomomas sp.* GM79 (Schaefer et al., 2016)are also associated with *pip* genes and these LuxR solos regulate genes that contribute to symbiosis traits including plant nodulation and plant carbon source utilization, respectively.

In comparison to the aforementioned plant-pathogenic and plant-symbiotic bacteria, the genomes of many well-studied PGPR contain numerous genes annotated as encoding solo LuxR-family homologs. For example the genomes of PGPR strains *P. chlororaphis* subsp. *aureofaciens* O6 and 30-84, *P. protegens* Pf-5, *P. brassicacearum* Q8r1-96, *P. fluorescens* Pf0-1, Q2-87, SBW25, A506, SS101 and *P. synxantha* BG33R (Loper et al., 2012) contain 12 to 28 solo *luxR*-type genes based on gene annotations in the National Center for Biotechnology Information (NCBI) database (https://www.ncbi.nlm.nih.gov/). Also, in comparison to the aforementioned examples, the context of the genes encoding the LuxR solos studied in PGPR thus far present distinctive features. For example, the promoter of the *pip* gene adjacent to *psoR* in *P. protegens* CHA0 and *P. protegens* Pf-5 lacks a *lux*-box palindrome (Subramoni et al., 2011) and there is no *pip* adjacent to *lesR* in *L. enzymogenes* (Qian et al., 2013) or *viscAR* and *viscBCR* in *P. fluorescens* SBW25(de Bruijn and Raaijmakers, 2009). LuxR-type regulators ViscAR and ViscBCR have a HTH DNA-binding domain but lack a classic autoinducer domain. All these LuxR solos activate genes important for biological control activity. For example, PsoR activates anti-microbial-related genes in response to plant signals from wheat and rice but not cucumber. LesR activates the production of heat-stable antifungal factor (HSAF) and ViscAR and ViscBCR regulate viscosin production important for antagonism of oomycete plant pathogens(de Bruijn and Raaijmakers, 2009; Qian et al., 2013).

Based on these few examples, I hypothesize LuxR solos may enable PGPR to sense and respond to interkingdom signals, facilitating bacterial adaptation to a symbiotic lifestyle and the expression of traits important for plant growth-promoting activities.

1.2.3.3. Investigations of LuxR-type regulators in *P. chlororaphis* 30-84

In the present study, I used bioinformatics to identify the LuxR-type regulators in the well characterized PGPR strain *Pseudomonas chlororaphis* subsp. *aureofaciens* 30-84 (hereafter *P. chlororaphis* 30-84). In my survey for potentially plant-responsive LuxR solo candidates in the genome of *P. chlororaphis* 30-84, I identified one atypical LuxR solo (PcsR2) based on the observation that *pcsR2* expression was highly upregulated when bacteria were grown on wheat roots compared to planktonic culture. In Chapter 2, I provide evidence that the expression of *pcsR2* and the adjacent operon respond to wheat root-derived signals rather than endogenous AHLs, and that a functional PcsR2 is required for this response. I describe bacterial phenotypes modulated by PcsR2. PcsR2 is involved in the expression of bacterial traits that contribute to the utilization of carbon and nitrogen from wheat roots, phenazine biosynthesis, and biofilm production, traits that promote the plant-associated lifestyle and plant growth promoting activity of the bacteria. I discuss the novelty of PcsR2 in relation to other known plant responsive LuxR solos. In Chapter 3, I provide evidence that PcsR2 is involved in cell membrane fluidity and show that deletion of *pcsR2* results in altered anisotropy (light scattering from the bacterial cell membrane) and altered fatty acid production. Key alterations include loss in the ability to produce two key unsaturated fatty acids 12:1 3OH and 16:1. Expression of the fatty acid desaturases encoded by Pchl3084_4801, not

Pchl3084_4803 *in trans* on a plasmid (under the control of a constitutive promoter) restored the 16:1w7c production in the *PcsR2* mutant. Additionally, the alteration of cell-membrane fluidity in the *PcsR2* mutant resulted in alterations in stress tolerances. In Chapter 4, I summarize my findings and provide thoughts on the impact of the work and future directions. I hypothesize membrane alteration provides a global mechanism for adaptation to the plant niche and the plant-associated life-style. Appendix 5 includes an appendix of preliminary data that I feel contribute to the story but require further investigation. For example, preliminary studies indicate PcsR2 regulation of fatty acid desaturation appears to be associated with plant immunity response. PcsR2 mutation also resulted in broad alterations in the capacity of the bacteria to utilizate amino acids, carbohydrate and especially fatty acid derivatives as nutrient.

CHAPTER II

PCSR2 IS A LUXR-TYPE REGULATOR THAT IS UPREGULATED ON WHEAT ROOTS AND IS UNIQUE TO *PSEUDOMONAS CHLORORAPHIS*[*]

2.1 Introduction

Albeit bacteria are unicellular organisms, they communicate with each other via small diffusible molecules to orchestrate their behaviors in a population density-dependent manner, which facilitates rapid adaptation to the environment (Fuqua et al., 1994; Mukherjee and Bassler, 2019). This cell-cell communication mechanism, known as quorum sensing (QS), enables populations to solve problems that single cells cannot, such as colonization, conjugation, biosynthesis of secondary metabolites, biofilm formation, and effective invasion of host organisms (Piper et al., 1993; Fuqua and Winans, 1994; Pierson and Weller, 1994; Wood et al., 1997; Maddula et al., 2006; Schuster and Greenberg, 2006). In Gram-negative bacteria the diffusible signals are primarily fatty acid-derived whereas in Gram-positive bacteria they are most often derived from small peptides (Waters and Bassler, 2005; Brameyer et al., 2015a). To date, QS systems utilizing *N*-acyl homoserine lactones (AHLs) are the most common in Gram-negative bacteria, although other types of QS signals continue to be discovered (Pierson and Pierson, 2007; Schaefer et al., 2008; Ahlgren et al., 2011; Brachmann et al., 2013; Brameyer et al., 2015b; Corral-Lugo et al., 2016; Papenfort et al., 2017). The prototypical AHL-based QS system

[*] Reprinted with permission from "PcsR2 is a LuxR-type regulator that is upregulated on wheat roots and is unique to *Pseudomonas chlororaphis*" by Pan, H., Pierson, L.S., and Pierson, E.A. 2020. Frontiers in Microbiology 11, 2731.

consists of a LuxI-type AHL synthase that produces an AHL signal and a cognate LuxR-type transcriptional regulator that senses the signal and regulates the expression of target genes, and autoinduction is a common feature of quorum sensing. Once the intracellular concentration of the signal exceeds a specific threshold, the signal binds to the LuxR, which in turn binds to a relatively conserved DNA sequence known as the *lux* box in the promoter region of regulated genes, activating or repressing their expression (Waters and Bassler, 2005). Quorum sensing was first identified in *Vibrio fischeri*, where LuxR binds the signal and directly activates transcription of the *luxICDABE* operon, resulting in an exponential increase in the production of both the signal and bioluminescence (Nealson and Hastings, 1979; Engebrecht et al., 1983; Fuqua and Greenberg, 2002).

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XccR and Pip are important for plant virulence (Zhang et al., 2007). Likewise, in other pathogenic bacteria, LuxR solos such as OryR in *Xanthomonas oryzae* pv. *Oryzae* (Ferluga et al., 2007; Ferluga and Venturi, 2009; González et al., 2013), XagR in *Xanthomonas axonopodis* pv. *glycines* (Chatnaparat et al., 2012), XocR in *Xanthomonas oryzae* pv. *oryzicola* (Xu et al., 2015), PsaR2 in *P. syringae* pv. *Actinidiae (Patel et al., 2014)*responds to plant signals and are involved in bacterial pathogenicity, mobility, or host colonization. The genes encoding the LuxR solo NesR in the symbiotic plant-beneficial bacteria *Sinorhizobium meliloti* (Patankar and González, 2009) and PipR in the cottonwood endophyte *Pseudomomas sp.* GM79 (Schaefer et al., 2016) are also associated with *pip* genes and these LuxR solos regulate genes that contribute to symbiosis traits including plant nodulation and plant carbon source utilization, respectively.

In comparison to the aforementioned plant-pathogenic and plant-symbiotic bacteria, the genomes of many well-studied plant growth-promoting rhizobacteria (PGPR) contain numerous genes annotated as encoding solo LuxR-family homologs. For example the genomes of PGPR strains *P. chlororaphis* subsp. *aureofaciens* O6 and 30-84, *P. protegens* Pf-5, *P. brassicacearum* Q8r1-96, *P. fluorescens* Pf0-1, Q2-87, SBW25, A506, SS101 and *P. synxantha* BG33R (Loper et al., 2012) contain 12 to 28 solo *luxR*-type genes based on gene annotations in the National Center for Biotechnology Information (NCBI) database (https://www.ncbi.nlm.nih.gov/). Also, in comparison to the aforementioned examples, the context of the genes encoding the LuxR solos studied in PGPR thus far present distinctive features. For example, the promoter of the *pip* gene adjacent to *psoR* in *P. protegens* CHA0 and *P. protegens* Pf-5 lacks a *lux*-box palindrome (Subramoni et al.,

2011) and there is no *pip* adjacent to *lesR* in *L. enzymogenes* (Qian et al., 2013) or *viscAR* and *viscBCR* in *P. fluorescens* SBW25 (de Bruijn and Raaijmakers, 2009). LuxR-type regulators ViscAR and ViscBCR have a HTH DNA-binding domain but lack a classic autoinducer domain. All these LuxR solos activate genes important for biological control activity. For example, PsoR activates anti-microbial-related genes in response to plant signals from wheat and rice but not cucumber. LesR activates the production of heat-stable antifungal factor (HSAF) and ViscAR and ViscBCR regulate viscosin production important for antagonism of oomycete plant pathogens (de Bruijn and Raaijmakers, 2009; Qian et al., 2013).

Based on these few examples, we hypothesize LuxR solos may enable PGPR to sense and respond to interkingdom signals, facilitating bacterial adaptation to a symbiotic lifestyle and the expression of traits important for plant growth-promoting activities. In the present study, we examined the LuxR-type regulators in the well characterized PGPR strain *P. chlororaphis* 30-84. This strain was selected as a biological control strain for take-all disease of wheat and was isolated from the roots of wheat grown in a take-all disease suppressive soil (Pierson and Thomashow, 1992; Pierson et al., 2013) Production of phenazines, heterocyclic nitrogen-containing secondary metabolites produced by *P. chlororaphis* 30-84 were shown to be the primary mechanism of disease suppression (Pierson and Weller, 1994; Pierson and Pierson, 1996). Phenazine production by *P. chlororaphis* 30-84 is necessary for effective inhibition of the take-all pathogen *Gaeumannomyces graminis var. tritici* (Pierson and Thomashow, 1992), persistence of *P. chlororaphis* 30-84 in the wheat rhizosphere (Mazzola et al., 1992), biofilm formation

(Maddula et al., 2006), and wheat seedling drought tolerance (Mahmoudi et al., 2019). Phenazines are produced by a diversity of prokaryotes and have been shown to play roles in Reactive Oxygen Species (ROS) generation, electron shuttling, and iron chelation in other *Pseudomonas* species (Pierson and Pierson, 2010; Wang et al., 2010; Wang et al., 2011; Recinos et al., 2012; Das et al., 2015). Phenazine biosynthesis is controlled by a complex network of regulatory genes organized in a hierarchical manner (Pierson et al., 1998b; Whistler and Pierson III, 2003; Pierson and Pierson, 2010; Wang et al., 2012; Wang et al., 2013; Yu et al., 2018b). The heart of this network is control by the LuxR-type transcriptional regulator (PhzR), part of the PhzR/PhzI QS system that directly regulates phenazine biosynthesis (Pierson et al., 1994; Wood and Pierson, 1996). A second set of QS genes CsaR/CsaI, indirectly influences phenazine production (Zhang and Pierson, 2001).

In our survey for potentially plant-responsive LuxR solo candidates in the genome of *P. chlororaphis* 30-84, we identified one atypical LuxR solo (PcsR2) based on the observation that *pcsR2* expression was highly upregulated when bacteria were grown on wheat roots compared to planktonic culture. We provide evidence that the expression of *pcsR2* and the adjacent operon respond to wheat root-derived signals rather than endogenous AHLs, and that a functional PcsR2 is required for this response. We describe bacterial phenotypes modulated by PcsR2. PcsR2 is involved in the expression of bacterial traits that contribute to the utilization of carbon and nitrogen from wheat roots, phenazine biosynthesis, and biofilm production, traits that promote the plant-associated lifestyle and

plant growth promoting activity of the bacteria. We discuss the novelty of PcsR2 in relation to other known plant responsive LuxR solos.

2.2 Materials and Methods

2.2.1 Bacterial strains, plasmids and growth conditions

Bacterial strains and plasmids used in this study are described in **Table 2.1**, and primers are listed in **Table 2.2.** A spontaneous rifampicin-resistant derivative of *P. chlororaphis* 30-84 was used in all studies and is hereafter referred to as 30-84WT. *P. chlororaphis* was grown at 28˚C in Luria-Bertani (LB) medium (Fisher BioReagents™, Hampton, NH), pigment production medium D (PPMD), AB minimal media (AB), AB amended with 0.4% glucose (AB+G), or AB+G amended with 2% casamino acids (AB+CAA) media (CAA is from BD Bacto™, San Jose, CA), as described previously (Yu et al. 2018). *Escherichia coli* (*E. coli*) was grown at 37° C in LB medium. *E. coli* and *P. chlororaphis* were grown in liquid culture with agitation (200 rotations per minute) or on solid medium (amended with agar at 15g/l). Antibiotics were used in the following concentrations for *E.coli:* kanamycin (Km), gentamicin (Gm), ampicillin (Ap), 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) at 50, 15, 100, and 40 μg/ml, respectively; and for *P. chlororaphis:* Km, Gm, Ap, rifampicin (Rif), Cycloheximide (Cyclohex) at 50, 50, 100, 100, and 100 μg/ml, respectively.

Table 2.1 Strains and plasmids used in Chapter II

Strains and plasmids	Descriptions	References
P. chlororaphis		
30-84WT	Phz^+, Rif^R, wild-type (WT)	Whistler and Pierson, 2003
30-84ΔpcsR2	Km^R, *pcsR2* replaced with Km^R cassette	This study
30-84ΔpcsR2(pGT2PcsR2)	Complemented mutant containing plasmid pGT2PcsR2	This study
30-84WT(pGT2PcsR2)	WT with plasmid pGT2PcsR2	This study
30-84ZN	Phz^-, Rif^R, *phzB*::*lacZ* genomic fusion	Wood et al., 1997
30-84R	Phz^+ Rif^R *phzR::Tn5* genomic fusion, Km^R	Pierson et al., 1994
E. coli		
DH5α	F^-*rec*A1 *end*A1 *hsdR*17 *sup*E44 *thi*-1 *gyr*A96 *rel*A1 Δ*(arg*F-*lac*ZYA*)* I169 Φ80lacZΔM15λ^-	GIBCO-BRL
Plasmid		
pGT2PcsR2	Promoter *tac::pcsR2* fusion in pGT2P created via replacement of *lacZ* with *pcsR2* in pGT2Ptac:lacZ	This study
pGT2PpcsR2:gfp	pGT2Ptac:lacZ with promoter of the *pcsR2* operon replacing Ptac promoter	This study
pGT2P4806:gfp	pGT2Ptac:lacZ with operon 2 promoter replacing Ptac	
pGT2Ptac:lacZ	pGT2 containing a constitutive promoter p*tac::lacZ* fusion (Ptac promoter drives *lacZ* and *gfp* expression)	Yu et al., 2017
pGT2	pProbe-GT′: pVS1 replicon, p15a origin of replication, *gfp* transcriptional fusion; Gm^R	Miller et al., 2000
pEX18Ap	Ap^R	Hoang et al., 1998
pUC4K	Km^R, Ap^R	Grindley and Joyce, 1981
pGT2Ptac:phzR	*lacZ* in pGT2Ptac:lacZ is replaced with 0.9 kb DNA fragment containing *P. chlororaphis* 30-84 *phzR*	Wang et al., 2013
pUCRpeB	1.2 kb DNA fragment containing *rpeB* in pUCP20G	Yu et al., 2017
pUCPip	784 bp DNA fragment containing *pip* in pUCP20G	Yu et al., 2017
pUCRpoS	1.4 kb DNA fragment containing *rpoS* in pUCP20G	Yu et al., 2017
pUCP20G	Gm^R, pUCP20 derivative containing constitutive promoter pLac with *Sma*I-flanked Gm^R cassette inserted into the unique *Sca*I site within *bla*	Chiang and Burrows, 2003

Ap^R, Km^R, Gm^R, Rif^R, indicate ampicillin, kanamycin, gentamicin, and rifampin resistance, respectively; gfp indiacates green fluorescent protein

Table 2.2 Oligonucleotides used for gene cloning and qRT-PCR in Chapter II

Oligonucleotide	Sequence (5'-3')
4807KO-1	CGgaattcCCTGATTATCGTCAATGGCG (*Eco*RI)
4807KO-2	TCACTCGTCctgcagACTGGTCATGCTTCCACGA (*Pst*I)
4807KO-3	ATGACCAGTctgcagGACGAGTGAGTGCTGCCTG (*Pst*I)
4807KO-4	GGaagcttCTTTGGCTTTTGATCTACCCG (*Hin*dIII)
4807Check1	ACGGCGAGCTGTTCGTCA
4807Check2	CTACAGGGTTTAGCGCCGA
4807qPCR-R	TGTTGTCCATCCTGTCCAGC
4807-F-BamHI	CGGggatccCAGGTCGTGGAAGCAT (*Bam*HI)
4807-R-HindIII	CCCaagcttTCACTCGTCGCAGAACAA (*Hin*dIII)
4807pr-EcoRI-F	CGgaattcTGCGCACGCTGCTGGCGATA (*Eco*RI)
4807pr-BamHI-R	CGggatccCAGCCGTACTTAGCCATCTC (*Bam*HI)
KmPstI-F	CGCGCGCctgcagTGTGTCTCAAAATC (*Pst*I)
KmPstI-R	CGCGCGCctgcagTTTAGAAAAACTCATCG (*Pst*I)
phzI**RT**1	CTACCTCCTGGCGTTCAATG
phzI**RT**2	GAAGCGAGTCATTTCCCAGA
phzR**RT**1	CGCAAGGATAATCCCATCAG
phzR**RT**2	CACATTCCCTACCGCTGAAC
pip**RT**1	AAAAGACCCGCGAGAACATT
pip**RT**2	ACGTACAGCTGCTCCTTGCT
rpeB**RT**1	CATCCTTCTGGTCGAAGACG
rpeB**RT**2	AGGTCGAGAATCACCAGGTC
rpoD**RT**1	ACGTCCTGAGCGGTTACATC
rpoD**RT**2	CTTTCGGCTTCTTCTTCGTC
rpoS**RT**1	ATCAGTGGCTTTCCGAATTG
rpoS**RT**2	GACCTTCGACCTGGATCTGA

Lowercase indicates nucleotides within restriction sites added to the primer for cloning purposes. The type of restriction enzyme sites at the end of primers is indicated. RT in bold indicates primers used for qRT-PCR.

2.2.2 Phylogenetic analysis of PcsR2

LuxR-family homologs within the *P. chlororaphis* 30-84 genome were identified based on annotation (GenBank: CM001559.1) in the National Center for Biotechnology Information (NCBI) database (https://www.ncbi.nlm.nih.gov/). The amino acid sequences for *P. chlororaphis* 30-84 LuxR homologs and other characterized LuxR sequences were retrieved from the *Pseudomonas* database (http://www.pseudomonas.com/) or NCBI. Protein domain analysis was performed using the NCBI conserved domain search (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) and Pfam (https://pfam.xfam.org/). Sequence alignments and amino acid identity comparisons were performed using Clustal-omega (http://www.ebi.ac.uk/Tools/msa/clustalo/). Representative LuxRs were displayed graphically using Boxshade (http://www.ch.embnet.org/software/Box_form.html). A maximum likelihood (ML) phylogenetic tree was constructed from multiple-sequence alignments of PcsR2 homologs in 48 fully sequenced strains of *P. chlororaphis* with MEGA7 (Kumar et al., 2016) using MUSCLE (MUltiple Sequence Comparison by Log- Expectation). The Jones, Taylor, and Thorton (JTT) model in MEGA 7 and bootstrap analysis with 1000 replicates were used. Values greater than 50 are indicated at the nodes.

2.2.3 Measuring gene expression levels on wheat roots

The four *luxR* solo genes that had the fewest number of substitutions among the nine highly conserved amino acid residues involved in autoinducer-binding and DNA-binding identified by (Gonzalez and Venturi, 2013) were selected for testing (**Table 2.3**).

Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was used to compare the expression levels of the encoding genes when bacteria were grown in planktonic culture or on wheat roots. For the planktonic culture treatment, bacteria were grown in AB+CAA to a standard optical density (OD_{620} = 1.0, 28^{o} C, with agitation). A 1 ml volume of the culture was mixed with 2 ml of Qiagen RNA Protect reagent (Qiagen, Hilden, Germany) to stabilize bacterial RNA. Bacterial cells were collected by centrifugation (16,000 x g). Whole RNA was extracted, and cDNA was obtained as described below. For the wheat root treatment, the hard red winter wheat cultivar TAM 112 (Reddy et al., 2014; Rudd et al., 2014) was used for all studies. Wheat seeds were surface-disinfested and grown in CYG^{TM} (Mega International, Newport, MN) germination pouches (in the dark, 28^{o} C, receiving 10 ml distilled water every two days) until the roots were 10-15 cm in length (6-10 days). Bacterial inoculum was prepared by growing bacteria in AB+CAA to a standard optical density (OD_{620} = 1.0, at 28^{o} C, with agitation). Plant roots were immersed in bacterial inoculum for 10 min, and then inoculated plants were transferred to modified germination pouches (roots wrapped in a single moistened sheet of germination paper) and grown for 16 h (8 h light/8 h dark, 28^{o} C). The entire root system (6 plants/rep) was rinsed with 5 ml of Qiagen RNA Protect reagent (Qiagen, Hilden, Germany) to stabilize bacterial RNA and collect the bacteria. The cells were collected by centrifugation (1,200 x g). Total RNA was extracted using a Qiagen RNeasy Mini Kit (Qiagen) according to the manufacturer's recommended protocol. The genomic DNA was removed using on-column DNase-I digestion (Qiagen) for 15 min. RNA concentration and purity were determined using a GE NanoVue spectrophotometer (GE Healthcare,

Pittsburgh, PA). Total RNA (1μg was reverse transcribed using random primers (Promega, Madison, WI) and AMV Reverse Transcriptase (Promega, Madison, WI) according to the Promega Reverse Transcription System manufacturer's protocol.

A second experiment compared the expression levels of *pcsR2* and two genes in the operon immediately downstream of *pcsR2* (Pchl3084_4801, Pchl3084_4803) in 30-84WT, 30-84ΔpcsR2, and 30-84ΔpcsR2(pGT2PcsR2) when bacteria were grown in planktonic culture or on wheat roots using qRT-PCR as described above.

SYBR Green reactions were performed using the CFX384™ Real-Time System (Bio-Rad, Hercules, CA) in Optical 384-Well Reaction Plates (Applied Biosystems, Foster City, CA). Quantitative PCR (qRT-PCR) assays were performed to measure the expression levels of the target genes as previously described with minor modifications (Yu et al., 2018b). Briefly, synthesized cDNA (5 ng/reaction) or a negative control was used for qRT-PCR with Fast SYBR Green® PCR Master Mix (Thermo Fisher, Waltham, MA) and gene-specific primers (250 nM final concentration). qRT-PCR amplification was used to detect the expression of the four *luxR* solo genes in the planktonic and wheat root growth treatments. Expression of *rpoD* was used as the housekeeping control. qRT-PCR amplifications were carried out at 50˚C for 2 min, 95˚C for 10 min, followed by 40 cycles of 95˚C for 15 sec and 60˚C for 1 min, and a final dissociation curve analysis step from 65˚C to 95˚C. Amplification specificity for each reaction was confirmed by dissociation curve analysis. The relative expression of the target gene was determined based on the mean of cycle threshold (Ct) values and ΔΔCt was calculated by normalizing target gene

expression to the expression of *rpoD* as the reference gene. All values are the means of three replicates. The primers for qRT-PCR are listed in **Table 2.2**.

