Plant growth promotion by *Pseudomonas fluorescens*

*mechanisms, genes and regulation*

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This research was conducted under the auspices of the Graduate School of Experimental Plant Sciences.
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Xu Cheng

*Thesis*

submitted in fulfilment of the requirements for the degree of doctor
at Wageningen University
by the authority of the Rector Magnificus
Prof. Dr A.P.J. Mol
in the presence of the
Thesis Committee appointed by the Academic Board
to be defended in public
on Monday 19 September 2016
at 1.30 p.m. in the Aula.
Xu Cheng

Plant growth promotion by *Pseudomonas fluorescens*: mechanisms, genes and regulation, 192 pages.

PhD thesis, Wageningen University, Wageningen, NL (2016)

With references, with summary in English

**ISBN**: 978-94-6257-875-3

**DOI**: 10.18174/387191
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Chapter 1

General Introduction

Xu Cheng
Chapter 1

The rhizosphere is the narrow zone of soil that surrounds and is influenced by plant roots (Raaijmakers et al., 2009). It harbours an overwhelming variety of organisms, in particular microorganisms such as bacteria, fungi, oomycetes, archaea, protozoa and algae, as well as nematodes and arthropods. Their numbers per gram of soil range from $\sim 10^8$-$10^9$ for bacteria to $\sim 10^1$-$10^2$ for nematodes (Mendes et al., 2013). These organisms are part of the complex food web and can have profound effects on plant growth, nutrition and health. In return, plant roots release low molecular weight compounds (amino acids, organic acids and sugars), mucilage and proteins, which feed the organisms and stimulate their activity (Mendes et al., 2013).

In the rhizosphere, numerous interactions occur not only between plants and microorganisms, but also between microorganisms. The most studied mutualistic plant-microbe associations to date are those involving rhizobia and mycorrhizal fungi. Rhizobia are hosted in nodules and fix nitrogen by converting atmospheric nitrogen ($N_2$) into ammonia when nitrogen is deficient (Gualtieri & Bisseling, 2000). Arbuscular mycorrhizal fungi (AMF) form mutualistic associations with their host plants under phosphorus-limited conditions (Yoshimura et al., 2013). Studies on the interplay between rhizosphere (micro)organisms and their plant hosts have indicated that also various other microbial genera can be beneficial to plants. In this context, specific emphasis is given to the plant growth-promoting rhizobacteria (PGPRs).

PGPRs colonize plant roots, protect plants by competitive exclusion of pathogens from the rhizosphere and/or promote plant growth (Moenne-Loccoz et al., 2001, Weller et al., 2002, Haas & Defago, 2005, de Bruijn & Raaijmakers, 2009, Mazurier et al., 2009, Mendes et al., 2011, Raaijmakers & Mazzola, 2012). The modes of action by which PGPRs protect plants include antibiosis (Haas & Defago, 2005, Gross & Loper, 2009, Raaijmakers et al., 2010, Raaijmakers & Mazzola, 2012, Philippot et al., 2013, Chowdhury et al., 2015) and induction of systemic resistance (Pieterse et al., 1996, Ryu et al., 2004, Lugtenberg & Kamilova, 2009, van de Mortel et al., 2012, Zamioudis & Pieterse, 2012, Pieterse et al., 2014, Chowdhury et al., 2015, Pieterse et al., 2016). The latter, known as induced systemic resistance (ISR) is defined as an enhanced disease resistance in plants that allows plants to inhibit pathogens without direct physical interaction between the PGPR and the pathogen. Antibiosis, which is the ability to directly inhibit growth of other microorganisms by
producing antimicrobial compounds, has been shown to be of great importance for warding off plant pathogens. Many PGPR genera, such as *Pseudomonas*, *Bacillus* and *Streptomyces*, produce a chemically diverse array of antimicrobial compounds (Raaijmakers & Mazzola, 2012). For example, the lipopeptides (LPs) produced by diverse bacterial genera have inhibitory activity against a wide range of organisms, including viruses (Huang et al., 2006), bacteria (Kuiper et al., 2004), fungi (Mendes et al., 2011) and oomycetes (Mendes et al., 2011, Van Der Voort et al., 2015). Some of the antimicrobial compounds produced by PGPRs, like 2,4-DAPG, not only directly inhibit growth of other microorganisms but can also trigger ISR.

PGPR-mediated ISR has been demonstrated in the model plant Arabidopsis, and also in a variety of economically important crops and ornamentals like bean (*Phaseolus vulgaris*), carnation (*Dianthus caryophyllus*), cucumber (*Cucumis sativus*), radish (*Raphanus sativus*), tobacco (*Nicotiana tabacum*) and tomato (*Solanum lycopersicum*) (van Loon et al., 1998, Compant et al., 2005, Berendsen et al., 2015). In many cases, ISR is regulated by the phytohormones jasmonic acid (JA) and ethylene (ET) (Van Oosten et al., 2008). However, some PGPRs do not induce systemic resistance via the JA/ET signalling pathway, but via the salicylic acid (SA) signalling pathway (Maurhofer et al., 1994, De Meyer & Hofte, 1997, Maurhofer et al., 1998, Audenaert et al., 2002, van de Mortel et al., 2012). Induction of the SA signalling pathway leads to activation of the regulatory protein nonexpressor of pathogenesis-related genes1 (NPR1) and further affects pathogenesis-related proteins (PR), like PR1, PR2 and PR5 (Ward et al., 1991, Van Loon & Van Strien, 1999, van de Mortel et al., 2012, Zamioudis & Pieterse, 2012). However, PR gene expression is not a hallmark of ISR. To date, many studies have shown that ISR by beneficial microbes is commonly based on defense priming (Pieterse et al., 2014). Priming involves the plant’s response to a pathogen challenge, leading to an enhanced level of resistance (Pieterse et al., 2014).

Other mechanisms by which PGPRs affect plant growth and health are nitrogen fixation, phosphate solubilization, sequestration of iron by siderophores and the production of indole acetic acid (IAA) and cytokinins (Kennedy et al., 1997, Richardson et al., 2001, Patten & Glick, 2002, Kennedy et al., 2004). As free-living N\textsubscript{2}-fixers, *Azospirillum* fertilizes wheat and maize leading to enhanced root development and increased water and nutrient uptake (Burdman et al., 1998). Many PGPRs produce and secrete siderophores under iron-limiting conditions.
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Siderophores inhibit pathogen growth directly by competing for iron, protect plant health via ISR and promote plant growth by iron acquisition (Loper & Buyer, 1991, Moon et al., 2008, Cornelis, 2010). Also the production of IAA by Pseudomonas and Streptomyces has been shown to stimulate plant growth (Patten & Glick, 2002, Tokala et al., 2002, Hayat et al., 2010). Furthermore, there is a growing body of evidence that microbial volatile organic compounds (VOCs) affect plant growth and health (Ryu et al., 2003, Blom et al., 2011, Cordovez et al., 2015). VOCs, like 2,3-butanediol, tridecane and acetoin, were shown to promote plant growth and elicit ISR in Arabidopsis (Ryu et al., 2004, Han et al., 2006). VOCs may also exhibit direct antimicrobial activities and act as chemical weapons against other microorganisms in competition for niches and nutrients in the plant rhizosphere. For instance, bacterial VOCs have been shown to inhibit spore germination and mycelial growth, and cause abnormal morphological changes in fungi (Effmert et al., 2012, Cordovez et al., 2015). Moreover, 2,3-butanedione and glyoxylic acid emitted by Bacillus subtilis mediate global changes in gene expression and substantially influence motility and antibiotic resistance in other bacteria (Kim et al., 2013). These examples indicate that VOCs are very interesting with respect to the interactions of microorganisms with plants, with competing microbes, and with other organisms such as protists (Song et al. 2015). Long-distance dispersal of VOCs through a soil matrix has been proposed as an ecological advantage as compared to the presumed short-distance effects of non-volatile secondary metabolites, like antibiotics and exoenzymes. Hence, microbial VOCs are viewed as important infochemicals in mediating both short- and long-distance interorganismal interactions.

The experimental chapters in this thesis focus on rhizospheric Pseudomonas species and the mechanisms underlying plant protection and plant growth promotion (Figure 1). In the following sections, I will give a brief overview of the current status of Pseudomonas genomics and phylogeny, followed by a description of the mechanisms and the many different modes of action of Pseudomonas in antagonism against pathogens, in plant growth promotion and in eliciting ISR. I will specifically address the role of the GacS/GacA two-component regulatory system in the production of bioactive metabolites (including VOCs) by Pseudomonas.

Pseudomonas ‘multipurpose’ soil inhabitants

The genus Pseudomonas is ubiquitous in soil and rhizosphere environments.
Pseudomonas species possess many traits to do so including high growth rates, a broad substrate utilization spectrum and the production of an array of bioactive metabolites that are antagonistic to other microorganisms (Weller, 2007). Pseudomonas species display a diversity of life styles exemplified by the complexity of interactions with multiple hosts (Ramos et al., 2004, Raaijmakers et al., 2006, Malfanova et al., 2013). Interest in Pseudomonas is for a large part due to the production of secondary metabolites, including exoenzymes, volatiles, bacteriocins, toxins, antibiotics, and cyclic lipopeptides (CLPs) (Raaijmakers et al., 2002, Haas & Defago, 2005, Gross & Loper, 2009, Loper et al., 2012, Song et al., 2015, Van Der Voort et al., 2015). Via the production of phytohormones and other metabolites, such as 2,4-DAPG and nitric oxide, certain plant-associated Pseudomonas strains can positively influence plant growth and development (Patten & Glick, 2002, Spaepen et al., 2007, Remans et al., 2008).

**Figure 1** Overview of multitrophic interactions in the rhizosphere. The Pseudomonas genus represents PGPRs as predominant inhabitants of the rhizosphere. Pseudomonas PGPR strains positively affect plant growth and health by inhibiting the growth of plant pathogens and/or indirectly by inducing systemic resistance (ISR) in the plant. Pseudomonas PGPR strains may also promote plant growth by acquisition of iron and by increasing the availability of other nutrients and trace-elements. In this thesis, chapters 2 and 3 address the direct growth-inhibitory effects of Pseudomonas strains on different plant pathogens; chapters 4, 5 and 6 focus on the mechanisms and genes involved in plant growth promotion and ISR by Pseudomonas fluorescens.
Chapter 1

Pseudomonas genomics and phylogeny

The genus *Pseudomonas* currently comprises more than 144 species. Based on multilocus sequence typing (MLST) analysis, these have been divided into ten major groups: *P. fluorescens*, *P. syringae*, *P. lutea*, *P. putida*, *P. anguilliseptica*, *P. straminea*, *P. aeruginosa*, *P. oleovorans*, *P. oryzihabitans* and *P. stutzeri* (Yamamoto et al., 2000, Mulet et al., 2010, Redondo-Nieto et al., 2013, Gomila et al., 2015, Van Der Voort et al., 2015). Comparative genomics studies have shown that the *Pseudomonas* genus is highly diverse not only among species but also between strains belonging to the same species (Silby et al., 2011, Wu et al., 2011, Loper et al., 2012, Redondo-Nieto et al., 2013, Van Der Voort et al., 2015). This enormous heterogeneity emphasizes the importance of obtaining and studying individual genomes in order to reveal features associated with specific lifestyles and biological functions. The first complete *Pseudomonas* genome sequence (*P. aeruginosa* strain PAO1) was published already 15 years ago (Stover et al., 2000). To date (May 2016), 572 complete *Pseudomonas* genomes and 1762 draft genome assemblies are available in the Pseudomonas Genome Database (http://www.pseudomonas.com/). With this enormous and increasing amount of genome sequences, it is now possible to get a detailed insight into the evolution and genetics of *Pseudomonas* by comparative genomics approaches. Silby et al. (2011) compared 20 *Pseudomonas* genomes and showed substantial variation in genome size (4.6Mb for *P. stutzeri* to 7.1Mb *P. protegens* Pf-5) and a remarkably large genome diversity among strains that belong to the *P. fluorescens* group. Loper et al. (2012) performed comparative genomics on 10 strains from the *P. fluorescens* group and showed that these strains fall into three sub-clades based on MLST analysis (Figure 2). As a consequence, *P. fluorescens* Pf-5 was re-classified as *P. protegens* Pf-5. Subsequent comparative genomics of 51 strains belonging to the *P. fluorescens* group (Redondo-Nieto et al., 2013) revealed an additional clade that comprises nine strains including Pf0-1, R124, and NZ011. The two *P. fluorescens* strains SBW25 and SS101 that are the subject of study in this thesis are grouped in sub-clade 3 (Figure 2).

Comparative genome analysis of the plant-associated *Pseudomonas* strains revealed a pan genome of approximately 14,000 genes and a core genome of only 1491 genes, representing 24-36% of any of the genomes of an individual *Pseudomonas* strain (Loper et al., 2012). In each of the genomes, selected
biosynthetic or catabolic genes and gene clusters were discovered with putative roles in biocontrol or other ecological functions. These included genes for insecticidal toxins and novel type II, III and VI secretion systems as well as gene clusters with unknown functions. Interestingly, most of the genes involved in the production of bioactive compounds mapped outside of the core genome, including the Non-Ribosomal Peptide Synthetase (NRPS) genes (Loper et al., 2012). NRPSs are responsible for the production of a broad spectrum of antimicrobial compounds, including lipopeptides that display enormous structural diversity and different natural functions (Gross & Loper, 2009, Marahiel & Essen, 2009, Raaijmakers & Mazzola, 2012, Van Der Voort et al., 2015). The study by Loper et al. (2012) as well as other genome analyses of different bacterial genera (Gross et al., 2007, Zerikly & Challis, 2009) further indicated that the number of biosynthetic pathways without an assigned function is much higher than the number of bioactive compounds currently identified. Collectively, the increasing number of complete genomes allows us to further study the genus Pseudomonas, their physiology and ecological functions.

Figure 2 This figure is reprinted from Loper et al. 2012. A) Phylogenetic analysis of Pseudomonas species. The 10 plant associated strains fall into tree clades (highlighted). The numbers on the right refer to the number of genes shared by the species and strains grouped; B) Venn diagram based on comparative genomics of 10 plant associated Pseudomonas strains. Numbers in brackets represent the total number of genes in each strain. The number of genes shared among different strains and the number of the unique genes of each strain are also indicated in the figure.
Chapter 1

Plant protection by *Pseudomonas* and the role of secondary metabolites

*Pseudomonas* species are involved, at least in part, in the natural suppressiveness of soils to specific soil-borne pathogens (Weller, 2007). For example, *Pseudomonas protegens* (formerly *P. fluorescens*) CHA0 was isolated from the roots of tobacco grown in a soil near Payern, Switzerland, that is naturally suppressive to black root rot in tobacco caused by the fungus *Thielaviopsis basicola* (Stutz et al., 1986). *P. fluorescens* 2-79 and *P. chlororaphis* 30-84 were isolated from wheat grown in soils suppressive to take-all disease caused by the fungus *Gaeumannomyces graminis var. tritici* (Thomashow & Weller, 1988, Cook et al., 1995, Mazzola et al., 1995). *P. fluorescens* 2-79 showed 50-90% suppression of take-all via production of phenazine-1-carboxylate (Thomashow & Weller, 1988) and similar results have been obtained with *P. chlororaphis* 30-84, which produces three phenazines (Pierson & Thomashow, 1992). *P. fluorescens* strain Q2-87, which produces 2,4-DAPG, also showed suppressiveness of take-all disease (Vincent et al., 1991, Harrison et al., 1993). Recently, evidence was found that a specific group of *Pseudomonas* species, exemplified by *Pseudomonas* sp. SH-C52, plays a role in soils suppressive to damping-off disease of sugar beet caused by *Rhizoctonia solani* (Mendes et al., 2011, Van Der Voort et al., 2015). In almost all these suppressive soil studies, the mechanism of plant protection by the rhizospheric *Pseudomonas* species was attributed, at least in part, to antibiosis.

To date, a wide variety of compounds with antagonistic activities against plant pathogens have been identified for Pseudomonads (Gross & Loper, 2009, Raaijmakers et al., 2010, Raaijmakers & Mazzola, 2012). These include siderophores, hydrogen cyanide, 2,4-DAPG, pyrrolnitrin, pyoluteorin, phenazines, 2,5-dialkylresorcinol, quinolones, gluconic acid, rhamnolipids, and various, structurally diverse lipopeptides (LPs) (Gross & Loper, 2009, D’Aes et al., 2010). The pyoverdines and pseudobactin, siderophores are well known for their high affinity for iron and this provides a competitive advantage in competition under iron-limiting conditions (Ambrosi et al., 2000, Meyer, 2000). The antibiotic compounds 2,4-DAPG, pyrrolnitrin, pyoluteorin and phenazines, and the LPs have been intensively studied with respect to their biosynthesis, regulation and activities against a range of plant pathogenic fungi, oomycetes, bacteria and nematodes.
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(Raaijmakers & Mazzola, 2012). For example, the biosynthesis of 2,4-DAPG is governed by the *phl* gene cluster and regulated by the GacS/GacA two-component system (Schnider-Keel et al., 2000). Certain LPs have a significant impact on zoospores of oomycete pathogens like *Pythium* and *Phytophthora* species. The lytic effects of LPs on zoospores have been well characterized for the viscosin-group of LPs, but were also described for putisolvins and orfamide A (De Souza et al., 2003, Gross et al., 2007, Tran et al., 2008, Krujt et al., 2009, van de Mortel et al., 2009). Interestingly, several *Pseudomonas* species and strains harbour more than one gene cluster for LP biosynthesis. For example, *Pseudomonas* sp. strain SH-C52 has four LP biosynthesis gene clusters, encoding the synthesis of at least six distinct LPs, namely the antifungal 9-amino-acid chlorinated LP thanamycin (Mendes et al., 2011, Van Der Voort et al., 2015), the three antibacterial and anti-oomycete 2-amino-acid LPs, brabantamide A-C (Reder-Christ et al., 2012, Schmidt et al., 2014, Van Der Voort et al., 2015), the anti-oomycete 22-amino acid LP thanapeptin, and a putative 8-amino acid LP with yet unknown structure and activity spectrum (Van Der Voort et al., 2015).

A second mode of action involved in plant protection by *Pseudomonas* is the induction of systemic resistance (van Loon et al., 1998, Ryu et al., 2003, Ryu et al., 2004, De Vleesschauwer et al., 2009, van de Mortel et al., 2012, Pieterse et al., 2014). ISR is activated by PGPRs (Van der Ent et al., 2009) and functions throughout the plant (Conrath et al., 2002). ISR responses are often regulated via SA-independent mechanisms, but several *Pseudomonas* strains are able to trigger a SA-dependent ISR. For example, *Pseudomonas aeruginosa* 7NSK2 triggered ISR in tomato plant against infection by the grey mould fungus *Botrytis cinerea* but not in a *NahG* mutant of tomato that does not accumulate SA (Audenaert et al., 2002). PGPRs trigger ISR by priming the plant for activation of various cellular defence responses (Conrath, 2006), including oxidative burst (Iriti et al., 2003), cell wall reinforcement (Benhamou & Belanger, 1998), and the production of secondary metabolites (Yedidia et al., 2003). The bacterial determinants that trigger ISR include LPs, flagella and also metabolites like siderophores, SA and VOCs. To date, however, only a few *Pseudomonas* metabolites are known for eliciting ISR. One example is 2,4-DAPG produced by *P. fluorescens* CHA0 that triggers ISR in Arabidopsis against infection by the oomycete downy mildew pathogen *Hyaloperonospora arabidopsidis*. This was based on the observation that the 2,4-DAPG-deficient
mutant of *P. fluorescens* Q2-87 showed reduced ISR activity compared to the wild type strain (Weller et al., 2012). Another example is salicylate produced by *P. fluorescens* strain P3. Overproduction of this compound showed stimulation of the defence of tobacco against tobacco necrosis virus (Maurhofer et al., 1998). Moreover, a combination of pyocyanin and pyochelin produced by *P. aeruginosa* 7NSK2 triggered ISR in tomato against infection by *Botrytis cinerea* (Audenaert et al., 2002) and the LP massetolide A produced by *P. fluorescens* SS101, contributed to ISR in tomato against *P. infestans* (Tran et al., 2007). In addition, structural changes in plant tissue have been implicated in ISR triggered by PGPRs. For example, *P. fluorescens* 63-28R treated pea plants showed enhanced cell wall apposition, which is the first line of defence at the *Pythium ultimum* infection site on the roots (Benhamou et al., 1996). Also *P. fluorescens* WCS417r treated Arabidopsis plants showed an increased frequency of callose depositions when plants were infected by *Hyaloperonospora arabidopsidis* (Van der Ent et al., 2009). Overall, these studies show that *Pseudomonas* produces several secondary metabolites that can act as ISR-elicitors. Further research will likely lead to the discovery of other, yet unknown *Pseudomonas* metabolites involved in ISR elicitation.

**Pseudomonas** species promote plant growth

Certain *Pseudomonas* strains have biofertilizing capacity and are applied as seed or soil inoculants. These *Pseudomonas* strains promote plant growth and development directly by producing plant hormones like IAA and cytokinins (Lambrecht et al., 2000, Cartieaux et al., 2003). *P. putida* strains that produce IAA under *in vitro* conditions were shown to enhance shoot growth of black pepper plants as well as root growth and the number of roots formed per stem cutting (Lambrecht et al., 2000). Also *P. fluorescens* strains WCS417 and WCS374, and *P. putida* strain WCS358 were reported to affect developmental plasticity of the roots of *Arabidopsis thaliana* by inhibiting primary root elongation and promoting lateral root and root hair formation via auxin signalling (Zamioudis & Pieterse, 2012). *Pseudomonas* strains can also benefit plant growth by facilitating resource acquisition, providing plants with macro- and micro-nutrients, including nitrogen, iron and phosphorus (Pieterse et al., 2014). Nitrogen fixation is widespread among bacteria that possess the nitrogenase enzyme complex, which is responsible for the reduction of nitrogen gas to ammonia. The genes coding for nitrogenase, the
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*nif* genes, have also been found in some *Pseudomonas* species. By analysis of the bacterial population in the rhizosphere of cord grass (*Spartina alterniflora*), *nifH* sequences were detected in *P. stutzeri* strains A1501 and CMT.9.A (Lovell *et al.*, 2000). When *Pseudomonas* strain Pf-5, and the *Pseudomonas* spp., *P. putida*, *P. veronii* and *P. taetrolens* were genetically modified with the *nif* genes from *P. stutzeri* A1501, they showed constitutive nitrogenase activity and high ammonium production. Furthermore, inoculation of Arabidopsis, alfalfa, tall fescue and maize with the modified Pf-5 derivative increased the ammonium concentration in soil and plant productivity under nitrogen-deficient conditions (Setten *et al.*, 2013).

Iron (Fe) is a vital element for almost all forms of life. In aerobic environments, Fe is typically present as Fe$^{3+}$ and generally unavailable to both plants and microorganisms (Cornelis, 2010, Pieterse *et al.*, 2012). Bacterial siderophores can act as an important source of Fe for plant growth in soils where iron availability is limited (Loper & Buyer, 1991, Pieterse *et al.*, 2012). Most of the siderophores are water-soluble and along with their strong Fe-binding capacity and chemical stability, they contribute to an increased mobility of Fe in the soil and in the rhizosphere in particular, thus making Fe better accessible for plants. Plants can assimilate iron from bacterial siderophores by means of different mechanisms, for instance by the direct uptake of siderophore-Fe complexes or by a ligand exchange reaction (Schmidt, 1999). For example, application of siderophore-producing *Pseudomonas* sp. GRP3A and PRS9 to maize seeds resulted in an increase in shoot and root length and dry weight of maize plants grown under iron limited conditions (Sharma & Johri, 2003). Similarly, the Fe-pyoverdine complex synthesized by *Pseudomonas fluorescens* C7 was taken up by *Arabidopsis thaliana* plants, leading to an increase of iron inside plant tissues and to improvement of plant growth (Vansuyt *et al.*, 2007). Collectively, these findings show that siderophores are common products of *Pseudomonas* spp. and can promote plant growth.

Phosphorus (P) is one of the most important nutrients for plant growth. The majority of soil P is found in insoluble forms and only two soluble forms, the monobasic ($\text{H}_2\text{PO}_4^{-1}$) and the dibasic ($\text{HPO}_4^{2-}$) ions, can be absorbed by plants (Bhattacharyya & Jha, 2012). Therefore, P-deficiency is a major constraint to crop production. Phosphate-solubilizing bacteria are considered as promising biofertilizers since they can increase the availability of P to plants (Zaidi *et al.*, 2009). Although several mechanisms can be involved, typically the mechanism of
phosphate solubilization is through the production of low molecular weight organic acids by the soil bacteria (Zaidi et al., 2009). *Pseudomonas* is one of the genera harboring phosphate-solubilizing species and strains (Bhattacharyya & Jha, 2012). For example, *P. fluorescens* strain RAF15, which was isolated from the rhizosphere of ginseng, solubilizes P by lowering the pH (Park et al., 2009).

A series of recent studies showed that multiple bacterial genera and species can affect other microorganisms, plant growth and plant health via the production of VOCs. Although most studies to date focus on VOCs-producing *Bacillus* spp., also *Pseudomonas* spp. have been found to produce VOCs with plant growth promoting activity (Blom et al., 2011)(Table 1). Recent studies with plant growth-promoting *P. fluorescens* SS101 showed that the VOCs produced by this strain promote plant growth both *in vitro* and in soil and that the bioactive VOCs involved in this plant growth promotion are 13-tetradecadien-1-ol, 2-butanone and 2-methyl-n-1-tridecene (Park et al., 2015). Still many features of VOCs produced by Pseudomonads remain to be studied, including their biosynthesis and genetic regulation.

**Regulation of secondary metabolism in *Pseudomonas* by the GacS/GacA two-component system**

The biosynthesis of secondary metabolites in *Pseudomonas* is tightly regulated and several pathway-specific regulators and global regulatory systems have been identified. In certain *Pseudomonas* species, LP biosynthesis has been shown to be under the regulation of quorum sensing (QS) via acyl-homoserine lactones (AHLs). A typical AHL-mediated QS-system involves two major components: an AHL synthase (which belongs to the LuxI protein family) and a modular transcriptional response-regulator (which belongs to the LuxR protein family). The LuxI protein is responsible for the synthesis of the AHLs, which are in turn detected by the LuxR protein (Steindler & Venturi, 2007). For example, studies on *P. fluorescens* 5064 and *P. putida* PCL1445 showed that accumulation of AHLs correlates with cell density increases. Subsequently, the cell density increase corresponded with the activation of viscosin and putisolvin production, respectively (Dubern et al., 2006). However, for various other *Pseudomonas* strains, including *P. fluorescens* strains SBW25 and SS101, no evidence was found for AHL-mediated regulation of LP biosynthesis (de Bruijn et al., 2007, de Bruijn et al., 2008, Raaijmakers et al., 2010). For LP biosynthesis also pathway-specific LuxR-type transcriptional regulators are involved.
as was shown for syringomycin, putisolvin, viscosin, massetolide, arthrofaction and sessilin (Roongsawang et al., 2003, Berti et al., 2007, de Bruijn et al., 2007, de Bruijn et al., 2008, Dubern et al., 2008, D’Aes et al., 2014). In Pseudomonas, also unpaired LuxR family proteins, referred to as LuxR ‘solos’, have been identified (Steindler et al., 2008). For instance, the LuxR solo PpoR binds AHLs and has been proposed to have a role in the detection and response to endogenous and/or exogenous signalling compounds (Brameyer & Heermann, 2015).

Next to these typical pathway-specific regulators, the GacS/GacA two-component system (referred to as the Gac-system) is a well-studied global regulator of gene expression in Pseudomonas. The Gac-system consists of the sensor kinase GacS and the response regulator GacA. It was first described as the global activator of antibiotic and cyanide synthesis, hence the abbreviation Gac (Laville et al., 1992). GacS belongs to the histidine sensor kinases, is activated upon yet unknown environmental signals by autophosphorylation and subsequently transmits the phosphoryl group to the response regulator GacA. GacA contains a DNA-binding helix-turn-helix motif, which recognizes specific DNA regions in those genes that are regulated, via small RNAs, by the Gac-system (Heeb & Haas, 2001, Lapouge et al., 2008, Song et al., 2015). In Pseudomonas, a mutation in either gacS or gacA results in the loss of more than one extracellular metabolite or enzyme as was initially described for P. fluorescens CHA0 (Laville et al., 1992). Also P. protegens Pf-5 produces many antibiotic compounds, including pyrrolnitrin, pyoluteorin, 2,4-DAPG, hydrogen cyanide, siderophores and the LP orfamide (Gross et al., 2007, Loper et al., 2007). Interestingly, a mutation in gacA of the Pf-5 strain resulted in a deficiency in all these metabolites, except for siderophore production (Hassan et al., 2010). By performing a genome-wide transcriptome analysis on P. Protegens Pf-5 and its gacA mutant, Hassan et al. (2010) identified two novel gene clusters encoding two yet unknown compounds based on down-regulation in the gacA mutant. This approach, referred to as global-regulator-based genome mining, was proposed as an efficient strategy to identify structurally novel bioactive compounds (Hassan et al., 2010).

Mutations in the gacS or gacA genes are known to occur spontaneously both in vitro and in situ. For example, Gac-mutations in the plant-beneficial bacterium Pseudomonas brassicacearum were observed in the rhizosphere (Achouak et al., 2004, Lalaouna et al., 2012) and in the alfalfa rhizosphere for P. fluorescens F113.
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Table 1 Examples of volatile organic compounds (VOCs) produced by *Pseudomonas* PGPRs and their biological functions in antagonism and plant growth promotion.

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(Sanchez-Contreras et al., 2002). Under *in vitro* conditions, these mutations typically lead to the formation of opaque and translucent colonies, designated as phase I (wild type strain) and phase II colonies (with Gac-mutation), respectively (van den Broek et al., 2003, Martinez-Granero et al., 2006). Also for *P. fluorescens*SBW25 it has been described that it switches phenotypes under natural selection by spontaneous mutation in the Gac system, mainly affecting biofilm formation (Spiers et al., 2002). Song et al. (2016) recently showed that spontaneous Gac-mutations occur predominantly on the edge of swarming colonies of *P. Protegens* strain PF-5 benefiting from the LP surfactants secreted by the wild type strain.
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Interestingly, cells of the Gac-mutant of Pf-5 were approximately 1.5 times longer and more flagellated than the wild type strain, phenotypic changes that together with a series of other altered genetic traits were proposed to confer local benefits in swarming motility when confronted with competitors and when exploring new environments (Song et al., 2016).

Scope of this thesis

In this thesis, two P. fluorescens strains (SBW25, SS101) were studied for their antagonistic activities against plant pathogens and their abilities to induce systemic resistance and promote plant growth. By taking an interdisciplinary approach and combining in vitro and in vivo bioactivity tests with genome mining, mutagenesis, metabolomics and imaging mass spectrometry, different mechanisms, bioactive genes and pathways involved in plant growth promotion and ISR were identified.

As a first step (Chapter 2) I conducted a genome-wide transcriptome comparison between Pf.SBW25 and a Tn5-mutant disrupted in the gacS gene. This gacS mutant is deficient in the sensor kinase that is a key component in the Gac-system and thus anticipated to show global changes in gene expression. The microarray-based transcriptional profiling indeed revealed around 700 genes that were differentially regulated in the gacS mutant when compared to Pf.SBW25. Subsequently, the function of several of these differentially expressed genes was studied and unexpectedly revealed an enhanced antimicrobial activity of the gacS mutant against several economically important plant and fish pathogens. To further unravel the mechanisms underlying the enhanced antimicrobial activity of the gacS mutant, the role of a specific nonribosomal peptide synthetase (NRPS) gene cluster was investigated in more detail by site-directed mutagenesis (Chapter 2). The gacS mutant was also subjected to random mutagenesis to find novel genes with a role in the unexpected broad-spectrum antimicrobial activity (Chapter 3). The results showed that a mutation in gacS enhances the production of gluconic acid and 2-keto gluconic acid, which leads to acidification of the extracellular medium that in turn suppresses growth of oomycete, fungal and bacterial pathogens (Chapter 3).

I then investigated the effects of volatile organic compounds (VOCs) (Chapter 4) and direct interactions (Chapter 5) on plant growth. The role of the Gac-system in regulation of VOCs production by Pf.SBW25 was also investigated (Chapter...
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4). Out of 205 putative VOCs, 24 were significantly different in their abundance between *Pf.*SBW25 and the *gacS*-mutant, which included 3 acyclic alkenes. These alkenes, 3-nonene, 4-undecyne and 1-undecene, were significantly reduced by the Gac-mutation independently of the growth media and of the incubation time. We subsequently determined if and how a mutation in the Gac-regulatory system affects VOCs-mediated growth promotion and rhizosphere acidification of Arabidopsis and tobacco, and ISR in Arabidopsis. Results indicate that the Gac-system is involved in the regulation of VOCs production in *Pf.*SBW25, which in turn affects plant growth promotion in a plant species-dependent manner. Subsequently we conducted MALDI-Dried Droplet and MALDI-Imaging Mass Spectrometry analyses to study the chemistry in the interactions between Arabidopsis roots, *Pf.*SBW25 and the *gacS* mutant. The results showed that *Pf.*SBW25 affects root architecture of Arabidopsis and tobacco, and also significantly increased biomass of Arabidopsis and cucumber. MALDI-IMS results showed that several of bioactive compounds, including the LP viscosin, are indeed produced in *Pf.*SBW25-Arabidopsis interactions. Viscosin was located mostly at the site where *Pf.*SBW25 was inoculated and absent in the *gacS* mutant-Arabidopsis interactions. MALDI-IMS analysis revealed other metabolites putatively regulated by the Gac-system. Structure elucidation and subsequent bioassays will be needed to further identify these metabolites and to resolve their potential effects on plant growth and root architecture.

