Plant growth promotion by *Pseudomonas fluorescens*

mechanisms, genes and regulation

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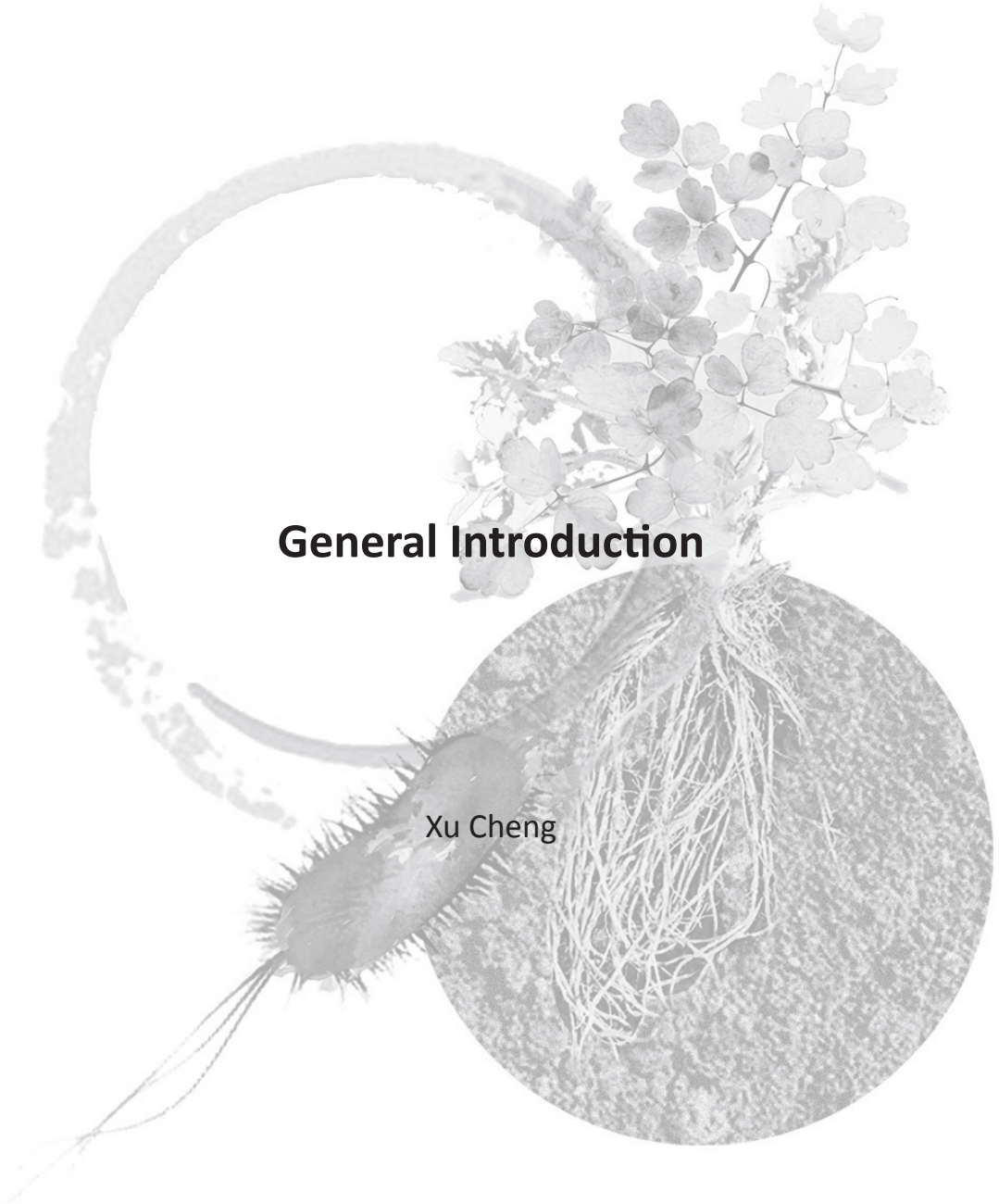
Chapter 1



General Introduction

Xu Cheng

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 ~10^8-10^9 for bacteria to ~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 ammoniawhen 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 toplants. 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 system 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 (*Cucumissativus*), radish (*Raphanussativus*), tobacco (*Nicotianatabacum*) 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_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.

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 info-chemicals 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.

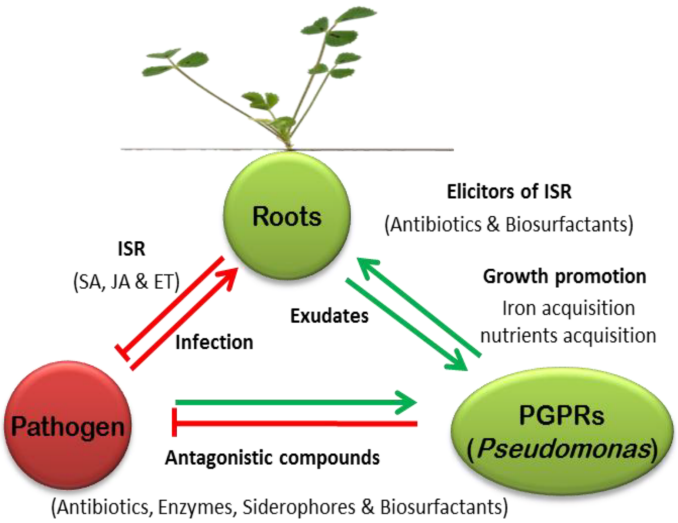


Figure 1 Overview of multitrophic interactions in the rhizosphere. The *Pseudomonas* genus represents PGPRs as predominant inhabitants of the rhizosphere. *Pseudomonas* PGPR strains positively affect plant growth and health by inhibiting the growth of plant pathogens and/or indirectly by inducing systemic 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*.

Pseudomonas species possess many traits to do so including high growth rates, a broad susbtrate 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).

Chapter 1

Pseudomonas genomics and phylogeny

1

The genus *Pseudomonas* currently comprises more than 144species. Based onmultilocus sequence typing (MLST) analysis, these have been divided into tenmajor 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 fo rthe 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.

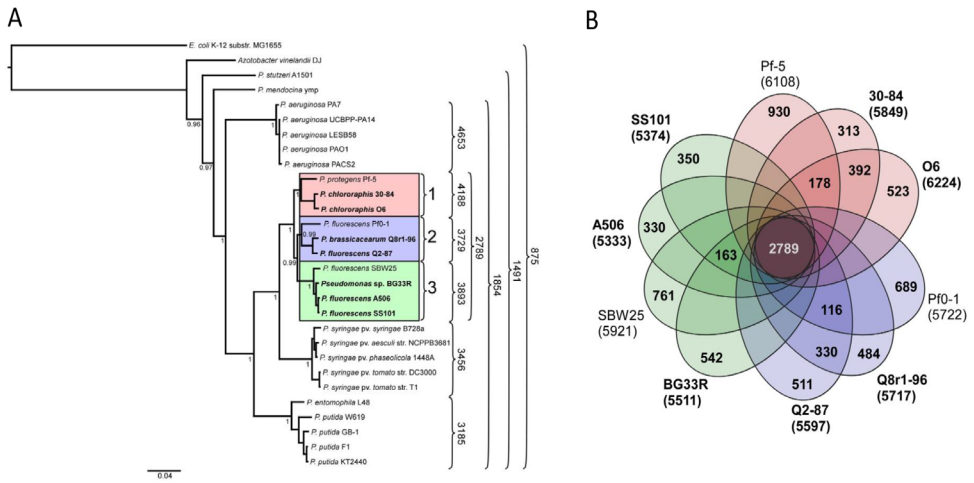


Figure 2 This figure is reprinted from Loper et al. 2012. A) Phylogenetic analysis of *Pseudomonas* species. The 10 plant associated strains fall into tree clades (highlighted). The numbers on the right refer to the number of genes shared by the species and strains grouped; B) Venn diagram based on comparative 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.

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 *Geaumannomyces 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

(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, Kruijt *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

nif genes, have also been found in some *Pseudomonas* species. By analysis of the bacterial population in the rhizosphere of cord grass (*Spartinaalterniflora*), *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 ($H_2PO_4^{-1}$) and the dibasic (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 solubilizationis 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

Table 1 Examples of volatile organic compounds (VOCs) produced by *Pseudomonas* PGPRs and their biological functions in antagonism and plant growth promotion.

Pseudomonas PGPRs	VOCs	Biological functions	References
P. chlororaphis	Benzothiazole	Antifungal	Fernando et al., 2005
	Cyclohexanol	Antifungal	
	n-Decanal	Antifungal	
	Dimethyl trisulfide	Antifungal	
P. chlororaphis & *P. aurantiaca*	Nonanal	Antifungal	Fernando et al., 2005
P. fluorescens *P. corrugata* *P. chlororaphis* *P. aurantiaca*	2-Ethyl,1-hexanaol	Antifungal	Fernando et al., 2005
	1-Heptedecanol		
	1-Undecene	Plant growth promotion	
	2-Methyl pyrazine		
	2-Tridecanone		
	2-Undecanone		
	4-Octybenzoic acid		
	Benzaldehyde		
	Decane		
	Dodecane		
	Hexadecane		
	Nonadecane		
	Nonane		
	Pentadecane		
	Phenylenediamine		
	Pyrazine		
	Tetradecane		
	Undecane	Plant growth promotion	
P. fluorescens SS101	2-Methyl-1-propanol, 3-methyl-1-butanol, 13-Tetradecadien-1-ol, 1-Cyclohexyl-1-pentanol Trans-9-Hexadecen-1-ol	Plant growth promotion	Park et al., 2015
	1-Nonene		
	2-Methyl-n-1-tridecene	Plant growth promotion	
	2-Butanone	Plant growth promotion	
	Methylthiolacetate		
	Decyl-Oxirane		
	2,5-dimethylpyrazine		
P. chlororaphis O6	2,3-butanediol	Induction of drought tolerance	Cho et al., 2008
P. fluorescens B-4117 *P. fluorescens Q8r1-96*	Methanethiol	Inhibit growth of *Agrobacterium tumefaciens* and *A. vitis*	Dandurishvili et al., 2010
	Methylthiolacetate		
	Dimethyldisulfide		
	1-Undecene		
P. fluorescens strain ALEB 7B	Dimethyldisulphide 2-Piperidinone	Controls southern blight caused by *Athelia rolfsii*	Zhou et al., 2014
P. aeruginosa PUPa3	2-aminoacetophenone	Plant growth promotion	Blom et al., 2011
P. chlororaphis *P. fluorescens* WCS 417r	1-hexanol, 1-heptanol	Plant growth promotion	Blom et al., 2011

(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.

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 Tn*5*-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**

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

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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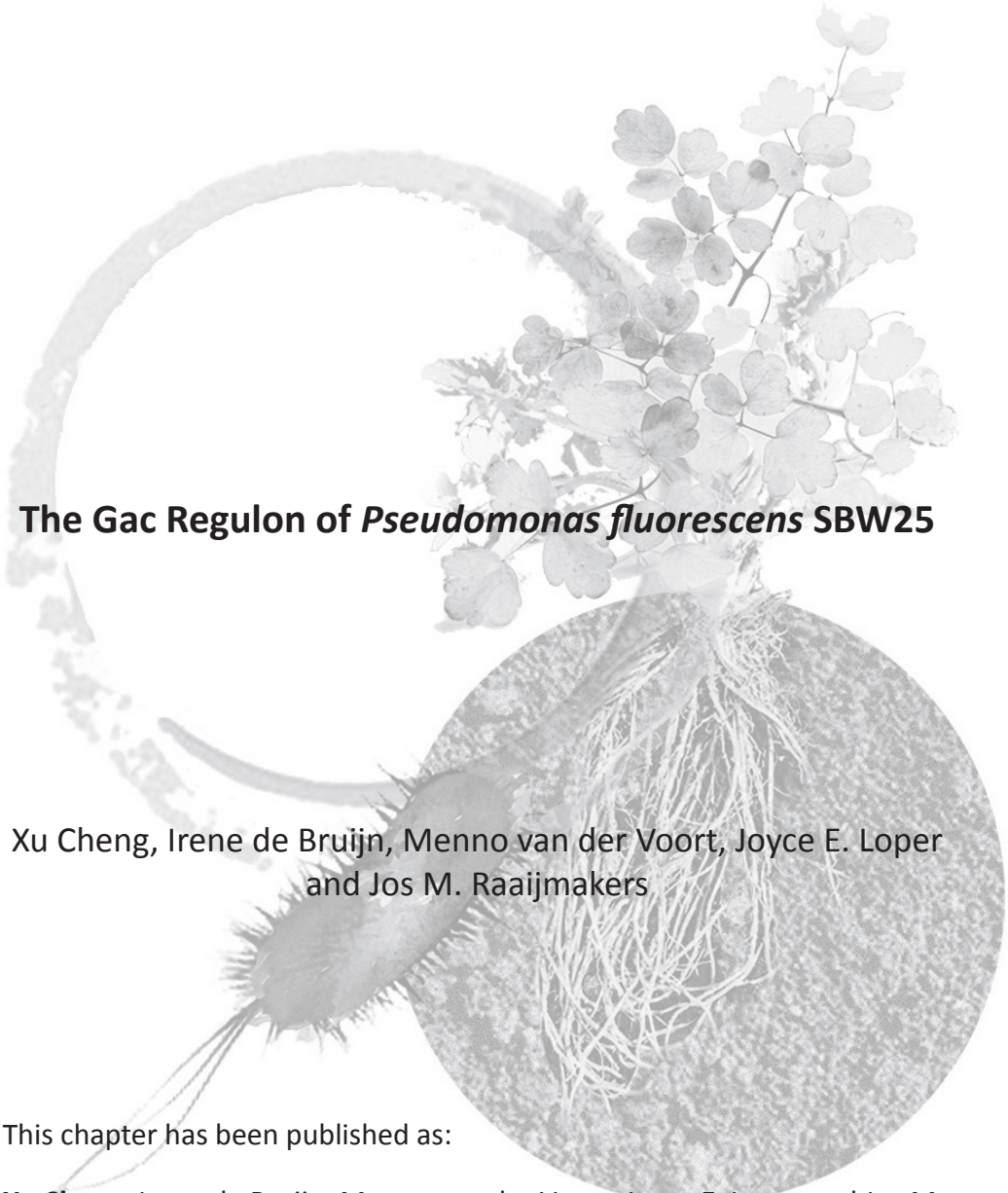
Chapter 2



The Gac Regulon of *Pseudomonas fluorescens* SBW25

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Abstract

Transcriptome analysis of *Pseudomonas fluorescens* SBW25 showed that 702 genes were differentially regulated in a *gacS*::Tn*5* 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, t*he 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 Tn*5*-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 Tn*5* plasposon mutagenesis using plasmid pTn*Mod*Okm (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* SCRI1043 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^{o}C; *Pythium aphanidermatum*, *Aphanomyces cochloides* at 25^{o}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 ^{o}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 C_{18} 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).

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 *Eco*RI. 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 1/5th 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 1/5th 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 1/5th 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

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 Brencic 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: Δ*CT* = *CT* (gene) - *CT* (*rpoD*). The relative quantification (RQ) values were calculated with the formula $RQ = 2^{[\Delta CT \text{ (mutant)} - \Delta CT \text{ (wild type)}]}$ (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 PLFU3225 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 *Bam*HI and *Hin*dIII, cloned into *Bam*HI and *Hin*dIII digested NucleoSpin-purified pEX18Tc (Choi and Schweizer, 2005) and

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 $MgSO_4$ 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 K_2HPO_4, 22 mM KH_2PO_4, 7.6 mM $(NH_4)_2SO_4$, 0.8 mM $MgSO_4$, 34 mM succinic acid, adjusted to pH 7 with NaOH). Overnight cultures were washed once and 5 ml of a cell suspension ($1*10^{10}$ 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

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 1/5th strength PDA plate with different amounts of H_2O_2 (0, 0.2, 0.5, 1.0 µM, respectively). Inoculated plates were incubated at 25 ^oC 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 1/5th 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 1/5th 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 1/5th 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 1/5th 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 ^oC 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.*

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 10min. 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 8mm thick sections using a sterile knife. Each section was placed into a Petri dish with sterile filter paper that has been inoculated with 1ml 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 Tn*5*-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).

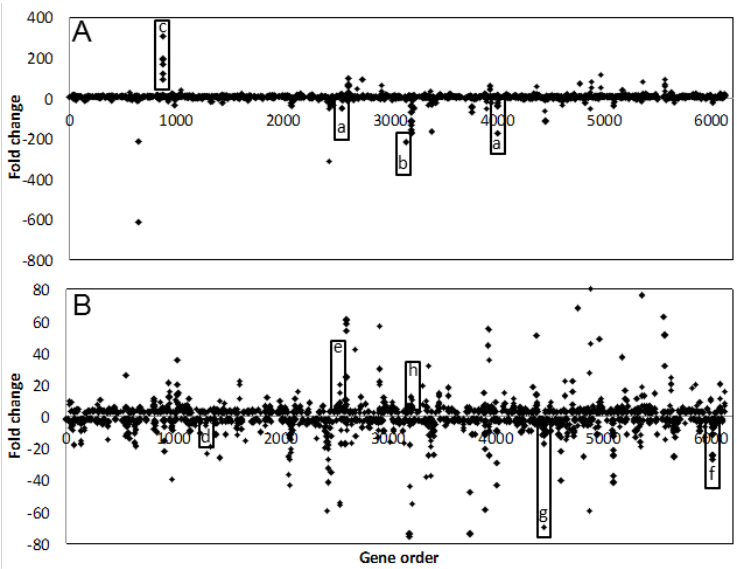


Figure 1 Genome-wide representation of the genes most differentially expressed in the *gacS* mutant of *Pseudomonas fluorescens* SBW25. Each point is one of the annotated genes in the SBW25 genome, with the X-axis showing gene order (the origin of replication at 0 and 6009), and the Y-axis showing the 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.

Role categories	# of CDSs	% of CDSs	Transcript level in *gacS* mutant vs. wildtype			
			Up-regulated		Down-regulated	
			#	%	#	%
A Amino acid synthesis	167	2.8	4	2.4	10	6.0
B Biosynthesis of cofactors, prosthetic groups, and carriers	203	3.4	4	2.0	6	3.0
C Cell envelope	499	8.3	31	6.2	50	10.0
D Cellular processes	502	8.4	31	6.2	77	15.3
E Central intermediary metabolism	297	4.9	12	4.0	23	7.7
F DNA metabolism	170	2.8	3	1.8	8	4.7
G Energy metabolism	709	11.8	32	4.5	49	6.9
H Fatty acid and phospholipid metabolism	180	3.0	14	7.8	17	9.4
I Hypothetical proteins	827	13.8	41	5.0	58	7.0
J Mobile and extrachromosomal element functions	97	1.6	5	5.2	6	6.2
K Protein fate	283	4.7	18	6.4	22	7.8
L Protein synthesis	201	3.3	2	1.0	4	2.0
M Purines, pyrimidines, nucleosides, and nucleotides	73	1.2	0	0.0	4	5.5
N Regulatory functions	719	12.0	29	4.0	31	4.3
O Signal transduction	138	2.3	8	5.8	8	5.8
P Transcription	101	1.7	13	12.9	3	3.0
Q Transport and binding proteins	1030	17.1	90	8.7	59	5.7
R Unclassified	129	2.1	10	7.8	7	5.4
S Unknown function	704	11.7	11	1.6	19	2.7
Total number of CDS	6009		300		402	

Comparison of the Gac regulons of *P. fluorescens* SBW25 and other *Pseudomonas* species

Comparison of our transcriptome data with that of *P. protegens* Pf-5 (Hassan et al., 2010) and of *P. aeruginosa* (Brencic et al., 2009) showed that most type VI secretion system (T6SS) genes were significantly down-regulated in the *gacA/S* mutants of all three species (Table S4) and represent one of the few conserved effects. Similar to the *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 *gacS* mutant of SBW25. Other functions that were affected include TonB-dependent signalling and energy metabolism (Tables S4, S5). In contrast to *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 *P. brassicacearum* subsp. *brassicacearum* NFM421 (Lalaouna et al., 2012), genes involved in chemotaxis (PFLU5092-5093; PSEBR_a3441-3442) were also down-regulated like in SBW25. For *P. syringae* pv. *phaseolicola* 1448A (De la Torre-Zavala et al., 2011), the gene encoding adenylyl-sulfate kinase (PFLU2097; PSPPH_4301), and for *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 *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 *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 *gacA* mutant instead of a *gacS* mutant. Even though the inactivation of *gacS* might result in downstream effects that differ from those of a *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

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 *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.