2.2.4 Generation of *pcsR2* mutant, complementation, and overexpression

Mutant Cloning Strategy: A derivative of 30-84WT containing a *pcsR2*-deletion mutation was generated using strategies and methods described previously (Hmelo et al., 2015) with minor modifications. Briefly, fragments containing the upstream and downstream coding sequences (459 base pair each) flanking *pcsR2* were amplified by PCR using the primer pairs 4807KO-UP-F-EcoRI (4807KO-1) and 4807K0-UP-R-PstI (4807KO-2), and 4807KO-DN-F-PstI (4807KO-3) and 4807KO-DN-R-HindIII (4807KO-4), respectively. The two amplicons each have 24 nt overlap at the 3' and 5' ends, respectively, due to the overlap of 4807KO-2 and 4807KO-3. Using the primers 4807KO-1 and 4807KO-4 and the products of the previous PCR as a template, overlap PCR was performed. The new product was a DNA fragment that fused the upstream and downstream coding sequence of *pcsR2* with a *Pst*I site at their junction. This fragment was ligated into the *Eco*RI and *Hin*dIII restriction enzyme sites in the multiple-cloning region of the suicide vector pEX18Ap (Hoang et al., 1998). The modified plasmid was then transformed into *E. coli* and screened via blue-white color on LB with ampicillin and Xgal. A Km resistance cassette with its promoter (916 bp) was amplified from pUC4K (Grindley and Joyce, 1981) using primer pairs KmPstI-F and KmPstI-R**.** Following *Pst*I digestion, the resistance cartridge was inserted into the *Pst*I site of the modified pEX18Ap construct via T4 ligation. The final construct was conjugated into 30-84WT, and positive transformants were selected on LB (amended with Kan and Rif). A double-crossover

mutant (30-84ΔpcsR2) was obtained by counter-selection with LB amended with Km and 6% sucrose and checked by PCR primers 4807KO-UP-UP-F(4807Check1), 4807-DN-DN-R(4807Check2), KmPstI-F, 4807qRT-PCR-R PCR was performed using FideliTaq™ DNA Polymerase (Affymetrix, Santa Clara, CA) or GoTaq® Green Master Mix (Promega, Madison, WI) according to the manufacturer's instructions. Vector constructions were verified via Sanger sequencing using an ABI 3130xl Genetic Analyzer (Laboratory for Genome Technology, Texas A&M University). *E. coli* transformation and *P. chlororaphis* conjugation were performed as described previously (Pierson and Thomashow, 1992; Wang et al., 2012).

Strategy for complementation and overexpression: For complementation of 30-84ΔpcsR2, a plasmid constitutively expressing *pcsR2* via the P*tac* promoter was created. This was done by replacing the coding sequence of *lacZ* with *pcsR2* in the expression vector pGT2Ptac:lacZ (Miller et al., 2000). The coding sequence of *pcsR2* was PCR amplified with the primers 4807-F-BamH1 and 4807-R-HindIII. The resulting fragment was digested with *Bam*HI and *Hind*III and cloned into the GT2Ptac:lacZ expression vector creating a *Ptac::pcsR2* fusion, and the plasmid (pGT2PcsR2) was introduced into *E. coli*. The sequence was confirmed by Sanger sequencing. The expression vector was transformed to 30-84ΔpcsR2 by conjugation to generate 30-84ΔpcsR2(pGT2PcsR2), the complemented version of the mutant. A *pcsR2* overexpression derivative also was obtained by conjugating the plasmid into 30-84WT to generate 30-84WT(pGT2PcsR2). For all comparisons utilizing the complement strain, 30-84WT and 30-84ΔpcsR2 containing the plasmid with no insert (NI), e.g. 30-84WT(NI) and 30-84ΔpcsR2(NI) were

used. Standard growth curves were obtained for strains grown in AB+CAA at 28^{o} C, with agitation.

2.2.5 Effect of AHLs and plant macerate on gene expression and bacterial growth

AHLs were extracted from 30-84ZN (lacks phenazine production) in ethyl acetate and quantified as described previously (Whistler and Pierson III, 2003). Wheat macerate was extracted as described previously, with minor modification (Schaefer et al., 2016). Briefly, wheat seeds (variety TAM 112) were surface disinfected and grown in growth pouches as described above. After 6-10 days, 2.5 g of roots or leaves were macerated in the presence of liquid nitrogen and resuspended in 100 ml distilled water, which then was filtered to remove plant tissue.

A reporter of *pcsR2* transcription (pGT2PpcsR2:gfp) was constructed by PCR amplification of the promoter sequence of the *pcsR2* operon (Pchl3084_4808-4807) using primers 4808pr-EcoRI-F and 4808pr-BamHI-R. The PCR product was then ligated into the *Eco*RI and *Bam*HI sites replacing the Ptac promoter within the reporter vector pGT2Ptac:lacZ (thus the promoter drives both *lacZ* and *gfp* expression, **Table 2.1)**. Similarly, a reporter of operon 2 transcriptional activity (pGT2P4806:gfp) was constructed by PCR amplification of the promoter sequence of operon 2 (Pchl3084_4806-4800) using primers 4806pr-EcoRI-F and 4806pr-BamHI-R. The reporters then were introduced into 30-84WT and 30-84ΔpcsR2 by conjugation.

The GFP green fluorescent protein reporter was used to determine whether certain plant extracts activated gene expression as described previously (Schaefer et al., 2016) with minor modifications. Strains containing reporter plasmid pGT2PpcsR2:gfp were incubated overnight in AB+G (AB minimal media supplemented with 0.4% glucose) at 28°C with agitation and cells were sub-cultured 1:10 into fresh AB+G supplemented with and without AHLs or different concentrations (1%, 10%, 20% v/v) of root macerate (RM) or leaf macerate (LM) in individual wells of a 96-well microtiter. The plates were incubated at 28°C with minimal agitation (10 sec mixing, 3 times every 3 hr). GFP intensity (excitation at 485 nm and emission at 535 nm) and growth (OD_{620}) were assessed using a Tecan Infinite M200 Pro with I-control software (Tecan, Mannedorf, Switzerland) at 12 h. Data were plotted as relative fluorescence units (RFU) per OD unit. The ß-galactosidase activity of the reporter was also used to verify gene expression levels as described previously (Yu et al., 2018b).

To determine whether wheat root macerate could be used as the primary carbon or nitrogen source, four types of modified bacterial media were prepared: AB without glucose as the carbon source (AB-C), AB without NH_4Cl as the nitrogen source (AB-N), AB-C media supplemented with 80% root macerate (v/v) as the carbon source (AB-C+M), and AB-N media supplemented with 80% root macerate (v/v) as the nitrogen source (AB-N+M). The three strains 30-84WT, 30-84ΔpcsR2, and 30-84ΔpcsR2(pGT2PcsR2) were grown separately overnight in AB+G and the cells were collected via centrifugation (16,000 x g), washed three times, and resuspended in sterile distilled water. The final cell density was standardized to $OD_{620} = 0.8$. The bacterial cultures were inoculated separately

at a 1:100 dilution into a 96 well plate. After 24 h (28º C, with agitation), cell density (OD_{620}) was measured. A minimum of three replicates per experiment were used and experiments were repeated at least twice.

2.2.6 Root Persistence Assay

Root persistence assays measuring the populations of 30-84WT or 30-84ΔpcsR2 on roots were conducted as described previously with minor modification (Mazzola et al., 1992; Dorosky et al., 2018). Soil used for these experiments was a Pullman clay loam collected from the USDA-ARS, Bushland, TX dryland wheat plots at a depth of 1 to 15 cm. Prior to use, it was necessary to sieve (2 mm mesh) and mix the soil with sand (soil: sand, 2:1, v:v) to facilitate drainage. The soil-sand mix, hereafter referred to as soil, was autoclaved twice (121° C, 15 PSI, 1 h, 24 h pause between cycles). For bacterial inoculum, 30-84WT and 30-84ΔpcsR2 were grown in AB+CAA, collected via centrifugation (16,000 x g), washed, and resuspended in distilled water to a final OD_{620} = 1.0. Aliquots of the cell suspension (2 ml) were diluted with distilled water (18 ml) and added to 500 g of soil and mixed thoroughly. Soil was dispensed into plastic tubes (Ray Leach Cone-tainers, 2.5-cm diameter × 16.5-cm, 10 g soil/tube). Prior to planting, wheat seeds (TAM112) were surface disinfested and germinated on sterilized germination paper. Two-day-old seedlings were planted into each container and covered with autoclaved vermiculite four days after the bacteria were inoculated into the soil. Each treatment had 10 replicate plants and soil without bacterial inoculation was used as the control. After 6 weeks of growth (8 h dark/16 h light cycle at 28° C), the entire root system (and loosely

adhering soil) was collected and immersed in 10 ml of phosphate-buffered saline (PBS) solution, and bacteria were collected by sonicating and vortexing. Bacterial populations were enumerated via serial dilution plating onto media (amended with Rif and Cyclohex). CFU was calculated after 48 h and normalized to root dry weight (48 h, 65° C).

2.2.7 Growth, phenazine, and biofilm quantification

For growth curves in AB+CAA, LB, and PPMD, populations were quantified spectrophotometrically (at OD_{620}) using an Opsys MR Microplate Reader (ThermoFisher Waltham, MA) or via serial dilution plating and colony counting (CFU/ml). To quantify phenazine production, phenazine was firstly extracted as described previously with minor modification (Wang et al., 2016). Briefly, three replicates of each of strains were started from single colonies and inoculated into 3 ml of broth and cultured overnight at 28°C with agitation. Cell densities were normalized to 0.8 at OD_{620} and the bacteria were sub-cultured at a 1: 100 dilution into 10ml fresh media broth in 125ml flasks. After 48h incubation with aeration, the cells were transferred to 50ml tubes and pelleted via centrifugation (1250 g, for 20 min). Supernatants of each sample (9ml) were decanted into a new 50ml tube and acidified with 2 drops concentrated HCl to pH 2. An equal volume of benzene was added in the tubes, which then were gently mixed using a rotating mixer for 1h. To separate water and benzene phase, the preparations were centrifuged at (1250g, for 10min). The benzene phase (8ml) was transferred to new tubes and evaporated under a stream of air. Phenazines were resuspended in 1 ml of 0.1N NaOH, serial dilutions were quantified by an absorbance of OD_{367} by Nanovue plus (GE Healthcare Life Sciences,

Pittsburgh, PA). The absorbance for each sample was normalized to the total absorbance of 10 mL culture. The assays were repeated twice.

Surface attached biofilms of cultures grown in 96 well plates were quantified using the crystal violet method as described previously (Maddula et al., 2006) and biofilm populations were quantified spectrophotometrically (OD_{540}). The concentration of extracellular (eDNA) present in floating biofilms was quantified (μg/ml) using a Qubit dsDNA Assay Kit and Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions, as described previously (Wang et al., 2012)**.** Matrix from floating biofilms of cultures grown in 24-well plates was collected and quantified as previously described with minor modification (Wang et al., 2016). At the time of sampling, the entire culture (including floating biofilm) was transferred to 1.5 ml Eppendorf tubes and biofilm matrix was collected by centrifugation (16,000 x g for 5 min). The supernatants were removed, and the mass of cells and hydrated matrix weighed. For DNase treatment, 30 units of water-dissolved DNase I (Qiagen) were added to 24 h bacterial cultures and then incubated an additional 24 h. The biofilm matrix was quantified as above.

2.2.8 qRT-PCR of other regulators of phenazine production

Expression levels of *phzI, phzR, rpeB, pip* (phenazine inducing protein, not proline iminopeptidase), and *rpos* were measured via qRT-PCR in 30-84WT(NI), 30-84ΔpcsR2(NI) and 30-84ΔpcsR2(pGT2PcsR2). The protocol for extraction of whole RNA

and qRT-PCR was as described above. The primers for qRT-PCR are listed in **Table 2.2**. Gene expression levels were expressed as ΔΔCt, relative to *rpoD*.

2.2.9 Constitutive expression of *phzR, rpeB, pip, rpos* in 30-84ΔpcsR2

The previously constructed plasmids pGT2PhzR, pUCPip, pUCRpeB and pUCRpoS with constitutive promoters were introduced into 30-84WT and 30-84ΔpcsR2 by either electroporation or triparental conjugation. After 48 h incubation, phenazine production was quantified as described above. 30-84WT(NI) and 30-84ΔpcsR2(NI), with the empty vector (pGT2 or pUCP20) (Chiang and Burrows, 2003)were used as controls. A minimum of three replicates per experiment were used and experiments were repeated at least twice.

2.2.10 Statistical analysis

All data presented are mean ± the standard error from at least two experiments. Multiple comparisons were analyzed using ANOVA and Turkey HSD and significant differences ($P<0.05$) are indicated by lowercase letters. Two-group comparisons (WT versus mutant) were performed using Student's t test and asterisks indicate significant differences ($P<0.05$). All data were analyzed using JMP Version 14 Software (SAS Institute In., Cary, NC).

2.3 Results

2.3.1 Identification of a novel LuxR solo with elevated gene expression on roots

Twenty-five genes are annotated by NCBI as encoding LuxR-family transcriptional regulators in the *P. chlororaphis* 30-84 genome, including those encoding the two previously described QS LuxRs (PhzR and CsaR). The other twenty-three solo LuxR-type

homologs we refer to by their locus tag and are listed in **Table 2.3**. Protein domain analysis indicated that one of the solo LuxR homologs (Pchl3084_3368) is a typical LuxR solo, i.e., having an autoinducer-binding domain (PFAM03472) at the N-terminus (hereafter PcsR1, **Table 2.3**). The other twenty-two LuxR-type homologs possess a HTH DNA-binding domain (PF00196) at the C-terminus, typical of LuxR-family regulators. However, they are atypical in that the N-terminal domains lack autoinducer-binding domains and instead have either Per-ARNT-Sim (PAS) signal-sensing, REC/CheY signal receiver, AAA ATPase domains or unidentified domains in place of the autoinducer-binding domains **(Table 2.3)**. Several of the solo LuxR-family homologs have predicted protein lengths that are considerably smaller than or greater than known LuxR solos (Pchl3084_4696, Pchl3084_0961, and Pchl3084_4880). The typical LuxR solo (Pchl3084_3368) has two substitutions among these nine conserved amino acids. Three of the atypical LuxR solos (Pchl3084_4807, Pchl3084_3111, Pchl3084_3136) have three substitutions among the nine and the others have as many as eight. In our survey for potentially plant-responsive LuxR solo candidates, we focused first on the four homologs with the fewest amino acid substitutions among the conserved amino acids involved in the signal-binding and DNA-binding domains (**Table 2.3**).

In a comparative analysis, relative gene expression (ΔΔCt, measured using qRT-PCR standardized to *rpoD*) of one of the four solo LuxR homologs (Pchl3084_4807) was highly upregulated when 30-84WT was grown on wheat roots compared to growth in planktonic culture (9.5 ± 1.4 vs 1.0 ± 0.1, respectively). In contrast, differences in the gene expression of the other three LuxR homologs were not as pronounced (data not shown).

Table 2.3 Representative LuxR regulators and LuxR-family regulators annotated by NCBI in the genome of *Pseudomonas chlororaphis* 30-84 and their protein characteristics.

	Protein or Locus tag	Species	Type	Protein(aa)	N-terminal domain	C-terminal	Signal-binding domain						HTH-domain		
							57	61	70	71	85	113	178	182	188
	TraR	*A. tumefaciens*	QS	234	Autobind-bind	HTH	W	Y	D	P	W	G	E	L	G
	LuxR	*V. fischeri*	QS	250	Autobind-bind	HTH	W	Y	D	P	W	G	E	L	G
	SdiA	*E. coli*	QS	240	Autobind-bind	HTH	W	Y	D	P	W	G	E	L	G
	LasR	*P. aeruginosa*	QS	239	Autobind-bind	HTH	W	Y	D	P	W	G	E	L	G
	RhlR	*P. aeruginosa*	QS	241	Autobind-bind	HTH	W	Y	D	P	W	G	E	L	G
	XccR	*X. campestris*	Solo	254	Autobind-bind	HTH	M	W	D	P	W	G	E	L	G
	OryR	*X. oryzae*	Solo	254	Autobind-bind	HTH	M	W	D	P	W	G	E	L	G
	XagR	*X. axonopodis*	Solo	254	Autobind-bind	HTH	M	W	D	P	W	G	E	L	G
	XocR	*X. oryzae*	Solo	254	Autobind-bind	HTH	M	W	D	P	W	G	E	L	G
	PsaR2	*P. syringae*	Solo	254	Autobind-bind	HTH	W	W	D	P	W	G	E	L	G
	PipR	*P. sp. GM79*	Solo	289	Autobind-bind	HTH	W	W	D	P	W	G	E	L	G
	NesR	*S. meliloti*	Solo	260	Autobind-bind	HTH	M	W	D	P	W	G	E	L	G
	PsoR	*P. fluorescent*	Solo	252	Autobind-bind	HTH	W	W	D	P	W	G	E	L	G
	LesR	*L. enzymogenes*	Solo	247	Autobind-bind	HTH	W	Y	D	P	W	G	E	L	G
	SmoR	*S. maltophilia*	Solo	234	Autobind-bind	HTH	W	G	C	P	W	G	E	L	G
1	PhzR	*P. chlororaphis* 30-84	QS	241	Autobind-bind	HTH	W	Y	D	P	W	G	E	L	G
2	CsaR	*P. chlororaphis* 30-84	QS	241	Autobind-bind	HTH	W	Y	D	P	W	G	E	L	G
3	Pchl3084_0961	*P. chlororaphis* 30-84	Solo	847	unidentified	HTH	F	N	D	A	Y	Y	E	L	G
4	Pchl3084_1089	*P. chlororaphis* 30-84	Solo	220	REC/CheY/Response_reg	HTH	-	-	-	-	L	I	E	I	G
5	Pchl3084_1239	*P. chlororaphis* 30-84	Solo	216	REC/CheY/Response_reg	HTH	-	-	G	P	W	-	Q	L	G
6	Pchl3084_1430	*P. chlororaphis* 30-84	Solo	209	REC/CheY/Response_reg	HTH	H	A	Q	V	-	G	E	L	G
7	Pchl3084_2371	*P. chlororaphis* 30-84	Solo	499	PAS	HTH	-	F	V	P	V	N	E	L	G
8	Pchl3084_3060	*P. chlororaphis* 30-84	Solo	224	REC/CheY/Response_reg	HTH	L	L	R	M	-	G	E	L	G
9	Pchl3084_3111	*P. chlororaphis* 30-84	Solo	225	unidentified	HTH	Y	Y	D	P	S	G	E	A	G
10	Pchl3084_3130	*P. chlororaphis* 30-84	Solo	267	unidentified	HTH	C	Y	E	V	F	G	E	A	G
11	Pchl3084_3136	*P. chlororaphis* 30-84	Solo	225	unidentified	HTH	Y	Y	D	P	S	G	E	A	G
12	Pchl3084_3179	*P. chlororaphis* 30-84	Solo	190	PAS	HTH	W	N	-	-	Y	G	E	S	G
13	Pchl3084_3242	*P. chlororaphis* 30-84	Solo	325	unidentified	HTH	G	F	N	N	L	G	E	A	G
14	Pchl3084_3368 (PcsR1)	*P. chlororaphis* 30-84	Solo	239	Autobind-bind	HTH	W	V	C	P	W	G	E	L	G
15	Pchl3084_3391	*P. chlororaphis* 30-84	Solo	238	unidentified	HTH	L	E	-	-	-	G	E	I	G
16	Pchl3084_3909	*P. chlororaphis* 30-84	Solo	201	REC/CheY/Response_reg	HTH	-	A	D	R	-	D	Q	L	G
17	Pchl3084_4081	*P. chlororaphis* 30-84	Solo	219	REC/CheY/Response_reg	HTH	I	L	H	P	L	G	Q	L	G
18	Pchl3084_4100	*P. chlororaphis* 30-84	Solo	208	REC/CheY/Response_reg	HTH	-	-	G	E	-	L	E	L	G
19	Pchl3084_4146	*P. chlororaphis* 30-84	Solo	209	REC/CheY/Response_reg	HTH	N	-	-	-	-	G	E	L	G
20	Pchl3084_4649	*P. chlororaphis* 30-84	Solo	208	REC/CheY/Response_reg	HTH	-	-	D	N	I	I	E	L	G
21	Pchl3084_4696	*P. chlororaphis* 30-84	Solo	78	unidentified	HTH	-	-	-	-	-	-	I	N	G
22	Pchl3084_4807 (PcsR2)	*P. chlororaphis* 30-84	Solo	289	unidentified	HTH	L	I	D	P	W	G	E	S	G
23	Pchl3084_4880	*P. chlororaphis* 30-84	Solo	911	AAA_16	HTH	W	L	H	P	-	A	E	L	G
24	Pchl3084_4931	*P. chlororaphis* 30-84	Solo	208	REC/CheY/Response_reg	HTH	L	Y	-	-	L	-	E	L	G
25	Pchl3084_5375	*P. chlororaphis* 30-84	Solo	209	REC/CheY/Response_reg	HTH	L	Y	-	-	L	-	E	L	G

Representative QS LuxR and LuxR solos appear at the top of the table and the 25 solo LuxR-family homologs identified by annotation from P. chlororaphis 30-84 are numbered at the bottom of the table and referred to by their locus tag. N-terminal domains are identified using Pfam database nomenclature. The nine conserved amino acids in the signal binding domain and the helix-turn-helix (HTH) domains are indicated (numbering relative to TraR). Amino acid substitutions are indicated in red and red dashes indicate no amino acids aligned at that position. One P. chlororaphis 30-84 solo LuxR homolog has a typical autoinducer binding domain (PcsR1, highlighted in green). Highlighted in yellow are atypical candidate LuxR solos in P. chlororaphis 30-84 with only three amino acids substitutions among the nine conserved amino acids

Based on gene expression patterns, Pchl3084_4807, hereafter referred to as PcsR2 became the focus of subsequent work. The *pcsR2* coding sequence is 870 nucleotides in length and thus predicted to encode a LuxR-type regulator somewhat larger than a typical LuxR (e.g., 289 amino acids vs. 250, respectively), but the same size as the LuxR solo PipR in the cottonwood endophyte *Pseudomonas* sp. GM79 (Schaefer et al., 2016) .

2.3.2 PcsR2 belongs to a novel subfamily of LuxR-family transcriptional regulators conserved in *P. chlororaphis*

Analysis of conserved LuxR protein domains of PcsR2 indicated that it possesses a typical HTH DNA-binding domain (PF00196) at the C-terminus, but no AHL-binding domain (PFAM03472) or any other conserved binding domain at the N-terminus. Amino acid sequence alignment of PcsR2 with other representative LuxR transcriptional regulators was used to compare the nine highly conserved amino acids (red asterisk) within the N-terminal signal-binding and C-terminal HTH domains (**Figure 2.1, indicated by blue vs. black lines, respectively and Table 2.3**). PcsR2 has one substitution out of three highly conserved residues in the HTH domain. Despite the lack of an AHL-binding domain, PcsR2 retains four of six highly conserved amino acid residues within the signal binding domain, which are also conserved in known QS LuxRs (such as LuxR and TraR) as well as previously published plant-responsive LuxR solos such as LesR, PipR, PsoR, NesR, XccR and OryR(Zhu and Winans, 2001; Zhang et al., 2007; Ferluga and Venturi, 2009; Patankar and González, 2009; Subramoni et al., 2011; Qian et al., 2013; Schaefer et al., 2016). The differences between PcsR2 and the other LuxR solos in the substitutions

of the conserved amino acid in the signal-binding domain suggest that PcsR2 may respond to different signals than those reported previously for plant-responsive LuxR solos.