*Pf.*SS101 is genetically close to *Pf.*SBW25 and represents the second PGPR strain studied in this thesis. *Pf.*SS101 was previously shown to induce systemic resistance in tomato and Arabidopsis. Here, we further elucidated the underlying mechanisms of plant growth promotion and ISR by *Pf.*SS101 by using targeted and untargeted approaches (Chapter 6). In the targeted approaches, we investigated the potential role of the GacS/GacA two-component regulatory system, IAA, ACC deaminase and VOCs production in plant growth promotion by *Pf.*SS101. Since none of these ‘usual suspects’ had a major role in the growth promotion or ISR activities by *Pf.*SS101, a library of 7,488 random mutants of *Pf.*SS101 was screened for reduced ISR and plant growth-promoting activities. We identified 21 mutants defective in both ISR and plant growth-promoting abilities. These mutants harbored a plasposon insertion in genes involved in amino acid biosynthesis, glucose utilization or sulfur assimilation. The results suggest that amino acid biosynthesis and sulfur assimilation are important mechanisms involved in *Pf.* SS101-induced
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plant growth and systemic resistance.

Collectively, the major results obtained in this PhD thesis are summarized in chapter 7. Particular emphasis is given to the role of the GacS/GacA two-component system as a global regulator of the expression of genes involved in antagonism of *Pseudomonas fluorescens* toward plant pathogenic microbes as well as in plant growth promotion and ISR.
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Chapter 2

The Gac Regulon of *Pseudomonas fluorescens* SBW25

Xu Cheng, Irene de Bruijn, Menno van der Voort, Joyce E. Loper and Jos M. Raaijmakers

This chapter has been published as:

Chapter 2

Abstract

Transcriptome analysis of *Pseudomonas fluorescens* SBW25 showed that 702 genes were differentially regulated in a gacS::Tn5 mutant, with 300 and 402 genes up- and down-regulated, respectively. Similar to the Gac-regulon of other *Pseudomonas* species, genes involved in motility, biofilm formation, siderophore biosynthesis and oxidative stress were differentially regulated in the gacS mutant of SBW25. Our analysis also revealed, for the first time, that transcription of nineteen rhizosphere-induced genes and of genes involved in type II secretion, (exo)polysaccharide and pectate lyase biosynthesis, twitching motility and an orphan nonribosomal peptide synthetase (NRPS) were significantly affected in the gacS mutant. Furthermore, the gacS mutant inhibited growth of oomycete, fungal and bacterial pathogens significantly more than wild type SBW25. Since RP-HPLC analysis did not reveal any potential candidate metabolites, we focused on the Gac-regulated orphan NRPS gene cluster that was predicted to encode an 8-amino acid ornicorrugatin-like peptide. Site-directed mutagenesis indicated that the encoded peptide is not involved in the enhanced antimicrobial activity of the gacS mutant but may function as a siderophore. Collectively, this genome-wide analysis revealed that a mutation in the GacS/A two-component regulatory system causes major transcriptional changes in SBW25 and significantly enhances its antimicrobial activities by yet unknown mechanisms.
Introduction

Comparative genome analyses of different plant-associated *Pseudomonas* species and strains revealed substantial heterogeneity within the *P. fluorescens* group, with a pangenome of close to 14,000 genes (Silby et al., 2009; 2011; Loper et al., 2012). A core genome of only 1491 genes was identified for all of the sequenced *Pseudomonas* species, representing 24-36% of any individual genome (Loper et al., 2012). In each of the genomes, orphan genes were discovered for traits that were not known previously, including genes for insect toxins, novel type II, III and VI secretion systems as well as gene clusters with unknown functions. Interestingly, almost all of the traits associated with biological control of plant pathogens or other multitrophic interactions mapped outside of the core genome to variable genomic regions present in only individual strains or a limited subset of strains. The study by Loper et al. (2012) as well as previous genome analyses of other bacterial genera (Gross et al., 2007; Zerikly and Challis, 2009) further indicated that the orphan biosynthetic pathways outnumber by far the number of bioactive compounds currently identified.

Approaches to explore and exploit microbial (meta)genomes for orphan biosynthetic pathways and novel bioactive compounds are numerous and include: 1) heterologous expression of genomic fragments obtained from culturable or unculturable microorganisms (i.e. metagenomics) followed by activity assays and metabolic profiling, 2) *in silico* mining of microbial genomes for consensus sequence motifs or modules followed by structure prediction, gene inactivation studies and/or genomisotopic approaches, or 3) inactivation of orphan biosynthetic pathways by manipulation of regulatory genes or by challenging microorganisms with specific culture conditions or other external cues (Gross et al., 2007; McAlpine, 2009; Scherlach and Hertweck, 2009; Li et al., 2012). Following a combination of the two latter approaches, Hassan et al. (2010) presented the global-regulator-based genome mining strategy to decipher the metabolome of *P. protegens* (formerly *P. fluorescens*) strain Pf-5 and to identify orphan gene clusters for novel secondary metabolites.

In *Pseudomonas*, the GacS/GacA two-component system is a highly conserved global regulatory system and comprises the membrane-bound sensor kinase GacS that, upon recognition of a yet unknown environmental signal, is
activated and phosphorylates the response regulator GacA. The GacS/GacA two-component system controls the biosynthesis of numerous secondary metabolites in *Pseudomonas* and mutations (spontaneous or site-directed) in the gacS or gacA genes generally lead to a substantial reduction in antimicrobial activity of *Pseudomonas* strains (Haas and Defago, 2005). In *P. protegens* Pf-5, it also has broad effects on iron homeostasis, enhancing transcript levels of genes functioning in siderophore biosynthesis and various mechanisms for iron uptake (Hassan et al. 2010). The GacS/GacA two-component system also regulates the expression of several genes involved in virulence, biofilm formation, motility, quorum sensing, stress responses and survival (Kinscherf and Willis, 1999; Haas and Keel, 2003; Raaijmakers et al., 2010; Yamazaki et al., 2012).

Here we investigated the Gac regulon of *P. fluorescens* SBW25, a well-known model strain for studying bacterial evolution and adaptation (Rainey, 1999; Gal et al., 2003; Kassen et al., 2004; Silby et al., 2009; Scanlan and Buckling, 2012). Strain SBW25 also promotes plant growth and controls plant diseases caused by fungal and oomycete pathogens (Rainey, 1999; Naseby et al., 2001; de Bruijn et al., 2007). To date, however, little is known about the genes and metabolites involved in plant growth promotion and antimicrobial activity of SBW25: it does not produce the typical antibiotic compounds 2,4-diacetylphloroglucinol, phenazines, pyrrolonitrin or pyoluteorin (Loper et al., 2012), but produces siderophores (Timms-Wilson et al., 2000; Moon et al., 2008) and the cyclic lipopeptide viscosin (de Bruijn et al., 2007). Strain SBW25 shares only 2/3 of its genome with that of *P. protegens* strain Pf-5 and harbors multiple gene clusters for which the functions are yet unknown (Loper et al., 2012). Here, we conducted a genome-wide transcriptome comparison between wild type strain SBW25 and a Tn5-mutant disrupted in the gacS gene. The functions of several of the differentially expressed genes were studied. The *in vitro* activity assays unexpectedly showed an enhanced antimicrobial activity of the gacS mutant against several economically important plant and fish pathogens. The role of a specific orphan NRPS gene cluster in this enhanced antimicrobial activity was studied in more detail by site-directed mutagenesis.

**Materials and Methods**

**Strains and culture conditions.** *Pseudomonas fluorescens* SBW25, *Pseudomonas aeruginosa* PAO1, *Pseudomonas syringae* pv. *tomato* DC3000 and *Bacillus* sp.
S18F11 were grown on Pseudomonas agar F (PSA, Difco) or in King’s medium B (KB; King et al., 1954). When needed, growth media were supplemented with 50 μg/ml rifampicin, 100 μg/ml kanamycin, and/or 25 μg/ml tetracycline. The gacS mutant was obtained by random Tn5 plasposon mutagenesis using plasmid pTnModOkm (de Bruijn et al., 2007). Transformants were screened for single insertions by Southern blot analysis. Plasmid rescue and sequence analysis of the regions flanking the plasposon was performed to verify the position of the transposon (Figure S1). The mutation was complemented with a plasmid containing a 3763-bp insert containing the gacS gene from SBW25 with putative promoter and terminator sites (see below). Escherichia coli, Pectobacterium atrosepticum SCR1043 and Xanthomonas campestris pv. campestris were cultured on Luria Bertani (LB) agar plates or LB broth. All bacteria were grown at 25 °C, except for E. coli at 37 °C. All fungal and oomycete strains were cultured on Potato Dextrose Agar (PDA, Difco, Becton, Dickinson and Company, Sparks, Maryland, USA), except for Phytophthora infestans strain 88069 and Phytophthora capsici LT3239 which were cultured on Rye Sucrose Medium and on V8 medium (Latijnhouwers et al., 2004), respectively. P. infestans and Saprolegnia parasitica were cultured at 18°C; Pythium aphanidermatum, Aphanomyces cochloides at 25°C; P. capsici, Rhizoctonia solani, Botrytis cinerea, Alternaria brassicicola, Fusarium solani and Verticillium dahliae were cultured at room temperature.

Reversed-Phase HPLC (RP-HPLC) analysis. P. fluorescens strains were grown on 1/5th strength PDA plate for 48 to 72 h and plates were cut into 6-8 big pieces, suspended in 80% (v/v) acetone and shaken vigorously for 1 hour at room temperature. Agar pieces were removed with a forceps and cells were removed by centrifugation. Extracts were dried in the fume hood and the remaining water fraction was acidified with 0.1 % (v/v) trifluoracetic acid (TFA) and extracted with two volumes of ethylacetate. The solution was shaken vigorously and incubated at -20 °C until the water phase was frozen. The upper ethylacetate phase was transferred to new tube and evaporated under continuous nitrogen flow. The extract was dissolved in 100% methanol and subjected to reverse phase high performance liquid chromatography (RP-HPLC) using a 5-μm C₁₈ column (Waters Symmetry column, Waters, Etten-Leur, Netherlands), a 55-min linear gradient of 0 to 100% acetonitrile + 0.1% TFA with a flow rate of 0.5ml/min. Detection was performed at wavelengths from 200 to 450 nm with a photodiode array detector (Waters).
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GacS complementation. For complementation, a fragment of 3763 bp containing the *gacS* gene with putative promoter and terminator, was obtained by PCR with specific primers (Table S1) and Phusion DNA polymerase (Finnzymes). The PCR fragment was subcloned in pGEM-T Easy (Promega) and transformed into *E. coli* by heat shock transformation in heat shock competent cells obtained according to the method described by Inoue et al. (1990). The obtained plasmid was isolated and digested with EcoRI. The obtained *gacS* fragment was cloned into the shuttle vector pME6031 (Heeb et al., 2000), and the obtained pME6031-*gacS* construct and pME6031 empty vector control were electroporated into the *gacS::Tn5* mutant of *P. fluorescens* SBW25. Electrocompetent cells and electroporation was performed according to Choi et al. (2006). Cells transformed with pME6031-*gacS* or pME6031 were selected on KB agar medium supplemented with tetracycline (25 μg/ml); the presence of the vectors was verified by PCR with pME6031 specific primers (Table S1).

Growth of SBW25 and the *gacS* mutant for microarray analysis. To prepare inoculum for the microarray experiments, *P. fluorescens* SBW25 and the *gacS* mutant were grown on PSA plates for 48 h at 25 °C and cells were collected and washed 3 times in sterile milliQ water. The cell density was set spectrophotometrically to an OD$_{600}$ of 1.0, corresponding to approximately $10^9$ cells/ml. Seven 5-μl droplets of each strain were spot-inoculated on 1/5$^{th}$ strength PDA agar in four Petri plates, to provide four biological replicates for the microarray experiment. After incubation at 25 °C for 48 h, cells from the seven inoculation spots on each plate were scraped from the 1/5$^{th}$ PDA plates, pooled, resuspended in milliQ water, and spun down; the cell pellets were flash frozen in liquid N$_2$ prior to extraction of total RNA.

Microarray analysis. *P. fluorescens* SBW25 has a genome of 6.7 Mb which is predicted to encode 6,009 Coding DNA Sequences (CDSs), with a coding density of 88.3% (Silby et al., 2009). Microarray Chips were generated by Roche NimbleGen Inc. (Reykjavik, Iceland) based on the current annotation of the *P. fluorescens* SBW25 genome (NC_012660.1) and contained nineteen 60-mer probes per gene with each probe spotted in triplicate. *P. fluorescens* SBW25 and the *gacS* mutant were grown on 1/5$^{th}$ strength PDA agar plates at 25 °C for 48 h. For each, four biological replicate plates were used. We chose these culture conditions for the transcriptome analysis to match the conditions used to determine the antimicrobial activity of SBW25 and mutants (see below). Total RNA was extracted from the
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bacterial cells according to De Bruijn et al. (2008), resulting in four independent RNA samples for both SBW25 and the gacS mutant. Probe preparations and hybridizations were performed according to protocols of Roche NimbleGen Inc. The Arraystar 4.1 software (DNASTAR, Madison, Wisconsin, USA) was used for analyzing the pre-normalized array data. Statistical analyses were carried out with the normalized data using a moderated t test with false discovery rate (FDR) multiple-test correction (Benjamini-Hochberg) to determine differential transcript abundance. Genes with a fold change > 2 and p-value < 0.0001 were considered to be differentially regulated. Functional grouping of the differentially regulated genes was based on the role categories of JCVI (http://cmr.jcvi.org/tigr-scripts/CMR/CmrHomePage.cgi). For the match table, a list of gene sequences of P. protegens Pf-5 and P. aeruginosa PAO1 that were orthologous to the SBW25 genes were obtained from the work of Bencic et al. (2009) and Hassan et al. (2010), respectively. For P. brassicacearum subsp. brassicacearum NFM421 (Lalaouna et al., 2012), P. syringae pv. syringae B728a (Records and Gross, 2010) and P. syringae pv. phaseolicola 1448A (De la Torre-Zavala et al., 2011), genes with more than 70% identity were considered orthologous. Descriptions of the microarray experiments, array design and data analysis are deposited in GEO (http://www.ncbi.nlm.nih.gov/geo/) and can be accessed with accession number GSE43443. Microarray results were validated by Q-PCR of selected genes according to protocols described below.

qPCR analysis. RNA isolation (as described above) and quantitative PCR (Q-PCR) analysis were performed as described previously (de Bruijn et al., 2008). The threshold cycle (CT) value for genes of interest was corrected for that of the housekeeping gene rpoD, as follows: ΔCT = CT (gene) - CT (rpoD). The relative quantification (RQ) values were calculated with the formula RQ = 2\(^{ΔCT (mutant) - ΔCT (wild \ type)}\) (Pfaffl, 2001). The primers used for Q-PCR are described in Table S1. Q-PCR analysis was performed in duplicate (technical replicates) on four independent RNA isolations (biological replicates). Statistically significant differences were determined for log-transformed RQ values by analysis of variance (P < 0.05).

Site-directed mutagenesis of PFLU3225. Site-directed mutagenesis of the NRPS gene PFLU3225 in the gacS mutant was performed according to Link et al. (1997) by using plasmid pEX18Tc (Hoang et al., 1998). Primers used are indicated in Table S1. The PCR fragments were digested with BamHI and HindIII, cloned into BamHI and HindIII digested NucleoSpin-purified pEX18Tc (Choi and Schweizer, 2005) and
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transformed into *E. coli* (Inoue et al., 1990). The inserts were verified by PCR analysis with pEX18Tc primers (Table S1), restriction analysis, and sequencing of the inserts. Introduction of the construct into the host strain was performed by triparental mating. Equal volumes of cell suspensions of the *gacS* mutant, *Escherichia coli* DH5α strain carrying the construct, and DH5α strain carrying a helper plasmid pRK2013 (Ditta et al., 1980) were resuspended in 100 µl of LB media after centrifugation at 5,500 rcf for 5 min. The mixture of the three strains was incubated on an LB agar plate at 30°C for 6 h. Cells were collected and resuspended in 10mM MgSO₄ and single cross-over transformants were selected on KB supplemented with tetracycline (25 µg/ml), kanamycin (100 µg/ml) and rifampicin (50 µl/ml). Obtained colonies were grown in LB broth medium for 2 h at 25°C and plated on LB agar medium supplemented with kanamycin (100 µg/ml), rifampicin (50 µl/ml) and 5% sucrose to select for double cross-over recombinants. By PCR with primers PFLU3225 FL-F-1 and R-1, colonies were identified to either have the deletion of PFLU3225 or to have reverted back to the host strain genetic background. Positive colonies for PFLU3225 deletions (designated *gacS/Δ3225*) were selected and the deletion was confirmed by PCR and sequencing of the obtained PCR fragments. Site-directed mutagenesis of the PFLU3225 gene in wild type strain SBW25 was performed based on the method described by Choi and Schweizer (2005). The primers used for amplification are described in Table S1.

**Phenotypic traits of *P. fluorescens* SBW25 and mutant strains.** Production of extracellular proteases was tested on Skimmed Milk agar (15 g/l skimmed milk agar (Oxoid), 4 g/l blood agar base (Difco), 0.5g/l yeast extract (Oxoid), 13.5 g/l agar (Difco) (DeShazer et al., 1999). Viscosin production, biofilm formation, swimming and swarming were tested according to the methods described previously (de Bruijn et al., 2007). In summary, viscosin production was determined by a drop collapse assay by spotting a drop of cell suspension on parafilm; motility was assessed on soft (0.6% agar, w/v) standard succinate medium (SSM; 32.8 mM K₂HPO₄, 22 mM KH₂PO₄, 7.6 mM (NH₄)₂SO₄, 0.8 mM MgSO₄, 34 mM succinic acid, adjusted to pH 7 with NaOH). Overnight cultures were washed once and 5 ml of a cell suspension (1*10¹⁰ cells ml⁻¹) were spotted in the centre of the soft SSM agar plates and incubated for 48 h at 25°C. Biofilm formation was assessed according to the method described by O’Toole et al. (1999) using flat-bottom 96-wells plates (Greiner) with 200 ml of King’s medium B broth per well. Plates were incubated
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for 24h and non-attached cells were removed by several washes with tap water. Attached cells were stained with 0.1% Crystal violet and after dissolving the stain in 100% EtOH, absorbance was measured at 600 nm.

For siderophore production, SBW25 and mutants were grown in KB broth overnight at 25 °C. Cells were washed twice with KB broth and the cell density was set to an OD$_{600}$ of 1.0. Five μl cell suspension was spotted on a CAS agar plate (Schwyn and Neilands, 1987). After 48 h of incubation at 25 °C, siderophore production was visualized by a color change of the CAS medium from blue to orange or a halo around the colony under UV light (Hartney et al., 2011).

For the oxidative stress response, cell suspensions of SBW25 and the gacS mutant (OD$_{600}$=1.0) were diluted 10, 10$^2$, 10$^3$, 10$^4$ and 10$^5$ times, and for each dilution 5 μl was spotted on a 1/5$^{th}$ strength PDA plate with different amounts of H$_2$O$_2$ (0, 0.2, 0.5, 1.0 μM, respectively). Inoculated plates were incubated at 25 °C and colony growth was checked after 2 days.

The antifungal and anti-oomycete activities of SBW25 and mutants were tested as follows: SBW25 and mutant strains were grown in 5 ml KB broth overnight at 25°C. Two μl bacterial suspension (OD$_{600}$ = 1.0) was spotted on a 1/5$^{th}$ strength PDA plate near the edges of the plate. After two days of incubation at 25 °C, a mycelial plug of 4-mm diameter of each fungal or oomycete pathogen was placed in the centre of the 1/5$^{th}$ PDA plate and incubated at their appropriate temperature. Radial hyphal growth was monitored for several days depending on the pathogen’s growth rate. For V. dahliae, spores were mixed into 1/5$^{th}$ PDA (Trifonova et al., 2009). Additionally, we also tested growth inhibition of P. infestans strain 88069 under different iron conditions (0, 25, 50, 100 and 200 μM) by adding FeSO$_4$ or FeCl$_3$ into the medium. To test the antibacterial activity of SBW25 and its mutants, 2 ul of cell suspension was spotted on 1/5$^{th}$ PDA plates and incubated at 25 °C for 2 days. Subsequently, overnight cultures of P. aeruginosa PAO1, P. syringae pv. tomato DC3000, Bacillus sp. S18F11, P. atrosepticum SCRI1043 and X. campestris pv. campestris were washed twice with MilliQ water, and cell suspensions (OD$_{600}$ = 1.0) were sprayed onto the SBW25/mutant inoculated agar surface and incubated at 25 °C for 2-3 days. The antimicrobial activity was quantified by measuring radial hyphal growth or the radius of the inhibition zone in case of the bacterial pathogens.

Pectolytic activity of SBW25 and the gacS mutant was tested on potato slices. P.
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*fluorescens* strain A506 has no pectolytic activity and was included as a negative control. A potato was first peeled and then sterilized via soaking in a 10% bleach solution for 10 min. The potato was soaked in sterile MilliQ water for 5 minutes and then soaked in another container of sterile MilliQ water for another 5 minutes to remove all traces of bleach. The potato was sliced into 8 mm thick sections using a sterile knife. Each section was placed into a Petri dish with sterile filter paper that has been inoculated with 1 ml sterile MilliQ water. A depression was cut into each slice into which 50 μL of a bacterial suspension (OD=0.1) was pipetted. All cultures tested were grown for 24 h on KB media. Potatoes were incubated at 20°C for 3-5 days after which pectolysis appears as a soft rot.

Results and discussion

The Gac regulon of *P. fluorescens* SBW25

For the transcriptome analysis, we isolated RNA from cells grown for 48 h at 25°C on 1/5th PDA agar plates to match the conditions used to assess the antimicrobial activity of SBW25 and mutants. The results showed that Tn5-based inactivation of *gacS* significantly affected the transcriptome of strain SBW25: a total of 1807 genes were differentially regulated (fold change > 2.0, p < 0.0001) in the *gacS* mutant, with a total of 935 and 872 genes up- or down-regulated, respectively (Figure 1). For our analyses, we focused mostly on genes with a fold change > 4.0 and a p-value < 0.0001. Using these more stringent criteria, 702 genes were differentially expressed in the *gacS* mutant of SBW25, with 300 and 402 genes significantly up- and down-regulated (Table S3). In general, the diversity in role categories showed comprehensive genome-wide effects of the *gacS* mutation (Figure 1, Table 1). The most affected role category was transcription, which accounted for 12.9% of the up-regulated genes (Table 1). Among the down-regulated genes, the most abundant role category was cellular processes (Table 1). Consistent with our previous study (de Bruijn et al., 2007), the viscosin biosynthesis genes *viscA* (PFLU4007), *viscB* (PFLU2553) and *viscC* (PFLU2552), as well as the exoprotease gene *aprA* (PFLU3146) were significantly down-regulated in the *gacS* mutant by -44.0, -54.8, -56.1 and -220.5 fold, respectively (Table S2). Transcription of *gacA* (PFLU2189) or the small RNA binding proteins RsmE (PFLU4165), CsrA (PFLU4324) and RsmA (PFLU4746) was not significantly affected by the *gacS* mutation (Table S2).
The Gac regulon of SBW25

Figure 1 Genome-wide representation of the genes most differentially expressed in the gacS mutant of Pseudomonas fluorescens SBW25. Each point is one of the annotated genes in the SBW25 genome, with the X-axis showing gene order (the origin of replication at 0 and 6009), and the Y-axis showing the fold change of transcript abundance of each gene in the gacS mutant relative to the wild type. Panels A and B represent different scales of the Y-axis. Annotated functions of boxed genes further discussed in the text are: a) viscosin biosynthesis cluster (PFLU2552, PFLU2553, PFLU4007), b) metalloprotease aprA (PFLU3146), c) sod gene cluster (PFLU0873-0877), d) sigma factor rpoS (PFLU1302), e) siderophore biosynthesis genes (PFLU2534-2550), f) type VI secretion system (PFLU6014-6026), g) flagellar biosynthesis genes (PFLU4437-4456), h) the putative ornicorrugatin NRPS gene cluster (PFLU3220-3225).

Table 1 Grouping of differentially regulated genes (fold change > 4.0, p < 0.0001) of the gacS mutant of P. fluorescens SBW25 into role categories. Certain genes fall in more than one role category and therefore are presented more than once in the table.
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Comparison of the Gac regulons of \textit{P. fluorescens} SBW25 and other \textit{Pseudomonas} species

Comparison of our transcriptome data with that of \textit{P. protegens} Pf-5 (Hassan et al., 2010) and of \textit{P. aeruginosa} (Brencic et al., 2009) showed that most type VI secretion system (T6SS) genes were significantly down-regulated in the \textit{gacA/S} mutants of all three species (Table S4) and represent one of the few conserved effects. Similar to the \textit{gacA} mutant of Pf-5 (Hassan et al. 2010; Kidarsa et al., 2013), genes involved in biofilm formation, pyoverdine biosynthesis and oxidative stress were differentially regulated in the \textit{gacS} mutant of SBW25. Other functions that were affected include TonB-dependent signalling and energy metabolism (Tables S4, S5). In contrast to \textit{P. aeruginosa} (Brencic et al., 2009), several genes involved in oxidative stress, iron homeostasis and siderophore production, and TonB-dependent receptors were up-regulated in SBW25 and Pf-5 (Table S4). For \textit{P. brassicacearum} subsp. \textit{brassicacearum} NFM421 (Lalaouna et al., 2012), genes involved in chemotaxis (PFLU5092-5093; PSEBR_a3441-3442) were also down-regulated like in SBW25. For \textit{P. syringae pv. phaseolicola} 1448A (De la Torre-Zavala et al., 2011), the gene encoding adenylyl-sulfate kinase (PFLU2097; PSPPH_4301), and for \textit{P. syringae pv. syringae} B728a (Records and Gross, 2010) an alginate biosynthesis protein (PFLU0979; Psyr_1052), were both down-regulated like in SBW25. The limited overlap of the Gac regulons of these different \textit{Pseudomonas} species may be due to the different growth conditions and transcript profiling methods applied in these studies and/or may be in line with the large genomic variation among different \textit{Pseudomonas} species (Silby et al., 2011; Loper et al., 2012). For example, Hassan et al. (2010) used different culture conditions (nutrient broth, 20 °C) for Pf-5, worked with a different microarray and studied the transcriptome in a \textit{gacA} mutant instead of a \textit{gacS} mutant. Even though the inactivation of \textit{gacS} might result in downstream effects that differ from those of a \textit{gacA} mutation, due to the interaction of GacS with other sensors and regulators, and despite the methodological differences, still 87 orthologous genes were similarly affected by the GacS/A mutations in strains SBW25 and Pf-5 (Table S4).

Next to the genes known to be under the control of GacA/S, our analysis also revealed, for the first time, several other genes/gene clusters to be regulated by the Gac two-component system. These include genes involved in type II secretion (T2SS), rhizosphere-induced genes, and genes involved in (exo)polysaccharide and
pectate lyase biosynthesis, twitching motility and an orphan nonribosomal peptide synthetase (NRPS) (Table S2). The functions of several of these ‘old’ and ‘new’ Gac-regulated SBW25 genes are addressed in more detail in the following sections.

The oxidative stress response

The sod gene cluster (PFLU0873-PFLU0877) was among the most differentially regulated loci in the gacS mutant of P. fluorescens SBW25, with an up-regulation of 87.4 to 300.6 fold (Table S2), which was confirmed by Q-PCR (Figure S2). The sod cluster consists of the superoxide dismutase gene sodA, the fumarate hydratase gene fumC1, a putative transport-related membrane protein, and two conserved hypothetical proteins (Figure S2). Several of these genes are known to function in oxidative stress adaptation in P. aeruginosa (Polack et al., 1996; Hassett et al., 1997a, b). While several of these transcriptional changes are consistent with the diminished capacity of gac mutants to survive oxidative stress, other alterations in the gac transcriptome reflect responses typically expressed by a bacterial cell experiencing oxidative stress. For example, the oxidative stress response in strains Pf-5 and CHA0 is mediated by the sigma factor RpoS, which is under the regulation of the Gac two-component system (Whistler et al., 1998; Heeb et al., 2005; Stockwell et al., 2009). Also in the gacS mutant of SBW25, rpoS (PFLU1302) was significantly down-regulated -6.1 fold (Table S2). When grown on agar media supplemented with more than 0.2 mM hydrogen peroxide, growth of the gacS mutant was inhibited when compared to wild type SBW25 (Figure 2A). It is not yet known which of the genes/enzymes involved in the oxidative stress response are responsible for the enhanced susceptibility of the gacS mutant of SBW25 to hydrogen peroxide. The transcriptional data obtained here suggest that sodA (up-regulated ~300 fold) does not play a role in this. The results further showed that the genes encoding for superoxide dismutase sodB (PFLU4855) and katE catalase HP II (PFLU0071) were down-regulated (Table S2). Catalases are ubiquitous enzymes that react with hydrogen peroxide to form water and oxygen (Lim et al., 2012). We postulate that KatE and/or SodB are involved in the increased susceptibility of the gacS mutant to hydrogen peroxide.

Iron homeostasis and siderophore biosynthesis

Iron is a common co-factor for redox-dependent enzymes and an essential element for living organisms (Moon et al., 2008). To facilitate iron uptake under
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Iron limiting conditions, *Pseudomonas* species produce a variety of siderophores such as the pyoverdines (PVDs) (Andrews et al., 2003; Meyer, 2000; Cornelis and Matthijs, 2002; Ravel and Cornelis, 2003; Moon et al., 2008; Cornelis, 2010). The microarray data showed that 16 genes associated with PVD biosynthesis were significantly up-regulated in the *gacS* mutant of strain SBW25 (Table S2). TonB-dependent outer membrane receptors, a group of proteins that facilitate siderophore uptake (Gal et al., 2003; Moon et al., 2008; Silby et al., 2009; Cornelis, 2010), were also up-regulated in the *gacS* mutant (Tables S2, S3 and S5). Also other genes involved in iron uptake and transport were up-regulated, including heme uptake proteins, cytochrome oxidase, and several iron-related transporters (Table S2). Assays conducted on CAS agar medium, which changes from blue to orange in the presence of siderophores (Meyer, 2000; Hartney et al., 2011), confirmed that the *gacS* mutant of SBW25 produced more siderophores than the wild type (Figure 2B). Complementation of the *gacS* mutation restored siderophore production back to wild type level (Figure S3A). These results indicate that the Gac two-component system negatively regulates siderophore production in *P. fluorescens* SBW25, confirming and extending results obtained for other *P. fluorescens* strains (Schmidli-Sacherer et al., 1997; Duffy and Defago, 2000; Hassan et al., 2010).

Secondary metabolism and exoenzyme production

The genes involved in viscosin (viscABC), exoprotease (aprA) and phospholipase C production were significantly down-regulated in the *gacS* mutant of SBW25 (Table S2). Also the LuxR-type regulators *viscAR* (PFLU4008) and *viscBCR* (PFLU2557), which flank *viscABC*, were significantly down-regulated in the *gacS* mutant (Table S2). In agreement with the down-regulation of *aprA*, a loss of extracellular protease activity was observed (Figure 2C). Complementation of the *gacS* mutation restored exoprotease production to wild type level (Figure S3B). Interestingly, the pectate lyase gene (PFLU3229) was down-regulated -55.3 fold in the *gacS* mutant (Table S2). In a potato tuber assay, we confirmed that a *gacS* mutation strongly reduced the pectolytic activity of SBW25: inoculation of the potato tuber slices with SBW25 showed discoloration (browning) and tissue degradation, whereas no such effects were observed for the *gacS* mutant and the negative control *P. fluorescens* strain A506 (Figure 2F). This pectate lyase is not present in many other *Pseudomonas* genomes and was proposed to be excreted by a yet unknown type II secretion system (Loper et al., 2012).
Type II, III and type VI secretion systems

Many extracellular enzymes are transported via type II secretion systems (T2SSs) such as exotoxin A and elastase (Durand et al., 2003; Cianciotto, 2005). In SBW25, three T2SSs gene clusters were predicted by Loper et al. (2012). Two of these gene clusters (PFLU2415-2425, PFLU3230-3240) were down-regulated in the \( gacS \) mutant (Table S2) and appear to be related to the Hxc (homologous to extracellular protein-deficient) of \( P. aeruginosa \) (Loper et al., 2012). For the third and novel T2SS (Loper et al. 2012), several genes (PFLU4070, 4075, 4078, 4080) were more than two-fold up-regulated in the \( gacS \) mutant. This is the first recognition that the Gac-system affects the regulation of T2SSs.

Type III and type VI secretion systems (T3SSs and T6SSs, respectively), which function in the delivery of effector molecules into plant, animal or bacterial cells, are prevalent in Gram-negative bacteria, including environmental \( Pseudomonas \) strains that have no known pathogenic or symbiotic associations with eukaryotic cells (Rezzonico et al., 2005; Barret et al., 2011). In \( P. fluorescens \) SBW25, T3SS has been shown to operate in the sugar beet rhizosphere and inactivation of some T3SS regulators affected growth \textit{in vitro} and thus short term fitness to colonize plant root tips (Rainey, 1999; Preston et al., 2001; Jackson et al., 2005). T3SS also plays a role in other reciprocal interactions between beneficial \( Pseudomonas \) and eukaryotes, including protozoa and oomycetes (Rezzonico et al., 2005; Matz et al., 2008). Our microarray analysis showed that the \( gacS \) mutation did not significantly affect the expression of T3SS genes in SBW25 under the conditions tested (Table S2). However, the T6SS gene cluster PFLU6014 - PFLU6026 was significantly down-regulated (-3.0 to -27.6 fold) in the \( gacS \) mutant (Table S2). As indicated earlier, also in Pf-5 (Hassan et al., 2010) and in \( P. aeruginosa \) (Brecnic et al., 2009) T6SS genes are down-regulated and represent one of the few conserved effects of a \( gacA/S \) mutation in all three species (Table S4). In \( P. aeruginosa \), chronic infections are associated with T6SS (Mougous et al., 2006; Barret et al., 2011). Although the functions of the Gac-regulated T6SS genes in SBW25 are yet unknown, their homology with the H1-T6SS of \( P. aeruginosa \), that delivers effectors targeting bacterial cells (Loper et al. 2012), suggests that they may play a role in interactions between SBW25 and other plant-associated bacteria.
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Biofilm formation and motility

The gacS mutant is impaired in biofilm formation and surface motility (Figure 2D, 2E). Complementation of the gacS mutation restored biofilm formation and motility to wild type level (Figure S3C and S3D). Genes known to be required for biofilm formation of SBW25 include the tol-pal cluster, the wsp chemosensory cluster and wss cellulose biosynthesis cluster (Spiers and Rainey, 2005). Transcription of the genes in these clusters was not significantly (> 4-fold altered in the gacS mutant, except for tolQ (PFLU4911; Table S2). The alg and psl gene clusters (PFLU0979-0990, PFLU2071-2082, respectively) for exopolysaccharide biosynthesis (Ghafoor et al., 2011) were significantly down-regulated in the gacS mutant of SBW25 (Table S2). Both clusters were also Gac-regulated in P. syringae pv. syringae B728a (Records and Gross, 2010) and P. aeruginosa (Ventre et al., 2006). In the gacA mutant of Pf-5, the psl genes were also down-regulated but not the alg genes (Kidarsa et al., 2013). In SBW25, down-regulation was observed for a four gene operon (PFLU0143-PFLU0146) putatively involved in the biosynthesis of a biofilm adhesin polysaccharide (Table S2; Itoh et al., 2008). Together these array data suggest that the reduced biofilm formation in the gacS mutant of SBW25 (Figure 2E) could be due to reduced exopolysaccharide production, in combination with the loss of viscosin production (de Bruijn et al., 2007; Figure S4).