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, swarming 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

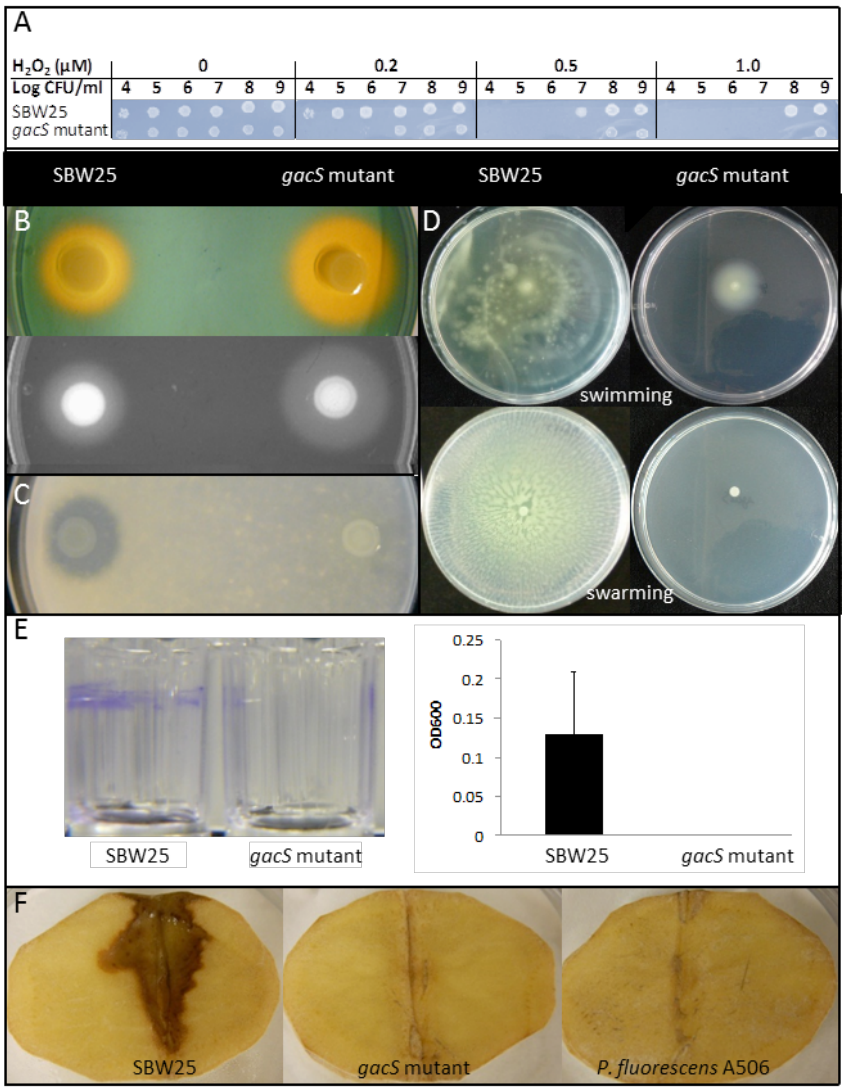
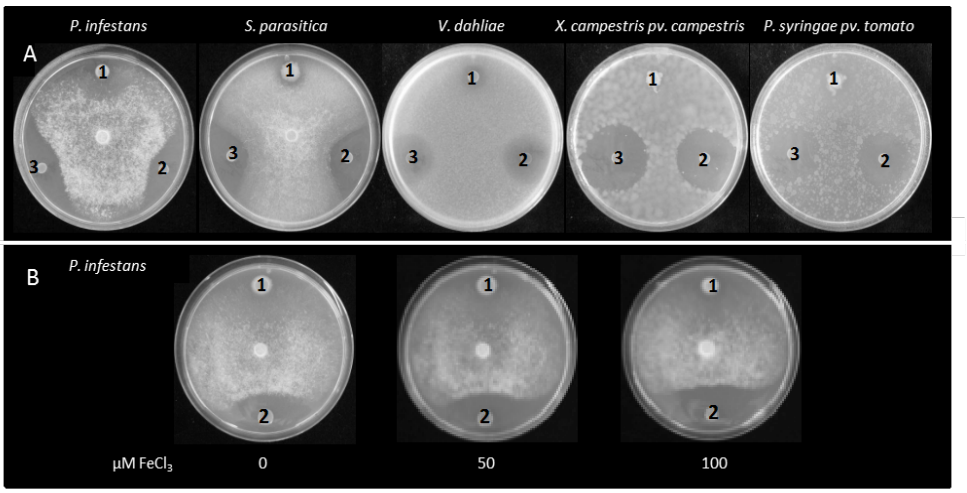


Figure 2 Phenotypic characterization of the wild type strain and the *gacS* mutant of *P. fluorescens* SBW25. (A) Sensitivity of SBW25 and the *gacS* mutant to hydrogen peroxide. Five µl of cell suspensions (initial densities ranging from 4 to 9 Log CFU/ml) of SBW25 and the *gacS* mutant were spot inoculated 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 *gacS* mutant exhibited stronger and more distinct inhibition of mycelial growth of *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 *gacS* mutant (Figure 3A, Table S6). Collectively, these results show that inactivation of *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 *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).



1. SBW25 wild type strain; 2. *gacS* mutant; 3. *gacS* mutant/Δ*3225*

Figure 3 (A) *In vitro* activity of wild type *P. fluorescens* SBW25, its *gacS* mutant and the *gacS*/Δ3225 double mutant against the oomycetes *Phytophthora infestans* and *Saprolegnia parasitica*, the fungus *Verticillium dahliae* and the bacteria *Xanthomonas campestris* pv. *campestris* and *Pseudomonas syringae* pv. *tomato*. (B) Activity of wild type SBW25 and the *gacS* mutant against *Phytophthora infestans* on agar plates supplemented with 0, 50 or 100µM of $FeCl_3$.

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 1/5th 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 *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 *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 *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

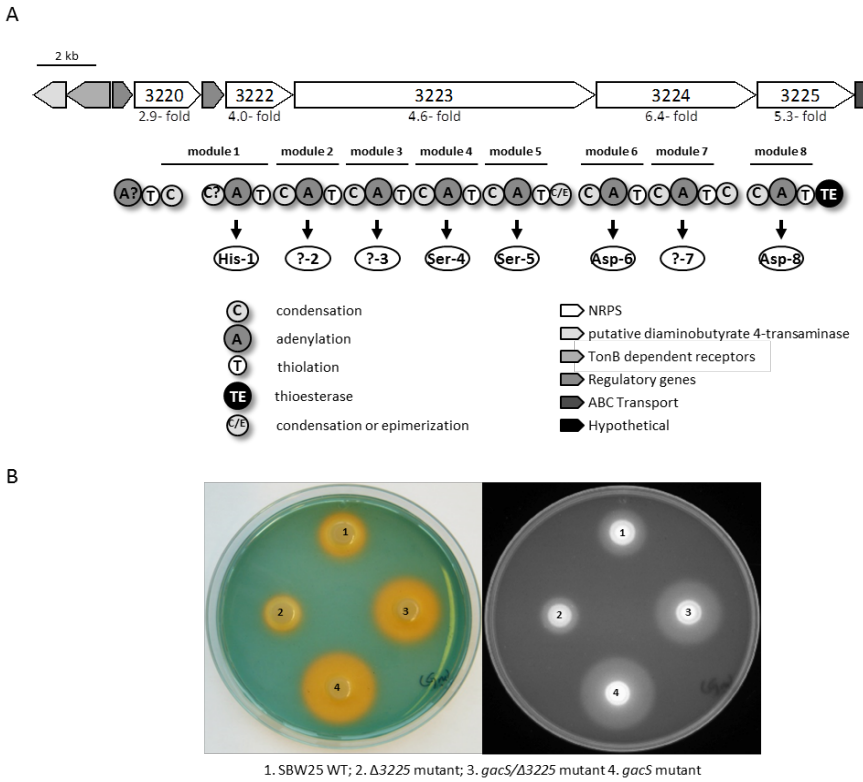


Figure 4 Organisation and predicted peptide configuration of the putative ornicorrugatin NRPS gene cluster in *P. fluorescens* SBW25 based on *in silico* analysis of the genome sequence (A). The gene codes 3220, 3222, 3223, 3224 and 3225 refer to PFLU3220, PFLU3222, PFLU3223, PLFU3224, PLFU3225 in the 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.

a siderophore or as a regulator of siderophore biosynthesis. In terms of antimicrobial activity, however, no significant effects were observed: the *gacS/Δ3225* double mutant had the same enhanced inhibitory activity as the *gacS* mutant against the oomycete, fungal and bacterial pathogens tested (Figure 3A). The enhanced activity of the *gacS* mutant against *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 *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 *gacS* mutant. Although *gacS/A* mutants of *Pseudomonas* species generally have a reduced activity against other microorganisms, our study revealed an enhanced, broad-spectrum activity of the *gacS* mutant of SBW25. Various pathogens, including oomycetes, fungi and bacteria, were substantially more inhibited in growth by the *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.

Acknowledgements

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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:

http://onlinelibrary.wiley.com/doi/10.1111/1758-2229.12061/abstract

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 Tn*5* 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.

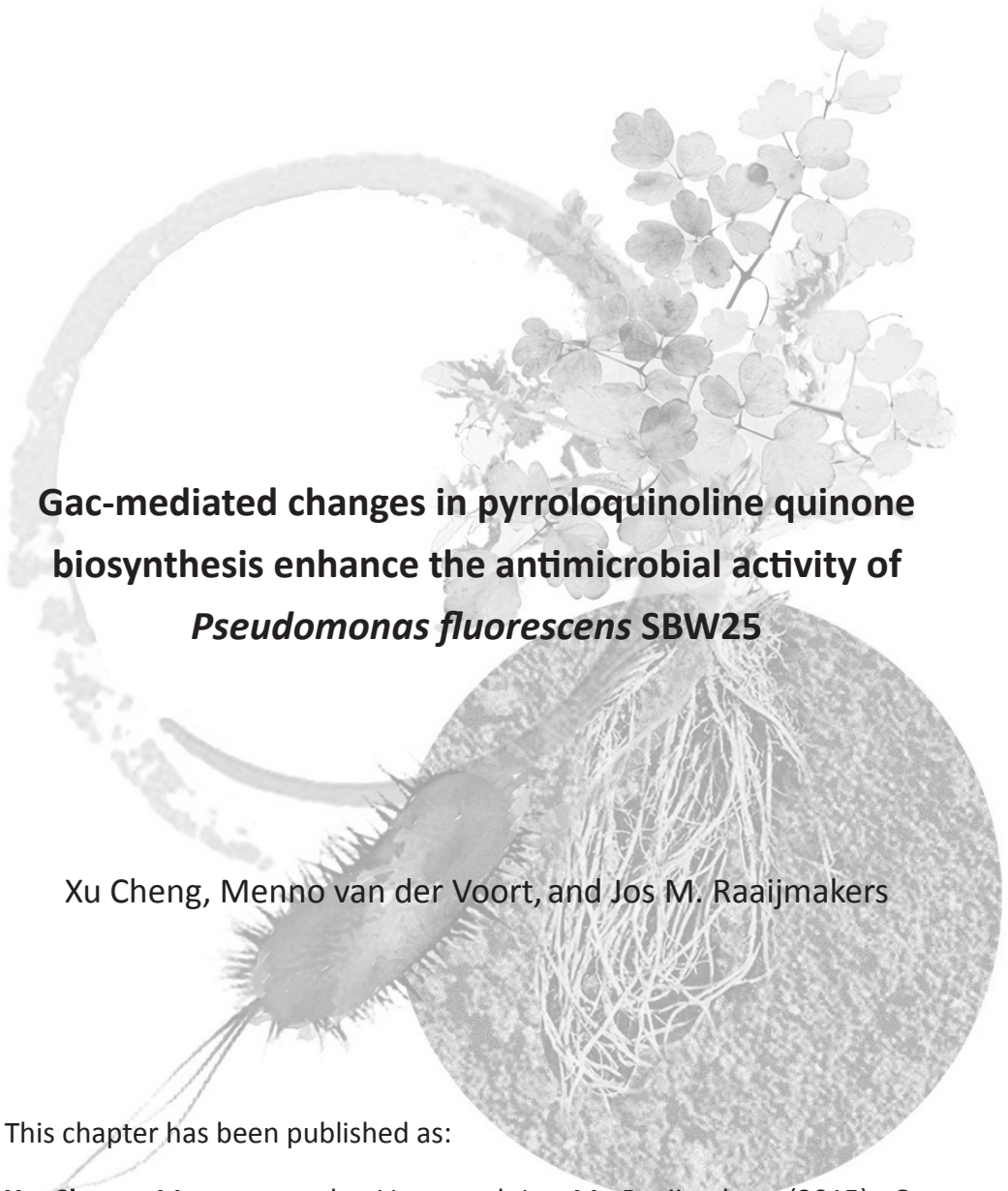
Figure S3 Phenotypic characterization of wild type strain SBW25, *gacS* mutant, complemented *gacS* mutant (*gacS* compl.) and *gacS* + empty vector (*gacS* ev). For complementation, a 3763-bp fragment containing the *gacS* gene with putative promoter and terminator was obtained by PCR with specific 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 *P. fluorescens* 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 $FeSO_4$.

2

Chapter 3



Gac-mediated changes in pyrroloquinoline quinone biosynthesis enhance the antimicrobial activity of *Pseudomonas fluorescens* SBW25

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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.

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 *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.

Materials and methods

Strains and growth conditions. *Pseudomonas fluorescens* SBW25 (spontaneous rifampicin resistant derivative) and *Pseudomonas syringae* pv. *tomato* DC3000 (*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 tetracyclin, and/or 40 µg/ml gentamycin. All fungal and oomycete species/strains were cultured on Potato Dextrose Agar (PDA, Difco). *Phytophthora infestans* and *Saprolegnia parasitica* were cultured at 18°C and 25°C, respectively; *Verticillium dahliae* JR-2 was cultured at room temperature (Cheng et al., 2013).

Random mutagenesis and selection of mutants deficient in antimicrobial activity. The *gacS*::Tn*5* mutant of strain SBW25 (de Bruijn and Raaijmakers, 2009) was subjected to random plasposon mutagenesis with plasmid pTn*Mod*OTc (Dennis and Zylstra, 1998). Transformants were selected and purified on KB agar plates supplemented with kanamycin (100µg/ml) and tetracyclin (25µg/ml), and subcultured in 96-well plates containing 200µl KB broth supplemented with kanamycin (100µg/ml) and tetracyclin (25µg/ml) at 25°C overnight. The mutant library was initially screened in *in vitro* assays for loss of activity against the oomycete pathogen *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 *in vitro* assay. The *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 *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 *S. parasitica* strain C65 and *P. infestans* strain 88069 by SBW25 and mutants was tested in liquid broth (1/5th PDB). *P. fluorescens* SBW25 and its mutants were cultured in 1/5th 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.

qPCR analysis. RNA isolation and quantitative PCR (qPCR) 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: Δ*CT* = *CT* (gene) - *CT* (*rpoD*). The relative quantification (RQ) values were calculated with the formula RQ = $2^{[\Delta CT \text{ (mutant)} - \Delta CT \text{ (wild type)}]}$ (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).

Genetic complementation of the mutants. For complementation of the *pqq* mutants, a 6.9-kb fragment containing the PQQ genes *pqqF-E* and their putative promoter and terminator regions, were obtained by PCR with primers pBBR-pqqF-F2-AccIII/pBBR-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 *Acc*III and *Cla*I. The obtained *pqqF-E* fragments were cloned into the shuttle vector pBBR1mcs-5 (Kovach et al., 1995; Heeb et al., 2000), and the obtained pBBR1mcs-5-*pqqF-E* construct was electroporated into the *gacS*/*pqqB* or *gacS*/*pqqE* double mutant according to methods described by Choi and Schweizer (Choi et al., 2006). Cells transformed with pBBR1mcs-5-*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-*pqqF-E* construct was also introduced

Table 1 Plasmids and oligonucleotide primers used in this study.

Plasmid or primers	Relevant genotype or description	Source
Plasmid		
pTnMod-OTc	plasposon for Gram-negative bacteria, TcR	Dennis and Zijlstra, (1998)
pBBR1mcs-5	shuttle/cloning vector for complementation, inducible GmR	Kovach, et al., (1995)
Primers*		
Q19-pqqA-F	CCT GGT CCA AAC CTG CTT AC	This study
Q20-pqqA-R	TAA CGG CTG GCG AAG TAC A	This study
Q23-pqqC-F	ATC CAT CAC CCG TAC CAT GT	This study
Q24-pqqC-R	GCG GGA TGT TCA CCT GAT AG	This study
Q25-pqqD-F	AGG CCA TGT GTT GCT CTA CC	This study
Q26-pqqD-R	GTT TAT CCA GCT CGG CAA TG	This study
Q31-gcd-F	ATC CGG CAG TTT GAA CGC	This study
Q32-gcd-R	CGC AAA CCA CAC CGA TGA	This study
pBBR-F-N	ATG CTT CCG GCT CGT ATG	Kovach, et al., (1995)
pBBR-R-N	CTC ATC GCA GTC GGC CTA	Kovach, et al., (1995)
pBBR-GmF	GACGCACACCGTGGAAA	Kovach, et al., (1995)
pBBR-Gm-R	GCGGCGTTGTGACAATTT	Kovach, et al., (1995)
pBBR-pqqF-F2-AccIII	GG tccgga GACCTGGACCATTCGATGTT	This study
pBBR-pqqE-R1-ClaI	GG atcgat GGTGCTGGAGTACGGCTTTC	This study
pBBR-gcd-F1-BamHI	GG ggatcc TAACGAGAGCGGCACCGATG	This study
pBBR-gcd-R2-BamHI	GG ggatcc TACCGCGAGAAGCCCGACAA	This study

* 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.

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

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

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.

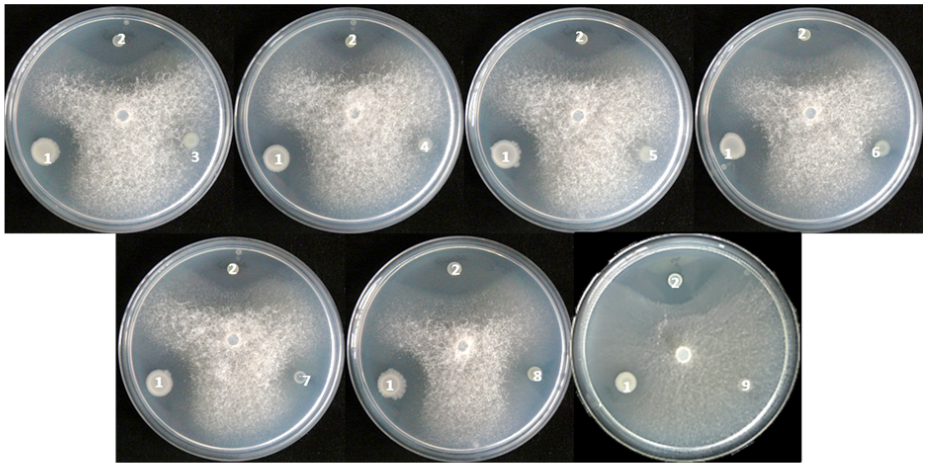
Sensitivity of bacterial, fungal and oomycete pathogens to pH. The pH of 1/5th PDB was set to 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5 and 7.0 with HCL or NaOH. After sterilization, the pH of the media was checked again and 5ml was added to each of the wells in a 6-well plate. A 4-mm-diameter mycelial 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.

Results and Discussion

Identification of genes involved in the enhanced antimicrobial activity of the *gacS* mutant of *P. fluorescens* SBW25

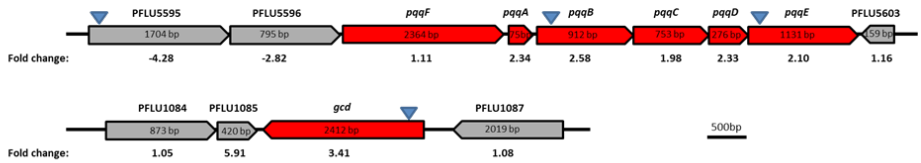
The *gacS* mutant of *P. fluorescens* SBW25 was subjected to random plasposon mutagenesis. Screening of an initial number of 1,248 random mutants resulted in 7 mutants with reduced or loss of activity against the oomycete pathogen *S. parasitica* (Figure 1, Table 2). Southern blot hybridization showed a single Tn*Mod*OTc insertion for all 7 mutants. Subsequent *in vitro* assays revealed that these 7 mutants also had reduced or loss of activity against other pathogens including *P. infestans, V. dahliae,* and *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 *S. parasitica*, the plasposon insertion was located in the pyrroloquinoline quinone (PQQ) biosynthesis genes *pqqB* (mutant 13D7, PFLU5599) and *pqqE* (mutant 6A6, PFLU5602), in the *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 *pqqB* (PFLU5599)*, pqqE* (PFLU5062) and *gcd* (PFLU1086) were significantly up-regulated in the *gacS* mutant (Table 2). In total, four out of the six PQQ biosynthesis genes (p*qqF, pqqA-E*) in SBW25 were up-regulated more than 2.0-fold in the *gacS* mutant (Table 2, Figure 1B). The up-regulation of the *pqq* and *gcd* genes in the *gacS* mutant was confirmed by qPCR (not shown). In *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 *P. fluorescens* SBW25 led to a significant transcriptional up-regulation of *pqq* biosynthesis genes and *gcd* gene. Genetic

A



1.SBW25 wild type; 2. *gacS* mutant; 3. *gacS/pqqB* mutant; 4. *gacS/pqqE* mutant; 5. *gacS/gcd* mutant; 6. *gacS/5595* mutant; 7. *gacS/5987* mutant; 8. *gacS/0969* mutant; 9. *gacS/2985* mutant.