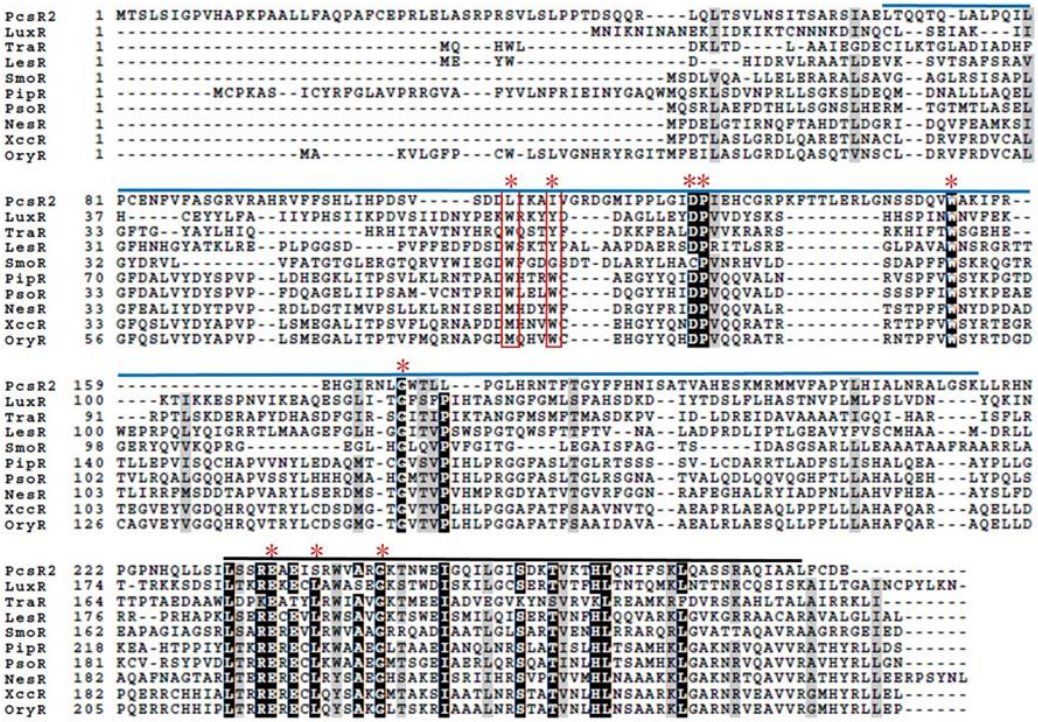

Figure 2.1 Amino acid sequence alignments of PcsR2 and other representative LuxR-type transcriptional regulators

Sequence alignments and amino acid identity comparisons were performed using Clustal-omega, and Boxshade was used to highlight the degree of amino acid identity (90-100%). The blue and black lines above the sequences indicate the signal-binding and helix-turn-helix domains of QS LuxRs, respectively. Red asterisks indicate the positions of nine conserved amino acids in QS LuxRs. The two residue positions boxed in red are the most common residues that differ between QS LuxRs (such as LuxR and TraR) and the LuxR solo homologs from plant-associated species (such as PipR, PsoR, NesR, XccR, and OxyR). The PcsR2 sequence also varies in these two positions (L_{57}, I_{61} instead of W_{57}, Y_{61} in canonical LuxRs; positions numbered as in TraR). Included in this table with PcsR2 are QS LuxR homologs: LuxR in *Vibrio fischeri* and TraR in *Agrobacterium tumefaciens*, and LuxR solos: LesR in *Lysobacter enzymogenes;* SmoR in *Stenotrophomonas maltophilia*; PipR in *Pseudomonas* sp. strain GM79; PsoR in *P. fluorescens and P. protegens*; NesR in *Sinorhizobium meliloti*; XccR in *Xanthomonas campestris* pv. *campestris;* OryR in *X. oryzae* pv. *Oryzea*.

Analysis of the *pcsR2* gene context indicated that different from previously characterized *luxR* solo genes such as *oryR*, *xccR*, *xocR*, or *xagR* in plant-pathogenic strains and *pipR*, or *psoR* in plant-beneficial strains (Gonzalez and Venturi, 2013), the

pcsR2 locus is not associated with a proline iminopeptidase (*pip*) gene (although there is a gene locus annotated as encoding this protein elsewhere in the genome (Pchl3084_0411), it is not associated with a transcriptional regulator). Instead, *pcsR2* is part of a two gene operon with an AMP-binding protein belonging to a family of adenylate-forming enzymes that generate an acyl-AMP intermediate **(Figure 2.2).** Members of this family include long chain fatty acid-CoA ligase, acetyl-CoA synthetase, and various other closely related synthetases. Located downstream is an operon containing two genes annotated as being involved in fatty acid desaturation. PcsR2 bears low amino acid identity with other well-characterized QS LuxR proteins such as LuxR (22%), TraR (15%) or even PhzR in *P. chlororaphis* 30-84 (18%) or LuxR solos such as LesR (20%), PipR (20%), and PsoR (20%) in other plant-associated bacteria. By contrast, it bears extremely high amino acid sequence identity (96%-100%, over the entire amino acid sequence) with PcsR2 homologs found in other sequenced strains of *P. chlororaphis* (**Table 2. 4**) and the organization of these LuxRs in operons with an AMP-binding protein is conserved. Interestingly, based on amino acid sequence similarity, the PcsR2 homologs in the sequenced *P. chlororaphis* strains generally cluster by subspecies (**Figure 2.3**).

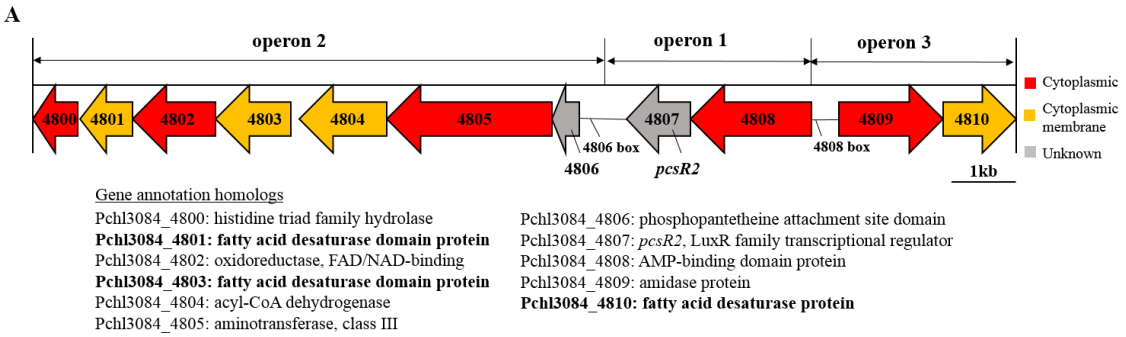

Figure 2.2 The genomic region surrounding the LuxR homolog *pcsR2* (Pchl3084_4807) in *P. chlororaphis* 30-84.

(A) The schematic was adapted from the *Pseudomonas* Genome Database (http://www.pseudomonas.com/). This region from Pchl3084_4800 to Pchl3084_4810 spans 14,475bp in total and contains eleven genes in three operons, among which are three fatty acid desaturase domain proteins. Arrows indicate orientation of transcription. Red indicates proteins predicted to be in the cytoplasm, orange indicates proteins predicted to be in the cytoplasmic membrane, the cellular location of products in gray is unknown. The location of putative *lux* box sequences in the promoter regions of Pchl3084_4808 (operon containing *pcsR2*) and Pchl3084_4806 (operon 2) are indicated. **(B)** Sequences of the predicted Pchl3084_4808 and Pchl3084_4806 *lux* boxes are provided in comparison to the *lux* box from *Vibrio fischeri* and the *phz* box from *P. chlororaphis* 30-84. The bases indicated in bold are shared among all sequences and the underlined bases are shared among at least two sequences.

In support of the hypothesis PcsR2 is a LuxR-type transcriptional regulator of adjacent operon 2 and its own operon, we identified putative *lux* box homologs in the promoter regions of both operons. In operon 2, the putative *lux* box sequence is centered 73 bp upstream of the translation start codon and in operon 1 it is centered 80 bp upstream of the translation start codon **(Figure 2.2).** Together, these results are consistent with the hypothesis that PcsR2 is a member of a novel subfamily of LuxR-type transcriptional regulators that is found thus far only in *P. chlororaphis.*

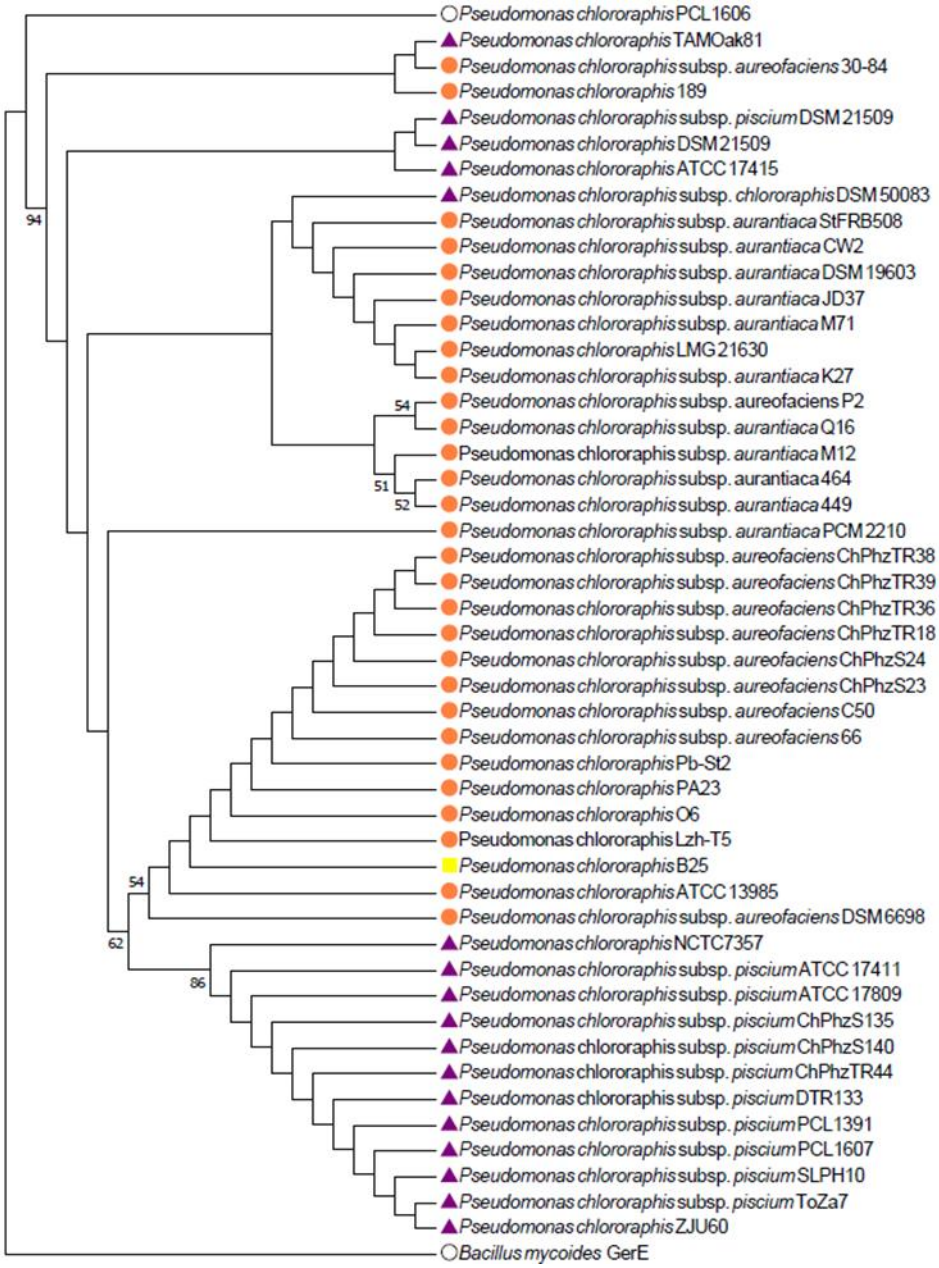

Figure 2.3 Molecular phylogenetic analysis of PcsR2 in *Pseudomonas*

The maximum likelihood (ML) phylogenetic tree was constructed from multiple-sequence alignments of PcsR2 homologs in 48 fully sequenced strains of *P. chlororaphis* with MEGA7 using MUSCLE (MUltiple Sequence Comparison by Log- Expectation) and bootstrap analysis with 1000 replicates. Values greater than 50 are indicated at the nodes. The closed circles (orange) indicate strains producing 2-hydroxypenzaine (2-OH-PHZ) and/or 2-hydroxypenazine-1-carboxylic acid (2-OH-PCA). The closed triangles (purple) indicate strains producing only pheanzine-1-carboxamide (PCN). The closed rectangles (yellow) indicate strains producing pheanzine-1-carboxylic acid (PCA). The open circle indicates strains that are not known to produce phenazines. GerE (a LuxR homlog) from *Bacillus mycoides* is used as a outgroup protein. Phenazine production is primarily inferred by Biessy et al, 2019.

Table 2.4 Amino acid identity of PcsR2 with other LuxR family regulators

Locus Tag	strains	% Identity
C4K25_RS24230	*P. chlororaphis* ATCC 17415	100.0
A3218_RS18615	*P. chlororaphis* isolate 189	100.0
PCHL3084_RS24535	*P. chlororaphis* subsp. *aureofaciens* 30-84	100.0
C4K26_RS24620	*P. chlororaphis* TAMOak81	100.0
BLU44_RS02205	*P. chlororaphis* ATCC 13985	99.7
C4K04_RS26015	*P. chlororaphis* B25	99.7
BLU06_RS10355	*P. chlororaphis* subsp. *piscium* DSM 21509	99.7
CXP47_RS24680	*P. chlororaphis* Lzh-T5	99.7
PchlO6_5060	*P. chlororaphis* O6	99.7
EY04_RS24920	*P. chlororaphis* PA23	99.7
C4K23_RS24070	*P. chlororaphis* Pb-St2	99.7
PCAU_RS24800	*P. chlororaphis* StFRB508	99.7
C4K20_RS25530	*P. chlororaphis* subsp. *aurantiaca* CW2	99.7
C4K17_RS26260	*P. chlororaphis* subsp. *aurantiaca* DSM 19603	99.7
JM49_RS06140	*P. chlororaphis* subsp. *aurantiaca* JD37	99.7
C4K19_RS24980	*P. chlororaphis* subsp. *aurantiaca* M71	99.7
C4K10_RS24990	*P. chlororaphis* subsp. *aureofaciens* 66	99.7
C4K11_RS24555	*P. chlororaphis* subsp. *aureofaciens* C50	99.7
C4K09_RS24480	*P. chlororaphis* subsp. *aureofaciens* ChPhzS23	99.7
C4K07_RS25360	*P. chlororaphis* subsp. *aureofaciens* ChPhzS24	99.7
C4K06_RS25320	*P. chlororaphis* subsp. *aureofaciens* ChPhzTR18	99.7
C4K12_RS25290	*P. chlororaphis* subsp. *aureofaciens* ChPhzTR36	99.7
C4K05_RS25645	*P. chlororaphis* subsp. *aureofaciens* ChPhzTR38	99.7
C4K08_RS25855	*P. chlororaphis* subsp. *aureofaciens* ChPhzTR39	99.7
C4K13_RS26055	*P. chlororaphis* subsp. *aureofaciens* DSM 6698	99.7
C4K38_RS26145	*P. chlororaphis* subsp. *piscium* DSM 21509	99.7
C4K18_4867	*P. chlororaphis* subsp. *aurantiaca* k27	99.7
C4K24_RS24590	*P. chlororaphis* subsp. *aurantiaca* M12	99.3
C4K16_RS24955	*P. chlororaphis* subsp. *aurantiaca* PCM 2210	99.3
C4K15_RS25095	*P. chlororaphis* subsp. *aurantiaca* Q16	99.3
C4K27_RS25250	*P. chlororaphis* subsp. *chlororaphis* DSM 50083	99.3
EL332_RS12475	*P. chlororaphis* NCTC7357	99.0
C4K22_RS25625	*P. chlororaphis* subsp. *aurantiaca* 449	99.0

Table 2.5 continued

Locus Tag	strains	% Identity
C4K14_RS27040	*P. chlororaphis* subsp. *aureofaciens* P2	99.0
C4K37_RS26530	*P. chlororaphis* subsp. *piscium* ATCC 17411	99.0
C4K36_RS26520	*P. chlororaphis* subsp. *piscium* ATCC 17809	99.0
C4K31_RS25185	*P. chlororaphis* subsp. *piscium* ChPhzS135	99.0
C4K29_RS26345	*P. chlororaphis* subsp. *piscium* ChPhzS140	99.0
C4K28_RS25195	*P. chlororaphis* subsp. *piscium* ChPhzTR44	99.0
C4K34_RS25405	*P. chlororaphis* subsp. *piscium* DTR133	99.0
C4K33_RS24820	*P. chlororaphis* subsp. *piscium* PCL1391	99.0
C4K32_RS25175	*P. chlororaphis* subsp. *piscium* PCL1607	99.0
C4K35_RS27045	*P. chlororaphis* subsp. *piscium* SLPH10	99.0
C4K30_RS25710	*P. chlororaphis* subsp. *piscium* ToZa7	99.0
C6Q18_RS24930	*P. chlororaphis ZJU60*	99.0
PCL1606_RS06075	*P. chlororaphis* PCL1606	96.5
Protein	**strains**	**% Identity**
LuxR	*Vibrio fischeri*	22.1
LesR	*Lysobacter enzymogenes*	20.5
PipR	*Pseudomonas sp. strain GM79*	23.8
PsoR	*Pseudomonas fluorescens*	20.1
CsaR	*P. chlororaphis* subsp. *aureofaciens* 30-84	20.0
XagR	*Xanthomonas axonopodis pv. glycines*	18.6
PhzR	*P. chlororaphis* subsp. *aureofaciens* 30-84	18.2
NesR	*Sinorhizobium meliloti*	18.1
RhlR	*Pseudomonas aeruginosa* PAO1	17.8
XocR	*Xanthomonas oryzae* pv. *oryzae*	17.7
XccR	*Xanthomonas campestris* pv. *campestris*	17.2
OryR	*Xanthomonas oryzae* pv. *oryzae*	17.2
LasR	*Pseudomonas aeruginosa* PAO1	17.1
TraR	*Agrobacterium tumefaciens*	15.0

P. chlororaphis strains used for comparison with PcsR2 are in black;proteins in blue are QS LuxRs proteins in red are LuxR solos from plant-associated bacteria.

2.3.3 Expression of *pcsR2* and the downstream operon are upregulated on wheat roots

A derivative of 30-84WT containing a *pcsR2*-deletion mutation (30-84ΔpcsR2) and the mutant complemented via constitutive expression (30-84ΔpcsR2(pGT2PcsR2)) were used to verify *pcsR2* response to plant signals from roots (**Table 2.1**). For all comparisons utilizing the complement, 30-84WT and 30-84ΔpcsR2 containing the plasmid with no insert (NI), e.g. 30-84WT(NI) and 30-84ΔpcsR2(NI) were used. All strains grew similarly to each other in planktonic culture consisting of AB+CAA and reached stationary phase by 12 h (**Figure 2.4A**). The relative gene expression of *pcsR2* (ΔΔCt, measured using qRT-PCR standardized to *rpoD*) was highly upregulated when 30-84WT was grown on wheat roots as compared to within planktonic culture consisting of AB+CAA. In contrast, there was almost no expression of *pcsR2* in the mutant in either condition, indicating the specificity of the primers. The relative gene expression of *pcsR2* in 30-84ΔpcsR2(pGT2PcsR2) was greater than 30-84WT in both conditions due to the higher copy number and constitutive expression of *pcsR2*, but the fold difference in gene expression between the two conditions was less (**Figure 2.4B**). These results verified that *pcsR2* is upregulated when 30-84WT is grown on wheat roots and that the mutant and complemented strains performed as expected with respect to *pcsR2* gene expression. Transcript abundances of two genes in operon 2 (Pchl3084_4801 and Pchl3084_4803) were also measured via qRT-PCR when bacteria were grown on wheat roots and planktonically (**Figure 2.4C-D**). Relative gene expression of Pchl3084_4801 and Pchl3084_4803 in 30-84WT was ~10 fold higher when bacteria were grown on wheat

roots as compared to liquid culture, whereas the relative expression of both genes in operon 2 in the *pcsR2* mutant was not upregulated on roots, consistent with PcsR2 involvement in the upregulation of operon 2 on wheat roots. In both conditions, expression of Pchl3084_4801 and Pchl3084_4803 in 30-84ΔpcsR2(pGT2PcsR2) was higher than in 30-84WT, consistent with the higher copy number of *pcsR2*, and similar to 30-84WT, relative gene expression of both genes in operon 2 in 30-84ΔpcsR2(pGT2PcsR2) was ~6-10 fold higher when bacteria were grown on roots as compared to liquid culture. These data are also consistent with PcsR2 involvement in the upregulation of operon 2 on wheat roots. The observation that the expression of Pchl3084_4801 and Pchl3084_4803 in the 30-84ΔpcsR2(pGT2PcsR2) was higher than in 30-84WT in AB+CAA, may indicate that the ligandsignal is present at a low level in the absence of wheat roots.

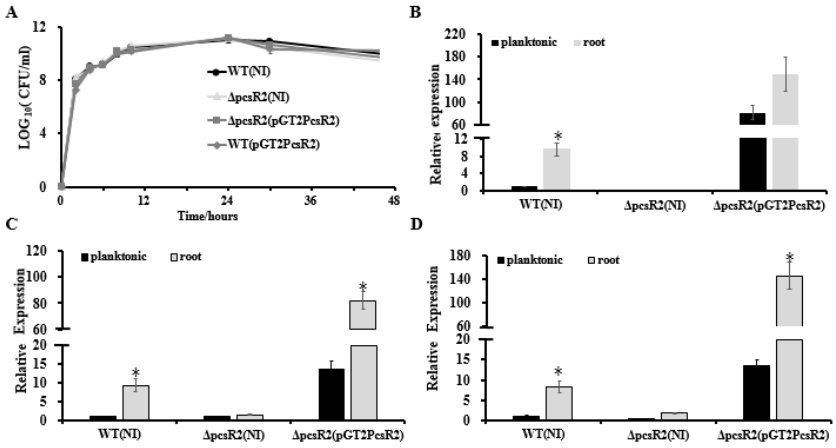

Figure 2.4 Expression of *pcsR2* and the downstream operon were upregulated on wheat roots

(A) Growth curves of strains grown in AB+CAA at 28 ℃, for 48 h, with agitation. Populations were enumerated via serial dilution and colony counts (CFU). **(B-D)** Expression of *pcsR2* and two genes in operon 2 (Pchl3084_4801and Pchl3084_4803) in 30-84WT, 30-84ΔpcsR2, or 30-84ΔpcsR2(pGT2PcsR2) when strains were grown on wheat roots as compared to within planktonic culture (AB+CAA). Expression levels were measured via qRT-PCR (where relative gene expression is expressed as ΔΔCt, with *rpoD* as an internal control) and data are the mean and standard error of nine replicates. Gene expression levels were compared by strain using a student's t-test. Asterisks indicate whether treatments are significantly different ($P<0.05$).

2.3.4 PcsR2 needed for root macerate utilization and rhizosphere colonization

We were curious whether *pcsR2* gene expression was responding specifically to root-derived substrates. The transcriptional reporter pGT2PpcsR2:gfp (containing the *pcsR2* operon promoter fused to *gfp*) transformed into 30-84WT and 30-84ΔpcsR2 was used to quantify the promoter activity of *pcsR2* when strains were grown planktonically in AB+G medium supplemented with or without AHLs, root macerate, or leaf macerate. In a preliminary experiment, we measured the expression of *pcsR2* over 24 h and observed that *pcsR2* expression plateaued at 12 h **(Figure 2.5)**, so subsequent assays were performed at this time point. Addition of AHLs (extracted from 30-84ZN) had no effect on the expression of the reporter in either 30-84WT or 30-84ΔpcsR2 (data not shown). Moreover, the expression of pGT2PpcsR2:gfp or pGT2P4806:gfp (containing the operon 2 promoter fused to *gfp*) in QS mutant 30-84R (disrupted in *phzR*) was not significantly different from expression in 30-84WT, indicating neither *pcsR2* nor operon 2 is regulated by PhzR (data not shown).