With respect to motility, our results showed substantial down-regulation of the flagellar biosynthesis gene clusters (PFLU4437-PFLU4456 and PFLU4728-PFLU4731) in the gacS mutant (Table S2). The effects of GacS/A on swimming and swarming motility appear to vary among species and even strains of Pseudomonas (Murray and Kazmierczak, 2008; Navazo et al., 2009; Hassan et al. 2010). In Pf-5, swimming but not swimming was affected in the gacA mutant (Hassan et al. 2010), whereas in SBW25 both swimming and swarming motility were reduced in the gacS mutant. Table S2 and the work by Hassan et al. (2010) point to flagella and other motility genes as an important difference between the Gac-regulons of Pf-5 and SBW25. For example, the flagellar genes were significantly down-regulated in the gacS mutant of SBW25 (Table S2) but not in the gacA mutant of Pf-5 (Hassan et al., 2010). In addition, many genes involved in twitching motility (Mattick, 2002; de Bentzmann et al., 2006) and pilus assembly (PFLU5762, PFLU0639, PFLU0641-PFLU0651) were down-regulated in the gacS mutant of SBW25 (Table S2). Combined with the lack of viscosin production, these results most likely explain the lack of swarming motility
and the reduced swimming motility of the gacS mutant of SBW25 (Figure 2D).

Inactivation of gacS influences rhizosphere induced genes of P. fluorescens SBW25

Rhizosphere competence of plant-associated bacteria is essential for promoting plant growth and protecting plants from pathogen infection. The gacS mutation in SBW25 had no significant effect on expression of PFLU5241 (panB) and PFLU5242 (panC) (Table S2), two pantothenate biosynthesis genes that are involved in sugar beet colonization (Rainey, 1999). Based on in vivo expression technology (IVET) analyses, more than 100 rhizosphere-induced genes were identified for P. fluorescens SBW25 (Rainey, 1999; Silby et al., 2009). Similarly, in P. protegens Pf-5 many genes expressed on seed surfaces are under Gac control (Kidarsa et al., 2013). For SBW25, 19 of the 100 plant/soil-induced genes were differentially expressed in the gacS mutant (Table S2). These include genes involved in regulation, cell envelope synthesis, nutrient acquisition, stress and detoxification, transport, biosynthesis of cofactors and prosthetic groups, as well as genes with unknown function (Table S2). Specifically, PFLU0144 was down-regulated -17.5 fold in the gacS mutant (Table S2, section ‘exopolysaccharides’); PFLU0144 is part of an operon (PFLU0143-PFLU0146) involved in the biosynthesis of the biofilm adhesin polysaccharide PGA (Itoh et al., 2008). Furthermore, PFLU4610, which was down-regulated -22.3 fold in the gacS mutant, is part of an operon (PFLU4607-PFLU4611) involved in nitrate/nitrite assimilation (Romeo et al., 2012). Finally, PFLU2750, up-regulated 6.3 fold in the gacS mutant, is in an operon (PFLU2749-PFLU2752) encoding a multidrug efflux system (Table S2, section ‘stress and detoxification’). Collectively, these results suggest that the GacS/A two-component system may act as an important regulator of traits that contribute to the competitive ability of SBW25 in the rhizosphere.

Inactivation of gacS leads to enhanced antimicrobial activity

The GacS/A two-component system controls bioactive metabolite production in many Pseudomonas species and strains (Haas and Keel, 2003; Raaijmakers et al., 2010). For P. fluorescens SBW25, the lipopeptide viscosin was shown to lyse zoospores of oomycete plant pathogens like Phytophthora infestans (de Bruijn et al., 2008). Since the gacS mutant of SBW25 does not produce viscosin (Figure S4) and does not lyse zoospores of P. infestans (De Bruijn et al., 2007), we expected that the antimicrobial activity would be impaired in the gacS mutant like in other gacA/S-mutants of Pseudomonads (Haas and Keel, 2003). However, the dual culture assays
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Figure 2 Phenotypic characterization of the wild type strain and the gacS mutant of *P. fluorescens* SBW25. (A) Sensitivity of SBW25 and the gacS mutant to hydrogen peroxide. Five μl of cell suspensions (initial densities ranging from 4 to 9 Log CFU/ml) of SBW25 and the gacS mutant were spot inoculated on 1/5th strength PDA agar medium supplemented with different concentrations of hydrogen peroxide. Plates were incubated at 25 °C for 2 days. (B) siderophore detection on CAS media: top, observation under normal light; bottom, under UV light; the size of the orange halos is a measure for the amount of siderophores produced. (C) Extracellular protease activity on skimmed milk agar plates; a halo around the bacterial colony represents protease activity. (D) Surface motility (swimming and swarming) on soft SSM agar medium (0.3% and 0.6 % agar (w/v), respectively). (E) Biofilm formation in KB liquid media in a 96-well PVC plate: the cells in the biofilm were stained with crystal violet (left panel); biofilm formation was quantified spectrophotometrically (right panel), (F) Pectolytic activity of wild type strain SBW25 and the gacS mutant on potato slices; *P. fluorescens* strain A506 was included as a negative control.
showed that the \textit{gacS} mutant exhibited stronger and more distinct inhibition of mycelial growth of \textit{P. infestans} than wild type SBW25 (Figure 3A, Table S6). Also, for several other oomycetes, fungi and bacteria, we observed an increased growth inhibition by the \textit{gacS} mutant (Figure 3A, Table S6). Collectively, these results show that inactivation of \textit{gacS} leads to an enhanced, broad-spectrum antimicrobial activity of strain SBW25. The complementation of the antimicrobial activity was less clear, ranging from no to partial restoration. Partial complementation of phenotypes associated with \textit{gacA/S} mutations by the corresponding wild type gene has been observed previously (Whistler et al., 1998). The mechanisms for this are unknown, but we speculate that proper function of the GacA/S system might require a stoichiometric balance between the sensor kinase and response regulator that is disrupted by higher copy numbers of the plasmid-borne GacS. Sensor kinases like GacS have phosphatase activity that, at higher levels, can dephosphorylate the corresponding response regulator (Kenney, 2010). Possibly, the copy number of GacS may be more important for re-establishing the biosynthesis of antimicrobial compounds than for the other phenotypes described above (Figure S3).

\textbf{Figure 3} (A) \textit{In vitro} activity of wild type \textit{P. fluorescens} SBW25, its \textit{gacS} mutant and the \textit{gacS/Δ3225} double mutant against the oomycetes \textit{Phytophthora infestans} and \textit{Saprolegnia parasitica}, the fungus \textit{Verticillium dahliae} and the bacteria \textit{Xanthomonas campestris pv. campestris} and \textit{Pseudomonas syringae pv. tomato}. (B) Activity of wild type SBW25 and the \textit{gacS} mutant against \textit{Phytophthora infestans} on agar plates supplemented with 0, 50 or 100\,\mu\text{M} of FeCl$_3$.
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Elucidation of the enhanced antimicrobial activity

To identify potential extracellular metabolites associated with the enhanced antimicrobial activity, the gacS mutant and wild type SBW25 were cultured on 1/5\textsuperscript{th} strength PDA agar plates followed by chemical extractions described previously for the isolation and detection of other secondary metabolites produced by Pseudomonas (De Souza et al., 2003). Subsequent reversed phase high performance liquid chromatography (RP-HPLC) coupled with photodiode array spectroscopic analysis (wavelength ranges from 200-450nm) did not show any differences between the metabolite profiles of the gacS mutant and wild type strain, except for the lipopeptide viscosin which was detected in extracts from wild type SBW25 but not in the extracts from the gacS mutant and the viscosin biosynthesis mutant \textit{viscA} (Figure S4). Given the roles of NRPS genes in the antimicrobial activities of a wide range of microorganisms including Pseudomonads (Raaijmakers et al., 2010), we subsequently focused on an orphan NRPS gene cluster (PFLU3217-3228) that was up-regulated in the gacS mutant of SBW25 (Figure 4A). This cluster is not present in the genomes of many other strains in the \textit{P. fluorescens} group (Loper et al. 2012) and at least one gene (PFLU3223) in this cluster was identified earlier in the IVET analysis (Table S2) of SBW25 genes expressed in the rhizosphere (Silby et al. 2009). This gene cluster contains five NRPS genes (PFLU3220, 3222-3225) that are predicted to encode an eight-amino-acid peptide (Figure 4A). The predicted peptide resembles ornicorrugatin, a putative siderophore produced by \textit{P. fluorescens} AF76 (Matthijs et al., 2008; Cornelis, 2010). No classic loading domain exists, indicating that the first module tethers a starter unit upon which the peptide chain elongates. In the case of ornicorrugatin, this is suggested to be octanoic acid (Risse et al., 1998). Two genes in the cluster (PFLU3219, PFLU3221) are predicted to encode proteins in the TauD family that could function in hydroxylation of the OL-threo-OH-His (position 1) and L-OH Asp (positions 6 & 8) of the peptide (Singh et al., 2008). PFLU3217 is a putative diaminobutyrate 4-transaminase (related to PvdH) that could be involved in the biosynthesis of diaminobutyrate, which is then incorporated into the peptide chain (Vandenende et al., 2004). The presence of a TonB-dependent receptor (PFLU3218) could enable uptake of the subsequent ferric-siderophore complex (Moon et al., 2008), whereas the two putative ABC transporters (PFLU3226, PFLU3228) could provide machinery for efflux.
Site-directed mutagenesis of the NRPS gene PFLU3225 in the wild type (SBW25/Δ3225) resulted in a decrease of the halo on CAS medium and of the fluorescence under UV (Figure 4B). Also in the gacS mutant, which itself has an increased production of siderophores compared to the wild type, a mutation in the PFLU3225 gene led to a reduced halo on CAS medium (Figure 4B). These results strongly suggest that the peptide encoded by this orphan NRPS gene cluster acts as

**Figure 4** Organisation and predicted peptide configuration of the putative ornicorrugatin NRPS gene cluster in *P. fluorescens* SBW25 based on *in silico* analysis of the genome sequence (A). The gene codes 3220, 3222, 3223, 3224 and 3225 refer to PFLU3220, PFLU3222, PFLU3223, PFLU3224, PFLU3225 in the Pseudomonas Genome Database, respectively. Underneath the genes are 1) the fold-changes in their expression in the gacS mutant, and 2) the predicted module and domain organization of the NRPSs according to analyses by PFAM, PKS/NRPSs and NRPS predictor2 software packages. Underneath the domains are the amino acids predicted to be incorporated into the peptide moiety based on specific signature sequences in the A-domains. The number associated with the amino acid refers to the position of the amino acid in the predicted peptide chain. (B) detection of siderophore production by wild type SBW25, the Δ3225 mutant, the gacS mutant and the gacS/Δ3225 double mutant on CAS agar medium; left panel, observation under normal light; right panel, observation under UV light; the sizes of the orange (left panel) and fluorescent halos (right panel) is a measure for the amount of siderophores produced.
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a siderophore or as a regulator of siderophore biosynthesis. In terms of antimicrobial activity, however, no significant effects were observed: the \textit{gacS}/\Delta3225 double mutant had the same enhanced inhibitory activity as the \textit{gacS} mutant against the oomycete, fungal and bacterial pathogens tested (Figure 3A). The enhanced activity of the \textit{gacS} mutant against \textit{P. infestans} was also not affected by addition of iron (Fe$^{3+}$, Fe$^{2+}$; Figure 3B, Figure S5) indicating that other iron-regulated pyoverdine-like siderophores do not play a major role in the enhanced antimicrobial activity.

Conclusions

The genome-wide microarray analysis of \textit{P. fluorescens} SBW25 presented here showed that inactivation of the GacS/A two-component system strongly influences expression of a large number of genes. The genes affected are involved in a broad range of biological functions, including oxidative stress response, iron acquisition, secondary metabolism, biofilm formation and motility. Next to these ‘usual suspects’, our analysis revealed, for the first time, that transcription of nineteen rhizosphere-induced genes and of genes involved in type II secretion, (exo) polysaccharide and pectate lyase biosynthesis, twitching motility and an orphan nonribosomal peptide synthetase were significantly affected in the \textit{gacS} mutant. Although \textit{gacS}/A mutants of \textit{Pseudomonas} species generally have a reduced activity against other microorganisms, our study revealed an enhanced, broad-spectrum activity of the \textit{gacS} mutant of SBW25. Various pathogens, including oomycetes, fungi and bacteria, were substantially more inhibited in growth by the \textit{gacS} mutant than by wild type SBW25. No candidate metabolites could be detected by RP-HPLC analysis and also the orphan NRPS gene cluster, which displays siderophore activity or acts as a regulator of siderophore biosynthesis, did not play a role in the enhanced antimicrobial activity. Hence, the mechanism(s) involved in the Gac-associated enhanced antimicrobial activity are yet unknown and subject of future studies.
The Gac regulon of SBW25

Acknowledgements

This project was financially supported by the Netherlands Genomics Initiative (NGI-EcoLinc project) and by the OECD Cooperative Research Program (JA00042522). We would like to acknowledge Ester Dekkers for technical assistance in the mutant constructions, and Sierra Hartney and Ed Davis for the NRPS prediction. We are also grateful to Brenda Schaeffer and Jennifer Lee for conducting the pectolytic activity assays.
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**Supplementary Tables and Figures**

Additional supporting information can be found in the online version of this article at the publisher’s web-site:


**Table S1** Plasmids and oligonucleotide primers used in this study.

**Table S2** Specific functions, corresponding genes and their relative expression levels in the *gacS* mutant of *P. fluorescens* SBW25 that are highlighted in the manuscript.

**Table S3** Genes (#702) differentially expressed (fold change > 4.0, p < 0.0001) in the *gacS* mutant of *P. fluorescens* SBW25. Certain genes fall in more than one role category and therefore are presented more than once in the table.

**Table S4** Comparison of genes significantly affected in expression in the *gacS* mutant of *P. fluorescens* SBW25 with orthologous genes significantly affected in expression in *gacS/A* mutants of *P. protegens* Pf-5 and *P. aeruginosa* PAO1. For Pf-5 and PA01, data were obtained from Hassan *et al.* (2010) and Brencic *et al.* (2009), respectively.

**Table S5** TonB-dependent receptors and linked extracytoplasmic function (ECF) sigma factors and anti-sigma factor regulators that were transcriptionally modulated in the *gacS* mutant of *P. fluorescens* SBW25.

**Table S6** Quantification of the antimicrobial activity of wild type *P. fluorescens* SBW25 and the *gacS* mutant against different oomycetes, fungi and bacteria.

**Figure S1** Location of the Tn5 transposon insertion (A) and the 19 microarray 60-mer probes in the *gacS* gene of *P. fluorescens* SBW25 (B). Probes are in grey and the
overlap between probes is depicted in dark grey.

**Figure S2** Organization of the sod gene cluster in *P. fluorescens* SBW25, including sodA, *fumC1*, a transport related membrane protein (PFLU0877), and two hypothetical proteins (PFLU0873, PFLU0875). For each of the genes, fold changes in transcription ± standard error of the mean were determined for the gacS mutant by Q-PCR analysis; these are indicated below each of the genes.

**Figure S3** Phenotypic characterization of wild type strain SBW25, gacS mutant, complemented gacS mutant (gacS compl.) and gacS + empty vector (gacS ev). For complementation, a 3763-bp fragment containing the gacS gene with putative promoter and terminator was obtained by PCR with specific primers (Table S1). The PCR fragment was first subcloned in pGEM-T Easy (Promega) and subsequently cloned into the shuttle vector pME6031 (Heeb et al., 2000). The obtained pME6031-gacS construct and pME6031 empty vector control were electroporated into the gacS::Tn5 mutant of *P. fluorescens* SBW25 and the presence of the vectors was verified by PCR with pME6031 specific primers (Table S2). (A) siderophore detection on CAS media: top, observation under normal light; bottom, under UV light; the size of the orange halos is a measure for the amount of siderophores produced. (B) Extracellular protease activity on skimmed milk agar plates; a halo around the bacterial colony represents protease activity. (C) Biofilm formation in KB liquid media in a 96-well PVC plate: the cells in the biofilm were stained with crystal violet (left); biofilm formation was quantified spectrophotometrically (right). (D) Swimming motility (top) and swarming motility (bottom) on soft SSM agar medium (0.3% and 0.6% agar (w/v), respectively).

**Figure S4** RP-HPLC analysis of culture extracts of wild type strain SBW25, the gacS mutant and the viscA mutant grown on 1/5th strength PDA agar medium for 48 h. RP-HPLC chromatograms at 210nm are shown for wild type SBW25, the gacS mutant and the viscA mutant.

**Figure S5** Activity of wild type SBW25, the gacS mutant and the viscA mutant against *Phytophthora infestans* on agar plates supplemented with 0, 50 or 100 μM of FeSO₄.
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Gac-mediated changes in pyrroloquinoline quinone biosynthesis enhance the antimicrobial activity of *Pseudomonas fluorescens* SBW25

Xu Cheng, Menno van der Voort, and Jos M. Raaijmakers

This chapter has been published as:

Chapter 3

Abstract

In *Pseudomonas* species, production of secondary metabolites and exoenzymes is regulated by the GacS/GacA two-component regulatory system. In *P. fluorescens* SBW25, mutations in the Gac-system cause major transcriptional changes and abolished production of the lipopeptide viscosin and of an exoprotease. In contrast to many other *Pseudomonas* species and strains, inactivation of the Gac-system in strain SBW25 significantly enhanced its antimicrobial activities against oomycete, fungal and bacterial pathogens. Here, random plasposon mutagenesis of the *gacS* mutant led to the identification of seven mutants with reduced or loss of antimicrobial activity. In four mutants, the plasposon insertion was located in genes of the pyrroloquinoline quinone (PQQ) biosynthesis pathway. Genetic complementation, ectopic expression, activity bioassays and RP-HPLC analyses revealed that a *gacS* mutation in SBW25 leads to enhanced expression of *pqq* genes, resulting in an increase in gluconic and 2-ketogluconic acid production, which in turn acidified the extracellular medium to levels that inhibit growth of other microorganisms. We also showed that PQQ-mediated acidification comes with a growth penalty for the *gacS* mutant in the stationary phase. In conclusion, PQQ-mediated acidification compensates for the loss of several antimicrobial traits in *P. fluorescens* SBW25 and may help *gac* mutants to withstand competitors.
PQQ-mediated antimicrobial activity in SBW25

Introduction

Plant growth-promoting rhizobacteria, including *Pseudomonas fluorescens* strain SBW25, can protect plants from pathogens by the production of secondary metabolites and exoenzymes, including toxins, antibiotics and siderophores (Howell and Stipanovic, 1979; Handelsman and Stabb, 1996; Loper et al., 2007) and by induction of systemic resistance (ISR) (Pieterse et al., 1996; Van Wees et al., 1997). In *Pseudomonas* species, production of many secondary metabolites and exoenzymes is regulated by the GacS/GacA two-component regulatory system (the Gac-system) (Haas and Keel, 2003; Mavrodi et al., 2006; Loper et al., 2007; Lapouge et al., 2008; de Bruijn and Raaijmakers, 2009). The Gac-system comprises the membrane-bound sensor kinase GacS, which is activated upon recognition of yet unknown environmental signals, and the response regulator GacA. Mutations in *gacS* or *gacA* generally lead to a substantial reduction in antimicrobial activity of *Pseudomonas* strains (Haas and Defago, 2005).

In *P. fluorescens* SBW25, mutations in the Gac-system caused major transcriptional changes and abolished the production of the lipopeptide surfactant viscosin and of an exoprotease (Hassan et al., 2010; Cheng et al., 2013). However, in contrast to several other *Pseudomonas* species and strains, inactivation of the Gac-system in strain SBW25 significantly enhanced its antimicrobial activities against oomycete, fungal and bacterial plant pathogens, including *Phytophthora infestans*, *Verticillium dahliae* and *Pseudomonas syringae* pv. *tomato* (Cheng et al., 2013). Genome-wide transcriptome analysis of strain SBW25 revealed that a mutation in *gacS* led to changes in the expression of genes involved in iron homeostasis, biofilm formation, motility, stress response and survival (Cheng et al., 2013). Among the 300 up-regulated genes, we focused on the ornicorrugatin-gene cluster (PFLU3220, 3222-3225) as a putative candidate for the enhanced, broad-spectrum antimicrobial activity of the *gacS* mutant. Site-directed mutagenesis and bioassays, however, showed that this gene cluster was not involved in the enhanced antimicrobial activity of the *gacS* mutant (Cheng et al., 2013).

To elucidate the underlying mechanism(s) of the enhanced antimicrobial activity of the *gacS* mutant of *P. fluorescens* SBW25, we performed a random mutagenesis of the *gacS* mutant and screened for double mutants with reduced or loss of antimicrobial activity. The candidate genes were characterized and their
role in the broad-spectrum antimicrobial activity was investigated in detail. The results showed that a mutation in \textit{gacS} enhances the production of gluconic acid and 2-keto gluconic acid, which leads to acidification of the extracellular medium that in turn suppresses growth of oomycete, fungal and bacterial plant pathogens.

\textbf{Materials and methods}

\textbf{Strains and growth conditions.} \textit{Pseudomonas fluorescens} SBW25 (spontaneous rifampicin resistant derivative) and \textit{Pseudomonas syringae} pv. \textit{tomato} DC3000 (\textit{Pst}) were grown on King’s B (KB) agar or in KB broth at 25°C (De Souza et al., 2003). When needed, growth media were supplemented with 50 μg/ml rifampicin, 100 μg/ml kanamycin, 25 μg/ml tetracycllin, and/or 40 μg/ml gentamycin. All fungal and oomycete species/strains were cultured on Potato Dextrose Agar (PDA, Difco). \textit{Phytophthora infestans} and \textit{Saprolegnia parasitica} were cultured at 18°C and 25°C, respectively; \textit{Verticillium dahliae} JR-2 was cultured at room temperature (Cheng et al., 2013).

\textbf{Random mutagenesis and selection of mutants deficient in antimicrobial activity.} The \textit{gacS::Tn5} mutant of strain SBW25 (de Bruijn and Raaijmakers, 2009) was subjected to random plasposon mutagenesis with plasmid pTn\textit{ModOTc} (Dennis and Zylstra, 1998). Transformants were selected and purified on KB agar plates supplemented with kanamycin (100μg/ml) and tetracycllin (25μg/ml), and subcultured in 96-well plates containing 200μl KB broth supplemented with kanamycin (100μg/ml) and tetracycllin (25μg/ml) at 25°C overnight. The mutant library was initially screened in \textit{in vitro} assays for loss of activity against the oomycete pathogen \textit{S. parasitica} strain C65. Selected mutants were screened for single insertions by Southern blot analysis. For the single insertion mutants, loss of antioomycete activity was confirmed in an \textit{in vitro} assay. The \textit{in vitro} dual culture assay was performed, with three replicates, as follows: SBW25 and mutant strains were grown in 5ml KB broth overnight at 25°C. Five μl bacterial suspension (OD$_{600}$ = 1.0) was spotted on a 1/5$^{th}$ strength PDA plate near the edges of the plate. After two days of incubation at 25°C, a 4-mm-diameter mycelial plug of \textit{S. parasitica} was placed in the centre of the 1/5$^{th}$ PDA plate and incubated at their appropriate temperature. Radial hyphal growth was monitored for several days depending on the pathogen’s growth rate. For plasposon mutants with reduced or loss of
antioomycete activity, plasmid rescue and sequence analysis of the regions flanking the plasposon were performed according to the methods described previously (Dennis and Zylstra, 1998). Additionally, growth inhibition of \textit{S. parasitica} strain C65 and \textit{P. infestans} strain 88069 by SBW25 and mutants was tested in liquid broth (1/5\textsuperscript{th} PDB). \textit{P. fluorescens} SBW25 and its mutants were cultured in 1/5\textsuperscript{th} PDB for 24h at 25°C. Filtrates of the bacterial cultures were collected by filter sterilization (0.22μm) of culture supernatants. To test the antimicrobial activities of these culture filtrates, each well of a 6-well plate was filled with 5ml of culture filtrate. Following, a 4-mm diameter mycelial plug of each oomycete pathogen was placed in the centre of the well and incubated at their appropriate temperature. Growth inhibition was scored visually and tested, for each pathogen, three times in duplicate.

\textbf{qPCR analysis.} RNA isolation and quantitative PCR (qPCR) analysis were performed as described previously (de Bruijn et al., 2008). The threshold cycle (\textit{CT}) value for genes of interest was corrected for that of the housekeeping gene \textit{rpoD}, as follows: 
\[ \Delta CT = CT (\text{gene}) - CT (rpoD). \]
The relative quantification (RQ) values were calculated with the formula 
\[ \text{RQ} = 2^{\Delta \text{CT} (\text{mutant}) - \Delta \text{CT} (\text{wild type})}, \]
(Pfaffl, 2001). The primers used for the Q-PCR are described in Table 1. Q-PCR analysis was performed in triplicate (technical replicates) on two independent RNA isolations (biological replicates).

\textbf{Genetic complementation of the mutants.} For complementation of the \textit{pqq} mutants, a 6.9-kb fragment containing the PQQ genes \textit{pqqF-E} and their putative promoter and terminator regions, were obtained by PCR with primers pBBR-\textit{pqqF-F2-AccIII}/pBBR-\textit{pqqE-R1-ClaI} (Table 1) and Phusion DNA polymerase (Finnzymes). The PCR fragments were subcloned in pGEM-T Easy (Promega) and the obtained plasmids were digested with \textit{AccIII} and \textit{ClaI}. The obtained \textit{pqqF-E} fragments were cloned into the shuttle vector pBBR1mcs-5 (Kovach et al., 1995; Heeb et al., 2000), and the obtained pBBR1mcs-5-\textit{pqqF-E} construct was electroporated into the \textit{gacS/pqqB} or \textit{gacS/pqqE} double mutant according to methods described by Choi and Schweizer (Choi et al., 2006). Cells transformed with pBBR1mcs-5-\textit{pqqF-E} were selected on KB agar medium supplemented with kanamycin (100μg/ml), tetracyclin (25μg/ml) and gentamycin (40μg/ml). The presence of the vector was verified by PCR with pBBR1mcs-5 specific primers pBBR-F-N/pBBR-R-N and pBBR-GmF/pBBR-Gm-R (Table 1). In addition, the pBBR1mcs-5-\textit{pqqF-E} construct was also introduced
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Table 1 Plasmids and oligonucleotide primers used in this study.

<table>
<thead>
<tr>
<th>Plasmid or primers</th>
<th>Relevant genotype or description</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pTnMod-OTc</td>
<td>plasposon for Gram-negative bacteria, TcR</td>
<td>Dennis and Zijlstra, (1998)</td>
</tr>
</tbody>
</table>

**Primers**

<table>
<thead>
<tr>
<th>Primers*</th>
<th>Sequence</th>
<th>Source</th>
</tr>
</thead>
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<tr>
<td>Q19-pqqA-F</td>
<td>CCT GGT CCA AAC CTG CTT AC</td>
<td>This study</td>
</tr>
<tr>
<td>Q20-pqqA-R</td>
<td>TAA CGG CTG GCG AAG TAC A</td>
<td>This study</td>
</tr>
<tr>
<td>Q23-pqqC-F</td>
<td>ATC CAT CAC CGG TAC CAT GT</td>
<td>This study</td>
</tr>
<tr>
<td>Q24-pqqC-R</td>
<td>GCG GGA TGT TCA CCT GAT AG</td>
<td>This study</td>
</tr>
<tr>
<td>Q25-pqqD-F</td>
<td>AGG CCA TGT GTT GCT CTA CC</td>
<td>This study</td>
</tr>
<tr>
<td>Q26-pqqD-R</td>
<td>GTT TAT CCA GCT CGG CAA TG</td>
<td>This study</td>
</tr>
<tr>
<td>Q31-gcd-F</td>
<td>ATC CGG CAG TTT GAA CGC</td>
<td>This study</td>
</tr>
<tr>
<td>Q32-gcd-R</td>
<td>CGC AAA CCA CAC CGA TGA</td>
<td>This study</td>
</tr>
<tr>
<td>pBBR-pqqF-F2-AccIII</td>
<td>GG tcgcga</td>
<td>This study</td>
</tr>
<tr>
<td>pBBR-pqqE-R1-ClaI</td>
<td>GG atcatg</td>
<td>This study</td>
</tr>
<tr>
<td>pBBR-gcd-F1-BamHI</td>
<td>GG ggatcc</td>
<td>This study</td>
</tr>
<tr>
<td>pBBR-gcd-R2-BamHI</td>
<td>GG ggatcc</td>
<td>This study</td>
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</table>

* Restriction sites incorporated into the primers are underlined.

into the *P. fluorescens* SBW25 wild type strain (Table 2). The antimicrobial activity of these strains was tested in the *in vitro* dual culture assay with *S. parasitica* as described above.

**Phenotypic analysis of *P. fluorescens* SBW25 and mutant strains.** Acidification of the growth medium was also tested for broth cultures. Wild type strain SBW25 and mutants were grown in 5ml KB broth overnight at 25°C. Then, 50μl of bacterial suspension (OD$_{600} = 1.0$) was inoculated into 5ml 1/5th PDB and incubated at 25°C; Every two hours 200μl of bacterial suspension was transferred into a 96-well plate. Cell density and pH were measured spectrophotometrically (Microplate reader Model 680, Bio-Rad) and by pH meter, respectively. This test was performed three times in duplicate.
PQQ-mediated antimicrobial activity in SBW25

Table 2 Seven antimicrobial activity deficient double mutants, which were obtained by phenotypically screening with *S. parasitica*.

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Bioactivity</th>
<th>Gene</th>
<th>Fold change in the gacS mutant (Microarray)</th>
<th>Role</th>
</tr>
</thead>
<tbody>
<tr>
<td>M7B6</td>
<td>Reduced</td>
<td>PFLU5987 (argB)</td>
<td>2.14</td>
<td>ATP and nucleotide binding</td>
</tr>
<tr>
<td>M9H3</td>
<td>Reduced</td>
<td>PFLU0969 (lysR)</td>
<td>1.30</td>
<td>Activator of lysine biosynthesis</td>
</tr>
<tr>
<td>M13D9</td>
<td>Reduced</td>
<td>PFLU5595 (oxidoreductase)</td>
<td>-4.28</td>
<td>Tryptophan metabolism</td>
</tr>
<tr>
<td>M13D7</td>
<td>Loss</td>
<td>PFLU5599 (pqqB)</td>
<td>2.58</td>
<td>Transporter</td>
</tr>
<tr>
<td>M6A6</td>
<td>Loss</td>
<td>PFLU5602 (pqqE)</td>
<td>2.10</td>
<td>Biosynthesis cofactor and carrier</td>
</tr>
<tr>
<td>10E1</td>
<td>Loss</td>
<td>PFLU1086 (gacD)</td>
<td>3.41</td>
<td>Membrane-bound dehydrogenase</td>
</tr>
<tr>
<td>M12G7</td>
<td>Loss</td>
<td>PFLU2985 (UTP)</td>
<td>-1.08</td>
<td>Precursor cell wall synthesis</td>
</tr>
</tbody>
</table>

Chemical analysis of *P. fluorescens* SBW25 and mutant strains. Wild type strain SBW25 and mutants were grown in 10ml 1/5th strength PDB broth supplemented with 5% glucose at 25°C for 4 days. Culture filtrates of each of the strains were collected at every 6h during 24h incubation. Detection and quantification of organic acids was performed on a Waters 996 High Performance Liquid Chromatogram (HPLC) equipped with PDA detector, Waters 717 plus autosampler, Waters 600 controller, Waters™ pump, Waters inline degasser AF, and Lichrosphere RP-18 column 250mm × 4.6mm and 5μm particle size (Merck, Germany). The mobile phase was 5mM sulphuric acid (Merck, Germany) in a gradient with a flow rate of 0.7ml/min. The organic acids were quantified by peak areas obtained for the authentic standards for Potassium D-gluconate (Sigma-Aldrich), 2-keto-D-gluconic acid hemi-calcium salt hydrate (Sigma-Aldrich), which were dissolved in the same medium as used for bacterial cultivation. The standards were mixed in a 1:1 ratio with different concentrations: 0, 0.25, 0.5, 0.75, 1.0, 1.25 and 1.5mM. Detection was performed at wavelengths from 200 to 450 nm.

