SIGNALING IN THE PHYTOMICROBIOME

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PUBLISHED IN: Frontiers in Plant Science and Frontiers in Microbiology

frontiers Research Topics

frontiers

ISSN 1664-8714
ISBN 978-2-88945-216-3
DOI 10.3389/978-2-88945-216-3

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SIGNALING IN THE PHYTOMICROBIOME

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An elm tree, approximately 200 years old, on Cape Breton Island, Nova Scotia. This tree is a community hosting an extensive phytomicrobiome as well as insects, birds and other animals. Image by Donald L. Smith

A plant growing under field conditions is not a simple individual; it is a community. We now know that there is a community of microbes associated with all parts of the plant, and that the root associated community is particularly large. This microbial community, the phytomicrobiome, is complex, regulated and the result of almost half a billion years of evolution. Circumstances that benefit the plant generally benefit the phytomicrobiome, and vice versa. Members of the holobiont modulate each other's activities, in part, through molecular signals, acting as the hormones of the holobiont. The plant plus the phytomicrobiome constitute the holobiont, the resulting entity that is that community. The phytomicrobiome is complex, well developed and well-orchestrated, and there is considerable potential in managing this system. The use of "biologicals" will develop during the 21st century and play as large a role as agro-chemistry did in the 20th century. Biologicals can be deployed to enhance plant pathogen resistance, improve plant access to nutrients and improve stress tolerance. They can be used to enhance crop productivity, to meet the expanding demands for plant material as food, fibre and fuel. They can assist crop plants in dealing with the more frequent and more extreme episodes of stress that will occur as climate change conditions continue to develop. The path is clear and we have started down it; there is a considerable distance remaining.

Citation: Smith, D. L., Gravel, V., Yergeau, E., eds. (2017). Signaling in the Phytomicrobiome. Lausanne: Frontiers Media. doi: 10.3389/978-2-88945-216-3

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frontiers in Plant Science

EDITORIAL
published: 24 April 2017
doi: 10.3389/fpls.2017.00611

Editorial: Signaling in the Phytomicrobiome

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Keywords: molecular signals, plant growth promoting rhizobacteria, phytomicrobiome, holobiont, crop

Editorial on the Research Topic

Signaling in the Phytomicrobiome

Over the last decade we have come to appreciate that there are close relationships between all "higher" organisms and communities of microbes. The human microbiome and its role in human metabolism and health, is being widely investigated. In a similar way, plant-associated microbial communities are now coming under scrutiny.

Plants have probably had associated microbes since they colonized the land about 0.5 billion years ago. The terrestrial environment presented water and nutrient acquisition challenges resulting in the evolution of sophisticated plant root systems. However, associated microbes also help address these hurdles, and at lesser energetic costs (Smith et al.). Because most energy enters the terrestrial biosphere at the green leaves of plants, organisms associated with plants have advantageous access to reduced carbon from photosynthesis. So, when plants prosper, associated microorganisms benefit. Microbes are associated with all plant structures, but roots are in constant contact with generally humid, microbe-laden soil, and so are associated with the greatest number and range of microbes. The earliest evidence we have of plant-microbe interactions are fossils showing mycorrhizal relationships from almost 400 million years ago (Smith et al.).

We now realize that a plant growing under field conditions is community, not just an individual. While the circumstances of associated microbes are improved when the plants are doing well, the plants must at the same time control their associated microbes, to minimize the presence of those that are potentially detrimental. The microorganisms that colonize plants are collectively termed "the phytomicrobiome". The genomes of the phytomicrobiome expand the genetic repertoire of the plant. This association has led to the redefinition of Karl August Möbius' biocenosis (metaorganisms comprising the macroscopic host and its synergistic interdependence with microbes) concept into the holobiont (an individual host and its microbial community) concept (Theis et al., 2016). The holobiont collective genome is the hologenome, the evolutionary unit; the phytomicrobiome is much more flexible than the plant genome and more readily modified than the hologenome (Nogales et al., 2016).

PLANT-PHYTOMICROBIOME SIGNALING

It is becoming clear that plants exert control over the composition of their phytomicrobiome (Smith et al.). This is reviewed extensively in the recent Frontiers in Plant Science Research Topic "Signaling in the Phytomicrobiome." Some of the regulatory activity by the plant is through availability of metabolites, but it is also increasingly evident that signals (exo-hormones or hormones of the holobiont) are being exchanged between the plant

OPEN ACCESS

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Specialty section:
This article was submitted to Plant Microbe Interactions, a section of the journal Frontiers in Plant Science

Received: *05 October 2016*
Accepted: *04 April 2017*
Published: *24 April 2017*

Citation:
Smith DL, Gravel V and Yergeau E (2017) Editorial: Signaling in the Phytomicrobiome. Front. Plant Sci. 8:611. doi: 10.3389/fpls.2017.00611

and members of its phytomicrobiome. Activities within the phytomicrobiome are also regulated through signaling, for instance through quorum sensing (Hartmann et al., 2014; Sitaraman; Smith et al.; Smith et al.), and other less well characterized signaling systems (e.g., Hagai et al., 2014).

Members of the phytomicrobiome can assist plant growth in a range of ways (Smith et al.). For instance, establishment of a specific phytomicrobiome on plants, such as willows, can allow them to better tolerate soil contamination, and so allow them to play a more effective role in phytoremediation (Bell et al., 2015; Yergeau et al., 2015). Some soil nutrients are relatively immobile (e.g., phosphorus and zinc) and some microbes, such as arbuscular mycorrhizal fungi (AMF), facilitate uptake of these nutrients by increasing effective root surface area; other microbes use chelators and other molecular interventions to help mobilize plant nutrients. Another key role of the phytomicrobiome is atmospheric nitrogen fixation. Indeed, nitrogen is the plant nutrient required in the greatest amounts; it is quite mobile in soils and it can become rapidly depleted.

The best understood example of signaling between a plant and elements of the phytomicrobiome occurs between leguminous plants and associated nitrogen-fixing rhizobia (Lira et al.; Nelson and Sadowsky; Smith et al.; Tóth and Stacey). Isoflavonoids secreted by plant roots guide rhizobial cells to the roots and activate key genes within the rhizobial cells, including the genes encoding production of lipo-chitooligosaccharides (LCOs) that signal back to the plant. Each legume species produces its own characteristic suite of isoflavonoids and it is generally the case that only the correct rhizobia respond to these. In a similar way, each type of rhizobia produces distinct LCOs, to which only the correct legume species responds (Smith et al.). The LCOs turn on a set of nodulation-related genes within the legume, initiating nodulation. In a few cases, the correct LCOs induce formation of completely differentiated nodules, in the absence of rhizobial cells. The presence of other phytomicrobiome members can enhance the nodulation process (Maymon et al.), although the mechanism is not understood. LCOs also serve as signals in the mycorrhizal relationship, suggesting that this is an ancient signaling system. However, the plant-to-mycorrhizal fungi signal is distinct from the plant-to-rhizobia signal, being strigolactone (Smith et al.), not an LCO and more related to the homoserine lactone used in quorum sensing among bacterial populations. Interestingly, rhizobia can also produce the plant growth promoting compound lumichrome, another phytomicrobome signal (Dakora et al.).

MANIPULATING THE PHYTOMICROBIOME

A better understanding of plant-microbiome signaling could help find novel ways to manipulate the microbiome to improve the plant holobiont's nutrition and resistance to stress. For instance, we have learned that the correct isoflavonoids can be added to rhizobial inoculants, to activate the nodulation genes prior to application onto plants (Smith et al.). This can overcome environmental stresses disrupting signal exchange and enhance the establishment of the nitrogen-fixing symbiosis. We have also learned that LCOs can stimulate plant growth directly, particularly under stressful conditions (Smith et al.; Subramanian et al., 2016a,b). Interestingly, it has been shown that jasmonate, a plant hormone which regulates plant responses to stressful conditions, can be excreted from plant roots and can activate genes that produce LCOs in some rhizobia; this has been shown to ameliorate plant response to stress (Smith et al.). Commercial products based on these understandings are now available for application to a range of crops (Smith et al.).

When one isolates bacteria from plant roots, *Bacillus* species are generally present. Recently, a strain of *Bacillus* which enhances plant growth under a range of conditions was isolated (Subramanian and Smith). This microbe was found to produce a small protein (thuricin 17) that, like the LCOs, stimulates plant growth at very low concentrations, and particularly when the plants are stressed. This protein is a bacteriocin that has a dual action by removing closely related competitors from the niche space, and promoting plant growth, thus enlarging the niche space, for the producing strain (Subramanian and Smith).

It is clear that the role of the phytomicrobiome is large, well developed and well-orchestrated. It is also clear that there is considerable potential in managing this system (Smith et al.; Quiza et al.) and that the use of "biologicals" will develop during the twenty first century and play as large a role as agro-chemistry did in the twentieth century. Biologicals can be deployed to enhance plant pathogen resistance (Ravichandran et al.). They can be used to enhance crop productivity, to meet the expanding demands for plant material as food, fiber and fuel. They can assist crop plants in dealing with the more frequent and more extreme episodes of stress that will occur as climate change conditions continue to develop. The path is clear and we have started down it; there is a considerable distance remaining.

AUTHOR CONTRIBUTIONS

DS was the overall editor of the theme volume. EY and VG were junior editors of the theme volume and contributed to the writing of this editorial.

FUNDING

Funding was provided for basic and applied research through the Natural Sciences and Engineering Research Council of Canada (grant number RGPIN-2015-06328) and from the Canadian Networks of Centres of Excellence (grant number G234970).

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

MINI REVIEW
published: 09 September 2015
doi: 10.3389/fpls.2015.00709

Signaling in the phytomicrobiome: breadth and potential

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Higher plants have evolved intimate, complex, subtle, and relatively constant relationships with a suite of microbes, the phytomicrobiome. Over the last few decades we have learned that plants and microbes can use molecular signals to communicate. This is well-established for the legume-rhizobia nitrogen-fixing symbiosis, and reasonably elucidated for mycorrhizal associations. Bacteria within the phytomircobiome communicate among themselves through quorum sensing and other mechanisms. Plants also detect materials produced by potential pathogens and activate pathogen-response systems. This intercommunication dictates aspects of plant development, architecture, and productivity. Understanding this signaling via biochemical, genomics, proteomics, and metabolomic studies has added valuable knowledge regarding development of effective, low-cost, eco-friendly crop inputs that reduce fossil fuel intense inputs. This knowledge underpins phytomicrobiome engineering: manipulating the beneficial consortia that manufacture signals/products that improve the ability of the plant-phytomicrobiome community to deal with various soil and climatic conditions, leading to enhanced overall crop plant productivity.

Keywords: molecular signals, plant growth promoting rhizobacteria, phytomicrobiome, holobiont, crop

OPEN ACCESS

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Specialty section:
This article was submitted to Plant Biotic Interactions, a section of the journal Frontiers in Plant Science

Received: *20 April 2015*
Accepted: *24 August 2015*
Published: *09 September 2015*

Citation:
Smith DL, Subramanian S, Lamont JR and Bywater-Ekegärd M (2015) Signaling in the phytomicrobiome: breadth and potential. Front. Plant Sci. 6:709. doi: 10.3389/fpls.2015.00709

Background

Most energy in the terrestrial biosphere enters it through photosynthesis (Imhoff et al., 2004) carried out by plant leaves (Luo et al., 2006). Non-photosynthetic organisms with reliable access to plant energy are in an advantaged situation. Under natural conditions higher plants are always associated with a complex and relatively constant microflora (Rout and Southworth, 2013; Turner et al., 2013a). Terrestrial plants release ∼20% of photosynthetically fixed carbon as root exudates, resulting in an energy rich rhizosphere (Kuzyakov and Domanski, 2000), and a rich, generally compositionally consistent phytomicrobiome (Bulgarelli et al., 2012; Hirsch and Mauchline, 2012; Lundberg et al., 2012). These exudates vary among species, specific genotypes within species, stages of plant development and growing conditions, and influence the composition of the rhizomicrobiome (Bascom-Slack et al., 2012; Marasco et al., 2012; Badri et al., 2013a,b; Turner et al., 2013a,b; Chaparro et al., 2014).

Phytomicrobiome associations are analogous to the animal microbiome (Koenig et al., 2011); microbiome diversity, stability, and resilience play a large role in human health and disease (Cho and Blaser, 2012). Plants have likely had associated microbes since they colonized the land, almost half a billion years ago; roots of the first terrestrial plants were almost certainly less sophisticated than those that followed, making these early plants more in need of microbial assistance (Knack et al., 2015). Fossil endomycorrhizal associations occur in the early Devonian period,

demonstrating association of plant roots with fungal elements of the rhizomicrobiome (Taylor, 1995; Bonfante and Genre, 2008; Porras-Alfaro and Bayman, 2011). Mycorrhizal relationships are sophisticated and their presence >400 million years ago indicates that the phytomicrobiome had already been developing for some time; it seems likely that bacterial associations have been present for at least as long. As plants adapted to and spread through diverse terrestrial environments, evolving to grow under a range of conditions, it is probable that their associations with microbes also evolved. This community of microbes is the phytomicrobiome (Smith and Zhou, 2014), with its root associated (Hirsch and Mauchline, 2012; Lundberg et al., 2012; rhizomicrobiome), above ground associated (Rastogi et al., 2012, 2013; Badri et al., 2013b; Kembel et al., 2014; phyllomicrobiome) and interior (Berg et al., 2014; endosphere) components. Even "lower plants" such as *Sphagnum* sp. have complex phytomicrobiomes, including highly specific associations with diazotrophs (Bragina et al., 2013).

Hence, a plant growing in nature is not a single organism; it is a community: a holobiont (Hartmann et al., 2014). While a plant growing in isolation can be very useful for research purposes, it is an anomaly. Like the human microbiome, the phytomicrobiome constitutes an underappreciated biological aspect (physiology, genome, metabolome, etc.) of plants. Plants and their associated phytomicrobiome affect each other in various and subtle ways (Berendsen et al., 2012); a field-grown plant is a meta-organism (Berg et al., 2013), having a persistent and regulated relationship with its phytomicrobiome. The composition of the phytomicrobiome is regulated by numerous biotic and abiotic factors including the complex matrix of plant–microbe and microbe–microbe communications. This communication is carried out through the release of signaling compounds, the forms and functions of which are currently being elucidated. This new understanding can be exploited to: (1) develop new approaches to crop growth promotion, (2) optimize related fermentation and formulation processess, and (3) develop novel and more consistent biocontrol mechanisms for field crops (East, 2013).

The Phytomicrobiome and Plant Growth

There has been an upsurge in phytomicrobiome publications; this community of microbes is now seen as key to the growth and health of plants (Schmidt et al., 2014); there is still a great deal to be learned about the composition and nature of interactions among members of this community, and its interactions with the host plant.

Microbes associate with the phyllosphere (as both epi- and endophytes, of leaves and stems), rhizosphere and reproductive structures such as flowers, fruits and seeds. In grape, *Pseudomonas* and *Bacillus* spp. colonize the epidermis and xylem of the ovary and ovules, while *Bacillus* spp. colonize berries and seed cell walls (Lugtenberg and Kamilova, 2009; Compant et al., 2010a,b). Nitrogen-fixing plant growth promoting rhizobacteria (PGPR; Loiret et al., 2004; Quecine et al., 2012; e.g., *Acetobacter diazotrophicus, Pantoea agglomerans* 33.1) associate with plant roots (Pisa et al., 2011), and stems of sugarcane (Velázquez et al., 2008), residing in the apoplast in a low-nitrogen, high-sucrose environment (Dong et al., 1994). Other nitrogen-fixing bacteria (*Azotobacter*, *Enterobacter*, *Bacillus*, *Klebsiella*, *Azospirillum*, *Herbaspirillum*, *Gluconacetobacter*, *Burkholderia, Azoarcus*) are found in grasses such as rice and maize (Von Bulow and Dobereiner, 1975; James, 2000; Baldani et al., 2002; Boddey et al., 2003; Santi et al., 2013). Phyllomicrobiome communities influence plant development and ecosystem function, while the host controls aspects of phytomicrobiome composition and function. Environmental factors are known to alter biosynthesis of many metabolites within plants; specific members of the rhizomicrobiome also alter plant development, growth, and composition. Treatment of leaves with specific phyllomicrobiome components suppresses feeding by insect larvae (Badri et al., 2013b). The distribution and community composition of microbes in the phyllosphere is thought to be somewhat random, whereas plants create niches in the rhizosphere and endosphere to accommodate specific microbial communities (Lebeis, 2015).

The rhizomicrobiome is comprised of diverse root endophytes (Gaiero et al., 2013), some of which are PGPRs. Compositionally the rhizomicrobiome is dynamic in time and space, in response to environmental conditions, the presence of other soil organisms, soil physical conditions, plant species and genotype and interactions between a specific microbe and a specific plant type. The best characterized microbes in the rhizomicrobiome are the PGPR. These include bacteria in the soil near plant roots, on the surface of plant root systems, in spaces between root cells or inside specialized cells of root nodules; they stimulate plant growth through a wide range of mechanisms (Gray and Smith, 2005; Mabood et al., 2014), such as: (1) nutrient solubilization (particularly phosphorus – Boddey et al., 2003; Kennedy et al., 2004; Trabelsi and Mhamdi, 2013), (2) production of metal chelating siderophores, (3) nitrogen fixation (Vessey, 2003; Bhattacharyya and Jha, 2012; Drogue et al., 2012), (4) production of phytohormones, (5) production of 1-aminocyclopropane-1-carboxylate deaminase, (6) production of volatile organic compounds, (7) induction of systemic resistance [induced systemic resistance (ISR) and systemic required resistance (SAR) – Jung et al., 2008b, 2011], and (8) suppression of disease through antibiosis (Bhattacharyya and Jha, 2012; Spence et al., 2014). It has also been shown that "signal" compounds produced by bacteria in the phytomicrobiome stimulate plant growth (Prithiviraj et al., 2003; Mabood et al., 2006a; Lee et al., 2009), particularly in the presence of abiotic stress (Wang et al., 2012; Subramanian, 2014; Prudent et al., 2015). In the broadest sense PGPR include legume-nodulating rhizobia. PGPR reside outside plant cells (extracellular – ePGPR) or, like rhizobia, live inside them (intracellular – iPGPR; Gray and Smith, 2005). Application of PGPR to crops, except for rhizobia, has met with mixed results in the field, causing increased growth sometimes and not others (Nelson, 2004). Elements of the phytomicrobiome also assist plants in dealing with abiotic stress. The *Arabidopsis* phytomicrobiome, for instance, can sense drought stress and help the plant maintain productivity (Zolla et al., 2013). Further, mycorrhizal associations enhance crop

salinity tolerance (Porcel et al., 2012; Ruiz-Lozano et al., 2012). At a time when we are looking to crop plants to provide biofuels and other bioproducts while still feeding the world's growing population, against a background of climate change, understanding and developing technologies that can increase overall plant productivity is imperative (Ragauskas et al., 2006; Babalola, 2010; Dutta and Podile, 2010; Beneduzi et al., 2012; Orrell and Bennett, 2013).