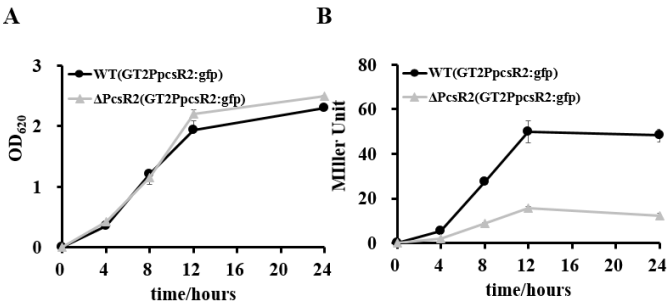

Figure 2.5 The growth curve and promoter activity of *pscR2* of 30-84WT and 30-84ΔPcsR2 in AB+G.
(A) growth curve of 30-84WT and 30-84ΔpcsR2 with the reporter(pGT2P4807: lacZ) in AB+G media **(B)** The promoter activity of pcsR2 in 30-84WT and 30-84ΔpcsR2 with reporter in AB+G media over 24h based on beta-galactosidase activity in Miller Units

Relative GFP intensity (standardized to cell density) of pGT2PpcsR2:gfp in 30-84WT increased significantly with increasing concentration of root macerate up to 10%, but not with leaf macerate even at the highest concentration (**Figure 2.6 A, B**). The relative GFP intensity of the reporter in the mutant was unchanged in both conditions, suggesting the mutant may be defective in signal uptake or signal sensing. These findings indicated *pcsR2* gene expression responds to unknown signals present in high enough concentration in wheat roots to be detected by the reporter, but not in leaves.

To test whether PcsR2 affects traits involved in utilization of root macerate as a carbon or nitrogen source, the three strains (30-84WT, 30-84ΔpcsR2, 30-84ΔpcsR2(pGT2PcsR2) were grown separately in modified AB medium supplemented with root macerate as the sole carbon (AB-C+M) or nitrogen (AB-N+M) source for 24 h. The modified AB without a carbon (AB-C) or nitrogen (AB-N) source was used as controls. None of the strains grew well without a supplemental carbon or nitrogen source. 30-84WT and 30-84ΔpcsR2(pGT2PcsR2) grew significantly better (as measured by OD_{620}) than 30-84ΔpcsR2 in media supplemented with macerate (AB-C+M) and (AB-N+M). These results suggest PcsR2 effects traits involved in the uptake and/or utilization of carbon and nitrogen sources from wheat root exudates (**Figure 2.6 C, D**).

To examine whether PcsR2 contributes to rhizosphere colonization and persistence, 30-84WT and 30-84ΔpcsR2 were inoculated separately into soil and after 4 days surface sterilized, pre-germinated wheat seedlings were planted. Bacteria were isolated from roots after 6 weeks and relative CFU (standardized to root dry weight) was calculated.

Populations of 30-84WT on roots were significantly greater (~10 fold) than populations of 30-84ΔpcsR2, (i.e., $\log_{10}$ (CFU/g) = 7.9 ± 0.2 vs 6.8 ± 0.1, respectively).

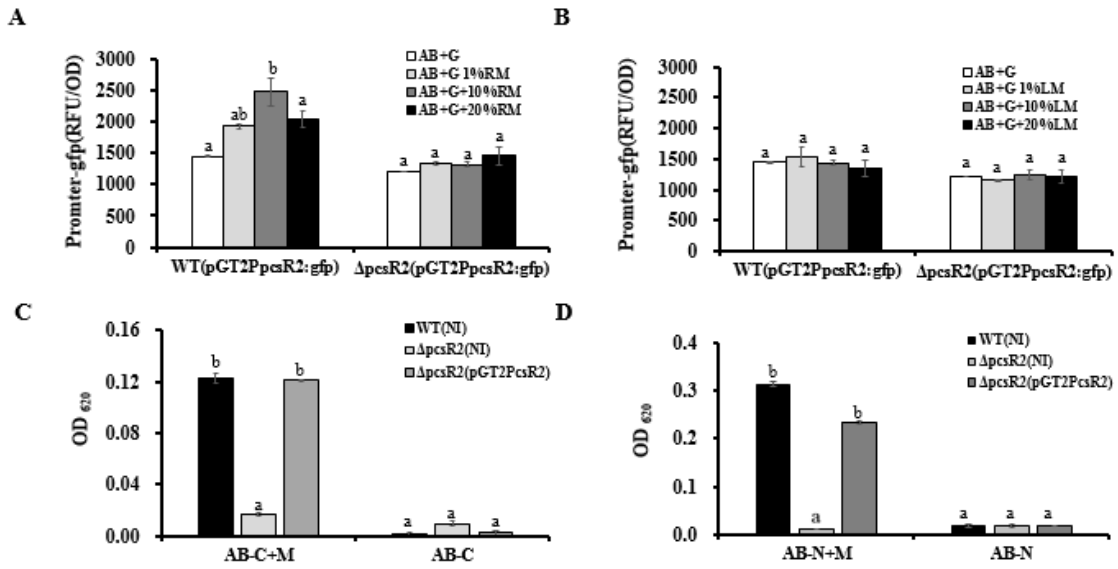

Figure 2.6 PcsR2 is involved in plant-microbe interactions
(A-B) Expression of the transcriptional pcsR2 GFP reporter (pGT2PpcsR2:gfp) in 30-84WT and 30-84ΔpcsR2 when grown in the presence of different concentrations of root or leaf macerate. 30-84WT and 30-84ΔpcsR2 carrying the reporter plasmid were grown in 96 well plates for 12 h in AB medium with glucose (AB+G) without or with varying concentrations (1-20%) of root macerate (RM) (A) or leaf macerate (LM) (B). GFP fluorescence is expressed as relative fluorescence units (RFU) (fluorescence units standardized to population density). (C-D) Growth of 30-84WT, 30-84ΔpcsR2, and 30-84ΔpcsR2(pGT2PcsR2) in AB medium without a carbon **(C)** or nitrogen (**D)** source and with 80% root macerate (AB-C+M or AB-N+M) or without it (AB-C or AB-N). Population density was measured spectrophotometrically (OD620) at 24 h of growth. NI means no insert control plasmid. Data are the means and standard errors of three replicates. Letters indicate whether treatments are significantly different (P<0.05, ANOVA and Tukey HSD).

2.3.5 Loss of PcsR2 affects phenazine production and biofilm traits

An unexpected phenotype of the 30-84ΔpcsR2 was diminished phenazine production (**Figure 2.7**). When grown in AB+CAA media (**Figure 2.7A**), total phenazines produced by 30-84WT and 30-84ΔpcsR2(pGT2PcsR2) were more than two-fold greater than the amount of phenazines produced by the mutant (i.e. OD_{367} =1.94 ± 0.20 and 1.84

± 0.20 vs. 0.68 ± 0.02, respectively). However, when grown in media supporting higher phenazine production (e.g., LB or PPMD), the difference in phenazine production between wild type and the mutant strain was not as pronounced (**Figure 2.7 B, C**). All strains (30-84WT, 30-84ΔpcsR2, 30-84ΔpcsR2(pGT2PcsR2), and 30-84WT(pGT2PcsR2)) grew at a similar rate to each other in planktonic culture regardless of media type, indicating this reduction in phenazine production was not a function of population density (**Figure 2.7 D, E, F**). These data indicate that a functional PcsR2 is required for wild type levels of phenazine production, that the defect is more pronounced in certain media, and that the complementation of the defect restores phenazine production to wild type levels.

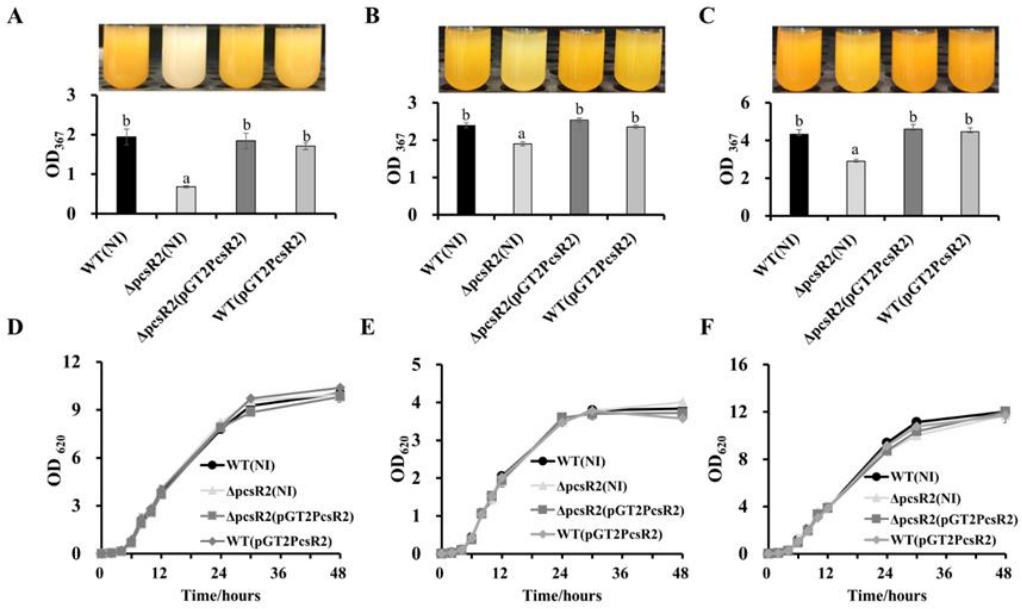

Figure 2.7 Phenazine production and growth of 30-84WT(NI), 30-84ΔpcsR2(NI), and 30-84ΔpcsR2(pGT2PcsR2) and 30-84WT(pGT2PcsR2) in different media
Strains were grown in **(A, D)** AB+CAA, **(B, E)** LB, and **(C, F)** PPMD at 28 ℃, for 48 h, with agitation. **(A-C)** Phenazines were quantified spectrophotometrically (OD_{367}) and **(D-F)** growth curves were calculated spectrophotometrically (OD_{620}). NI means no insert control plasmid. Data are the means and standard errors of at least three replicates; some error bars do not exceed the size of symbol. Letters indicate whether treatments are significantly different ($P<0.05$, ANOVA and Tukey HSD).

Given the effect of root macerate on pcsR2 gene expression, we were curious whether root macerate would stimulate phenazine production. Phenazine production by 30–84WT was enhanced when this strain was grown in AB + G media with increasing root macerate supplementation, however phenazine production by 30–841pcsR2 was almost unmeasurable and was unchanged by the addition of root macerate (**Figure 2.8**). These data are consistent with PcsR2 having an indirect role on phenazine production.

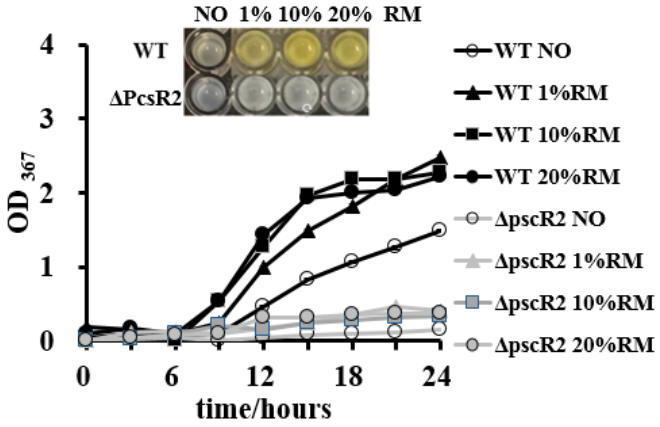

Figure 2.8 Phenezine production of 30-84WT and 30-84ΔPcsR2 with and without root macerate (RM).
30-84WT and 30-84ΔpcsR2 were grown in AB+G supplemented with and without difference concentration of root macterate (1%, 10% and 20%) for 24 h and phenazine was quantified every 3 h at OD_{367}. The picture was taken at 24 h.

Because production of phenazines was shown previously to be essential for surface attached biofilm formation (Maddula et al., 2006) and to promote extracellular DNA (eDNA) release and extracellular matrix production (Wang et al., 2016), we hypothesized that a functional PcsR2 may contribute to these phenotypes. When grown in AB+CAA, no differences were measured between 30-84WT, 30-84ΔpcsR2, and 30-84ΔpcsR2(pGT2PcsR2) in the number of surface-attached cells after 24 h growth in static culture or the amount of eDNA released into floating biofilm cultures **(Figure 2.9A, B)**.

However, by 48 h the surface-attached populations observed for 30-84WT and 30-84ΔpcsR2(pGT2PcsR2) increased dramatically and were significantly greater than observed for 30-84ΔpcsR2 **(Figure 2.9A)**. eDNA production by 30-84WT and 30-84ΔpcsR2(pGT2PcsR2) was also significantly greater than the mutant after 48 h (**Figure 2.9B**). However, by 48 h, 30-84ΔpcsR2 produced significantly more extracellular matrix than 30-84WT or 30-84ΔpcsR2(pGT2PcsR2) and the difference was dramatic after 72 h in static culture (**Figure 2.9C**). As expected, the extracellular matrix of all strains was completely disrupted by the addition of DNase1 (data not shown). Greater matrix production by the mutant was somewhat surprising given that it produced less eDNA, but eDNA was vital to extracellular matrix integrity. Taken together, these results indicate that the reduction in phenazine production associated with the loss of PcsR2 is likely responsible for most, but not all of the changes in biofilm characteristics.

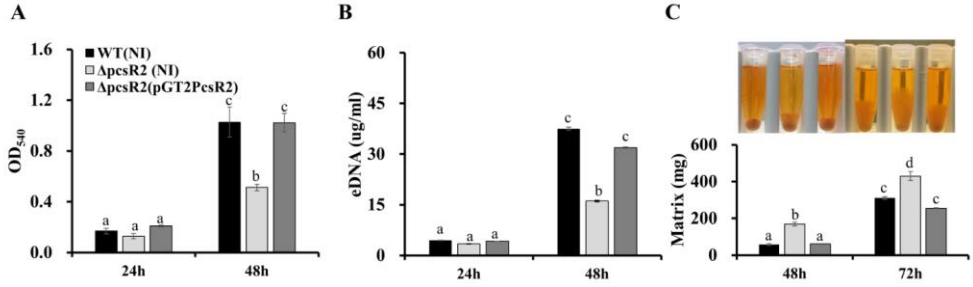

Figure 2.9 Biofilm traits of 30-84WT(NI), 30-84ΔpcsR2(NI), and 30-84ΔpcsR2(pGT2PcsR2).

(A) Spectrophotometric quantification of surface-attached biofilms by the crystal violet staining method (at OD_{540}) after 24 h and 48 h. **(B)** Quantification of extracellular (eDNA) when strains were grown in static culture after 24 h and 48 h. **(C)** Image of biofilm matrix and mass of cells and hydrated matrix when strains were grown in static culture measured by weight after 48 h and 72 h.. All figures use the same labeling for each strain. NI refers to the no insert control plasmid. For all experiments, bacteria were grown in AB+CAA. Data are the means and standard errors of at least three replicates. Letters indicate whether treatments are significantly different ($P<0.05$, ANOVA and Tukey HSD)

2.3.6 Disruption of PcsR2 reduced the expression of *phzR/phzI* and other phenazine regulators

Phenazine biosynthesis is regulated by a hierarchical network of regulatory genes. For example, the quorum sensing genes *phzR/phzI* are regulated by the phenazine inducing protein Pip (not proline iminopeptidase), which in turn is regulated by the two-component signal transduction system RpeA/RpeB and under nutrient limited conditions the stationary sigma factor RpoS (Wang et al., 2012; Wang et al., 2013; Whistler and Pierson III, 2003). In order to examine whether PcsR2 affects the expression of these regulators, we measured their transcript abundance in 30-84WT(NI), 30-84ΔpcsR2(NI) and 30-84ΔpcsR2(pGT2PcsR2) using qRT-PCR. The relative expression of regulators *phzI*, *phzR*, *pip*, and *rpeB* **(Figure 2.10A)** were significantly lower in 30-84ΔpcsR2(NI) compared to 30-84WT (NI) or 30-84ΔpcsR2(pGT2PcsR2). In contrast, the relative expression of *rpoS* did not differ significantly among the three strains. These results indicate that PcsR2 influences phenazine production via activity of RpeB, Pip, and PhzR/PhzI, but not RpoS.

To confirm the influence of PcsR2 on the RpeB/Pip/PhzR/PhzI regulatory network, extra copies of these regulatory genes under the control of a constitutive promoter were introduced into 30-84WT and 30-84ΔpcsR2. As expected, in 30-84WT constitutive expression of *phzR*, *pip* or *rpeB*, but not *rpoS* resulted in a substantial increase in phenazine production. Significantly, in 30-84ΔpcsR2 constitutive expression of *phzR*, *pip*, and *rpeB*, but not *rpoS*, restored phenazine production to wild type levels (**Figure 2.10 B-E).** Consistent with these results, supplementation of growth media with AHLs purified from

30-84ZN (defective in phenazine production) restored phenazine production in 30-84ΔpcsR2 to wild type levels (data not shown).

The promoter region for the operon encoding the two component signal transduction system RpeA/RpeB at the top of the network lacks a *lux* box. These data suggest that PcsR2 modulates the production of phenazines via an as yet undetermined, indirect effect on the RpeA/RpeB regulatory network that includes Pip and PhzR/PhzI.

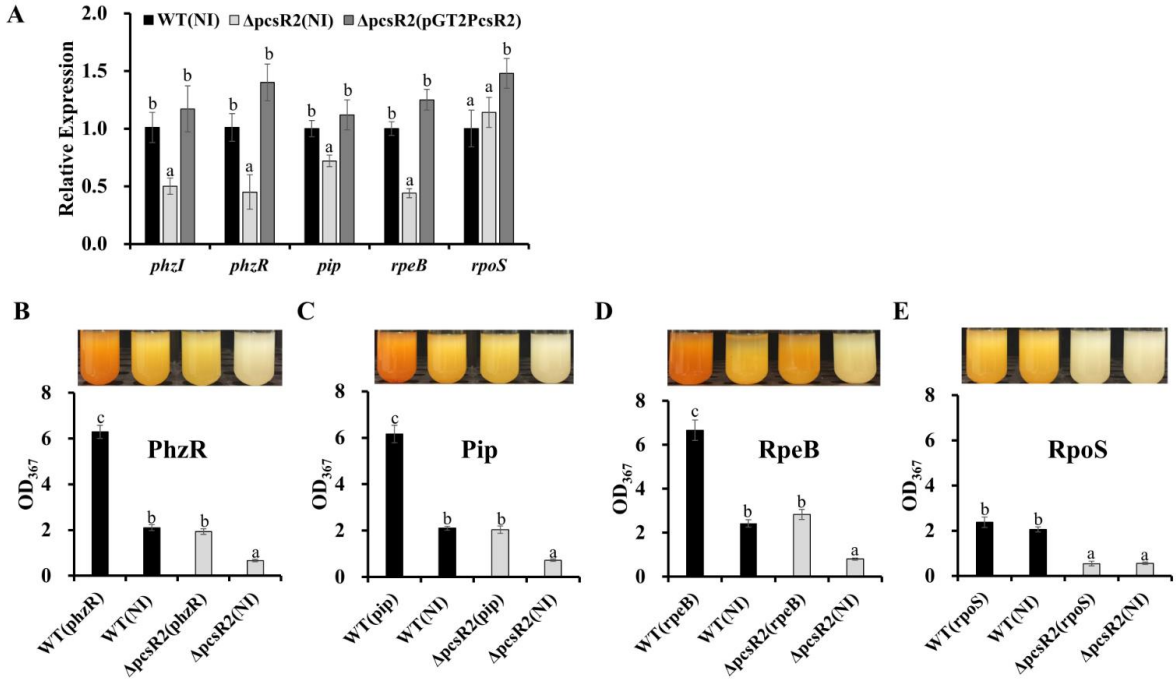

Figure 2.10 Gene expression of phenazine-regulators and phenazine production by 30-84WT and 30-84ΔpcsR2 with and without additional copies of some phenazine regulatory proteins *in trans*.

(A) Relative gene expression of some phenazine regulatory genes measured by qRT-qRT-PCR (ΔΔCt method with *rpoD* as internal control) after 24 in AB+CAA media. Relative fold change of these genes is compared to 30-84WT expression levels (which are set to 1). **(B-E)** Phenazine production of strains with and without additional copies of *phzR*, *pip*, *rpeB*, or *rpoS*, via expression of the following plasmids *in trans*: pGT2Ptac:phzR, pUCPip, pUCRpeB, and pUCRpoS, respectively. NI means no insert control plasmid (pGT2 or pUCP20). Images above graphs are of cultures after 48 h growth in AB + CAA media when phenazines were extracted and quantified spectrophotometrically (OD_{367}). Data are the means and standard errors of five replicates. Letters indicate whether treatments are significantly different ($P<0.05$, ANOVA and Tukey HSD).

2.4 Discussion

Plants and prokaryotes have coexisted and coevolved over millions of years in the rhizosphere. In this specific ecological niche, eukaryotes and prokaryotes communicate efficiently via chemically-mediated interkingdom signaling, shaping their evolution and contemporary ecology (Hughes and Sperandio, 2008). Bacterial QS regulation via the production of AHLs, the prototypical QS signal, has been shown to be important for regulating key bacterial traits for bacteria-plant interactions, and the production of AHL mimics by plants highlights the importance of LuxR-based signal-response regulators as contributing to the recognition of the chemical language involved in interkingdom communication (Loh et al., 2002). LuxR solos comprise an interesting subgroup of LuxR regulators that may not necessarily respond to an AHL type molecule, and recent observations suggest that LuxR solos are widespread among Gram negative bacteria and may be more common than QS LuxRs in bacterial genomes (Subramoni and Venturi 2009a, Hudaiberdiev et al., 2015). Thus, LuxR solos are good candidates for being mediators of host-PGPR interactions by regulating traits involved in host recognition, host adaptation, or host growth-promoting activities in response to plant signals. In the present study, we focused on LuxR-type regulators in the genome of the well-characterized PGPR strain *P. chlororaphis* 30-84, which has a diversity of LuxRs, including QS LuxRs and typical and atypical solo LuxR-type regulators. We were particularly interested in plant-responsive gene regulation by any of the LuxR solos.

The twenty-five genes annotated as encoding LuxR-family transcriptional regulators in the *P. chlororaphis* 30-84 genome include the two previously described QS LuxRs (PhzR and CsaR) and twenty-three solo LuxR-type homologs. As is characteristic of LuxR-family proteins, they typically bear little amino acid sequence identity to one another and are annotated based on the presence of signal-binding and HTH DNA-binding domains. Only one of the LuxR solo homologs is predicted to have an autoinducer binding domain at the N-terminus (PcsR1, **Table 2.3**). PcsR1 has two substitutions of the nine highly conserved amino acids in typical QS LuxRs, suggesting it may not bind to AHLs produced by PhzI or CsaI (i.e., N-(3-OH-hexanoyl)- and N-(hexanoyl)-HSL or N-(butanoyl)-HSL, respectively) (Khan et al., 2007), but may bind to exogenously produced AHLs (i.e., produced by other strains of bacteria) or other types of signals, although this remains to be tested. Interestingly, SmoR in *Stenotrophomonas maltophilia*, which bears the highest amino acid sequence identity to PcsR1 (53%) and has amino acid substitutions at the same two positions, is reported to respond to exogenously produced AHL, specifically oxo-C8-homoserine lactone (Martínez et al., 2015). The other twenty-two solo *P. chlororaphis* 30-84 LuxR-family homologs are atypical in that in place of the autoinducer binding domain, they have either Per-ARNT-Sim (PAS) signal-sensing domains, REC/CheY signal receiver domains, or unidentified domains, indicating that they may respond to other types of exogenously-derived signals or stimuli. The N-terminal domains of one half of the atypical solo LuxR-family homologs are annotated as a REC/CheY family of response regulator domains. Response regulators that combine the REC/CheY domain with a DNA binding HTH domain are common in prokaryotic

genomes. Typically, these response regulators are part of two-component signal transduction systems that respond to a variety of environmental stimuli or intracellular signals (Galperin, 2006). Interestingly, solo LuxR-family homologs Pchl3084_2371 and Pchl3084_3179 are annotated as having PAS-domains. PAS domains are a family of sensor protein domains involved in signal transduction and are found in all kingdoms of life. They have been shown to bind chemically diverse small-molecule metabolites and to serve as sensors of external stimuli (Henry and Crosson, 2011). For example, the gene downstream of Pchl3084_3179 is annotated as a chitinase. Thus, it may be reasonable to hypothesize that Pchl3084_3179 plays a sensory role in bacteria-fungi interactions by regulating the expression of this gene in direct response to fungal metabolites, although this has yet to be tested.