B



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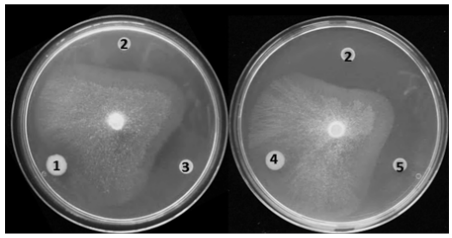

1. SBW25 WT; 2. *gacS*; 3. SBW25 WT+ *pqqF-E*; 4. *gacS/pqqB* ; 5. *gacS/pqqB+pqqF-E*

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 *pqqB* and *pqqE* by introduction of plasmid pBBR-*pqqF-E* restored activity against *S. parasitica* to the level of the *gacS* mutant. When the PQQ gene cluster (*pBBR-pqqF-E*) was ectopically expressed in wild type strain SBW25 (WT+*pqqF*-E), this led to enhanced activity against *S. parasitica* to levels similar to that of the *gacS* mutant (Figure 1C). Similar results were obtained in liquid broth assays in which *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 *pqq* biosynthesis genes play a key role in the enhanced antimicrobial activity of the *gacS* mutant.

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/pqqB* and *gacS/pqqE,* the pH of their culture filtrates was 5.9 and 5.8, respectively. Genetic complementation of *pqqB* and *pqqE* resulted in pH values of 3.8 and 3.7, respectively, similar to that of the *gacS* mutant (Figure 2A). When *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 a reduced or lack of mycelium growth of *S. parasitica* (Figure 2A). Subsequent assays showed that mycelial growth of *S. parasitica* was inhibited at a pH of 4.5 or lower (Figure 2B). Also for *P. infestans*, *V. dahliae* and *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 *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 *S. parasitica* was not inhibited (Figure 3A). Similar results were obtained for *P. infestans, V. dahliae,* and *P. syringae* (Figure 3B-D). The role of *pqq* and *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 *P. protegens* strain CHA0, a *gcd* mutant was shown to be more effective in biological

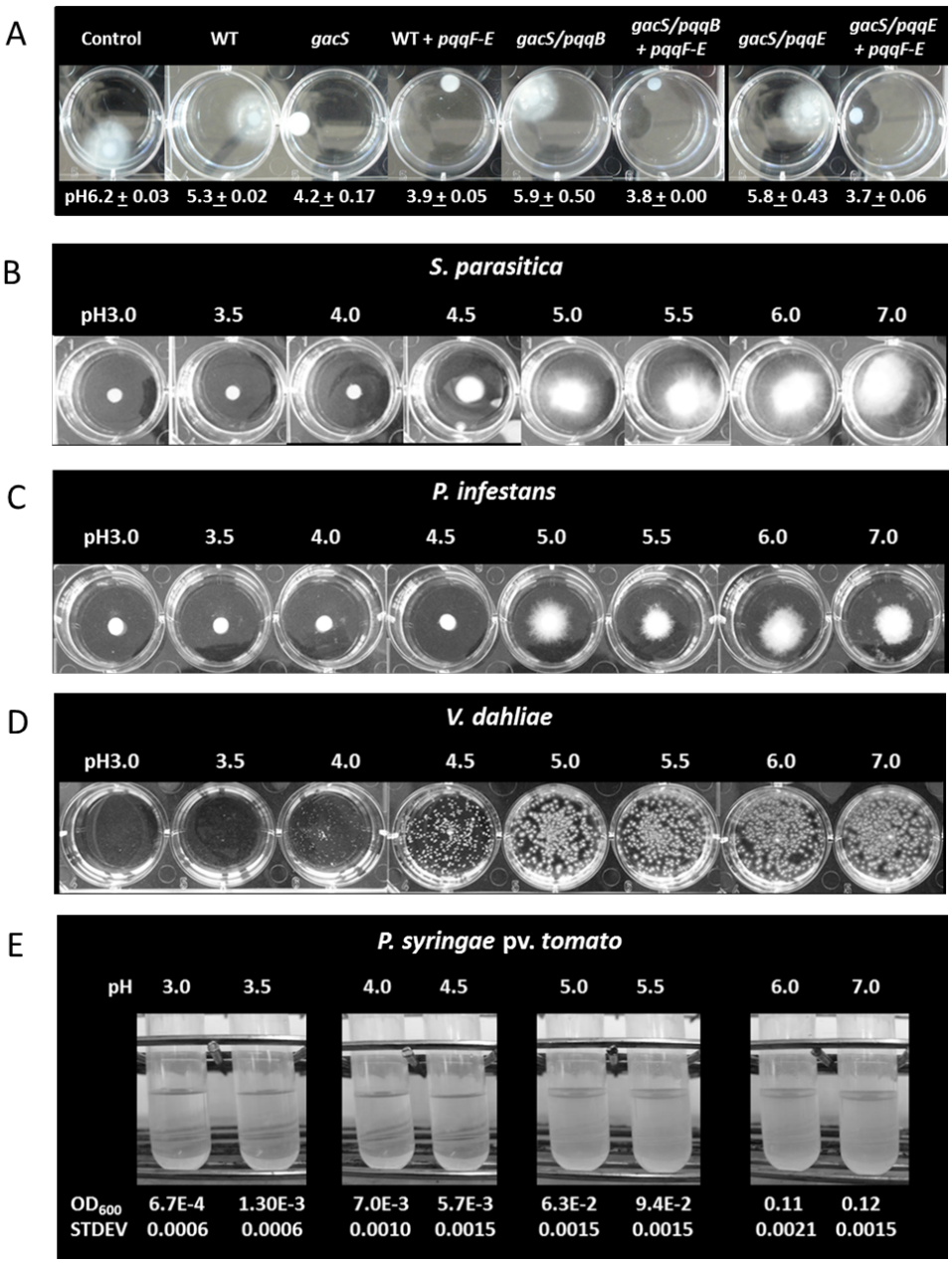

Figure 2 (A) Activity of culture filtrates of wild type *P. fluorescens* SBW25 and its mutants against *S. parasitica*. Underneath the wells are the pH values measured for each of the culture filtrates of each of the strains. Averages of 3 replications are given, ± represents the standard deviation. pH sensitivity of *S. parasitica* (B), *P. infestans* (C), *V. dahliae* (D) and *P. syringae* pv. *tomato* (E), grown in 1/5th 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.

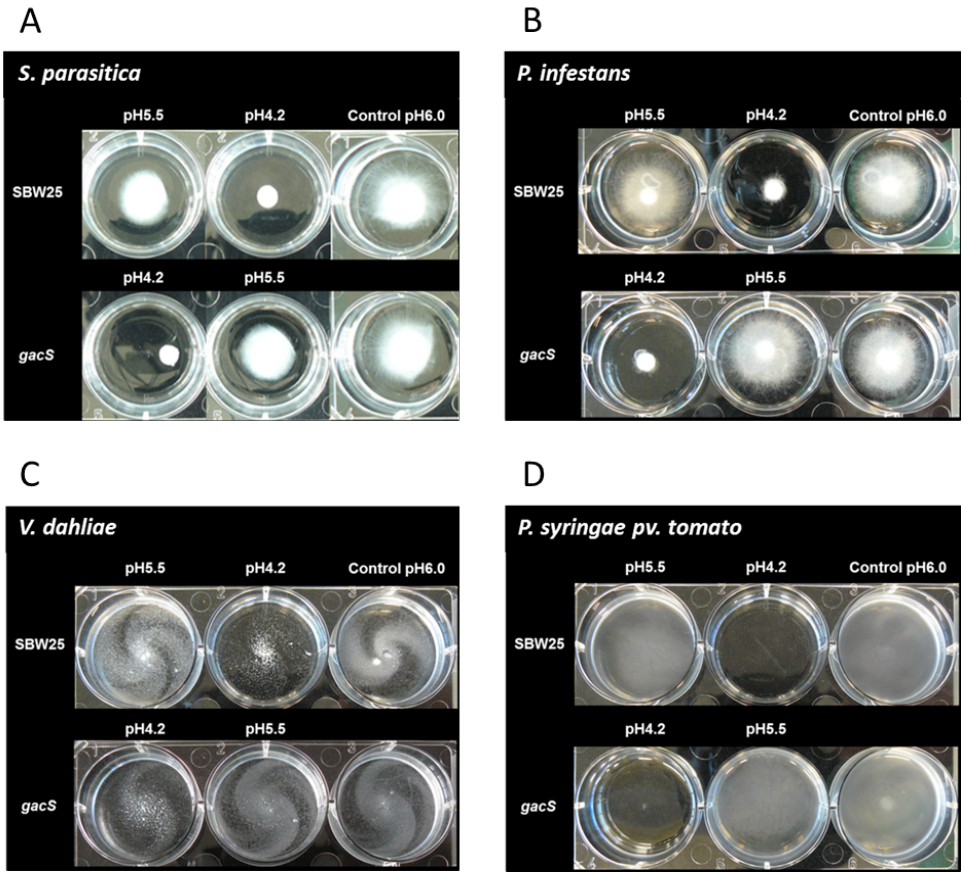

Figure 3 Activity of culture filtrates of wild type *P. fluorescens* SBW25 and the *gacS* mutant with initial pH and re-adjusted pH against *S. parasitica*. The pH of culture filtrates of wild type *P. fluorescens* SBW25 and the *gacS* mutant were measured and re-adjusted prior inoculation of *S. parasitica* (A), *P. infestans* (B), *V. dahliae* (C) and *P. syringae* pv. *tomato* (D). Filtrate of 1/5th PDB with pH6.0 was used as a control.

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*

and *gacS/pqqE* mutants did not produce detectable levels of 2-ketogluconic acid and gluconic acid, which was restored in the complemented mutants (*gacS/pqqB*+*pqqF-E* and *gacS/pqqE*+*pqqF-E*). Introduction of the *pqqF-E* genes in wild type SBW25 (WT+*pqqF-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-keto gluconic 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 *pqq* 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 *pqqF* 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/pqqB* mutant and could be restored by genetic complementation (Figure 5). These results indicate that a mutation in *gacS*

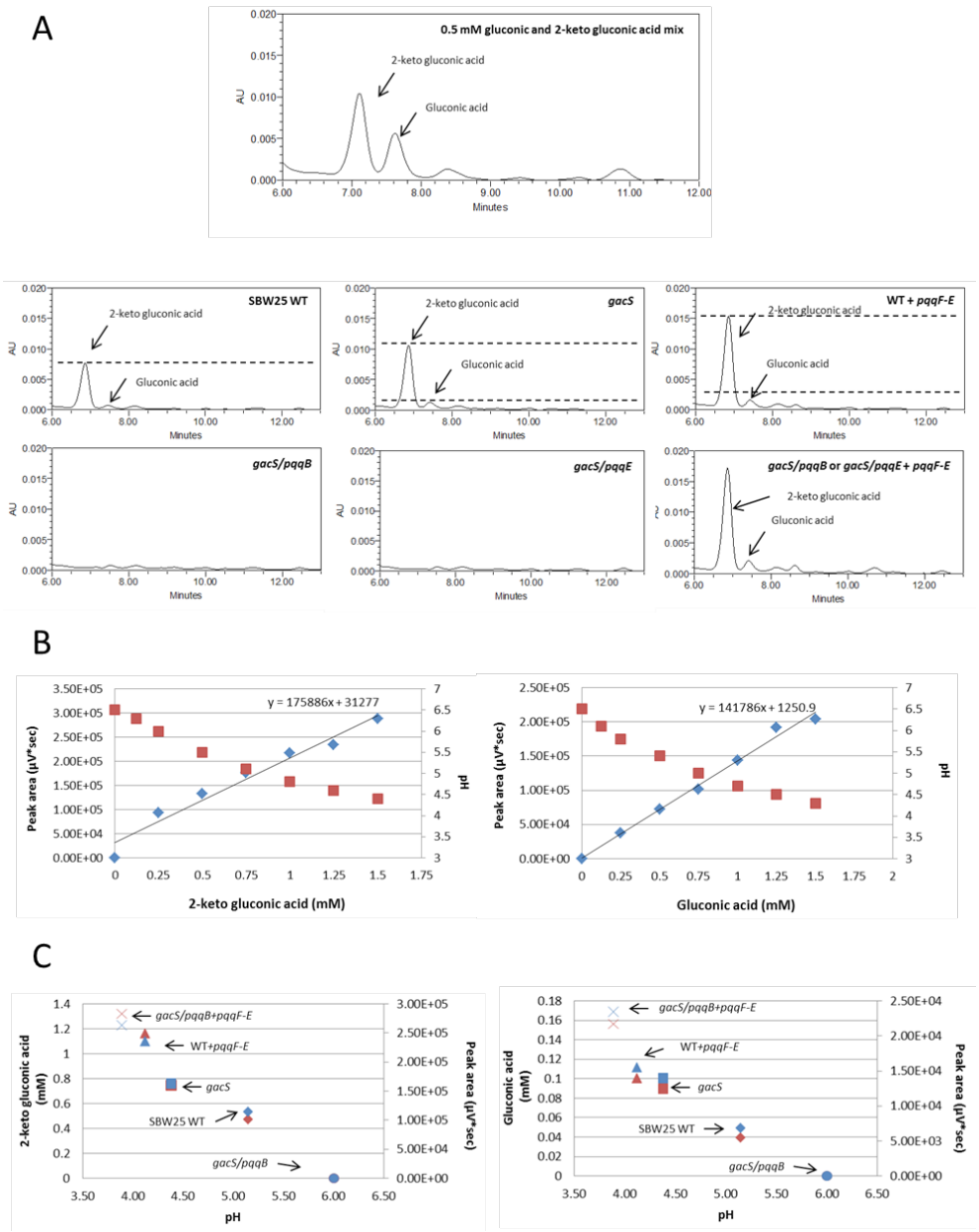

Figure 4 RP-HPLC analysis of culture filtrates of wild type *P. fluorescens* SBW25, the *gacS* mutant, PQQ genes ectopic expression strain WT+*pqqF*-E, double mutants *gacS/pqqB* and *gacS/pqqE,* and PQQ complemented strain *gacS/pqqB+pqqF-E* and *gacS/pqqE* + *pqqF-E* grown in 1/5th PDB supplemented 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 tese two acids; (C) correlation of gluconic acid (right) and 2-keto gluconic acid (left) production (mM and peak area) and pH changes.

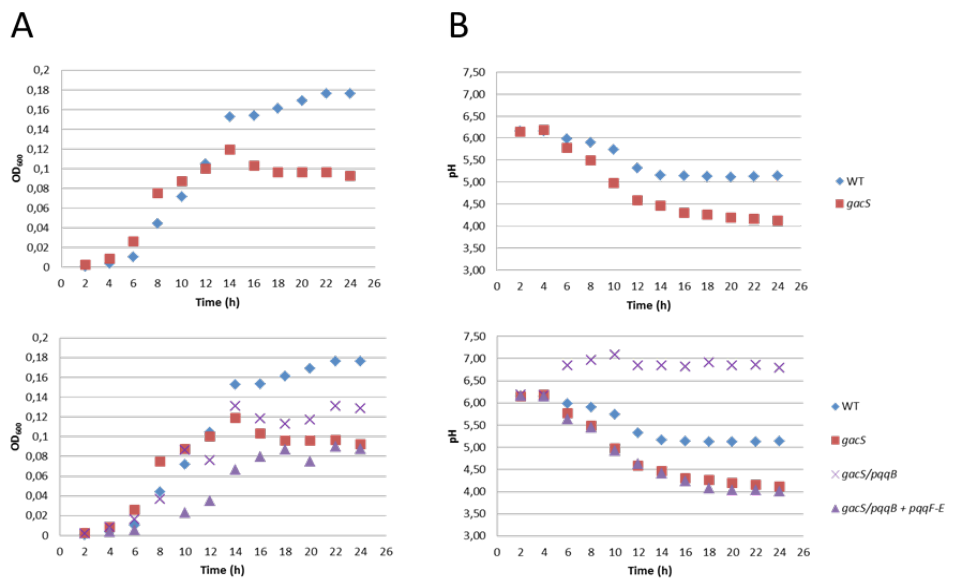

Figure 5 Growth of wild type *P. fluorescens* SBW25 and its mutants in 1/5th PDB broth. Cell density (OD_{600}) was measured every 2 hours and growth curves were generated (A); at every time point, pH were measured in cultures of each of the strains (B).

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

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

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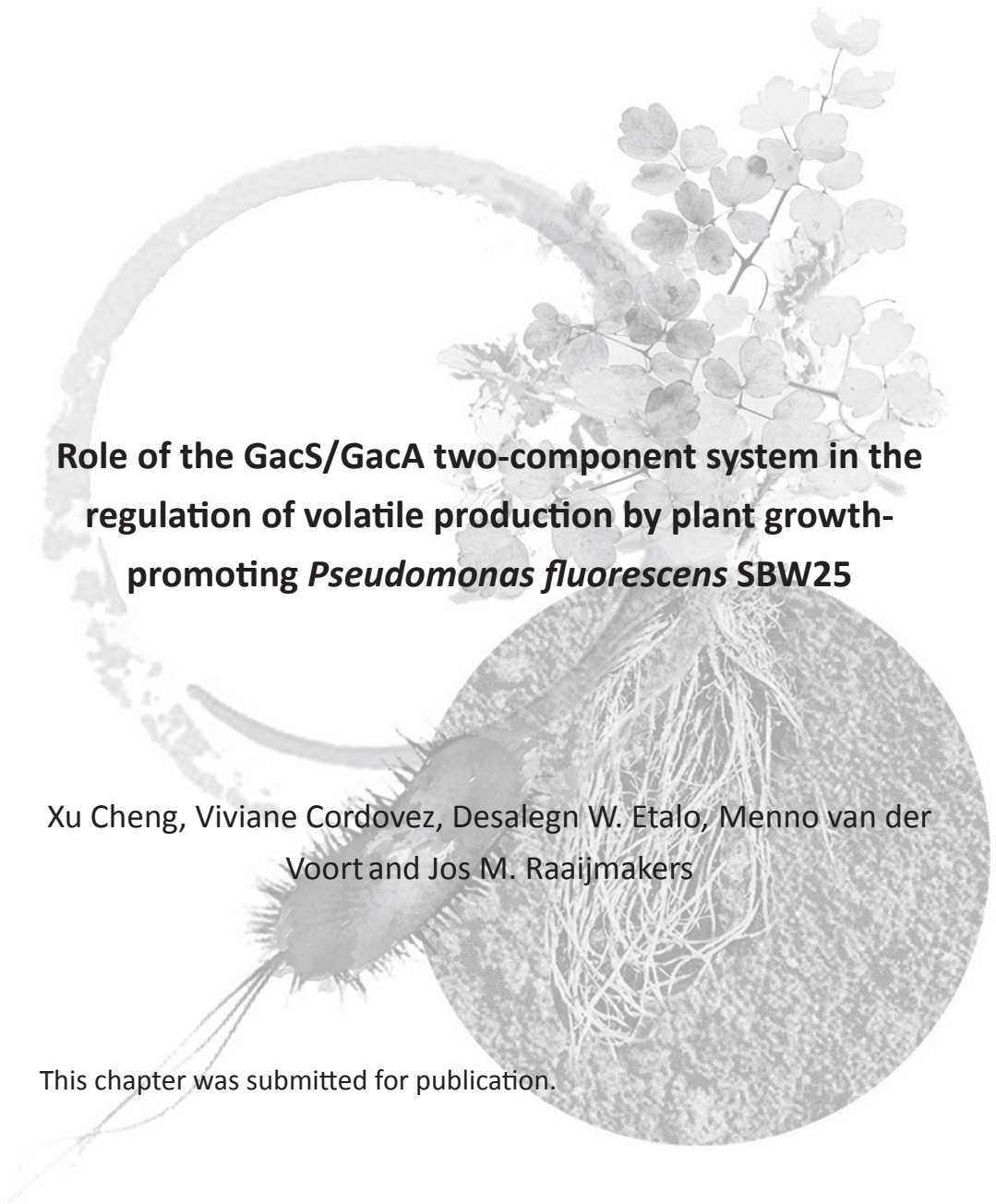
Chapter 4



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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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.