Newer deployments of PGPR and/or arbuscular mycorrhizal fungi (AMF) consortia that promote crop productivity by mimicking, or partially reconstructing, the phytomicrobiome are being developed. Application of a PGPR consortium (*Bacillus amyloliquefaciens* IN937a, *Bacillus pumilus* T4, AMF *Glomus intraradices*) to greenhouse tomato resulted in full yield with 30% less fertilizer (Adesemoye et al., 2009). Co-inoculation of *B. japonicum* 532C, RCR3407 and *B. subtilis* MIB600 increased biomass for two soybean cultivars (Atieno et al., 2012). Co-inoculation of *B. japonicum* E109 and *Bacillus amyloliquefaciens* LL2012 improved soybean nodulation efficiency. Phytohormone production by *B. amyloliquefaciens* LL2012 improved nodulation efficiency for *B. japonicum* E109 (Masciarelli et al., 2014). A consortium of *B. megaterium*, *Enterobacter* sp., *B. thuringiensis* and *Bacillus* sp., plus composted sugar beet residue, on *Lavandula dentata* L. helped restore soils by increasing phosphorus availability, soil nitrogen fixation and foliar NPK content (Mengual et al., 2014).

Signaling in the Phytomicrobiome

The complex community formed by the plant and its phytomicrobiome is carefully orchestrated; there is signal exchange among the various microbes involved, and also between the host plant and the microbe community (Engelmoer et al., 2014). These signals regulate aspects of each other's activities and the community overall. Microbial chemical signals can help plants initiate immune responses to harmful pathogens or allow the entry of beneficial endophytes (Hartmann et al., 2014). Microbe associated molecular patterns (MAMPs) play a key role in plant immune response and antibiotic secretion in microbes. Plant associated *Bacillus* strains have been shown to down-regulate MAMP-regulated immune response including antibiotic secretion in the presence of plant root exudates to better facilitate root infection (Lakshmanan et al., 2012). Bacteria can also interfere with signaling between plants and other microbial strains. LCOs are similar in structure to chitin and can be cleaved by bacterially produced chitinases, thus interfering with plant microbe symbioses (Jung et al., 2008a). Other aspects plant–microbe symbiosis follow pathways similar to pathogen infection (Barea, 2015).

Signaling compounds produced by plants include a variety of root exudates such as primary metabolites (carbohydrates, proteins, organic acids, etc.) and secondary metabolites (flavonoids, phenol, phytohormones, etc.). Plants often excrete more of these signaling compounds in response to stress. PGPR-to-plant signaling compounds include phytohormones, acyl homoserine lactones, phenols and peptides and can also act as microbe to microbe signals (Barea, 2015). Root exudates signal and recruit specific microbial communities. Secretion of malic acid in *Arabidopsis thaliana* in response to foliage pathogen attack stimulates the formation of beneficial biofilms in the rhizosphere (Rudrappa et al., 2008).

That plants and microbes use signal compounds to communicate during establishment of beneficial plant-microbe interactions (Desbrosses and Stougaard, 2011), is well-described for the legume-rhizobia nitrogen fixing symbiosis (Oldroyd et al., 2010; Giles et al., 2011; Oldroyd, 2013), and somewhat elucidated for mycorrhizal associations (Gough and Cullimore, 2011). In the legume-rhizobia relationship the plant releases flavonoid signals to rhizobia (Hassan and Mathesius, 2012) or, in some cases, jasmonate signals (Mabood et al., 2006a,b; Mabood et al., 2014), followed by rhizobial production of lipo-chitooligosaccharides (LCOs) as return signals (Oldroyd, 2013). The LCOs are bound by LysM receptors, which have kinase activity (Antolin-Llovera et al., 2012), changing root hormone profile (Zamioudis et al., 2013) and triggering development of root nodules. Plants also communicate with, or otherwise influence the phytomicrobiome, affecting its composition and structure (Delaux et al., 2012; Badri et al., 2013a; Bálint et al., 2013; Peiffer et al., 2013; Turner et al., 2013b; Venkateshwaran et al., 2013; Chaparro et al., 2014; Evangelisti et al., 2014). Bacteria also communicate among themselves (Cretoiu et al., 2013); quorum sensing via *N*-acyl homoserine lactone (Teplitski et al., 2000) is well-characterized, and there are likely other, as of yet unknown, mechanisms (Lv et al., 2013). Quorum sensing signals can trigger immune responses and changes in hormone profiles in plants, leading to growth responses (Hartmann and Schikora, 2012). Quorum sensing in the phytomicrobiome will be the subject of an upcoming Frontiers in Plant Science theme volume (Plant responses to bacterial quorum sensing signal molecules, topic editors Schikora A, Hartmann A, and Munchen HZ). This sort of signaling almost certainly occurs in the phytomicrobiome. Plants also detect materials produced by potential pathogens and respond by activating response systems (Tena et al., 2011). Phytomicrobiome intercommunication in the rhizosphere dictates aspects of above-ground plant architecture and above-ground symbiotic/pathogenic microbial communities (Segonzac and Zipfel, 2011; Tena et al., 2011). Similarly, pathogen or herbivore attacks above ground can effect microbial community composition in the rhizosphere. Above ground injury has been shown to stimulate the production of signaling compounds in plant roots (Lakshmanan et al., 2012). Greater photosynthetic rates under elevated CO_2 conditions have been shown to change microbial community composition in the rhizosphere (Berlec, 2012; He et al., 2012). Understanding plant responses to microbial signals via proteomics (Elmore et al., 2012; Nguyen et al., 2012; Rose et al., 2012) and metabolomics (Watrous et al., 2012; Zhang et al., 2012) studies has added valuable knowledge toward developing effective low-cost and eco-friendly practices to reduce fossil-fuel dependent crop inputs, leading to interest in phytomicrobiomes engineered to enhanced plant growth under

variable soil and climatic conditions, improving global crop productivity.

Surprisingly, LCOs are also able to stimulate plant growth directly (Souleimanov et al., 2002; Prithiviraj et al., 2003; Almaraz et al., 2007; Khan et al., 2008; Wang et al., 2012); confirmed by Oláh et al. (2005) for root growth in *Medicago truncatula*, Chen et al. (2007) for accelerated flowering (a typical response to stress) and increased yield in tomato, and stimulation of early somatic embryo development in Norway spruce (Dyachok et al., 2002). Enhanced germination and seedling growth, along with the mitogenic nature of LCOs, suggest accelerated meristem activity. Products based on LCOs are now used to treat seed sown into several 10s of million ha of crop land each year, largely corn and soybean. A similar jasmonate product is now available. The effects of LCOs are much greater when stress (salt, drought, cold) is present than under optimum conditions (Smith, 2009, 2010; Subramanian et al., 2009, 2010, 2011; Schwinghamer et al., 2014; Subramanian, 2014; Prudent et al., 2015). Thuricin 17, a bacteriocin produced by *Bacillus thuringiensis* NEB17 isolated from soybean roots, improves plant growth and resilience to stress (Schwinghamer et al., 2014; Subramanian, 2014). Inhibition of legume nodulation, and of overall plant growth, by stressful conditions can be overcome by LCOs (nodulation – Zhang and Smith, 1995, 2002; plant growth – Schwinghamer et al., 2014; Prudent et al., 2015); Estévez et al. (2009) showed that at least one rhizobial strain produce different LCOs when grown under salt stress, and that salt stress itself can induce the *nod* genes of this strain (Guasch-Vidal et al., 2013).

Future Directions

We now understand that the phytomicrobiome is a complex, structured and dynamic community with a relatively constant set of potential members, whose relative abundances can shift within plant species and their genotypes, and in response to both abiotic conditions and plant development, leading to dynamism in the communications among the microbial community and the host plants. Methods, such as high throughput genotyping, are allowing us to determine the taxonomic diversity of the phytomicrobiome (Hirsch and Mauchline, 2012; Peiffer et al., 2013; Turner et al., 2013b). A better understanding of plant signaling may also become a tool for investigating community composition of the phytomicrobiome. Root exudates play an important role in the formation of microbial communities in the rhizosphere and can be useful in predicting community compositions (Berg et al., 2014). Correlations between phytomicrobiome bacterial diversity and host growth, mortality, and function suggest that incorporating information on plant–microbe associations will improve our ability to understand plant functional biogeography and drivers of variation in plant and ecosystem function (Kembel et al., 2014). It has even been suggested that beneficial effects of the phytomicrobiome could be enhanced through plant breeding, developing genotypes that encourage best membership in the phytomicrobiome (Bakker et al., 2012). More effective methods to study plant MAMP receptors are being developed (Wittulsky et al., 2014) and could lead to ways to engineer plant recognition receptors.

Novel methods of manipulating signaling in the phytomicrobiome could lead to crop production practices that are less reliant on non-renewable resources and crops more resilient in the face of stresses (Marasco et al., 2012), most crucially, those associated with climate change. Plant stress response seems to play an important role in the release of signaling compounds in the rhizosphere but the specifics of this interaction are still unclear. A better understanding of the relationship between environmental plant stress and signaling could help in developing technologies that utilize plant signaling in crop stress alleviation (Barea, 2015).

Recent developments have shown that temperature (Schwinghamer et al., 2014) and water stress (Prudent et al., 2015) can influence plant microbe communication. Environmental factors likely play an important and underdescribed role in signaling in the phytomicrobiome. Variable environmental factors may account for some of the inconsistency observed in field trials of microbial products that previously yielded favorable results in laboratory conditions. A more complete understanding of how plant–microbe communication is influenced by environmental factors will likely be useful in achieving more consistent results with agricultural microbial products.

Despite being at an early stage in understanding these communities, it is clear that there is considerable potential for application of coordinated microbial consortia to crop agriculture and, thus, to enhancing global food security. While advances in methods and technologies in microbiology used to investigate non-culturable microbial strains have led to a stronger focus on a community level approach to plant–microbe interaction research (Berlec, 2012; Rastogi et al., 2013), isolated, culturable microbial strains are still required for most plant–microbe signaling research, particularly if the research is aimed at developing commercial microbial products. Culturable strains are needed both to produce a consistent product and to verify growth promotion through plant growth trials. There are clear opportunities for development of products for more sustainable agronomic production systems (Kloepper et al., 2004; De-la-Peña and Loyola-Vargas, 2014). A range of PGPR have been identified, and even developed into products utilized in crop production. Signaling compounds that directly stimulate plant growth or improve stress tolerance have great potential because they can be produced by microbes in a controlled bioreactor rather than in variable field conditions as with inoculants. The global market for biostimulants has been projected to reach $2.241 million by 2018 and to have a compounded annual growth rate of 12.5% from 2013 to 2018 (Calvo et al., 2014). Products based on multispecies consortia may address consistency in performance observed in single species inoculants. Industry is working to harness the knowledge surrounding the phytomicrobiome, to quickly bring sustainable, consortia-based products to production agriculture.

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Conflict of Interest Statement: The fourth author is Executive Vice President of Technology and Innovation at Inocucor Technologies, a company that manufactures and sells microbial consortia for plants; the first author conducts research in collaboration with this company, where the research is funded through a Canadian Federal Government program (Mitacs) which levers industrial funding.

frontiers in Microbiology

MINI REVIEW
published: 08 September 2015
doi: 10.3389/fmicb.2015.00945

Legume-rhizobia signal exchange: promiscuity and environmental effects

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Although signal exchange between legumes and their rhizobia is among the best-known examples of this biological process, most of the more characterized data comes from just a few legume species and environmental stresses. Although a relative wealth of information is available for some model legumes and some of the major pulses such as soybean, little is known about tropical legumes. This relative disparity in current knowledge is also apparent in the research on the effects of environmental stress on signal exchange; cool-climate stresses, such as low-soil temperature, comprise a relatively large body of research, whereas high-temperature stresses and drought are not nearly as well understood. Both tropical legumes and their environmental stress-induced effects are increasingly important due to global population growth (the demand for protein), climate change (increasing temperatures and more extreme climate behavior), and urbanization (and thus heavy metals). This knowledge gap for both legumes and their environmental stresses is compounded because whereas most temperate legume-rhizobia symbioses are relatively specific and cultivated under relatively stable environments, the converse is true for tropical legumes, which tend to be promiscuous, and grow in highly variable conditions. This review will clarify some of this missing information and highlight fields in which further research would benefit our current knowledge.

Keywords: tropical legumes, broad spectrum, soil acidity, soil temperature, salinity

OPEN ACCESS

Edited by:
Etienne Yergeau, National Research Council Canada, Canada

Reviewed by:
Oswaldo Valdes-Lopez, National Autonomous University of Mexico, Mexico
Dong Wang, University of Massachusetts Amherst, USA

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Specialty section:
This article was submitted to Plant Biotic Interactions, a section of the journal Frontiers in Microbiology

Received: *10 April 2015*
Accepted: *27 August 2015*
Published: *08 September 2015*

Citation:
Lira MA Jr., Nascimento LRS and Fracetto GGM (2015) Legume-rhizobia signal exchange: promiscuity and environmental effects. Front. Microbiol. 6:945. doi: 10.3389/fmicb.2015.00945

Legume-Rhizobia Signal Exchange Importance and General Information

Biological nitrogen fixation is one of the main biological cycles worldwide (Canfield et al., 2010) and is estimated to contribute close to half (Herder et al., 2010) of the world's biologically available nitrogen. Most of that fixed nitrogen comes from the legume-rhizobia symbiosis, which is based on a very large and constantly changing group of bacteria generically called rhizobia, including *Allorhizobium, Aminobacter, Azorhizobium, Bradyrhizobium, Devosia, Ensifer (Sinorhizobium), Mesorhizobium, Methylobacterium, Microvirga, Ochrobactrum, Phyllobacterium, Rhizobium,* and *Shinella* among the α-Proteobacteria; *Burkholderia, Cupriavidus,* and *Herbaspirillum* among the β-Proteobacteria (Vinuesa, 2015); and at least one *Pseudomonas* sp. from the γ-Proteobacteria (Shiraishi et al., 2010). This usage of rhizobia as a catch-all name has been challenged recently because it was based initially on the *Rhizobium* genus (then the Rhizobiaceae family), whereas

we now know that at least three classes of the Proteobacteria include at least one genus with this capability. In contrast, this well-recognized term has been used extensively and, as such, is used throughout this review.

This symbiosis begins with an elaborate signal exchange process that is among the best studied between bacteria and plants (Hirsch and Fujishige, 2012). Initially, the legume root releases exudate compounds such as sugars, amino acids, several classes of proteins classes (De-la-Peña et al., 2008, 2010; Badri and Vivanco, 2009; Badri et al., 2009), and flavonoids, and phenolic compounds (Broughton et al., 2003), such as flavone, flavonones, isoflavones, and betains (Cooper, 2007). These compounds induce chemoostatic reactions from the bacteria and act as nodulation gene inducers (Hirsch and Fujishige, 2012; Ryu et al., 2012).

These compounds may act as weak or strong inducers, whereas others are inhibitors or have no effect on nodulation (Mulligan and Long, 1985; Firmin et al., 1986; Peters et al., 1986; Redmond et al., 1986; Hartwig et al., 1989, 1990; Hungria et al., 1992; Bolaños-Vásquez and Werner, 1997; Begum et al., 2001; Mabood et al., 2006; Subramanian et al., 2007).

Which compounds, or class of compounds, induce nodulation the strongest varies among symbiotic pairs. For common beans (*Phaseolus vulgaris*), the strongest inducers are genistein-3-*O*-glucoside, eriodictyol, naringenin, daidzein, genistein, and coumesterol (Hungria et al., 1991a; Dakora et al., 1993b); this plant also releases other classes of compounds such as anthocyanidins, flavonols, isoflavonoids, and flavones (Hungria et al., 1992). For soybeans (*Glycine max*), the most effective plant-to-bacteria signal has been variously found to be an isoflavone (Subramanian et al., 2006), jasmonic acid and its derivatives (Mabood and Smith, 2005), or genistein (Zhang and Smith, 1995).

After the nodulation genes are activated, the rhizobia release nod factors, lipochitooligosaccharides specific to each symbiotic association that are sufficient to activate nodule organogenesis at least under some conditions, and these factors may induce cellular modifications associated with early rhizobial root infection (Oldroyd and Downie, 2004; Cooper, 2007; Jones et al., 2007). In addition to the nod factors, several other bacterial compounds affect several stages of the interaction, including exopolysaccharides (EPS), lipopolysaccharides, K-antigen polysaccharides, cyclic β-glucan, high-molecular-weight neutral polysaccharides (glucomannan), and gel-forming polysaccharides (Fraysse et al., 2003; Laus et al., 2006; Downie, 2010; Janczarek, 2011).

Signal Exchange Diversity and Legume Promiscuity

The complex signal exchange between plant and bacterial partners in symbiosis is also a key component of symbiotic specificity, which varies from highly specific to highly promiscuous. For example, although *Sinorhizobium* sp. NGR234 nodulates 232 legume species from 112 distantly related genera, with varying efficacy, some strains of *Rhizobium leguminosarum* bv. *viciae* do not nodulate pea (*Pisum sativum*) cultivars from different origins (Ovtsyna et al., 1998; Masson-Boivin et al., 2009).

The lack of effective signal exchange between legumes and bacteria precludes symbiosis establishment for incompatible partners, but in some situations, nodules may be formed in which the rhizobia do not enter, are not liberated from the infection thread, or do not fix nitrogen (Miller et al., 2007). This lack of recognition may occur even after the initial signal exchange. For example, *R. leguminosarum* bv. *trifolii* (*Rlt*) strain ICC105 does not fix nitrogen with white clover (*Trifolium repens*), whereas this strain is effective when paired with Caucasian clover (*T. ambiguum*). According to Miller et al. (2007), this difference is due to a region between the *nifH* gene and the *fixA* promoter that is differentially activated when in symbiosis with the two *Trifolium* species. It is not clear if this difference is due to positive or negative regulation by a specific plant signal, nor is it clear how NifA activity is regulated (Miller et al., 2007).

The combination of a vast range of compounds secreted by both plants and bacteria is one of the main characteristics of this symbiotic compatibility. Because the first step is exudation by the plant, this step may be considered the most important one. These exudates are continuously secreted into the rhizosphere, but both the number and concentration of these compounds increases when compatible bacteria are detected by the plant (Zaat et al., 1989; Dakora et al., 1993a,b; Hassan and Mathesius, 2012).