In the present study, we focused on the LuxR solo PcsR2 because its gene expression is upregulated ~10-fold on roots. We showed that *pcsR2* expression is enhanced specifically in response to root-derived substrates as compared to leaf-derived substrates and does not respond to endogenous AHLs produced by wild type *P. chlororaphis* 30-84. Gene expression of the downstream operon also was highly upregulated on roots in wild type but not in the mutant, consistent with PcsR2 being involved in regulation of this operon. As was shown via the contrasting behavior of the *pcsR2* mutant versus the wild type and complemented mutant, PcsR2 is involved in the metabolism of root-derived carbon and nitrogen, although it is unclear if this is due to PcsR2 effects on metabolite uptake or catabolism. The specificity of the *pcsR2* transcriptional response is similar to the specificity of LuxR solos in some plant-beneficial

Pseudomonas strains. For example, PipR in *Pseudomonas* sp. GM79, an endophyte of *Populus*, responds specifically to metabolites in leaf but not root macerates. One particular component of leaf macerate, ethanolamine derivatives, was identified as specifically interacting with PipR (Schafer et al., 2016; Coutinho et al., 2018). For PsoR in the biological control strains *P. protegnes* CHA0 and *P. protegens* Pf-5, the specificity is for plant signals derived from certain plant species, i.e., plant-produced molecules in rice and wheat but not in cucumber (Subramoni et al., 2011). In the present study, we show that *pcsR2* is upregulated in the presence of wheat root macerate but have yet to demonstrate a specific plant-derived signal interaction with PcsR2 on a protein level. However, it is also possible that a plant-derived signal is modified by *P. chlororaphis* 30-84 and that PcsR2 responds to this modified product. The observation that the expression of Pchl3084_4801 and Pchl3084_4803 in 30-84ΔpcsR2(pGT2PcsR2) was higher than in 30-84WT in AB+CAA media, may indicate that the ligand is present at a low level in the absence of root exudates, that there is some induction of operon 2 by PcsR2 in the absence of ligand, or be an unknown consequence of the higher expression *pcsR2* in 30-84ΔpcsR2(pGT2PcsR2) than 30-84WT and warrants further study.

Our results suggest that PcsR2 belongs to a novel subfamily of LuxR-type transcriptional regulators found in *P. chlororaphis* that may be involved in the regulation of a conserved fatty acid desaturation locus. PcsR2 bears low amino acid identity with other well-characterized LuxR solos in plant-beneficial species such as LesR, PipR, and PsoR (~20%) but high amino acid sequence identity (96%-100%) with PcsR2 homologs only found in other sequenced strains of *P. chlororaphis* (**Table 2.4).** Additionally, unlike

the other plant responsive LuxR solos characterized to date, *pcsR2* is not associated with a proline iminopeptidase (*pip*) gene. It is part of a two-gene operon with an AMP-binding protein. Located downstream is a fatty acid desaturase containing operon **(Figure 2.2)** and this operon is present and topologically organized in the same manner in all other sequenced *P. chlororaphis* strains with PcsR2 homologs (NCBI database, as of this date).

An interesting discovery was the involvement of PcsR2 in phenazine production. We observed that compared to wild type or the complemented mutant, phenazine production by the *pcsR2* mutant was significantly reduced even after cultures attained a high cell density and entered into stationary phase. Compared to wild type and the complemented mutant, the formation of surface attached biofilm communities by the *pcsR2* mutant was also diminished. We hypothesize this biofilm phenotype is related to the reduced phenazine production by the mutatnt. This hypothesis is supported by previous reports showing that phenazine production is required for the formation of surface attached biofilm communities (Maddula et al., 2006). Phenazine production also was shown previously to contribute to eDNA release associated with autolysis, resulting in more extensive biofilm matrix production and viscosity (Das and Manefield, 2012; Das et al., 2015; Wang et al., 2016). Somewhat surprisingly, although the wild type and complemented mutant released large quantities of eDNA and produced extensive biofilm matrix, we found that the *pcsR2* mutant produced significantly more extracellular matrix, but significantly less eDNA. As expected, the extracellular matrix of the wild type, mutant and complemented mutant were completely disrupted by the addition of DNase1,

indicating eDNA is required for matrix integrity, but the mutant must be contributing as yet unidentified component(s) to the matrix.

Our data suggest that PcsR2 affects phenazine production in *P. chlororaphis* 30-84 indirectly via the network of regulatory genes that control phenazine biosynthesis. Data presented in this study indicate that mutation of *pcsR2* results in a reduction in the expression of key phenazine regulatory genes *phzR*, *pip* (gene encoding phenazine inducing protein, not proline iminopeptidase), and *rpeB*, and that phenazine production can be restored to wild-type levels by expressing any of these genes *in trans* in the mutant. These findings are in agreement with previous work that demonstrated phenazine biosysnthesis in *P. chlororaphis* 30-84 is regulated by the PhzR/PhzI QS system, which in turn is positively regulated by Pip (phenazine inducing protein) and the response regulator RpeB, part of the RpeA/RpeB two component signal transduction system (Wang et al., 2012). According to the model proposed in that study, the RpeA/RpeB two component system may function as a sensor of the metabolic or environmental stress condition of the cell, governing the production of secondary metabolites under stress conditions via the control of additional regulatory proteins. In that model, the membrane bound sensor kinase protein RpeA is proposed to control the level of active, phosphorylated RpeB, which in turn promotes the expression of *pip* (gene encoding phenazine inducing protein), and Pip in turn promotes the expresion of *phzR* (Wang et al., 2012). Our data suggest PcsR2 acts upstream of RpeA/RpeB via an as yet undetermined mechanism. Although the function and role of the genes in the downstream operon have yet to be determined, we hypothesize that PcsR2 regulation of fatty acid desaturation may

affect membrane fluidity. If so, this may affect the function of proteins bound in the outer membrane, including transport proteins or the receptors of two component systems such as RpeA that are likely to be involved in secondary metabolite production. Indeed, previous work using *Pseudomonas aeruginosa* has shown that mutations that disrupt the fatty acid profile of phospholipids alter membrane fluidity, resulting in altered production of QS signals, secondary metabolites, and traits associated with survival (Baysse et al., 2005). Future work will focus on the function of the PcsR2-regulated genes that appear to be unique to *P. chlororaphis* and their roles in *P. chlororaphis* interactions with plants.

CHAPTER III

A LUXR HOMOLOG PCSR2 IS INVOLVED IN CELL MEMBRANE FLUIDITY AND ABIOTIC STRESSES IN PSEUDOMONAS CHLORORAPHIS

3.1 Introduction

Bacteria must sense and respond to changes in a complex and dynamic environment for survival. LuxR solos, a subgroup of transcriptional regulators, are becoming recognized as one of the important components for this adaption (Patel et al., 2013; Venturi and Ahmer, 2015). Bacterial taxa carrying one to multiple LuxR solos (e.g *Pseudomonas*, *Burkholderia* and *Photorhabdus*) have been shown to exist in diverse ecological host environments, including plants, nematodes, as well as humans, suggesting a role of these LuxR solos in adaption to diverse habitats and the potential for interkingdom signaling (Brameyer et al., 2014; Subramoni et al., 2015; Truong et al., 2015). To date, only a few LuxR solos have been characterized (Gonzalez and Venturi, 2013; Subramoni et al., 2015; Xu, 2020). Most of these characterized LuxR solos regulate bacterial traits associated with interkingdom interactions, such as production of virulence factors, antagonism, colonization of hosts or response to novel signals from the host (Chugani et al., 2001; Zhang et al., 2007; Ferluga and Venturi, 2009; Qian et al., 2013; Gardiner et al., 2015; Truong et al., 2015; Xu et al., 2015; Coutinho et al., 2018; Mosquito et al., 2020). LuxR solos typically regulate neighboring genes by binding to a *lux box* (a 20-base inverted repeat) in their promoter sequences of the controlled genes. In Chapter 2, I identified a solo *luxR, pcsR2* in the genome of a member of the plant growth promoting rhizobacteria (PGPR) *Pseudomonas chlororaphis* 30-84 that was highly expressed on

plant roots. Bioinformatic analysis revealed that PcsR2 is a member of a novel subfamily of LuxR-type transcriptional regulators found to date only in *P. chlororaphis* that may be involved in the regulation of a conserved fatty acid desaturation locus. PcsR2 bears low amino acid identity with other well-characterized LuxR solos in plant-beneficial species such as LesR, PipR, and PsoR (~20%) but high amino acid sequence identity (96%-100%) with PcsR2 homologs only found in other sequenced strains of *P. chlororaphis.* Additionally, unlike the other plant responsive LuxR solos characterized to date, *pcsR2* is not associated with a proline iminopeptidase (*pip*) gene. It is part of a two-gene operon with an AMP-binding protein. Located downstream is an operon that appears to encode genes involved in fatty acid desaturation, and this operon is present and topologically organized in the same manner in all other sequenced *P. chlororaphis* strains with PcsR2 homologs (NCBI database, as of this date). Because LuxR solos are typically found in close proximity with the genes they regulate (Subramoni et al., 2015) , I hypothesized that PcsR2 may regulate the expression of the neighboring operon encoding genes annotated as having a function in the desaturation of fatty acids. In bacteria alterations in the production of desaturated fatty acids are typically associated with changes in cell membrane fluidity via alteration of unsaturated fatty acids (UFA) in the phospholipid bilayer.

Adaptable fluidity of cell membranes is crucial to the maintenance of biological functions under various environments. Bacterial membranes typically consist of a lipid bilayer and other embedded membrane components including proteins (e.g porins, receptors, and enzymes) and glycolipids (e.g. lipopolysaccharide)(Silhavy et al., 2010).

The predominant lipid component of the lipid bilayer are phospholipids such as phosphatidylethanolamine (PE) and phosphatidylglycerol (PG), but a limited number (15%) of bacteria (e.g *Streptococcus* and *Pseudomonas*) also may have a low amount of phosphatidylcholine (PC) in cell membranes. In constrast, PC is common in cell membranes of eukaryotes (Geiger et al., 2013; Joyce et al., 2019) . It has been well-documented that elevated UFAsin the aliphatic chain of these phospholipids lead to greater cell membrane fluidity due to a decrease in the gel-liquid-phase transition temperature (Hagve, 1988). More recently, cyclopropane fatty acids (cFAs) also showed to affect cell membrane fluidity. Increased cFAs create a more rigid membrane because cFAs enhance the packing (density) within lipid bilayers (Pandit and Klauda, 2012). By stringently controlling the composition of fatty acids comprising phospholipids, bacteria are capable of adapting to challenging environmental conditions brought on by temperature, osmotic stress, or toxic compounds (Wada et al., 1990; Aguilar et al., 1998; Los and Murata, 2004; Mansilla et al., 2004; Schweizer, 2004; Mendoza, 2014; Poger and Mark, 2015). Moreover, previous work has shown that mutations that alter the fatty acid profile of phospholipids change cell membrane fluidity, resulting in altered metabolic networks (due to alteration in the production of QS signals or expression of signal transduction systems), altered flagella-mediated adhesion, and other traits associated with survival (Baysse et al., 2005; Flechard et al., 2018b; Cazzola et al., 2020).

Bacteria UFAs are generally biosynthesized by two pathways. One is the anaerobic FabAB-mediated pathway exemplified by *E.coli*, and the other is the aerobic inducible desaturase (Des) pathway exemplified by *Bacillus spp* and cyanobacteria (Los and Murata,

2004). In the anaerobic pathway, FabA dehydrates and isomerizes ß-hydroxydecanoyl-ACP to produce cis-3-decenoyl-ACP, diverting the recently formed acyl chain into the UFA synthetic pathway(Cronan et al., 1969). The cis -3-decenoyl-ACP then elongates by FabB (a type II fatty acid synthase) (Feng and Cronan, 2009). The FabAB pathway is a *de novo* UFA synthesis pathway and the reactions occur in the cytoplasm. In the aerobic pathway, UFAs are produced by desaturases such as DesA and/or DesB, iron-containing transmembrane desaturases (Aguilar et al., 1998). Some bacteria such as *P. aeruginosa* can produce UFAs under both aerobic and anaerobic pathways(Zhu et al., 2006). Specially, DesA is a phospholipid Δ 9-desaturase that converts exogenous stearate into 18:1 Δ 9, whereas DesB is an inducible acyl-CoA Δ 9-desaturase whose expression is repressed by the TetR transcriptional regulator DesT and is important for the production of virulence factors (Schweizer and Choi, 2011).

The *P. chlororaphis* 30-84 genome contains genes encoding proteins required for the FabAB anaerobic pathway, suggesting that UFAs can be biosynthesized *de novo*. In addition, there are five genes annotated as fatty acid desaturases: Pchl3084_0205, Pchl3084_4679, Pchl3084_4801, Pchl3084_4803, and Pchl3084_4810. All of these uncharacterized genes encode membrane-bound desaturases that could produce UFAs in the cell membrane. Among the five desaturases, Pchl3084_0205 has 84% amino acid identity with DesA in *P. aeruginosa* and is likely to be a Δ 9- fatty acid desaturase. The other four desaturases have no homologs in species other than *Pseudomonas chlororaphis.* Interestingly, 3 of the genes (Pchl3084_4801, Pchl3084_4803, and Pchl3084_4810) are co-located in the flanking operons of PcsR2, suggesting *P. chlororaphis* may pose a novel

alternative pathway for UFA biosynthesis and that PcsR2 may serve as a regulator of this pathway.

Results presented in Chapter 2 demonstrated that PcsR2 regulates the adjacent operon containing Pchl3084_4801 and Pchl3084_4803 by comparing the promoter activity of the operons in 30-84WT and a *pcsR2* deletion mutant. In the present study, I show that disruption of PsR2 leads to alteration of the fatty acid production profile. This includes, reduction in the production of certain UFAs such as 16:1 w7c, which is one of the main components of phospholipid and contributes to the fluidity of the cell membrane. Further, we confirmed that the fatty acid desaturase encoded by Pchl3084_4801, but not Pchl3084_4803, is responsible for the introduction of a double bond at the Δ9 position in palmitic acid. Chapter 2 demonstrated that PcsR2 was associated with the expression of several traits involved in interkingdom interactions, including the expression of phenazines, biofilm formation, and metabolism of root-derived nutrient. I hypothesized PcsR2 affects phenazine production in *P. chlororaphis* 30-84 indirectly via alterations in membrane fluiditiy that in turn influenced the network of regulatory genes that control phenazine biosynthesis. Consistent with this hypothesis, results presented in this Chapter demonstrate that overexpression of Pchl3084_4801 partially restored phenazine production in the PcsR2 mutant. As expected given the involvement of PcsR2 in the regulation of UFA production, the loss of a functional PcsR2 resulted in bacterial susceptibility to stress. I discuss these results in terms of the role of PcsR2 in bacterial adaptation to the plant niche.

3.2 Materials and Methods

3.2.1 Bacterial strains, plasmids and growth conditions

Bacterial strains and plasmids used in this study are described in **Table 3.1**, and primers are listed in **Table 3.2**. A spontaneous rifampicin-resistant of *P. chlororaphis* 30-84 is hereafter referred to as wild type. Wild type 30-84 and derivatives were grown at 28˚C in liquid media with agitation (200 rotations per minute) or on solid medium (amended with agar at 15g/L) in Luria-Bertani (LB) (Fisher BioReagentsTM, Hampton, NH), AB minimal media (AB) supplemented with 0.4 % glucose (AB+G), or AB+G supplemented with 2% casamino acids (AB+CAA) (CAA, from BD Bacto™, San Jose, CA). *E.coli* were grown at 37°C in LB medium with agitation (200 rpm). Supplements were used in the following concentrations for *E.coli:* kanamycin (K), gentamicin (Gn), ampicillin (Ap), 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) at 50, 15, 100, and 40 μg/ml, respectively; and for *P. chlororaphis*: Km, Gn, Ap, rifampicin (Rif), Cycloheximide (Cyclohex) at 50, 50, 100, 100, and 100 μg/ml, respectively. Basic molecular cloning and transformation of *E. coli* and *P. chlororaphis* 30-84 were performed as described previously (Pan et al, 2020).

Table 3.1 Strains and plasmids used in Chapter III

Strains and plasmids	Descriptions	References
P. chlororaphis		
30-84WT	Phz+, Rif^R, wild-type (WT)	Whistler and Pierson, 2003
30-84ΔpcsR2	Km^R, *pcsR2* replaced with Km^R cassette	Pan *et al*., 2020
30-84ΔpcsR2(pGT2PcsR2)	Complemented mutant containing plasmid pGT2PcsR	Pan *et al*., 2020
30-84WT(pGT2PcsR2)	WT with plasmid pGT2PcsR2	Pan *et al*., 2020
30-84ZN	Phz^-, Rif^R, *phzB*::*lacZ* genomic fusion	Wood *et al*, 1997
30-84WT(pUCP4801)	WT with plasmid pUCP4801	This study
30-84ΔpcsR2(pUCP4801)	30-84ΔpcsR2 with plasmid pUCP4801	This study
30-84WT(pUCP4803)	WT with plasmid pUCP4803	This study
30-84ΔpcsR2 (pUCP4803)	30-84ΔpcsR2 with plasmid pUCP4803	This study
30-84WT(pUCP20)	WT with plasmid pUCP20	This study
30-84ΔpcsR2(pUCP20)	30-84ΔpcsR2 with plasmid pUCP20	This study
E. coli		
DH5α	F-recA1 endA1 hsdR17 supE44 thi-1 gyrA96 relA1 Δ(argF-lacZYA) I169 Φ80lacZΔM15λ-	GIBCO-BRL
HB101	F^- *hsdS*20(r_B^- m_B^-) *sup*E44 *rec*A1 *ara*14 *pro*A2 *lac*Y1 *gal*K2 *rps*L20 *xyl*-5 *mtl*-5λ^-	GIBCO-BRL
Plasmids		
pUCP20G	Gm^R, pUCP20 derivative containing constitutive promoter pLac with *Sma*I-flanked Gm^R cassette inserted into the unique *Sca*I site within *bla*	Chiang and Burrows, 2003
pGT2Ptac:lacZ	pGT2 containing a constitutive promoter ptac::lacZ gfpfusion	Yu et al., 2017
pGT2PpcsR2:lacZ	pGT2 containing *pcsR2* promoter fused to lazgfp	This study
pGT2P4806:lacZ	pGT2 containing operon 2 promoter fused to lazgfp	This study
pGT2P4809:lacZ	pGT2 containing operon 3 promoter fused to lazgfp	This study
pUCP4801	993bp DNA fragment containing Pchl3084-4801 in Pucp20	This study
pUCP4803	1339bp DNA fragment containing Pchl3084-4803 in Pucp20	This study

Km^R, Gm^R, Rif^R = kanamycin, gentamicin, rifampin resistantrespectively

Table 3.2 Oligonucleotides used for gene cloning and qRT-PCR in Chapter III

Oligonucleotide	Sequence (5'-3')
4806pr-EcoRI-F	CGgaattcCTCCATACGATGTCCTCGACT (*Eco*RI)
4806pr-BamHI-R	CGggatccGCGTATTTCGCTTCCTTTGC (*Bam*HI)
4807pr-EcoRI-F	CGgaattcTGCGCACGCTGCTGGCGATA (*Eco*RI)
4807pr-BamHI-R	CGggatccCAGCCGTACTTAGCCATCTC (*Bam*HI)
4809pr-EcoRI-F	CGgaattcCAGCCGTACTTAGCCATCTC (*Eco*RI)
4809pr-BamHI-R	TTAggatccTGCGCACGCTGCTGGCGATA (*Bam*HI)
4801-F-EcoRI	CGgaattcGATACAGACAGAGGAAGGTC (*Eco*RI)
4801-R-HindIII	CCCaagcttAAATGTAGCTCACGCCGCT (*Hin*dIII)
4803-F-EcoRI	CGgaattcTCGGCAACATGCCTAATGGA (*Eco*RI)
4803-R-HindIII	CCCaagcttACGGTTCATGGCTGGCCCA (*Hin*dIII)
4806-F-SacI	CgagtctGAAATACGCTGGGAGGCGACT (*Sac*I)
4803-F-KpnI	GGggtaccCCTCGGCAACATGCCTAATGG (*Kpn*I)
4804-R-KpnI	GGggtaccCCGCTTCTACTCCATTAGGC (*Kpn*I)
4800-R-HindIII	CCaagcttGGTTAGGTTGACGTTGCGTGCA (*Hin*dIII)
4803-F	TCGGCAACATGCCTAATGG
4804-R	GCTTCTACTCCATTAGGC
rpoD**RT1**	ACGTCCTGAGCGGTTACATC
rpoD**RT2**	CTTTCGGCTTCTTCTTCGTC
4801**RT1**	GGTGTTCTTCGCCAGATCCA
4801**RT2**	CTGCTGGACAACAAGCCCTA
4803**RT1**	CTGCGCTTGTGTTTGCTCTT
4803**RT2**	GTTCTACATGCTGGTGCCCT
4807**RT1**	CATTTTGCTTTCGTGGGCCA
4807**RT2**	TGTTGTCCATCCTGTCCAGC
4810**RT1**	GCTGGGCGCCTATATGTTCT
4810**RT2**	GCACATGGTGGTAGCCGATA

Lowercase indicates nucleotides within restriction sites added to the primer for cloning purposes. The type of restriction enzyme site at the end of primers is indicated. RT in bold indicates primers used for qRT-PCR.

3.3.2 Phylogenetic analysis

The genome sequence from Pchl3084_4800 to Pchl3084_4810 (14,475bp) was retrieved from the National Center for Biotechnology Information (NCBI) database (https://www.ncbi.nlm.nih.gov/). BLASTn (Basic Local Alignment Search Tool for Nucleotides) in NCBI and the *Pseudomonas* database (http://www.pseudomonas.com/) were used to identify all fully sequenced *P.chlororaphis* strains with conserved sequence in this region. Sequence alignments and phylogenetic analyses were performed with MEGA7. Consensus sequences were generated using the WebLogo tool (http://weblogo.berkeley.edu/). Nucleotide alignments were performed with MUSCLE (MUltiple Sequence Comparison by Log- Expectation) and phylogenetic tress were built by the maximum likelihood (ML) method based on the Tamura-Nei Model (Tamura and Nei, 1993) in MEGA7 (Kumar et al., 2016). Bootstrap analysis with 1000 replicates was performed to assess the support of the clusters. Bootstrap values are indicated at the nodes and only ML bootstrap values >70% are shown. The analysis involved 48 nucleotide sequences. All positions containing gaps and missing data were eliminated.

3.3.3 Gas Chromatographic Analysis of Fatty Acid Methyl Esters (GC-FAME)

To determine the effect of PcsR2 and the proteins encoded by the fatty acid desaturases on fatty acid profiles, fatty acid analysis of 30-84WT(NI), 30-84ΔpcsR2(NI), 30-84 WT (pUCP4801 or pUCP4803), 30-84 ΔpcsR2(pUCP4801 or pUCP4803), and *E. coli* carrying pUCP4801 or pUCP4803 were compared by GC-FAME analysis provided by Microbial Identification Incorporated (MIDI), Newark, DE. Briefly, strains from glycerol stock were grown on AB+CAA plates for 48h. Two replicates of each strain were

overnight cultured in AB+CAA broth with agitation as above and sub-cultured in 3ml fresh AB+CAA for 48h. The whole cells were collected by centrifugation at 1250 x g for 10 min and the pellets were shipped with dry ice to MIDI, Inc. NI refers to pUCP20 lacking an insert in this assay.