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-butanone, 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 **m**ultiple **v**irulence **f**actor **r**egulator (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).

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 ℃ 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). MSClust 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) 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 (½MS) 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 ½ 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 (~10^9 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 1/5th 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 1/5th PDA and KB media.

VOCs detected for both *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 1/5th PDA (Figure 1). Student t-Test between the *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 *Pf.*SBW25 and its Gac-mutant areshown 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 n-decene (RI: 992) 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 *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).

RI[1]	Compound	Annotation[2]	KB medium				1/5th PDA medium			
			D3		D6		D3		D6	
			t-Test	FC	t-Test	FC	t-Test	FC	t-Test	FC
532	Bicyclo[2.1.0]pentane	2	0.04	1.84	0.59	1.43	0.02	3.77	0.02	4.29
608	Butanoic acid, methyl ester	4	0.16	1.32	0.66	1.12	0.00	2.22	0.00	2.66
701	Diethyl sulfide	2	0.53	5.48	0.17	4.05	0.00	8.43	0.03	3.89
715	Propanoic acid	4	0.95	1.00	0.07	-3.84	0.04	2.73	0.15	2.27
733	Propionic acid	4	0.89	1.04	0.65	-1.12	0.11	3.65	0.00	6.74
740	2-[(octyloxy)methyl]cyclobutanone	4	0.10	-1.46	0.66	1.27	0.21	1.59	0.01	2.84
796	[1-13C]-Butanol	4	0.20	18.85	0.11	8.14	0.00	35.73	0.56	1.40
801	Furan, 2-ethyl-5-methyl-	2	0.07	288.01	0.11	95.35	0.00	56.14	0.00	56.29
812	Ethanone, 1-(2-furanyl)	4	0.14	13.77	0.12	15.63	0.01	3.52	0.00	4.55
827	2,2-Dimethyl-4-pentenylamine	4	0.35	1.12	0.42	1.42	0.00	23.66	0.59	1.44
847	7-Fluoro-1-(trimethylsilyl)norcarane	4	0.36	4.41	0.40	1.82	0.00	2.23	0.85	-1.05
879	1-Butene, 2-methyl	4	0.08	2.14	0.16	2.94	0.00	32.00	0.08	1.57
886	S-2-S-Butylfuran	2	0.05	104.68	0.33	50.61	0.00	34.21	0.00	29.01
891	3-Nonene	2	0.00	48.62	0.00	28.49	0.00	37.31	0.02	38.00
992	n-Decene	2	0.01	94.18	0.07	24.66	0.92	-1.26	1.00	-1.00
1080	1-Undecyne	4	0.01	22.18	0.03	15.97	0.37	35.33	0.37	-15.40
1085	4-Undecyne	2	0.00	96.15	0.00	44.53	0.01	32.06	0.00	3055.71
1089	1-Undecene	2	0.00	6.29	0.01	2.74	0.00	35.84	0.00	27.15
1125	Methanamine, N-hydroxy-N-methyl-	4	0.53	5.21	0.70	2.44	0.14	3.34	0.04	2.11
1280	cis-1,2-Cyclohexanedimethanol	4	0.00	295.05	0.00	313.43	0.12	95.59	0.72	9.21
1310	4-(p-Methoxyphenyl)-butan-2-one	4	0.37	-4.18	0.39	3.63	0.33	1.87	0.05	6.40
1596	Methyl 5-hydroxy-5-methyl-5,6,7,8-tetrahydronaphthalene-1-carboxylate	4	1.00	-1.00	0.37	190.96	0.53	-1.18	0.00	365.27
1631	5-Chloro-1-(4'-hydroxy-2'-butynyl)-2(1H)-pyrimidinone	4	0.25	-1.31	0.85	-1.11	0.00	3.42	0.15	-4.89
1877	4-(2',6'-Difluorophenyl)-1-methyl-1H-pyrazole-3-carbonitrile	4	0.85	1.02	0.56	-1.77	0.41	-1.57	0.04	9.02

[1]Calculated linear retention index

[2]Annotation level according to the Metabolomics Standards Initiative (MSI)

Colored cells represent significant (P >0.05/FC< 2) differencials: 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 calulated using the compound peak height (*Pf.* SBW25 versus the Gac-mutant)

MF: library match factor

in the Gac-mutant (Table 1, Figure 2C). On 1/5th 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 1/5th 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

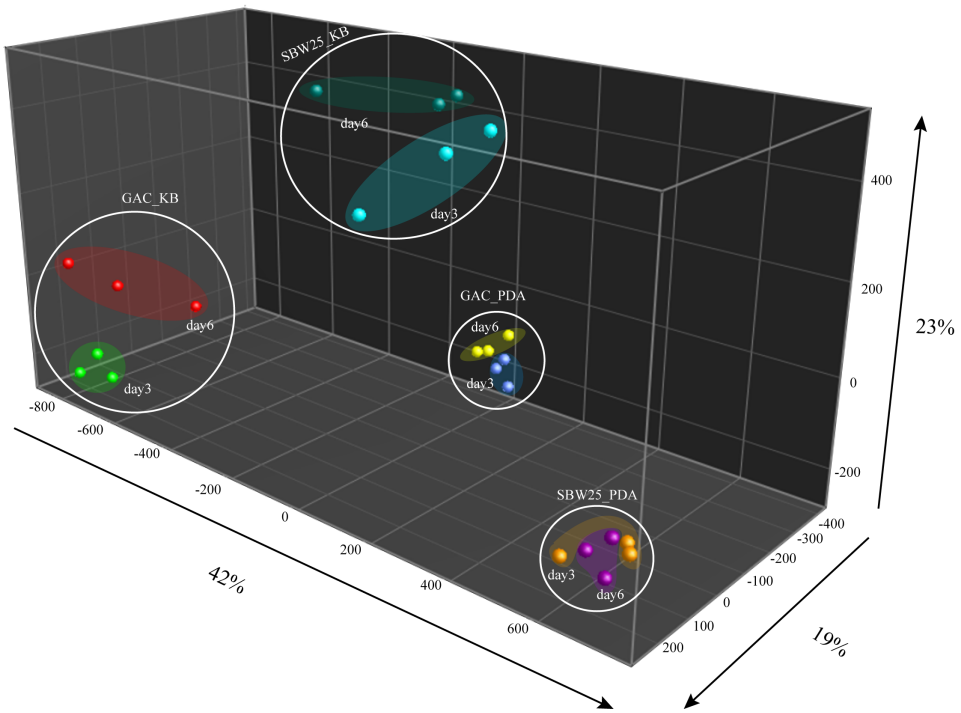

Figure 1 Discriminant function analysis based on 79 VOCs that were significantly different in their abundance at least under one of the conditions analyzed (ANOVA, *P<0.05*). The first component explained 42% of the total variation primarily associated to variation in the abundance of VOCs when *Pf.*SBW25 and the Gac-mutant are grown in 1/5th 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 1/5th PDA.

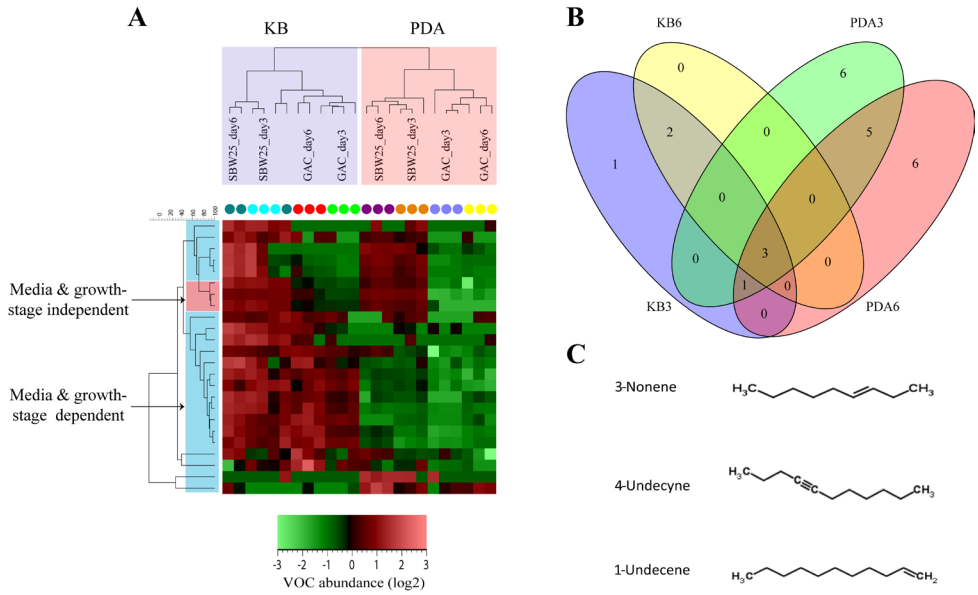

Figure 2 VOCs profiling of *Pf*.SBW25 and the *gacS*-mutant (Gac-mutant). (A) Hierarchical cluster analysis based on 24 VOCs that were significantly different ($P < 0.05$ and fold change > 2) for the *Pf*.SBW25 and the Gac-mutant grown on 1/5th PDA and KB media after 3 and 6 days of inoculation. Columns represent the different isolates (in triplicates), whereas rows represent the VOCs (green: low abundance, red: high abundance). (B) Venn diagram showing the unique and shared VOCs detected for *Pf*.SBW25 and the Gac-mutant grown on 1/5th PDA and KB media after 3 (D3) and 6 (D6) days of inoculation. Yellow and blue cycles represent VOCs detected for strains grown on KB at D3 and D6, respectively; Green and red cycles represent VOCs detected for strains grown on 1/5th PDA at D3 and D6, respectively. (C) Group of alkenes significantly affected by the Gac-mutation of *Pf*.SBW25 grown on KB and 1/5th PDA media at D3 and D6 after inoculation.

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

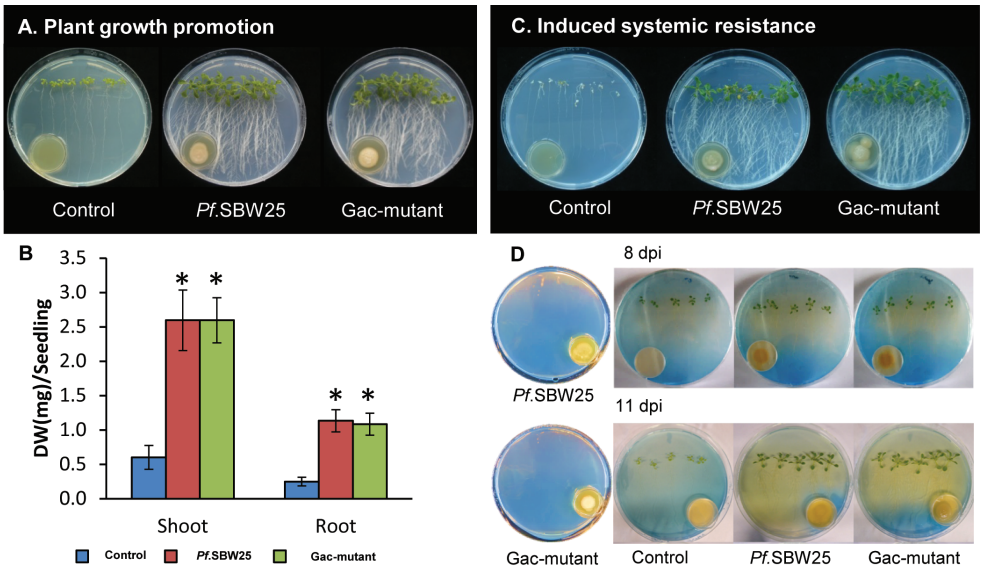

Figure 3. Effects on Arabidopsis growth after 11 days of exposure to VOCs from *Pf.*SBW25 and the Gac-mutant. Growth promotion: phenotypic observation (A) and biomass quantification (B). Bars represent the mean of 4 replicates (with 8-10 seedlings per replicate) ± standard deviation. Bars in blue (control), 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) ISR of Arabidopsis against *Pst* infection by *Pf.*SBW25 and the Gac-mutant. (D) Arabidopsis rhizosphere acidification, orange colour reflects pH decline.

incidence was significantly reduced in plant seedlings exposed to VOCs from either *Pf.*SBW25 or from the Gac-mutant (Figure 3C). Collectively, these results show that a mutation in the Gac-system of *Pf.*SBW*25* did not alter VOCs-mediated growth promotion of Arabidopsis seedlings nor ISR against *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 *Pf.*SBW25 or the Gac-mutant. However, acidification in the *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).

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.*SBW*25* and the

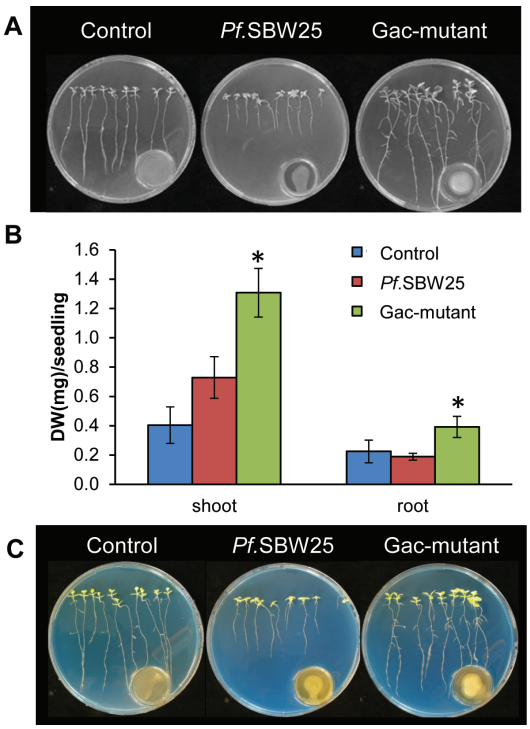

Figure 4. Effects on tobacco (*Nicotiana benthamiana*) growth after 11 days of exposure to VOCs from *Pf.*SBW25 and the Gac-mutant. (A) Growth promotion and (B) biomass quantification: Bars represent the mean of 4 replicates (with 8-10 seedlings per replicate) ± standard deviation**.** Bars in blue (control), 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.

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 *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 *Pf.*SBW25 is regulated by the GacS/GacA two-component system. Our results show that the Gac-system in *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 *Pseudomonas* species and have been reported to have plant growth promoting-effects on *Arabidopsis thaliana* (Blom et al., 2011) and tobacco (*Nicotiana tabacum* cv. Xanthi-nc) (Park et al., 2015). For 1-undecene, Rui et al. (2014) recently identified the biosynthetic gene *undA* in *P. aeruginosa* PA14. Heterologous expression of *undA* homologs of several other *Pseudomonas* strains in *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 *Pf.*SBW25 caused major transcriptomic changes (Cheng et al., 2013). Revisiting these microarray data showed that PFLU4307, the *undA* orthologue of *P. protegens* Pf-5, was 9.7-fold down regulated in the *gacS*-mutant of *Pf.*SBW25. In *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 *Pf.*SBW25, we compared all

the already known or published target genes of small RNA repressor proteins by using the conserved motif 5'-$^{A}/_{U}$ CANGGANG$^{U}/_{A}$-3' (N is any nucleotide) (Lapouge et al., 2008;Song et al., 2015). However, there was no so called GacA Box upstream of the *undA* in *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 *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 *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 *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 *Pf.*SBW25 and the Gac-mutant. While VOCs produced by *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 *Pf.*SBW25 can be due to Gac-regulated VOCs and/or due to specific VOCs that are emitted by *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-

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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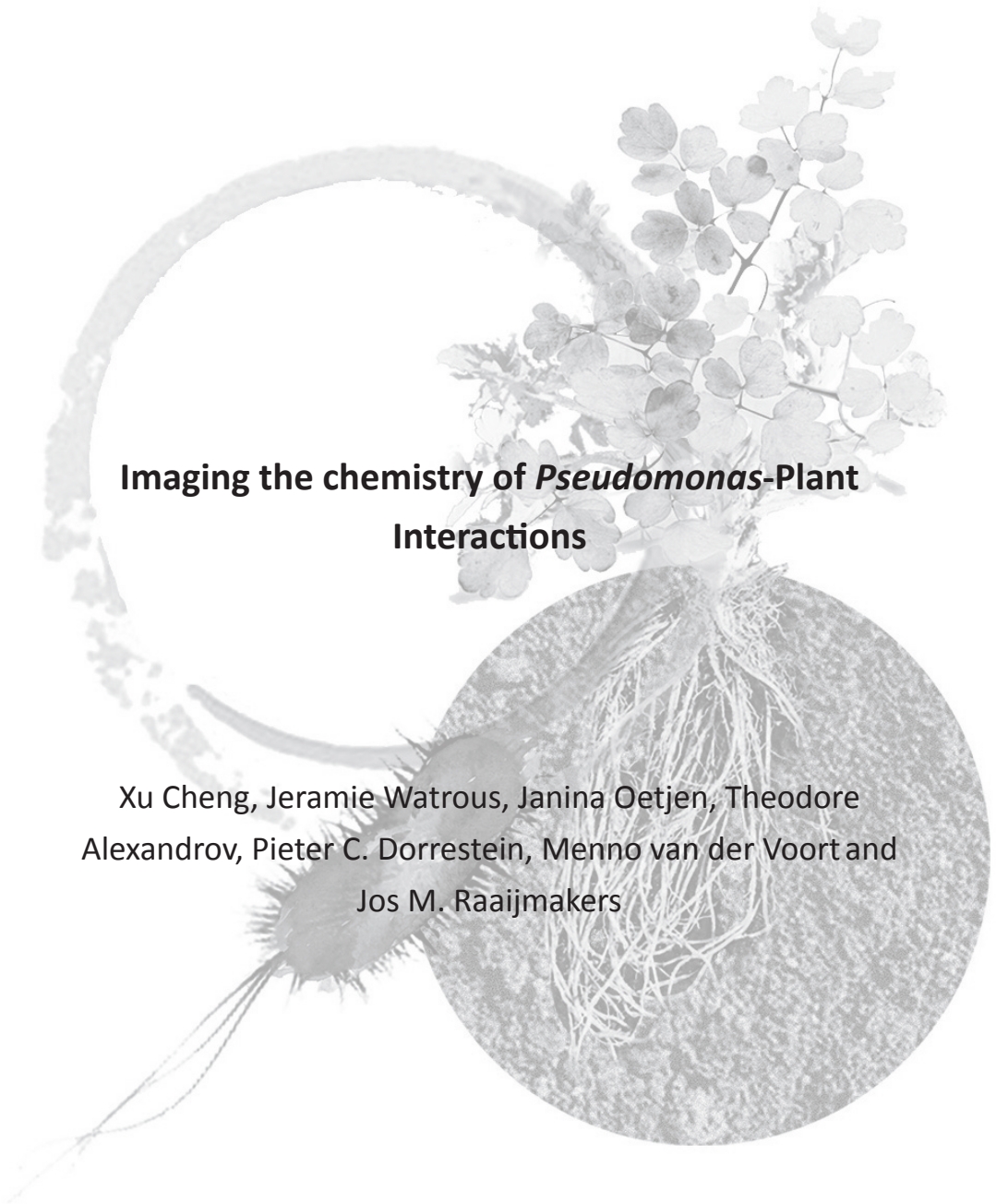
Chapter 5



Imaging the chemistry of *Pseudomonas*-Plant Interactions

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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.