These plant-bacteria signals activate three main groups of nodulation genes in the bacteria: the common *nodABC* genes that are present in almost all rhizobia (the exception being some photosynthetic bradyrhizobia and some *Burkholderia,* Giraud et al., 2007) and are required to produce the basic structure of the nod factors; host-specific *nod* genes that are linked to specific modifications of the basic nod factor structure that allows for symbiotic specificity, such as *nodEF, nodG, nodH, nodPQ,* and *nodRL*; and regulatory genes that are linked to the activation and transcription of both the common and specific *nod* genes (Horvath et al., 1986; Göttfert et al., 1990; Lerouge et al., 1990; Sanjuan et al., 1994; Moulin et al., 2001; Schlaman et al., 2006).

Nod factor perception is mediated by Nod factor receptors (NFRs), which are serine/threonine kinases that are located in the plasma membrane and that contain LysM motifs in their extracellular domains (Limpens et al., 2003; Madsen et al., 2003; Radutoiu et al., 2003; Arrighi et al., 2006). These NFRs correspond to the Nod factor structure and act as host determinants for symbiotic specificity. This specificity was shown by the transfer of *Lj-NFR1* and *Lj-NFR5* to *Medicago truncatula*, which enabled nodulation by the *Lotus japonicus* symbiont *Mesorhizobium loti* (Radutoiu et al., 2007); the specificity of two *Lotus* species is the function of a single amino acid residue in one of the LysM domains of Lj-NFR5 (Radutoiu et al., 2007).

In addition to Nod factors, some rhizobia secrete proteins that are involved in nodulation via a type III secretion system (T3SS; Fauvart and Michiels, 2008; Deakin and Broughton, 2009). These proteins, called nodulation outer proteins (Nops), are believed to contribute to legume immune response suppression or to modulate root cell cytoskeletal rearrangement during nodule development (Bartsev et al., 2004; Skorpil et al., 2005; Soto et al.,

2009). The *nopP* and *nopL* genes are found in *Rhizobium* sp. NGR234, *Sinorhizobium fredii* and *Bradyrhizobium japonicum* and are absent in pathogenic bacteria (Deakin and Broughton, 2009). In *Rhizobium* sp. NGR234, these genes are required for the nodulation of the tropical legumes *Tephrosia vogelii* and *Flemingia congesta* (Marie et al., 2003; Skorpil et al., 2005). Moreover, the nodulation of *Vigna unguiculata* by *S. fredii* is also affected by Nop proteins injected by *S. fredii* in a T3SS-dependent fashion (Schechter et al., 2010), but further studies on their effects on host specificity are still necessary.

Exopolysaccharides, bacterial cellular wall constituents, are also known to have important effects on symbiosis. For example, a defect on the EPS surface may induce failures both in the early and late stages of symbiosis, such as those observed in strains of *S. meliloti* presenting normal nodules in some ecotypes of *M. truncatula* but defective nodules in others, and this pattern may be transferred by a change in the EPS biosynthesis locus (Simsek et al., 2007). Because *M. loti* EPS mutants result in nonfunctional nodules in *L. leucocephala* but functional ones in *L. pedunculatus*, the EPS surface has also been linked to specificity in the nitrogen fixing phase (Hotter and Scott, 1991), as demonstrated by a *B. japonicum exoB* mutant fixing nitrogen in *G. max* but not in *G. soja* (Parniske et al., 1994) or some *R. leguminosarum* LPS mutants fixing nitrogen in peas (*Pisum sativum*), whereas other mutants do not (Kannenberg et al., 1992).

One point that deserves attention is the almost complete lack of literature on this signal exchange in tropical legumes, which are typically more promiscuous than temperate ones. Because of this knowledge gap, it is not known how the degree of promiscuity of a legume affects the signal exchange process because with the exception of *Phaseolus*, the best-studied legumes are all generally considered to nodulate with a few species or genera at the most (Michiels et al., 1998; Martínez-Romero, 2003; Rodríguez-Navarro et al., 2011; Rufini et al., 2013). A synthesis of a large portion of the literature identifying seed or root exudate compounds with known nod-gene activating properties (**Table 1**) indicates that more promiscuous (or less-selective) legumes may exhibit a broader range of these compounds, as per a comparison between *P. vulgaris* and *G. max*, which are less and more selective, respectively, for the rhizobial partner of the symbiosis. In contrast, the only paper we could find on *V. unguiculata* identifies only three compounds, although it has a very broad range of rhizobial partners. One further puzzle is that genistein is a known inducer for *G. max, P. vulgaris*, and *V. unguiculata*, although the rhizobia of these three species are not identical.

A lack of depth in the literature on this topic leads to ambiguity in how to relate legume promiscuity (or specificity) with the signal exchange process, although this relationship is expected to exist due to the specific nature of this exchange. Thus, this relationship might be an interesting line of future research; a better understanding of this relationship may lead to biotechnological approaches to enhance or reduce the compatibility profile of a given legume similarly, to soybean breeding for broad bacterial compatibility in Africa (Gwata et al., 2005).

TABLE 1 | Seed and root exudate compounds with known *nod* gene-activating factors, from legumes with broad or narrow ranges of symbiotic compatibility.

Species	Source	*nod* gene-activating factors	Source
Glycine max	Root exudates	Daidzein, genistein, coumestrol, isoliquiritigenin	Kape et al. (1992), Pueppke et al. (1998)
G. max	Seeds	Daidzein, genistein	Pueppke et al. (1998)
Medicago sativa	Seeds	Chryoseriol, luteolin, liquiritigenin	Maxwell et al. (1989), Hartwig et al. (1990)
M. sativa	Root exudates	4,7-dihydroxyflavone formononetinin	Maxwell et al. (1989)
Phaseolus vulgaris	Root exudates	Genistein, eriodictyol, naringenin, daidzein, coumesterol	Davis and Johnston (1990), Hungria et al. (1991b), Dakora et al. (1993b)
P. vulgaris	Seeds	Unidentified isoflavone, delphinidin, petunidin, malvidin, myricetin, quercetin, kaempferol	Hungria et al. (1991a)
Vigna unguiculata	Root exudates	Daidzein, genistein, and glycitein	Dakora (2000)

Environmental Effects on Signal Exchange

Although the interaction between environmental stresses and legume-rhizobia signal exchange has been investigated, as will be discussed, these studies have also centered on temperate climate pulses, and their stresses. Much work is still needed to understand how the signal exchange process of other legumes is affected by their more typical stresses.

Temperature

Much research has examined low root zone temperatures and their effects on signal exchange and nodulation, particularly in soybeans, but little is known about the effects of high root zone temperatures.

Low root zone temperatures inhibit the synthesis and secretion of plant-to-bacteria signals, as shown in *G. max*, in which the root exudation of genistein is strongly reduced below 17.5°C (Zhang and Smith, 1994, 1996a; Zhang et al., 1995; Pan and Smith, 1998). Low root zone temperatures also reduce nod factor synthesis and/or excretion in *R. leguminosarum* bv. *trifolii* (McKay and Djordjevic, 1993) and *B. japonicum* (Zhang et al., 2002). The molecular basis of this effect indicates that the *T3SS* gene cluster was progressively activated as temperatures increased, whereas the *nod* genes were rapidly induced at 15°C (Wei et al., 2010). Genistein has been proposed to induce this gene cluster through a regulatory cascade involving NodD1 and NodW (Krause et al., 2002).

These signal exchange effects combine to delay nodulation onset (Pan and Smith, 1998) and reduce the nodule growth rate, leading to smaller nodules (Lira Junior et al., 2005).

Further confirmation that these stresses are directly linked to signal exchange is that the exogenous application of genistein is sufficient to mitigate a delay in nodulation under environmental conditions in which the root system temperature is below this threshold and the shoot is above it (Zhang and Smith, 1995, 1997; Pan et al., 1997). This mitigation is stronger for lower soil temperatures or stronger stresses (Zhang and Smith, 1996b).

Salinity

Although salinity is known to affect Nod factor production by *R. tropici* CIAT 899 in the presence of apigenin (Estévez et al., 2009), there are indications that high salt concentrations may induce *nod* genes even in the absence of flavonoid inducers (Guasch-Vidal et al., 2012).

However, increased salinity reduces Nod factor production by *S. arboris*, which nodulates *Acacia* and *Prosopis*, both of which are legume trees tolerant to salt stresses (Penttinen et al., 2013). Similar effects were found for *R. tropici* and *R. etli*, which nodulate *P. vulgaris* (Dardanelli et al., 2012).

Similarly, to what is observed at low soil temperatures, as previously described, some of the salinity effects may be reduced if the bacteria are pre-incubated with their respective legume signals, such as genistein for *B. japonicum* (Miransari and Smith, 2009) or hesperetin and apigenin for *R. tibeticum* (Abd-Alla et al., 2013).

Soil pH

Soil pH affects symbiosis in several ways, including signal exchange (Hungria and Vargas, 2000). For example, both *G. max* and *P. vulgaris* isoflavonoid exudation from roots were reduced when the pH was lowered from 5.8 to 4.5 (Hungria and Stacey, 1997), and some nodulation genes, including *nodA*, are inactivated by reducing the pH in *R. leguminosarum* bv. *trifolii* (Richardson et al., 1988a,b). The production and excretion of Nod factors were also reduced in acidic soils (McKay and Djordjevic, 1993).

Another effect is a change in the profile of the Nod factors secreted by *R. tropici* CIAT 899, which is tolerant to acid conditions. A total of 52 different molecules were produced under an acidic pH and 29 at a neutral pH; only 15 are common to both conditions (Moron et al., 2005). This phenomenon might be linked to the reduction in *nodC* expression by the *Arachis hypogaea* bacterial symbionts under acidic conditions (Angelini et al., 2003).

In contrast to what is observed for low soil temperatures and salinity, the addition of flavonoids did not reduce the effects of low pH on acid-sensitive or acid-tolerant *A. hypogaea* (Angelini et al., 2003), which was apparently due to increased flavonoid uptake and toxicity.

Low pH also activates a systemic, shoot-controlled, and GmNARK-dependent (Nodulation Autoregulation Receptor Kinase) mechanism that negatively regulates initial nodule development in soybeans (Lin et al., 2012), as confirmed by the reduced expression of the *GmENOD40b*, *GmNIN-2b*, *GmRIC1*, *GmRabA2,* and cytochrome P450 genes, which are critical to early nodulation stages.

Iron and Phosphorus Deficiency

The legume-rhizobia symbiosis demands high levels of iron due to its inclusion in the compositions of leghemoglobin, nitrogenase, and cytochromes (Brear et al., 2013). Iron deficiency effects vary between legume species and may include altered nodule initiation, as seen in *Lupinus angustifolius* L. (Tang et al., 1990), or late development, as seen in peanuts (*A. hypogaea*), common beans (*P. vulgaris*), and soybeans (O'Hara et al., 1988; Soerensen et al., 1988; Slatni et al., 2011).

Iron absorption regulation by rhizobia in culture media has been extensively researched, and iron-responsive transcription regulators such as IrrA and RirA and the genes they control under iron deficiency and sufficiency have been determined (Viguier et al., 2005; Todd et al., 2006). Several of these genes encode siderophore production, heme biosynthesis, and transporters, such as the ferric siderophore ATP-binding cassette (ABC)-related genes.

Under iron-limiting conditions, free-living rhizobia express TonB-dependent receptors after activation by an iron regulator (Small et al., 2009), although bacteriod active siderophore transport is not necessary for symbiosis (Chang et al., 2007; Small et al., 2009). Mutations in ABC transporters, TonB-dependent receptors and TonB do not affect symbiosis establishment (Lynch et al., 2001; Nienaber et al., 2001), suggesting that bacteriods do not require a high affinity for siderophore absorption to obtain iron during symbiosis (Brear et al., 2013), although *S. meliloti* strains deficient in the siderophore absorption system exhibited lower nodule occupation rates under iron-deficient conditions than the corresponding wild types (Battistoni et al., 2002).

N_2 fixation has a high energy cost, and P deficiency is an important restriction for legume production, particularly in the low-P soils of most tropical regions (Sulieman and Tran, 2015). Organic phosphates are the main source to sustain nodule symbiotic activities (Li et al., 2012), and several genes involved in recycling P are up-regulated under low-P conditions (Hernandez et al., 2009), particularly those encoding acid phosphatases (Maougal et al., 2014; Zhang et al., 2014).

Generally, the specific activity of acid phosphatases in nodules strongly increases when P supply is reduced in the growth medium but is stable when P supply is high (Araujo et al., 2008). The expression of several genes of the purple acid phosphatase GmPAP family was highly induced in soybean nodules under low-P availability (Li et al., 2012); the expression of phytate and phosphoenol pyruvate phosphatase was also increased in nodules under these conditions (Araujo et al., 2008; Bargaz et al., 2012). Acid phosphatases may have multiple functions, such as carbon metabolism, nodule permeability for O_2 diffusion, and oxidative stress attenuation (Sulieman and Tran, 2015), which makes their study both more challenging and necessary.

Drought and Flood

The current literature lacks information on the effects of either drought or flooding on legume-rhizobia signal exchange, although both situations are well known to reduce nodulation and nitrogen fixation (Arayangkoon et al., 1990;

Marcar et al., 1991; Purwantari et al., 1995; Hatimi, 1999). Thus, further research is necessary on this topic. Nodule formation ceases completely under sufficiently long or severe drought conditions, and nitrogenase and nodule respiratory activities are also strongly diminished in soybeans and common beans (Gerosa-Ramos et al., 2003). In alfalfa, such nitrogenase activity reduction has been linked to diminished bacteriod metabolic capability and oxidative damage to nodule cell components (Naya et al., 2007).

At the other extreme, several legumes are highly sensitive to water-logged conditions, with nodule development and function being more impaired than infection. Some of these effects, including nitrogenase activity, may be even stronger than observed for drought conditions. This phenomenon appears to be mostly linked to reduced O_2 availability (Andres et al., 2012).

Heavy Metals and Pesticides

Although the literature contains little information on the effects of pesticides and heavy metals on signal exchange, some *in vitro* work with 30 different pesticides and environmental contaminants showed that *S. meliloti* NodD was affected, delaying nodulation, and reducing biological nitrogen fixation by *M. sativa* (Fox et al., 2001, 2004). *M. sativa* and *G. max* fungicide-treated seeds also exhibited reduced *nod* gene activity for their respective partners (Andrés et al., 1998).

More recently, it has been shown that *R. alamii*, an EPS producer, modulates its metabolism in response to cadmium (Schue et al., 2011) through the activation of biofilm formation, both in the wild type and in EPS-deficient mutants, which may reduce the effects of this heavy metal.

Overall Synthesis

Although signal exchange between legumes and their bacterial symbionts is a well-studied process, much still needs to be clarified, particularly in relation to tropical legumes, which have been barely studied, and environmental effects other than low soil temperature.

Under at least some conditions, a delay in nodulation onset and, therefore, biological nitrogen fixation may be reduced by the exogenous supply of the appropriate legume signal. Because current predictions indicate a probable reduction in global agricultural season lengths, this phenomenon should receive increased attention.

Another field that deserves more attention is the study of signal exchange with non-traditional rhizobia, such as *Burkholderia* and *Cupriavidus*, and its effects on the plant host, for which no literature was found.

Acknowledgments

We thank the Brazilian Funding Agencies CAPES, CNPq, and FACEPE for their funding through several different projects, including a Research Fellowship for the first author, a Post-Doctoral Fellowship for the second author, and joint research funding for all three authors.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

OPINION
published: 29 September 2015
doi: 10.3389/fpls.2015.00787

Pseudomonas spp. as models for plant-microbe interactions

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Keywords: *Pseudomonas*, microbiota, phytomicrobiome, plant-microbe interactions, phospholipase, quorum sensing, type VI secretion, lipopeptide

The Phytomicrobiome in Context

As is the case with other multicellular eukaryotes, plants are colonized by large numbers of unicellular microorganisms. They may be free-living commensals, epiphytes, symbionts (endophytes), or obligate parasites. The plant holobiont is in effect an ecosystem, and it is of interest to know how this assemblage is established and maintained, and reacts to both biotic and abiotic cues. The current view, initially elaborated in the context of coral-dwelling microbial communites, is that the multicellular organism is more inclusively described by the term "holobiont" that includes associated microbiota, and is a valid unit of natural selection (Rosenberg et al., 2007). The holobiont then, is often dependent on its microbiota for crucial functions, drastic imbalances in which, termed dysbiosis, are thought to lead to compromised or deficient functioning.

The association of plants with microbes is phylogenetically ancient, going back to the macroalgae (Marshall et al., 2006). The role of the microbiota of plants, collectively termed the "phytomicrobiome," in their overall life cycle is now under investigation, close on the heels of more extensive studies on animal, especially human, microbiota. The development of *Arabidopsis thaliana* (thale cress) and *Brachypodium distachyon* (purple false brome) as model systems for dicotyledonous and monocotyledonous plants respectively, and the availability of genome databases for Pseudomonads (Winsor et al., 2011) and plants (Duvick et al., 2008) indicate that the potential for both hypothesis-based and discovery science are indeed great.

The assembly, development and maintenance of the plant holobiont is not possible without an exchange and sensing of, and responses to, biomolecular cues between its constituents. Within this overall theme, we focus on a few recently discovered, novel inter- and intra-species interactions of some *Pseudomonas* spp., indicating their utility as model systems, and highlighting some previously unforeseen mechanisms that could have a bearing on plant-phytomicrobiome interactions. Note that, for purposes of this article, we use the word "signaling" to refer generically to the sensing and response of organisms to environmental cues of both biotic and abiotic origins.

Some Aspects of the Social Biology of *Pseudomonas* spp.

The genus *Pseudomonas* is the most numerous among the cataloged genera of Gram-negative bacteria (Gomila et al., 2015). The ubiquity and metabolic versatility of this genus allows it to colonize a wide range of natural habitats and adopt a variety of lifestyles. *Pseudomonas* spp. have been isolated from each of the ecological niches within a plant as stated earlier (for a compilation, see Table 1 of Mercado-Blanco and Bakker, 2007). Their known ability to interact with and influence other bacteria, fungi, and multicellular organisms in a variety of biological contexts, and the availability of experimental tools for their genetic manipulation, should greatly facilitate the translation of knowledge for a wide range of practical applications.