3.3.4 Anisotropy measurements

Fluorescence anisotropy analyses were performed as previously described (Flechard et al., 2018a) with a few modifications. Cells of 30-84WT(NI), 30-84ΔpcsR2(NI) and 30-84ΔpcsR2(pGT2PcsR2) were grown overnight in AB+CAA at 28°C and the OD_{620} was normalized to 1.0. The normalized cultures were sub-cultured 1:100 in fresh AB+CAA with or without 0.2% polysorbate 80 (PS80) and incubated at 28°C and 10°C, respectively. After 24 h and 48 h incubation, cell pellets were washed three times (8,000g, 5 min) in 10 mM Phosphate Buffered Saline (PBS) and re-suspended in the same wash solution to reach an OD_{620} of 0.015. Then20 µL of 1,6-diphenyl-1,3,5-hexatriene (DPH) stock solution (4mM) in tetrahydrofuran was added to half of the tubes and the remaining tubes without DPH were used as controls. The cultures were then incubated in the dark for 30 min at 30°C to allow the probe to incorporate into the cytoplasmic membrane. A 2 ml aliquot of culture and its control were transferred into cuvettes and measurement of the fluorescence anisotropy was performed using the ISS K2 Multifrequency Fluorometer (ISS, Inc., Champaign, IL) equipped with digital acquisition electronics and an active temperature regulator (Johnson et al., 2007). Excitation and emission wavelengths were set to 365 nm and 425 nm, respectively. The slit widths for the excitation and emission beams were 8 nm. The current for polarized light amplifiers was 20 amperes. Data were

recorded using Vinci2 software. The degree of polarization (r) was calculated from the emission fluorescence intensities measured parallel (Ivv) and perpendicular (Ivh) to the plane of excitation light according to the following equation (Marczak, 2009).

$$r = \frac{Ivv - Ivh * G}{Ivv + 2Ivh * G}$$

The correlation G-factor was the ratio of parallel and perpendicular emitted light when the excitation light was horizontal (G = Ihv/Ihh). The fluorescence anisotropy values are inversely proportional to cell membrane fluidity, where increasing anisotropy values correspond to a more rigid membrane and vice versa (Shinitzky and Barenholz, 1978) The ISS K2 multifrequency fluorometer was kindly provided by Dr. Gregory Reinhart's lab in the Department of Biochemistry and Biophysics at Texas A&M University.

3.3.5 Construction of plasmids containing genes Pchl3084_4801 and Pchl3084_4803 encoding fatty acid desaturases

The coding sequence of genes Pchl3084_4801 and Pchl3084_4803 encoding fatty acid desaturases were PCR amplified with primers 4801-F-EcoRI and 4801-R-HindIII, and 4803-F-EcoRI and 4803-R-HindIII, respectively (**Table 3.2**). The purified DNA fragments were ligated to pUCP20 at *Eco*RI and *Hind*III driven by promoter P*lac*. The constructs were transformed into *E. coli* and positive colonies were selected with blue-white screening and confirmed by PCR and DNA sequencing. The positive constructs pUCP4801, pUCP4803 as well as control plasmid pUCP20 were then transformed into 30-84WT and 30-84ΔpcsR2 by electroporation. DNA sequencing service to verity the constructs was provided by the Laboratory for Genome Technology, Texas A&M University.

3.3.6 Salt, temperature, and hydrogen peroxide stress assays

For salt stress assays, bacteria were cultured overnight in AB+G media and OD_{620} values were normalized to 1.0 and then subcultured 1:10 in fresh AB+G media. Aliquots of 100 µl were dispensed into PM9 Microplate™ Osmolytes from Biolog, Inc. These plates contain 12 osmolytes with concentration gradients as well as 23 osmoprotectants. For NaCl, there are 12 concentrations from 1% to 10%. The corresponding strains cultured in AB+G without salt were used as control. Bacterial growth and phenazine production were quantified spectrophotometrically (OD_{620} and OD_{367}, respectively) at 0 h, 3 h, 6 h, 9 h, 12 h,18 h, 24 h, 30 h, and 48 h after inoculation using a Tecan Infinite M200 Pro. Two measurements were taken at each time point. ZN is a derivative of *P. chlororaphis* 30-84, which cannot produce phenazines. Optical density measurements obtained using this strain were used to normalize phenazine production by 30-84WT(NI) and 30-84ΔpcsR2(NI) to remove the influence of cellular products at OD_{367}. NI refers to pGT2P*tac:lacZ* in this assay.

For temperature stress assays bacteria were grown in AB+CAA at 28°C with agitation and sub-cultured (1:100 dilution) into fresh AB+CAA and standardized to the same optical density (OD_{620}= 0.8). For temperature stress, strains were grown at 10°C up to 72 h, and growth and phenazine production measured spectrophotometrically (OD_{620} and OD_{367}, respectively) as described above using a Nanovue plus (GE Healthcare Life Sciences, Pittsburgh, PA). Growth and phenazine production at 28°C were used as the no-stress control. For phenazine quantification, bacterial populations were quantified by spectroscopy (OD_{620}) and then phenazines were extracted and quantified by spectroscopy

(OD_{367}, standardized to cell density) as described previously (Wang et al., 2016). Briefly, cell cultures were acidified and phenazines were extracted in benzene, benzene was removed via evaporation, and phenazines were resuspended on 0.1 N NaOH.

For the hydrogen peroxide stress assays, bacteria were grown to mid-log phase (OD_{620} = 0.4-0.8) in LB medium at 28°C with agitation and standardized to the same optical density ((OD_{620} = 0.5). The bacterial culture was diluted 1:100 in 30% H_2O_2 and cells were incubated in this condition at room temperature for 5-10 min with agitation. All serial dilutions in sterile water were spotted on LB agar and cultured at 28°C for 48h and percent survival was determined by standardizing the CFU of the H_2O_2-stressed cells to the CFU of non-stressed cells. NI refers to pGT2P*tac:lacZ* in this assay.

3.3.7 Statistical analysis

All data are presented as the mean ± the standard error of the mean (SE) from at least two experiments. Multiple comparisons were analyzed using ANOVA and Turkey HSD (honestly significant difference; $P<0.05$). Two group comparisons (30-84WT and30-84ΔpcsR2) were performed using Student's t test ($P<0.01$, $P<0.005$, and $P<0.001$). All analyses were performed using JMP14 software (JMP, Version 14. SAS Institute Inc. Cary, NC, 1989-2019).

3.3 Results

3.3.1 *pcsR2* is part of a highly conserved genomic region in *P. chlororaphis*

The transcriptional regulatory gene *pcsR2* (Pchl3084_4807) is located between two operons containing genes annotated as functioning in fatty acid desaturation, and altogether the sequence of this region spans 14,475 bp and contains eleven genes in three

operons **(Figure 2.2A in Chapter II)**. *pcsR2* is part of an operon (designated operon 1) that also contains Pchl3084_4808 (a putative AMP-binding domain protein/acyl-CoA synthetase). Acyl-CoA synthetases are proposed to catalyze the formation of fatty acyl-CoA by a two-step process including hydrolysis of ATP to yield pyrophosphate. They are important for the synthesis of complex membrane lipids and the synthesis and mobilization of storage lipids. They also function in the transport of exogenous fatty acids into the cell, the targeting of fatty acids into specific metabolic pathways, and in fatty acid-mediated transcriptional control (Li et al., 2007).

Operon 1 is located directly upstream of a larger seven-gene (Pchl3084_4800 to Pchl3084_4806) operon (Operon 2), encoding components of a fatty acid desaturase complex. This includes two genes encoding fatty acid desaturase proteins (Pchl3084_4801, Pchl3084_4803), a gene encoding an oxidoreductase-FAD/NAD-binding protein (Pchl3084_4802), and other related components e.g., genes encoding an acyl-CoA dehydrogenase (Pchl3084_4804), an aminotransferase class III (Pchl3084_4805), a phosphopantetheine attachment site protein (Pchl3084_4806) and a hydrolase (Pchl3084_4800). Upstream and divergently transcribed from Operon 1 is a two-gene operon (Operon 3) encoding a putative amidase protein and a third fatty acid desaturase protein (Pchl3084_4809, Pchl3084_4810, respectively).

Based on nucleotide sequence homology to the entire 14,475 bp region, the three operons are conserved almost exclusively (except for *P. chlororaphis* UFB2) among the 49 *P. chlororaphis* strains for which there is complete genome or chromosome sequence information in GenBank (National Center for Biotechnology Information, as of

10/26/2019). Bioinformatic analysis of the genomic region of the three operons by BLASTn in NCBI and the *Pseudomonas* Genome DB (http://www.pseudomonas.com/) indicates that this entire region is conserved in 50 *Pseudomonas* strains based on the presence of homologs with 91.62% to 100% nucleotide sequence identity with *P. chlororaphis 3*0-84. This includes 48 of 49 *P. chlororaphis* strains (with the exception of *P. chlororaphis* UFB2) and two unidentified *Pseudomonas* species (*Pseudomonas* sp.09C 129 and *Pseudomonas* sp. MRSN 12121, the phylogenetic tree does not include these two strains).

Phylogenetic analysis (**Figure 3.1**) of the 48 *P. chlororaphis* strains containing this region sorted them into two groups, strains that have the phenazine biosynthetic operon (47 strains, hereafter referred to as phenazine producers) and strains that do not (only *P. chlororaphis* PCL1606). The majority of phenazine producers were differentiated into four distinct subgroups with strong bootstrap support (100%). *P. chlororaphis* 30-84 clustered with a diversified subgroup of 5 strains, which based on bioinformatics produce PCN or 2-hydroxy PCA. The *piscium* subgroup contained 14 strains with 12 strains from *P. chlororaphis* subsp. *piscium,* the aurantiaca subgroup contained 12 strains with 11 strains from *P. chlororaphis* subsp. *aurantiaca,* and the aureofaciens subgroup contained 13 strains with 9 strains from *P. chlororaphis* subsp. *aureofaciens*. Three strains *(P. chlororaphis* Pb-St2, *P. chlororaphis* B25 and *P. chlororaphi* subsp. *aureofaciens* P2) did not cluster with any of these subgroups (**Figure 3.1**).

It is worth mentioning that the similarities in this region were somewhat related to the types of phenazines each strain is predicted to produce. For instance, the *aurantiaca*

and *aureofaciens* subgroups clustered to form a clade of strains that are predicted to produce 2-hydroxy PCA with strong bootstrap support (97%), which were differentiated from the PCN-producing *piscium* clade with strong bootstrap support (94%). In contrast, the strains of diversified subgroups were separated from the two clades above and are predicted to produce PCN or 2-hydroxy PCA. As expected, *P. chlororaphis* B25 predicted to produce only PCA (lacking any phenazine modifying genes) did not cluster in any of clades above. An intriguing finding is that the distribution of this phylogenetic tree based on the 14,475 bp region containing *pcsR2* bears a striking high similarity with the phylogenetic tree based on 2651 genes of the core genome of *P. chlororaphis* (Biessy et al., 2019).

3.3.2 Fatty acid profiles of 30-84 WT(NI) and 30-84ΔpcsR2(NI) detected by GC-FAME

Fatty acid profiles of 30-84WT(NI) and 30-84ΔpcsR2(NI) were detected by GC-FAME. As shown in **Figure 3.2**, after 48 h growth in AB+CAA media, a total 28 fatty acids were detected and the predominant fatty acids (>10%) in 30-84WT(NI) are 16:0 (24.51 ± 0.89%), 17:0 cyclo w7c (15.44 ± 0.61%), 12:0 3OH (12.63 ± 0.35%), and 10:0 3OH (10.86 ± 0.36%). Significantly, 18 of the 28 fatty acids detected were differentially expressed in the 30-84WT(NI) and 30-84ΔpcsR2(NI), including 6 saturated fatty acids (10:0, 12:0, 14:0, 15:0, 16:0, and 17:0 iso); one cyclopropane fatty acid (17:0 cyclo w7c); 5 three-hydroxyl saturated fatty acids (10:0 3OH, 12:0 3OH, 14:0 3OH, 15:0 3OH, and 16:0 3OH); and 6 unsaturated fatty acids (UFAs) (12:1 3OH, 16:1 w7c, 17:1 w7c, 15:1 iso F, 16:1 w7c alcohol, and16:1 w6c).

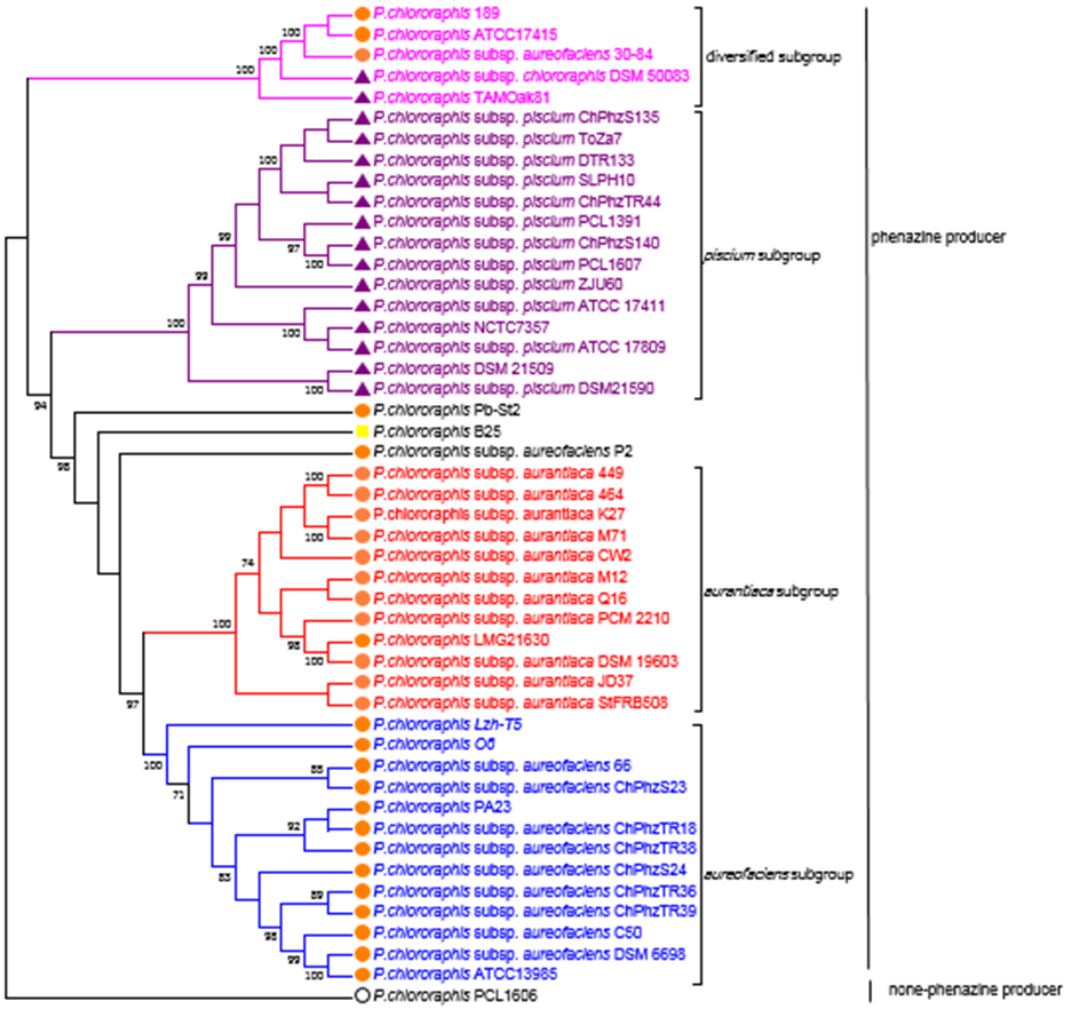

Figure 3.1 Phylogenetic analysis of the genomic region surrounding *pcsR2*
The maximum likelihood (ML) tree with Jones-Taylor-Thornton (JTT) model was constructed based on multiple sequence alignments (MUSCLE) of nucleotides homologs (14,475 bp homologs of Pchl3084_4800 to Pchl3084_4800) from 48 fully sequenced species of *P. chlororaphis*. Bootstrap values (1000 replicates) are indicated at the nodes and only the values >70% are shown. The closed circles (orange) correspond to strains producing 2-hydroxypenzaine (2-OH-PHZ) and/or 2-hydroxypenazine-1-carboxylic acid (2-OH-PCA). The closed triangles (purple) correspond to strains producing only pheanzine-1-carboxamide (PCN). The closed rectangle (yellow) indicates a strain producing pheanzine-1-carboxylic acid (PCA). The open circle corresponds to a strain that does not produce phenazines. Strains in fuchsia belong to a diversified subgroup, strains in purple clustered in the *piscium* subgroup, strains in red clustered in *aurantiaca* subgroup and strains in blue clustered in the *aurofaciens* subgroup. Strains in black did not cluster with any subgroup.

The two most prominent differences are the reductions in the two UFAs 16:1 w7c and 12:1 3OH in 30-84ΔpcsR2(NI) compared with 30-84WT(NI), suggesting these are the main UFAs regulated by PcsR2. UFA 16:1 w7c is the predominant UFA in the

phospholipid bilayer in the cell membrane of *Pseudomonas (*Flethcher et al., 2018), whereas the function of 12:1 3OH in *P. chlororaphis* is unknown.

. GC-FAME results revealed that the ratios of the total UFAs to SFAs were slightly greater in 30-84WT(NI) compared to 30-84 ΔpcsR2(NI) (26.20% and 22.82%, respectively), and the percentage of the total fatty acids that were comprised of cyclopropyl fatty acids (17:0 cyclo w7c + 19:0 cyclo w8c) were less in 30-84WT(NI) than in 30-84ΔpcsR2(NI) (17.68% and 21.88%, respectively). These results are consistent with the membrane of 30-84 ΔpcsR2(NI) being less fluid than in 30-84WT(NI).

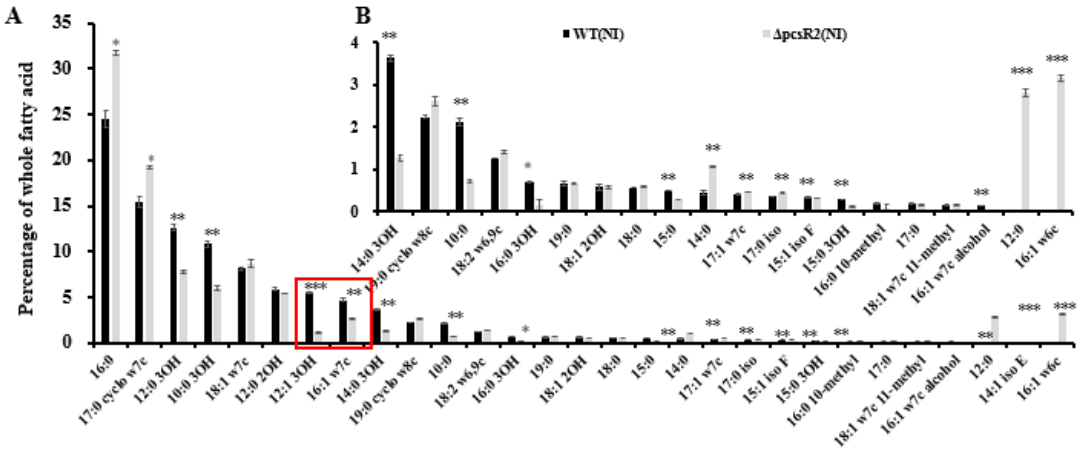

Figure 3.2 Comparison of fatty acid profiles in 30-84WT and 30-84ΔpcsR2.
Strains 30-84WT(NI) and30-84 ΔpcsR2(NI) were cultured in AB+CAA media for 48 h and the total fatty acids were quantified by Gas Chromatography analysis of Fatty Acid Methyl Esters (GC-FAME) from MIDI, Inc. (A) The table for all fatty acid profiles. (B) Fatty acids that are lower than 4%. NI refers to pUCP20 in this assay. The fatty acids boxed by the red rectangle are the predominant unsaturated fatty acids regulated by PcsR2. Data are the means and standard errors of two replicates. An asterisk indicates significant differences at P<0.05; two asterisks at P<0.01, and three asterisks at P<0.001

3.3.3 PcsR2 altered the fluidity of the cell membrane

To determine whether PcsR2 plays a role in cell membrane fluidity, fluorescence anisotropy between 30-84WT(NI) and 30-84ΔpcsR2(NI) was compared. In this assay, the fluorescent probe 1,6-diphenyl-1,3,5-hexatriene (DPH) was inserted into the cell

membrane and the intensity of the probe was measured when the cell culture was exposed to polarized light. The relationship between fluorescence anisotropy and membrane fluidity is inversely correlated, which means increasing anisotropy values correspond to a more rigid membrane and vice versa (Shinitzky and Barenholz, 1978). As shown in **Figure 3.3A**, as cells enter stationary phase (24 h, 28 °C), the anisotropy value of 30-84ΔpcsR2(NI) was strikingly higher than that of 30-84WT(NI) or the complemented mutant 30-84ΔpcsR2(pGT2PcsR2) (0.246 ± 0.002 vs 0.213 ± 0.001 and 0.199 ± 0.002, respectively), which indicates 30-84ΔpcsR2(NI) has a more rigid membrane than 30-84WT or the complemented mutant. This difference was somewhat obscured as time went on (48 h), likely due to the contribution of LPS matrix to the cell culture. In addition, the anisotropy of 30-84WT(NI) and 30-84ΔpcsR2(NI) under low temperature (10 °C) was also measured. The results **(Figure 3.3B)** showed that the anisotropy value of 30-84WT(NI) was significantly higher when cells were grown for 24 h at 10°C compared to 28 °C indicating an adaptive decrease in the fluidity of the cell membrane occurred at the lower temperature hypothetically due to the lower production of unsaturated fatty acids (Los and Murata, 2004). However, the value of anisotropy in 30-84ΔpcsR2(NI) did not change significantly between these two temperatures, consistent with there being less unsaturated fatty acids (UFAs) or more cyclopropyl-fatty acids (cFA) in cell membranes of the mutant compared to the wild type (Pandit and Klauda, 2012). Polysorbate 80 (PS80), a nonionic surfactant, which can increase the fluidity of cell membranes, was used as a control, and showed a significant change for both strains with temperature.

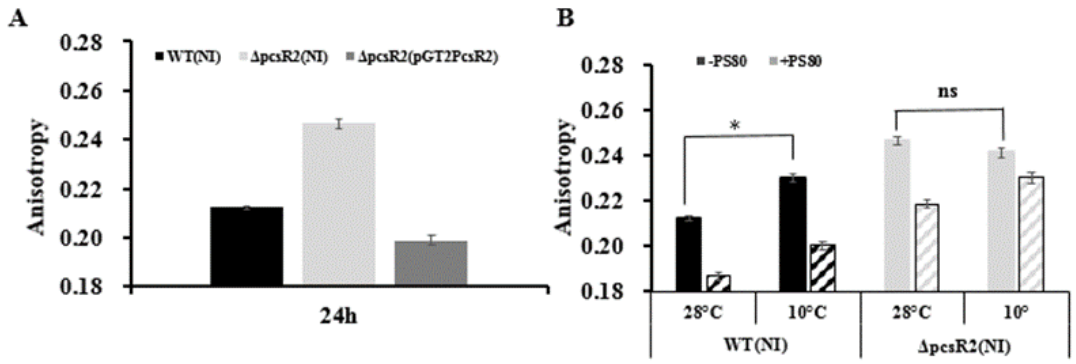

Figure 3.3 Anisotropy in response to temperature stress
(A) The cell membrane anisotropy of WT, 30-84ΔpcsR2 and complement strain 30-84ΔpcsR2 (pGT2PcsR2) were measured using the probe 1,6-diphenyl-1,3,5-hexatriene (DPH). (**B).** The anisotropy of WT and 30-84ΔpcsR2 after 24 h 30-84ΔpcsR2at 28℃ and 10℃ with and without the detergent polysorbate 80 (PS80), which moderately destabilizes the cell envelope. NI indicates no insert pGT2Ptac:lacZ in this assay. An asterisk indicates a significant difference at P<0.05, ns indicates not significant by Student's T-test.