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

& 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 $MgSO_4$ and resuspended in 10 mM $MgSO_4$ to a final density of 10^9 CFU ml^{-1} (OD_{600} = 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 (10^9 CFU ml^{-1}); in the control

treatment, seedlings were inoculated with 2 µl of 10 mM $MgSO_4$. 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 10^6 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 (OD_{600nm}= 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

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

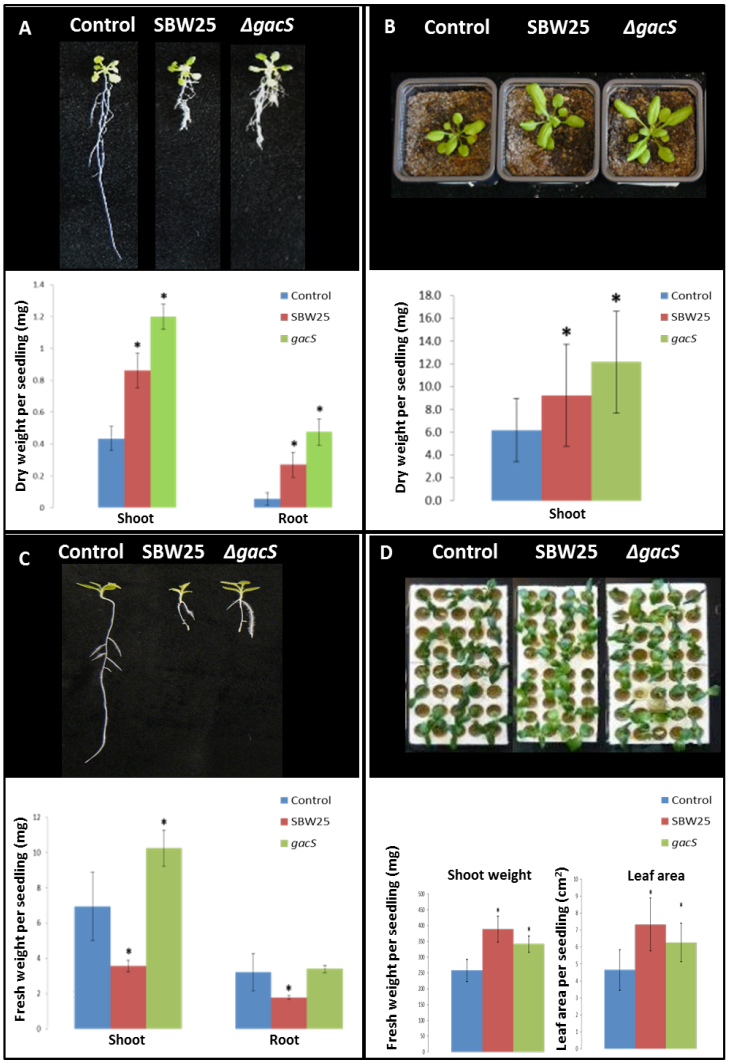


Figure 1 Effects of *Pf.*SBW25 and its *gacS* mutant on growth of multiple plant species. In *in vitro* conditions, *Pf.*SBW25 and its *gacS* mutant enhance plant growth, inhibit primary root growth and induce lateral root formation in *Arabidopsis thaliana* accession Columbia grown on 0.5x MS medium 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**).**

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.

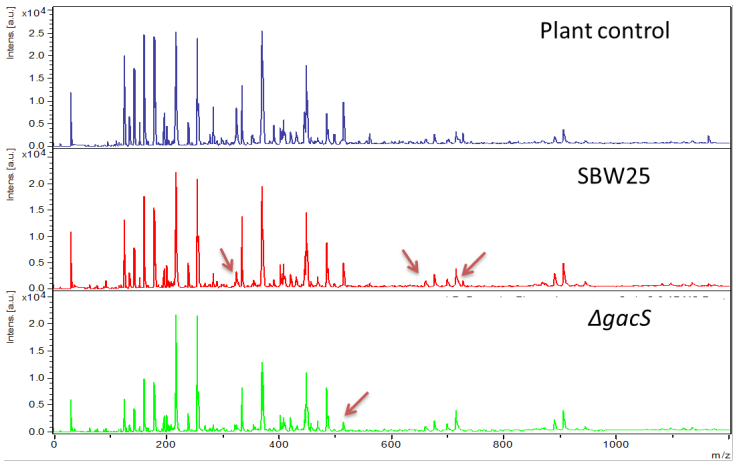


Figure 2 Chemical profiles generated by MALDI-DD analysis. Molecules detected on roots of nontreated Arabidopsis seedlings and on seedlings treated with *Pf.*SBW25 or its *gacS* mutant. The sample plates were subjected to MALDI-TOF mass spectrometry (Microflex from Bruker Daltonics, Billerica, MA, USA) 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.

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 Arabidospsis, 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

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.

Grown on 0.5x MS (m/z)		Grown on Arabidopsis roots (m/z)	
Pf. SBW25	*gacS mutant*	SBW25-Arabidopsis	*gacS* -Arabidopsis
		37	37
		63	63
		77	77
			89
		93	93
			118
			128
		161	
			200
			204
			208
			218
		372	372
		402	402
			421
		447	447
		451	451
			469
471			
477			
			487
492			
549			
593			
607			
638			
652			
		677	677
			699
		715	715
		890	890
	909		

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

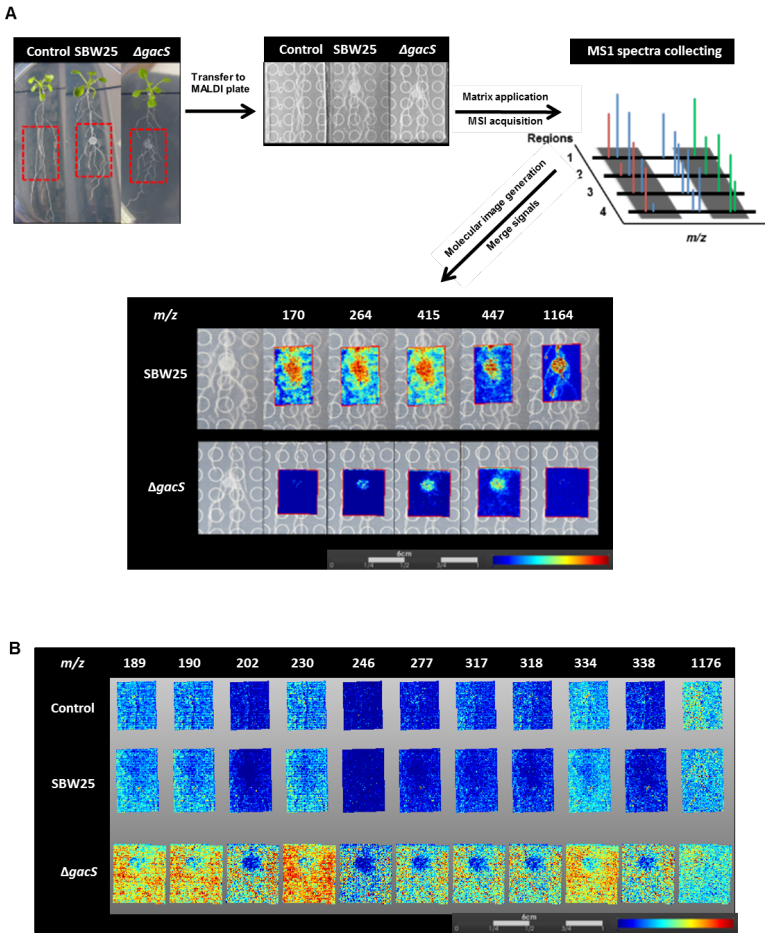


Figure 3 Chemical profiles of the *Pf*.SBW25 or the *gacS* mutant-Arabidopsis interaction detected by MALDI-IMS. (A) shows general procedures for generating and visualizing chemical productions during *Pseudomonas*-Arabidopsis interactions. Detected ions with masses ranging from 0 to 2kDa are shown. Several differential ions detected on roots treated with *Pf.*SBW25 or the *gacS* mutant. (B) 11 ions (AUC>0.75), which are more abundant in the *gacS* -Arabidopsis interaction.

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 interactions (Fig. 3A).

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.

Comparison	Centroid	± Da	Maximal AUC at m/z	Maximal AUC	Putatively identified compounds
SBW25 vs *gacS*[a]	37.646	0.047	37.646	0.949	
	107.723	0.135	107.723	0.753	
	125.639	0.157	125.639	0.944	
	128.814	0.161	128.814	0.837	
	135.617	0.17	135.617	0.939	
	138.565	0.173	138.565	0.963	
	143.214	0.179	143.101	0.906	
	144.688	0.181	144.688	0.966	Valine
	153.760	0.192	153.760	0.842	
	161.697	0.202	161.697	0.96	Glucose/fucose/galactose/mannose
	162.718	0.203	162.831	0.934	
	170.655	0.213	170.769	0.953	
	177.005	0.221	176.892	0.968	
	205.807	0.257	205.693	0.936	
	217.826	0.272	217.713	0.974	
	220.775	0.276	220.661	0.981	Cysteine
	226.671	0.283	226.784	0.961	
	248.783	0.311	248.783	0.977	
	264.544	0.567	264.657	0.979	
	266.925	0.334	266.925	0.95	
	302.984	0.379	302.984	0.968	
	334.167	0.418	334.053	0.959	
	343.125	0.429	342.898	0.951	
	353.33	0.442	353.33	0.958	
	364.102	0.455	364.216	0.954	
	410.026	0.513	410.026	0.964	
	435.540	0.544	435.426	0.920	
	437.807	0.567	438.374	0.784	
	448.806	1.587	448.353	0.888	
	470.464	0.588	470.351	0.970	
	491.328	1.247	491.895	0.952	
	492.122	0.615	492.349	0.831	
	521.944	0.652	521.831	0.980	
	1150.931	1.587	1150.704	0.884	
	1164.94	5.216	1165.445	0.961	Viscosin
gacS vs SBW25[b]	171.336	0.214	171.222	0.958	
	172.243	0.215	172.356	0.937	
	189.365	0.237	189.365	0.948	Glyceric acid
	190.272	0.238	190.272	0.935	Trehalose
	202.292	0.253	202.292	0.95	
	205.24	0.257	205.24	0.938	Erythrose/Glycerol
	211.25	0.264	211.363	0.966	
	212.27	0.265	212.27	0.969	
	227.351	0.284	227.238	0.974	
	228.372	0.285	228.372	0.984	
	229.392	0.287	229.279	0.975	
	230.526	0.288	230.413	0.952	L-hydroxyproline
	246.401	0.308	246.515	0.95	Glutamic acid
	249.463	0.312	249.463	0.968	
	250.37	0.313	250.37	0.943	
	251.504	0.314	251.504	0.951	
	265.565	0.332	265.565	0.952	
	267.492	0.334	267.379	0.941	Hydroxybenzoic acid
	277.584	0.347	277.584	0.954	Cystathiomine
	289.604	0.362	289.604	0.948	
	290.624	0.363	290.738	0.942	
	291.645	0.365	291.645	0.956	
	293.572	0.367	293.686	0.966	
	294.706	0.368	294.593	0.957	
	317.725	0.397	317.725	0.938	Lysine
	318.632	0.398	318.632	0.93	Mannitol/xylitol/arabitol
	334.734	0.418	334.734	0.939	Gluconic acid
	335.641	0.42	335.641	0.932	
	336.775	0.421	336.775	0.937	
	338.703	0.423	338.816	0.948	Tyramine
	350.609	0.438	350.609	0.944	
	351.743	0.44	351.743	0.938	
	400.955	0.501	400.955	0.962	
	413.028	0.456	412.974	0.938	
	414.821	0.486	414.789	0.932	
	416.943	0.521	416.83	0.982	
	418.778	1.134	418.871	0.981	
	1176.671	1.471	1176.558	0.779	Pyoverdine/ornicorrugatin

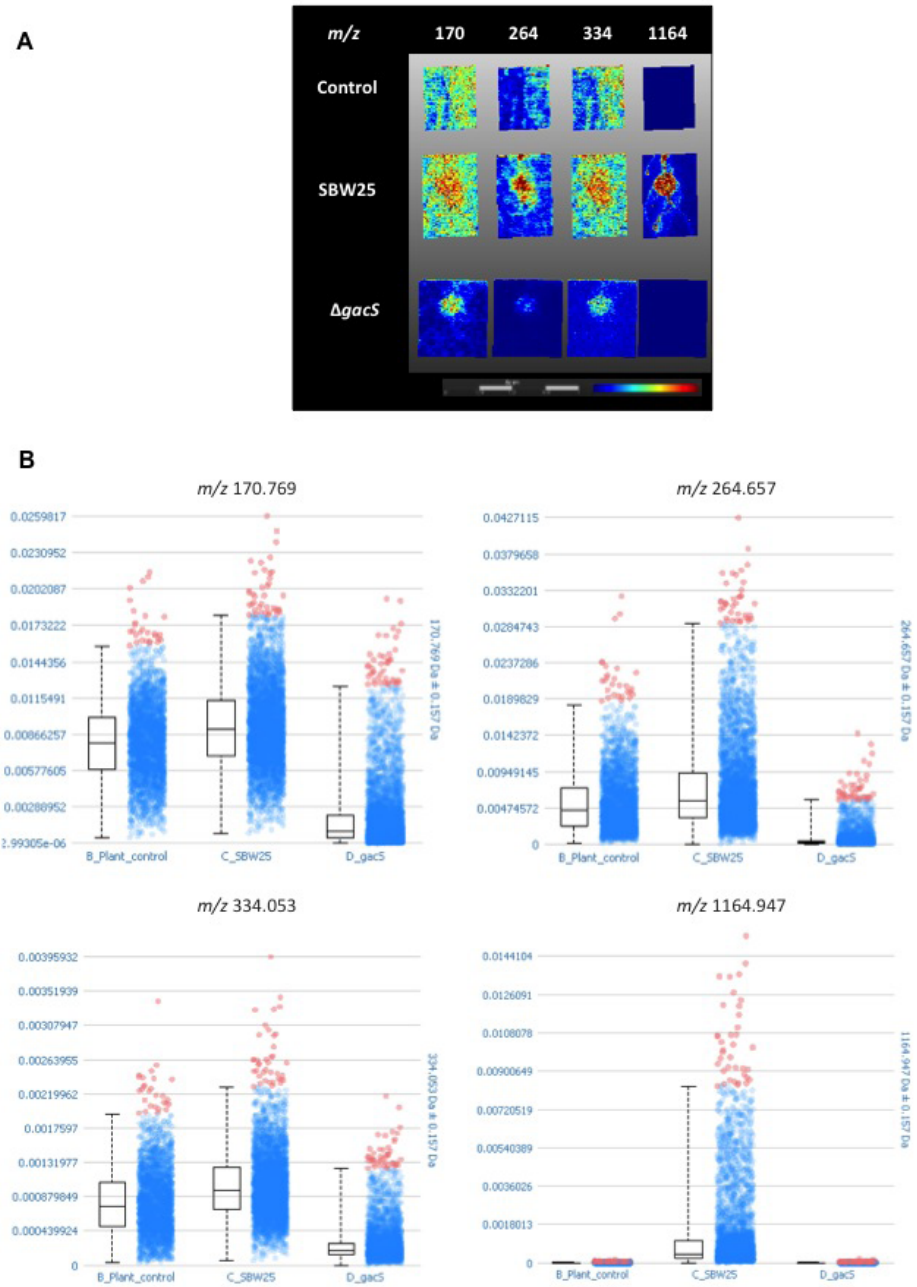
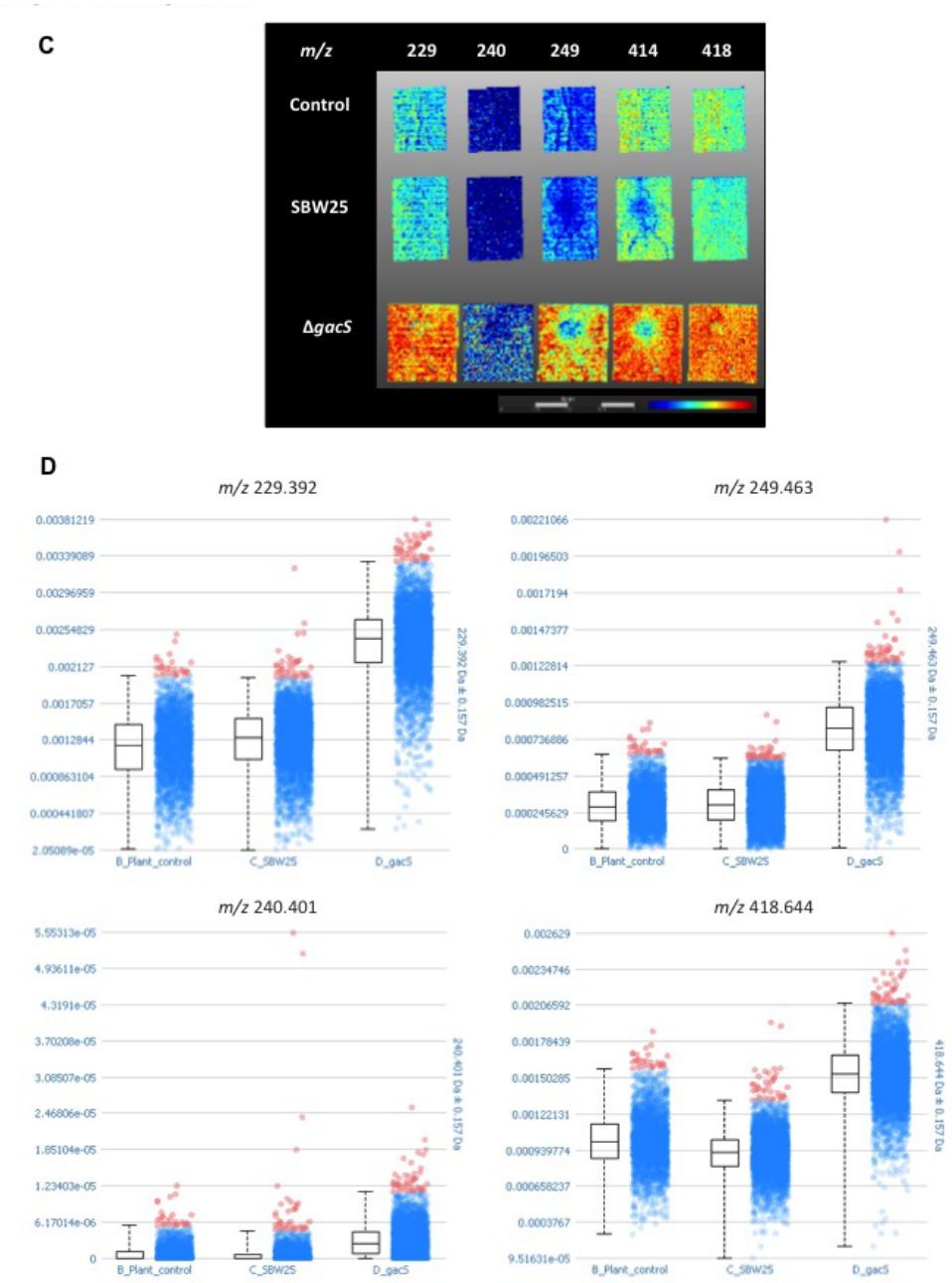


Figure 4 MALDI-IMS shows 4 ions (*m/z* of 170, 264, 334 and 1164) detected on roots treated with *Pf.*SBW25 (A) and 5 highlighted ions detected on root treated with the *gacS* mutant (C). The box plots represent the median intensity in arbitrary units after TIC normalization (horizontal line) (B, D), 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).