OPEN ACCESS

Edited by:
Valerie Gravel, McGill University, Canada

Reviewed by:
Vittorio Venturi, International Centre for Genetic Engineering and Biotechnology, Italy

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Specialty section:
This article was submitted to Plant Biotic Interactions, a section of the journal Frontiers in Plant Science

Received: *01 April 2015*
Accepted: *11 September 2015*
Published: *29 September 2015*

Citation:
Sitaraman R (2015) Pseudomonas spp. as models for plant-microbe interactions. Front. Plant Sci. 6:787. doi: 10.3389/fpls.2015.00787

At the outset, it is worth recalling that strains of *P. putida* and *P. aeruginosa* were the first living, genetically modified organisms to be patented for a specific application (biodegradation of petrochemicals—camphor, octane, salicylate, and naphthalene)—truly heralding the modern era of genetically modified organisms (Chakrabarty, 1981). The genus *Pseudomonas* is behaviorally very versatile, with free-living as well as parasitic forms capable of colonizing a wide variety of host organisms and ecological niches within hosts. For example, *P. aeruginosa* (PA) is a free-living soil bacterium that is also an opportunistic pathogen of both plants and animals, *P. syringiae* (PS) is an opportunistic plant pathogen, *P. putida* (PP) has been extensively used in bioremediation for its ability to utilize a wide range of hydrocarbons as carbon sources, and both *P. putida* and *P. fluorescens* (PF) are promising growth-promoting and biocontrol agents.

In order to understand the role of the bacterial component of the phytomicrobiome in plant physiology, the functional analysis of bacteria colonizing multiple ecological niches provided by the plant—the roots (rhizosphere), leaves (phyllosphere), surfaces (ectosphere), and tissues (endosphere)—needs to be undertaken, ideally *in situ* and over the several developmental stages of the plant. This is an understandably formidable task, and the utility of a model bacterium in this context is apparent. From a bacterial viewpoint, it has to sense the presence of, and stimuli from, potential hosts as well as competitors (of the same or different species), strategize in a manner that allows it to reach the host, survive competition, and colonize, gain access to resources, and persist for a reasonable length of time in the face of perturbations. The establishment and resilience of the plant-microbe interaction is therefore dependent on the exchange and sensing of a variety of signals by both types of partners, often simultaneously, and combinatorially.

Bacterial Quorum Sensing and Inter-species Competition

Pseudomonas spp. possess quorum-sensing (QS) systems that synthesize and sense hormone-like messages of diverse origins in their immediate environment. QS systems are often linked with other regulons, leading to different phenotypes (for a review, see Venturi, 2006). For example, PP produces cyclic lipopeptide surfactants putisolvin I and II, that are under the control of QS and disrupt biofilms (Kuiper et al., 2004; Dubern et al., 2006). Interestingly, this can happen not only at the stationary phase, but also stochastically in the early stages of growth resulting in swarming motility (Cárcamo-Oyarce et al., 2015), promoting colonization of fresh surfaces. Other putisolvin-like lipopeptides of PP have been found to exhibit lytic activity against the zoospores of the fungal pathogen *Phytophthora capsici* zoospores *in vitro*, inhibit growth of the fungal pathogens *Botrytis cinerea* and *Rhizoctonia solani* in addition to being involved in swarming motility (Kruijt et al., 2009). In more general terms such interactions could contribute to the overall composition of the phytomicrobiome by modifying its diversity, and contribute to its resilience to perturbation by invaders.

Plant growth promotion effects of *Pseudomonas* spp. may also be under QS control, as was demonstrated in the case of QS-controlled production of an *N*-acyl-L-homoserine lactone (AHL), cyclic dipeptides and their derivative diketopiperazines (DKPs) by PA. Exposure of *A. thaliana* seedlings to 3-oxo-C12-AHL produced by the LasI AHL synthase causes growth inhibition of the primary root, while DKP stimulated the growth of lateral roots (Ortiz-Castro et al., 2011). The presence of orphan AHL transcriptional regulators such as QscR in PA that lack a cognate AHL synthase and bind with relaxed specificity to both endogenously and exogenously produced AHLs adds another layer of complexity to plant-phytomicrobiota interactions (for a recent and detailed review, see Chugani and Greenberg, 2014). Likewise, pseudomonads as well as other plant-associated bacteria have been found to encode a unique family of orphan (or solo) AHL transcriptional regulators that are uniquely responsive to unknown plant and/or bacterial signal molecules (Patel et al., 2013).

That one component of the microbiota may influence another indirectly by modulating host signals and responses has been dramatically demonstrated recently in both plant and animal contexts. PS pathovar tomato (PSt) infection of *Arabidoposis thaliana* leaves induces the plant enzyme phospholipase Db1 (PLDb1) that is a negative regulator of the salicylic acid-dependent resistance to PS, but is a positive regulator of the jasmonic acid-dependent resistance to the fungal pathogen *Botrytis cinerea*. Even more interestingly, infection with an avirulent PSt strain that expresses the effector AvrRpt2 secreted by the type III secretion system can also lead to resistance against virulent Pst that does not express AvrRpt2 (Zhao et al., 2013). Thus, indirect microbial modulation of the host can cause subtle, even strain-level, shifts in the composition of microbiota, depending on the temporal sequence of host colonization. In what may well be a case of convergent survival strategies, PA infection of airways in human patients of cystic fibrosis induces airway cells of the airway epithelium to produce secretory phosopholipase A2, which is bactericidal for Gram-positive bacteria such as *Staphylococcus aureus* but relatively less so for PA (Pernet et al., 2014). This effectively allows PA to proliferate at the expense of *S. aureus*.

Identification of Putative Type VI Effectors in Plant-associated *Pseudomonas* spp.

The ability of PA to infect both plant and animal hosts, and the identification of a common set of virulence determinants during plant and animal infections (Rahme et al., 1997, 2000), along with genome sequence information can be exploited to identify potential effectors and predict putative mechanisms of interaction with the host plant in the context of other *Pseudomonas* spp. Recent, extensive analyses of the *A. thaliana*-associated microbiota indicate that *Pseudomonas* spp. are preferentially enriched in the endophytic compartment of the plant, as compared to the rhizosphere (Bulgarelli et al., 2012; Lundberg et al., 2012). Therefore, the identification of conserved effectors within the pseudomonad lineage can be used as a starting point to probe plant-microbe interactions. The type VI secretion systems (T6SS) merit special attention in this regard as they are widespread among diverse Gram-negative bacteria, both pathogenic and non-pathogenic including *Pseudomonas*

TABLE 1 | A representative list of putative effectors potentially targeting plant cells and encoded by T6SS in plant-associated *Pseudomonas* spp.

Effector molecule(s) of *P. aeruginosa*	Locus tag/Strain of PA	Known function and context in PA	Reference(s) for known functions	Plant-associated *Pseudomonas* spp.	Ortholog locus tag
Phospholipase D (PldB)	PA5089/PAO1	Encoded by the H3-T6SS. Elimination of compteting bacteria. Promoting PA internalization by host (human) epithelial cells	Jiang et al., 2014	*Pseudomonas* sp. UW4	PputUW4_03278
				P. syringae pv. *Phaseolicola* 1448A (pathogen)	PSPPH_0117
				P. syringae pv. *Syringae* B728a (pathogen)	Psyr_4970
Valine-glycine repeat protein (VgrG2b)	PA0262/PAO1	Encoded by the H2-T6SS. Delivered into host (human) epithelial cells, promotes microtubule-mediated PA internalization by direct interaction with microtubules	Sana et al., 2015	*P. syringae* pv. *syringae* B728a	Psyr_4080
				Pseudomonas sp. UW4	PputUW4_03083
				P. putida F1 (orthologs also present in strains HB3267, KT2440, H8234, ND6, GB-1, NBRC 14164, W619 and DOT-T1E)	Pput_2117

These have been identified based on two T6SS effectors in P. aeruginosa, PldB and VgrG2b, that are known to target eukaryotic host cells. Orthologs were identified by searching the Pseudomonas database (http://beta.pseudomonas.com); (Winsor et al., 2011) and the Pseudomonas ortholog database (http://pseudoluge.pseudomonas.com/pseudoluge/named/list/search?field=locus_tag&value=PCHL3084_RS00035); (Whiteside et al., 2013).

spp. (Barret et al., 2011), and can potentially deploy effectors targeting both prokaryotic and eukaryotic cells (Jiang et al., 2014). Two effectors secreted by T6SS in PA that are known to target host cells, and their orthologs identified in plant-associated *Pseudomonas* spp. are listed in **Table 1**. The functionality of these effectors on plant cells, if verified, can provide important information about the assembly and disruption of bacterial communities, as well as their interaction with the host plant.

Conclusions and Future Directions

The foregoing account suggests new lines of inquiry into the signals that drive the formation and maintenance of the plant microbiota. Can systemic effects on the host and/or microbiota be mediated by diffusible signals produced in one part of the plant? If so, over what distances do these effects extend, and how are they mediated? What is the role of conserved and functional T6SS effectors in diverse plant-bacteria associations that range from commensalism to symbiosis? In the effort to understand the relative contribution of different components of the microbiota to the plant holobiont, it may be remembered that abundance alone may not truly reflect the relative importance of the species/strain in question. Numerically less abundant species could be key players within the microbiota, assuming the role of "keystone" species, as has been suggested earlier (Saraswati and Sitaraman, 2014).

A potential limitation in reliance on Gram-negative pseudomonads as model systems is that their relative importance may depend on environmental conditions. For example, *Pseudomonas* spp. may be an important disease-suppressive agent in a moist and temperate environment in the Netherlands (Mendes et al., 2011), whereas the Gram-positive *Bacillus* spp. contribute to disease suppression in Egypt, a more arid zone (Köberl et al., 2011). Over reliance on *Pseudomonas* spp. as models could therefore potentially overlook unique interactions and mechanisms operative over large geographical areas and ecological zones. Also to be remembered is that most studies of microbiota (plant or animal) focus on the bacterial component alone, and the role of fungi and archaea is less studied and understood.

The microbiota of multicellular organisms, whether plant or animal, present a case wherein simultaneous and combinatorial interactions have to be identified, and their relative importance determined. To this end, the identification of effectors and the delineation of mechanisms of interaction are required. The predictive and inferential value of *Pseudomonas* spp.-based models that can be probed with conventional as well as high-throughput methods is therefore undeniable, and insights so gained have immense potential to inform and refine our efforts to dissect the mechanistic bases of interactions taking place in the plant holobiont.

Acknowledgments

This paper is dedicated to my parents, Mr. G. Sitaraman and Mrs. Indu Bala, for their unwavering encouragement and support of my studies. Dr. Jay Ram Lamichhane of INRA, France is thanked for a critical comment on the paper. The publication of this article has been facilitated by the grant of a full waiver of publishing fees by Frontiers. No funding was received for this work.

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Conflict of Interest Statement: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 04 August 2015
doi: 10.3389/fpls.2015.00568

Optimal level of *purple acid phosphatase5* is required for maintaining complete resistance to *Pseudomonas syringae*

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OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Plant Biotic Interactions, a section of the journal Frontiers in Plant Science

Received: 06 May 2015
Accepted: 09 July 2015
Published: 04 August 2015

Citation:
Ravichandran S, Stone SL, Benkel B, Zhang J, Berrue F and Prithiviraj B (2015) Optimal level of purple acid phosphatase5 is required for maintaining complete resistance to Pseudomonas syringae
Front. Plant Sci. 6:568.
doi: 10.3389/fpls.2015.00568

Plants possess an exceedingly complex innate immune system to defend against most pathogens. However, a relative proportion of the pathogens overcome host's innate immunity and impair plant growth and productivity. We previously showed that mutation in *purple acid phosphatase* (*PAP5*) lead to enhanced susceptibility of *Arabidopsis* to the bacterial pathogen *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000). Here, we report that an optimal level of *PAP5* is crucial for mounting complete basal resistance. Overexpression of *PAP5* impaired *ICS1*, *PR1* expression and salicylic acid (SA) accumulation similar to *pap5* knockout mutant plants. Moreover, plant overexpressing *PAP5* was impaired in H_2O_2 accumulation in response to *Pst* DC3000. *PAP5* is localized in to peroxisomes, a known site of generation of reactive oxygen species for activation of defense responses. Taken together, our results demonstrate that optimal levels of *PAP5* is required for mounting resistance against *Pst* DC3000 as both knockout and overexpression of *PAP5* lead to compromised basal resistance.

Keywords: ***purple acid phosphatase5* (*PAP5*), *Pseudomonas syringae* pv. tomato DC3000, disease resistance, *Arabidopsis*, reactive oxygen species (ROS)**

Introduction

Plants are constantly exposed to a diverse array of microbial pathogens. In nature, plants have evolved mechanism(s) to restrict most pathogens (non-host disease resistance) and also reduce pathogen ingress (basal resistance or PAMP-triggered immunity) (Bittel and Robatzek, 2007). Activation of defense response begins with the recognition of conserved molecular signatures or PAMP (Pathogen-Associated Molecular Pattern) by pattern recognition receptors (PRRs) localized on the plasma membrane and in the cytoplasm (Chisholm et al., 2006; Jones and Dangl, 2006). PAMPs are microbial molecular signatures (e.g., flagellin, bacterial lipopolysaccharides, elongation factor, chitin, and β-glucan) that are absent in the host (Boller and Felix, 2009; Schwessinger and Ronald, 2012). Following PAMP perception, the PRRs initiate complex signaling networks that are associated with rapid synthesis of reactive oxygen species, activation MAP kinase signaling and pathogenesis related (PR) genes leading to PAMP triggered immunity (PTI) (Chisholm et al., 2006). However, well-adapted microbial pathogens have evolved the means to subvert defense signaling and breach PTI.

Plants are exposed to pathogens that have diverse infection strategies and therefore activation of appropriate, pathogen specific defense responses is vital for plant growth and productivity (Glazebrook, 2005). Structural alteration in cell wall including waxy cuticle layer, deposition of callose, suberin, and lignifications of cell wall provide protection contributing to non-host disease resistance (Dangl and Jones, 2001). Upon pathogen recognition, immediate early response genes including glutathione S transferase 6 (GST) and immediate early induced glucosyltransferase (IEGT) detoxify and protect cells from oxidative stress (Uquillas et al., 2004 and references therein). Salicylic acid (SA) dependent defense marker gene such as PR1 is induced later during pathogenesis involving the key signal transducer NPR1 (non-expressor of pathogenesis related genes 1) (Schenk et al., 2000). Specific response to pathogens is also mediated by gene-for-gene recognition leading to the activation of resistance (R) genes or recently termed effector-triggered immunity (ETI) in host (Nimchuk et al., 2003; Chisholm et al., 2006). Activation of R gene is usually accompanied by production of reactive oxygen species (ROS) leading to hypersensitive responses (HR) to restrict the spread of pathogen (Glazebrook, 2005). The localized cell death triggers systemic acquired resistance (SAR) to confer resistance throughout the plant (Baker et al., 1997). Hypersensitive response is also associated with induction of diverse group of defense related and pathogenesis related (PR) genes.

Plants defense responses are primarily associated with salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) (Vlot et al., 2009). SA regulates the activation of defense responses against most biotrophic, hemi-biotrophic pathogens (Durner et al., 1997) and also mediates the establishment of systemic acquired resistance (SAR) (Grant and Lamb, 2006). By contrast, JA and ET operate synergistically to confer resistance against necrotrophic pathogens and herbivorous insects. JA is also associated with induced systemic resistance (ISR) elicited by rhizobacterial strains that promote plant growth and enhances resistance to various bacterial and fungal pathogens (Ton et al., 2002). In recent years, the role of other phytohormones including abscisic acid (ABA), auxins, gibberellins (GA), cytokines (CK), and brassinosteriods (BR) have started to emerge (Mutka et al., 2013) reviewed by Bari and Jones (2009). Apart from plant hormones, class of secondary metabolites including phenyl proponoid, glucosinolates, terpenoids, and phytoalexins aid in the protection of plant against most biotic stress (Kliebenstein, 2004). Synthesis and secretion of anti-microbial compounds confer selective advantage to curb pathogen invasion. Over 100,000 low-molecular-mass compounds derived from isoprenoid, polypropanoid, polyketide pathways are known to enhance defense against microbial infections (Dixon, 2001).

Purple acid phosphatases (*PAPs*) belong to a family of binuclear metalloenzymes and have been identified and characterized in numerous plants, animals, and a limited number of microorganisms (Mitic et al., 2006; Schenk et al., 2008). All PAPs contain dinuclear metal ions and a characteristic set of seven invariant residues, which coordinate the metal ions within the active site (reviewed by Mitic et al., 2006; Schenk et al., 2008). PAPs have been implicated in an array of biological functions. Most PAPs have been classified as non-specific acid phosphatases that catalyze the hydrolysis of inorganic phosphate (Pi) from various monoesters and anhydrides substrates (Olczak and Watorek, 2003). The physiological role of plant PAPs is predominantly associated with the regulation of Pi uptake and recycling (Li et al., 2002; Veljanovski et al., 2006). However, previous studies have also revealed roles for plant PAPs in other biological functions, including peroxidation (Del Pozo et al., 1999), ascorbate recycling (Zhang et al., 2008), mediation of salt tolerance (Liao et al., 2003), and regulation of cell wall carbohydrate biosynthesis (Kaida et al., 2009).

Recently, we demonstrated that the loss of *purple acid phosphatase5* (*PAP5*) in *Arabidopsis* leads to enhanced susceptibility to virulent *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000). *Arabidopsis* plants carrying a mutation in *PAP5* exhibited a defect in the expression of pathogenesis related (PR) genes including Pathogenesis Related gene 1 (*PR1*), Isochorismate synthase1 (*ICS1*) and plant defensin1.2 (*PDF1.2)*. This study also revealed that *pap5* plants failed to accumulate H_2O_2 in response to *Pst* DC3000 infection compared to wild-type plants (Ravichandran et al., 2013). One of the earliest responses to pathogen infection is generation of reactive oxygen intermediates ROIs (O_2^- and H_2O_2). Although, ROIs such as hydroxyl radical (.OH), superoxide radical (O_2^-) and hydrogen peroxide (H_2O_2) are produced under normal metabolic processes. The rapid accumulation of ROS (also known as oxidative burst) cause oxidative cross linking of cell wall, activation of cellular signaling (protein phosphorylation) and induction of pathogenesis related (PR) genes (Alvarez et al., 1998). It is widely assumed that ROS production after pathogen recognition is associated with membrane bound NADPH oxidase in the apoplast (Lamb and Dixon, 1997). H_2O_2 generated in response to pathogen recognition induces salicylic acid (SA) and PR protein accumulation (Chamnongpol et al., 1998). A number of studies have indicated that ROS produced in response to pathogen recognition is located in the apoplast (reviewed by Torres et al., 2002). It is also evident that plants can produce ROS in other inter-cellular organelles including chloroplast, mitochondria and peroxisomes. However, the cellular homeostasis and concentration of ROS is highly regulated by enzymes such catalase, peroxidase and superoxide dismutase (Foyer and Noctor, 2003).