Sensitivity of bacterial, fungal and oomycete pathogens to pH. The pH of 1/5th PDB was set to 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5 and 7.0 with HCL or NaOH. After sterilization, the pH of the media was checked again and 5ml was added to each of the wells in a 6-well plate. A 4-mm-diameter mycelial plug of the fungal and oomycete pathogens was placed in the centre of the well and incubated at their appropriate temperature. Growth inhibition was monitored visually. This test was performed in duplicate.
Chapter 3

Results and Discussion

Identification of genes involved in the enhanced antimicrobial activity of the \textit{gacS} mutant of \textit{P. fluorescens} SBW25

The \textit{gacS} mutant of \textit{P. fluorescens} SBW25 was subjected to random plasposon mutagenesis. Screening of an initial number of 1,248 random mutants resulted in 7 mutants with reduced or loss of activity against the oomycete pathogen \textit{S. parasitica} (Figure 1, Table 2). Southern blot hybridization showed a single Tn\textit{ModOTc} insertion for all 7 mutants. Subsequent \textit{in vitro} assays revealed that these 7 mutants also had reduced or loss of activity against other pathogens including \textit{P. infestans}, \textit{V. dahliae}, and \textit{P. syringae} (data not shown). For the three mutants with reduced activity, the plasposon insertion was located in genes involved in arginine and proline metabolism (mutant 7B6, PFLU5987), in tryptophan metabolism (mutant 13D9, PFLU5595), or in a gene encoding a LysR-family transcriptional regulator (mutant 9H3, PFLU0969). For the other four mutants with loss of activity against \textit{S. parasitica}, the plasposon insertion was located in the pyrroloquinoline quinone (PQQ) biosynthesis genes \textit{pqqB} (mutant 13D7, PFLU5599) and \textit{pqqE} (mutant 6A6, PFLU5602), in the \textit{gcd} gene encoding a glucose dehydrogenase (mutant 10E1, PFLU1086), or in a gene encoding a UTP-glucose-1-phosphate-uridylyltransferase (mutant 12G7, PFLU2985) (Table 2). Previous microarray analysis (Cheng et al., 2013) had shown that \textit{pqqB} (PFLU5599), \textit{pqqE} (PFLU5062) and \textit{gcd} (PFLU1086) were significantly up-regulated in the \textit{gacS} mutant (Table 2). In total, four out of the six PQQ biosynthesis genes (\textit{pqqF}, \textit{pqqA-E}) in SBW25 were up-regulated more than 2.0-fold in the \textit{gacS} mutant (Table 2, Figure 1B). The up-regulation of the \textit{pqq} and \textit{gcd} genes in the \textit{gacS} mutant was confirmed by qPCR (not shown). In \textit{Pseudomonas} and other Gram-negative bacteria, gluconic acid biosynthesis has been shown to be dependent on PQQ as an enzymatic cofactor of the membrane bound quinoprotein, glucose dehydrogenase (Gcd) (Goldstein, 1995; Kaur et al., 2006; de Werra et al., 2009; Kremmydas et al., 2013; Ponraj et al., 2013). Gcd acts as a glucose sensor and catalyses glucose into gluconic acid by periplasmic oxidation. A consecutive oxidation reaction converts gluconic acid to 2-keto gluconic acid (Schleissner et al., 1997; Hwangbo et al., 2003; de Werra et al., 2009; Ponraj et al., 2013). This study showed, for the first time, that a mutation in the Gac-system of \textit{P. fluorescens} SBW25 led to a significant transcriptional up-regulation of \textit{pqq} biosynthesis genes and \textit{gcd} gene. Genetic
Figure 1 (A) In vitro activity of wild type P. fluorescens SBW25, the gacS mutant and double mutants against the oomycete pathogen Saprolegnia parasitica. (B) Organization of the six pqq biosynthesis genes pqqF-E and the glucose dehydrogenase gene (gcd) of P. fluorescens SBW25. Underneath the genes are the fold-changes in their expression in the gacS mutant relative to wild type SBW25 as determined by microarray analysis. (C) In vitro activity against S. parasitica by the gacS mutant, wild type (WT) P. fluorescens SBW25, WT + pqqF-E, double mutant gacS/pqqB and the PQQ complemented mutant gacS/pqqB+pqqF-E.
complementation of the mutations in \textit{pqqB} and \textit{pqqE} by introduction of plasmid pBBR-\textit{pqqF-E} restored activity against \textit{S. parasitica} to the level of the gacS mutant. When the PQQ gene cluster (pBBR-\textit{pqqF-E}) was ectopically expressed in wild type strain SBW25 (WT+\textit{pqqF-E}), this led to enhanced activity against \textit{S. parasitica} to levels similar to that of the gacS mutant (Figure 1C). Similar results were obtained in liquid broth assays in which \textit{S. parasitica} was exposed to cell-free culture filtrates of wild type strain SBW25 and the filtrates of the different mutants (Figure 2A). These observations further confirmed that \textit{pqq} biosynthesis genes play a key role in the enhanced antimicrobial activity of the gacS mutant.

\textbf{PQQ-mediated acidification is involved in enhanced antimicrobial activity}

Wild type strain SBW25 acidified the 1/5th PDB medium from pH 6.2 to pH 5.3, whereas the gacS mutant acidified the medium to pH 4.2 (Figure 2A). This acidification was not only observed in 1/5th PDB medium but also in another growth medium used for P-solubilization (NPRIB). For the two double mutants, gacS/\textit{pqqB} and gacS/\textit{pqqE}, the pH of their culture filtrates was 5.9 and 5.8, respectively. Genetic complementation of \textit{pqqB} and \textit{pqqE} resulted in pH values of 3.8 and 3.7, respectively, similar to that of the gacS mutant (Figure 2A). When \textit{pqqF-E} were introduced in wild type strain SBW25, pH was reduced from 5.3 to 3.9 (Figure 2A). The acidification of the medium to a pH of 4.2 or lower coincided with a reduced or lack of mycelium growth of \textit{S. parasitica} (Figure 2A). Subsequent assays showed that mycelial growth of \textit{S. parasitica} was inhibited at a pH of 4.5 or lower (Figure 2B). Also for \textit{P. infestans}, \textit{V. dahliae} and \textit{P. syringae}, growth was strongly diminished at pH 4.5 or lower. (Figure 2C-E). When the pH of the cell-free culture filtrate of wild type strain SBW25 was adjusted from 5.5 down to 4.2, the same pH of the gacS mutant culture filtrate, a similar level of growth inhibition of \textit{S. parasitica} was obtained (Figure 3A). Conversely, when the pH of the gacS mutant culture filtrate was adjusted from 4.2 up to 5.5, the pH of the wild type culture filtrate, growth of \textit{S. parasitica} was not inhibited (Figure 3A). Similar results were obtained for \textit{P. infestans}, \textit{V. dahliae}, and \textit{P. syringae} (Figure 3B-D). The role of \textit{pqq} and \textit{gcd} in antimicrobial activity has been described before (Mavrodi et al., 2006; Driscoll et al., 2011; Lalaouna et al., 2012), but has, to our knowledge, not been directly linked with PQQ-mediated acidification of the extracellular environment. For example, in \textit{P. protegens} strain CHA0, a \textit{gcd} mutant was shown to be more effective in biological
Figure 2 (A) Activity of culture filtrates of wild type *P. fluorescens* SBW25 and its mutants against *S. parasitica*. Underneath the wells are the pH values measured for each of the culture filtrates of each of the strains. Averages of 3 replications are given, ± represents the standard deviation. pH sensitivity of *S. parasitica* (B), *P. infestans* (C), *V. dahliae* (D) and *P. syringae pv. tomato* (E), grown in 1/5 th PDB liquid media at different pH (3.0 to 7.0). For *P. syringae pv. tomato*, underneath the tubes are cell densities measured at OD$_{600}$. Averages of 3 replications are given and STDEV stands for standard deviation.
control of *G. graminis var. tritici* but this enhanced activity was not attributed to a change in pH but to an increased production of the antifungal compounds 2,4-diacetylphloroglucinol and pyoluteorin (de Werra et al., 2009). Together, these observations suggest that the enhanced, broad-spectrum antimicrobial activity of the *gacS* mutant of *P. fluorescens* SBW25 is due to PQQ-mediated acidification.

**Gac-mediated production of gluconic acid and 2-ketogluconic acid**

HPLC analysis showed that the *gacS* mutant produced more 2-ketogluconic acid and gluconic acid than wild type strain SBW25 (Figure 4A). The *gacS/pqqB*
PQQ-mediated antimicrobial activity in SBW25

and gacS/qqqE mutants did not produce detectable levels of 2-ketogluconic acid and gluconic acid, which was restored in the complemented mutants (gacS/qqqB+qqqF-E and gacS/qqqE+qqqF-E). Introduction of the qqqF-E genes in wild type SBW25 (WT+qqqF-E) resulted in an increase in 2-ketogluconic acid and gluconic acid production as compared to the wild type strain (Figure 4). The standard curves for 2-ketogluconic acid and gluconic acid further showed that with increasing concentrations of 2-ketogluconic acid or gluconic acid, pH decreases from pH 6.5 to pH 4.2 (Figure 4B). Combining the results of these standard curves (Figure 4B) with the areas of the (2-keto)gluconic acid peaks (Figure 4A) and the pH values of the culture filtrates of wild type SBW25 and mutants, indicate that wild type strain SBW25 and the gacS mutant produce approximately 0.50 mM and 0.75 mM 2-keto gluconic acid, respectively, and approximately 0.045 mM and 0.095 mM gluconic acid, respectively (Figure 4C, 4D). Expression of the qqq genes in wild type SBW25 led to an increase in (2-keto) gluconic acid and a decrease in pH (Figure 4C, 4D). Next to the PQQ-biosynthesis mutants of strain SBW25, we also analyzed the culture filtrates of the other four double mutants obtained in the initial screening. Based on HPLC analysis of the culture filtrates of these four mutants, gluconic acid and 2-ketogluconic acid production was also abolished in mutant 13D9 with a mutation in gene PFLU5595 involved in tryptophan metabolism. Given that this gene is located upstream of the qqqF gene (PFLU5597) suggests that the encoded putative oxidoreductase may play a role in PQQ biosynthesis. For the other three double mutants with reduced or loss of antimicrobial activity (7B6, 9H3, 12G7), gluconic acid and 2-ketogluconic acid production was not abolished, suggesting that additional mechanisms may play a role in the enhanced antimicrobial activity of the gacS mutant.

Effect of PQQ-mediated acidification on growth of P. fluorescens SBW25

Analysis of the growth showed that the gacS mutant reached the exponential phase earlier than wild type SBW25, but established a lower cell density in the stationary phase than wild type SBW25 (Figure 5). This reduced growth of the gacS mutant in the transition from the exponential to the stationary phase coincided with a decrease of the pH to 4.5 and lower. This alteration in growth dynamics and pH change was not observed in the gacS/qqqB mutant and could be restored by genetic complementation (Figure 5). These results indicate that a mutation in gacS
Figure 4 RP-HPLC analysis of culture filtrates of wild type *P. fluorescens* SBW25, the gacS mutant, PQQ genes ectopic expression strain WT+pqqF-E, double mutants gacS/pqqB and gacS/pqqE, and PQQ complemented strain gacS/pqqB+pqqF-E and gacS/pqqE+pqqF-E grown in 1/5th PDB supplemented with 5% glucose for 24h at 25°C. (A) RP-HPLC chromatograms are: gluconic acid and 2-keto gluconic acid detection at λ210nm of a 1:1 ratio (0.5mM) mixture (Top) and of each of the strains; (B) standard curve of gluconic acid (right) and 2-keto gluconic acid (left), which were used for quantification of the production of these two acids; (C) correlation of gluconic acid (right) and 2-keto gluconic acid (left) production (mM and peak area) and pH changes.
causes PQQ-mediated acidification and concomitant adverse effects on growth. Gac-mutations in *Pseudomonas* spontaneously occur in nutrient-rich cultures (Driscoll et al., 2011), but also in rhizosphere environments (Sanchez-Contreras et al., 2002; Redondo-Nieto et al., 2012). Spontaneous mutations in the Gac-system in *Pseudomonas brassicacearum* NFM421 (Achouak et al., 2004; Lalaouna et al., 2012) and *Pseudomonas fluorescens* F113 (Sanchez-Contreras et al., 2002) resulted in a higher motility and enhanced colonization abilities in the rhizosphere. Since mutations in the Gac-system generally lead to a reduction in antimicrobial activity, Gac-mutants are considered to be less competent than their wild type in competition with other microbes. Combined with the fact that Gac-mutants are generally poor biofilm formers, they most likely face strong counterselection in rhizosphere environments, where biofilms provide additional protection against competitors and predators. Our study shows that, at least for *P. fluorescens* SBW25, PQQ-mediated acidification may compensate for the loss of several antimicrobial traits and help the *gac* mutant to withstand competitors. However, in terms of growth, PQQ-mediated acidification comes with a penalty, especially in the transition from exponential to stationary growth. Preliminary *in vitro* assays showed
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that acidification by the gacS mutant also occur in the rhizosphere of Arabidopsis. Whether this is also the case in the rhizosphere of plants grown in the soil remains to be addressed. Furthermore, how PQQ-mediated acidification affects colonization and survival in soil and rhizosphere environments, and how this affects plant root architecture, plant growth and health will be subject of future studies.

Acknowledgements

This project was financially supported by the Netherlands Genomics Initiative (NGI-EcoLinc project). We would like to acknowledge Dr Emilie Pineau-Chapelle for technical assistance and advice with the RP-HPLC analysis. There is no conflict of interest to declare.
PQQ-mediated antimicrobial activity in SBW25

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PQQ-mediated antimicrobial activity in SBW25


Role of the GacS/GacA two-component system in the regulation of volatile production by plant growth-promoting *Pseudomonas fluorescens* SBW25

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This chapter was submitted for publication.
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Abstract

In plant-associated *Pseudomonas* species, the production of several secondary metabolites and exoenzymes is regulated by the GacS/GacA two-component regulatory system (the Gac-system). Here, we investigated if the Gac-system also regulates the production of volatile organic compounds (VOCs) in *P. fluorescens* SBW25 (*Pf.*SBW25) and how this impacts on VOCs-mediated growth promotion and induced systemic resistance of Arabidopsis and tobacco. A total of 205 VOCs were detected by using Gas Chromatography Mass Spectrometry (GC-MS) for *Pf.* SBW25 and the *gacS*-mutant grown on KB or 1/5th PDA media for 3 and 6 days. Discriminant function analysis followed by hierarchical clustering revealed 24 VOCs that were significantly different in their abundance between *Pf.*SBW25 and the *gacS*-mutant, which included three acyclic alkenes (3-nonene, 4-undecyne, 1-undecene). These alkenes were significantly reduced by the Gac-mutation independently of the growth media and of the incubation time. For Arabidopsis, both *Pf.*SBW25 and the *gacS*-mutant enhanced, via VOCs, root and shoot biomass, induced systemic resistance against leaf infections by *P. syringae* and induced rhizosphere acidification to the same extent. For tobacco, however, VOCs-mediated effects on shoot and root growth were significantly different between *Pf.*SBW25 and the *gacS*-mutant. While *Pf.*SBW25 significantly inhibited tobacco root growth, the *gacS*-mutant significantly enhanced root biomass and lateral root formation relative to the non-treated control plants. Collectively these results indicate that the Gac-system is involved in the regulation of VOCs production in *Pf.*SBW25, which in turn affects plant growth promotion in a plant species-dependent manner.
The Gac regulation of volatile production by SBW25

Introduction

Microorganisms produce a variety of volatile organic compounds (VOCs), which are defined as low molecular weight compounds with high vapour pressures (Cordovez et al., 2015; Schmidt et al., 2015). Their physical and chemical properties allow dispersal over longer distances than other extracellular microbial metabolites. VOCs are structurally diverse and include many yet unknown compounds. For example, from 12 *Streptomyces* strains isolated from the plant rhizosphere, a total of 381 VOCs were detected with most of them structurally unknown (Cordovez et al., 2015). To date, microbial VOCs have been grouped into hydrocarbons, ketones/alcohols, acids, sulfur compounds, nitrogen-containing compounds and terpenes (Audrain et al., 2015; Schmidt et al., 2015). Bacteria can also release inorganic volatiles such as hydrogen cyanide (HCN), ammonia and nitrous oxide (Audrain et al., 2015; Schmidt et al., 2015). Based on the structural diversity, several natural functions have been proposed for microbial VOCs. These include a role of VOCs as: 1) infochemicals in inter- and intraorganismal communications (Chernin et al., 2011; Schmidt et al., 2015), 2) antimicrobial agents (Kai et al., 2010; Cordovez et al., 2015), and 3) compounds that promote or inhibit plant growth (Blom et al., 2011; Cordovez et al., 2015). Indeed, several bacterial genera, including *Bacillus*, *Pseudomonas*, *Streptomyces*, *Serratia*, *Arthrobacter*, *Collimonas* and *Stenotrophomonas*, influence plant growth via VOCs (Ryu et al., 2003; Garbeva et al., 2014; Audrain et al., 2015; Cordovez et al., 2015; Kanchiswamy et al., 2015; Park et al., 2015). The bacterial VOCs acetoin and 2,3-butanediol from *Bacillus* are well-known for their role in plant growth promotion and induction of systemic resistance (ISR) against pathogen infection (Ryu et al., 2003; Ryu et al., 2004). Over the past years, several other bacterial VOCs, including indole, 1-hexanol, pentadecane, 13-tetradecadien-1-ol, 2-butanol, and 2-methyl-n-1-tridecene, have been implicated in plant growth promotion (Blom et al., 2011; Park et al., 2015).

Bacterial VOCs are synthesized via diverse pathways including aerobic, heterotrophic carbon metabolism, fermentation, amino-acid catabolism, terpenoid biosynthesis, fatty acid degradation or sulphur reduction (Penuelas et al., 2014). Because the production of certain VOCs appears to be dependent on cell density, quorum sensing (QS) has been suggested as a possible regulatory system of bacterial VOCs production (Audrain et al., 2015). For example, 2-amino-acetophenone (2-
AA) produced by *Pseudomonas aeruginosa* is controlled by the multiple virulence factor regulator (MvfR), a known quorum sensing system (Kesarwani et al., 2011; Que et al., 2013). In contrast, no significant effects on VOCs production were found for a QS-mutant of *Burkholderia ambifaria* LMG19182 (Groenhagen et al., 2013). In *Pseudomonas*, the production of various secondary metabolites and exoenzymes is under the regulation of the GacS/GacA two-component regulatory system (referred to here as the Gac-system). The Gac-system consists of the membrane-bound sensor kinase GacS and the cytoplasmic transcriptional response regulator GacA. Mutations (spontaneous or site-directed) in the *gacS* or *gacA* genes generally abolish secondary metabolite production (Zuber et al., 2003). In *P. protegens* Pf-5 and *P. fluorescens* SBW25 (*Pf.SBW25*), mutations in the *gacA* or *gacS* genes have significant effects on the iron homeostasis and the expression of multiple genes involved in virulence, biofilm formation, motility, stress responses and survival (Blom et al., 2011; Cheng et al., 2013). Production of the volatile HCN is regulated by the Gac-system in *P. protegens* strains CHA0 and Pf-5 (Duffy and Defago, 2000; Hassan et al., 2010) and also the production of 2R, 3R-Butanediol by *P. chlororaphis* O6 was shown to be Gac-dependent (Han et al., 2006). To date, however, the role of the Gac-system in the overall regulation of VOCs produced by plant growth-promoting rhizobacteria is not known. In this study, we analysed the VOC profiles of wildtype *Pf.SBW25* and its Gac-mutant grown on different media and after different incubation periods. We subsequently determined if and how a mutation in the Gac-regulatory system affects VOCs-mediated growth promotion and rhizosphere acidification of Arabidopsis and tobacco, and ISR in Arabidopsis.

**Materials and Methods**

**Bacterial strains, media and culture conditions**

*Pseudomonas fluorescens* SBW25 (*Pf.SBW25*) and its gacS-mutant (referred to here as the Gac-mutant) were pre-cultured in liquid King’s B (KB) broth supplemented with rifampicin (50µg/ml) and with rifampicin (50µg/ml) and kanamycin (100µg/ml), respectively, at 25 °C for 24 hours. The pathogen *Pseudomonas syringae pv. tomato* DC3000 (*Pst*) was cultured in KB broth supplemented with rifampicin (50µg/ml) at 25 °C for 24 hours. Bacterial cells were collected by centrifugation, washed three times with 10 mM MgSO$_4$ and resuspended in 10 mM MgSO$_4$ to a final density of OD$_{600}$ = 1.0 (~10$^9$ CFU/ml).
The Gac regulation of volatile production by SBW25

Collection and analysis of VOCs

For the collection of bacterial VOCs, 100 µL of a cell suspension of Pf.SBW25 and the Gac-mutant (OD$_{600}$ = 0.1) were inoculated individually in 90-mm-diameter glass Petri dish containing 20 ml of KB agar or 1/5th strength Potato Dextrose Agar (1/5th PDA, Oxoid) media with three replicates each. Plates containing the agar media only served as controls. To collect the headspace VOCs, the lid of these Petri dishes were designed with an outlet connected to the traps filled with an adsorbent (Tenax). The Tenax traps were pre-conditioned at 260°C with a Helium flow rate for 45 min and cooled afterwards to room temperature. Petri dishes connected to the Tenax traps were sealed with parafilm and incubated at 25°C. After 3 and 6 days of incubation, headspace VOCs was analyzed by GC-Q-TOF-MS (Agilent 7890B GC and the Agilent 7200A QTOF, Santa Clara, USA). VOCs were thermally desorbed from the Tenax traps using an automated thermodesorption unit (model UnityTD-100, Markes International Ltd., Llantrisant, UK) at 210°C for 12 min (He flow 50 ml/min) and captured on a cold trap at -10°C. The compounds released were transferred onto the analytical column (30 × 0.25 mm ID RXI-5MS, film thickness 0.25 μm - Restek 13424-6850, Bellefonte, PA, USA) with a split ratio of 1:20 (v/v). The temperature program of the GC oven started at 39°C (2-min hold) and rose to 95°C at a rate of 3.5°C min$^{-1}$, to 165°C at 6°C min$^{-1}$, to 250°C at 15°C min$^{-1}$ and finally to 300°C at 40°C min$^{-1}$ (20 min-hold). VOCs were detected by the MS operating at 70 eV in EI mode. Mass scanning was done from 30-400 m/z with a scan time of 4 scans s$^{-1}$.

Mass signals from GC-MS raw data that were generated using an untargeted metabolomics approach were extracted and aligned by MetAlign software (Lommen and Kools, 2012). MSclus was used to remove signal redundancy per metabolite and to reconstruct compound mass spectra as previously described (Tikunov et al., 2012). VOCs detected for both the media and the bacterial strains (Fold Change (FC) < 2) were removed from the analyses. VOCs were annotated by comparing their mass spectra with those of commercial NIST14 (National Institute of Standards and Technology, USA, [http://www.nist.gov](http://www.nist.gov)) and Wiley database 9th edition. The linear retention indices (RI) and the accurate mass of selected VOCs were compared with those in the library. Processed VOCs data were log transformed and auto-scaled using the average as an offset and the standard deviation as scale (raw value-average...
(offset)/SD (scale)] with GeneMaths XT Version 2.11 (Applied Maths, Belgium)). Log transformed data were subjected to One-Way ANOVA and VOCs that showed a significant difference ($P < 0.05$) at least under one of the conditions analysed were used in the hierarchical cluster analysis (Pearson’s correlation coefficient with UPGMA algorithm) and discriminant analysis. To select VOCs affected by the Gac-mutation, Student’s t-Test (for independent samples) was performed between wildtype and mutant for both media and time points following the criteria: $P < 0.05$ (t-Test), peak intensity of at least $10^4$ and fold change (FC) > 2.

**VOCs-mediated plant growth promotion**

To determine the role of the Gac-system in VOCs-mediated plant growth promotion of Arabidopsis and tobacco (*Nicotiana benthamiana*), seedlings were exposed to the VOCs emitted by the wild type *Pf.SBW25* or the Gac-mutant. Arabidopsis seeds (Col-0) were surface sterilized and were sown on square plates (100 x 100 mm) containing 50 ml of half-strength Murashige and Skoog ($\frac{1}{2}$MS) agar medium (Murashige and Skoog, 1962) supplemented with 0.5% (w/v) sucrose as previously described (van de Mortel et al., 2012). Plates were sealed with parafilm and incubated in a climate chamber (21°C/21°C day/night temperature; 250 μmol light m$^{-2}$ s$^{-1}$ at plant level during 16 h/d; 70 % relative humidity). Dual-dish plates were prepared with a large round Petri dish (145-mm-diameter) containing 100 ml $\frac{1}{2}$ MS medium fixed with a small Petri dish (35-mm-diameter) positioned inside the large Petri plate. Arabidopsis or tobacco seedlings were grown in the large Petri plate, whereas in the small Petri plate the bacteria were cultured on KB agar medium. Six days after sowing of surface-sterilized Arabidopsis seeds, 10 μl of bacterial suspension (~$10^9$ CFU/ml) was spot-inoculated in the small petri dishes and incubated at 25°C overnight. On the next day, the 7-day-old Arabidopsis seedlings were transferred from square Petri dish to half strength MS media in the large Petri plate. Using this experimental set-up, the plants and bacterial cultures were physically separated in the dual-dish plate and bacteria-plant interactions were only possible via VOCs. Plates were sealed with parafilm and then incubated in the climate chamber (21°C/21°C day/night temperature; 250 μmol light m$^{-2}$ s$^{-1}$ at plant level during 16 h/d; 70% relative humidity). After 11 days of incubation, plant fresh and dry weights were determined. Differences in shoot and root biomass were analysed statistically by one-way ANOVA, Tukey, $P < 0.05$). Experiments with
tobacco seedlings were performed as described above for Arabidopsis.

**VOCs-mediated induced systemic resistance**

For the induced resistance (ISR) assay, leaves of 14-day-old Arabidopsis seedlings, exposed (or not) to VOCs from wild type *Pf.* SBW25 or the Gac-mutant, were inoculated in the center of the leaf rosette with 2 µl cell suspension (~10⁹ CFU/ml) of the pathogen *Pst.* Five to 7 d after inoculation, disease incidence was assessed by determining the percentage of diseased leaves per plant. Leaves were scored as diseased when they exhibited necrotic or water soaked lesions surrounded by chlorotic or necrotic leaf tissue. Disease incidence was calculated for each plant with at least 20 plants per treatment. The experiment was performed at least twice. Statistically significant differences were determined by ANOVA (*P* < 0.05).

**VOCs-induced rhizosphere acidification**

Next to the plant growth promotion and ISR assays, pH changes in the rhizosphere of Arabidopsis and tobacco seedlings were monitored by supplementing bromocresol green (0.02% w/v) into the ½ MS medium on which the seedlings were grown. Bromocresol green acts as a pH indicator with a yellow color at pH 3.8 and a blue color at pH 5.4. The pH changes were monitored and captured during 11 days of co-cultivation of Arabidopsis and tobacco seedlings with the bacterial strains.

**Results**

**Gac-regulation of VOCs production by *Pf.* SBW25**

VOCs produced by wild type *Pf.* SBW25 and its Gac-mutant grown on KB or 1/5<sup>th</sup> PDA agar media were collected at different time points (3 or 6 days of incubation). A total of 205 putative VOCs were detected by GC-MS in the headspace of *Pf.* SBW25 and its Gac-mutant of which 79 VOCs differed significantly in abundance (One-way ANOVA, *P* < 0.05) in at least one of the growth conditions (Figure 1). Discriminant function analysis of these 79 VOCs resulted in a model with 3 principal components, which explained 84% of the total variation. The first component explained 42% of the total variation associated primarily with the variation in the abundance of VOCs when *Pf.* SBW25 and the Gac-mutant grown on 1/5<sup>th</sup> PDA and KB media.
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VOCs detected for both \textit{Pf.SBW25} and its Gac-mutant were clearly separated by the type of cultivation media (Figure 1). The second component explained 23\% of the total variation related to the effect of the Gac-mutation on VOCs when grown on KB medium. The third component explained 19\% of the total variation and is related to the effect of the Gac-mutation on VOCs when grown on 1/5\textsuperscript{th} PDA (Figure 1). Student t-Test between the \textit{Pf.SBW25} and the Gac-mutant (\(P < 0.05\), FC > 2) revealed a total of 24 VOCs that were significantly affected by the mutation at least under one of the growth conditions (Table 1). The VOCs profiles of \textit{Pf.SBW25} and its Gac-mutant are shown in the Hierarchical Cluster Analysis (HCA) (Figure 2A). For the majority of these metabolites, the effect of the Gac mutation on the VOCs emission was dependent on the cultivation media (Figure 2A, 2B).

On KB medium only, the emission of 3 VOCs was significantly reduced in the Gac-mutant, which belong to the class of acyclic alkenes (Table 1). These VOCs are 3-nonene (RI: 891), 1-undecene (RI: 1089) and 4-undecyne (RI: 1085). The VOC 891 3-nonene was only detected at 3 days of incubation and it was reduced.

Table 1 List of 24 VOCs significantly affected by the Gac-mutation of \textit{Pf.SBW25} at least under one of the growth conditions. Statistically significant changes were determined by Student’s t-Test and fold change (FC) calculated between wildtype and the Gac-mutant. VOCs listed were identified based on library match (Annotation level 4) or also checked with retention time index (RI) and accurate mass (Annotation level 2).

<table>
<thead>
<tr>
<th>RI</th>
<th>Compound</th>
<th>Annotation</th>
<th>KB medium</th>
<th>1/5\textsuperscript{th} PDA medium</th>
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</thead>
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<tr>
<td>532</td>
<td>Bicyclo[2.1.0]pentane</td>
<td>2</td>
<td>0.04 1.14 1.09 1.43</td>
<td>0.02 1.77 0.02 4.29</td>
</tr>
<tr>
<td>608</td>
<td>Butanoic acid, methyl ester</td>
<td>4</td>
<td>0.16 1.32 0.66 1.12</td>
<td>0.00 2.22 0.00 2.66</td>
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<tr>
<td>701</td>
<td>Dodecyl succinate</td>
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<td>0.53 5.48 0.17 4.05</td>
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<tr>
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<td>Propanoic acid</td>
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<td>0.95 1.00 0.07 -3.84</td>
<td>0.04 2.73 0.15 2.27</td>
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<tr>
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<td>Proprionic acid</td>
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<td>0.89 1.04 0.65 -1.12</td>
<td>0.11 3.65 0.00 6.74</td>
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<tr>
<td>740</td>
<td>2-(acetoxy)ethyl cyclohexanone</td>
<td>4</td>
<td>0.10 -1.46 0.66 1.27</td>
<td>0.21 1.59 0.01 2.84</td>
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<tr>
<td>796</td>
<td>(1-13C)-Butanol</td>
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<td>0.20 18.85 0.11 8.14</td>
<td>0.00 35.73 0.56 1.40</td>
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<td>0.00 56.14 0.00 56.29</td>
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<tr>
<td>812</td>
<td>Ethanone, 1-(2-furanyl)</td>
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<td>2,2-Dimethyl-4-pentenylamine</td>
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<tr>
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\[^1\text{Calculated linear retention index}\]
\[^2\text{Annotation level according to the Metabolomics Standards Initiative (MSI)}\]
\[\text{Colored cells represent significant (p<0.005/FC>2) differentials; VOCs reduced in the Gac-mutant are in red; VOCs increased in the Gac-mutant are in green.}\]

D3: 3 days after inoculation; D6: 6 days after inoculation

FC: Fold change calculated using the compound peak height (\textit{Pf.SBW25} versus the Gac-mutant)

MF: Library match factor
The Gac regulation of volatile production by SBW25 in the Gac-mutant (Table 1, Figure 2C). On 1/5\textsuperscript{th} PDA, the emission of 7 VOCs was significantly reduced in the Gac-mutant, independent of the incubation time (Table 1). These VOCs are bicyclo[2.1.0]pentane (RI: 532), dimethyl sulfide (RI: 701), S-2-S-butylfuran (RI: 886) and the three alkenes described above. Moreover, the emission of these 3 VOCs was significantly reduced in the Gac-mutant under all growth conditions (media and incubation time) tested (Figure 2B and Table 1).

**VOCs-mediated effects on plant growth and ISR**

Since the Gac-mutant established much lower cell densities than wildtype *Pf.*SBW25 on 1/5\textsuperscript{th} PDA medium but not on KB medium, the plant growth-promotion and ISR assays were performed only with the bacterial strains grown on KB. When

![Figure 1](image.png)

**Figure 1** Discriminant function analysis based on 79 VOCs that were significantly different in their abundance at least under one of the conditions analyzed (ANOVA, *P*<0.05). The first component explained 42% of the total variation primarily associated to variation in the abundance of VOCs when *Pf.*SBW25 and the Gac-mutant are grown in 1/5\textsuperscript{th} PDA and KB media. The second component explained 23% of the total variation and particularly related to the effect of the mutation on VOCs when *Pf.*SBW25 and the Gac-mutant are grown on KB medium. The third component explained 19% of the total variation and is related to the effect of the Gac-mutation on the VOCs when *Pf.*SBW25 and the Gac-mutant are grown on 1/5\textsuperscript{th} PDA.
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Arabidopsis seedlings were exposed to VOCs from *Pf*.SBW25 or from the Gac-mutant, plant growth and root architecture were altered: the primary roots were longer, and more lateral roots were formed than for non-exposed (medium only) control plants (Figure 3A). Both shoot and root biomass of Arabidopsis seedlings, exposed to either *Pf*.SBW25 or the Gac-mutant, increased approximately 4-fold as compared to control plants. Leaves of Arabidopsis plants exposed to VOCs from *Pf*.SBW25 or the Gac-mutant were also visually greener than those of control plants (Figure 3B). To test for VOCs-mediated ISR activity, Arabidopsis leaves were inoculated with a suspension of the pathogen *Pst*. Results showed that *Pst* disease
The Gac regulation of volatile production by SBW25

The incidence was significantly reduced in plant seedlings exposed to VOCs from \textit{Pf.SBW25} or from the Gac-mutant (Figure 3C). Collectively, these results show that a mutation in the Gac-system of \textit{Pf.SBW25} did not alter VOCs-mediated growth promotion of Arabidopsis seedlings nor ISR against \textit{Pst}.