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

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Supplementary figures and tables

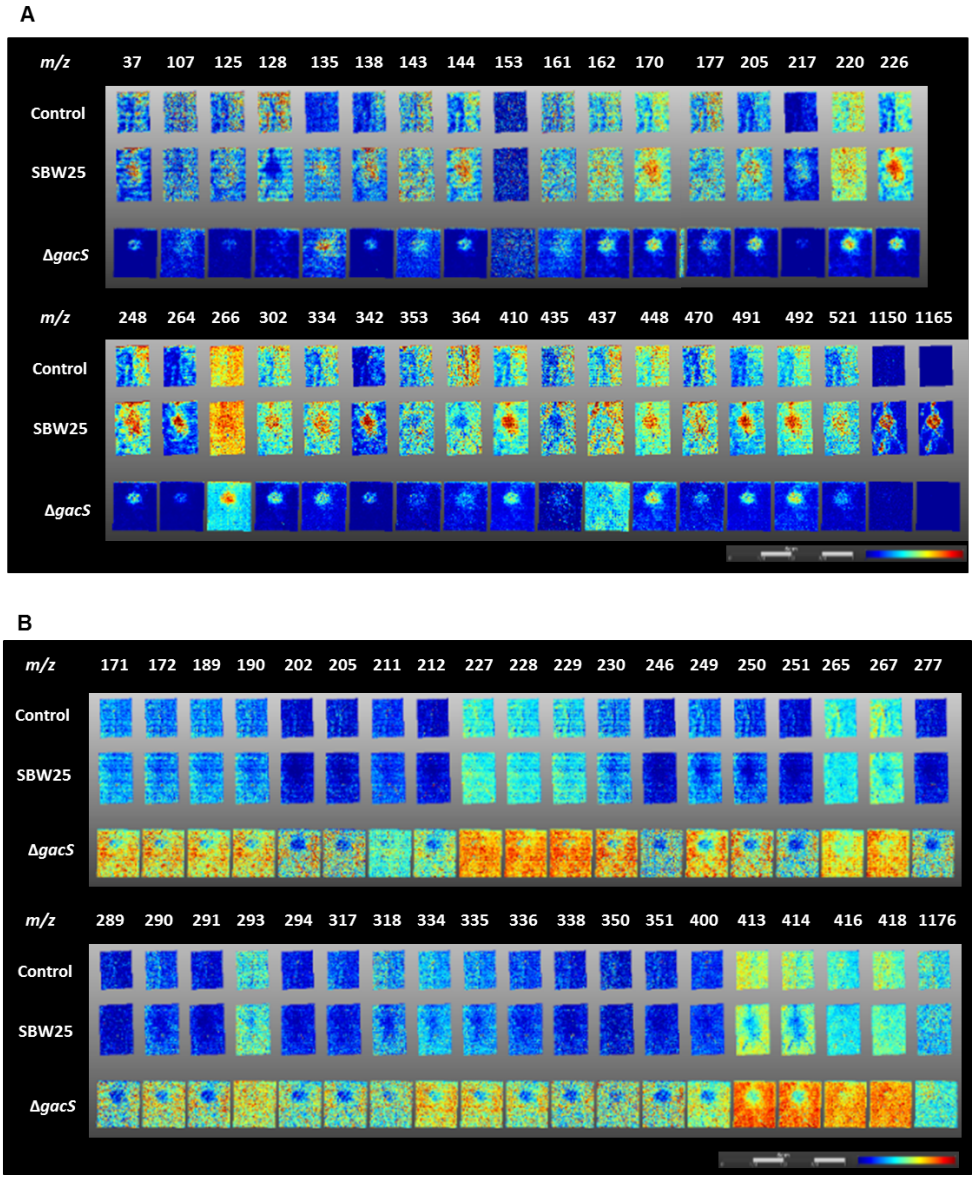
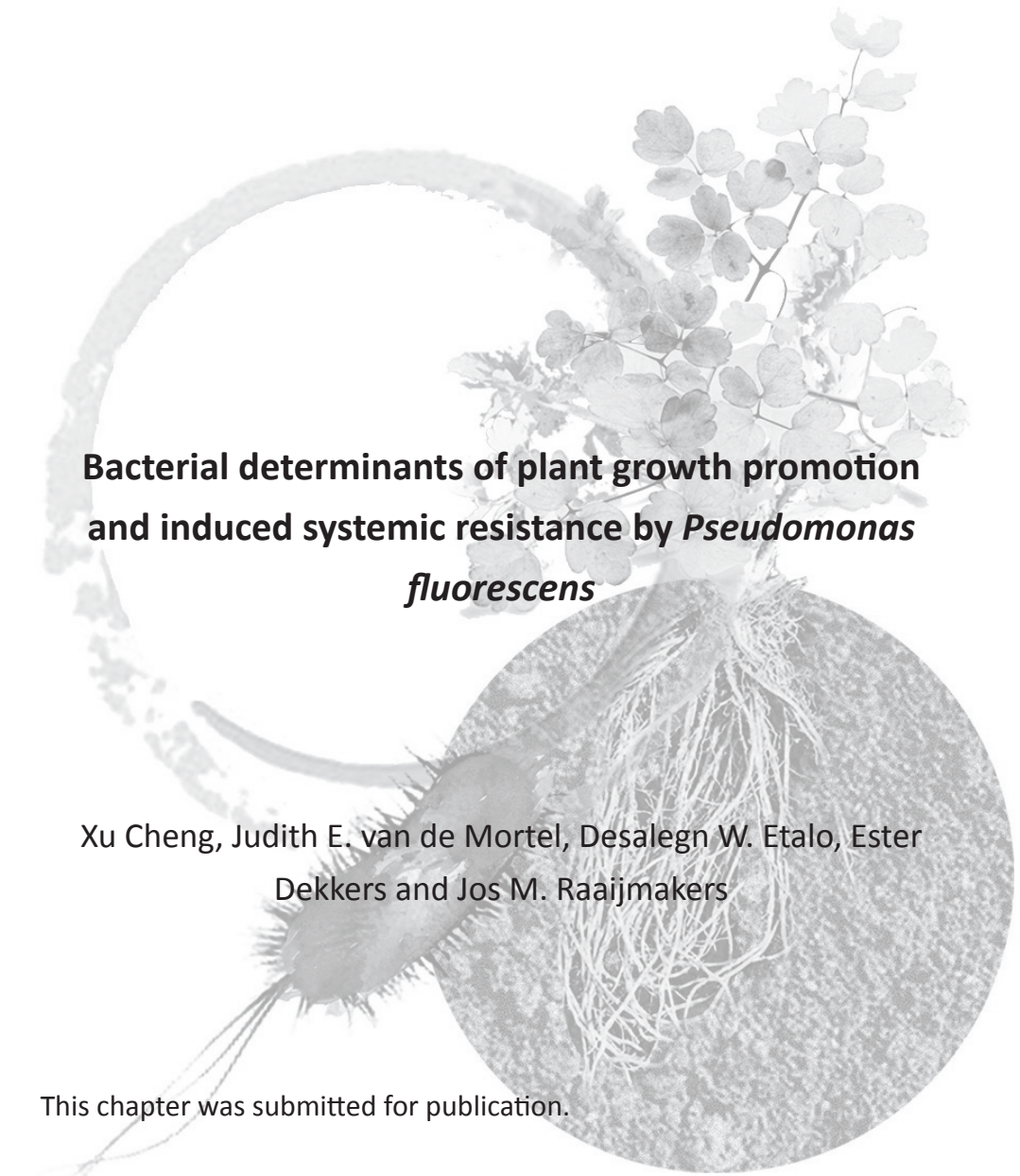


Figure S1 Chemical profiles of the *Pf*.SBW25 or the *gacS* mutant-Arabidopsis interactions detected by MALDI-IMS. Data were analyzed by using SCiLS Lab 2014b. Spatial distribution of detected ions were visualized and compared. (A) shows the selected 35 ions (AUC>0.75), which are more abundant in the *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.

Centroid	± Da	Maximal AUC at m/z	Maximal AUC
37.646	0.047	37.646	0.782
102.960	0.227	102.960	0.882
128.700	0.161	128.814	0.805
138.565	0.173	138.565	0.770
162.604	0.203	162.604	0.770
170.542	0.213	170.542	0.769
177.119	0.227	176.892	0.972
177.799	0.222	177.799	0.827
182.788	0.228	182.788	0.765
188.685	0.236	188.685	0.792
217.826	0.272	217.940	0.903
218.734	0.273	218.847	0.871
219.868	0.275	219.754	0.821
220.775	0.276	220.888	0.848
226.784	0.283	226.784	0.771
241.072	0.301	241.072	0.822
255.132	0.319	255.132	0.791
264.657	0.331	264.884	0.886
266.925	0.334	266.925	0.847
302.871	0.379	302.984	0.875
325.889	0.407	325.889	0.754
328.044	0.410	328.157	0.775
329.178	0.411	329.064	0.770
366.257	0.458	366.257	0.799
372.040	0.465	372.153	0.817
380.091	0.475	380.091	0.761
404.357	0.505	404.357	0.760
408.552	0.511	408.439	0.787
410.026	0.513	410.253	0.884
411.300	0.427	411.387	0.751
412.155	0.428	412.067	0.847
448.806	1.587	448.353	0.888
454.136	0.568	454.249	0.771
492.122	0.615	492.349	0.831
504.822	0.631	504.595	0.883
522.625	1.021	522.285	0.933
530.449	0.663	530.449	0.793
533.347	0.616	533.397	0.765
534.581	0.618	534.758	0.799
549.045	0.686	548.818	0.903
578.981	0.724	578.981	0.838
592.928	0.741	592.815	0.827
622.977	0.779	622.977	0.751
636.697	2.154	637.037	0.930
651.438	0.814	651.098	0.801
675.591	0.844	675.591	0.764
681.827	1.701	681.714	0.890
695.661	1.021	695.094	0.869
719.814	0.900	719.587	0.784
725.370	0.907	725.257	0.793
739.544	0.924	739.090	0.845
758.120	0.814	758.140	0.780
759.749	0.815	759.501	0.756
769.253	0.962	769.253	0.824
783.540	0.979	783.313	0.842
827.536	1.134	827.310	0.797
858.946	1.074	859.059	0.765
865.183	1.361	864.729	0.817
870.399	2.721	870.399	0.859
908.725	2.721	908.725	0.875
915.415	1.144	915.075	0.776
947.279	1.587	947.052	0.827
985.605	1.232	985.152	0.783
1046.950	1.309	1046.610	0.776
1053.074	1.928	1053.414	0.823
1097.410	2.268	1096.956	0.812
1135.396	1.419	1134.829	0.762
1211.858	1.211	1211.936	0.765
1214.282	1.213	1213.750	0.781
1324.648	1.656	1324.194	0.787

Chapter 6



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

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This chapter was submitted for publication.

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

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 $MgSO_4$ and resuspended in 10 mM $MgSO_4$ to a final density of 10^9 CFU ml^{-1} (OD_{600} = 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

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 (10^9 CFU ml^{-1}); in the control treatment, seedlings were inoculated with 2 µl of 10 mM $MgSO_4$. 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 (10^9 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 $MgSO_4$. For the root tip treatment, 2 µl *Pf.*SS101 (10^9 CFU ml^{-1}) was applied to the root tips of one-week-old seedlings. Control plants were inoculated with 2 µl of 10 mM $MgSO_4$. The challenge with *Pst* was performed by inoculation of 2 µl cell suspension (10^9 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-

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 *Pf.*SS101 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% toludine 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 $MgSO_4$ 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

elongation step at 72 °C for 5 min. All fragments were run on a 1% (w/v) agarose gel and purified with illustra™GFX™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. Electroporation 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 $MgSO_4$, vortexing and spinning down. The *Pf.*SS101 cells were frozen in liquid N_2 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 $MgCl_2$ 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 (C_T) was used to determine transcript levels. For each gene the threshold was fixed based on the exponential segment of the PCR curve. The C_T value for the gene of interest was corrected for the

housekeeping gene as follows: $\Delta C_T = C_{T(gene)} - C_{T(rpoD)}$. The relative quantification (RQ) values were calculated by the formula RQ = $2^{-[\Delta CT(\text{day 7, 10, 4 or 18}) - \Delta CT(\text{day 0})]}$. If there was no difference in transcript level between day 7, 10, 14 or 18 and day 0, then RQ = 1 (2^0) and log 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 α-ketobutyrate was generated. 200 µl of 0, 0.1, 0.25, 0.5, 0.75 and 1 µM α-ketobutyrate in 0.1 M Tris-HCl pH 8.5 was added in a sterile glass test tube. 300 µl 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 α-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 µl 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 µl of 0.1M Tris-HCl pH 8.5. 30 µl of toluene was added and the tubes were vortexed for 30 seconds at the highest setting. 200 µl of the samples were transferred into new 1.5 ml microcentrifuge tubes, 20 µl 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

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.

Conditions	Treatment	Leaves (mg)*	Roots (mg)*
Soil	Control	1120 ± 120^{a}	940 ± 200^{a}
	SS101	1940 ± 200^{b}	2730 ± 530^{c}
in vitro	Control	3.95 ± 0.32^{d}	7.3 ± 0.00^{e}
	SS101	14.6 ± 1.07^{f}	41.6 ± 5.70^{g}

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 1x10^{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 x10^{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.

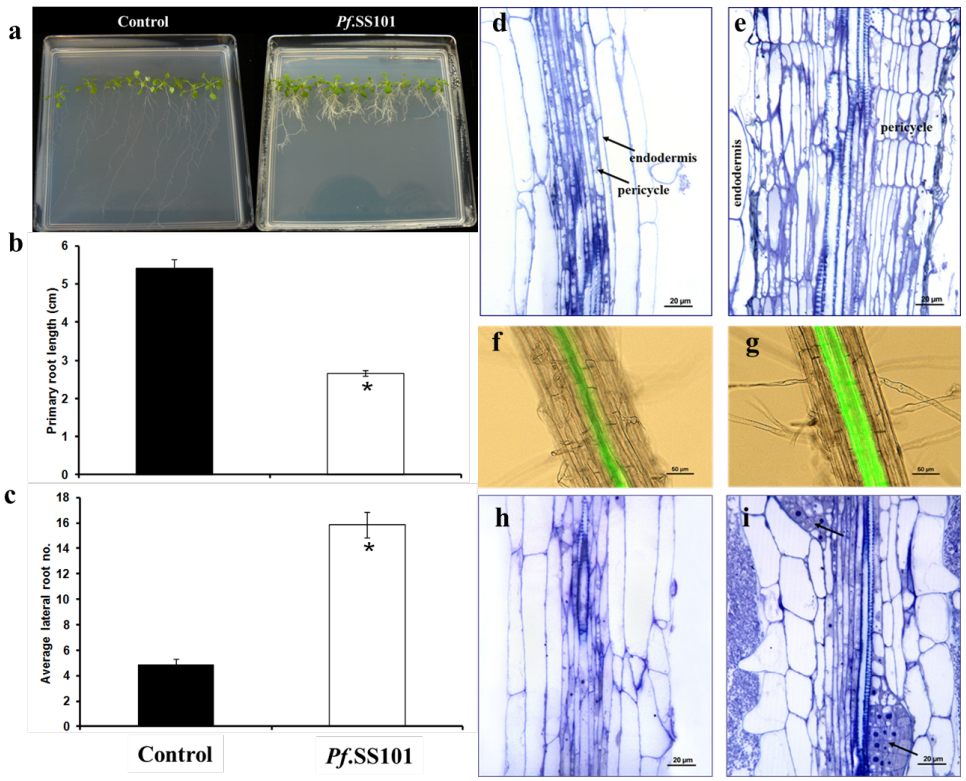

Figure 1 *Pf.*SS101 promotes growth of Arabidopsis grown on MS agar medium *in vitro* (a). Primary root length of mock- and *Pf.*SS101-treated Arabidopsis grown for 14 days *in vitro* (b). Average number of lateral roots of mock- and *Pf.*SS101-treated Arabidopsis grown for 14 days *in vitro* (c). Longitudinal 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 (± standard error) with 15-20 plants per replicate. Bars with asterisk are significantly different as determined by One-way ANOVA analysis (P<0.05).

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).

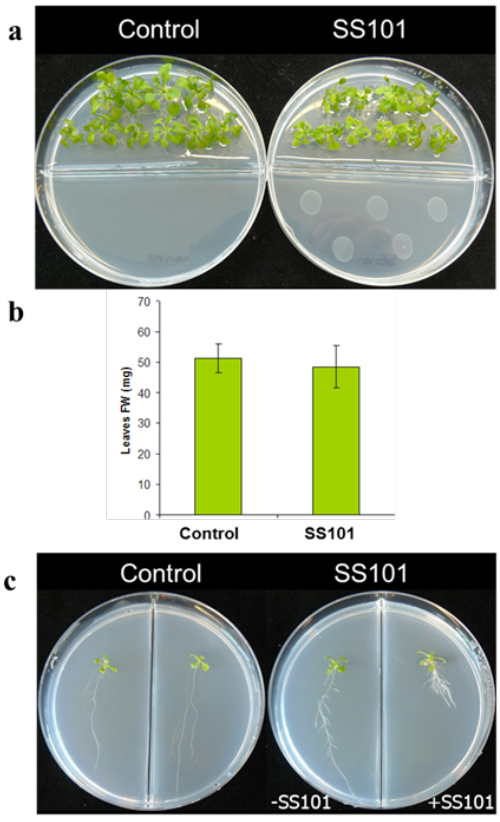

Figure 2 Effect of *Pf.*SS101 on Arabidopsis growth via volatile production. Phenotypic observation of Arabidopsis explored to *Pf.*SS101 grown on MS medium (a) and fresh weight of leaves were quantified (b); (c) Arabidopsis with root tip inoculation of *Pf.*SS101 (+SS101) was grown on one side of the 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).

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 massitolide A (EXP3), the siderophore mutant (61C8, Pflss101_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 masssetolide 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.

		Plant growth promotion		ISR
	Treatment	**Leaves (mg)**	**Roots (mg)**	**Chlorotic leaves (%)**
EXP1	Control	3,95±0,32[a]	7,3[a]	79,44 ± 9,73[a]
	Pf.SS101	14,59±1,07[b]	41,6[b]	7,77 ± 2.42[b]
	Dead cells	4,5±0,51[a]	8,6[a]	83,88 ± 4.94[a]
EXP2	Control	4,30 ± 0,73[a]	1,21 ± 0,54[a]	93.74 ± 1,81[a]
	Pf.SS101	7,29 ± 2,09[b]	2,85 ± 0,10[b]	0.00 ± 0,00[b]
	gacSΔ	10,39 ± 0,50[c]	3,65 ± 0,24[b]	16.31 ± 3,94[c]
	massAΔ	7,14 ± 1,16[b]	3,29 ± 0,98[b]	3.30 ± 1,19[b]
EXP3	massA0 *	7,40 ± 1,22[b]	2,49 ± 0,36[c]	89.73 ± 1,96[a]
	massA10*	5,77 ± 1,11[a]	2,48 ± 0,61[c]	94.11 ± 2,36[a]
	massA25*	5,87 ± 0,39[a]	2,07 ± 0,33[d]	85.14 ± 3,59[a]
	massA50*	5,25 ± 0,19[a]	2,52 ± 0,61[c]	84.78 ± 5,25[a]
EXP4	Control	10,61 ± 0,83[a]	3,56 ± 0,54[a]	79,44 ± 9,73[a]
	Pf.SS101	12,32 ± 0,12[a]	5,08 ± 0,53[b]	7,77 ± 2.42[b]
	61C8	10,85 ± 0,43[a]	5,13 ± 0,59[b]	9,44 ± 3.89[b]

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 *Pflss101_3099*), enhanced root biomass

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

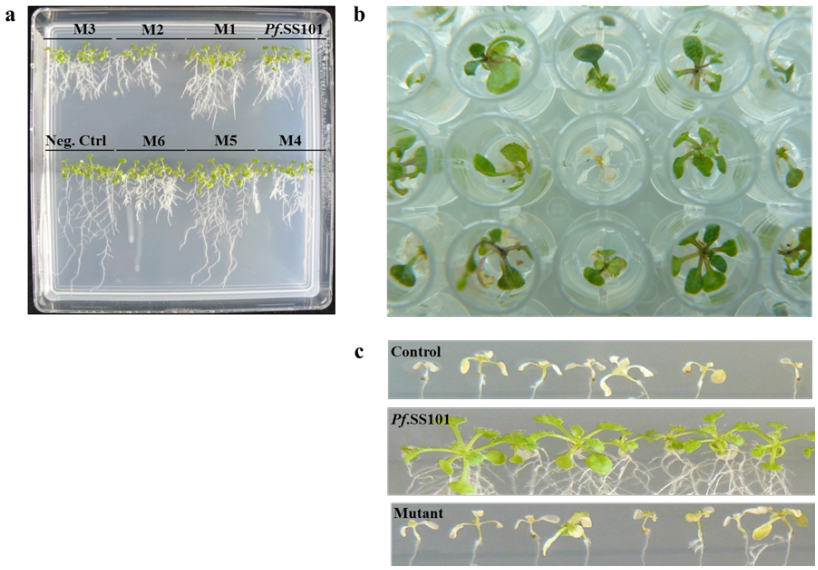

Figure 3 Screening of 7488 *Pf.*SS101 random plasposon mutants for lateral root formation on square plates (a) or for pathogen resistance in 96-well plates (b+c). Bacterial suspensions were inoculated on Arabidopsis seeds after sowing on plates carrying 0.5x MS. *P.syringae* pv. *Tomato* (*Pst*) was inoculated on rosset to infecting Arabidopsis plants.

Table 3 Phenotypic and genetic characterization of *Pf.*SS101 and mutants deficient in plant growth promotion and ISR. [a] COG Functional annotation. C = Energy production and conversion; E = Amino acid transport and metabolism; T = Signal transduction mechanisms. Cell density of *Pf.*SS101 and the mutants grown in KB and SSM media at 25°C were measured at OD_{600nm}; (+) = growth; (-) = no growth.

Strains	Colonisation Log (cfu mg^{-1}) roots	Locus_tag	Gene	Product	COG[a]	Growth curve	
						KB	SSM
Pf.SS101	5.54 ± 0.04^{a}					+	+
7H2	4.16 ± 0.03^{b}	PflSS101_4580	*ilvC*	ketol-acid reductoisomerase	E (valine, leucine, isoleucine)	+	-
44D8	3.55 ± 0.00^{c}	PflSS101_4908	*argC*	N-acetyl-gamma-glutamyl-phosphate reductase	E (arginine)	+	-
24B12	4.31 ± 0.07^{b}	PflSS101_0919	*hisD*	histidinol dehydrogenase	E (histidine)	+	-
22G5	3.95 ± 0.12^{b}	PflSS101_5114	*metX*	homoserine O-acetyltransferase	E (methionine)	+	-
71H9	873 ± 114^{d}	PflSS101_0035	*trpB*	tryptophan synthase, beta subunit	E (tryptophan)	+	-
13E4	2.93 ± 0.00^{e}	PflSS101_0355	*hisA*	1-(5-phosphoribosyl)-5-[(5- phosphoribosylamino)methylideneamino]imidazole-4- carboxamide isomerase	E (histidine)	+	-
18F11	4.17 ± 0.09^{b}	PflSS101_0437	*gltB*	glutamate synthase, large subunit	E (glutamate)	+	-
24A12	4.09 ± 0.30^{b}	PflSS101_4919	*trpD*	anthranilate phosphoribosyltransferase	E (tryptophan)	+	-
40H11	4.94 ± 0.06^{e}	PflSS101_1161	*argG*	argininosuccinate synthase	E (arginine)	+	-
51G1	4.20 ± 0.15^{b}	PflSS101_3512	*metZ*	O-succinylhomoserine sulfhydrylase	E (methionine)	+	-
59B6	3.08 ± 0.10^{d}	PflSS101_3523	*leuB*	3-isopropylmalate dehydrogenase	C (valine, leucine, isoleucine)	+	-
9F8	4.36 ± 0.09^{b}	PflSS101_3526	*leuC*	3-isopropylmalate dehydratase, large subunit	E (valine, leucine, isoleucine)	+	-
42B9	4.52 ± 0.06^{b}	PflSS101_3837	*cysM*	cysteine synthase B	E (cysteine)/sulfur metabolism	+	-
20H12	5.63 ± 0.02^{a}	PflSS101_3982	*cysH*	adenylylsulfate reductase, thioredoxin dependent	E (cysteine)/sulfur metabolism	+	-
25C8	5.60 ± 0.03^{a}	PflSS101_4354	*edd*	phosphogluconate dehydratase	E (glucose utilization)	+	+
16G6	3.73 ± 0.10^{c}	PflSS101_4370	*colR*	DNA-binding response regulator ColR	T	+	+

[a] 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 ($P<0.05$).