Having demonstrated that the loss of *PAP5* impaired plants innate immune responses (Ravichandran et al., 2013), we wanted to determine if overexpression of *PAP5* results in enhanced disease resistance. Further, *in silico* predictions failed to detect signal peptides on *PAP5*, hence we wanted to experimentally verify the prediction by tagging *PAP5* with a fluorescent label. Here, we report that the level of *PAP5* is crucial for mounting complete resistance against *Pst* DC3000. Optimal levels of *PAP5* are required for induction of PR genes and SA accumulation. Further, *PAP5* was found to be peroxisomal localized and aid the generation of reactive oxygen species for activation of defense responses.

Results

Optimal Level of *PAP5* Is Required for Complete Resistance to Pst DC3000

Previously, we reported that loss of *PAP5* resulted in enhanced susceptibility of *Arabidopsis* to the virulent *Pst* DC3000 (Ravichandran et al., 2013). Having demonstrated that the loss of *PAP5* lead to enhanced susceptibility, we tested if overexpression of *PAP5* will result in enhanced resistance. We generated transgenic plants overexpressing *PAP5* under the control of constitutive cauliflower mosaic virus (CaMV) 35S promoter. Plants exhibiting Basta resistance were chosen and plants were picked randomly to verify the abundance of *PAP5* transcripts. Among the transgenic lines tested, two independent overexpressor lines *35S:PAP5*-A and *35S:PAP5*-B were chosen for further studies. Overexpressor lines *35S:PAP5*-A and *35S:PAP5*-B showed ∼8 and 62-fold increase in *PAP5* transcripts respectively compared to wild-type (Col-0) plants (**Figure 1A**). There was no difference in the growth and development of both the overexpressing lines compared to wild-type plants.

For pathogenicity assay, plants were sprayed with suspension of *Pst* DC3000 as described in the methods. Interestingly, the overexpressor lines exhibited extensive chlorosis and increased susceptibility to *Pst* DC3000 compared to wild-type plants. Assessment of *Pst* DC3000 growth in plant apoplast revealed that both overexpressor lines had higher bacterial titers compared to wild-type plants (**Figure 1B**). Both overexpressor lines exhibited comparable levels of chlorosis and bacterial titers suggesting that the enhanced susceptibility is not due to the positional effects on insertion of the transgene. Although the overexpressor line *35S:PAP5*-B constitutively expressed higher levels (∼62 fold) of *PAP5* compared to *35S:PAP5*-A plants (**Figure 1A**) we did not observe any significant difference in susceptibility to *Pst* DC3000 (**Figure 1B**).

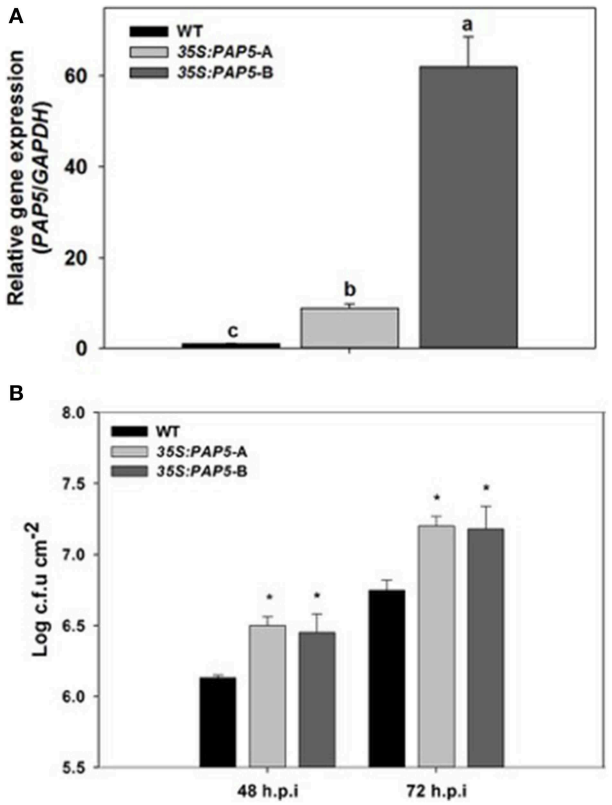

FIGURE 1 | Transgenic plants overexpressing *PAP5* exhibit enhanced disease susceptibility. (A) Transcript levels of *PAP5* in transgenic plants. Total RNA was extracted from wild-type and transgenic plants as described in materials and methods. Transcript levels of *PAP5* was normalized to the expression of GAPDH in the same samples and expressed relative to the normalized transcript levels of wild-type plants. The bars represent the mean and standard deviation from two independent experiments. Significant differences ($P < 0.05$) are indicated by different letters. **(B)** Growth of *Pst* DC3000 in wild type and transgenic plants. Plants were inoculated with *Pst* DC3000 (10-8 c.f.u ml^{-1}) and bacterial growth in plant apoplast was determined as described in the materials and methods. The bars represent the mean and standard deviation from values of three separate trials with six to eight replicates each trial. An asterisk indicates a significance increase in *Pst* DC3000 growth compared to wild-type plants (Student's *t*-test; $P < 0.05$).

Overexpression of *PAP5* Impairs Pathogenesis Related (PR) Gene Expression and Alters H_2O_2 and Salicylic Acid Accumulation

In contrast to our expectation, overexpression of *PAP5* did not result in enhanced resistance. Therefore, we tested if expression of defense related genes are impaired in the overexpressor lines (*35:PAP5*-A and *35S:PAP5*-B). The overexpressor lines and wild-type plants were spray inoculated with suspension of *Pst* DC3000 (10^8 c.f.u ml^{-1}) and leaf tissues were harvested for gene expression analysis. The expression of pathogenesis related protein gene1 (*PR1*), a commonly used marker gene associated with *Pst* DC3000 infection and SA-mediated defense responses was observed. There was no significant difference in the transcript abundance of *PR1* between the mock infected overexpressor lines and the wild-type plants. However, *PR1* was strongly induced in infected wild-type plants 24 h.p.i., whereas it was not induced in OE lines (**Figure 2A**). Although the expression of *PR1* was slightly higher in both overexpression lines at 48 h.p.i., the levels of *PR1* was significantly lower compared to infected wild-type plants (**Figure 2A**). We also tested the expression of *isocorismate synthase1* (*ICS1*), which is responsible for ∼90% of pathogen induced SA production (Wildermuth et al., 2002). As shown in **Figure 2B**, accumulation of *ICS1* was ∼4 fold higher in infected wild-type plants, whereas the expression of *ICS1* was strongly suppressed in both the OE1, OE2 overexpressor lines. However, the expression of *ICS1* in infected wild-type plants was similar to mock infected wild-type plants at 48 h.p.i. Moreover, the expression of *ICS1* correlated with the expression of *PR1*.

To determine whether the decrease in the expression of ICS1 altered the concentration of salicylic acid (SA), we quantified the SA in the infected leaf tissue. We quantified SA in wild-type, overexpressor lines (*35:PAP5*-A and *35S:PAP5*-B) and knockout line *pap5-1* following *Pst* DC3000 infection. The concentration of SA increased in all *Pst* DC3000 infected plants. However, the SA levels in both the overexpressor lines and *pap5-1* plants were only ∼60% of the wild-type plants (**Figure 3A**). In

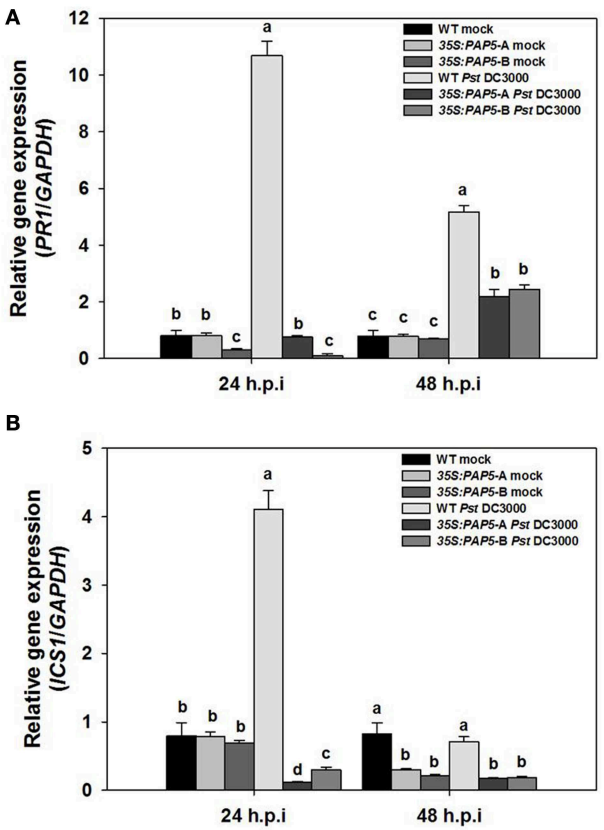

FIGURE 2 | Expression of *ICS1* and *PR1* is impaired in transgenic plants (*35S:PAP5*-A and *35S:PAP5*-B). Plants were spray inoculated with *Pst* DC3000 (10^8 c.f.u ml^{-1}) and RNA was extracted from leaf tissues sampled 24 and 48 h.p.i. Transcript levels were normalized to the expression of *GAPDH* in the same samples. The transcript levels were expressed relative to the normalized transcript levels of mock infected wild-type plants. The bars represent the mean and standard deviation from two independent experiments. Transcript levels of *ICS1* **(A)** and *PR1* **(B)** in mock and *Pst* DC3000 infected plants. Significant differences ($P < 0.05$) are indicated by different letters (Student's *t*-test; $P < 0.05$).

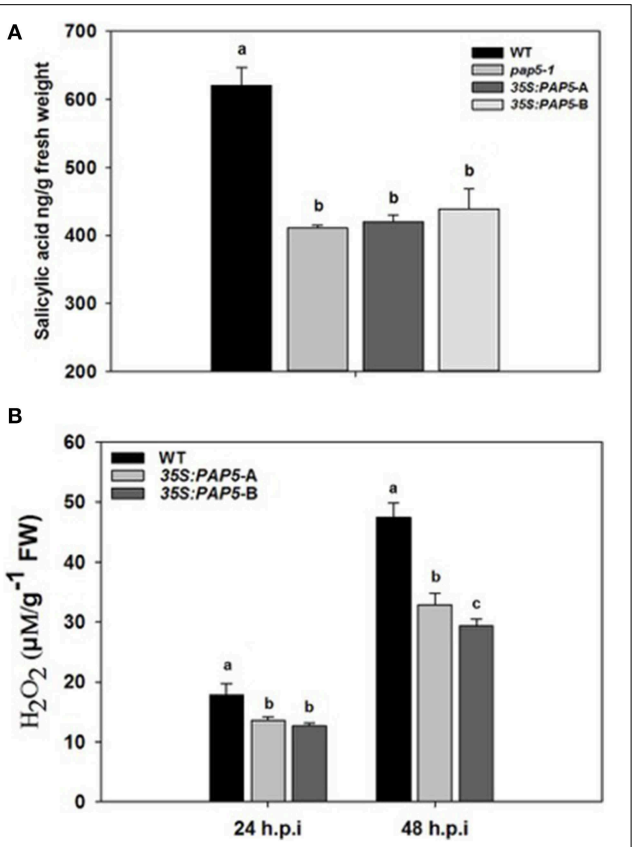

FIGURE 3 | Both loss and overexpression of *PAP5* affect salicylic acid (SA) and H_2O_2 accumulation in *Pst* DC3000 infected plants. (A) Quantification of SA in *pap5-1* and 35S:*PAP5* plants following *Pst* DC3000 infection. Plants were spray inoculated with *Pst* DC3000 (10^8 c.f.u ml^{-1}) and leaf tissues were excised 48 h.p.i. The bars represent the mean and standard deviation from three replicates. Significant differences ($P < 0.05$) are indicated by different letters (Student's *t*-test; $P < 0.05$). **(B)** Quantification of H_2O_2 following Pst DC3000 infection. The bars represent mean and SD of H_2O_2 accumulation. A significant difference ($P < 0.05$) in H_2O_2 production are indicated by different letters (Student's t-test; $P < 0.05$).

addition, accumulation of hydrogen peroxide (H_2O_2) generated in response *Pst* DC3000 was suppressed in both overexpressor lines (**Figure 3B**). These results suggest that the over expression of *PAP5* impaired the transcription of *ICS1*, *PR1,* and SA accumulation subsequently similar to that of the loss-of-function *pap5* mutant. Taken together, it is evident that optimal level of *PAP5* is required for expression of *ICS1* and *PR1* and for accumulation of H_2O_2 and SA after *Pst* DC3000 infection. Further, these results suggest that the enhanced growth of *Pst* DC3000 in *35S:PAP5*-A and *35S:PAP5*-B is dependent on SA accumulation and SA-mediated defense responses.

Sub-cellular Localization of *PAP5*

To identify the sub-cellular localization of *PAP5*, a comprehensive *in silico* prediction was performed. Most *in silico* prediction revealed that *PAP5* could be localized to the nucleus, cytosol, extracellular, endoplasmic reticulum, golgi bodies and to the extracellular space (http://suba.plantenergy.uwa.edu.au/). However, a search on http://www.cbs.dtu.dk/services/SignalP/ revealed that *PAP5* does not carry a signal peptide. To verify this contradictory *in silico* prediction, the coding region of *PAP5* was fused to the YFP reporter gene under the control of CaMV 35S promoter. Confocal microscopy reveled *YFP-PAP5* as rapidly moving punctate structures within the cytoplasm and faintly in the nucleus (**Figure 4**). To identify the cellular compartment, the agrobacterium strains carrying organelle specific markers (Nelson et al., 2007) were coinfiltered with *YFP-PAP5* and leaf tissues were harvested at different time points for confocal microscopy. As shown in **Figure 4**, *YFP-PAP5* showed a strong colocalization with peroxisomal specific marker (peroxisomal targeting signal 1; *PTS1-CFP*). Agrobacterium strains carrying *YFP-PAP5* was also cofiltered with golgi (GmMan1 cytoplasmic tail and transmembrane) and plastid (rubisco targeting sequence) specific markers. In contrast to *in silico* predictions, *YFP-PAP5* did not localize to golgi or plastid organelle specific markers (**Figure S1**).

Since the *in silico* prediction showed that *PAP5* lack signal peptides, we wanted to determine if removal of specific stretch

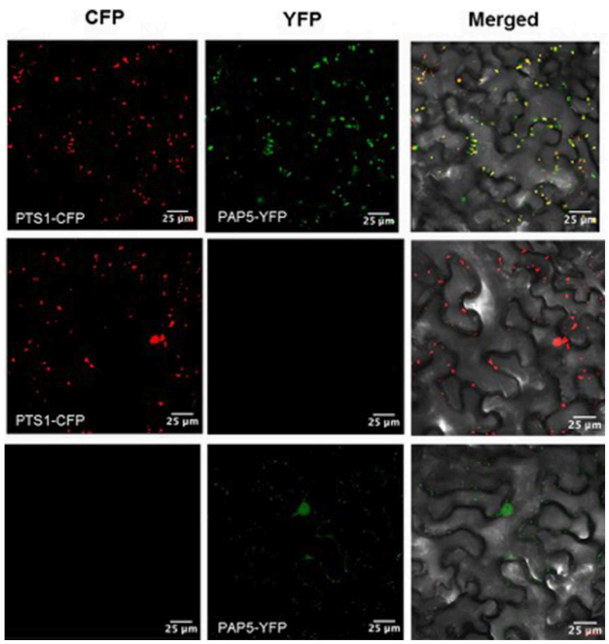

FIGURE 4 | Subcellular localization of *PAP5* in *N. benthamiana* leaves. Agrobacterium strains carrying the recombinant plasmids (*35S:YFP-PAP5*) and peroxisomal specific marker (*PTS1:CFP*) was transiently expressed in tobacco leaves. Left plane shows a single optical section of CFP fluorescence images, the middle plane show single optical section of YFP fluorescence and right planes shows transmitted light images with merged CFP and YFP fluorescence. Bar = 25 µm.

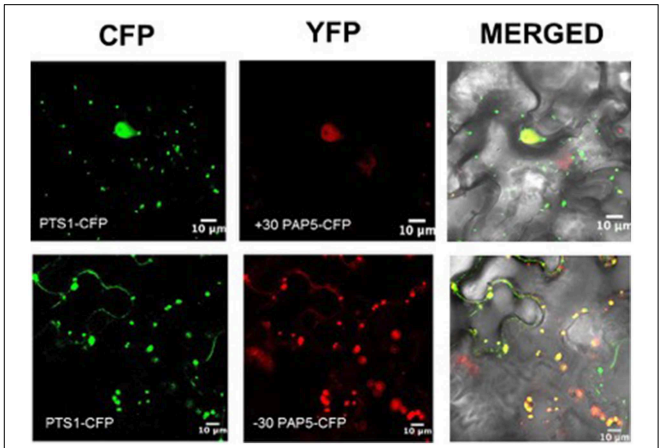

FIGURE 5 | Subcellular localization of *PAP5* in *N. benthamiana* leaves. Agrobacterium strains carrying the recombinant plasmids (*+30PAP5:YFP or +30PAP5:YFP*) and peroxisomal specific marker (*PTS1-CFP*) was transiently expressed in tobacco leaves. Left plane shows a single optical section of CFP fluorescence images, middle planes show a single optical section of YFP fluorescence and right planes shows transmitted light images with merged CFP and YFP fluorescence. *+30PAP5:YFP* lacks a stretch of N-terminal amino acids MSLETFPPPA. *−30PAP5:YFP* lack N-terminal amino acids MSLETFPPPA. Bar = 10 µm.

of amino acids from C- or N-terminals affect the peroxisomal localization of *PAP5*. *PAP5* constructs lacking a stretch of N-terminal amino acids (MSLETFPPPA), referred as *YFP:+30PAP5* failed to localize to peroxisomes (**Figure 5**). However, removal of amino acids RYYLPEEETI from the C-terminal (referred as *YFP:-30PAP5*) of *PAP5* did not prevent the localization of *PAP5* to peroxisomes. These results suggest that N-terminal amino acids MSLETFPPPA are required for subcellular localization of *PAP5*.