**VOCs-mediated acidification of the rhizosphere**

When bromocresol green was added to the plant growth medium as a pH indicator, we observed more rhizosphere acidification (yellowish area) in the VOCs-exposed Arabidopsis plants than the control plants (Figure 3D). This difference may, for a large part, be explained by the substantial increase in root biomass of the seedlings exposed to VOCs from \textit{Pf.SBW25} or the Gac-mutant. However, acidification in the \textit{Pf.SBW25}-exposed seedlings was observed not only around the roots but across the entire plate, whereas for the Gac-mutant-exposed plants acidification was located mostly in the area surrounding the roots (Figure 3D).
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In the absence of the Arabidopsis seedlings, *Pf.*SBW25 and the Gac-mutant did not cause acidification of the medium. These results suggest that *Pf.*SBW25 and the Gac-mutant induce a quantitatively different VOCs-mediated rhizosphere acidification in Arabidopsis.

**VOCs-mediated effects on tobacco**

To investigate if the observed VOCs-mediated phenotypic changes by *Pf.*SBW25 are typical for Arabidopsis or also appear in other plant species, we conducted similar assays with tobacco (*N. benthamiana*). When tobacco seedlings were exposed to VOCs of *Pf.*SBW25 or the Gac-mutant, the root architecture was differentially affected. When exposed to VOCs from *Pf.*SBW25, growth of the primary roots was inhibited (Figure 4A). In contrast, VOCs from the Gac-mutant promoted root growth and induced more lateral roots relative to *Pf.*SBW25 and the rhizosphere acidification, blue color represents no pH decline.

![Figure 4](image)

**Figure 4.** Effects on tobacco (*Nicotiana benthamiana*) growth after 11 days of exposure to VOCs from *Pf.*SBW25 and the Gac-mutant. (A) Growth promotion and (B) biomass quantification: Bars represent the mean of 4 replicates (with 8-10 seedlings per replicate) ± standard deviation. Bars in blue (control), red (*Pf.*SBW25) and green (the Gac-mutant) represent dry weight of Arabidopsis seedlings. Asterisks indicate a statistical difference as compared to control plants. (C) rhizosphere acidification, blue color represents no pH decline.
The Gac regulation of volatile production by SBW25 control (Figure 4A). Biomass quantifications of both shoot and root supported this observation (Figure 4B). For tobacco, no VOCs-mediated rhizosphere acidification was observed (Figure 4C). Collectively, these results indicate that VOCs-mediated plant growth promotion and rhizosphere acidification by \textit{Pf}.SBW25 is plant species specific.

**Discussion and conclusions**

Microbial VOCs act as infochemicals in microbe-microbe interactions influencing inter- and intraorganismal communications (Schmidt et al., 2015). They can also have antagonistic activities by inhibiting bacterial and fungal growth (Chernin et al., 2011; Garbeva et al., 2014; Audrain et al., 2015; Cordovez et al., 2015; Schmidt et al., 2015), or by affecting gene expression, motility, biofilm formation and antibiotic resistance of competing bacterial species (Kim et al., 2013; Audrain et al., 2015; Schmidt et al., 2015). Here, we show that VOCs production by plant growth-promoting \textit{Pf}.SBW25 is regulated by the GacS/GacA two-component system. Our results show that the Gac-system in \textit{Pf}.SBW25 regulates, independently from growth medium or incubation time, the production of acyclic alkenes, in particular 3-nonene, 1-undecene and 4-undecyne. These VOCs are produced by several \textit{Pseudomonas} species and have been reported to have plant growth promoting-effects on \textit{Arabidopsis thaliana} (Blom et al., 2011) and tobacco (\textit{Nicotiana tabacum} cv. Xanthi-nc) (Park et al., 2015). For 1-undecene, Rui et al. (2014) recently identified the biosynthetic gene undA in \textit{P. aeruginosa} PA14. Heterologous expression of undA homologs of several other \textit{Pseudomonas} strains in \textit{E.coli} conferred heterologous expression and production of 1-undecene (Rui et al., 2014). Our previous work showed that a mutation of the Gac-system of \textit{Pf}.SBW25 caused major transcriptomic changes (Cheng et al., 2013). Revisiting these microarray data showed that PFLU4307, the undA orthologue of \textit{P. protegens} Pf-5, was 9.7-fold down regulated in the gacS-mutant of \textit{Pf}.SBW25. In \textit{Pseudomonas}, most of the known small RNAs are under the control of the Gac/Rsm (repressor of secondary metabolites) signal transduction pathway (Song et al., 2015). The activated regulator GacA is suspected to bind to a conserved element termed the GacA box in the promoters of the small RNA genes, to activate their expression. These small RNA genes all share multiple unpaired GGA motifs, which are essential for sequestration of repressor proteins (Lapouge et al., 2008). To determine the potential targets of Rsm in the undA of \textit{Pf}.SBW25, we compared all
the already known or published target genes of small RNA repressor proteins by using the conserved motif 5′-\(\text{A}/\text{U}\) CANGANG\(\text{U}/\text{A}\)-3′ (N is any nucleotide) (Lapouge et al., 2008; Song et al., 2015). However, there was no so called GacA Box upstream of the \textit{unda} in \textit{Pf.} SBW25 (Data not shown). These results suggest that 1-undecene is not regulated by the Gac-system via small RNAs but presumably via modulation of primary metabolism (Sonnleitner and Haas, 2011). The production of VOCs by \textit{Pf.} SBW25 was also affected by the growth medium and growth stage (incubation time), confirming and extending earlier observations made for VOCs produced by other bacterial and fungal genera (Blom et al., 2011; Schmidt et al., 2016).

VOCs differentially produced by \textit{Pf.} SBW25 and the Gac-mutant had no apparent differential effects on growth promotion and ISR in Arabidopsis. This observation suggests that the observed phenotypic effects in Arabidopsis are caused by VOCs not regulated by the Gac-system. Another possible explanation for the lack of differential responses of Arabidopsis to VOCs from \textit{Pf.} SBW25 and the Gac-mutant may be that other VOCs compensate for the growth-promoting or ISR-inducing VOCs regulated by the Gac-system. Interestingly, tobacco plants did respond differentially to VOCs produced by \textit{Pf.} SBW25 and the Gac-mutant. While VOCs produced by \textit{Pf.} SBW25 repressed root growth of tobacco seedlings, VOCs from the Gac-mutant promoted tobacco growth relative to the untreated control. This observation suggests that some of the Gac-regulated VOCs could be toxic to the growth of tobacco seedlings growth and that the Gac-mutation might enhance the production of other VOCs that trigger an increase in plant biomass. VOCs-mediated effects on plant growth are dose-dependent and can range from deleterious to beneficial effects (Blom et al., 2011; Schmidt et al., 2016). For example, indole has been shown to promote plant growth at low concentrations but to kill plants at high concentrations (Blom et al., 2011). The same was observed for sulfur-containing compounds such as dimethyl disulfide (Kai et al., 2010; Meldau et al., 2013). Therefore, the growth repression of tobacco seedlings by wildtype \textit{Pf.} SBW25 can be due to Gac-regulated VOCs and/or due to specific VOCs that are emitted by \textit{Pf.} SBW25 at higher concentrations than by the Gac-mutant. The Gac-regulated VOCs that qualify for potential adverse effects on tobacco seedling growth include compounds putatively identified as 3-nonene (RI: 891), n-decene (RI: 992) 1-undecyne (RI: 1080), 4-undecyne (RI: 1085), 1-undecene (RI: 1089) and cis-1,2-cyclohexanedimethanol (RI: 1280). The absence of these plant growth-
The Gac regulation of volatile production by SBW25 inhibitory VOCs in the Gac-mutant may explain in part the growth promotion observed for the Gac-mutant as compared to the Pf.SBW25 treated plants. Plant bioassays with these identified VOCs, applied alone in a dose-dependent manner and in combination, should be performed to pinpoint those VOCs with toxic and growth-promoting effects on tobacco seedlings.

The observed plant-species-specific responses to the bacterial VOCs may also be due to differences in VOCs-receptors or down-stream signal transduction pathways between the two plant species tested. To our knowledge, ethylene receptors are the only type of putative VOCs receptors reported so far in VOCs perception (Ryu et al., 2003; Bailly and Weisskopf, 2012). Loss of the positive regulator of the ethylene pathway EIN2 led to different VOCs-mediated growth responses in Arabidopsis by Bacillus strains IN937a and GB03 (Ryu et al., 2003). Bacillus VOCs failed to promote growth of ein2 Arabidopsis mutants suggesting that the ethylene pathway is involved in the response to bacterial VOCs (reviewed by (Bailly and Weisskopf, 2012)). Whether similar or other genotypic differences affect the differential responses of the two plant species to VOCs produced by Pf.SBW25 will be subject of future analyses.

Next to plant growth promotion, rhizosphere acidification was also observed for Arabidopsis but not for tobacco seedlings. Farag and colleagues (2013) showed that Bacillus strain GB03 is able to induce rhizosphere acidification via VOCs, and the possible mechanisms proposed include elevating proton exudation from roots and direct acidification of the environment by the bacterial VOCs themselves (Farag et al., 2013). VOCs-triggered acidification was also reported to be associated with the enhancement of iron assimilation and photosynthetic efficiency (Farag et al., 2013). Rhizosphere acidification is an efficient way to enhance iron uptake and hence, could explain the enhanced greening of leaves of the VOCs-treated Arabidopsis seedlings observed in our study.

In conclusion, our study demonstrated the involvement of the Gac-system in the regulation of VOCs production in Pf.SBW25, which in turn affects plant growth promotion in a plant species-dependent manner. The Gac-system significantly affects alkene production in Pf.SBW25 independent of the cultivation medium and growth stage. Future studies will focus on the identification of the plant growth promoting VOCs of Pf.SBW25 as well as the mechanisms involved in the differential responses observed for Arabidopsis and tobacco.
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Acknowledgements

This project was financially supported by the Netherlands Genomics Initiative (NGI-EcoLinc project). We would like to acknowledge Hans Zweers for running the GC-MS.
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Chapter 5

Imaging the chemistry of *Pseudomonas*-Plant Interactions

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Chapter 5

Abstract

The bacterial genus *Pseudomonas* harbours multiple species and strains that promote plant growth and alter plant root architecture. Substantial advances have been made in recent years to identify the underlying molecular mechanisms of plant growth promotion, but the chemistry of these interactions is still largely unknown. Here, we studied the effects of *Pseudomonas fluorescens* strain SBW25 (*Pf.*SBW25) on the growth of Arabidopsis, tobacco and cucumber, and also determined the importance of the GacS/GacA two-component regulatory system in growth promotion of these three plant species. The results showed that *Pf.*SBW25 affects root architecture of Arabidopsis and tobacco, significantly increased biomass of Arabidopsis and cucumber, but reduced root biomass of tobacco. Effects of the gacS mutant on Arabidopsis and cucumber growth were similar to that of *Pf.*SBW25. The gacS mutant affected root architecture and shoot biomass of tobacco to the same extent as *Pf.*SBW25, but did not reduce tobacco root biomass. Subsequently, MALDI-Dried Droplet (DD) and MALDI-Imaging Mass Spectrometry (IMS) revealed a variety of compounds produced in *Pseudomonas*-Arabidopsis interactions, which were putatively identified as dodecane, acetoxypinoresinol, pentose and viscosin.
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**Introduction**

Plant growth-promoting rhizobacteria (PGPR) can directly or indirectly affect plant growth and health. Direct PGPR effects include enhanced nutrient acquisition, the production of plant growth hormones, and protection against pathogen infection (Bloemberg & Lugtenberg, 2001). Indirect effects include the induction of systemic disease resistance (Van Wees *et al.*, 2008) and the transformation of toxic compounds in soil like heavy metals (Wu *et al.*, 2006). Despite the advances in recent years to elucidate the underlying mechanisms of beneficial microbe-plant interactions, the chemistry of these interactions and their spatial-temporal patterns are largely unknown. Limited investigations into the chemistry in beneficial microbe-plant interactions are caused, in part, by a lack of available tools to connect the specific phenotypes with the underlying chemistry (Watrous & Dorrestein, 2011). The metabolites produced by PGPR strains in pure culture can be different from those produced during interactions with plants. Imaging mass spectrometry (IMS) approaches now enable to connect observations at the phenotypic level with specific changes at the chemical level *in situ* (Watrous & Dorrestein, 2011). Recently, by using matrix-assisted laser desorption/ionization imaging mass spectrometry (MALDI-IMS), an array of metabolites in the *Medicago-Sinorhizobium* interaction was detected and visualized. Also the spatial distribution of metabolites at the interface between roots and nodules could be investigated allowing a better insight in the chemistry of plant nodulation and nitrogen fixation by Rhizobia (Ye *et al.*, 2013, Gemperline & Li, 2014). For Bacilli, MALDI-IMS provided insight in the production and spatial distribution of lipopeptide surfactants (surfactins, iturins and fengycins) produced in biofilms on plant roots (Debois *et al.*, 2014).

The bacterial genus *Pseudomonas* harbours multiple species and strains that promote plant growth and alter plant root architecture. They are known to produce a range of secondary metabolites including plant hormones (Lambrecht *et al.*, 2000, Cartieaux *et al.*, 2003). For example, black pepper stem cuttings treated with IAA-producing *P. putida* strains showed an increase in shoot height and weight, root length and the number of roots (Lambrecht *et al.*, 2000). *P. fluorescens* strains WCS417 and WCS374, and *P. putida* strain WCS358 promote plant growth and drive developmental plasticity of *Arabidopsis* roots by inhibiting primary root elongation and promoting lateral root and root hair formation via auxin signaling (Zamioudis
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& Pieterse, 2012). Also P. fluorescens SBW25 (Pf.SBW25), the strain investigated in more detail in this study, promotes growth of pea plants and changes plant root architecture but the underlying mechanisms are yet unknown (Naseby et al., 2001).

In Pseudomonas, the GacS/GacA two-component system is highly conserved and regulates the biosynthesis of metabolites that play a role in interactions with other microbes and with plants (Haas & Defago, 2005, Gross & Loper, 2009). Inactivation of the Gac-system in Pf.SBW25 enhanced its antimicrobial activity, which was attributed to gluconic-acid-mediated acidification of its surrounding environment to pH levels that were detrimental to the growth of competing microbes (Cheng et al., 2013, Cheng et al., 2015). If and how Gac-mediated changes in secondary metabolite production impact plant growth promotion by Pf.SBW25 is yet unknown. Here, we investigated and compared the effects of Pf.SBW25 and the gacS mutant on growth of Arabidopsis, tobacco and cucumber. We subsequently used MALDI-DD and MALDI-IMS analyses to detect and visualize metabolites produced by Pf.SBW25 and the gacS mutant on Arabidopsis roots.

Materials and Methods

Bacterial strains and culture conditions

Pseudomonas fluorescens SBW25 (Pf.SBW25) and its gacS mutant (de Bruijn et al., 2007) were cultured in liquid King’s B medium (KB) at 25°C for 24 h. Bacterial cells were collected by centrifugation, washed three times with 10 mM MgSO₄ and resuspended in 10 mM MgSO₄ to a final density of 10⁹ CFU ml⁻¹ (OD₆₀₀ = 1.0).

Plant Material and Growth Conditions

Seeds of Arabidopsis thaliana Columbia-0 (Arabidopsis) were surface sterilized for 3 h by placing seeds in opened Eppendorf tubes in a desiccator jar. Two 100-mL beakers each containing 50 mL commercial bleach were placed inside and 1.5 mL 37% HCl was added to each beaker. The desiccator jar was closed and the seeds were sterilized by chlorine gas. After 4 h, seeds were transferred on water-saturated filter paper in petri dishes followed by a 3-day treatment at 4°C. Thereafter, 10-12 seeds were sown on plates containing 50 ml half-strength Murashige Skoog (MS) medium (Murashige & Skoog, 1962). One-week-old Arabidopsis seedlings were root tip inoculated with 2 µl bacterial cell suspensions (10⁹ CFU ml⁻¹); in the control
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Treatment, seedlings were inoculated with 2 µl of 10 mM MgSO₄. For soil assays, 10-day-old seedlings were transferred to 60-mL PVC pots containing a sand-potting soil mixture that was autoclaved twice for 20 min with a 24-h interval. Once a week, plants were supplied with modified half-strength Hoagland nutrient solution (Hoagland and Arnon, 1938). In the *in vitro* assays on half-strength MS medium, bacterial strains were applied to the root tips. Seeds were sterilized and sown on plates as described above. Germination and plant cultivation were performed in a climate chamber (21°C/21°C day/night temperatures; 250 µmol light m⁻² s⁻¹ at plant level during 12 h/d; 70% relative humidity). Plant phenotypes were captured and biomass was quantified. Statistically significant differences were determined by analysis of variance (P<0.05). The tobacco (*Nicotiana benthamiana*) assay was performed according to the same method as the Arabidopsis assay. For cucumber (cv. Chinese Slangen), seeds were soaked in 0.03N HCl on a rotator for 6 h, followed by soaking in 2% bleach for 5 min and then multiple washes with sterilized MQ water. This process was repeated twice. Then sterilized seeds were sown in a rockwool plug (1 seed/plug) and inoculated with bacterial suspension (5ml of 10⁶ CFU ml⁻¹). Germination and plant cultivation were performed in a climate chamber (21°C/21°C day/night temperatures; 250 µmol light m⁻² s⁻¹ at plant level during 12 h/d; 70% relative humidity). Plant leaf area and shoot biomass were quantified. Data were statistically tested by analysis of variance (P<0.05).

**Mass spectrometry analysis**

*Preparation of samples for MALDI-Dried Droplet*

*Pf.*SBW25 and its gacS mutant were grown in 5 ml LB broth overnight at 25°C. Five µl of the washed bacterial suspension (OD₆₀₀nm = 1.0) was spotted on a 0.5x MS agar plates without plants or inoculated on Arabidopsis root tips as described above. Bacterial plates and Arabidopsis seedlings inoculated with bacterial strains were incubated in a climate chamber as described above. After 11 days of incubation, a plug of 2-mm diameter of the bacterial colony or bacterial inoculation spot on the plant root was placed into 100 µl 75% methanol and the sample was vortexed and centrifuged briefly. The supernatant was collected and 0.7 µl of supernatant was mixed with 0.7 µl of saturated Universal MALDI matrix mixture (1:1) of 2,5-dihydroxybenzoic acid and α-cyano-4-hydroxy-cinnamic acid (Sigma-Aldrich). One µl of this mixture was deposited onto the MALDI target plate and
allowed to dry. The sample plates were subjected to MALDI-TOF mass spectrometry (Microflex, Bruker Daltonics, Billerica, MA, USA) for MS acquisition, and were run in positive reflector mode with a mass range of 0-4 kDa. The data was analysed using FlexAnalysis 3.3 (Bruker Daltonics) and ClinProTool 3.0 software (Bruker Daltonics).

**Preparation of Pseudomonas-Arabidopsis samples for MALDI-IMS**

Bacterial cultures preparation and Arabidopsis seed sterilization were done as described above. The 0.5x MS agar plates for this experiment were 1-1.5 mm thick which is equivalent to approximately 10 ml in petridish with 9-cm diameter. Sterilized Arabidopsis seeds were sown on these thin agar plates. Bacterial inoculation, plant germination and co-cultivation with bacterial strains were performed in a climate chamber (21°C/21°C day/night temperatures; 250 μmol light m⁻² s⁻¹ at plant level during 12 h/d; 70% relative humidity). After 11 days of co-cultivation, plant phenotypes were captured and regions of agar containing bacterial cells, plant roots and surrounding area were cut and placed on top of a MALDI-MSP-96 anchor plate (Bruker Daltonics, Billerica, MA, USA). A photograph was taken and a layer of Universal MALDI matrix (1:1 mixture of 2,5-dihydroxybenzoic acid and α-cyano-4-hydroxy-cinnamic acid (Sigma-Aldrich)) was applied to the sample using a 53 μm sieve. Samples were dried at 37°C for a minimum of 6 h until they were completely dry and adhered to the MALDI plate. The sample plates were subjected to MALDI-TOF mass spectrometry (Autoflex from Bruker Daltonics, Billerica, MA, USA) for IMS acquisition, and were run in positive linear mode, with 150 μm laser intervals in XY-direction and a mass range of 0-4 kDa. The data was analysed using FlexImaging 3.0 software (Bruker Daltonics) and the SCiLS lab 2014b software (SCiLS, Bremen, Germany).

**Data analysis with SCiLS Lab software version 2014b**

The software SCiLS Lab version 2014b (SCiLS, Bremen, Germany) was used for data analysis to detect ions which are overrepresented under a specific condition e.g. to determine which m/z-values have higher intensities in the bacteria-treated samples compared to the nontreated plant control. Receiver operating characteristics (ROC) were performed to detect these differences. ROC is an univariate measure to judge the discrimination quality of all m/z-values from two datasets and it is calculated based on the statistical specificity and sensitivity when the intensity of a single m/z-value represents the discrimination rule. The area
Imaging the chemistry of *Pseudomonas*-Plant Interactions under curve (AUC) measures the discrimination quality in the interval between 0.5 and 1.0. A perfect discrimination would yield an AUC equal to 1.0. The closer the AUC to 0.5, the less reliable is the $m/z$-value to discriminate the two groups, and the closer it is to 1.0, the more suitable it is to be used as a univariate criterion (Song et al., 2015).

Raw data from media control, plant control, *Pf.*SBW25 and its *gacS* mutant were first imported all together into the software SCiLS Lab 2014b. In total, the complete data set comprised of 18,492 spectra with 15,000 datapoints per spectrum in the mass range of 0-4 kDa. The data was processed using the Preprocessing Pipeline of SCiLS Lab 2014b using the default setting. This includes baseline reduction using iterative convolution with 20 interactions and sigma set to 20 and normalization to the total ion count (TIC). In order to compare the different conditions with each other, the imported data sets were grouped if necessary. The ROC tool of SCiLS Lab 2014b was run for the various combinations using all spectra from the data sets. We considered $m/z$-values with an AUC of at least 0.75. Individual $m/z$ images were created from the selected ions with a hotspot removal applied for better visualization. In order to compare the intensity of ions of interest in different samples, single $m/z$ values were also displayed in an intensity box plot. The low and high quantiles for the hotspot removal and the intensity box plot were set to 0.00% and 99.00%, correspondingly.

**Results and discussion**

**Effects of *Pf.*SBW25 on growth of Arabidopsis, tobacco and cucumber**

*Pf.*SBW25-treated Arabidopsis seedlings showed altered root growth, characterized by a reduction of primary root growth and an increased number of lateral roots (Fig. 1A). After 18 days of plant growth, *Pf.*SBW25-treated seedlings had a 1.9-fold and 4.9-fold increase in shoot and root biomass, respectively (Fig. 1A). Also in soil, similar effects of *Pf.*SBW25 on Arabidopsis growth were observed (Fig. 1B). *Pf.*SBW25-treated tobacco seedlings showed altered root growth with a reduction of primary root growth and an increased number of lateral roots (Fig. 1C). In contrast to Arabidopsis, however, fresh weights of both shoot and roots of tobacco seedlings were significantly reduced by *Pf.*SBW25 as compared to the non-treated control seedlings (Fig. 1C). For cucumber, introduction of cell suspensions of *Pf.*SBW25 into rockwool resulted in significant increases in shoot biomass and
Figure 1 Effects of *Pf*.SBW25 and its *gacS* mutant on growth of multiple plant species. In *in vitro* conditions, *Pf*.SBW25 and its *gacS* mutant enhance plant growth, inhibit primary root growth and induce lateral root formation in *Arabidopsis thaliana* accession Columbia grown on 0.5x MS medium for 18 days (A, top). Dry weight of shoot and root was quantified (A, bottom). In soil, *Pf*.SBW25 and its *gacS* mutant also promote plant growth (B, top) and leaf biomass was quantified (B, bottom). Effects of *Pf*.SBW25 and its *gacS* mutant on tobacco (*Nicotiana benthamiana*) seedling growth (C, top); fresh weights of both shoot and root were quantified (C, bottom); In rockwool, effects of *Pf*.SBW25 and its *gacS* mutant on cucumber growth (D, top); leaf area and leaf fresh weight were quantified (D, bottom). Bars represent the mean of 4 replicates (± standard error) with 10-15 plants per replicate. Blue bars represent control plants; Red bars represent the *Pf*.SBW25 treatment and light green bars represent the *gacS* mutant treatment. Bars with asterisk are significantly different as determined by One-way ANOVA analysis (*P*<0.05).
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leaf area with 1.5-fold and 1.6-fold after 20 days of incubation, respectively (Fig. 1D). Collectively, these results showed that *Pf.SBW25* significantly affected plant growth in a plant species-dependent manner (Fig. 1).

For *Arabidopsis*, the *gacS* mutant altered root architecture and enhanced plant biomass to the same extent as the wildtype *Pf.SBW25* (Fig. 1A, B). Also for cucumber, similar effects were observed for the *gacS* mutant and the wildtype (Fig. 1D). For tobacco, however, seedlings treated with the *gacS* mutant showed a significant increase in shoot fresh weight with 1.4-fold and 2.8-fold compared to the control seedlings and thus also to the *Pf.SBW25*-treated seedlings (Fig. 1C).

MALDI-DD analysis of metabolites produced by *Pf.SBW25* and the *gacS* mutant

*Pf.SBW25* produces a range of secondary metabolites and enzymes, including siderophores, lipopeptides, proteases and organic acids, several of which are under the control of the Gac-system (Cheng *et al.*, 2013, Cheng *et al.*, 2015). To begin to understand the chemistry of the interactions of *Pf.SBW25* and the *gacS* mutant with *Arabidopsis*, bacterial strains grown in interaction with *Arabidopsis* roots were extracted with methanol and analyzed in MALDI-Dried Droplet (DD) assays.

![Figure 2](image)

**Figure 2** Chemical profiles generated by MALDI-DD analysis. Molecules detected on roots of nontreated *Arabidopsis* seedlings and on seedlings treated with *Pf.SBW25* or its *gacS* mutant. The sample plates were subjected to MALDI-TOF mass spectrometry (Microflex from Bruker Daltonics, Billerica, MA, USA) for MS acquisition, and were run in positive reflector mode. The data was analysed using FlexAnalysis 3.3 (Bruker Daltonics) and ClinProTool 3.0 software (Bruker Daltonics). Detected ions with a mass range from 0 to 1.2 kDa were shown. Red arrows point to several differential molecules (peaks), as examples, which were detected in different sample sets.
Chapter 5

Bacterial strains grown on 0.5xMS medium without Arabidopsis were used as the reference. Several ions with a mass-to-charge ratio (m/z) between 0-1200 were detected. For the bacterial strains grown on 0.5xMS, 8 ions with m/z values of 471, 477, 492, 549, 593, 607, 638 and 652 were detected for Pf.SBW25 only, whereas an ion with m/z value 909 was only detected for the gacS mutant (Table 1).

When Pf.SBW25 or the gacS mutant were co-cultivated with Arabidopsis, the compounds detected were different from the ones detected for the bacteria grown in absence of Arabidopsis (Fig. 2, Table 1). In the Pf.SBW25-Arabidopsis interaction, ions with m/z 37, 63, 77, 93, 161, 372, 402, 447, 451, 677, 715 and 891 were detected. For the gacS mutant, m/z 161 was absent and ions with m/z 89, 118, 128, 200, 204, 208, 218, 421, 469, 487 and 699 were detected additionally (Table 1). Together, these results indicate that the the metabolite profile of Pf.SBW25 changes in interaction with Arabidopsis roots, and that the gacS mutation influences the production of metabolites produced in interaction with Arabidopsis.

MALDI-IMS analysis of the chemistry of Pseudomonas-Arabidopsis interactions

Eleven days after bacterial inoculation, plant phenotypes were captured and samples were prepared for MALDI-IMS analysis to visualize metabolites produced in interactions between the bacterial strains and Arabidopsis roots (Fig. 3A). The MALDI-IMS data was further analyzed with SCiLS Lab 2014b. On roots of Arabidopsis grown on MS agar medium without bacteria, 70 ions were detected at higher levels (AUC > 0.75) than in the bacteria-treated seedlings (Table S1). There were 35 ions predominant in the Pf.SBW25-treated Arabidopsis seedlings as compared to the gacS mutant-treated and the control seedlings (Table 2, Fig. S1A). Box plots further confirmed that the intensities of several of these ions were higher in the Pf.SBW25-treated Arabidopsis seedlings than in the control and Arabidopsis seedlings treated with the gacS mutant (Fig. 4B).

A total of 38 ions were predominant in the gacS-treated Arabidopsis seedlings as compared to the Pf.SBW25 and control treatments (Table 2, Fig. S1B). Eleven compounds with the most obvious visual discrimination in this pool compared to Pf.SBW25-treated seedlings were highlighted and further analyzed (Fig. 3B). These compounds with m/z values 189, 190, 202, 229, 246, 249, 277, 317, 338, 414 and 418 were detected in the treatment with the gacS mutant, but were not observed on the roots but in the surrounding area of the roots (Fig. 3B, Fig. 4C). Box plots
Imaging the chemistry of *Pseudomonas*-Plant Interactions

**Table 1** Highlighted ions detected by MALDI-DD for Pf.SBW25 and its *gacS* mutant grown on 0.5x MS medium and on Arabidopsis roots.

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<th>Grown on Arabidopsis roots (m/z)</th>
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also confirmed these results (Fig. 4D). The results suggested that mutation of the Gac-system in *Pf*.SBW25 abolishes production of certain compounds (Fig. 3A) but also leads to producing additional compounds (Fig. 3B) during the interaction with Arabidopsis seedlings.

Based on information available in databases and comparisons of the *m/z* values with those of the compounds detected by Roessner and co-workers (Roessner *et al*., 2000), several putative bioactive compounds were assigned to the ions detected. These include viscosin (*m/z* 1164), gluconic acid (*m/z* 334) and pyoverdine/ornicorrugatin (*m/z* 1176) (Fig. 3, Table 2). Viscosin is a lipopeptide known to be produced by *Pf*.SBW25 and known to be under the regulation of the Gac-system (De Bruijn *et al*., 2009). MALDI-IMS confirmed that (the ion putatively identified as) viscosin was indeed only detected in the *Pf*.SBW25-treated Arabidopsis seedlings (Fig. 3A, Table 2). In previous studies, we showed that a mutation of the Gac-system enhances gluconic acid and siderophore production by *Pf*.SBW25 (Cheng
et al., 2013, Cheng et al., 2015). MALDI-IMS analysis (putatively) confirmed that gluconic acid and pyoverdine are detected at a higher intensity on roots of gacS-treated Arabidopsis seedlings than on Pf.SBW25-treated and control seedlings (Fig. 3C, Table 2). A few more compounds of interest found in Pf.SBW25-Arabidopsis interactions are highlighted, such as those with \( m/z \) values 170, 264, 415 and 447. Based on database searching, these compounds are putatively dodecane (\( m/z \) 170), acetoxypinoresinol (\( m/z \) 415) and pentose (\( m/z \) 447). These compounds were not detected in the gacS mutant-Arabidopsis interaction (Fig. 3A).
Imaging the chemistry of *Pseudomonas*-Plant Interactions

Table 2 Comparison of ions detected in *Pf* SBW25 or the gacS mutant-Arabidopsis interactions. Ions detected by using MALDI-IMS and analyzed by SCiLS Lab 2014b with maximal AUC>0.93 are shown. a represents ions highly abundant in *Pf* SBW25-Arabidopsis interaction; b represents ions highly abundant in the gacS mutant-Arabidopsis interaction. The putative chemical annotations were only based on online databases and comparison of m/z values with those of compounds detected in Roessner et al., 2000.

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Figure 4 MALDI-IMS shows 4 ions (m/z of 170, 264, 334 and 1164) detected on roots treated with PfSBW25 (A) and 5 highlighted ions detected on root treated with the gacS mutant (C). The box plots represent the median intensity in arbitrary units after TIC normalization (horizontal line) (B, D), the upper and lower quartiles (box layout, spectra in which the intensities are within
a range of 25% - 75% of the data), the upper and lower quantiles (dashed lines, spectra in which the intensities are within a range of 0 - 99%) as well as the outliers (spectra with intensities greater than 99% of the data).
Chapter 5

Conclusions

The results showed that Pf.SBW25 affects root architecture of Arabidopsis and tobacco, and also significantly increased biomass of Arabidopsis and cucumber. In addition, it was observed that the gacS mutant was able to increase the growth of tobacco, whereas there was no effect of the Pf.SBW25 wild-type. In previous studies, we showed that mutations in the Gac-system caused major transcriptional changes in Pf.SBW25 and abolished the production of the lipopeptide viscosin and of an exoprotease, but also altered the expression and production of several other extracellular metabolites (Hassan et al., 2010, Cheng et al., 2013). By using MALDI-IMS, we putatively identified several of these compounds, including gluconic acid, pyoverdine/ornicorrugatin and viscosin, in the Pf.SBW25-Arabidopsis interactions. We also showed that the ion putatively identified as viscosin is located primarily at the site where Pf.SBW25 was inoculated. The results further suggested that other metabolites are produced in the interactions between Pf.SBW25 and roots of Arabidopsis. These metabolites tentatively are dodecane, acetoxypinoresinol and pentose. Structure elucidation will be needed to confirm these results and additional experiments will be needed to investigate the potential effects of these metabolites on plant growth and root architecture.

Acknowledgements

This project was financially supported by the Netherlands Genomics Initiative (NGI-EcoLinc project). We would like to acknowledge Margo Wisselink and Yingjie Jiang for their contributions of doing tobacco assay and cucumber assay, respectively.
Imaging the chemistry of *Pseudomonas*-Plant Interactions

References


Chapter 5


Supplementary figures and tables

Figure S1 Chemical profiles of the *Pf.SBW25* or the *gacS* mutant-Arabidopsis interactions detected by MALDI-IMS. Data were analyzed by using SCiLS Lab 2014b. Spatial distribution of detected ions were visualized and compared. (A) shows the selected 35 ions (AUC>0.75), which are more abundant in the *Pf.SBW25*-Arabidopsis interaction. (B) shows selected 38 ions (AUC>0.75) that are more abundant in the *gacS* mutant-Arabidopsis interaction as compared to the *Pf.SBW25*-Arabidopsis interaction.
Table S1 MALDI-IMS reveals 70 ions that are highly abundant on roots of Arabidopsis compared to the 0.5x MS medium alone. Detected ions with a mass range from 0 to 2 kDa are shown. These detected ions were further analyzed by SCiLS lab 2014b, AUC>0.75.