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

Table 4 Growth of *Pf.*SS101 and the 10 plasposon mutants in SSM supplemented with different L-/D-amino acids at 25°C; cell density was measured spectrophotometrically (600 nm) in six replicates. (+) = growth.

Strains	Locus_tags	Mutated genes	Supplementation of amino acids	
			L-	D-
Pf .SS101			+	+
44D8	PflSS101_4908	*argC*	+	+
24B12	PflSS101_0919	*hisD*	+	+
71H9	PflSS101_0035	*trpB*	+	+
13E4	PflSS101_0355	*hisA*	+	+
18F11	PflSS101_0437	*gltB*	+	+
24A12	PflSS101_4919	*trpD*	+	+
40H11	PflSS101_1161	*argG*	+	+
59B6	PflSS101_3523	*leuB*	+	+
42B9	PflSS101_3837	*cysM*	+	+
20H12	PflSS101_3982	*cysH*	+	+

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)-

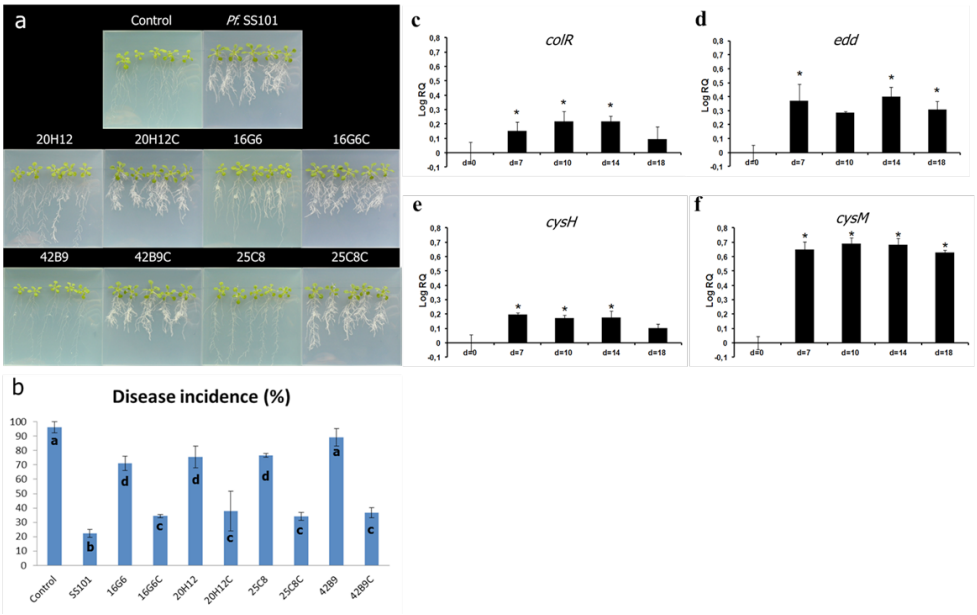

Figure 4 Effects of *Pf.*SS101 and the mutants (20H12, 25C8, 16G6, 42B9 and the complemented strains) on lateral root formation (a) and ISR (b). Q-PCR analysis of genes involved in plant growth promotion, lateral root formation and pathogen resistance by *Pf.*SS101 were showed: (c) expression level of *ColR*; (d) expression level of *edd*; (e) expression level of *cysH*; (f) expression level of *cysM*. Gene expression levels are measured in wild-type strain SS101 after 0, 7, 10, 14 and 18 days of growth on *Arabidopsis thaliana* Col-0. The transcript level of each gene was corrected for the transcript level of *rpoD* and is presented relative to the level at day 0 (logRQ). Black bars represent, for each time point, the mean values of gene expressions in four biological replicates. Blue bars represent the mean of disease incidence (%, ± standard deviation) of 4 replicates with 10-15 Arabidopsis seedlings per replicate. Experiments were performed at least twice and representative results are shown. The asterisk indicate statistically significant different relative to day 0 and letters represent a significant difference of the means according to One-way ANOVA (P<0.05).

lyase encoded by *cysM*. 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

Table 5 Genes involved in sulfur transport and assimilation in *Pf*.SS101. a Functional annotation. C = Energy production and conversion; E = Amino acid transport and metabolism; H = Coenzyme metabolism; J = Translation, ribosomal structure and biogenesis; K = Transcription; O = Posttranslational modification, protein turnover, chaperones; P = Inorganic ion transport and metabolism; R = General function prediction only; S = Function unknown.

Locus_tag	Gene	Product	COG[a]
PflSS101_0214	*sbp*	sulfate ABC transporter, periplasmic sulfate-binding protein	P
PflSS101_1778	*sbp2/cysP*	periplasmic sulfate-binding protein	P
PflSS101_0215	*cysT*	sulfate ABC transporter, permease protein CysT	O
PflSS101_0216	*cysW*	sulfate ABC transporter, permease protein CysW	P
PflSS101_0217	*cysA*	sulfate ABC transporter, ATP-binding protein CysA	P
PflSS101_0778	*cysD*	sulfate adenylyltransferase, small subunit	E
PflSS101_0779	*cysC/cysN*	putative sulfate adenylyltransferase, large subunit/adenylylsulfate kinas	P
PflSS101_0300	*cysQ*	3'(2'),5'-bisphosphate nucleotidase	P
PflSS101_2247	*cysI*	sulfite reductase (NADPH) hemoprotein, beta-component	P
PflSS101_3954	*cysJ*	Molybdopterin oxidoreductase	R
PflSS101_3184	*cysG*	siroheme synthase	H
PflSS101_4462	*cysE*	serine O-acetyltransferase	E
PflSS101_3837	*cysM*	cysteine synthase B	E
PflSS101_1560	*cysK*	cysteine synthase A	E
PflSS101_1716	*cysB*	HTH-type transcriptional regulator CysB	K
PflSS101_4454	*iscX*	FeS assembly protein IscX	S
PflSS101_4455	*fdx*	ferredoxin, 2Fe-2S type, ISC system	C
PflSS101_4456	*hscA*	Fe-S protein assembly chaperone HscA	O
PflSS101_4457	*hscB*	Fe-S protein assembly co-chaperone HscB	O
PflSS101_4458	*iscA*	iron-sulfur cluster assembly protein IscA	S
PflSS101_4459	*iscU*	FeS cluster assembly scaffold IscU	C
PflSS101_4460	*iscS*	cysteine desulfurase IscS	E
PflSS101_4461	*iscR*	iron-sulfur cluster assembly transcription factor IscR	K
PflSS101_4462	*cysE*	serine O-acetyltransferase	E
PflSS101_4463	*trmJ*	tRNA (cytidine/uridine-2'-O-)-methyltransferase TrmJ	J

medium supplemented with different concentrations of cysteine and methionine. 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 *Pst* in Arabidopsis (Fig. 5c). Preliminary LC-MS/MS analyses did not show detectable levels of cysteine and methionine production by *Pf.*SS101 when grown on MS agar medium or on Arabidopsis roots under *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 *Pf*.SS101 and the *cysH* mutant (20H12). To explore the expression pattern of Arabidopsis genes that were altered by *Pf.*SS101 or the *cysH* mutant, the expression of all 22,850 genes present on the ATH1 genome

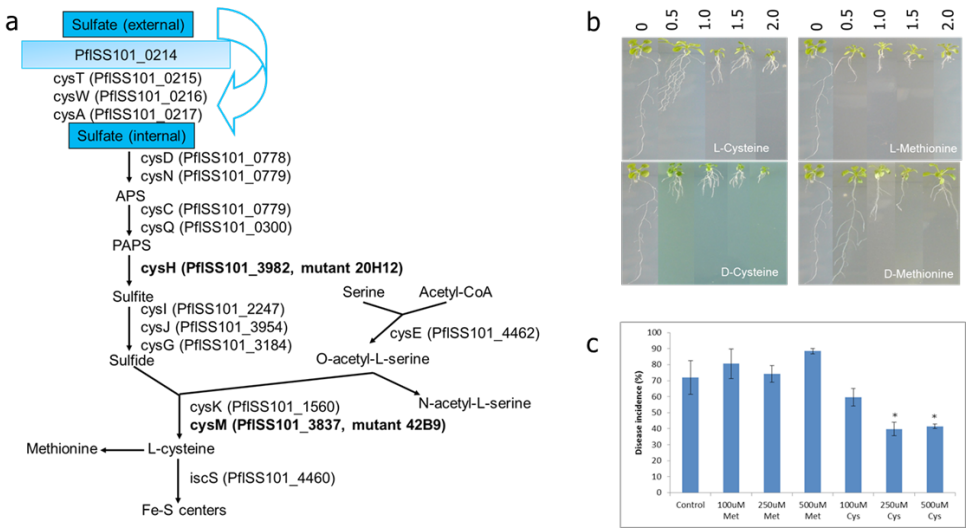

Figure 5 (a) Proposed metabolic model for the uptake and assimilation of sulfur in *Pf.*SS101. Candidate genes are shown in bold; (b) effects of L-/D-cysteine and methionine on root architecture of Arabidopsis; (c) effects of cysteine on ISR of Arabidopsis against *Pst*. Bars represent the mean of disease incidence (%, ± standard deviation) of 4 replicates with 10-15 Arabidopsis seedlings per replicate. Bars with asterisk are significantly different from each other as determined by One-way ANOVA analysis (*P*<0.05**).**

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

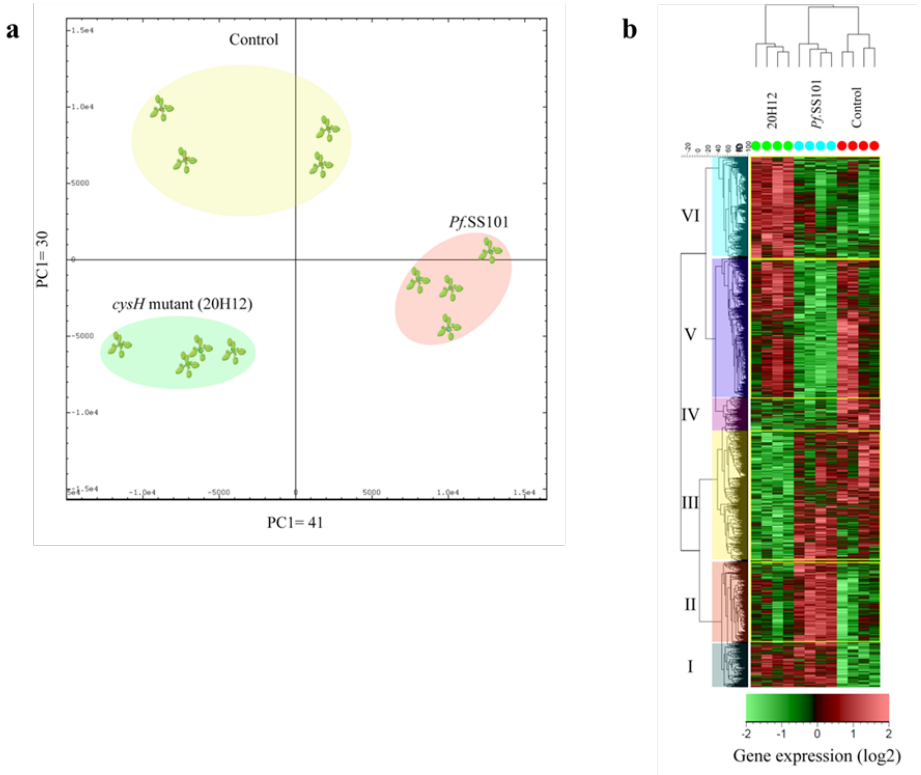

Figure 6. Genome wide transcriptome analysis of Arabidopsis treated with cell suspensions of *Pf.*SS101, the *cysH* mutant (20H12) or not treated (control). Principal component analysis (PCA) (a) based on 6308 genes that showed differential regulation ($P<0.05$, with no FDR correction) between Arabidopsis 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.

Gene Set Name (#*)	Gene orthology (GO)	NO. Genes in Overlap (k)$	p value	FDR
CYSTEINE_BIOSYNTHETIC_PROCESS(210)	GO:0019344	34	1.44E-19	1.54E-16
SERINE_FAMILY_AMINO_ACID_BIOSYNTHETIC_PROCESS(222)	GO:0009070	34	6.70E-19	5.72E-16
CARBOHYDRATE_BIOSYNTHETIC_PROCESS(1069)	GO:0016051	71	1.84E-18	1.47E-15
CELLULAR_AMINO_ACID_BIOSYNTHETIC_PROCESS(501)	GO:0008652	48	1.99E-18	1.50E-15
PRIMARY_METABOLIC_PROCESS(11672)	GO:0044238	321	1.91E-15	7.63E-13
SECONDARY_METABOLIC_PROCESS(1241)	GO:0019748	70	8.62E-15	3.35E-12
CARBOXYLIC_ACID_BIOSYNTHETIC_PROCESS(1113)	GO:0046394	65	1.90E-14	6.77E-12
RESPONSE_TO_SALT_STRESS(780)	GO:0009651	52	8.27E-14	2.72E-11
GLYCERALDEHYDE-3-PHOSPHATE_METABOLIC_PROCESS(232)	GO:0019682	28	1.85E-13	5.78E-11
ISOPENTENYL_DIPHOSPHATE_BIOSYNTHETIC_PROCESS,_MEVALONATE-INDEPENDENT_PATHWAY(229)	GO:0019288	27	8.27E-13	2.36E-10
LIPID_BIOSYNTHETIC_PROCESS(1156)	GO:0008610	62	2.65E-12	7.07E-10
PHOSPHOLIPID_BIOSYNTHETIC_PROCESS(403)	GO:0008654	34	5.70E-12	1.46E-09
CATABOLIC_PROCESS(2175)	GO:0009056	91	1.03E-11	2.58E-09
RESPONSE_TO_STRESS(4037)	GO:0006950	139	1.56E-11	3.85E-09
COENZYME_METABOLIC_PROCESS(507)	GO:0006732	37	3.36E-11	7.84E-09
SMALL_MOLECULE_CATABOLIC_PROCESS(931)	GO:0044282	52	4.40E-11	9.41E-09
MONOCARBOXYLIC_ACID_METABOLIC_PROCESS(1480)	GO:0032787	69	5.85E-11	1.23E-08
GLUCOSINOLATE_BIOSYNTHETIC_PROCESS(172)	GO:0019761	21	1.63E-10	3.11E-08
GLUCOSE_CATABOLIC_PROCESS(474)	GO:0006007	34	3.27E-10	5.91E-08
STARCH_BIOSYNTHETIC_PROCESS(191)	GO:0019252	21	9.28E-10	1.51E-07
REGULATION_OF_BIOLOGICAL_QUALITY(1637)	GO:0065008	68	7.85E-09	1.09E-06
PHOTORESPIRATION(161)	GO:0009853	18	1.16E-08	1.57E-06
HETEROCYCLE_METABOLIC_PROCESS(1020)	GO:0046483	49	1.78E-08	2.31E-06
NUCLEOTIDE_METABOLIC_PROCESS(685)	GO:0009117	38	2.39E-08	3.00E-06
TRANSPORT(3558)	GO:0006810	115	4.79E-08	5.68E-06
PENTOSE-PHOSPHATE_SHUNT(200)	GO:0006098	19	5.06E-08	5.86E-06
CALCIUM_ION_TRANSPORT(121)	GO:0006816	15	5.61E-08	6.36E-06
LOCALIZATION(3799)	GO:0051179	119	1.37E-07	1.45E-05
RESPONSE_TO_ORGANIC_SUBSTANCE(2739)	GO:0010033	91	5.29E-07	5.47E-05
ORGANELLE_ORGANIZATION(2037)	GO:0006996	73	6.06E-07	6.22E-05
JASMONIC_ACID_BIOSYNTHETIC_PROCESS(135)	GO:0009695	13	5.68E-06	5.24E-04
GENERATION_OF_PRECURSOR_METABOLITES_AND_ENERGY(730)	GO:0006091	33	1.34E-05	1.15E-03
CELLULAR_CATION_HOMEOSTASIS(172)	GO:0030003	14	1.49E-05	1.26E-03
STEROID_BIOSYNTHETIC_PROCESS(221)	GO:0006694	16	1.47E-05	1.26E-03
HORMONE_BIOSYNTHETIC_PROCESS(277)	GO:0042446	18	1.74E-05	1.46E-03
GLYCINE_CATABOLIC_PROCESS(53)	GO:0006546	8	2.03E-05	1.69E-03
BIOLOGICAL_REGULATION(6172)	GO:0065007	162	2.32E-05	1.90E-03
RESPONSE_TO_DESICCATION(39)	GO:0009269	7	2.50E-05	2.03E-03
SERINE_FAMILY_AMINO_ACID_CATABOLIC_PROCESS(55)	GO:0009071	8	2.58E-05	2.08E-03
GLUCONEOGENESIS(169)	GO:0006094	13	5.15E-05	3.86E-03
RESPONSE_TO_NEMATODE(82)	GO:0009624	9	6.17E-05	4.55E-03
MICROTUBULE_NUCLEATION(66)	GO:0007020	8	8.37E-05	5.96E-03
RESPONSE_TO_GLUCOSE_STIMULUS(86)	GO:0009749	9	8.65E-05	6.13E-03
INDOLEACETIC_ACID_BIOSYNTHETIC_PROCESS(108)	GO:0009684	10	9.15E-05	6.41E-03
PROTEIN_COMPLEX_ASSEMBLY(577)	GO:0006461	26	1.15E-04	7.90E-03
PROTEIN_COMPLEX_BIOGENESIS(577)	GO:0070271	26	1.15E-04	7.90E-03
UNSATURATED_FATTY_ACID_BIOSYNTHETIC_PROCESS(70)	GO:0006636	8	1.22E-04	8.35E-03
ANTHOCYANIN_ACCUMULATION_IN_TISSUES_IN_RESPONSE_TO_UV_LIGHT(113)	GO:0043481	10	1.29E-04	8.59E-03
CHLOROPHYLL_METABOLIC_PROCESS(188)	GO:0015994	13	1.41E-04	9.26E-03
GLUTAMATE_METABOLIC_PROCESS(22)	GO:0006536	5	1.42E-04	9.29E-03
PROTEOLYSIS(940)	GO:0006508	36	1.48E-04	9.60E-03
RESPONSE_TO_BIOTIC_STIMULUS(1675)	GO:0009607	55	1.56E-04	1.01E-02
RESPONSE_TO_LIGHT_STIMULUS(1182)	GO:0009416	42	2.02E-04	1.29E-02
FLAVONOID_BIOSYNTHETIC_PROCESS(225)	GO:0009813	14	2.23E-04	1.41E-02
CELL_WALL_ORGANIZATION(609)	GO:0071555	26	2.57E-04	1.56E-02
ORGAN_DEVELOPMENT(2037)	GO:0048513	63	2.56E-04	1.56E-02
TRYPTOPHAN_CATABOLIC_PROCESS(79)	GO:0006569	8	2.61E-04	1.57E-02
PROTEASOME_CORE_COMPLEX_ASSEMBLY(126)	GO:0080129	10	2.94E-04	1.75E-02
CELLULAR_RESPONSE_TO_STIMULUS(2302)	GO:0051716	69	3.06E-04	1.80E-02
DEVELOPMENTAL_GROWTH(781)	GO:0048589	30	4.94E-04	2.78E-02
GLYCOLYSIS(219)	GO:0006096	13	5.63E-04	3.14E-02
RESPONSE_TO_CADMIUM_ION(470)	GO:0046686	21	5.63E-04	3.14E-02
ORGANIC_ANION_TRANSPORT(17)	GO:0015711	4	6.07E-04	3.34E-02
THYLAKOID_MEMBRANE_ORGANIZATION(198)	GO:0010027	12	7.65E-04	4.10E-02
RESPONSE_TO_COLD(622)	GO:0009409	25	7.74E-04	4.14E-02
AUXIN_POLAR_TRANSPORT(95)	GO:0009926	8	8.16E-04	4.34E-02
MERISTEM_GROWTH(173)	GO:0035266	11	8.67E-04	4.57E-02
PROTON_TRANSPORT(147)	GO:0015992	10	9.09E-04	4.76E-02

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

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 *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 *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 *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

also significantly enriched in *Pf.*SS101-treated seedlings.