Discussion

In this study, we showed that an optimal level of *PAP5* is critical for mounting appropriate defense responses. Previous studies have revealed several molecular cues that regulate plant defense responses. Often, genes identified as positive regulator of defense responses are overexpressed to generate disease tolerant crops (Zhang et al., 2014). Interestingly, in some instances, both reduced and overexpression of a gene could result in the same phenotype. For example, *OXI1* (*Oxidative Signal-Inducible1*), encoding a serine/threonine kinase has been shown to be required for complete activation of mitogen-activated protein kinase (MAPKs). *oxi-1* null mutants showed enhanced susceptibility to virulent *Hyaloperonospora arabidopsidis* (formerly *Paranospora parasitica*) (Menke et al., 2004). Interestingly, transgenic plants overexpressing *OXI1* (*35S::OXI1*) displayed enhanced susceptibility to both virulent *Hyaloperonospora arabidopsidis* and *Pst* DC3000 (Petersen et al., 2009). Since both reduced and overexpression of *PAP5* lead to enhanced susceptibility to *Pst* DC3000, we hypothesize that *PAP5* could exist in complex with other proteins. Thus, constitutive expression of *PAP5* (*35S:PAP5*) alone may not be sufficient for complete resistance. It is also possible that the prolonged expression of *PAP5* could negatively affect basal resistance against *Pst* DC3000. Previously, we identified that *PAP5* is not expressed under normal growth conditions and are selectively induced only during prolonged Pi starvation and early stage of *Pst* DC3000 (6 h post inoculation) (Ravichandran et al., 2013). Hence, constitutive overexpression of *PAP5* is not optimal and may perturb and impair its function following *Pst* DC3000. Previous studies have also shown that both overexpression and partial loss of *FIP1* [FIN (Far-red insensitive) 219 Interacting Protein] resulted in hyposensitive hypocotyl phenotype under continuous Far Red (FR) light (Chen et al., 2007). *FIP1* was also shown to exhibit glutathione S-transferse activity which lead to delayed flowering phenotype under long-day conditions. Similarly, loss and overexpression of *EBS* (*Early Bolting in Short Days*) showed early flowering, a dwarf phenotype and reduced fertility (Pineiro et al., 2003). *EBS* encodes a nuclear protein with homeodomain Zn finger that regulate chromatin remodeling and repress the initiation of flowering in short days.

Pathogen recognition triggers generation of reactive oxygen intermediates (ROIs), which is required for activation of defense responses (Torres et al., 2002). It is also evident that the generation of ROI occurs within hours of pathogen infection (Alvarez et al., 1998). Since *PAP5* is induced only during the earlier stages (6 h.p.i) of *Pst* DC3000 infection and the localization of *PAP5* in peroxisome (**Figure 4**) suggests that *PAP5* may act as a component of ROI generation. Hence we hypothesized that *PAP5* might exist in complex with other

proteins, a comprehensive *in silico* prediction was performed to identify proteins that may potentially interact with *PAP5*. Most *in silico* prediction searches on Bio-Analytic Resource for Plant Biology (BAR), Biological General Repository for Interaction Datasets (BioGRID) and GeneMANIA failed to detect any physical interaction. Moreover, most subcellular tools including SUBA, TargetP, and WoLF PSORT failed to identify the peroxisomal targeting sequences of *PAP5*. Previous studies have also shown that most *in silico* prediction programs fail to identify signature peroxisomal targeting sequences (Nelson et al., 2007). Few *in silico* prediction showed that *PAP5* is targeted to the extracellular space (http://suba.plantenergy.uwa.edu.au/; http://wolfpsort.org/).

In mammals, the high expression of PAPs in macrophages and increased ROS production that mediates microbial killing has been demonstrated (Kaija et al., 2002). Although ROS are produced under normal metabolic processes, the role of ROS in signaling is largely dependent on the rate of synthesis and is controlled by antioxidative enzymes such as catalase and peoxidases in peroxisomes (Nyathi and Baker, 2006). Previously, catalase deficient plants have been shown to display marked perturbation of intracellular redox and cellular homeostasis (Vandenabeele et al., 2004). Such perturbation is associated with accumulation of salicylic acid (SA) and induction of pathogenesis related (PR) genes (Chamnongpol et al., 1998). Similarly, catalase deficient *Arabidopsis* (*cat2*) plants showed increased peroxisomal H_2O_2 (Chaouch et al., 2010). Peroxisomal β-oxidation is also attributed to the degradation of various straight and branched chain fatty acids (Baker et al., 1997). Derivatives of β-oxidation such as cyclic oxylipins also play a significant role in the synthesis of plant hormones jasmonic acid (JA) and salicylic acid (SA) which are important signaling molecules (Theodoulou et al., 2005). Following *Pst* DC3000, infection the SA levels in both overexpressor lines and *pap5-1* plants were ∼60% of the wild-type plants. These results also suggest that SA accumulation is not completely abolished in both transgenic (*35S:PAP5*) and *pap5-1* plants.

It is well recognized that majority of the eukaryotic proteins undergo reversible phosphorylation via protein kinase (PK) and phosphatase (PP) to control major cellular processes. A large family of protein kinases has been characterized and their function in various cellular processes has been well established (País et al., 2009). However, the physiological role of its counter partner protein phosphates has been poorly understood. Activation of sucrose phosphate synthase (SPS) and nitrate reduction (NR) has been associated with decrease in phosphorylation status of SPE and NR (Huber and Huber, 1996). Interestingly, phosphorylation of Ser158 is sufficientfor inactivation of spinach SPS *in vitro* (Huber and Huber, 1992). Similarly, *PAP5* may be required for complete activation of vital enzymes such as glycolate oxidase in peroxisomes that modulate H_2O_2 generation (**Figure 6**). Since ROS is generated under normal metabolic processes, a highly regulated mechanism must exist to control ROS generation on pathogen recognition.

Several PAPs (SAP1, SAP2, AtPAP17, and AtPAP26) induced under Pi starvation are secreted to the extracellular space to hydrolyze Pi containing substrates and also exhibit peroxidase activity (Del Pozo et al., 1999; Bozzo et al., 2002; Hurley et al., 2010). However, the role of PAPs and its peroxidase activity in the extracellular space is not clear. Previously, PAPs exhibiting peroxidase activity has been speculated to function in ROS production similar to the oxidative burst that occurs in response to pathogen recognition (Bozzo et al., 2002; Hurley et al., 2010). Bacterial pathogens including *Pst* DC3000 reaches the apoplastic region to acquire nutrients (Alfano and Collmer, 1996). It is possible that the PAPs secreted to the extracellular space have dual functions, i.e., the hydrolytic activity under Pi starvation and microbial killing during pathogenesis. Hence, H_2O_2 generated in response to *Pst* DC3000 infection is necessary for activation of basal defense responses. Further, H_2O_2 secreted to the extracellular space and apoplast may restrict *Pst* DC3000 growth directly. Taken together, these evidences suggest that peroxisomal localized *PAP5* plays a vital role in basal defense response. Moreover, an optimal level of *PAP5* is critical for maintaining complete basal resistance during pathogenesis. It is evident that the isoform of PAP have evolved to attribute various biological functions in plants.

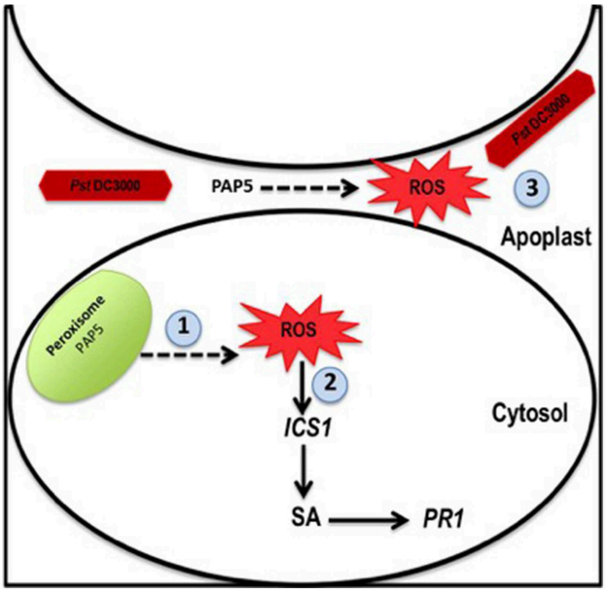

FIGURE 6 | Model for role of *PAP5* during *Pst* DC3000 infection. When plants are infected with virulent *Pst* DC3000 1. Peroxisomal localized *PAP5* may be required for activation of glycolate oxidase, which modulate H_2O_2 generation. 2. ROS induces several defense responsive genes including *PR1* and *ICS1*. 3. ROS secreted to the apoplast may directly affect *Pst* DC3000. Recognition of *Pst* DC3000, induce expression of *PAP5* only during the early stages of infection (6 h) and triggers ROS synthesis which subsequently activates other defense related signals down stream for complete resistance.

Materials and Methods

Biological Materials and Growth Conditions

Arabidopsis thaliana (L.) Heynh, ecotype Columbia (Col-0) seeds were purchased from Lehle seeds (Round Rock, TX, USA) and *pap5* T-DNA insertion mutant line was obtained from

Arabidopsis Biological Resource Center (Columbus, OH, USA). Stratified seeds were planted on Jiffy peat pellets (Halifax seeds, Canada) and seedling were grown at 22 ± 2°C with a photoperiod of 16 h light at 125 μmol $m^{-2}s^{-1}$ and 8 h dark cycle. Virulent *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000) was a kind gift from Dr. Diane Cuppels, Agriculture and Agri Food Canada (AAFC), ON, Canada. *Pst* DC3000 strains was maintained on King's medium B supplemented with rifampicin (50 μg ml^{-1}).

Pathogen Inoculation

For pathogenicity assay, 4–5 week old plants were spray inoculation with bacterial suspension of virulent *Pst* DC3000. Plant inoculation and bacterial growth in plant apoplast was determined as described earlier (Ravichandran et al., 2013). In brief, strains of *Pst* DC3000 was cultured in King's medium B supplemented with rifampicin (50 μg ml^{-1}) at 28°C until OD_{600} of 0.8. Bacterial cells were collected by centrifugation and resuspended in water containing 0.02% Silwet L-77 (Lehle seeds, USA) to a final concentration of 10^8 c.f.u ml^{-1}. Plants were spray inoculated and kept under high humidity for disease development. Leaves were excised (8-10 replicates) from different infected plants and were surface sterilized with ethanol (75% v/v). Four to five samples were made by pooling 2 leaf discs (0.5 cm^2) and the samples were ground in sterile water with microfuge tube pestle. The ground tissues were serially diluted and plated on King's B medium containing rifampicin (50 μg/ml). The plates were incubated at 28°C and colonies were counter after 48 h. For *Pst* DC3000 induced gene expression, plants were spray inoculated with bacterial suspension (10^8 c.f.u ml^{-1}) and leaf tissues were frozen in liquid nitrogen at the time points indicated.

RNA Extraction and Quantitative Real-time PCR

Total RNA was extracted from frozen leaf tissues (3 plants per replicate) for two biological replicates using a monophasic extraction method (Chomczynski and Sacchi, 1987). Total RNA was treated with DNase (Promega, WI, USA) and Reverse Transcription was performed with 1 μg of total RNA using High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, ON, Canada). Relative transcript levels were assayed by Real-Time PCR using gene specific primers (**Table 1**) on a StepOnePlus Real-Time PCR system (Applied Biosystems, ON, Canada), using SYBR Green reagent (Applied Biosystems, ON, Canada). To determine relative expression levels, the amount of target gene (three technical replicates/sample) was normalized over the abundance of constitutive *GAPDH* as endogenous controls. Primers were generated spanning an intron if possible using Primer3Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/).

Quantification of Salicylic Acid (SA) and Hydrogen Peroxide (H_2O_2)

For SA and JA quantification 4–5 week plants were spray inoculated with *Pst* DC3000 (10^8 c.f.u ml^{-1}). Leaves were excised (five plants per sample) in triplicates at 48 h.p.i and were snap frozen in liquid nitrogen. Leaf tissues (250 mg) were ground with liquid nitrogen and extracted with 5–10 ml of MeOH-H_2O-HOAc (90:9:1). After 15 min the samples were centrifuged at 12,000 g for 10 min and the supernatant was collected. The extraction procedure was repeated twice. The pooled supernatant was dried under steam of N_2 and suspended in 5 ml of 0.05% HOAc in H_2O-MeCN (85:15). The samples were then filtered through 0.4 5 μm filter and meanwhile the SampliQ SAX (Aligent technologies, USA) cartridge was conditioned with 2 ml of MeOH. The cartridge was then equilibrated with 5 ml of water. The filtered samples were loaded on to SampliQ SAX cartridge and were washed with 5 ml of 50 mM sodium acetate in 5% methanol. The interphase (IP) was removed with 5 ml of methanol. SA and JA were eluted with 5 ml of 2% formic acid in methanol and dried under N_2.

TABLE 1 | List of primer sequences used in RT-qPCR.

Gene	Locus	Primer sequences (5′–3′)
GAPDH	At1g13440	TTGGTGACAACAGGTCAAGCA AAACTTGTCGCTCAATGCAATC
ICS1	At1g74710	GCGTCGTTCGGTTACAGG ACAGCGAGGCTGAATATCAT
PAP5	At1g52940	AACAGGTCGCTCCACTAGACA TGGTTAGAGGCATATGTTTGTCC
PR1	At2g14610	TGATCCTCGTGGGAATTATGT TGCATGATCACATCATTACTTCAT

After optimization of the liquid chromatography conditions and the tuning of the Orbitrap mass spec for negative mode ionization, a dilution series of salicylic acid (SA) were injected. The detection was performed in negative mode monitoring for the exact mass of the pseudomolecular ion $[M\text{-}H]^-$ at 137 amu. The samples (500 μg/ml) were analyzed with the same optimized LC/MS method using a Agilent analytical C18 (3.5 μm, 2.1 × 100 mm) with a gradient elution from 5% ACN in water to 100% ACN using 0.1% formic acid in both solvents.

For H_2O_2 quantification, leaf tissues were harvested (4–5 plants per replicate) for 4 biological replicate and was frozen and ground in liquid nitrogen. To 50 mg of ground frozen tissue, 500 μl of phosphate buffer (50 mM, sodium phosphate, pH-7.4) was added. The samples were centrifuged and 50 μl of the aliquot was used for H_2O_2 quantification, using an Amplex red hydrogen peroxide/peroxidase assay kit (Molecular Probes, Life Technologies, Canada).

Cloning and Plant Transformation

The clone of interest was obtained from ABRC and cloned to gateway compatible expression vectors (Earley et al., 2006) using LR Clonase II Gateway Technology (Invitrogen, ON, Canada). Briefly, the clone DQ459170, containing full length *PAP5* (At1g52940) cDNA was obtained from ABRC. The full length cDNA was amplified without the stop codon via polymerase chain reaction (PCR) using TaKaRA Ex Taq® Polymerase (Clontech, USA). The PCR primers were designed to contain *att*B sites to enable Gateway® technology compatible cloning (Gateway® Technology, Life Technologies, Canada). Shine-Dalgarno and Kozak consensus sequences were included between the *att*B1 site and the start codon to allow protein expression in *E. coli* and mammalian cells. The fusion constructs lacking either N/C-terminal of *PAP5* was

generated via PCR. The *PAP5* gene lacking N-terminal amino acids MSLETFPPPA (*YFP:+30PAP5*) was generated using primers 5′-GGTTATAACGCTCCTGAACAAGTT-3′ (forward) and 5′-GGTTATAACGCTCCTGAACAAGTT-3′ (reverse). *PAP5* gene lacking C-terminal amino acids RYYLPEEETI was amplified using primers (*-30PAP5:YFP*) using primers 5′-ATGTCACTCGAAACATTTCCTC-3′ (forward) and 5′-ATTTTTCAACCAAATAGAGTCTGCA-3′. The PCR product was purified and introduced to pDONR™ 221 vector as per manufactures instruction to generate entry clones (Gateway® Technology, Life Technologies, Canada). The recombinant plasmids were sequenced using the M13 sequencing primers to confirm the insert position and orientation.

The entry clone containing the full length *PAP5* or was introduced to the expression vector pEarleyGate 104 (Earley et al., 2006) and pMDC32 (Curtis and Grossniklaus, 2003) using LR clonase (Gateway® Technology, Life Technologies) to generate *35S:YFP-PAP5* and 35S:*PAP5* fusion constructs, respectively. Similarly, the fusion constructs lacking either N/C-terminal of *PAP5* was introduced to the expression vector pEarleyGate 104. The recombinant plasmids were introduced in to *Agrobacterium* strain GV310 (pMB90) using the freeze thaw method (Weigel and Glazebrook, 2005). The *Agrobacterium* strain carrying the fusion construct was used to transform *Arabidopsis* plants by floral dip method (Clough and Bent, 1998) or infiltrated in to tobacco plants for subcellular localization studies (Sparkes et al., 2006). The floral dip inoculation medium contained 0.5X Murashige and Skoog medium with 5.0% sucrose and 0.05% Silwet (Lehle seeds, TX, USA). Plants were selected for Hygromycin and Basta resistance for pMDC and pEarley vectors, respectively.

Transient Protein Expression and Subcellular Localization

For subcellular localization 6 week old *Nicotiana benthamiana* (tobacco) plants were used. Tobacco plants were grown at 22 ± 2°C with a photoperiod of 16 h light at 125 μmol $m^{-2}s^{-1}$ and 8 h dark cycle. Plant organelle specific markers were obtained from ABRC (Nelson et al., 2007) and the plasmids were transformed to *Agrobacterium* strain GV310 (pMB90) using the freeze thaw method (Weigel and Glazebrook, 2005). Agrobacterium strains carrying the recombinant plasmids were grown in liquid Luria-Bertani (LB) media supplemented with appropriate antibiotics. Cells were harvested by centrifugation (5500 g for 10 min) and resuspended in infiltration medium to OD_{600} of 0.8. The infiltration medium contained 0.5% glucose, 50 mM MES, 2 mM Na_3PO_4, 0.1 mM acetosyringone (Sparkes et al., 2006). For co-expression of different constructs *Agrobacterium* suspension was mixed in equal proportion and the *Agrobacterium* suspension mixtures were infiltrated to the tobacco leaves using a needleless syringe. The leaf samples were excised 48 h after infiltration and mounted on a microscope slide in water. The images were obtained (single optical sections and Z-stack) using a Zeiss LSM 510 META inverted confocal laser scanning microscope (Carl Zeiss MicroImaging GmbH). For CFP fluorescence, excitation wavelength of 458 nm was used and emissions were collected between 475, 471 and 525 nm. For YFP fluorescence, an excitation wavelength of 514 nm was used and emissions were 472 collected between 530 and 600 nm. The fluorescence images were processed using Zeiss LSM Image Browser (Carl Zeiss MicroImaging GmbH).

Acknowledgments

We would like to thank Dr. Hongxia Liu (Department of Biology, Dalhousie University) for her help and advice with cloning and confocal imaging experiments. We also thank Ms. Margie Tate (Faculty of Agriculture, Dalhousie University) for her assistance with SA quantification. This work was supported by Natural Science and Engineering Research Council of Canada Discovery grant to BP.