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Chapter 6

Bacterial determinants of plant growth promotion and induced systemic resistance by *Pseudomonas fluorescens*

Xu Cheng, Judith E. van de Mortel, Desalegn W. Etalo, Ester Dekkers and Jos M. Raaijmakers

This chapter was submitted for publication.
Chapter 6

Abstract

*Pseudomonas fluorescens* strain SS101 (*Pf.*SS101) promotes growth of *Arabidopsis thaliana*, enhances greening and lateral root formation, and induces systemic resistance (ISR) against the bacterial pathogen *P. syringae* pv. *tomato* (*Pst*). Here, targeted and untargeted approaches were adopted to identify bacterial determinants and underlying mechanisms involved in growth promotion and ISR by *Pf.*SS101. No evidence was found for ACC deaminase, volatiles, lipopeptides and siderophores in plant growth promotion by *Pf.*SS101. Subsequent high-throughput analyses of 7,488 random transposon mutants of *Pf.*SS101 led to the identification of 21 mutants defective in both plant growth promotion and ISR. Most of these 21 mutants, had mutations in amino acid metabolism genes, were auxotrophic and impaired in root colonization. However, two mutants were not auxotrophic and one mutant was not affected in root colonization. These three mutants had transposon insertions in the phosphonate dehydratase gene *edd*, the response regulator gene *colR* and the adenylsulfate reductase gene *cysH*, respectively. Site-directed mutagenesis and genetic complementation confirmed the role of these three genes in growth promotion and ISR in *Pf.*SS101-Arabidopsis interactions. Subsequent bioassays and comparative plant transcriptomics analyses indicated that modulation of auxin biosynthesis and transport, steroid biosynthesis, carbohydrate metabolism and sulfur assimilation in Arabidopsis are key mechanisms associated with growth promotion and ISR by *Pf.*SS101.
Introduction

*Pseudomonas* represents one of the most abundant bacterial genera in the plant rhizosphere (Pieterse et al. 2002; Loper and Gross, 2007; Raaijmakers et al., 2009, 2010; Mendes et al., 2011; Raaijmakers and Mazzola, 2012; Zamioudis et al., 2013; Mendes et al., 2013; Philippot et al., 2013; Chowdhury et al., 2015). Certain *Pseudomonas* strains promote plant growth via nutrient and iron acquisition or by protection against pathogen infection via competition, antibiosis or induction of systemic resistance (ISR) (van Loon et al., 1998; Ryu et al., 2003; Haas and Défago 2005; Berendsen et al., 2012; Zamioudis et al., 2013; Pieterse et al., 2014, 2016; Chowdhury et al., 2015). To date, several bacterial traits have been identified for their role in plant growth promotion and ISR by *Pseudomonas*: (1) production of 1-aminocyclopropane-1-carboxylate (ACC) deaminase, an enzyme that reduces ethylene levels in the root thereby increasing root length and growth (Li et al., 2000; Penrose and Glick, 2001); (2) production of hormones like indole acetic acid (IAA) (Patten and Glick, 2002), abscisic acid (ABA) (Dangar and Basu, 1987; Dobbelaere et al., 2003), gibberellic acid (GA) and cytokinins (Dey et al., 2004); (3) solubilization and mineralization of nutrients, particularly mineral phosphates (de Freitas et al., 1997; Richardson, 2001); (4) production of vitamins including niacin, pantothenic acid, thiamine, riboflavine and biotin (Martinez-Toledo et al., 1996; Sierra et al., 1999; Revillas et al., 2000); (5) cell-surface components including flagella and lipopolysaccharides (Peter et al., 2007); (6) secondary metabolites including lipopeptides (Peter et al., 2007; Audenaert et al., 2002; Tran et al. 2007), 2,4-diacetylphloroglucinol (Iavivoli et al., 2003; Weller et al. 2012), siderophores (Bakker et al., 2007; Pieterse et al., 2014) and salicylic acid (Maurhofer et al., 1994, 1998; Audenaert et al., 2002; De Vleesschauwer et al., 2014), and (7) volatile organic compounds (Blom et al., 2011; Park et al., 2015).

In this study, we identified several bacterial genes involved in plant growth promotion and ISR by the rhizobacterial strain *P. fluorescens* SS101 (*Pf*.SS101). *Pf*.SS101 was originally isolated from the wheat rhizosphere (de Souza et al., 2003; de Bruijn et al., 2008) and has biocontrol activities either directly or via ISR against *Pythium* root rot of flower bulb crops (de Souza et al., 2003), tomato late blight caused by *Phytophthora infestans* (Tran et al., 2007), the bacterial pathogen *P. syringae* pv. *tomato* (*Pst*) and the insect *Spodoptera exigua* on Arabidopsis (Van...
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de Mortel et al. 2012). To investigate the underlying mechanisms and bacterial traits involved in plant growth promotion, induction of lateral root formation and ISR, several known mechanisms such as ACC deaminase activity, volatile and lipopeptide production were studied first. We further investigated the role of the GacS/GacA two-component regulatory system of Pf.SS101 in plant growth promotion and ISR. To identify other, potentially novel bacterial traits, a total of 7,488 random transposon mutants of Pf.SS101 were screened for loss or reduced ability to induce lateral root formation and/or ISR in Arabidopsis. Results of high-throughput assays showed that 21 out of the 7,488 mutants did not induce lateral root formation nor were able to induce resistance in Arabidopsis against P. syringae pv. tomato (Pst). These Pf.SS101 mutants harbored a transposon insertion in genes involved in amino acid biosynthesis, glucose utilization, transcription, or sulfur assimilation. Site-directed mutagenesis, genetic complementation, phenotypic and plant transcriptional analyses were performed to assess the functions of these genes in the Pf.SS101-Arabidopsis interaction in order to unravel the underlying mechanisms of growth promotion and ISR.

Material and Methods

Bacterial strains and culture conditions

Pseudomonas fluorescens SS101 (Pf.SS101) was cultured in liquid King's B medium (KB) at 25 °C for 24 h. Bacterial cells were collected by centrifugation, washed three times with 10 mM MgSO₄ and resuspended in 10 mM MgSO₄ to a final density of 10⁹ CFU ml⁻¹ (OD₆₀₀ = 1.0). Pseudomonas syringae pv. tomato DC3000 (Pst) was cultured in KB broth supplemented with rifampicin (50 µg ml⁻¹) at 25 °C for 24 h. Escherichia coli strain DH5α was used as a host for the plasmids for site-directed mutagenesis and complementation. E. coli strains were grown on Luria-Bertani (LB) plates or in LB broth amended with the appropriate antibiotics. The random plasposon mutants of Pf.SS101 were obtained by biparental mating with E. coli strain S17 λ pir harboring the TnModOKm element in plasmid (Dennis and Zylstra, 1998), according to protocols described by Sambrook and Russel (Sambrook et al., 2001). Transformants were selected on KB supplemented with rifampin (100 µg ml⁻¹) and kanamycin (100 µg ml⁻¹).

Plant Material and Growth Conditions
Bacterial determinants plant growth by *Pf.*SS101

Seeds of *Arabidopsis thaliana* Columbia-0 (Arabidopsis) were surface sterilized for three hours by placing seeds in opened Eppendorf tubes in a desiccator jar. Two 100-mL beakers each containing 50 mL commercial bleach was placed inside and 1.5 mL concentrated HCl was added to each beaker. The desiccator jar was closed and the seeds were sterilized by chlorine gas. After 4 h, seeds were transferred on water-saturated filter paper in petri dishes followed by a 3-day treatment at 4 °C. Thereafter, 10-12 seeds were sown on plates containing 50 ml half-strength Murashige Skoog (MS) medium (Murashige and Skoog, 1962). One-week-old *Arabidopsis* seedlings were inoculated at the root tip with 2 µl *Pf.*SS101 cell suspensions (10⁹ CFU ml⁻¹); in the control treatment, seedlings were inoculated with 2 µl of 10 mM MgSO₄. After an additional three days of plant growth, the 10-day-old seedlings were transferred to 60-mL PVC pots containing a sand-potting soil mixture that was autoclaved twice for 20 min with a 24-h interval. Once a week, plants were supplied with modified half-strength Hoagland nutrient solution (Hoagland and Arnon, 1938).

In the *in vitro* assays with half-strength MS medium, *Pf.*SS101 was applied to the seeds or to the root tips. For both treatments, seeds were sterilized and sown on plates as described above. For the *Pf.*SS101 seed treatment, a cell suspension (10⁹ CFU ml⁻¹) of *Pf.*SS101 was added to the sterilized seeds in a Petri dish and incubated for 30 minutes at room temperature. For the control, seeds were incubated for 30 min with sterile 10 mM MgSO₄. For the root tip treatment, 2 µl *Pf.*SS101 (10⁹ CFU ml⁻¹) was applied to the root tips of one-week-old seedlings. Control plants were inoculated with 2 µl of 10 mM MgSO₄. The challenge with *Pst* was performed by inoculation of 2 µl cell suspension (10⁹ CFU ml⁻¹) in the centre of the leaf rosette of 14-day-old plants. Five to seven days after challenge inoculation, disease incidence was assessed by determination of the percentage of diseased leaves per plant. Leaves were scored as diseased when they exhibited necrotic or water-soaked lesions surrounded by chlorotic tissue. From the number of diseased and non-diseased leaves, the disease incidence was calculated for each plant (20-30 plants per treatment). The experiment was performed at least twice. Colonization levels of the rifampicin-resistant *Pst* and *Pf.*SS101 bacteria were determined at the end of bioassays with the same method as described by Pieterse et al. (1996). Statistically significant differences were determined by analysis of variance (*P*<0.05).

For the *in vitro* assays with amino acids, the amino acid was added to half-
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strength MS medium at concentrations range from 0 to 2.0 mM. Germination and plant cultivation were performed in a climate chamber (21 °C/21 °C day/night temperatures; 250 µmol light m⁻² s⁻¹ at plant level during 12 h/d; 70% relative humidity).

Microscopic analysis

Seeds of Arabidopsis were pre-treated with PfSS101 and grown for 18 days vertically in plates containing half-strength MS medium. Then longitudinal and cross sections of the roots were made. Parts of the tip and base of the roots were fixed in 2% glutaraldehyde in 0.1 M phosphate buffer at pH7.2 for 2h at room temperature. Tissues were dehydrated in ethanol and propylene oxide and embedded in Spurr’s resin. Sections of 1µm were stained with 0.1% toluidine blue in 1% borax. Sections were viewed and photographed with a Leitz Orthoplan microscope, equipped with a Leica camera DFC 420C. The ND filter 8 and FGP(R)-LP filter set were used in combination with the NIS-Elements imaging software version 2.3.

Auxotrophy

Selected plasposon mutants were grown O/N in 5 ml KB supplemented with the appropriate antibiotics and shaken at 220 rpm at 25 °C. Next day cultures were washed three times with 10 mM MgSO₄ and set to OD600= 1. Then a starting culture was inoculated at a concentration of 0.5% (v/v) in 200 µl KB or the minimal medium SSM in a 96 well plate. Growth was determined in a Bio-Rad 680 micro plate reader at 600 nm with settings at 25 °C, high shaking, 1 min mixing and 2 min measurement over a period of 24 hours.

Site-directed mutagenesis

Site-directed mutagenesis of cysH, cysM and pvdI was performed based on the method described by Choi and Schweizer (2005). The primers used for amplification are described in supporting information Table S1. The FRT-Gm-FRT cassette was amplified with pPS854-GM, a derivative of pPS854, and FRT-F and FRT-R were used as primers (Supporting information Table S1). The first-round PCR was performed with KOD polymerase (Novagen), according to the manufacturer’s protocol. PCR reactions were carried out under the following conditions: an initial denaturation step for 2 min at 95 °C followed by denaturation for 15 s at 95 °C, annealing for 20s at 58 °C and extension for 30 min at 72 °C for 30 cycles, followed by a final
Bacterial determinants plant growth by Pf.SS101

elongation step at 72 °C for 5 min. All fragments were run on a 1% (w/v) agarose gel and purified with illustraTMGFX™PCR DNA and Gel Band Purification Kit (GE Healthcare Life Sciences). The overlap extension PCR was performed with Verbatim High Fidelity DNA polymerase (Thermoscientific) according to the manufacturer’s protocol by addition of equimolar amounts of the 5-end fragment, FRT-Gm-FRT, and 3-end fragment. PCR reactions were carried out under the following conditions: an initial denaturation step for 2 min at 95 °C followed by denaturation for 20s at 98 °C, annealing for 15s at 58 °C and extension for 2 min at 72 °C for 30 cycles, followed by a final elongation step at 72 °C for 5 min and the PCR fragments were purified as described above. The fragments were digested with BamHI and cloned into BamHI-digested plasmid pEX18Tc and transformed colonies were selected on LB medium supplemented with 25 µg ml⁻¹ gentamicin (Sigma). Integration of the inserts was verified by PCR analysis with pEX18Tc primers (Supporting information Table S1) and by restriction analysis of isolated plasmids. The pEX18Tc-cysH and pEX18Tc-cysM constructs were subsequently transformed to Pf.SS101. Competent cells were obtained by washing the cells three times with 300 mM sucrose from a 6-ml overnight culture and finally dissolving the cells in 100 µl of 300 mM sucrose. Electroploration occurred at 2.4kV and 200F and after incubation in SOC medium for 2 h at 25 °C cells were plated on KB supplemented with gentamicin (40 µg ml⁻¹) and rifampicin (50µg ml⁻¹). Six obtained colonies were grown in LB for 2-3 h at 25 °C than diluted 10 times and plated on LB supplemented with gentamicin (40µg ml⁻¹) and 5% sucrose to accomplish the double crossover. The plates were incubated at 25°C for at least 48 h and colonies were re-streaked on LB supplemented with gentamicin and 5% sucrose. Twelve colonies per transformation were transferred to KB plates supplemented with tetracycline (25 µg ml⁻¹) and KB plates with gentamycin and rifampicin. Colonies that grew on LB with gentamicin and rifampicin but not on LB with tetracycline were selected and subjected to colony PCR to confirm the presence of the gentamicin resistance cassette and the absence of the tetracycline resistance cassette. Positive colonies were confirmed by sequencing the PCR fragments obtained with the Up forward and Dn reverse primers (Supporting information Table S1). The mutants obtained were tested for induction of lateral root formation in the in vitro assay with Arabidopsis.

Construction of pME6031-based vectors for genetic complementation

A fragment of approximately 2 kb containing the cysH or cysM gene, including
the promoter and terminator, was obtained by PCR with specific primers (Table S1) and Phusion DNA polymerase (Finnzymes) according to the manufacturers protocols. The PCR fragments were isolated from gel with Illustra™GFX™PCR DNA and Gel Band Purification Kit (GE Healthcare Life Sciences) and digested with HindIII and cloned into the shuttle vector pME6031 (Heeb et al., 2000). E. coli DH5α was transformed with the constructs by heat shock transformation (Inoue et al., 1990) and transformed colonies were selected on LB agar plates supplemented with tetracycline (25 µg ml⁻¹). Correct integration of the fragments was verified by PCR analysis and restriction analysis of the isolated plasmids. The pME6031-cysH, pME6031-cysM constructs were subsequently transformed into the cysH or cysM plasposon mutant. Transformed cells were plated on KB supplemented with tetracycline (25 µg ml⁻¹) and the presence of pME6031-cysH or pME6031-cysM was verified by PCR analysis with primers specific for pME6031. The complemented mutants obtained were tested for their ability to induce lateral root formation in the in vitro assay with Arabidopsis.

Q-PCR analysis

Arabidopsis was grown with Pf.SS101 on half-strength MS medium for 18 days. Cells of Pf.SS101 were collected at day 0, 7, 10, 14 and 18 by collecting Arabidopsis roots in 1 ml 10 mM MgSO₄, vortexing and spinning down. The Pf.SS101 cells were frozen in liquid N₂ and stored at –80°C. For the RNA isolations and cDNA synthesis, four biological replicates were used for each time point. RNA was isolated from the frozen bacterial cells with Trizol reagent (Invitrogen) followed by DNase I (GE Healthcare) treatment. One microgram of RNA was used for cDNA synthesis with Superscript III (Invitrogen) according to the manufacturer’s protocol. For the Q-PCR, conducted with the 7300SDS system from Applied Biosystems, the SensiMix™ SYBR kit (Bioline) with a final concentration of 3.0 mM MgCl₂ was used according to the manufacturer’s protocol. The concentrations of the primers were optimized (400 nM final concentration for all) and a dissociation curve was performed to check the specificity of the primers. The primers used for the Q-PCR are listed in Supporting Information Table S2. To correct for small differences in template concentration, rpoD was used as the housekeeping gene. The cycle where the SYBR green fluorescence crosses, a manually set threshold cycle (Cₚ) was used to determine transcript levels. For each gene the threshold was fixed based on the exponential segment of the PCR curve. The Cₚ value for the gene of interest was corrected for the
Bacterial determinants plant growth by Pf.SS101 

housekeeping gene as follows: \( \Delta C_r = C_{\text{r}(\text{gene})} - C_{\text{r}(\text{rpoD})} \). The relative quantification (RQ) values were calculated by the formula \( \text{RQ} = 2^{-\left[\Delta C_T(\text{day 7, 10, 14 or 18}) - \Delta C_T(\text{day 0})\right]} \). If there was no difference in transcript level between day 7, 10, 14 or 18 and day 0, then \( \text{RQ} = 1 \left(2^0\right) \) and \( \log \text{RQ} = 0 \). Q-PCR analysis was performed on four independent RNA isolations (biological replicates). Statistically significant differences were determined for log-transformed RQ values by analysis of variance (\( P < 0.05 \)) followed by the Bonferroni and Dunnet post hoc multiple comparisons.

**ACC deaminase activity**

Initially a standard concentration curve of \( \alpha \)-ketobutyrate was generated. 200 \( \mu \text{L} \) of 0, 0.1, 0.25, 0.5, 0.75 and 1 \( \mu \text{M} \) \( \alpha \)-ketobutyrate in 0.1 M Tris-HCl pH 8.5 was added in a sterile glass test tube. 300 \( \mu \text{L} \) of 2,4-dinitrophenyl-hydrazine reagent (0.2 \% 2,4-dinitrophenyl-hydrazine in 2 N HCl) was added to the tube, vortexed and incubated at 30°C for 30 minutes. During this process \( \alpha \)-ketobutyrate is derivatized as phenylhydrazone. 2 ml 2N NaOH was added in order to color phenylhydrazone. Solution was mixed and the light absorption at 540 nm was measured.

Bacterial ACC deaminase activity was measured as follow: bacteria were grown O/N in KB liquid medium, shaking at 200 rpm at 25°C. The supernatant is removed and the cells are washed with 5ml Dworkin and Foster (DF) (1958) salts minimal medium. Following an additional centrifugation for 10 min at 8000 g at 4°C, the cells were suspended in 7.5 ml DF salts minimal medium in a fresh culture tube. Just prior to incubation, the frozen 0.5 M ACC solution was thawed, and an aliquot of 45 \( \mu \text{L} \) was added to the cell suspension to obtain a final ACC concentration of 3.0 mM. The bacterial cells were returned to the shaking water bath to induce the activity of ACC deaminase at 200 rpm for 24 h at 25°C. The bacterial cells were harvested by centrifugation at 8000 g for 10 min at 4°C. The supernatant was removed, and the cells were washed with suspending the cell pellet in 5 ml 0.1M Tris-HCl, pH7.6. An additional centrifugation step followed, the supernatant was discarded and the cells were suspended in 1 ml of 0.1M Tris-HCl pH 7.6 and transferred into 1.5 ml microcentrifuge tubes. Tubes were centrifuged at 16000g for 5 minutes; the supernatant was removed and were resuspended in 600 \( \mu \text{L} \) of 0.1M Tris-HCl pH 8.5. 30 \( \mu \text{L} \) of toluene was added and the tubes were vortexed for 30 seconds at the highest setting. 200 \( \mu \text{L} \) of the samples were transferred into new 1.5 ml microcentrifuge tubes, 20 \( \mu \text{L} \) of 0.5M ACC was added in each tube, briefly
vortexed and incubated at 30°C for 15 minutes. 1 ml of 0.56M HCl was added; the tubes were centrifuged at 16000 g for 5 minutes and 1 ml of the supernatant was transferred into glass test tubes. 800 μl of 0.56M HCl was added at each tube and the mixtures were vortexed. 300 μl of the 2,4-dinitrophenylhydrazine was added to each tube, the tubes were vortexed and were incubated at 30°C for 30 minutes. Finally, 2 ml of 2M NaOH was added and the light absorbance at 540 nm was measured. The absorbance of light at 540 nm of the reagents participating at the final solution was measured and subtracted from the measurements in order to estimate the phenylhydrazone absorbance (Penrose and Glick, 2003).

Transcriptome analysis of Arabidopsis treated with Pf.SS101 or the cysH mutant

Total RNA was extracted from roots and shoots of untreated, Pf.SS101-treated and cysH mutant-treated plants after 18 d of growth. Four biological replicates with 30 plants per replicate were used for each treatment. RNA was isolated from the frozen tissues with Trizol reagent (Invitrogen). The RNA samples were further purified using the NucleoSpin RNA II kit (Macherey-Nagel). For the Affymetrix Arabidopsis genome GeneChip array analysis (ServiceXS), amplification and labeling of the RNA samples as well as hybridization, staining, and scanning were performed according to the manufacturer’s specifications. The raw array data (CEL files) were normalized using the RMA probe summarization algorithm in R programme using Bioconductor package; the processed data was used for further analysis. ANOVA without false discovery rate (FDR) correction was performed to identify significantly altered transcripts between Arabidopsis plants treated with Pf.SS101, the cysH mutant (20H12) and non-treated control plants. Using the transcripts that were significantly altered ($P < 0.05$, without FDR correction) between the treatments, discriminant function analysis (DFA) and hierarchical cluster analysis (HCA) were performed in Genemaths XT software (Applied Maths, Inc. Austin, TX, USA). For HCA, Pearson’s correlation coefficients were used to calculate the distance or similarity between two entries and the resulting clusters were summarized using a complete linkage algorithm. To compare the expression values, the raw values of each sample were auto-scaled by the use of the average as an offset and the standard deviation as scale (raw value-average (offset)/SD (scale)). Clusters of genes that showed altered expression patterns between the contrasting treatments in the HCA were selected. Independent t-Test was performed to compare the expression of these genes in Pf.SS101-treated plants.
Bacterial determinants plant growth by *Pf*.SS101

with gene expression in plants treated with either the *cysH* mutant or control plants. Genes that showed significant alteration in their expression in plants treated with *Pf*.SS101 were further investigated by gene set enrichment analysis (GSEA) using the web-based Plant GeneSet Enrichment Analysis toolkit (http://structuralbiology.cau.edu.cn/PlantGSEA/) with the standard settings. The gene X GO matrix was used to perform HCA to reduce the number of redundant biological processes described by a group of genes.

**Results**

*Pf*.SS101 promotes plant growth and changes root architecture

Introduction of *Pf*.SS101 in soil resulted in a significant growth promotion of Arabidopsis seedlings with 1.7- and 2.9-fold increases in leaf and root biomass, respectively (Fig. 1a, Table 1). Comparable but more pronounced effects on shoot and root biomass by *Pf*.SS101 were observed for Arabidopsis seedlings grown under *in vitro* conditions on vertically oriented MS agar plates (Fig. 1a, Table 1). *Pf*.SS101-treated seedlings also showed altered shoot and root development, exemplified by enhanced greening, a 2-fold reduction of primary root growth and a 3-fold increase in the number of lateral roots (Fig. 1b, c). Microscopic analysis showed an increased number of pericycle cells in roots of *Pf*.SS101-treated seedlings (Fig. 1d, e). Furthermore, the roots of *Pf*.SS101-treated plants appeared to switch earlier into secondary growth (Fig. 1i) than control plants (Fig. 1h). Using a *gfp*-tagged derivative of *Pf*.SS101 we found no evidence for endophytic colonization: *Pf*.SS101 was only found on the root surface and not detected inside the root tissue of

**Table 1** Effects of *Pf*.SS101 on growth of Arabidopsis cultivated *in vitro* or in soil. Plant fresh weights were quantified. The asterisk indicates that plant growth is expressed as milligrams of plant fresh weight. The “±” represents the standard error of the mean of, *in vitro* assay, 4 replicates (± standard error) with 15-20 plants per replicate; and means of, soil assay, at least 35 Arabidopsis plants with or without *Pf*.SS101. Letters represent a significant difference of the means at *P*<0.05 according to One-way ANOVA.

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<td>Control</td>
<td>3.95 ± 0.32&lt;sup&gt;d&lt;/sup&gt;</td>
<td>7.3 ± 0.00&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>SS101</td>
<td>14.6 ± 1.07&lt;sup&gt;f&lt;/sup&gt;</td>
<td>41.6 ± 5.70&lt;sup&gt;g&lt;/sup&gt;</td>
</tr>
</tbody>
</table>
Chapter 6

Arabidopsis (Fig. 1f, g). When surface-sterilized Arabidopsis seeds were inoculated with a *Pf.* SS101 cell suspension, *Pf.* SS101 established a population density on the roots of $1 \times 10^6$ CFU mg$^{-1}$ root fresh weight after 18 days of plant growth. In the *in vitro* assays on MS agar plates, root tip inoculation with *Pf.* SS101 resulted in a density of $5.5 \times 10^5$ CFU mg$^{-1}$ root fresh weight after 18 days of plant growth (Table 3). In these assays, *Pf.* SS101 was not detected on or in the leaves.

![Figure 1](image_url)

**Figure 1** *Pf.* SS101 promotes growth of Arabidopsis grown on MS agar medium *in vitro* (a). Primary root length of mock- and *Pf.* SS101-treated Arabidopsis grown for 14 days *in vitro* (b). Average number of lateral roots of mock- and *Pf.* SS101-treated Arabidopsis grown for 14 days *in vitro* (c). Longitudinal root sections captured by light-microscopy (d, e, h, i) or GFP fluorescence (with the DR5 reporter) (f, g) of Arabidopsis seedlings grown on half-strength MS medium for 18 days without (d, f, h) or with *Pf.* SS101 (e, g, i). Black bars represent mock-inoculated plants and white bars represent *Pf.* SS101-inoculated plants. Bars represent the mean of 4 replicates ($\pm$ standard error) with 15-20 plants per replicate. Bars with asterisk are significantly different as determined by One-way ANOVA analysis (P<0.05).
Bacterial determinants plant growth by *Pf.*SS101

**Targeted identification of bacterial traits involved in growth promotion and ISR**

Arabidopsis treated with heat-killed cells of *Pf.*SS101 did not result in growth promotion, enhanced lateral root formation or ISR (Table 2, EXP1), indicating that live *Pf.*SS101 cells are required to induce these plant phenotypes. Next, we conducted a series of experiments to determine if traits described previously for other *Pseudomonas* strains and other rhizobacterial genera are involved in growth promotion and ISR by *Pf.*SS101. These traits include siderophore, lipopeptide (i.e. massetolide), ACC deaminase, and volatile production. To test the role of these bacterial traits, several approaches were adopted including site-directed mutagenesis. To study the role of ACC deaminase in growth promotion, we first analyzed the *Pf.*SS101 genome but did not find the required *acdS* gene. Also, spectrophotometric analysis (with *P. fluorescens* F113 as a positive control) revealed that *Pf.*SS101 did not exhibit ACC deaminase activity (Fig. S1).

**Figure 2** Effect of *Pf.*SS101 on Arabidopsis growth via volatile production. Phenotypic observation of Arabidopsis exposed to *Pf.*SS101 grown on MS medium (a) and fresh weight of leaves were quantified (b); (c) Arabidopsis with root tip inoculation of *Pf.*SS101 (+SS101) was grown on one side of the petri dish; the non-treated Arabidopsis seedling (-SS101) was grown on the other side of the same petri dish. Non-treated Arabidopsis seedlings grown on MS medium on both sides of petri dish were controls. Effect of *Pf.*SS101 treated Arabidopsis on non-treated Arabidopsis via volatile compounds production was shown. Bars represent the mean of 4 replicates with at least 10 plants per replicate (± standard error).
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Table 2 Analysis of the role of known bacterial traits in growth promotion and ISR of Arabidopsis by Pf.SS101. Effects of Pf.SS101 and heat-killed Pf.SS101 cells (EXP1), the gacS and massA mutants (EXP2), different concentrations of the lipopeptide massetolide A (EXP3), the siderophore mutant (61C8, Pfss101_3099) (EXP4) on plant growth and ISR. Different letters indicate statistically significant differences among the treatments according to One-way ANOVA analysis (P<0.05). The asterisk indicates different concentrations of massetolide A (µg ml⁻¹) supplemented in 0.5x MS medium. The “±” represents the standard error of the mean of 4 replicates (± standard error) with at least 10 plants per replicate.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Leaves (mg)</th>
<th>Roots (mg)</th>
<th>Chlorotic leaves (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>EXP1</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>3.95±0.32</td>
<td>7.3⁵</td>
<td>79.44±9.73</td>
</tr>
<tr>
<td>Pf.SS101</td>
<td>14.59±1.07</td>
<td>41.6⁵</td>
<td>7.77±2.42</td>
</tr>
<tr>
<td>Dead cells</td>
<td>4.5±0.51</td>
<td>8.6⁵</td>
<td>83.88±4.94</td>
</tr>
<tr>
<td><strong>EXP2</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>4.3±0.73</td>
<td>1.21±0.54⁵</td>
<td>93.74±1.81</td>
</tr>
<tr>
<td>Pf.SS101</td>
<td>7.29±2.09</td>
<td>2.85±0.10⁵</td>
<td>0.00±0.00</td>
</tr>
<tr>
<td>gacΔ</td>
<td>10.39±0.50</td>
<td>3.65±0.24⁵</td>
<td>16.31±3.94</td>
</tr>
<tr>
<td>massAΔ</td>
<td>7.14±1.16</td>
<td>3.29±0.98⁵</td>
<td>3.30±1.19</td>
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<tr>
<td><strong>EXP3</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>massAO*</td>
<td>7.40±1.22</td>
<td>2.49±0.36⁵</td>
<td>89.73±1.96</td>
</tr>
<tr>
<td>massA10*</td>
<td>5.77±1.11</td>
<td>2.48±0.61⁵</td>
<td>94.11±2.36</td>
</tr>
<tr>
<td>massA25*</td>
<td>5.87±0.39</td>
<td>2.07±0.33⁵</td>
<td>85.14±3.59</td>
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<tr>
<td>massA50*</td>
<td>5.25±0.19</td>
<td>2.52±0.61⁵</td>
<td>84.78±5.25</td>
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<tr>
<td><strong>EXP4</strong></td>
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<td></td>
</tr>
<tr>
<td>Control</td>
<td>10.61±0.83</td>
<td>3.56±0.54⁵</td>
<td>79.44±9.73</td>
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<tr>
<td>Pf.SS101</td>
<td>12.32±0.12</td>
<td>5.08±0.53⁵</td>
<td>7.77±2.42</td>
</tr>
<tr>
<td>61C8</td>
<td>10.85±0.43</td>
<td>5.13±0.59⁵</td>
<td>9.44±3.89</td>
</tr>
</tbody>
</table>

To determine the potential role of volatile organic compounds (VOCs), a split-plate assay was used where Pf.SS101 was grown on MS agar medium on one side and the Arabidopsis seedlings on the other side of the plate. After 14 days of plant growth, no enhancement of shoot biomass was observed (Fig. 2A, B). Since Pf.SS101 did not grow well on MS agar medium and assuming that specific VOCs may be produced only when Pf.SS101 is colonizing plant roots, we also setup an assay where Pf.SS101 was inoculated on roots of Arabidopsis seedlings grown on one side of the plate and non-treated seedlings growing on the other side. Also in this experimental setup, no growth promotion was observed for the non-treated Arabidopsis seedlings. However, lateral root formation in the non-treated seedlings exposed to VOCs from the Pf.SS101-treated seedlings appeared to be enhanced (Fig. 2C).

For extracellular metabolites, the results showed that the siderophore-deficient mutant of Pf.SS101, generated in this study by site-directed mutagenesis and designated 61C8 (mutated in gene Pfss101_3099), enhanced root biomass...
Bacterial determinants plant growth by *Pf.*SS101 and induced resistance to the same extent as wildtype *Pf.*SS101 (Table 2, EXP4). Similar results were obtained for the *Pf.*SS101 mutant deficient in the production of the lipopeptide massetolide (Table 2, EXP2). To further exclude the role of massetolide in growth promotion and ISR, we also grew Arabidopsis seedlings on plates amended with different concentrations of massetolide. Also in this experiment, no growth promotion or ISR (Table 2, EXP3) was observed. We further made a mutation in the GacS/GacA two-component regulatory system (the Gac-system) to examine if other yet unknown extracellular metabolites regulated by the Gac-system in *Pf.*SS101 were involved. When roots of Arabidopsis seedlings were treated with the *gacS* mutant, leaf biomass was enhanced compared to the control and even more enhanced than in the *Pf.*SS101-treated seedlings. The *gacS* mutant still showed ISR activity but less than that observed for *Pf.*SS101 (Table 2, EXP2). This observation suggests that yet unknown Gac-regulated metabolites of *Pf.*SS101 play a role in plant growth promotion and ISR.