3.3.4 Role of Pchl3084_4801 and Pchl3084_4803 desaturated fatty acid production

To determine whether either of the two genes identified as encoding desaturases are responsible for differences between the wild type and the *pcsR2* mutant in fatty acid production profiles or the more easily measured phenotype, phenazine production, plasmids carrying Pchl3084_4801 or Pchl3084_4803 were introduced separately into the wild type and 30-84ΔpcsR2 strains. Fatty acid profiles were also compared in *E. coli* strains transformed with plasmids carrying Pchl3084_4801 or Pchl3084_4803 or the empty vector. As shown in **Figure 3.4A,** phenazine production by 30-84WT(pUCP4801) and 30-84ΔpcsR2(pUCP4801) measured using spectroscopy (OD_{367}) were 2.95 ± 0.14 and 1.17 ± 0.1, respectively, which was significantly higher than 2.06 ± 0.06 and 0.50 ± 0.05 in the corresponding control strains 30-84WT(NI) and 30-84ΔpcsR2(NI), respectively, indicating Pchl3084_4801 expression is correlated with phenazine production. GC-FAME revealed that in *E. coli*(pUCP4801), 16:1 w7c comprised 10 ±

0.1% of all fatty acids, which is 42.5% higher than ± in the control, *E. coli*(NI) **(Figure 3.4B)**. Consistent with this result, overexpression of Pchl3084_4801 in 30-84WT led to a 67.5% increase of 16:1 w7c compared to 30-84WT(NI) (**Figure 3.4C**). In both *E. coli* and 30-84WT, elevated 16:1 w7c production was negatively correlated with cFA (17:0 cyclo and/or 19:0 cyclo) production. Interestingly, no 12:1 3OH is made in *E. coli* and there was no significant difference associated with Pchl3084_4801 overexpression in 30-84WT, indicating Pchl3084_4801 is not responsible for the difference in expression of 12:1 3OH between 30-84WT and 30-84ΔpcsR2. In contrast, in 30-84WT(pUCP4803) and neither phenazine production nor the production of 16:1 w7c or 12:1 3OH were altered relative to the controls **(Figure 3.4 D-F),** although the relative abundance of some SFAs were altered (date not shown). Together, these data suggested that alteration in the expression of Pchl3084_4801 is responsible for the difference between 30-84WT and 30-84ΔpcsR2 in the production of 16:1w7c, and that reduced 16:1w7c is correlated with reduced phenazine production in 30-84ΔpcsR2.

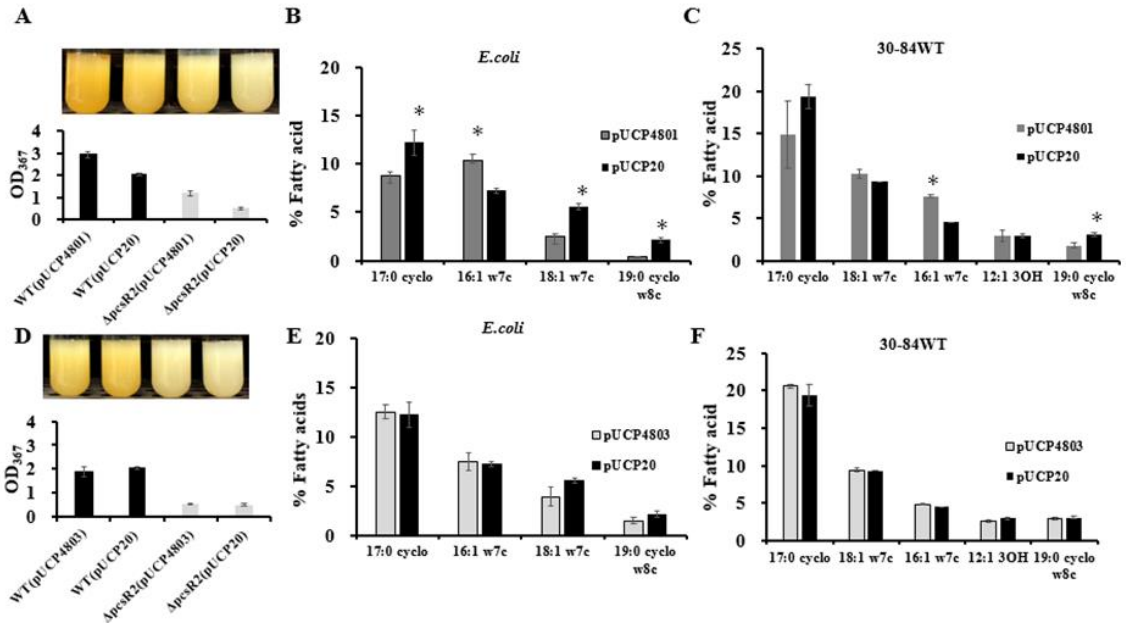

Figure 3.4 Effect of expression of Pchl3084_4801 and Pchl3084_4803 on fatty acid production profiles and phenazine production

The coding sequence of the two genes were ligated into pUPC20 driven by the constitutive pLac promter and transformed into *E.coli*, 30-84WT, or ΔPcsR2. The strains were cultured in AB+CAA media for 48 h. **(A, D)** Phenazine production was quantified spectrophotometrically (OD_{367}) with corresponding strains transformed with empty plamids. Fatty acids were quantified by Gas Chromatography analysis of Fatty Acid Methyl Esters (GC-FAME) from MIDI, Inc. in *E.coli* **(B, E)** and 30-84WT **(C.F)**. Data are the means and standard errors of two replicates. An asterisk indicates significant differences at $P<0.05$; two asterisks at $P<0.01$, and three asterisks at $P<0.001$

3.3.5 PcsR2 plays a role in salt, temperature and hydrogen peroxide tolerance.

Because cell membrane fluidity is important for cell adaptation to stress (Los and Murata, 2004), we examined the role of PcsR2 in salt, temperature, and redox stress tolerance by comparing the growth of wild type and the *pcsr2* mutant under stress conditions. For salt stress, the function of PcsR2 in osmoregulation was investigated using PM9 MicroPlate™ Osmolytes from Biolog, Inc. Salt concentrations in excess of 4% inhibited the growth of both strains, but there were no significant differences in the growth of wild type and the mutant even in the highest salt conditions (data not shown).

It has been reported that some osmoprotectants (e.g. proline and betaine) could relieve osmotic stresses as osmotic balancers and effective stabilizers of enzymatic functions (Sleator and Hill, 2002). The effect of 22 osmopretectants on the growth of 30-84WT(NI) and 30-84ΔpcsR2(NI) under 6% NaCl stress were evaluated using the PM 9 plates. Our data show that at 6% NaCl, growth of the wild type and mutant were significantly reduced compared to the no salt control, but addition of choline as an osmoprotectant significantly improved the survival of wild type but not the mutant, and this effect was only observed for choline and no other osmoprotectants (**Figure 3.5A, B,** showing a subset of osmoprotectants).

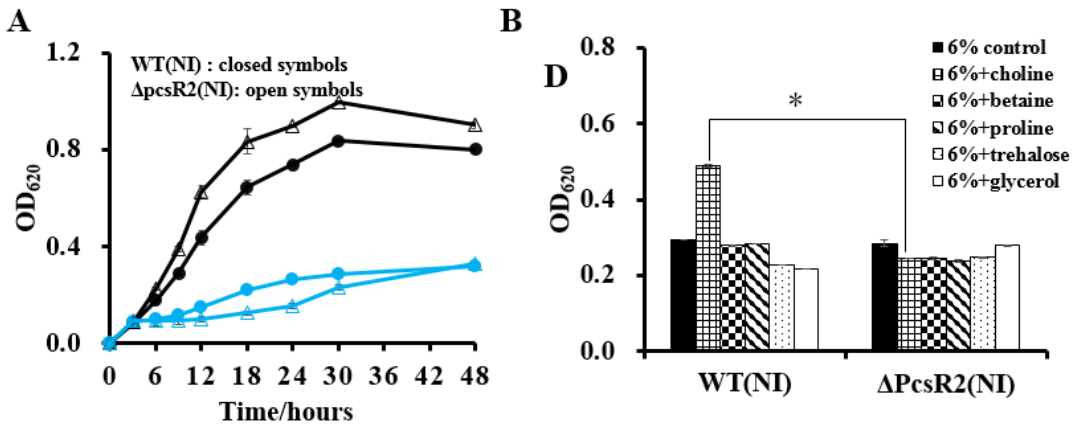

Figure 3.5 Comparison of 30-84WT(NI) and 30-84ΔpcsR2(NI) under salt stress with and without osmoprotectants

30-84WT(NI) and 30-84ΔpcsR2(NI) strains were grown overnight in AB+G media and the OD_{620} was standardized before the cells were inoculated 1:10 into 100 µl fresh AB+G media supplemented with 12 osmolytes with concentration gradients as well as 23 osmoprotectants in PM9 Microplate™ Osmolytes from Biolog Inc., **(A)** growth curve of 30-84WT(NI) and 30-84ΔpcsR2(NI) with and without NaCl stress. black, no NaCl; blue, 6% NaCl. **(B)** growth curve of 30-84WT(NI) and 30-84ΔpcsR2(NI) with osmoprotectants with 6% NaCl stress at 48 h. Asterisk indicates significant difference at P>0.05 by Student's t-test

Previous work demonstrated that UFA components of cell membranes play an essential role on membrane fluidity and cold stress tolerance (Los and Murata, 2004).

Therefore, we compared the growth of wild type, mutant, and complemented mutant at 28°C and 10°C for three days. The result showed that growth rates of 30-84WT(NI), 30-84ΔpcsR2(NI) and 30-84ΔpcsR2(pGT2PcsR2) were not significantly different at either temperature. All three grew much slower at 10°C compared to 28°C, but reached the same final cell density at 72 h **(Figure 3.6 A)**. These results indicate that loss of PcsR2 in the mutant does not impair growth at low temperature. Interestingl, the production of phenazine in 30-84ΔpcsR2(NI) was not measurable at 10°C during the entire incubation **(Figure 3.6 B-C)**. In contrast, phenazine production of 30-84WT(NI) and 30-84ΔpcsR2(pGT2PcsR2) at 10°C ultimately accumulated to the same level observed for growth at 28°C, although cells started to produce phenazine much later. Collectively, these results suggested that under cold stress PcsR2 did not affect primary growth but affected phenazine secondary metabolites production.

Membrane lipid composition was shown previously to affect oxidative stress tolerance by regulating transport of hydrogen peroxide (H_2O_2) via aquaporins in cell membranes (Bienert et al., 2006; Bienert et al., 2007; Tong et al., 2019). To verify the role of PcsR2 in oxidative stress, cell densities of 30-84WT(NI), 30-84ΔpcsR2(NI) and 30-84ΔpcsR2(pGT2PcsR2) with and without exposure to H_2O_2 were measured at 48 h and survival rates determined by standardizing growth in H_2O_2 to growth without H_2O_2. Survival of 30-84WT(NI) and 30-84ΔpcsR2(pGT2PcsR2) were 35.4 ± 4.6% and 25.7 ± 4.3%, respectively, whereas the survival rate of 30-84ΔpcsR2 was significantly lower (4.1 ± 0.8%), indicating PcsR2 was important for redox stress tolerance. **(Figure 3.6 D-E).**

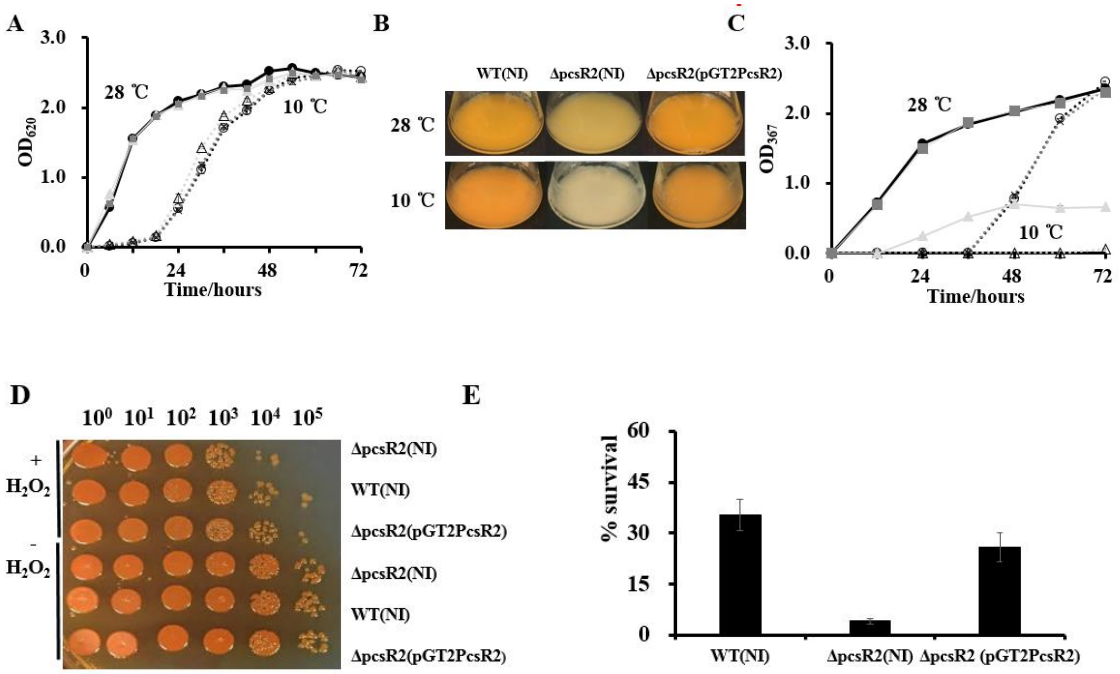

Figure 3.6 PcsR2 is associated with temperature and H_2O_2 stress tolerance
(A) Overnight cultures of WT and pcsR2 derivative strains were sub-cultured 1:100 in fresh AB+CAA media and cultured at 10℃ and 28℃, respectively. The growth curves were calculated spectrophotometrically (OD_{620}) every 6 h over a 72 h period. **(B-C)** Phenazines were quantified spectrophotometrically (OD_{367}) every 12 h and the images of the strains were taken at 72 h. solid line, 28℃; dashed line, 10℃. Markers with circle: 30-84WT(NI); triangle: 30-84ΔpcsR2(NI); square: 30-84ΔpcsR2(pGT2PcsR2). Overnight cultures of 30-84WT(NI) 30-84ΔpcsR2(NI) and 30-84ΔpcsR2(pGT2PcsR2) were sub-cultured in fresh AB+CAA media to mid-log phase. 30% H_2O_2 was added 1:100 (v/v). **(D)** Serial dilutions were made 5 min after the addition of H_2O_2. Bacteria were grown on LB plates for 48 h. **(E)** Percent survival was normalized by the CFU of the cells that were not stressed at 10^4. NI indicates no insert pGT2Ptac:lacZ in this assay. Data are the means and standard errors of at least three replicates; some error bars do not exceed the size of symbols

3.4 Discussion

Fatty acids are indispensable for a myriad of biological functions. They provide the precursors not only for the biosynthesis of cellular components (e.g. phospholipid, LPS and lipoproteins in cell membranes), but also certain signal chemicals (e.g. AHLs and oxylipins) (Schweizer, 2004; Wang et al., 2019). In the present study, I discuss the organization and conservation of a region of the bacterial chromosome that contains two operons with genes annotated as encoding enzymes involved in fatty acid desaturation in

P. chlororaphis species. Previous work (Chapter 2) demonstrated that the solo LuxR, PcsR2, is involved in the regulation of one of these operons (operon 2), and that the expression of *pcsR2* and operon 2 is highly upregulated on plant roots. My results demonstrate that deletion of *pcsR2* resulted in alteration of the fatty acid profile, especially reduction in the production of UFAs such as 16:1 w7c, which is one of the main components of phospholipid and contributes to the fluidity of cell membranes, and nearly all the fatty acids having a 3-hydroxyl group. Importantly deletion of *pcsR2* resulted in changes in anisotropy measurements, consistent with changes in membrane fluidity. Interestingly, LuxR solos wereshown previously to be involved in the regulation of fatty acid production, especially as it relates to cell membranes. For example, VarR in the marine macroalgal pathogen *Nautella italica* is a LuxR solo involved the regulation of traits related to membrane integrity as well as lipid metabolism and transport systems (Gardiner et al., 2015). QscR in the human pathogen *P.aeruginosa* was also demonstrated to regulate a downstream operon, which includes a gene encoding fatty acid desaturase that is associated with cell membranes and phenazine production(Ding et al., 2018). Additionally, previous studies have shown that alterations in cell membranes can have broad effects on bacterial traits that rely on membrane-bound transport systems or signal transduction systems, resulting in altered metabolism and survival (Baysse et al., 2005; Flechard et al., 2018; Cazzola et al., 2020).

UFAs are a primary determinant of bacterial cell membrane fluidity and while most unsaturated fatty acid production occurs via conserved fatty acid desaturation mechanisms some bacteria contain additional genes encoding novel fatty acid desaturases

that may provide fine-tuning in response to particular environments. For example, inactivation of *desA* and *desD* in *Synechocystis* were reported to result in a dramatic decrease in membrane fluidity and the ability to acclimate to low temperatures (Tasaka et al., 1996). Operons 1-3 in *P. chlororaphis* containing multiple desaturases, appear to be unique to *P. chlororaphis* species and the potential functions of these fatty acid desaturation have not been described previously. Our previous work suggested PcsR2 was more directly involved in the regulation of operon 2, so I focused on this operon. Constitutive expression of each of the two fatty acid desaturase genes (Pchl3084_4801 and Pchl3084_4803) in 30-84WT and mutant 30-84ΔpcsR2 demonstrated that Pchl3084_4801 was involved in the production of 16:1 w7c, but Pchl3084_4803 was not involved in production of any fatty acids. I hypothesize that Pchl3084_4803 together with other enzymes encoded by the genes in that operon operate as a biosynthetic unit to alter fatty acid production and that individually they may have little or no ability to effectively alter fatty acid production Future work will examine whether expression of the entire operon restores wild type fatty acid production and other associated bacterial traits in the mutant. An alternative hypothesis is that PcsR2 also indirectly affects the expression of operon 3, and together the two operons contribute to the phenotype of the mutant.

Adaptation via membrane fluidity has been previously associated with adaptation to temperature stresses, especially low temperature, as well as osmotic and redox stress (Los and Murata, 2004). Indeed, the discovery of novel fatty acid desaturases that protect against cold stress have led to useful applications, such as the introduction of the bacterial genes encoding fatty acid desaturases that confer cold tolerance into plants as a way to

enhance their chilling tolerance (Wada et al., 1990; Ishizaki-Nishizawa et al., 1996; Orlova et al., 2003). In the present study, my data demonstrates that mutation of *pcsR2* did not play a significant role in bacterial sensitivity to cold stress, or osmotic stress caused by excess NaCl, because both 30-84WT and the *pcsR2* mutant were equally susceptible. However, it was interesting that the osmoprotectant choline relieved the osmotic stress from excessive NaCl in 30-84WT, but not in 30-84ΔpcsR2 Based on these data I speculate that choline uptake or potential utilization in lipid biosynthesis was impaired in 30-84ΔpcsR2. Choline-derived phosphatidylcholine (PC) is a phospholipid ubiquitous in cell membranes of eukaryotes. However, it has been found that a small amount (4%) of PC also exists in a limited of number (10%) of prokaryotes (e.g. rhizobia, *Streptococcus* and *Pseudomonas*). Previous work showed that PC is involved in functions important for symbiotic or pathogenic microbe-host interactions(de Rudder et al., 1999; Wilderman et al., 2002; Aktas et al., 2010; Geiger et al., 2013; Joyce et al., 2019). For example, it has been reported that plant-exudate choline was used for biosynthesis of cellular membrane PC in *rhizobia* (de Rudder et al., 1999). PC is synthesized mainly by the phospholipid N-methyltransferases (Pmt)-dependent pathway (PC *de novo*) in eukaryotes, during which PC is converted from methylation of phosphatidylethanolamine (PE) by PmtA. In contrast, bacteria can synthesize PC by a unique phosphatidylcholine synthase (Pcs)-dependent pathway in addition to a Pmt-dependent pathway. In the Pcs-dependent pathway, Pcs catalyzes the condensation of choline directly with cytidine diphosphate diacylglycerol (CDP-DAG) to form PC. Because results showed that chloline supplementation alleviated NaCl osmotic stress, we postulate that PcsR2 may be involve in biosynthesis of PC in

Pseudomonas chlororaphis. Subsequently, the defect in PC in 30-84ΔpcsR2 alters the fluidity of cell membranes, and therefore brings about a wide range of compromised biological functions including bacteria metabolism, stress tolerance, production of phenazine and proteases. Interestingly, in comparison to low temperature and osmotic stress, growth of 30-84ΔpcsR2 was impaired under redox stress as compared to 30-84WT. Previous work has shown that redox stress tolerance is correlated with phenazine production, which is also reduced in 30-84ΔpcsR.

Interestingly, nearly all the fatty acids having a 3-hydroxyl group were significantly decreased in the *pcsR2* mutant compared to 30-84 WT. These 3-hydroxyl fatty acids have been shown to be critical substrates for biosynthesis of the LipA moiety in lipopolysaccharide (LPS), which is the outermost part of the outer membrane of Gram negative bacteria (Raetz and Whitfield, 2002). Recently, the synthetic and bacterial LPS-purified medium-chain 3-hydroxy fatty acids were demonstrated to trigger plant immunity in *Arabidopsis* plants (Kutschera et al., 2019). Appendix examines whether alterations in 3-hydroxyl fatty acid profiles in the *pcsR2* mutant compared to 30-84 WT alter plant immunity.

In summary, PcsR2 is a plant responsive LuxR-transcriptional regulator that is involved in the expression of genes involved in the production of UFAs by a previously unreported mechanism conserved in *P. chlororaphis*. The production of these UFAs plays a role in cell membrane fluidity, which in turn likely controls other metabolic and regulatory networks that contribute to the traits important for plant-beneficial interactions. We propose a model for the function of PcsR2 via the regulation of fatty acids in

P.chlororaphis **(Figure 3.7)**. PcsR2 is mainly involved in regulation of two types of FA biosynthesis: 16:1 w7c (in blue) and 3-hydroxy FA (in green). On the one hand, via regulation of Pchl3084_4801, PcsR2 turns 16:0 (palmitic acid) into 16:1w7c, which is the predominant UFA in phospholipid of cell membrane and contributes to fludity of cell membrane, resulting in a broad range of alterations in bacteria such as adaptation to stressess (e.g salt, cold and redox), phenazine produciton and a global alteration of metobolism (Appendix). We speculate the specific lipid in cell membrane regulated by PcsR2/Pchl3084_4801 is phosphatidylcholine and it needs to be further confirmed. On the other hand, PcsR2 may divert ß-hydroxyacyl-ACP into biosynthesis of 3-hydroxy FAs such as 12:0 3OH, which is finally converted to another important UFA 12:1 3OH. All 3-hydroxy FAs may be invovled in lipidA biosynthesis of LPS in outermembrane of gram-neagtive bacteia, whereas the function of 12:1 3OH is unkown. We hypothsize 12:1 3OH may play role in three ways. First, it may serve as the normal UFA in cell membrane or the normal 3-hydroxy FAs as component of LPS in outermembrane. Second it may be an interkingdom signal for plant-microbe interactions such as plant immunity response. Third, it may be a novel interaspecies signal since it is structurely similar with a diffusible fatty acid signal molecule, cis-2-dodecenoic acid (BDSF), in *Burkholderia cenocepacia* (Ryan et al., 2009). However, further research is needed to test all the three ways.