Supplementary Material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2015.00568

Figure S1 | Subcellular localization of *PAP5* in *N. benthamiana* leaves. Agrobacterium strains carrying the recombinant plasmids (*35S:YFP-PAP5*) and Golgi or plastid specific markers were transiently expressed in tobacco leaves. Left plane shows a single optical section of CFP fluorescence images, middle planes show a single optical section of YFP fluorescence and right planes shows transmitted light images with merged CFP and YFP fluorescence. Bar = 25 μm.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

REVIEW
published: 14 September 2015
doi: 10.3389/fpls.2015.00700

Rhizosphere ecology of lumichrome and riboflavin, two bacterial signal molecules eliciting developmental changes in plants

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OPEN ACCESS

Edited by:
Valerie Gravel, McGill University, Canada

Reviewed by:
Yusuke Saijo, Max Planck Institute for Plant Breeding Research, Germany
Jens Kossmann, Stellenbosch University, South Africa

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Specialty section:
This article was submitted to Plant Biotic Interactions, a section of the journal Frontiers in Plant Science

Received: *02 April 2015*
Accepted: *21 August 2015*
Published: *14 September 2015*

Citation:
Dakora FD, Matiru VN and Kanu AS (2015) Rhizosphere ecology of lumichrome and riboflavin, two bacterial signal molecules eliciting developmental changes in plants. Front. Plant Sci. 6:700. doi: 10.3389/fpls.2015.00700

Lumichrome and riboflavin are novel molecules from rhizobial exudates that stimulate plant growth. Reported studies have revealed major developmental changes elicited by lumichrome at very low nanomolar concentrations (5 nM) in plants, which include early initiation of trifoliate leaves, expansion of unifoliate and trifoliate leaves, increased stem elongation and leaf area, and consequently greater biomass accumulation in monocots and dicots. But higher lumichrome concentration (50 nM) depressed root development and reduced growth of unifoliate and second trifoliate leaves. While the mechanisms remain unknown, it is possible that lumichrome released by rhizobia induced the biosynthesis of classical phytohormones that caused the observed developmental changes in plants. We also showed in earlier studies that applying either 10 nM lumichrome, 10 nM ABA, or 10 ml of infective rhizobial cells (0.2 OD_{600}) to roots of monocots and dicots for 44 h produced identical effects, which included decreased stomatal conductance and leaf transpiration in Bambara groundnut, soybean, and maize, increased stomatal conductance and transpiration in cowpea and lupin, and elevated root respiration in maize (19% by rhizobia and 20% by lumichrome). Greater extracellular exudation of lumichrome, riboflavin and indole acetic acid by N_2-fixing rhizobia over non-fixing bacteria is perceived to be an indication of their role as symbiotic signals. This is evidenced by the increased concentration of lumichrome and riboflavin in the xylem sap of cowpea and soybean plants inoculated with infective rhizobia. In fact, greater xylem concentration of lumichrome in soybean and its correspondingly increased accumulation in leaves was found to result in dramatic developmental changes than in cowpea. Furthermore, lumichrome and riboflavin secreted by soil rhizobia are also known to function as (i) ecological cues for sensing environmental stress, (ii) growth factors for microbes, plants, and humans, (iii) signals for stomatal functioning in land plants, and (iv) protectants/elicitors of plant defense. The fact that exogenous application of ABA to plant roots caused the same effect as lumichrome on leaf stomatal functioning suggests molecular cross-talk in plant response to environmental stimuli.

Keywords: plant growth promoting molecules, IAA, ABA, rhizosphere, rhizobial exudates

Introduction

In nature, plants and soil microbes seem to have co-evolved to overcome environmental stress in their habitats. Outside pathogenesis and allelopathy, many plant–plant or plant–bacterial interactions have tended to be facilitative in providing benefits to both partners (He et al., 2013). Thus, the rhizosphere is generally regarded as the hotspot of interactive events between soil microbes and plants, which occur through perception of signals released in the form of simple chemical molecules. In nutrient-poor soils, a typical rhizosphere consists of mixtures of molecules secreted by both plants and microbes for promoting nutrient mobilization and increased mineral uptake (Marschner, 1995; Dakora and Phillips, 2002). Under Fe-limiting conditions, bacterial species can secrete specialized compounds such as siderophores to enhance Fe acquisition (Jurkevitch et al., 1986). In times of abiotic stress such as drought, soil microbes, including rhizobia and other diazotrophs, produce chemical molecules in their exudates that effect changes in plant development. In an exhaustive review, Mehboob et al. (2009) found that applying 29 different rhizobial species/strains to 11 non-legume crops led to an increase in plant growth, plant height and plant biomass, as well as greater tissue concentration of N, P, K, Ca, Mg, Na, Fe, Zn, and Cu in plant organs. The growth-promoting molecules released by the test rhizobia included indole acetic acid (IAA) by 13 strains, gibberellins by four strains, exopolysaccharides by three strains, followed by lipopolysaccharides, hydrocyanic acid, abscisic acid (ABA), phenolics and lumichrome by one strain each. This review assesses lumichrome and riboflavin, and to some extent IAA, as rhizobial signals influencing plant growth, and discusses their roles in the rhizosphere of monocots (cereals) and dicots (legumes) in relation to plant growth and mineral nutrition.

Molecular Signals From Rhizobial Exudates and Their Effects on Plant Growth

Species and strains of rhizobia are reported to synthesize various metabolites for bacterial cell growth. These include the vitamins thiamine, niacin, biotin, ascorbic acid, and pantothenic acid, as well as the amino acids glutamate, lysine, arginine, tryptophan, and methionine purified from culture filtrates of *Sinorhizobium meliloti*, *Rhizobium leguminosarum* bv. *viceae*, *Azospirillum brasilense*, *Azotobacter vinelandii*, and *Pseudomonas fluorescens* (Rodelas et al., 1993; Sierra et al., 1999; Yang et al., 2002). In addition to IAA, simple nitrogenous molecules such as cytokinins, gibberellins, lumichrome, and riboflavin (**Figure 1**) have also been purified from bacterial culture filtrates and proven to be active signals controlling plant development. Gibberellins and cytokinins isolated from symbiotic rhizobia (Phillips and Torrey, 1970, 1972; Dart, 1974; Lynch and Clark, 1984) are known to promote bacterial cell growth, as well as stimulate root hair production in plants for increased uptake of water and mineral nutrients (Yanni et al., 2001).

In addition to traditional bacterial hormones such as gibberellins, cytokinins and IAA, lipo-chitooligosaccharide molecules (**Figure 1**) represent a new group of biologically-active compounds that stimulate cell growth and induce nodule organogenesis (De Jong et al., 1993; Dyachok et al., 2000). In the absence of rhizobial cells, purified Nod factors can morphogenically elicit nodule formation in legumes (Dénarié et al., 1996). Furthermore, exogenously applied rhizobial Nod factors have been reported to stimulate seed germination (Zhang and Smith, 2002) and promote seedling development in both monocots and dicots (Smith et al., 2002). Applying Nod factors (10^{-7} M or 10^{-9} M) to soybean plants increased root mass by 7–16%, and root length by 34–44% (Smith et al., 2002). Similarly, spraying sub-micromolar concentrations (10^{-6}, 10^{-8}, or 10^{-10} M) of Nod factors on leaves of soybean, common bean, maize, rice, canola, apple, and grape plants increased photosynthetic rates by 10–20%, and caused a 40% increase in grain yield of field-grown soybean (Smith et al., 2002). But more importantly, Nod factors also induce the expression of genes involved in the phenylpropanoid pathway (Savouré et al., 1994; Spaink and Lugtenberg, 1994), and in so doing increase phytoalexin biosynthesis, which has the potential to protect the host plant against pathogens (Dakora and Phillips, 1996). It has also been shown that, even at low concentrations (10^{-7} nM), Nod factors can promote AM colonization of both nodulating and non-nodulating plants (Xie et al., 1995), suggesting a role for this rhizobial metabolite in the establishment of mycorrhizal symbiosis (Parniske, 2008). In fact, it has now been shown that after the initial Nod factor and Myc factor perception, both nodulation and mychorrhization processes share a common symbiotic pathway (Parniske, 2008; Maillet et al., 2011).

Although lumichrome is considered a novel molecule that stimulates plant development (Phillips et al., 1999; Matiru and Dakora, 2005a), the discovery that rhizobia are capable of synthesizing riboflavin (a precursor of lumichrome biosynthesis) and thiamine for cell growth occurred almost 80 years ago (West and Wilson, 1938). Carpenter (1943) later isolated riboflavin from field soil and showed its uptake by plant roots and translocation to shoots. However, the role of this molecule in plant growth stimulation was only reported thirty five years later (Rao, 1973). Today, the findings of new studies have shown that lumichrome and riboflavin purified from rhizobial exudates can promote plant growth and alter stomatal function (Phillips et al., 1999; Matiru and Dakora, 2005a,b). What was however unclear is whether the exudation of lumichrome and riboflavin is unique to N_2-fixing rhizobia.

Lumichrome and Riboflavin are Symbiotic Signals Involved in Plant Development

Lumichrome is a molecule commonly synthesized by both microbes and plants, but is also a known degradation product of the vitamin riboflavin (Phillips et al., 1999). As a result, the role of lumichrome is often linked with riboflavin as the latter is easily converted enzymatically or photochemically into lumichrome (Yanagita and Foster, 1956; Yagi, 1962). Applying purified lumichrome from *S. meliloti* exudates to roots of alfalfa seedlings increased root respiration by 11–30%, and promoted plant growth by 8–18% (Phillips et al., 1999). The enhanced plant growth was attributed to increased net C assimilation, possibly via PEP carboxylase activity (Phillips et al., 1999). Later studies

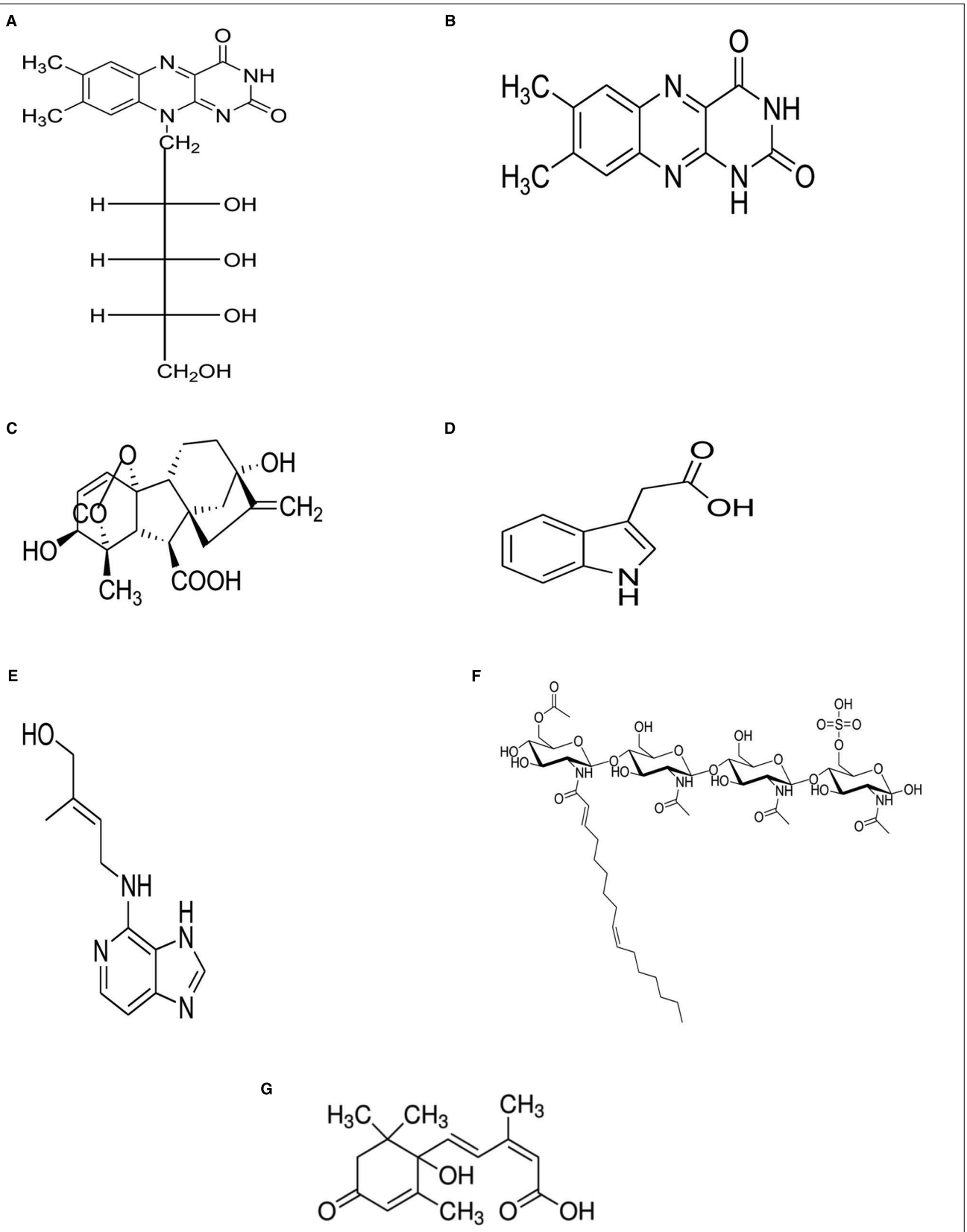

FIGURE 1 | Structures of selected rhizobial molecules functioning as plant growth promoters. (A) Riboflavin, **(B)** Lumichrome, **(C)** Indole acetic acid, **(D)** Gibberelline, **(E)** Cytokinins, **(F)** Nod factors, and **(G)** Abscisic acid.

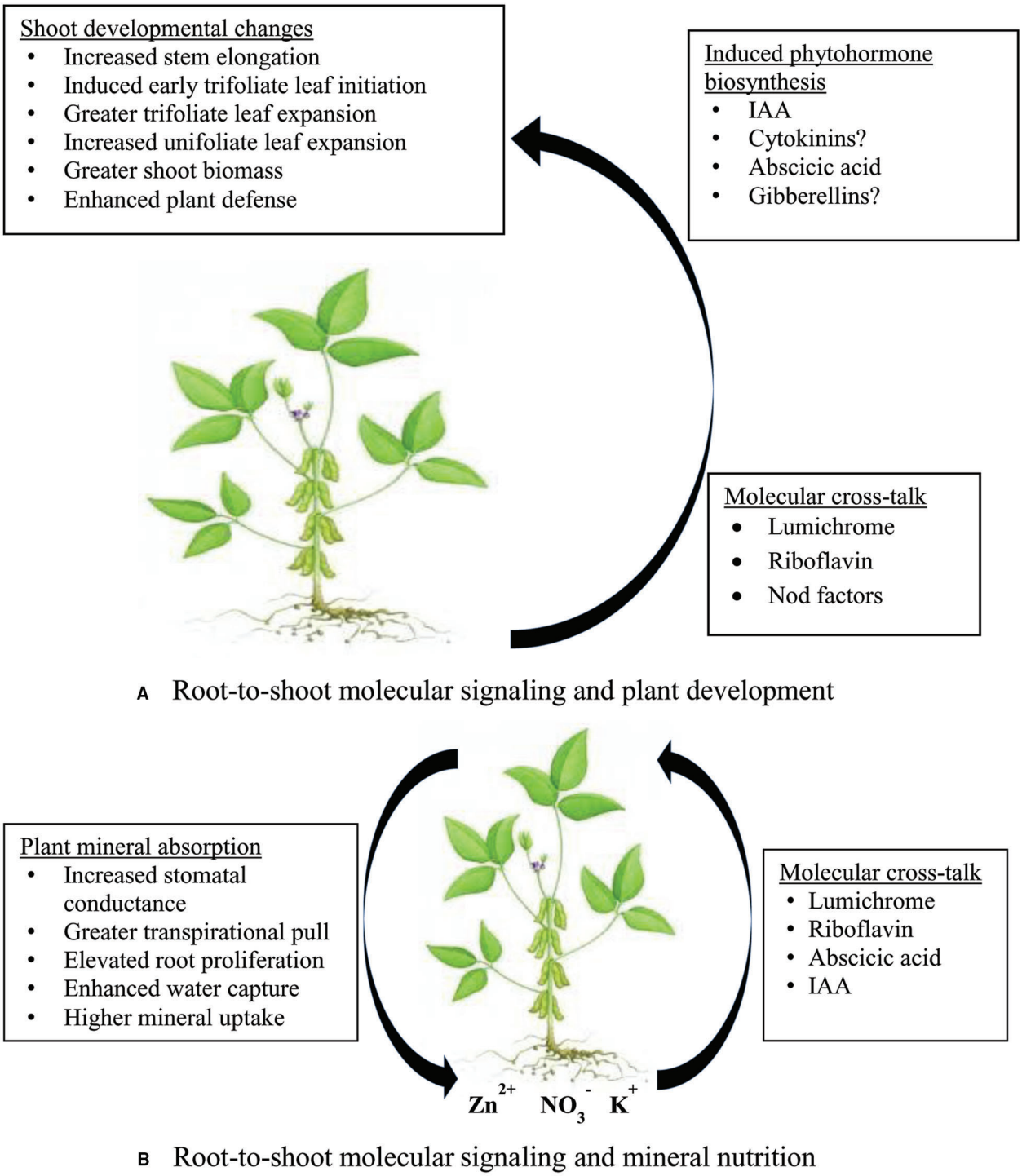

FIGURE 2 | A model describing the effect of root-to-shoot signaling by rhizobial molecules on (A) shoot developmental changes, and (B) symbiosis-induced mineral uptake in the rhizosphere.

have shown that plants exhibit a mixed response to lumichrome and riboflavin application (see **Figure 2A**). While this molecule significantly increased root respiration in maize plants (Phillips et al., 1999; Matiru and Dakora, 2005b), it decreased it in lupin, and had no affect on cowpea, soybean, Bambara groundnut, pea, and sorghum (Matiru and Dakora, 2005b). Inoculating the roots of these monocots and dicots with ineffective rhizobial cells produced the same results as obtained with lumichrome application, in that, maize showed significantly increased rate of root respiration, and lupin a decreased rate, while cowpea,

soybean, Bambara groundnut, pea, and sorghum were unaffected in their root respiration (Matiru and Dakora, 2005b). These responses by both monocots and dicots to rhizobia and purified lumichrome clearly indicate that the observed changes in root respiration with bacterial inoculation were caused by lumichrome released by the applied rhizobia. Other studies have similarly found increased root respiration and dry matter accumulation following lumichrome supply to lotus and tomato (Gouws et al., 2012). Furthermore, both lumichrome and riboflavin have been implicated as quorum-sensing molecules in rhizobial bacteria (Rajamani et al., 2008). But the independent role of riboflavin as a signal molecule was underscored by the finding that *S. meliloti* strains carrying extra copies of the riboflavin biosynthesis gene *ribBA* could release 15% more riboflavin than wild-type, and were 55% more efficient in alfalfa root colonization for nodule formation (Yang et al., 2002).