**Untargeted identification of bacterial traits involved in growth promotion and ISR**

A library of 7,488 random *Pf.*SS101 mutants was generated via random plasposon mutagenesis and each of these mutants was tested individually in

**Figure 3** Screening of 7,488 *Pf.*SS101 random plasposon mutants for lateral root formation on square plates (a) or for pathogen resistance in 96-well plates (b+c). Bacterial suspensions were inoculated on Arabidopsis seeds after sowing on plates carrying 0.5x MS. *P. syringae* pv. *Tomato* (*Pst*) was inoculated on rosette to infecting Arabidopsis plants.
<table>
<thead>
<tr>
<th>Strain</th>
<th>Colonisation Log (cfu mg(^{-1}))</th>
<th>Locus_tag</th>
<th>Gene</th>
<th>Product</th>
<th>COG*</th>
<th>Growth curve</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pf.SS101</strong></td>
<td>5.54 ± 0.04(^b)</td>
<td>COG 1.4580</td>
<td>iheC</td>
<td>ketol-1-ald reductoisomerase</td>
<td>E (valine, leucine, isoleucine)</td>
<td>+</td>
</tr>
<tr>
<td>7E12</td>
<td>4.36 ± 0.01(^a)</td>
<td>COG 1.4908</td>
<td>argC</td>
<td>N-acetyl-γ-glutamyl-phosphate reductase</td>
<td>E (arginine)</td>
<td>+</td>
</tr>
<tr>
<td>44E8</td>
<td>3.35 ± 0.00(^a)</td>
<td>COG 1.0919</td>
<td>hisD</td>
<td>histidinol dehydratase</td>
<td>E (histidine)</td>
<td>+</td>
</tr>
<tr>
<td>2B12</td>
<td>4.31 ± 0.07(^b)</td>
<td>COG 1.5114</td>
<td>mattX</td>
<td>homoserine O-acetyltransferase</td>
<td>E (methionine)</td>
<td>+</td>
</tr>
<tr>
<td>7E19</td>
<td>8.73 ± 1.14(^d)</td>
<td>COG 1.0035</td>
<td>trpB</td>
<td>tryptophan synthase, beta subunit</td>
<td>E (tryptophan)</td>
<td>+</td>
</tr>
<tr>
<td>1E14</td>
<td>2.91 ± 0.00(^e)</td>
<td>COG 1.0355</td>
<td>hisA</td>
<td>1-(5-phosphoribosyl)-5-(5-phosphoribosylamino)-5-(5-phosphoribosylamino)-midazol-4-carboxamidase isomerase</td>
<td>E (histidine)</td>
<td>+</td>
</tr>
<tr>
<td>1E14</td>
<td>4.17 ± 0.06(^c)</td>
<td>COG 1.0437</td>
<td>gldB</td>
<td>glutamate synthase, large subunit</td>
<td>E (glutamate)</td>
<td>+</td>
</tr>
<tr>
<td>2A12</td>
<td>4.09 ± 0.30(^c)</td>
<td>COG 1.4919</td>
<td>trpD</td>
<td>anthranilate phosphoribosyltransferase</td>
<td>E (tryptophan)</td>
<td>+</td>
</tr>
<tr>
<td>40E11</td>
<td>4.94 ± 0.06(^c)</td>
<td>COG 1.3161</td>
<td>argG</td>
<td>argininosuccinate synthase</td>
<td>E (arginine)</td>
<td>+</td>
</tr>
<tr>
<td>5E11</td>
<td>4.20 ± 0.13(^a)</td>
<td>COG 1.3515</td>
<td>metZ</td>
<td>O-succinylhomoserine sulfohydrylase</td>
<td>E (methionine)</td>
<td>+</td>
</tr>
<tr>
<td>5B11</td>
<td>3.09 ± 0.10(^a)</td>
<td>COG 1.3523</td>
<td>lauB</td>
<td>3-isopropylmalate dehydratase</td>
<td>C (valine, leucine, isoleucine)</td>
<td>+</td>
</tr>
<tr>
<td>9F8</td>
<td>4.34 ± 0.09(^b)</td>
<td>COG 1.3526</td>
<td>leuC</td>
<td>3-isopropylmalate dehydratase, large subunit</td>
<td>E (valine, leucine, isoleucine)</td>
<td>+</td>
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<tr>
<td>4B19</td>
<td>4.52 ± 0.06(^b)</td>
<td>COG 1.3837</td>
<td>cyAM</td>
<td>cysteine synthase B</td>
<td>E (cysteine)/sulfur metabolism</td>
<td>+</td>
</tr>
<tr>
<td>20E11</td>
<td>5.63 ± 0.03(^c)</td>
<td>COG 1.3982</td>
<td>cySH</td>
<td>adenyllyl sul fate reductase, threonine-dependent</td>
<td>E (cysteine)/sulfur metabolism</td>
<td>+</td>
</tr>
<tr>
<td>2C18</td>
<td>5.60 ± 0.03(^b)</td>
<td>COG 1.4354</td>
<td>addD</td>
<td>phosphoglucomutase dehydratase</td>
<td>E (glucose utilization)</td>
<td>+</td>
</tr>
<tr>
<td>1E06</td>
<td>3.71 ± 0.01(^d)</td>
<td>COG 1.4370</td>
<td>colR</td>
<td>DNA binding response regulator ColR</td>
<td>T</td>
<td>+</td>
</tr>
</tbody>
</table>

*COG Functional annotation. C = Energy production and conversion; E = Amino acid transport and metabolism; T = Signal transduction mechanisms. (+) = growth; (-) = no growth. Letters indicate significant differences among the treatments according to One way ANOVA analysis (P<0.05).
Bacterial determinants plant growth by *Pf.*SS101

different high-throughput bioassays: a plate assay for plant growth promotion and root architecture, and a 96-well plate assay for ISR (Fig. 3). A total of 21 potential mutants were identified that were not able to promote plant growth, alter root architecture and induce resistance to *Pst* (Table 3). The lack of effects on plant growth and ISR by these 21 mutants was confirmed in the *in vitro* bioassay described above (Fig. 3). The results of these bioassays also showed that many of the 21 mutants were impaired in root colonization and established significantly lower cell densities on roots of Arabidopsis than wildtype *Pf.*SS101. Only two mutants (20H12, 25C8) established rhizosphere population densities similar to that of wildtype *Pf.*SS101 (Table 3). These results suggest that for most mutants, except 20H12 and 25C8, the lack of effects on plant growth and ISR of mutants may be due to poor root colonization.

**Genetic characterization of *Pf.*SS101 mutants**

For all 21 mutants, the regions flanking the plasposon insertion were cloned and sequenced. In 19 of the 21 mutants, the plasposon insertion was located in genes involved in biosynthesis of different amino acids, including arginine (40H11; 44D8), cysteine (42B9, 20H12), glutamate (18F11), histidine (13E4; 13H6; 24A12; 32H11), tryptophan (24B12; 24D10; 71H9; 74F8), methionine (22G5; 51G1) and valine, leucine, isoleucine (7H2; 9F8; 59B6; 76G8) (Table 3). For the remaining two mutants, the plasposon was inserted in the genes coding for the DNA-binding response regulator ColR (16G6) and for phosphogluconate dehydratase (25C8), respectively (Table 3). All mutants were able to grow in KB broth to the same density as *Pf.*SS101, but only 16G6 and 25C8 were able to grow in minimal (SSM) medium alike *Pf.*SS101 (Table 3). All mutants were able to grow to the same density as *Pf.*SS101 when minimal medium was supplemented with the amino acid whose biosynthesis was blocked by the mutation (Table 4). These results indicate that most mutants, except 25C8 and 16G6, were auxotrophic.

Southern-blot hybridization showed that 19 mutants had a single plasposon insertion except the two mutants 20H12 and 42B9 where two insertions were found. To confirm the role of *cysH* (20H12) or *cysM* (42B9) in plant growth promotion and ISR, site-directed mutagenesis of each of these genes was performed to obtain single knockout mutants for *cysH* and *cysM*. Consistent with the phenotype of the random mutants, also the site-directed mutants lacked the
ability to induce lateral root formation and ISR against Pst. The in vitro bioassay confirmed that mutants 16G6 and 25C8 did not promote plant growth, alter root architecture and induce systemic resistance against Pst (Fig. 4b). Mutants 20H12 (cysH, PflSS101_3982), 42B9 (cysM, PflSS101_3837), 16G6 (colR, PflSS101_4370) and 25C8 (edd, PflSS101_4354) were selected for further functional analysis. For each of the 4 mutants, genetic complementation with the respective gene restored plant growth promotion, alteration of root architecture and ISR to the same level as observed for Pf. SS101 (Fig. 4a).

Next, we studied if the genes mutated in these 4 mutants were expressed in wildtype Pf. SS101 when colonizing Arabidopsis roots. Over a course of 7-18 days of plant growth, the genes edd (PflSS101_4354) (Fig. 4d) and cysM (PflSS101_3837) (Fig. 4f) were indeed expressed in Pf. SS101 on roots of Arabidopsis. Also cysH (PflSS101_3982) and colR (PflSS101_4370) showed higher expression in Pf. SS101 on Arabidopsis roots after 7, 10 and 14 days but not at 18 days (Fig. 4c,e).

Role of sulfur in plant growth promotion and ISR by Pf. SS101

The cysH and cysM genes are essential in the biosynthesis of the amino acids cysteine and methionine (Fig. 5a). More specifically, cysH in Pf. SS101 encodes a predicted protein with the conserved (KRT)ECG(LS)H signature of the APS/PAPS reductase families and the critical two cysteine pairs found in APS reductases (Fig. S2). The final step of sulfur assimilation into cysteine is the synthesis of L-cysteine from O-acetyl-L-serine and sulfide catalyzed by O-acetyl-L-serine(thiol)-

<table>
<thead>
<tr>
<th>Strains</th>
<th>Locus_tags</th>
<th>Mutated genes</th>
<th>Supplementation of amino acids</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pf. SS101</td>
<td>PflSS101_4908</td>
<td>argC</td>
<td>+</td>
</tr>
<tr>
<td>44D8</td>
<td>PflSS101_0919</td>
<td>hisD</td>
<td>+</td>
</tr>
<tr>
<td>71H9</td>
<td>PflSS101_0035</td>
<td>trpB</td>
<td>+</td>
</tr>
<tr>
<td>13E4</td>
<td>PflSS101_0355</td>
<td>hisA</td>
<td>+</td>
</tr>
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<td>24A12</td>
<td>PflSS101_0437</td>
<td>glnB</td>
<td>+</td>
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<td>trpD</td>
<td>+</td>
</tr>
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<td>40H11</td>
<td>PflSS101_1161</td>
<td>argG</td>
<td>+</td>
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<td>+</td>
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<td>42B9</td>
<td>PflSS101_3837</td>
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<td>+</td>
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<tr>
<td>20H12</td>
<td>PflSS101_3982</td>
<td>cysH</td>
<td>+</td>
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</tbody>
</table>
Bacterial determinants plant growth by *Pf.*SS101

A putative *cysE* gene was also detected in the *Pf.*SS101 genome, embedded in a gene cluster predicted to be involved in the formation of Fe-S clusters (Table 5, Fig. 5a). Cysteine serves as the main source of sulfur for the biosynthesis of Fe-S centers. Based on these genomic analyses, we hypothesized that modulation of the plant’s sulfur metabolism is one of the mechanisms underlying growth promotion and/or ISR by *Pf.*SS101.

To experimentally provide support for this hypothesis, two major approaches were taken. First, we conducted *in vitro* assays with Arabidopsis grown on MS agar.
The results showed that both L- and D-cysteine and methionine induced lateral root formation in Arabidopsis in a concentration dependent manner (Fig. 5b). Moreover, cysteine at relatively high concentrations induced disease resistance against \textit{Pst} in Arabidopsis (Fig. 5c). Preliminary LC-MS/MS analyses did not show detectable levels of cysteine and methionine production by \textit{Pf}.\textit{SS101} when grown on MS agar medium or on Arabidopsis roots under \textit{in vitro} conditions (data not shown).

The second approach to provide support for a role of sulfur metabolism in plant growth promotion and ISR was to analyze the transcriptional changes in Arabidopsis treated with \textit{Pf}.\textit{SS101} and the \textit{cysH} mutant (2OH12). To explore the expression pattern of Arabidopsis genes that were altered by \textit{Pf}.\textit{SS101} or the \textit{cysH} mutant, the expression of all 22,850 genes present on the ATH1 genome...
Bacterial determinants plant growth by *Pf*.SS101

Array were subjected to one-way ANOVA without FDR correction. A total of 6,308 genes showed differential regulation (*P*<0.05, without FDR correction) between Arabidopsis plants treated with *Pf*.SS101, the *cysH* mutant or the non-treated control. Hierarchical cluster analysis (HCA) and principal component analysis (PCA) were performed with the 6,308 differential genes to explore the pattern of their expression and amount of total variation in expression attributed to *Pf*.SS101 or the *cysH* mutant, respectively (Fig. 6). In the HCA, six major clusters were found that explain the total variation in gene expression in the different treatments. These clusters represent genes induced or repressed in plants treated with *Pf*.SS101 or the *cysH* mutant (Fig. 6). Clusters II and V represent Arabidopsis genes induced or repressed by *Pf*.SS101, respectively. Similarly, clusters VI and III represent Arabidopsis genes induced or repressed by the *cysH* mutant, respectively. The remaining clusters I and IV correspond to Arabidopsis genes induced or repressed by both *Pf*.SS101 and the *cysH* mutant, respectively (Fig. 6). In the PCA, the first principal component (PC1) explained 41% of the total variation in gene expression that is attributed to the unique clusters of genes that were altered in plants treated with *Pf*.SS101.
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Figure 6. Genome wide transcriptome analysis of Arabidopsis treated with cell suspensions of *Pf.*SS101, the *cysH* mutant (20H12) or not treated (control). Principal component analysis (PCA) (a) based on 6308 genes that showed differential regulation (*P*<0.05, with no FDR correction) between Arabidopsis plants treated with *Pf.*SS101, the 20H12 mutant and control seedlings. The first component explained 41% of the total variation primarily associated to induction and repression of the expression of clusters of genes on plants that are treated either by the *Pf.*SS101 or the 20H12 mutant (See the HCA, Clusters, II, III, V and VI). The second component explained 30% of the total variation and corresponds to clusters of genes that are either induced or repressed similarly in plants that were treated with *Pf.*SS101 or the 20H12 mutant (See the HCA, Clusters I and IV). Hierarchical cluster analysis (HCA) (b) were performed using the same 6308 genes to explore the pattern of their expression and amount of total variation in expression explained by the treatment of the plants with *Pf.*SS101 and the 20H12 mutant. Six major clusters were formed, which explain the total variation that corresponds to the above mentioned principal components. These clusters represent active induction and repression of genes in control Arabidopsis seedlings and the seedlings treated with *Pf.*SS101 and the 20H12 mutant.
Table 6: Gene set enrichment analysis (GSEA) representing significantly enriched biological processes in *A. thaliana* treated with Pf.SS101. The asterisk represents the number of Arabidopsis genes belonging to the indicated biological process. The dollar sign represents the number of genes in the selected cluster (cluster II, Fig.6) belonging to the indicated biological process. The GO stands for gene ontology.

<table>
<thead>
<tr>
<th>Gene Set Name (GO)</th>
<th>GO orthology</th>
<th>NO. Genes in Overlap (k)</th>
<th>$p$ value</th>
<th>FDR</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYSTEINE_BIOSYNTHETIC_PROCESS(210)</td>
<td>GO:0019444</td>
<td>34</td>
<td>1.44E-19</td>
<td>1.54E-16</td>
</tr>
<tr>
<td>SERINE_FAMILY_AMINO_ACID_BIOSYNTHETIC_PROCESS(222)</td>
<td>GO:0007070</td>
<td>34</td>
<td>6.71E-19</td>
<td>5.72E-16</td>
</tr>
<tr>
<td>CARBONIC_HYDROXYLASE_BIOSYNTHETIC_PROCESS(1069)</td>
<td>GO:0016051</td>
<td>71</td>
<td>1.84E-18</td>
<td>1.47E-15</td>
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<tr>
<td>CELLULAR_AMINO_ACID_BIOSYNTHETIC_PROCESS(501)</td>
<td>GO:0008652</td>
<td>48</td>
<td>1.99E-18</td>
<td>1.50E-15</td>
</tr>
<tr>
<td>PRIMARY_METABOLIC_PROCESS(5172)</td>
<td>GO:0044238</td>
<td>321</td>
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<td>GO:0019748</td>
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Bacterial determinants plant growth by Pf.SS101
either with Pf.SS101 or the cysH mutant (Fig. 6, clusters II, III, V and VI). The second principal component (PC2) explained 30% of the total variation and is attributed to clusters of genes that were altered similarly in plants that were treated by Pf.SS101 and the cysH mutant (Fig. 6, clusters I and IV).

In order to understand the major growth and defence related biological processes (BPs) that are altered in Arabidopsis by Pf.SS101, we performed gene set enrichment analysis (GSEA) specifically on genes in Cluster II of the HCA, representing genes in Arabidopsis whose expression was significantly induced by Pf.SS101 (Fig. 6b). Prior to performing the GSEA, we selected the genes in this cluster and computed independent t-Tests by comparing the mean expression value for each of the genes in Pf.SS101-treated plants treated with the genes in the cysH mutant-treated plants. Cluster II contains a total of 967 genes and 547 were significantly different (P<0.05, with FDR correction) between plants treated with Pf.SS101 and plants treated with the cysH mutant. The GSEA on these 547 genes revealed 246 BPs significantly enriched. However, these long lists of BPs are largely redundant and were reduced to 68 BPs by performing HCA on the gene X GO matrix, an output from the GSEA (Table 6). These 68 BPs fall into the following major categories: biosynthesis, transport, catabolism, response to stimulus and growth. From the processes associated with biosynthesis, sulfur compounds and specifically serine, cysteine and glucosinolate biosynthetic processes were the most significantly enriched (Table 6). The BPs involved in plant growth such as indole acetic acid biosynthetic process and auxin transport, steroid biosynthesis and isopentenyl diphosphate biosynthesis also showed significant enrichment in this cluster. Another significantly enriched BP was carbohydrate biosynthetic processes, specifically the biosynthesis of starch. In line with this, also glucose catabolic processes were significantly enriched (Table 6).

Discussion

In the present study, we show that Pf.SS101 enhances Arabidopsis growth, changes the root architecture and induces systemic resistance against P. syringae pv. tomato (Pst). Arabidopsis seedlings treated with Pf.SS101 showed inhibition of primary root growth and initiation of lateral root formation. Zamioudis et al. (2013) reported similar results for P. fluorescens WCS417 and further showed a role of auxin signalling in altered root development. In line with this, the transcriptome
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profiling performed in our study showed significant enrichment of biological processes that play a critical role in plant growth, including processes related to auxin biosynthesis, auxin polar transport and steroid biosynthesis. The Pf.SS101 genome does not harbor the typical genes for IAA biosynthesis, but it can produce IAA \textit{in vitro} when fed with L-tryptophan (not shown). Whether L-tryptophan is exuded by Arabidopsis roots and if this will then lead to IAA production by Pf.SS101 \textit{in situ} and results in growth promotion remains to be investigated.

Previous studies have shown that different rhizobacterial genera enhance plant growth and induce systemic resistance via the production of phytohormones, siderophores, lipopeptides or volatiles (VOCs) (Ryu et al., 2003; Tran et al. 2007; Raaijmakers et al., 2010; Van de Mortel et al. 2012; Bakker et al., 2013). Our results indicate that the siderophore and the lipopeptide massetolide produced by Pf.SS101 are not involved in plant growth promotion and ISR. The role of VOCs in plant growth promotion and ISR by Pf.SS101 is not fully clear from this study. When grown on MS agar medium alone, physically separated from the Arabidopsis seedlings, Pf.SS101 did not enhance plant growth. However, lateral root formation appeared to be enhanced when non-treated seedlings were exposed to VOCs from Pf.SS101-treated seedlings.

Results from the screening of 7,488 Pf.SS101 random mutants led to the selection of 21 mutants deficient in both growth promotion and ISR. This result seems to be in contrast to the results of Zamioudis et al. (2013) who showed that for Pf.WCS417 plant growth promotion and ISR are mediated by different signaling pathways. Genetic analysis of the 21 Pf.SS101 mutants showed that most Pf.SS101 mutants were deficient in amino acid biosynthesis and also poorly colonized the roots of Arabidopsis. The role of amino acids in rhizobacteria-plant interactions is not well studied, although some amino acids such as methionine and tryptophan may act in soil as precursors for the biosynthesis of the phytohormones ethylene and indole-3-acetic acid, respectively (Murcia et al., 1997; González-López et al., 2005). Whether Pf.SS101 produces cysteine and methionine \textit{in vitro} or in the rhizosphere of Arabidopsis is not fully clear yet. Also the ability of Arabidopsis to take up cysteine and methionine from the rhizosphere has not been investigated in detail. Nevertheless, our transcriptome data revealed that biosynthetic processes associated to sulfur compounds and specifically serine, cysteine and glucosinolates were the most significantly enriched in seedlings treated with Pf.SS101 as compared
to the control plants and plants treated with the cysH mutant. These results indicate that Pf.SS101 modulates sulfur metabolism in plants. Recently, studies by Meldau et al. (2013) also attributed modulation of sulfur metabolism as a key mechanism in growth promotion and induction of lateral roots of tobacco by Bacillus sp. strain B55. In their study, they further showed that these effects were mediated by the production of the S-containing volatile dimethyl disulphide. Whether Pf.SS101 modulates sulfur metabolism in Arabidopsis by dimethyl disulfide or via other S-containing metabolites is not yet known and will be subject of future studies.

In plants, sulfur is important in various stress responses (Bloem et al., 2005; Kertesz et al., 2007). Elemental sulfur itself can also be used directly by plants, via deposition in the xylem parenchyma, to combat fungal infection (Cooper and Williams, 2004). The metal-chelating properties of sulfur in phytochelatins help alleviate heavy metal stress and sulfur is also important to the plant in responding to pathogen attack, since many defense compounds, in particular the glucosinolates, contain sulfur (Brader et al., 2006). Cysteine biosynthesis in plants involves the incorporation carbon backbone from serine with reduced inorganic sulfur (Neuenschwander et al., 1991; Saito et al., 1994; Bonner et al., 2005). Cysteine might enter into the glucosinolate biosynthesis pathway in three routes. The first route involves direct donation of reduced sulfur to glucosinolate biosynthesis. The second route involves the incorporation of cysteine into methionine and through a series of side chain elongation, S-glycosilation and other secondary modification it ends up in the glucosinolate pool. The third route could involve the conjugation of cysteine, glutamate and glycine to form glutathione (GSH) (Meister, 1995). Geu-Flores et al. (2011) recently showed that GSH acts as a sulfur donor for glucosinolate biosynthesis. In line with this, metabolic processes related to all the aforementioned amino acids showed significant enrichment among the genes that are induced by Pf.SS101, indicating that this route is the most probable means of reduced sulfur channelling mechanism into the glucosinolate pool.

Carbohydrate biosynthetic processes in general and starch biosynthetic processes in particular were highly induced by Pf.SS101 and these processes are critically important for biomass formation. The recycling of glucose is the primary step before its incorporation into starch through the enzymes of glycolytic, glucogenic and pentose phosphate pathways (Glawischnig et al., 2002). Interestingly, the transcriptome data showed that genes involved in these biological processes are
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also significantly enriched in \textit{Pf}.SS101-treated seedlings.

In addition to the \textit{cysH} and \textit{cysM} mutants, two other \textit{Pf}.SS101 mutants with mutations in the \textit{colR} and \textit{edd} genes were identified in this study. The CoIR-ColS pathway was first characterized in \textit{P. fluorescens} for its role in competitive colonization of plant roots (Dekkers et al., 1998). Subsequent studies have shown that mutations in the CoIR-ColS two-component system lead to several other defects in different \textit{Pseudomonas} strains (Hörak et al., 2004; Kivistik et al., 2006). \textcite{De Weert and colleagues (2006)} showed that a putative methyltransferase/wapQ (\textit{inaA}) operon is located downstream of CoIR-ColS in \textit{P. fluorescens} WCS365 and that this operon is regulated by CoIR-ColS. Since \textit{wapQ (inaA)} encodes a putative lipopolysaccharide (LPS) phosphatase, the possibility was studied that the integrity of the outer membrane of \textit{P. fluorescens} WCS365 mutant PCL1210 was altered. PCL1210 was identified as a colonization mutant with an insertion in the CoIR-ColS two-component system (Dekkers et al., 1998). Mutants in the methyltransferase/\textit{wapQ} operon were also altered in their outer membrane permeability and defective in competitive tomato root tip colonization (De Weert et al., 2006). In \textit{Pf}.SS101, we also identified a putative methyltransferase/\textit{inaA} (\textit{wapQ}) operon downstream of CoIR-ColS but its role in plant growth promotion and ISR is not yet known.

The \textit{edd} gene codes for 6-phosphogluconate dehydratase, an enzyme that catalyzes the first step in the Entner-Doudoroff (ED) pathway (Wanken et al., 2003), the dehydration of 6-phospho-D-gluconate into 6-phospho-2-dehydro-3-deoxy-D-gluconate (Peekhaus and Conway, 1998; Kim et al., 2007). Many bacteria possess genes for the ED pathway (Kim et al., 2007). For \textit{P. chlororaphis} O6, Kim et al. (2007) showed that the \textit{edd} gene contributes to root colonization and ISR. They concluded that metabolism of sugars through the ED pathway in \textit{P. chlororaphis} O6 may be important because it facilitates the production of the effectors involved in ISR (Kim et al., 2007). In our study we showed that the \textit{edd} gene was significantly higher expressed in \textit{Pf}.SS101 on Arabidopsis and that the \textit{Pf}.SS101 \textit{edd} mutant 25C8 showed no induction of lateral root formation and systemic resistance in Arabidopsis, similar to what was shown for \textit{P. chlororaphis} O6 (Kim et al., 2007). In contrast to \textit{P. chlororaphis} O6, however, elimination of the \textit{edd} gene in \textit{Pf}.SS101 had no effect on colonization of Arabidopsis. A major determinant for ISR elicited by \textit{P. chlororaphis} O6 against \textit{E. carotovora} was the volatile 2R, 3R-butanediol (Han et al., 2006; Kim et al., 2007). Butanediol likely is produced from pyruvate
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(Ryu et al., 2003), which is a direct product from the ED pathway. Kim et al. (2007) hypothesized that the loss of carbon flux through this pathway in the \textit{edd} mutant would limit pyruvate, reducing butanediol production and ISR.

In conclusion, modulation of auxin biosynthesis and transport, steroid biosynthesis, carbohydrate metabolism and sulfur assimilation in Arabidopsis appear to be key mechanisms linked to growth promotion and ISR by \textit{Pf}.SS101. In particular sulfur metabolism was shown to be an important biological process altered by \textit{Pf}.SS101 in Arabidopsis. The molecular signals and sulfur-containing compounds involved have not yet been identified and need further investigation. Also identification of the bacterial traits associated with the ColR-ColS two-component system and the ED pathway in \textit{Pf}.SS101 will be required to shed more light on the other mechanisms of plant growth promotion and ISR.

Acknowledgements

This research was financially supported by the Dutch Technology Foundation and by the Netherlands Genomics Initiative (NGI) ECOLINC program. The authors would like to thank Mieke Wolters-Arts and Dragana Kocevski for technical assistance.
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Bacterial determinants plant growth by Pf.SS101


Van Loon LC, Bakker PA & Pieterse CM (1998) Systemic resistance induced by


Supplementary figures

**Figure S1.** Measuring ACC deaminase activity in *Pf.*SS101. *Pf.*F113 was used as a positive control and *P. protegens* CHA0 was used as a negative control. α-ketobutyrate production by strains F113, CHA0 and SS101 tested after incubation of their cell extracts with ACC. Averages of 4 replicates are given. Letters indicate significant differences among these strains tested according to One-way ANOVA analysis (*P*<0.05).
Figure S2. Comparison of amino acid sequences of APS reductase from *Pf.SS101* with *P. putida*, *P. entomophila*, *P. syringae*, *Pf.SBW25*, *Pf01*, *Pf5* and *P. aeruginosa*. The sequences were aligned with the program Clustal W. Asterisks identify identical residues, and arrows mark the additional Cys in APS reductases. The conserved APS reductase signature is underlined.
Chapter 7

Summarizing discussion and future perspectives

Xu Cheng
Chapter 7

Soil microorganisms represent an important resource for the discovery of novel genes and bioactive compounds, including antimicrobial metabolites and enzymes. Traditional methods to discover microbial natural products involve isolation and cultivation of a microorganism, followed by extraction, fractionation and structure elucidation of the bioactive compounds (Scherlach & Hertweck, 2009, Zerikly & Challis, 2009). In the 20th century, this approach was successfully used for the discovery of a wide range of antibiotics and enzymes, but the chances of identifying novel bioactive molecules have decreased considerably over the past decades (Wright & Sutherland, 2007). The recent increase in the availability of whole genome sequences of microorganisms, however, has substantially changed our fundamental knowledge of the metabolic capabilities of microorganisms. There are several approaches to explore and exploit microbial genomes for the discovery of orphan biosynthetic pathways and novel bioactive compounds (Gross et al., 2007, McAlpine, 2009, Scherlach & Hertweck, 2009). These include (1) in silico mining of microbial genomes for consensus sequence motifs or modules followed by structure prediction, gene inactivation studies and/or genomisotopic approaches, (2) heterologous expression of genomic fragments obtained from culturable or unculturable microorganisms (i.e. metagenomics) followed by activity assays and comparative metabolic profiling, and (3) activation of orphan biosynthetic pathways by manipulation of regulatory genes or by challenging microorganisms with specific culture conditions or other external cues.

In the research presented in this thesis, several of these approaches were adopted to identify pathways and bioactive compounds in *Pseudomonas fluorescens* strains SBW25 (*Pf*.SBW25) and SS101 (*Pf*.SS101). These strains are well known for their beneficial effects on plant growth and their abilities to produce lipopeptide surfactants (LPs) with strong activities against oomycete pathogens. In *Pseudomonas*, many of the genes involved in the production of antimicrobial secondary metabolites are under regulation of the GacS/GacA two-component regulatory system (Kitten et al., 1998, Koch et al., 2002, Dubern et al., 2005, de Bruijn & Raaijmakers, 2009, Vallet-Gely et al., 2010). We therefore exploited a mutant strain of *Pf*.SBW25 that has a deficiency in the Gac-regulatory system to elucidate novel/bioactive metabolites and orphan biosynthetic pathways (Chapters 2, 3). Subsequently, the functions of these metabolites and pathways in the control of plant pathogens (Chapter 3), in plant growth promotion and in induced systemic
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resistance (ISR) by strain *Pf.SBW25* (Chapters 4, 5) were analysed. Finally, novel traits involved in plant growth promotion and ISR by strain *Pf.SS101* were identified (Chapter 6).

Genome-wide transcriptional analysis and bioassays revealed that a mutation in the Gac-system causes major transcriptional changes in *Pf.SBW25* and significantly enhances its antimicrobial activities (Chapter 2). Previously, it was shown that *Pf.SBW25* produces viscosin, a lipopeptide with anti-oomycete activity, and that the production of viscosin is under regulation of the Gac-system (de Bruijn et al., 2007). Therefore it was expected that mutating the Gac-system in *Pf.SBW25* would lead to either a reduction or a complete loss of antimicrobial activity, but we observed the opposite. We then revealed that a GacS-mutation in *Pf.SBW25* leads to enhanced expression of genes involved in the pyrroloquinoline quinone (PQQ) biosynthesis pathway, resulting in an increase in gluconic and 2-keto gluconic acid production, which in turn acidified the extracellular medium to levels that inhibit growth of other microorganisms (Chapter 3). Medium acidification may lead to an increase of phosphate (P) solubilization that could affect plant growth (Goldstein, 1995, Hwangbo et al., 2003). To test this, *Pf.SBW25* and its *gacS* mutant were applied to multiple plant species including Arabidopsis, tobacco and cucumber and plant growth promotion and ISR traits were analyzed (Chapter 5).

Extracting metabolites from bacterial monocultures and screening their biological activity is the classic way of investigating and discovering microbial bioactive compounds that play roles in promoting plant growth and/or ISR. However, since the metabolites produced by bacteria may change when grown in association with their plant hosts, we searched for a more advanced method that allows the detection of metabolites produced during the interaction with the plant. By using matrix-assisted laser desorption/ionization imaging mass spectrometry (MALDI-IMS) and dried droplet (MALDI-DD), we visualized the chemistry in *Pseudomonas*-Arabidopsis interactions and showed that MALDI-DD/IMS is a powerful tool for analyzing the chemistry profiles and visualizing the spatial distribution of metabolites produced during plant-microbe interactions (Chapter 5). With the aim to further unravel the role of the Gac-system in plant growth promotion we also used MALDI-IMS to examine the metabolites produced by the *Pf.SBW25 gacS* mutant when applied to Arabidopsis roots (Chapter 5). The GacS-mutation in *Pf.SBW25* also affected the production of volatile organic compounds
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(VOCs), which in turn influenced plant growth promotion and ISR in a plant species-dependent manner (Blom et al., 2011, Schmidt et al., 2016) (Chapter 4). In addition, new high-throughput screening methods were developed to identify novel genes and bioactive compounds in Pf.SS101, a strain known for its exceptional effects on plant growth, root development and ISR. The results suggest that modulation of auxin biosynthesis and transport, steroid biosynthesis, carbohydrate metabolism and sulfur assimilation in Arabidopsis are key mechanisms associated with growth promotion and ISR by Pf.SS101 (Chapter 6). Future studies involving a combination of chemical elucidation and biofunctional analysis of the detected metabolites and VOCs, may lead to the identification of (novel) bioactive compounds that affect plant growth promotion.