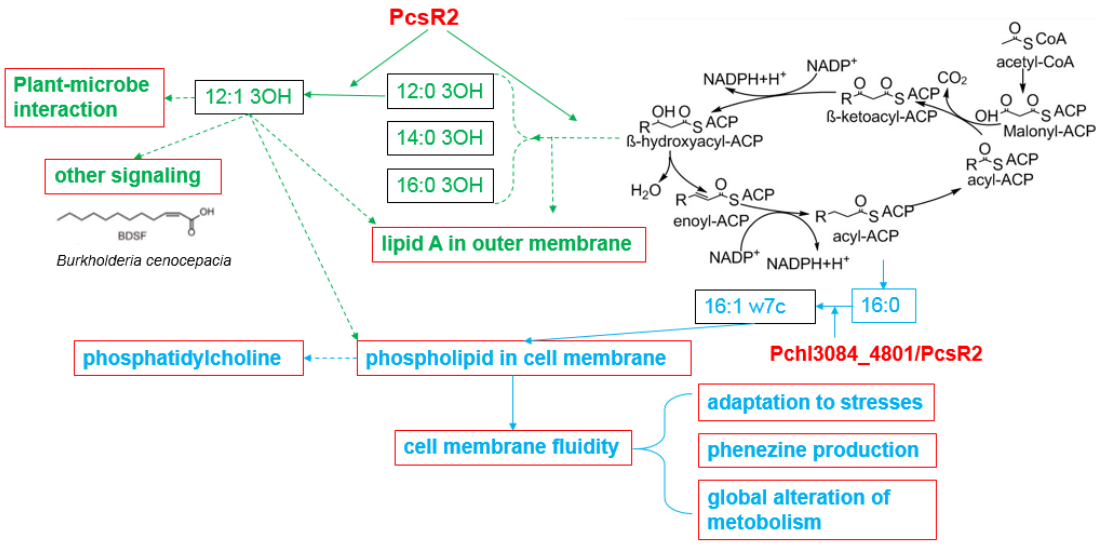

Figure 3.7 Proposed function of PcsR2 via the regulation of fatty acids in *P.chlororaphis*

PcsR2 is mainly involved in regulation of two types (16:1 w7c in blue and 3-hydroxy FA in green) of FA biosynthesis and plays role on the rhizosphere adaptation and bacteria-plant interaction. The circular pathways in black indicate the common FA biosynthesis pathway. Texts and arrows in blue indicate 16:1 w7c-medicated functions, whereas those in blue indicate 3-hydroxy FA- medicated functions. The rectangular text boxes with black borders indicate biosynthesis pathway, whereas those with red borders indicate potential functions. Solid arrows means the functions has been confirmed and dash arrows means functions need to be further studies. Some abbreviations are as follows. FA: fatty acids, BDSF cis-2-dodecenoic acid.

CHAPTER IV

SUMMARY AND CONCLUSION

Interkingdom signaling between microbes and plants plays a fundamental role both for pathogenesis and beneficial interactions. Interkingdom communication can be categorized into three groups by orientation: ① intra-/inter-species signaling among microbes; ② signaling from microbes to plants; and ③ signaling from plants to microbes (Venturi and Keel, 2016). Over the past few decades, many microbiological studies have focused on signaling among or from microbes. However, limited information is available concerning the effects of these signals on bacterial gene expression and the role of those genes with altered expression in plant–microbe interactions.

LuxR solos have emerged to be increasingly important players not only in cell-cell communication, but interkingdom -signaling as the receptor for alternative plant-derived communication molecules to AHLs (Kan et al., 2017). Unlike the LuxR-type transcriptional regulators of prototype LuxR/LuxI quorum sensing systems, LuxR solos do not have a LuxI-type autoinducer synthase associated with them. LuxR solos play important roles in virulence and symbiosis in plant-pathogenic bacteria and plant-beneficial bacteria respectively. A better understanding of plant-responsive LuxR solos and the compounds to which they respond is of general importance, as it may provide great potential for new strategies to promote plant health or to combat plant disease. Here, in this dissertation we elucidate the molecular function of a LuxR, PcsR2, in bacterial intracellular communication as well as plant-microbe interaction through a common interactive system of wheat and PGPR *P.chlororaphis* 30-84.

In chapter II, we identified PcsR2 among 21 unknown LuxR solos in the biological control species *P. chlororaphis* 30-84. Quantitative RT-PCR showed that the expression of *pcsR2* and genes in the operon immediately downstream was upregulated ~10-fold when the wild type strain was grown on wheat roots compared to planktonic culture. Bioinformatics analysis showed that, unlike the LuxR solos in most of the plant-associated bacteria characterized to date (Gonzalez and Venturi, 2013), PcsR2 is not associated with a proline iminopeptidase (*pip*) gene and belongs to the subfamily of LuxR solos with undefined N-terminal binding domains (Brameyer et al., 2014). Moreover, it is conserved specifically in *P. chlororaphis*.

Using a GFP transcriptional reporter, we found that *pcsR2* did not respond to endogenously produced AHLs as expected due to the two substitutions in the nineamino acid conserved motif in the C-terminal domain where normally AHLs bind to. Rather, *PcsR2* responded specifically to root-derived substrates as compared to leaf-derived substrates. We created a *pcsR2* deletion mutant and found it was impaired in the ability to utilize root carbon and nitrogen sources in wheat root macerate and the CFU of *P.chlororaphis 3*0-84 in the wheat rhizosphere decreased ~10 fold in the *PcsR2* mutant compared to the wild type (WT) strain. Together, these results indicated that PcsR2 plays a role in plant-microbe interaction.

In addition, PcsR2 was involved in phenazine regulation. It was revealed that phenazine production was diminished greatly in30-84ΔpcsR2, especially in AB+CAA. Also, most biofilm traits (e.g. attachment ability, production of extracellular DNA and

biofilm matrix) previously shown to be correlated with phenazine production (Wang et al., 2016) were also diminished in 30-84ΔpcsR2. In addition, the regulatory position of PcsR2 for phenazine production was determined. qRT-PCR revealed that expression of several genes encoding proteins in the phenazine regulatory network including Quorum Sensing (QS) receptor PhzR, Pip (phenazine inducing protein) and RpeA/RpeB was reduced in the mutant, and that overexpression of these genes *in trans* could restore phenazine production in the mutant to wild-type levels, indicating PcsR2 acts upstream of these regulatory genes.

In chapter III, we aimed to figure out the molecular mechanisms of PcsR2 on phenazine regulation and plant microbe interactions. First, we found that the 14,475 bp genomic region surrounding *PcsR2* from Pchl3084_4800 to Pchl3084_4810 which contains eleven genes in three operons was conserved in *P.chlororaphis*. Interestingly, the adjacent operons contain three genes encoding fatty acid desaturases (Pchl3084_4801, Pchl3084_4803, and Pchl3084_4810). Since most LuxR solos regulate the adjacent genes/operons via the binding to the *lux* box, and there are *lux*-box like sequenvces both in the promoter of the downstream operon (operon 2, Pchl3084_4800-Pchl3084_4806) and the operon containing PcsR2*PcsR2* (operon 1, Pchl3084_4807-Pchl3084_4808).We hypothesized that PcsR2 is involved in regulation of a broad range of traits via the regulation of the biological function of the cell membrane.

Consistent to our hypothesis, mutation of *pcsR2*PcsR2 led to alteration of the anisotropy in the cell membrane and the contents of 18 of 28 (64%) fatty acids detected from GC-FAME. Specifically, the percentage of nearly all the 3-hydroxy-fatty acids (e.g

12:1 3OH) and the predominate unsaturated fatty acid (UFA) 16:1 w7c, which contributes to fluidity of the membranes, were significantly different between the *PcsR2* mutant and the WT strain. In line with this finding, overexpression of Pchl3084_4801, but not Pchl3084_4803, of operon 2 restored the level of 16:1w7c and partial pheanzine production in the PcsR2*PcsR2* mutant, indicating the regulation of PcsR2 on operon 2 and the alteration of cell membranes affected phenazine production, probably by compromising the function of the Two-Component Signal transduction RepA/RepB in the cell membrane. Accordingly, the alteration of cell-membrane fluidity resulted in the alteration of a variety of biological traits including stress tolerances from H_2O_2, temperature, and saline solutions.

In conclusion, we identified a novel LuxR solo, PcsR2, that regulates the fluidity of cell membranes and is highly conserved among *P. chlororaphis* species. It is not only involved in bacteria cell-cell communication such as Quorum sensing (QS) and phenazine production, but is also associated with abiotic stress (e.g. H_2O_2, temperature and saline) as well as plant-microbe interactions. Further work should focus on identifying theproductsencoded by Operon 2 and the chemical signals from root exudate.

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APPENDIX

SOME FURTHER RESEARCH

Introduction

Recent research has been revealed that changes in the fluidity of bacterial membrane result in a global modification on the expression of genes involved in QS, nutrient transport, carbon and energy metabolisms (Baysse et al., 2005; Flechard et al., 2018). To test the effect of pcsR2 on bacterial metabolism and the potential influence on bacteria-plant interaction, we did the test global nutrient utilization, extracellular protease production of bacteria and its influence on plant immunity response.

Materials and Methods

Nutrient metabolite utilization, protease activity

To test the influence of PcsR2 on nutrient uptake as carbon source, nutrient metabolites assays were carried out in AB media supplemented with 190 compounds as the sole carbon source. Briefly, WT (NI) and 30-84ΔpcsR2 (NI) were overnight cultured in AB+G and standardized to OD_{620} =0.4 as described for the saline stress assay. Aliquots of 100ul of cell culture were dispensed separate wells of the PM1-2 MicroplateTM from Biolog, Incorporated, Hayward, CA. Cell density was measured (OD_{620}) every 3h up to 48h using a Tecan Infinite M200 Pro. The bacteria were regards as none-growth when the value of OD_{620} was less than 0.015.

Extracellular protease activity of 30-84WT(NI),30-84ΔpcsR2(NI) and 30-84ΔpcsR2 (pGT2PcsR2) were quantified as described previously with minor modification (Schweizer and Choi, 2011). ZN(NI) was the control to exclude the effect of phenazine

for this assay. Briefly, bacteria were grown overnight in AB+CAA, collected, and washed resuspended in sterile water to a standard optical density (OD_{620}=0.8). An aliquot 2ul of cells was stab-inoculated into 0.8% nutrient agar supplemented with 1.5% slim milk. The diameters of the clear zone around the inoculated sites were measured at 12h and 24h after inoculation.

Plant immunity response assay

I hypothesize that changes in UFA production associated with having a functional PcsR2 may affect plant recognition of the bacteria. To test this hypothesis, immunity response assays based on promoter-luciferase activity were performed as described previously with minor modification(Cheng et al., 2013). Three genotypes of plants were used in this assay: *Arabidopsis thaliana* WT (Col-0), transgenic *A. thaliana* FRK1 (carrying the FRK1 promoter fused with a luciferase reporter gene) and *A. thaliana* WRKY46 (carrying WRKY promoter fused with a luciferase reporter gene). FRK1 (flg22-induced receptor-like kinase 1) represents a specific and early immune responsive gene activated by multiple PAMPs (He et al., 2006). WRKY46 is a transcriptional regulator induced by effector AvrRpt2 (Gao et al., 2013). The plants were grown in pots containing soil-less planting medium (Metro Mix 360, Sun Gro Horticulture, Agawam, MA) in growth chambers at 23°C , 60% relative humidity and 75 uE m-2 s –l light with a 12h light/12h dark for ~4 weeks. Three derivative strains of *Pseudomonas syringae* DC3000 and two derivative strains of *P. chlororaphis* 30-84 were used in this assay. *P. syringae* DC3000 WT has a Type III Secretion Systems (T3SS) and Hop effector, which can block Pathogen-associated molecular patterns (PAMP) and Pathogen Triggered Immunity (PTI)

and is therefore used as the negative control of PTI luciferase assay; *P. syringae* DC3000 hrcC has a defective T3SS and which cannot deliver effectors and cannot block PTI, and thus used as positive control of PTI. *P. syringae* DC3000 avrRpt2 has the avrRpt2 effector, which can activate Effector-Triggered Immunity (ETI) and is therefore used as positive control of ETI luciferase assay, whereas *P. syringae* DC3000 lacking the avrRpt2 effector was used as negative control of ETI assay. The two *P. chlorarphis* strains included in the assay were 30-84WT(NI) and 30-84ΔpcsR2(NI). All strains were cultured overnight in KB medium containing rifamycin (50ug/ml) or in AB+CAA media containing kanamycin (50ug/ml) at 28°C respectively. Cells were pelleted by centrifugation, washed twice and normalized to OD_{620} =0.4. The leaves of 4 week-old *A. thaliana* were hand-inoculated with bacteria using a 1ml needleless syringe until water-soaked and then incubated again in the growth chamber for 16h. Inoculated leaves were excised and put into 96 well plate, sprayed with 0.2mM luciferin solution with 0.2% Silwet L-77 (OSi Specialties, Greeley, CO) and then the intensity of fluorescence was read using a GloMax Luminometer (Promega, Madison, WI). The plant, soil, equipment and growth chambers were kindly provided by Dr. Libo Shan Department of Plant Pathology and Microbiology, Texas A&M University.

Results

Knocking out *pcsR2* leading to global altering of nutrient metabolites and protease activity

As the integrity of the cell membrane is essential for a serial of biological function partly in that one-fifth of bacteria proteins such as enzymes, prions and sensory proteins

are in cell membrane (Chevalier et al., 2017). The influence of PcsR2 on nutrient uptake and catabolites were tested by measuring the cell density (OD_{620}) of 30-84WT(NI) and30-84ΔpcsR2 (NI) on the PM1-2 MicroplateTM from Biolog, Inc, where 190 compounds were supplemented as the sole carbon sources for the bacteria. The results showed both strains could not use 49/190 compounds as carbon source (OD_{620}<0.15 at 24h) and the population of the two strains were significant difference when 68/190 compounds were supplemented as the sole carbon source. For example, population of 30-84WT(NI) were greatly higher than 30-84ΔpcsR2 (NI) supplemented with Tween 20 and Tween 40, but not Tween 80, where the predominant components of these compounds were lauric acid (12:0), palmitic acid (16:0) and Oleic acid (18:1 w7c), respectively **(Figure A.1,A-C).** These results indicating the transportation and/or metabolism of C12 and C16, but not C18 fatty acids pathway were compromised in30-84ΔpcsR2 (NI), which was consistent with the results of GC-FAME in that the contents of C12 and C16 were significantly difference, but all C18 fatty acids were none significant difference between the two strains. In contrast to the fatty acids, the population of 30-84WT(NI) decreased significantly compared to30-84ΔpcsR2 (NI), including dozens of saccharides **(Figure A.1D)**, amino acids **(Figure A.1E)** and some intermediate metabolites involved in TCA cycle (data no shown). Likewise, the extracellular protease activities of 30-84WT(NI) also decreased compared to30-84ΔpcsR2 (NI) **(Figure A.2)**, which may be an indication that the protein secretion systems for exporting extracellular proteins were affected due to the alteration of cell membrane. Together, PcsR2 may play roles on global metabolic reorientation and protein secretion systems via regulation of cell membrane.

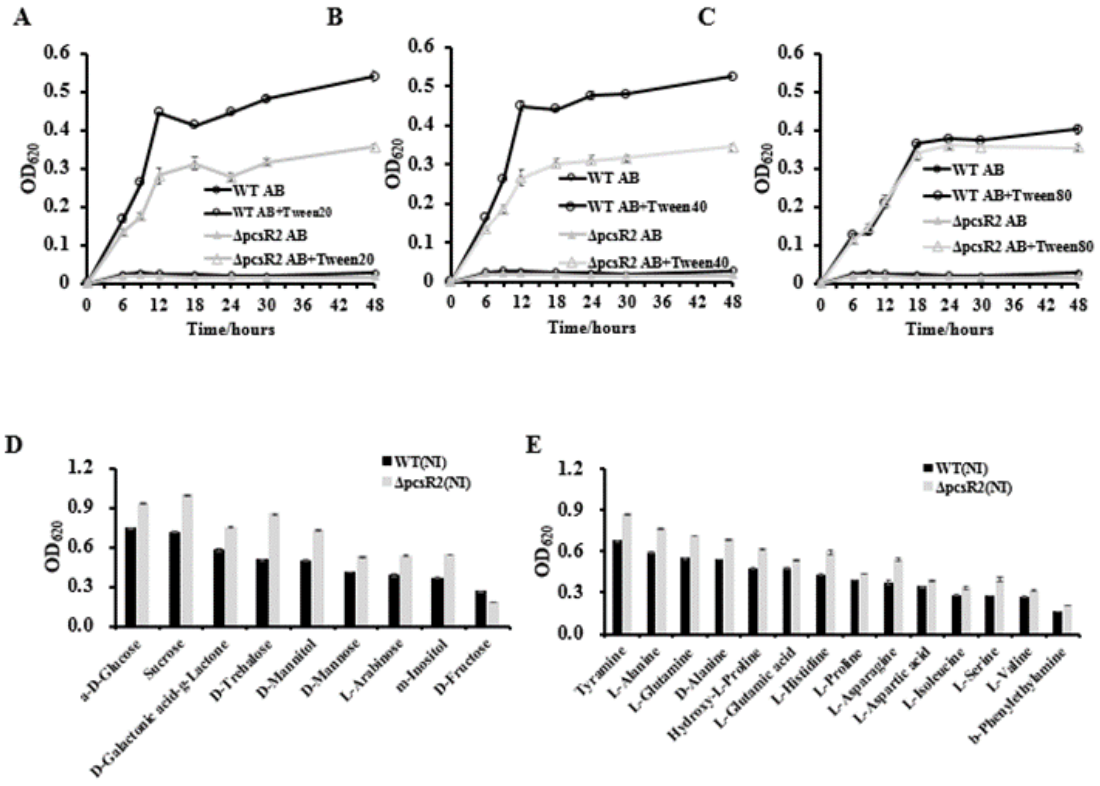

Figure A.1 PcsR2 affects metabolism of biological pathways

The cell density (OD_{620})of 30-84WT(NI) and 30-84ΔpcsR2 (NI) grown on AB minimal media supplemented with Tween 20 **(A)**, Tween 40 **(B)**, Tween 80 **(C)**, saccharide (**D)** and amino acids **(E)** as the sole carbon source. The hydrophobic tail of Tween 20, Tween 40 and Tween 80 are lauric acid (12:0), palmitic acid (16:0) and Oleic acid (18:1 w7c) respectively. The value of OD_{620} are all significant difference by student t test in JMP ($P<0.5$).

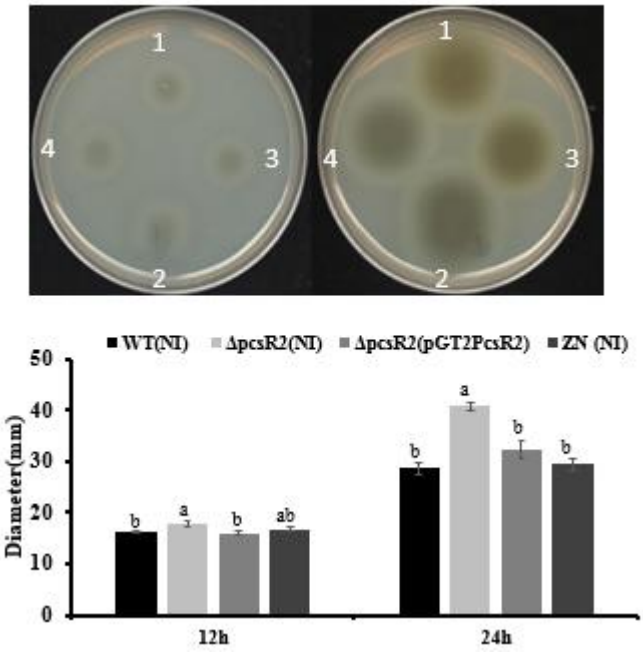

Figure A.2 PcsR2 is associated with protease activity
Bacteria were grown overnight in AB+CAA and OD_{620} was standardized after ddH_2O rinsing. An aliquot of 2ul cell was stab-inoculated in to a 0.8% nutrient agar supplemented with 1.5% slim milk. The diameters of the clear zone around the inoculated sites were measured at 12h and 24h after inoculation. The strains labeled with number 1-4 were 30-84WT(NI), 30-84ΔpcsR2(NI), 30-84ΔpcsR2 (pGT2PcsR2) and ZN(NI) in order. NI means no insert plasmid pGT2Ptac:lacZ. Data are the means and standard errors of at least three replicates. Letters indicate whether treatments are significantly different ($P<0.05$, ANOVA and Tukey HSD).

PcsR2 helps *P.chlororaphis* 30-84 suppress plant immunity response

Like pathogens, beneficial microbes need to overcome or evade plant immune responses in order to establish a prolonged and intimate mutualistic interaction with the host (Pieterse et al, 2014). Plant immunity responses (PTI and ETI) are mounted after microbe-associated molecular patterns (MAMPs) of plant pathogen such as flagellin or elongation factors are perceived by plants. It is well known that PGPR like *P. choloraphis* 30-84 also contain those MAMPs and thus we wonder whether plants can also perceive these patterns and what the role of PcsR on this recognition. The result

revealed that **(Figure A.3A)**, the intensity of FRK1-Luciferase and WRKY46 were also triggered as positive control. However, the value of FRK1-Luciferase of plants with ΔPcsR2 (NI) inoculation was 660 ± 85, which was 53% higher compared to that with 30-84WT(NI) inoculation (408 ± 62). Likewise, the value of WRKY46-Luciferase of plants with ΔPcsR2 (NI) inoculation (125 ± 5) increased 44% compared to that with 30-84WT(NI) inoculation (87 ± 4) **(Figure A.3 B)**. These results indicate that *P. choloraphis* 30-84 can trigger plant immunity response as well as *P. syringae* DC3000 and PcsR2 contributes to suppression of plant immunity. These results were reasonable since *P. choloraphis* 30-84 has 100% identity in flg22 (22 conserved amino acid in flagellin) and some fatty acids is showing to play role on plant immunity.

In line with these findings, the population (CFU) of 30-84WT (NI) ($\sim 10^8$) was ten folds of 30-84 30-84ΔpcsR2 (NI) ($\sim 10^7$) enumerated from the roots of wheat plants in the autoclaved soil. However, the population of the two strains from rhizosphere (closely adhering soil) and bulk soil (no direct contact with root) did not show significantly difference, both at about 10^6 and 10^5, respectively **(chapter II**). This suggested that PcsR2 contributes to the survival of bacteria on root by relieving the immunity pressure from plants and this function did not triggered at the bulk soil without bacteria-plant interaction. We believe this contribution probably enact by some chemical signals such as 3-hydoxy fatty acids.

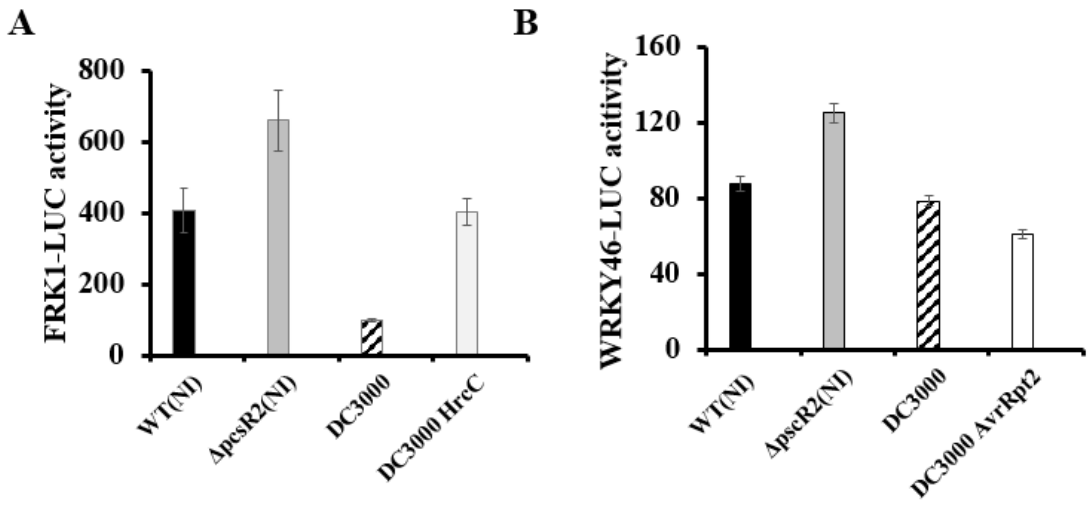

Figure A.3 PcsR2 is associate with plant immunity activity

4 weeks of *Arabidopsis thaliana* WT (Col-0), transgenic Col-0 plants FRK1 and WRKY46 were inoculated with 30-84WT(NI), 30-84 30-84ΔpcsR2(NI), *P.syringae* DC3000, DC3000 *HrcC* and *AvrRpt2* at OD=0.4, after 16h incubation the intensity of promoter FRK1 **(A)** and WRKY46 **(B)** were quantified with the Promega Luminometer. Transgenic plants FRK1 carry FRK1 promoter fused with a luciferase reporter gene and WRKY46 carry WRKY promoter fused with a luciferase reporter gene. Each treatments have 12 replicates. In Figure B, the leave with strain DC3000 atrophied after inoculation therefore the WRKY46_Luc acticity was not relabile.