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

The *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 *P. chlororaphis* O6, Kim et al. (2007) showed that the *edd* gene contributes to root colonization and ISR. They concluded that metabolism of sugars through the ED pathway in *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 *edd* gene was significantly higher expressed in *Pf*.SS101 on Arabidopsis and that the *Pf*.SS101 *edd* mutant 25C8 showed no induction of lateral root formation and systemic resistance in Arabidopsis, similar to what was shown for *P. chlororaphis* O6 (Kim et al., 2007). In contrast to *P. chlororaphis* O6, however, elimination of the *edd* gene in *Pf*.SS101 had no effect on colonization of Arabidopsis. A major determinant for ISR elicited by *P. chlororaphis* O6 against *E. carotovora* was the volatile 2R, 3R-butanediol (Han et al., 2006; Kim et al., 2007). Butanediol likely is produced from pyruvate

(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 *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 *Pf.*SS101. In particular sulfur metabolism was shown to be an important biological process altered by *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 *Pf*.SS101 will be required to shed more light on the other mechanisms of plant growth promotion and ISR.

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Supplementary figures

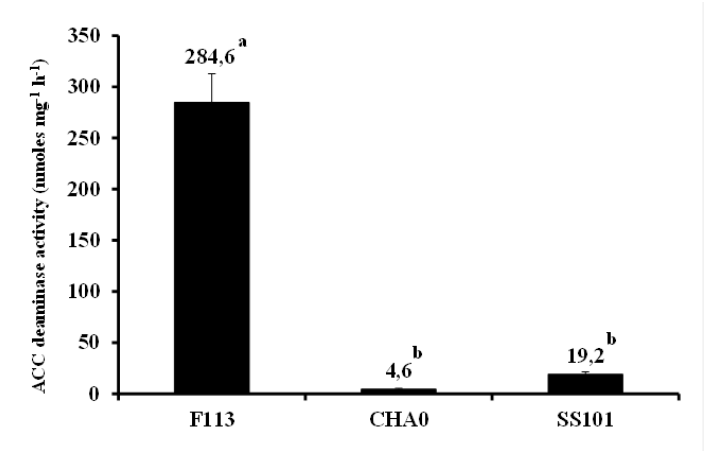

Figure S1. Measuring ACC deaminase activity in *Pf.*SS101. *Pf.*F113 was used as a positive control and *P. protegens* CHA0 was used as a negative control. α-ketobutyrate production by strains F113, CHAO and SS101 tested after incubation of their cell extracts with ACC. Averages of 4 replicates are given. Letters indicate significant differences among these strains tested according to One-way ANOVA analysis ($P<0.05$).

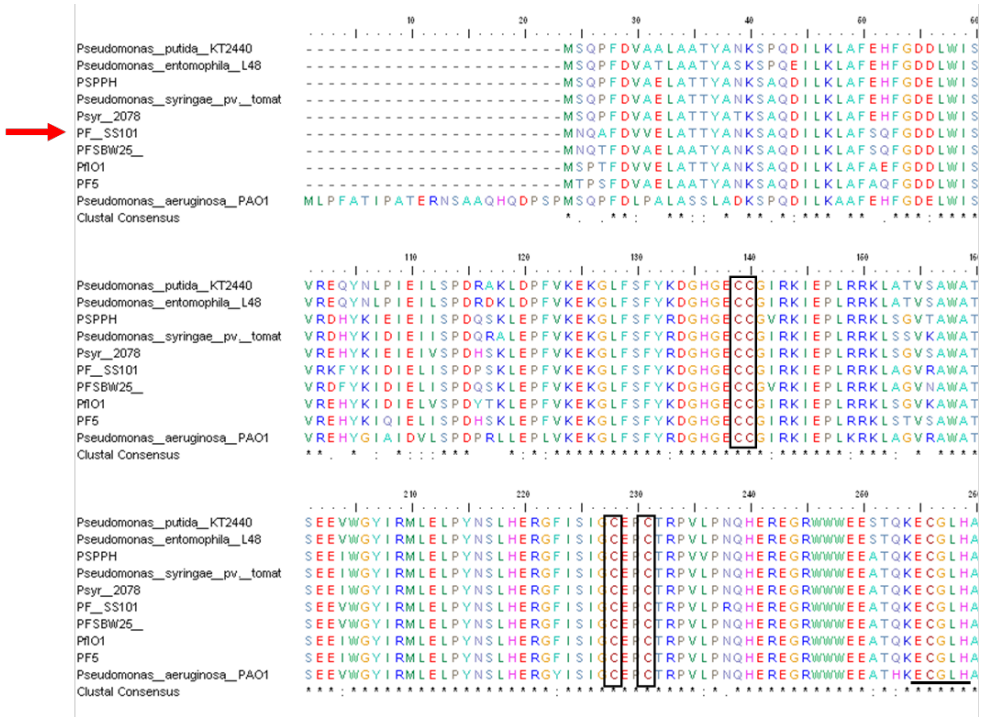

Figure S2. Comparison of amino acid sequences of APS reductase from *Pf*.SS101 with *P. putida*, *P. entomophila*, *P. syringae*, *Pf*.SBW25, *Pf*01, *Pf*5 and *P. aeruginosa*. The sequences were aligned with the program Clustal W. Asterisks identify identical residues, and arrows mark the additional Cys in APS reductases. The conserved APS reductase signature is underlined.

Chapter 7

Summarizing discussion and future perspectives

Xu Cheng

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

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

(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*

(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 PQQ 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-

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.

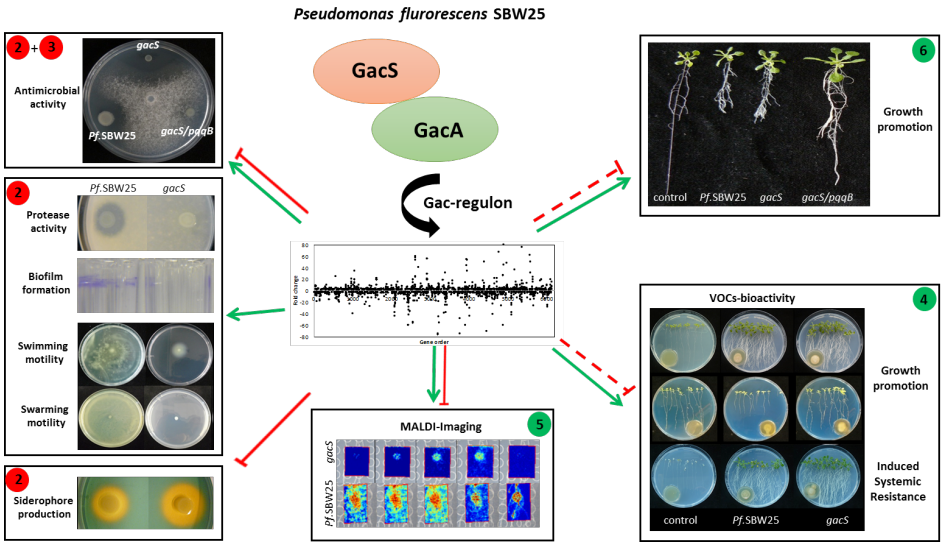

Figure 2 Importance of the Gac-system of *Pf.*SBW25 in the regulation of bioactive compound production, antagonism against different plant pathogens, plant growth promotion and ISR. The numbered circles refer to the corresponding thesis chapters. Circles with a red colour refer to chapters with a focus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

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.

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

To obtain a better understanding of the mechanisms of plant growth promotion,

the effects of *Pf*.SS101 on Arabidopsis growth and ISR were studied (**Chapter 6**). In previous studies it was shown that *Pf*.SS101 enhances resistance in Arabidopsis against several pathogens, including *Pst* and the insect pest *Spodoptera exigua* (van de Mortel *et al.*, 2012). In **Chapter 6**, we showed that Arabidopsis seeds or seedlings treated with *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 *Pf.*SS101. Screening of a random mutant library (7488 mutants) of *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 *Pf.*SS101. Arabidopsis transcriptome analysis showed that genes involved in the cysteine biosynthesis and sulfur-containing compounds biosynthesis were induced by *Pf*.SS101 but not by a mutant of *Pf.*SS101 (20H12, *ΔcysH*). Van de Mortel *et al.* (2012) showed that *Pf*.SS101 induces resistance against *Pst* and the insect *Spodoptera exigua* by boosting the levels of indolic and aliphatic glucosinolates in Arabidopsis. All together, the transcriptome analyses suggest that also *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 *Pseudomonas*. Recently, Song *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 *Pf.*SS101 (Song *et al.*, 2015). Identification of new metabolites by exploiting mutants with defects in biosynthetic or regulatory genes is a promising strategy (Rigali *et al.*, 2008, Laureti *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.

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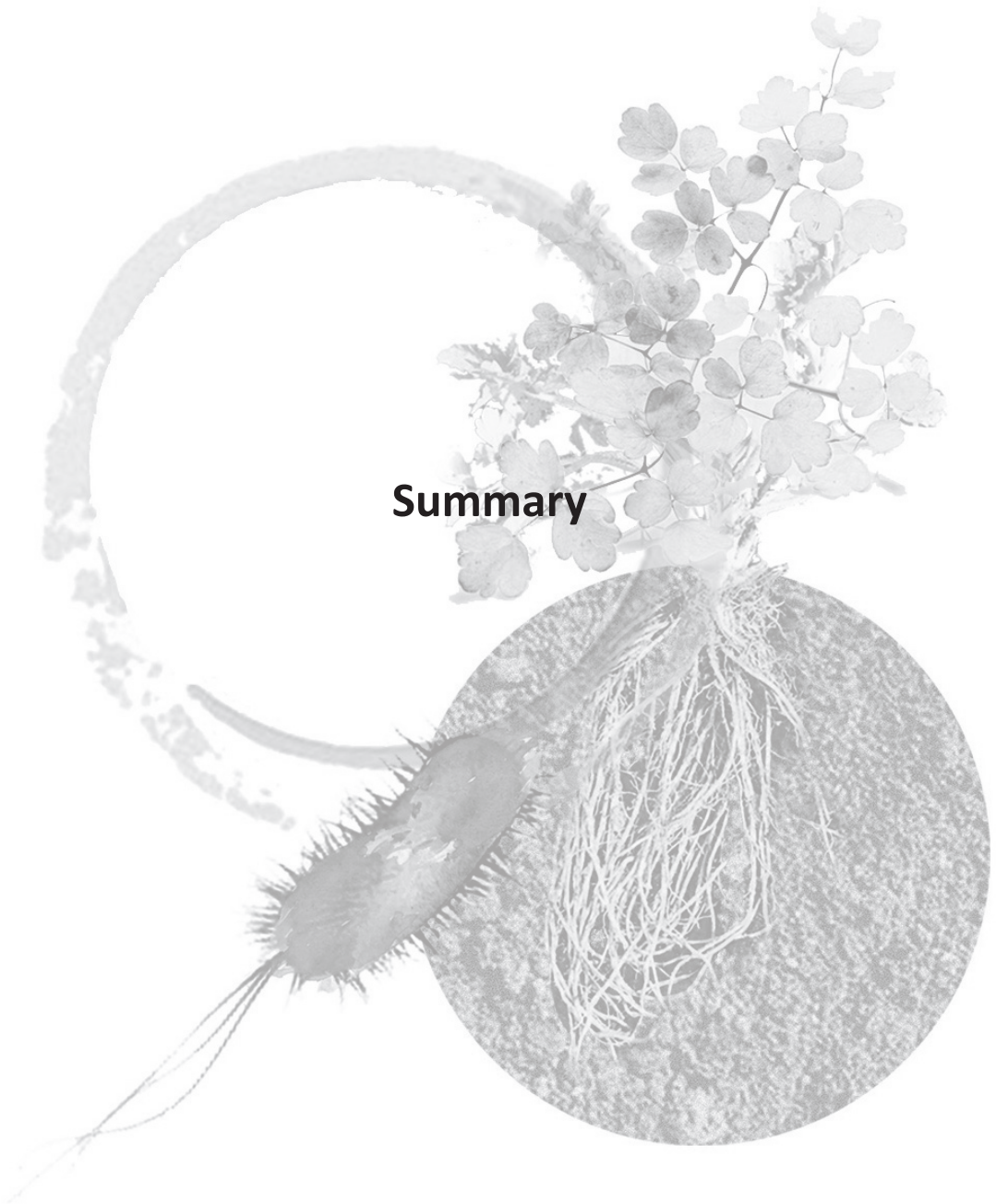

Summary

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

to pH levels that inhibited the growth of other microorganisms. Further analysis of the *gacS* mutant showed that the Gac-system also regulates the production of volatile organic compounds (VOCs) in *Pf.*SBW25, in particular the acyclic alkenes 1-undecene, 3-nonene and 4-undecyne. Moreover, Gac-mediated VOCs production by *Pf*.SBW25 affected plant growth promotion in a plant species-dependent manner. We also studied the effects on plants by directly inoculating *Pf*.SBW25 and the *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 *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 *Pf.*SBW25 changes when grown on Arabidopsis roots and that it produces viscosin and several other metabolites, tentatively identified as dodecane and acetoxypinoresinol. Comparison of the metabolites produced by *Pf*.SBW25 and the *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 *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 *Pf.*SS101.

In conclusion, the results presented in my thesis showed that the Gac-system in *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 *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 *P. fluorescens* and *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

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.

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Publications

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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！

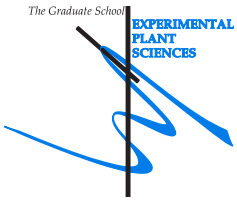
Curriculum Vitae

Xu Cheng (程旭) was born on 17th of April, 1983 in Handan of China. In 2001, he stared 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 Eco*L*inc 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

1. **Xu Cheng**, Jeramie Watrous, Janina Oetjen, Theodore Alexandrov, Pieter C. Dorrestein and Jos M. Raaijmakers (2016) Accumulation of multiple mutations leads to enhanced plant growth promotion by *Pseudomonas* spp. ***In preparation***.
2. **Xu Cheng**, Viviane Cordovez da Cunha, Desalegn W. Etalo, Menno van der Voort and Jos M. Raaijmakers (2016). Role of the GacS/GacA two-component system in regulation of volatile production by plant growth-promoting *Pseudomonas fluorescens* SBW25. ***Submitted.***
3. **Xu Cheng**[‡], Judith E. van de Mortel[†], Desalegn W. Etalo[†], Ester Dekkers and Jos M. Raaijmakers (2016). Bacterial determinants of plant growth promotion and induced systemic resistance by *Pseudomonas fluorescens*. ***Submitted***.
4. Xiaojie Qin[†], Hongmei Xiao[†], **Xu Cheng**, Hailian Zhou and Linyuan Si (2016). *Hanseniaspora uvarum* prolongs shelf life of strawberry via volatile production. ***Submitted.***
5. Irene de Bruijn, **Xu Cheng**, Ruth Gomez Exposito, Nrupali Patel, Joeke Postma, Donald Kobayashi, Jeramie Watrous, Pieter Dorrestein and Jos M. Raaijmakers (2015). Comparative genomics and chemical profiling of the soil bacteria *Lysobacter. BMC Genomics* 2015.
6. Chunxu Song[†], Mark Mazzola[†], **Xu Cheng**, Janina Oetjen, Theodore Alexandrov, Pieter Dorrestein, Jeramie Watrous, Menno van der Voort and Jos M Raaijmakers (2015). Molecular and chemical dialogues in bacteria-protozoa interactions. *Scientific Reports* 2015; 5:12837.
7. **Xu Cheng,** Menno van der Voort, and Jos M. Raaijmakers (2015). Gac-mediated changes in pyrroloquinoline quinone biosynthesis enhance the antimicrobial activity of *Pseudomonas fluorescens* SBW25. *Environ Microbiol Rep*. 2015, 7, 139–147.
8. **Xu Cheng[†],** Irene de Bruijn[†], Menno van der Voort[†], Joyce E. Loper and Jos M. Raaijmakers (2013). The Gac Regulon of *Pseudomonas fluorescens* SBW25. *Environ Microbiol Rep*. 2013, 4,608-19.
9. Petra Worm, Alfons J. M. Stams, **Xu Cheng** and Caroline M. Plugge (2011). Growth- and substrate-dependent transcription of formate dehydrogenase and hydrogenase coding genes in *Syntrophobacter fumaroxidans* and *Methanospirillum hungatei*. *Microbiology* 2011*,* 157, 280–289.
10. Jennifer L. Nelson, Jennifer M. Fung, Hinsby Cadillo-Quiroz, **Xu Cheng** and Stephen H. Zinder (2011). A Role for *Dehalobacter* spp. in the Reductive Dehalogenation of Dichlorobenzenes and Monochlorobenzene. *Environ. Sci. Technol.* 2011, 45, 6806–6813.
11. **Xu Cheng**, Petra Worm, Alfons J.M. Stams and Caroline M. Plugge (2008). The expression of formate dehydrogenases and hydrogenases in *Syntrophobacter fumaroxidans* in different growth phases and at different pHs. *Journal of Biotechnology* 2008, 136.

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		*date*
►	**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		*date*
►	**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 Commelinn 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**	

	Phytopathology Seminars	2010-2014
	Invited seminar Prof. Nick Panopoulos	Jan 11, 2010
	Invited seminar Laurent Zimmerli	Jan 25, 2010
	Plant Sciences Seminar Prof. Holger Meinke and Prof. Paul Struik	Apr 13, 2010
	Plant Sciences Seminar Prof. Louise Vet and Just Vlak	May 11, 2010
	Wageningen Evolution and Ecology Seminars Toby Kiers	Jun 17, 2010
	Invited seminar Prof. Naoto Sibuya	Sep 09, 2010
	Invited seminar Prof. David Baulcombe	Sep 27, 2010
	Invited seminar Dr. Kirsten Bomblies	Nov 18, 2010
	1st meeting WUR-NIOO Centre for Soil Ecology, Wageningen	Jun 27, 2011
	Invited seminar Rosie Bradshaw	Aug 04, 2011
	Invited seminar Birgit Piechulla : The smell of rhizobacteria: biological and chemical aspects	Oct 04, 2012
	Invited seminar Gabriele Berg: Plant microbes: Specificity and impact on plant health	Oct 09, 2012
	WEES seminar Bertus Beaumont: Adaptive radiation, flagella and the evolution of biological complexity	Jan 24, 2013
	Seminar: Evolution in the laboratory	Mar 14, 2013
	WEES seminar David Berry: Ecological and evolutionary aspects of the gut microbiota in health and inflammation	Mar 21, 2013
	Invited seminar Kathrin Riedel: Metaproteomics - novel insights into old questions in medical microbiology and microbial ecology	Mar 25, 2013
	WEES seminar Martin Ackermann: An evolutionary perspective on bacterial individuality	Jun 20, 2013
	Invited seminar Sara Mitri: Modelling the evolution of competition and cooperation between strains and species of bacteria	Sep 24, 2013
	Invited seminar Ross Mann: Endophytes in agriculture - evaluating their application via metabolomics and genomics	Oct 04, 2013
	Invited seminar Gabriele Berg	Oct 14, 2013
	PacBio Seminar	Mar 26, 2014
	EPS Symposium 'Omics Advances for Academia and Industry - Towards True Molecular Plant Breeding'	Dec 11, 2014
►	**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

	The 14th International Symposium on Microbial Ecology (ISME), Copenhagen, Denmark (Poster)	Aug 19-24, 2012
	The 2nd Annual Progress Meeting EcoLinc Consortium (Talk)	Apr 13, 2012
	The 3rd Annual Progress Meeting EcoLinc Consortium (Talk)	Apr 19, 2013
	The 10th International Congress of Plant Pathology (Invited talk)	Aug 25-30, 2013
	The 4th Rhizosphere conference (Rhizo4), Maastricht, NL (Talk)	Jun 21-25, 2015
►	**IAB interview**	
	Meeting with a member of the International Advisory Board of EPS	Dec 03, 2014
►	**Excursions**	
	CBSG Matchmaking Event for Young Plant Scientists	Oct 18, 2012
	Rijk Zwaan excursion	Sep 27, 2013
	Subtotal Scientific Exposure	*22.2 credits**

3) In-Depth Studies		*date*
►	**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		*date*
►	**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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