The Gac-system in Pf.SBW25 regulates the production of bioactive compounds

To date, little is known about the genes and metabolites of Pf.SBW25 involved in antimicrobial activity and plant growth promotion. Pf.SBW25 does not produce the antibiotic compounds 2,4-DAPG, phenazines, pyrrolonitrin and pyoluteorin (Loper et al., 2012), but does produce siderophores (Timms-Wilson et al., 2000, Moon et al., 2008) and the lipopeptide viscosin (de Bruijn et al., 2007). The Gac-system is a two-component regulatory system that regulates the production of a broad spectrum of secondary metabolites and exoenzymes in Pseudomonas: a mutation in either one of the two genes, gacS or gacA, abolishes the production of these secondary metabolites and enzymes (Kitten et al., 1998, Koch et al., 2002, Dubern et al., 2005, de Bruijn & Raaijmakers, 2009, Vallet-Gely et al., 2010, Song et al., 2015). To reveal the regulatory effects of a mutation in the Gac-system in Pf.SBW25, a genome-wide transcriptome analysis was performed. Mutation of gacS in Pf.SBW25 affected the expression of a total of 1807 genes (fold change > 2.0, p<0.0001), 935 of which were up-regulated and 872 down-regulated (Chapter 2). By using more stringent criteria (fold change > 4.0, p<0.0001), the total number was reduced to 702 differentially expressed genes, including 300 up- and 402 genes down-regulated genes. These results are comparable to those reported by Hassan et al. (2010) who analysed the genome-wide changes in the transcriptome due to a GacS-mutation in Pseudomonas protegens PF-5 (Hassan et al., 2010). In the gacS mutant of Pf.SBW25, the viscosin biosynthesis genes viscA (PFLU4007), viscB
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(PFLU2553) and viscC (PFLU2552), as well as the exoprotease gene aprA (PFLU3146), were significantly down-regulated (by 44-, 54-, 56- and 220-fold, respectively), and this is consistent with previous findings by de Bruijn et al. (de Bruijn et al., 2007) who showed that the gacS mutant has a viscosin-deficient phenotype. They found that the production of viscosin in the gacS mutant was indeed abolished and this was confirmed by HPLC analysis (De Bruijn et al., 2007). Among the upregulated genes in the gacS mutant, we identified an orphan NRPS gene cluster that was predicted to synthesize an eight-amino-acid peptide (Chapter 2). The predicted peptide resembled ornicorrugatin, a putative siderophore also produced by the P. fluorescens strain Pf.AF76 (Matthijs et al., 2008, Cornelis, 2010). A siderophore detection assay showed that the Gac-system negatively regulates siderophore production in Pf.SBW25, confirming and extending results obtained for other P. fluorescens strains (SchmidliSacherer et al., 1997, Duffy & Defago, 2000, Hassan et al., 2010). Remarkably, the GacS-mutation also led to a significant transcriptional up-regulation of genes involved in PQ biosynthesis, resulting in enhanced production of two organic acids, gluconic acid and 2-keto gluconic acid (Chapter 3).

To further identify bioactive compounds and biosynthetic pathways involved in Pseudomonas-plant interactions, Pf.SBW25 and the gacS mutant were applied to roots of Arabidopsis seedlings. Chemical profiles of Pseudomonas-Arabidopsis interactions were generated and this revealed the importance of the Gac-system. A gasS mutation not only caused reduction in the production of certain metabolites, it also led to the production of additional metabolites. Mass spectrometric analysis (MALDI-DD and MALDI-IMS) showed that many metabolites produced during Pf.SBW25-Arabidopsis interactions were absent when Arabidopsis roots were challenged with the gacS mutant. Some of these compounds (including lipopeptides) were putatively identified based on their m/z values (Chapter 5). Spatial distribution of the detected compounds showed that they were either associated with bacterial colonization of the root surface (like m/z 1164, matching viscosin) or were released as diffusible compounds by the plant or the bacterial strains to the surrounding area. MALDI-IMS/DD analysis visualized a variety of metabolites in the Pf.SBW25-Arabidopsis interaction, including metabolites that are regulated by the GacS/GacA two-component system. Although chemical elucidation and functional assays are required, visualization of the spatial distribution of these compounds provided valuable information to better understand the role of these compounds in plant-
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*Pseudomonas* interactions (*Chapter 5*).

Plant growth-promoting rhizobacteria (PGPRs) can also affect plant growth via the production of VOCs (Ryu *et al.*, 2003, Blom *et al.*, 2011). For instance, the VOCs 1-hexanol, indole, pentadecane, acetoin and 2,3-butanediol (2,3-BD) have been shown to promote plant growth and the latter two also induce systemic resistance in Arabidopsis (Ryu *et al.*, 2003, Rudrappa *et al.*, 2010). Interestingly, a mutation in the GacS/GacA two-component regulatory system significantly altered the emission of VOCs by *Pf.*SBW25. We showed for example, that the Gac-system in *Pf.*SBW25 significantly affects the production of specific alkenes (*Chapter 4*), in particular three acyclic alkenes (3-nonene, 4-undecyne, 1-undecene). These three were significantly reduced by the GacS-mutation independently of the cultivation media and of the cultivation time. When testing the effects of *Pf.*SBW25 and the *gacS* mutant on plant growth of two unrelated plant species, Arabidopsis and tobacco, we observed different responses. This indicates that Gac-regulated VOCs affect plant growth promotion in a plant species-dependent manner.

**Gac-mediated traits of *Pf.*SBW25**

PGPRs, like *Pseudomonas*, are able to inhibit the growth of pathogens and also promote plant growth via the production of bioactive compounds and/or via ISR. For *Pf.*SBW25, the Gac-regulated metabolites described above also contribute to interactions of *Pf.*SBW25 with other microorganisms. Mutating the Gac-system in *Pf.*SBW25 reduced or abolished protease activity, motility, biofilm formation and oxidative stress responses, and enhanced siderophore biosynthesis (*Chapter 2*). Moreover, the large number of genes that was affected in the *gacS* mutant, includes genes involved in (exo)polysaccharide, type II secretion and transcription of nineteen rhizosphere induced genes (Rainey, 1999, Naseby *et al.*, 2001). Genes involved in pectate lyase biosynthesis were significantly down-regulated in the *gacS* mutant of *Pf.*SBW25, which was confirmed by the loss of pectate lyase activity (*Chapter 2*). In contrast to other *Pseudomonas* strains, in which the effect of a Gac mutation has been studied, the *gacS* mutant of *Pf.*SBW25 showed significantly enhanced antimicrobial activities. This was shown to be due to the overexpression of PQQ biosynthesis genes and the subsequent enhanced production of gluconic acid and 2-keto gluconic acid that leads to acidification. This PQQ-mediated acidification may compensate for the loss of other antimicrobial traits in the
gacS mutant and may help the mutant to withstand competitors (Chapter 3). As a global regulator, the Gac-system may also influence plant growth by regulating the production of certain bioactive compounds. Therefore, effects of a deficient Gac-system in Pf.SBW25 on plant growth promotion were tested (Chapter 5). PQQ is known as antioxidant and plant growth promotion factor (Choi et al., 2008). When the pqqB mutant of Pf.SBW25 was applied to Arabidopsis roots, the root architecture and biomass of the seedlings were similar to that of seedlings treated with Pf.SBW25. Strikingly, exposure of the roots to the double mutant gacS/pqqB, which had lost antimicrobial activity (Chapter 3), caused a significant increase in plant biomass as compared to the seedlings treated with Pf.SBW25 and the gacS mutant (Figure 1). The plants also had many more lateral roots and greener leaves as compared to the control plants and to Arabidopsis plants treated with either Pf.SBW25 or the gacS mutant (Figure 1). This showed that impairment of the Gac-system in combination with PQQ biosynthesis led to enhanced plant growth promotion. However, the underlying mechanisms of this unexpected plant growth promotion are as yet unknown and will be subject of future studies.

Figure 1 Effect of Pf.SBW25 and several mutants on Arabidopsis growth. The mutants include two single knock-out mutants (gacS and pqqB), a double mutant (gacS/pqqB) and the double mutant in which the mutation in pqqB is complemented (gacS/pqqB+pqqF-E) (A) in vitro assays with Arabidopsis seedlings treated with Pf.SBW25 or its mutants. (B) Arabidopsis plants grown in soil and inoculated with Pf.SBW25 or its mutants.
In another line of research, we showed that *Pf.*SBW25 significantly promotes Arabidopsis growth and induces systemic resistance against the bacterial pathogen *Pseudomonas syringae* pv. *tomato* (*Pst*) via VOCs production. The involvement of VOCs in plant growth promotion by certain *P. fluorescens* strains has also been shown by others (Blom *et al.*, 2011, Rui *et al.*, 2014, Park *et al.*, 2015). Mutation of the Gac-system caused major transcriptional changes and resulted in altered production of VOCs. Nevertheless, VOCs produced by *gacS* mutant and by the wild type strain *Pf.*SBW25 showed a similar effect on plant growth and ISR. On tobacco, however, VOCs of *Pf.*SBW25 repressed root growth whereas those produced by the *gacS* mutant promoted growth. This study indicated that the Gac-system affects VOCs production of *Pf.*SBW25, which in turn affects plant growth in a plant species-dependent manner (Chapter 4). The importance of the Gac-system for the antagonistic properties of *Pf.*SBW25, and for its ability to promote plant growth are summarized in Figure 2.

**Figure 2** Importance of the Gac-system of *Pf.*SBW25 in the regulation of bioactive compound production, antagonism against different plant pathogens, plant growth promotion and ISR. The numbered circles refer to the corresponding thesis chapters. Circles with a red colour refer to chapters with a focus on microbe-microbe interactions, whereas the green-coloured circles represent the chapters with a focus microbe-plant interactions.

Putative mechanisms underlying plant growth promotion and ISR induced by *Pf.*SS101

To obtain a better understanding of the mechanisms of plant growth promotion,
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the effects of \textit{Pf}.SS101 on Arabidopsis growth and ISR were studied (Chapter 6). In previous studies it was shown that \textit{Pf}.SS101 enhances resistance in Arabidopsis against several pathogens, including \textit{Pst} and the insect pest \textit{Spodoptera exigua} (van de Mortel \textit{et al.}, 2012). In Chapter 6, we showed that Arabidopsis seeds or seedlings treated with \textit{Pf}.SS101 showed inhibition of primary root growth and initiation of lateral root formation. Next we showed that the Gac-system and possibly VOCs might have a (minor) role in plant growth promotion of \textit{Pf}.SS101. Screening of a random mutant library (7488 mutants) of \textit{Pf}.SS101 for plant growth promoting properties and ISR, resulted in the selection of four mutants (20H12, 42B9, 25C8 and 16G6). Site-directed mutagenesis, genetic complementation and phenotypic analysis indicated that modulation of sulfur assimilation plays a role in plant growth promotion and ISR by \textit{Pf}.SS101. Arabidopsis transcriptome analysis showed that genes involved in the cysteine biosynthesis and sulfur-containing compounds biosynthesis were induced by \textit{Pf}.SS101 but not by a mutant of \textit{Pf}.SS101 (20H12, \textit{ΔcysH}). Van de Mortel \textit{et al.} (2012) showed that \textit{Pf}.SS101 induces resistance against \textit{Pst} and the insect \textit{Spodoptera exigua} by boosting the levels of indolic and aliphatic glucosinolates in Arabidopsis. All together, the transcriptome analyses suggest that also \textit{Pf}.SS101 modulates auxin biosynthesis and transport, steroid biosynthesis, carbohydrate metabolism next to sulfur assimilation in Arabidopsis (Chapter 6).

Future perspectives

The GacS/GacA two-component system (the Gac-system) plays a central role in the regulation of the production of several extracellular bioactive metabolites and exoenzymes. Overall, the Gac-system mediates a wide range of biological functions in many bacteria, including \textit{Pseudomonas}. Recently, Song \textit{et al.} (2015) showed that RsmY/RsmZ, controlled by the Gac-system, regulates lipopeptide biosynthesis and transcription of other genes that play important roles in motility, competition and survival of \textit{Pf}.SS101 (Song \textit{et al.}, 2015). Identification of new metabolites by exploiting mutants with defects in biosynthetic or regulatory genes is a promising strategy (Rigali \textit{et al.}, 2008, Laureti \textit{et al.}, 2011). In this thesis, we followed this strategy and demonstrated that mutagenesis of a global regulatory gene is a powerful tool for identifying novel genes, bioactive compounds and biosynthetic pathways involved in microbe-microbe interactions and microbe-plant interactions. It is important to find the yet unknown signals or signal
molecule(s) that are sensed by GacS and further activate the downstream signal transduction pathways. Comparative genomic analysis has shown 761 unique genes in the Pf. SBW25 genome as compared to nine other P. fluorescens genomes (Loper et al., 2012). Comparison of biosynthetic/catabolic genes or gene clusters in these P. fluorescens strains showed that the Pf.SBW25 genome carries much less genes involved in biosynthesis of well-known bioactive compounds (Loper et al., 2012). However, by analysing the properties of the gacS mutant of Pf.SBW25, we observed enhanced antimicrobial activity. In addition, we found that a mutation in PQQ biosynthesis genes, in combination with the GacS-mutation, results in a strongly enhanced capacity to promote Arabidopsis growth as compared to the wild type and the single mutant strains. These results indicate that there are other mechanisms and metabolites involved in plant growth promotion that are yet to be discovered.

Microbial VOCs attract more and more attention in the last decades. These VOCs have been reported to promote plant growth and to inhibit pathogen growth, directly as antagonistic compounds or indirectly as elicitors of ISR (Ryu et al., 2003, Blom et al., 2011, Kim et al., 2013, Cordovez et al., 2015, Sharifi & Ryu, 2016). The bioactive VOCs are mainly detected by GC/MS analysis of the headspace of bacterial cultures. However, to date little is known about the regulation of VOC biosynthesis genes or pathways and VOC production in situ. In this thesis, we showed that in bacteria the Gac-system not only regulates production of secondary metabolites and exoenzymes but also production of VOCs. By combining various approaches, including mutant analysis, chemical analysis, targeted mutagenesis and comparative genomics more VOCs biosynthesis genes and novel VOCs will be discovered. Given the specific features of VOCs, such as their proposed ability to travel over long distances in a soil matrix, they will give rise to new types of applications in agricultural systems. In conclusion, a better understanding of yet unknown mechanisms exploited by the various Pseudomonas fluorescens strains will lead to new opportunities for the discovery and application of natural bioactive compounds for both industrial and agricultural purposes. To further extend our knowledge from a more ecological perspective, effects of bacterial consortia on disease control and plant growth need to be studied to achieve a more robust and durable strategy to enhance plant growth and health.
Summarizing discussion and future perspectives

References


Chapter 7


Summarizing discussion and future perspectives


Chapter 7


Summary
Pseudomonas fluorescens is a Gram-negative rod shaped bacterium that has a versatile metabolism and is widely spread in soil and water. *P. fluorescens* strain SBW25 (*Pf.*SBW25) is a well-known model strain to study bacterial evolution, plant colonization and biocontrol of plant diseases. It produces the biosurfactant viscosin, a lipopeptide that plays a key role in motility, biofilm formation and activity against zoospores of *Phytophthora infestans* and other oomycete pathogens. In addition to viscosin, *Pf.*SBW25 produces other metabolites with activity against plant pathogens. The production of these yet unknown metabolites appeared to be regulated by the GacS/GacA two-component regulatory system (the Gac-system). The second *P. fluorescens* strain SS101 (*Pf.*SS101) studied in this thesis is known for its plant growth-promoting activities but the underlying mechanisms and genes are largely unknown. The objectives of this PhD thesis were to identify novel metabolites and biosynthetic genes in *Pf.*SBW25 and *Pf.*SS101, and to investigate their role in plant growth promotion and biocontrol. To this end, a multidisciplinary approach involving bioinformatic analysis of the genome sequences of strains *Pf.*SBW25 and *Pf.*SS101, microarray-based expression profiling, screening of genomic libraries, bioactivity assays, mass spectrometric image analysis (MALDI-IMS) and GC/MSMS analysis was adopted.

In *Pseudomonas*, the two-component system GacS/GacA (the Gac-system) regulates the biosynthesis of multiple extracellular antimicrobial compounds. In this thesis, we investigated the effects of a *gacS* mutation on the transcriptome and antimicrobial activities of *Pf.*SBW25. Unexpectedly, the *gacS* mutant of *Pf.*SBW25 showed enhanced and broad-spectrum antimicrobial activity despite the fact that it no longer produces several of the known antimicrobial compounds. Transcriptional analysis of the *gacS* mutant showed that expression of approximately 700 genes was significantly up- or down-regulated. These included genes involved in type II secretion, (exo)polysaccharide and pectate lyase biosynthesis, twitching motility, and an orphan nonribosomal peptide synthetase (NRPS) gene cluster predicted to encode an 8-amino acid ornicorrugatin-like peptide. To unravel the underlying mechanism of the increased antimicrobial activity of the *gacS* mutant, a random mutant library was screened for loss of function mutants. This led to the identification of genes involved in the pyrroloquinoline quinone (PQQ) biosynthesis pathway. Their enhanced expression in the *gacS* mutant resulted in an increase of gluconic and 2-ketogluconic acid, which in turn acidified the surrounding medium.
Summary
to pH levels that inhibited the growth of other microorganisms. Further analysis of the \textit{gacS} mutant showed that the Gac-system also regulates the production of volatile organic compounds (VOCs) in \textit{Pf}.SBW25, in particular the acyclic alkenes 1-undecene, 3-nonene and 4-undecyne. Moreover, Gac-mediated VOCs production by \textit{Pf}.SBW25 affected plant growth promotion in a plant species-dependent manner. We also studied the effects on plants by directly inoculating \textit{Pf}.SBW25 and the \textit{gacS} mutant on roots of Arabidopsis, tobacco and cucumber and observed changes in root architecture as well as root biomass. To better understand the importance of the Gac-system in the chemical interplay between \textit{Pf}.SBW25 and plants, we subsequently used MALDI-IMS to detect and visualize metabolites produced by the bacteria during the interaction with Arabidopsis roots. This revealed that the metabolite profile of \textit{Pf}.SBW25 changes when grown on Arabidopsis roots and that it produces viscosin and several other metabolites, tentatively identified as dodecane and acetoxyphloretin. Comparison of the metabolites produced by \textit{Pf}.SBW25 and the \textit{gacS} mutant suggests that a mutation in the Gac-system abolishes production of certain compounds such as viscosin, but at the same time boosts production of other compounds, including gluconic acid and pyoverdine. For \textit{Pf}.SS101, high-throughput screening of a random mutant library (7,488 mutants) led to the identification of several mutants without plant growth promoting and ISR activities. These loss of function mutants were disrupted in genes involved in amino acid biosynthesis, glucose utilization, transcription or sulfur assimilation. Subsequent bioassays and comparative plant transcriptomics analyses indicated that modulation of auxin biosynthesis and transport, steroid biosynthesis, carbohydrate metabolism and sulfur assimilation in Arabidopsis are key mechanisms associated with growth promotion and ISR by \textit{Pf}.SS101.

In conclusion, the results presented in my thesis showed that the Gac-system in \textit{P. fluorescens} regulates the biosynthesis of various secondary metabolites that play a role in microbe-microbe and microbe-plant interactions. We further showed that plant growth promotion by \textit{P. fluorescens} is associated with alterations in auxin biosynthesis and transport, steroid biosynthesis, carbohydrate metabolism and sulfur assimilation. Moreover, advanced chemical profiling allowed us to compare the metabolite profiles of free-living \textit{P. fluorescens} and \textit{P. fluorescens} living in association with plant roots. The identity of only a few of these metabolites could be elucidated. Many more are waiting to be identified and to be tested for their
Summary

biological activities in the rhizosphere. How the plant genotype shapes the dynamics and activities of *P. fluorescens* and other microorganisms in the rhizosphere will be subject of future studies.
Acknowledgements
Curriculum Vitae
Publications
Acknowledgements

Acknowledgements

When the first time I arrived in Wageningen to continue my B.S.c study in December of 2003, I could have never imagined that this small city would be my second hometown in the following 13 years. I was very lucky for meeting many people and made friends with many of them during these years! Thank you all very much! In this thesis, I would like to specifically express my profound gratitude to those who have helped and supported me to find my way out during my PhD period in both scientific work and my personal life.

First of all, I would like to thank my supervisor and promoter, Prof. Jos Raaijmakers, for appointing me as the PhD student in the EcoLinc project. I still remember the first time of meeting and talking with you. I was inspired by your knowledge and passion for science. And also, the way how you present things are very attractive. I’m very appreciate for the trainings I got from you on presentation skills, communication and team work skills, and writing skills. Thank for your guiding me patiently during my PhD period and also thank you very much for all helps and support you have provided to me!

Second, I would like to thank my promoter, Prof. Francine Govers, for the critical comments on my conference presentations, propositions and my thesis writing. Besides that, thank you also for your attention and suggestions on both my work and life.

Third, I would like to thank my co-promoter, Menno van der Voort. We met each other on basketball court a few years ago, I was very happy for working with you in these years during my PhD study. Thank you very much for all your critical comments on my work, for spending so much time with me discussing research and thank you for supporting me on writing papers and this thesis.

I also want to thank my external supervisor, Prof. Fons Stams, for the discussion and support on my research progress. Thanks to our fantastic collaborators, Joyce Loper, Pieter Dorrestain, Jeramie Watrous, Janina Oetjen, Theodore Alexandrov. Thank you for all contributions to the thesis.

Then, I would also like to thank all my colleagues from the Laboratory of Phytopathology, it was my pleasure to conduct my PhD with all of you around. Special thanks to all the Beggers (B&EG group members). Rodrigo Mendes, thank you for
Acknowledgements

being my first daily supervisor, although you left to Brazil around half a year after
I start my PhD. I definitely expressed by the way how you work and I learned a lot
from you! Judith van de Mortel, thank you for training me on plant assays and also
thank you for being supportive when I experiencing difficulties during my PhD life.
Chunxu Song宋春旭, thank you for sharing knowledge and experiences with me
during work. Also, thank you and Wei覃伟, for the encouragement and support
to conquer the difficulties in my work and also my personal life. I will never forget
the nice food you cooked, especially the hotpot! Thank you our technician, Ester
Dekkers, for all kind of help on the work during my PhD life. Emilie Chapelle, Vic-
tor Carrion, Desalegn Etalo, Yiying Liu刘懿莹, Juan Esteban Pérez Jaramillo, Allison
Jack, Kumar Aundy, Le Nhu Cuong, Manuela Vicente Dominguez, Melissa Sarmien-
to, Anne Coon, Christin Zachow, Janne Louize. Thank you for making our group a
family! I had a great time with all of you! I would like to thank all my B.S.c and M.S.c
students as well for your contribution to this thesis!

Thanks to all the Phyto-Chinese. Lisha Zhang张丽莎, Chenlei Hua华辰雷, Yu
Du杜羽, Yan Wang王燕, Chunxu Song宋春旭, Zhao Zhang张钊, Miao Han韩淼,
Yin Song宋银, Guozhi Bi毕国志, Xiaolin Wu吴晓林, Xiaoqian Shi史小倩, Guodong
Wang王国栋, Jinling Li李金岭, Huayi Li李华一, Jinbin Wu吴锦斌. It was great for
having all of you around. Thanks for forming the great atmosphere at work, all nice
dinners and wonderful time together.

Special thanks to Lisha Zhang张丽莎, Chenlei Hua华辰雷 and Feng Zhu朱峰,
my buddies, I’m so lucky because I meet you in Wageningen. Thank you for your full
support at my work and also in my daily life. I will not forget our agreement that to
ruan a conteen for our future Faculty of Life Sciences! And for sure, we will go to
KFC together again and again, because I like the taste when you are there!

Many thanks to my Chinese friends: Chunxu Song宋春旭, Wei Qin覃伟, Ke
Peng彭珂, Chunhui Zhou周春晖, Lisong Ma马利松, Fang Xu徐方, Tingting Xiao
肖婷婷, Wei Liu刘巍, Qiushi Wang王秋实, Si Wang王思, Hequn Li李鹤群, Yuan-
chuan Zhang张塬川, Xin Li李欣, Kaile Sun孙凯乐, Xueping Chen陈雪平, Xiaoxue
Sun孙晓雪, Yajun Wang王亚军, Yiqian Fu傅一倩, Xiao Lin林啸, Jia Ning宁佳, Wei
He贺玮, Chidu Huang黄持都, Xingxing Wang王鑫鑫, Yang Yu于洋, Ming an Yao
要明天, Yong Hou候勇, Zhaohai Bai柏兆海, Junqi Zhu朱俊奇, Fang Guo苟芳,
Wenfeng Cong从汶峰, Jingying Jing荆晶莹, Junfei Gu顾俊飞, Guohua Li李国华,
Acknowledgements

Jingmeng Wang, Qian Liu, Xuan Xu, Fengfeng Wang, Yun-meng Zhang, Shuhang Wang, Chunzhao Zhao, Zheng Zheng, Dan Yan, Ningwen Zhang, Ke Lin, Ting Yang, Qing Liu, Cheng Liu, Pingping Huang, Wei Song, Lemeng Dong, Hui Li, Yanxia Zhang, Hanzi He, Bing Bai, Xianwen Ji, Jun He, Xi Cheng, Xi Chen, Weicong Qi, Juan Du, Lijin Tian, Zhaoying Li, Junyou Wang, Junli Guo, Si Zhou, Ting Hieng Ming, Lin Ya-Fen, Gao Wei, Songlin Xie, Suxian Zhu, Liping Gao, Zhengyi Lin, Qi Qi, Lu Yu, Yingying, Zhanguo Bai, Wenbiao Shi, Lu Chen, Jing Tang, Yuan He, Lu Luo, Hui Tian, Yuling Bai, Liping Weng, Yutian Lu, Wenbiao Shi, Lu Chen, Jing Tang, Yuan He, Lu Luo, Hui Tian, Yuling Bai, Liping Weng, Zhanguo Bai, Xi Wan, Xinyou Yin.

Thank you all for warm talks, happy hours and all kinds of supports during my stay in Wageningen. You made here like my home!

Thank you very much!

Thanks Dr Rene Geurts and Prof. Ton Bisseling for offering me the position to start my Post-doc research from the 1st of June, 2014 in the Laboratory of Molecular Biology, WUR. Thank you for giving me a lot of flexibility and support while the writing period. Thanks to our Rhizo group members: Carolien Franken, Martinus Schneijderberg, Yuda Roswanjaya and Zhichun Yan. Without your understanding and enormous support, I would not have finished writing my PhD thesis.

At the end, I would like to express my deepest gratitude to my parents for their love, understanding and support! 感谢父母的养育之恩。感谢我的爸爸妈妈，是你们的鼓励，支持，理解以及言传身教，伴随着我的成长！在我遇到困难的时候，你们总是给我无限的安全感和家的温暖，鼓励和支持我一路向前！从2001年至今这十五年，我很少在家陪伴你们。每周的视频聊天始终传递着你们对我的关心，理解和支持！感谢你们对我的包容，感谢你们对我人生选择的尊重和支持！希望我可以永远和你们分享人生的快乐！祝你们幸福，安康！
Xu Cheng (程旭) was born on 17th of April, 1983 in Handan of China. In 2001, he started his undergraduate study in Biotechnology at China Agricultural University in Beijing. In 2003, he moved to the Netherlands to continue his B.S.c study in Biotechnology at Wageningen University. After receiving his B.S.c degree with a major in Biotechnology and minor in Genetics, he continued with a M.S.c study in Biotechnology and specialized in Environmental Biotechnology at Wageningen University. During his master, Xu conducted his major thesis research under supervision of Dr Worm Petra, Dr Caroline Plugge and Prof. Fons Stams in the laboratory of Microbiology at Wageningen University. Afterwards, he spent a 6-month internship under supervision of Dr Jennifer Nelson and Prof. Stephen Zinder in the laboratory of Microbiology at Cornell University, U.S.A. From 2009, he started working on Plant Growth Promotion Rhizobacteria under supervision of Prof. Jos Raaijmakers and Dr Judith van der Mortel in the laboratory of Phytopathology at Wageningen University. From 2010, he worked as a PhD student of the EcoLinc project on Plant Growth Promotion by *Pseudomonas fluorescens* under supervision of promoters Prof. Jos Raaijmakers and Prof. Francine Govers, and co-promoter Dr Menno van der Voort. His PhD project is presented in this thesis. Since June 2014, he has been working as a Postdoc on microbial support for plant growth under abiotic stresses with Dr Rene Geurts and Prof. Ton Bisseling in the Laboratory of Molecular Biology at Wageningen University.
Publications

Publications


Education Statement of the Graduate School  
Experimental Plant Sciences

Issued to: Xu Cheng  
Date: 19 September 2016  
Group: Laboratory of Phytopathology  
University: Wageningen University & Research

1) Start-up phase  
- First presentation of your project  
  Title: ‘Mining bacterial genomes for novel traits and bioactive compounds’  
  Apr 26, 2010  
- Writing or rewriting a project proposal  
  Title: ‘Mining bacterial genomes for novel traits, bioactive compounds and biosynthetic pathways’  
  2010  
- Writing a review or book chapter  
- MSc courses  
- Laboratory use of isotopes  

Subtotal Start-up Phase 7.5 credits*

2) Scientific Exposure  
- EPS PhD student days  
  PhD student day, Utrecht University  
  Jun 01, 2010  
  PhD student day, Wageningen University  
  May 20, 2011  
  PhD student day, Amsterdam University  
  Nov 30, 2012  
- EPS theme symposia  
  EPS theme 2 symposium ‘Interactions between Plants and Biotic Agents’, Utrecht University  
  Jan 15, 2010  
  EPS theme 2 symposium ‘Interactions between Plants and Biotic Agents’, Amsterdam University  
  Jan 03, 2011  
  EPS theme 2 symposium ‘Interactions between Plants and Biotic Agents’, together with Willie Commelin Scholten Day, Wageningen University  
  Feb 10, 2012  
  EPS theme 2 symposium ‘Interactions between Plants and Biotic Agents’, together with Willie Commelin Scholten Day, Utrecht University  
  Jun 24, 2013  
  EPS theme 2 symposium ‘Interactions between Plants and Biotic Agents’, together with Willie Commelin Scholten Day, Amsterdam University  
  Feb 25, 2014  
- Lunteren days and other National Platforms  
  Omics Meeting, 2010, RIVM Bilthoven, the Netherlands  
  Apr 15, 2010  
  Annual meeting ‘Experimental Plant Sciences’, Lunteren, NL  
  Apr 14-15, 2014  
- Seminars (series), workshops and symposia
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<tr>
<td>Phytopathology Seminars</td>
<td>2010-2014</td>
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<td>Invited seminar Prof. Nick Panopoulos</td>
<td>Jan 11, 2010</td>
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<td>Invited seminar Laurent Zimmerli</td>
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<td>Plant Sciences Seminar Prof. Holger Meinke and Prof. Paul Struik</td>
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<td>Plant Sciences Seminar Prof. Louise Vet and Just Vlak</td>
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<td>Wageningen Evolution and Ecology Seminars Toby Kiers</td>
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<td>Invited seminar Prof. Naoto Sibuya</td>
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<td>Invited seminar Prof. David Baulcombe</td>
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<td>Invited seminar Dr. Kirsten Bomblies</td>
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<td>1st meeting WUR-NIOO Centre for Soil Ecology, Wageningen</td>
<td>Jun 27, 2011</td>
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<td>Invited seminar Rosie Bradshaw</td>
<td>Aug 04, 2011</td>
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<td>biological and chemical aspects</td>
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<td>Invited seminar Gabriele Berg: Plant microbes: Specificity and</td>
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<td>WEES seminar Bertus Beaumont: Adaptive radiation, flagella and</td>
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<td>Seminar: Evolution in the laboratory</td>
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<td>WEES seminar David Berry: Ecological and evolutionary aspects of</td>
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<td>the gut microbiota in health and inflammation</td>
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<td>and cooperation between strains and species of bacteria</td>
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<td>their application via metabolomics and genomics</td>
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<td>Invited seminar Gabriele Berg</td>
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<td>PacBio Seminar</td>
<td>Mar 26, 2014</td>
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<td>EPS Symposium ‘Oomics Advances for Academia and Industry -</td>
<td>Dec 11, 2014</td>
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<td>Towards True Molecular Plant Breeding’</td>
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► Seminar plus

► International symposia and congresses

Conference Ecology of Soil Microorganisms, Prague, the Czech Republic | Apr 27-May 01, 2011

The 14th International Symposium on Microbial Ecology (ISME), Copenhagen, Denmark | Aug 19-24, 2012

The 10th International Congress of Plant Pathology, Beijing, China | Aug 25-30, 2013

The 4th Rhizosphere conference (Rhizo4), Maastricht, NL | Jun 21-25, 2015

► Presentations

PhD Summer school: Rhizosphere signaling (Poster) | Aug 23-25, 2010

Conference Ecology of Soil Microorganisms, Prague, the Czech Republic (Poster) | Apr 27-May 01, 2011
### 3) In-Depth Studies

**EPS courses or other PhD courses**
- Ph.D Summerschool: Rhizosphere signaling
  - Aug 23-25, 2010
- PhD course ‘Ecological Genomics’
  - Feb 14-18, 2011
- Ph.D Autumnschool: Host-Microbe-Interactomics
  - Nov 1-3, 2011

**Journal club**
- Literature discussion - Molecular Ecology Group of Phytopathology
  - 2010-2015

**Individual research training**
- Mass spectrometric analysis (MALDI-Imaging analysis), Dorrestein’s Lab, University of California, San Diego, U.S.A
  - Oct 2013-Feb 2014

**Subtotal In-Depth Studies**

9.8 credits*

### 4) Personal development

**Skill training courses**
- Information Literacy PhD including EndNote Introduction
  - Apr 27-28, 2010
- Project and time management
  - Apr-Jun 2012
- Techniques for Writing and Presenting a Scientific Paper
  - Dec 11-14, 2012
- Last Stretch of the PhD Programme
  - Aug 02, 2013
- Career Orientation
  - Oct-Dec, 2013
- Reviewing a scientific paper
  - Mar 05, 2015

**Organisation of PhD students day, course or conference**
- 2011 Organizing labouting
  - May 24, 2011

**Membership of Board, Committee or PhD council**

**Subtotal Personal Development**

5.9 credits*

**TOTAL NUMBER OF CREDIT POINTS**

45.4

Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS which comprises of a minimum total of 30 ECTS credits

*A credit represents a normative study load of 28 hours of study.*

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This research was conducted in the Laboratory of Phytopathology of Wageningen University and was financially supported by the Netherlands Genomics Initiative (NGI-EcoLinc project).