Physiologically, lumichrome has been shown to influence plant growth, but with differing effects depending on the plant species and metabolite concentration. Treating the roots of cowpea, Bambara groundnut, soybean, pea, lupin, sorghum, and maize plants with 10 nM purified lumichrome and 10 mL of infective rhizobial cells (0.2 OD_{600}) for 44 h in growth chambers, increased stomatal conductance and leaf transpiration rates in cowpea, but decreased both parameters in Bambara groundnut, soybean, and maize, and had no effect on them in pea and sorghum (Matiru and Dakora, 2005b). In that study, the effect of bacterial inoculation closely mirrored that of 10 nM lumichrome application, again indicating that rhizobial effects on these physiological changes including stomatal functioning (whether in nature or under experimental conditions) were more likely due to the lumichrome molecule released by symbiotic rhizobia in the rhizosphere. Thus, the finding that rhizobial inoculation in the field alleviated the effects of water stress in symbiotic legumes (Figueiredo et al., 1999) could be attributed to strain secretion of lumichrome that decreased stomatal conductance and reduced plant water loss. More studies are needed to explore matching superior N_2-fixing ability in inoculant rhizobia with high lumichrome production as insurance for increased water-use efficiency and drought tolerance in food legumes.

Developmentally, the supply of 5 nM lumichrome to roots of cowpea and soybean seedlings elicited early initiation of trifoliate leaf development, expansion in unifoliate and trifoliate leaves, and increased stem elongation, which together caused an increase in shoot and plant total biomass relative to the control (Matiru and Dakora, 2005a). Even with monocots such as maize and sorghum, lumichrome application at 5 nM also induced leaf area expansion, and thus increased shoot and total biomass, but had no effect on the leaf area of some cereals. Similar plant growth data were also obtained with lumichrome supply to lotus and tomato (Gouws et al., 2012). Other developmental changes observed included an increase in root growth in sorghum, millet, lotus and tomato caused by the supply of 5 nM lumichrome to seedlings of these species (Matiru and Dakora, 2005a; Gouws et al., 2012). Higher doses of lumichrome at 50 nM however depressed the development of unifoliate leaves in soybean, the second trifoliate leaf in cowpea, and shoot biomass in soybean. Furthermore, the 50 nM concentration also consistently decreased root development in cowpea and millet, but had no effect on the other species (Matiru and Dakora, 2005a). These findings also showed that the developmental effect of lumichrome on plant species was not age-specific as growth of both 11- and 37-day-old sorghum, 23- and 37-day-old soybean, 23- and 37-day-old millet, as well as 11- and 37-day-old cowpea were significantly increased by lumichrome supply at 5 nM concentrations. Unlike the legumes, however, the supply of 5 nM lumichrome markedly increased ($P < 0.05$) root growth in cereals such as sorghum and millet (Matiru and Dakora, 2005a). From these results, lumichrome is no doubt a rhizosphere signal molecule that affects seedling development in both monocots and dicots. It is likely that, in nature, lumichrome released by symbiotic rhizobia into the rhizosphere dictate the developmental path of plant species than is currently known, with potential for greater plant growth from increased water/mineral uptake and/or drought tolerance.

At the metabolic level, shoot and root application of lumichrome increased starch accumulation in roots of both lotus and tomato, which suggests a role for lumichrome in carbon partitioning and modulation of carbon fluxes in infected symbiotic plant cells (Gouws et al., 2012). This argument is re-inforced by the fact that lotus-treated roots showed a reduction in carbonaceous and nitrogenous solutes such as organic acids and amino acids. Root treatment with lumichrome also increased ethylene evolution rates in lotus, but not in tomato (Gouws et al., 2012). Taken together, these findings show that bacteria are capable of producing various simple organic molecules that serve as environmental cues in altering plant development. With the discovery of more active novel bacterial metabolites, it has become clear that besides the classical phytohormones such as auxins, cytokinins, gibberellins and abscisic acid, additional signal molecules exist that influence plant development. Although Phillips et al. (1999) attributed the enhanced plant growth from lumichrome application to increased net C assimilation via PEP carboxylase activity, the marked developmental changes (dramatic expansion in unifoliate and trifoliate leaves, and the increased stem elongation) observed with lumichrome application to cowpea and soybean would seem to suggest that this molecule stimulates plant growth via cell division and cell expansion, as happens with classical phytohormones (Mansfield, 1978; Ross et al., 2001; Campanoni et al., 2003; van der Graaff et al., 2003). In fact, it is our view that both lumichrome and riboflavin caused the developmental changes in plants by inducing the synthesis of classical phytohormones, which then modulate plant growth. However, experimental data are needed to support this claim.

Agronomic Benefits of Lumichrome and Riboflavin

The increase in stem elongation, early initiation and rapid expansion of trifoliate leaves with the provision of 5 nM lumichrome to cowpea and soybean plants resulted in a twofold accumulation of dry matter in trifoliate leaves relative to 0-lumichrome control (Matiru and Dakora, 2005a). Lumichrome could also stimulate seedling development in monocots such as millet, sorghum and maize, in addition to legumes. As a result,

whole-plant dry matter yield of these cereal species receiving 5 nM lumichrome was greater compared to control (Matiru and Dakora, 2005a). Root growth was also much greater in cereals (especially millet and sorghum) than legumes, suggesting that in the former, lumichrome application altered assimilate partitioning in favor of root development. Gouws et al. (2012) also observed an increase in dry matter accumulation following lumichrome application to lotus and tomato. The observed promotion in plant growth by lumichrome in both monocots and dicots suggests that, in addition to tapping symbiotic N contributed in cropping systems, cereals can also benefit from growth stimulation by lumichrome released by N_2-fixing rhizobia in the soil. Its growth-promoting effect on both monocots and dicots further suggests that lumichrome is capable of influencing plant rhizospheres in both natural and agricultural ecosystems.

Foliar application of lumichrome at 10^{-6} M concentration significantly increased shoot and total dry matter yield of field-grown soybean plants (Khan et al., 2008). The increased accumulation of dry matter was partly due to a marked increase in leaf area with lumichrome supply (Khan et al., 2008). As found with cowpea and soybean, the observed increase in plant growth (Matiru and Dakora, 2005b) and Fe uptake (Matiru and Dakora, 2004) following sorghum inoculation with infective rhizobia could be attributed to lumichrome secreted by the introduced bacterial cells. As an agronomic practice, lumichrome supply with rhizobial inoculants therefore has the potential to increase crop yields in agricultural systems.

Effect of N and P Nutrition on Rhizobial Exudation of Lumichrome, Riboflavin and IAA

There are a number of factors affecting the production and release of metabolites by soil bacteria. For example, the synthesis and extracellular release of lumichrome, riboflavin and IAA by rhizobia was found to differ between and among bacterial species and strains (Kanu et al., 2007). In some studies, there was generally greater production of lumichrome, riboflavin and IAA by N_2-fixing bacteria than those unable to nodulate legumes such as *Psoralea pinnata* and sirato (Shokri and Emtiazi, 2010; Kanu and Dakora, 2012), a finding consistent with their role in symbiotic N_2 fixation (Phillips et al., 1999; Lambrecht et al., 2000; Matiru and Dakora, 2005a,b; Gouws et al., 2012). In fact, Kanu and Dakora (2012) found that strain TUT57pp, which was effective in N_2 fixation, produced 2.2-fold and 3.2-fold more IAA than the non-nodulating isolates TUT65prp and TUT33pap, respectively. Furthermore, studies on the effect of lumichrome on N and P nutrition in rhizobial isolates showed that N_2-fixing strain TUT57pp consistently produced significantly more lumichrome, riboflavin and IAA than its non-nodulating counterpart TUT61pp (Kanu and Dakora, 2009, 2012). These results provide further evidence that the three molecules (lumichrome, riboflavin and IAA) are indeed rhizobial symbiotic signals.

Although we know the effect of N and P nutrition on Nod factor production in symbiotic rhizobia (McKay and Djordjevic, 1993), little information currently exists on the effects of these mineral nutrients on the biosynthesis of other symbiotically-important metabolites such as lumichrome, riboflavin and IAA. There are reports of marked variation in the secretion of lumichrome, riboflavin and IAA by symbiotic rhizobia compared to their non-nodulating bacterial counterparts (Shokri and Emtiazi, 2010; Kanu and Dakora, 2012). Kanu and Dakora (2012) measured much greater concentrations of lumichrome and riboflavin in the culture filtrate of five N_2-fixing and 11 non-nodulating bacterial strains grown at high P (5.7 mM) than at low P (1.4 mM). The five N_2-fixing isolates also differed in their levels of extracellular secretion of lumichrome, with TUT23prt releasing the most lumichrome at both low P and high P, and TUT18pac the least. Strain TUT23prt would therefore seem to be more adaptable to environments with a wide range of P concentrations, a trait very useful for selecting food legumes for P tolerance. The subtle differences in strain adaptation found between TUT23prt and TUT18pac point to why some legume/rhizobial symbioses perform well across environments with varying nutrient regimes, and hence in the case of P, the commonly encountered low-P tolerant and low-P sensitive symbioses.

Ammonium nutrition (whether at 28.1 mM or 112.0 mM NH_4^+) had no effect on the biosynthesis and release of riboflavin by rhizobia (Kanu and Dakora, 2012), a finding consistent with the reported lack of response of Nod factor secretion to ammonium supply (McKay and Djordjevic, 1993). However, lumichrome production was markedly affected by ammonium nutrition (Kanu and Dakora, 2012). While some strains produced more, or less, lumichrome with ammonium supply, strains TUT23prt and TUT33pap produced significantly large amounts of lumichrome at both low and high ammonium concentrations (Kanu and Dakora, 2012), a trait that could contribute to the strains' tolerance of high soil N. The level of lumichrome and riboflavin production by the test isolates from *Psoralea* species also differed with nitrate nutrition. Feeding these strains with 59.3 mM nitrate resulted in significantly decreased concentration of lumichrome and riboflavin in bacterial exudates (Kanu and Dakora, 2012), indicating an inhibitory effect of nitrate on the biosynthesis and extracellular release of the two metabolites by rhizobial bacteria. In fact, the levels of lumichrome in culture filtrate were decreased by high nitrate concentration for all the isolates. A similar decrease in Nod factor production was observed by McKay and Djordjevic (1993), following nitrate supply to *Rhizobium leguminosarum* bv. *trifolii*. It was interesting to note that, in the study by Kanu and Dakora (2012), the isolates which showed the least production of riboflavin at high nitrate (e.g., TUT10pm and TUT13pac), were also among the least in lumichrome production at high nitrate. More importantly, however, the observed inhibition of lumichrome and riboflavin biosynthesis and release by nitrate is in addition to its known depressive effect on nodulation and N_2 fixation in symbiotic legumes (Streeter and Wong, 1988; Ayisi et al., 2000). In nature, soil nitrate at high concentrations is therefore likely to inhibit nodulation in legumes via its repressive effect on the synthesis and secretion of lumichrome and riboflavin by rhizobia, given the fact that the former was found to increase nodulation in lotus plants (Gouws et al., 2012).

Effect of Rhizobial Strain, Temperature, Salinity, and pH on Bacterial Secretion of Lumichrome and Riboflavin

Metabolic adaptation plays a major role in the survival of legumes and their microsymbionts in harsh environments such as the nutrient-poor, acidic, dry and water-deficient soils of the Cape fynbos in South Africa. Kanu and Dakora (2009) found that bacterial isolates from *Psoralea* nodules collected from the fynbos differed in their levels of secretion of lumichrome, riboflavin and IAA, as well as in their exudation response to pH, salinity and temperature. For example, while isolate AS2 from *Psoralea* nodules could produce greater amounts of lumichrome at both pH 5.1 and 8.1, strains RT1 and P1 secreted more lumichrome per cell at only pH 8.1. Strains AP1 and RP2 were also found to produce more riboflavin at pH 8.1 than pH 5.1, while strain RT1 produced greater amounts of riboflavin at pH 8.1 than pH 5.1. Taken together, the estimated levels of lumichrome and riboflavin secreted by *Psoralea* bacterial isolates ranged from 0.1 to 15 nM (Kanu and Dakora, 2012). These variations in the concentration of lumichrome and riboflavin released by *Psoralea* isolates is consistent with the findings of earlier studies which showed significantly greater production of riboflavin by *Bradyrhizobium japonicum* Tal 110, *S. meliloti* RAKI and *Sinorhizobium fredii* 6217 relative to eleven other standard laboratory strains (Kanu et al., 2007). In contrast, *Rhizobium leguminosarum* bv. *viceae* 30, *Bradyrhizobium* CB756, and *Sinorhizobium arboris* lma 14919 exhibited the lowest production of lumichrome in culture filtrate when compared to the other test strains (Kanu et al., 2007).

As a further evidence of metabolic adaptation, two *P. repens* strains (RP1 and RP2) isolated from a very saline environment close to the Atlantic Ocean secreted large amounts of lumichrome and riboflavin at both low and high salinity levels (Kanu and Dakora, 2009). Although the concentration of IAA produced by *Psoralea* isolates was greater at high acidity and high temperatures, lumichrome production was more elevated at lower (10°C) than higher (30°C) temperature (Kanu and Dakora, 2009). The greater production of lumichrome at 10°C than 30°C was not surprising as Nod factors produced by *Bradyrhizobium aspalati* (now *Burkholderia tuberum*) isolated from *Aspalathus canosa* in the Cape fynbos was also greater at 12°C than 28°C (Boone et al., 1999). This can be explained by the fact that legume nodulation in the Mediterranean Cape region of South Africa occurs during the winter rains when temperatures are low, around 10–15°C. Thus, the biosynthesis and release of symbiotic signals such as Nod factors by rhizobia and flavonoid *nod* gene-inducers by the Cape legumes are likely to be metabolically more adapted to the lower (10°C) than higher (30°C) rhizosphere temperatures. As found with the biosynthetic response of lumichrome to salinity in the salt-tolerant *P. repens* from the Western Cape, legumes and their microsymbionts are generally metabolically-adapted to the environmental factors of their niches.

The observed variation in the secretion of lumichrome, riboflavin and IAA by bacterial isolates from *Psoralea* root nodules exposed to different pH, salinity and temperature regimes, or fed different levels of N (nitrate and ammonium) and P, was due to alteration in the number of bacterial cells. For example, the number of rhizobia measured as colony forming units (CFU) ranged from 0.91 to 121.48 $\times$ 10^7 cfu mL^{-1} at pH 5.1 and from 0.69 to 214.05 $\times$ 10^7 cfu mL^{-1} at pH 8.1 (Kanu and Dakora, 2009, 2012). This suggests that the genes encoding these metabolites are regulated differently by the imposed environmental factors. Furthermore, our findings indicate that natural changes in pH, salinity and/or temperature in plant rhizospheres could potentially elevate the concentrations of lumichrome, riboflavin and IAA in soils, with consequences for ecosystem functioning as both lumichrome and riboflavin (being vitamins) act as growth factors and developmental signals in plants, microbes and humans.

Riboflavin as a Defense Molecule in Plants

Besides riboflavin and lumichrome, bacteria and plants produce other vitamins such as thiamine, biotin, niacin and ascorbic acid for their growth and cellular functioning. Recent studies have however revealed a new role for these vitamins in plant–microbe interactions, one being protection against pathogens (Mehboob et al., 2009; Palacios et al., 2014). It has been shown, for example, that spraying riboflavin (0.1 up to 10 mM concentration) on tobacco or *Arabidopsis* leaves caused resistance to *Peronospora parasitica*, *Pseudomonas syringae* pv. *Tomato*, Tobacco mosaic virus (TMV), and *Alternaria alternata* (Dong and Beer, 2000). In that study, riboflavin was found to induce expression of pathogenesis-related genes, leading to systemic acquired resistance to pathogens without the involvement of salicylic acid (Dong and Beer, 2000; Zhang et al., 2009; Liu et al., 2011).

In addition to its requirement as a growth factor for microbes, plants and humans, thiamine (vitamin B1) has also been reported to function as a defense molecule in inducing systemic acquired resistance in the plant kingdom (Ahn et al., 2005). This discovery that vitamins protect plants from pathogens has led to the suggestion that most root-colonizing, non-pathogenic, biocontrol bacteria probably elicit systemic acquired resistance in plants that is independent of the salicylic acid signaling pathway (Van Wees et al., 1997). Clearly, vitamins (especially riboflavin and its degradation product lumichrome) produced by microbes in the rhizosphere are probably the major elicitors of plant defense against soil-borne pathogens in the real world.

For example, rhizosphere microbes such as the fungus *Ashbya gossypii*, which overproduces riboflavin (Lim et al., 2001) probably provides a blanket protection to plants from its copious production and release of this molecule into the rhizosphere. But given the commonly known role of isoflavonoid phytoalexins and phytoanticipins in plant defense (Dakora and Phillips, 1996), it is likely that the overall health of a legume is dependent on molecular cross-talk involving the total defense appertoire of isoflavones, anthocyanins, riboflavin, thiamine and other yet unknown molecules. Whatever the case, we now know that vitamins such as riboflavin produced by rhizobia and other microbes have multiple functions, which include serving as (i) growth factors for microbes, plants and humans, (ii) signals for stomatal functioning in land plants, and (iii) protectants/elicitors in plant defense. So far, however, no study has found a role for lumichrome in plant defense.

Is Mineral Nutrition in Nodulated Legumes Controlled by Multiple Symbiotic Signals via Molecular Cross-Talk?

One major finding from rhizobial interaction with monocot and dicot plant species is the effect of lumichrome on stomatal functioning. Matiru and Dakora (2005b) showed that applying 10 nM purified lumichrome, 10 nM ABA, or 10 ml of infective rhizobial cells at 0.2 OD_{600} to cowpea and lupin increased stomatal conductance and transpiration rates, but decreased them in soybean, Bambara groundnut and maize, and showed no effect in pea and sorghum. The decrease in stomatal conductance and transpiration with lumichrome supply to maize, soybean and Bambara groundnut closely mirrors the reduced stomatal conductance and leaf transpiration rates caused by elevated CO_2 in C3 plant species (40.5 and 3.6%, respectively, in soybean; see Madhu and Hatfield, 2014). In one study, the decrease in stomatal conductance and transpiration rates with elevated CO_2 led to reduced mineral ^{15}N uptake (Kanemoto et al., 2009), just as the reduced stomatal conductance, and hence lower transpirational pull in test legumes exposed to elevated CO_2 also resulted in significantly decreased uptake of Mg, Fe, Cu, and B (Duval et al., 2012). In contrast, where there was an increase in stomatal conductance and leaf transpiration, mineral nutrient uptake was also increased in roots. For example, Tani and Barrington (2005) reported an increase in the uptake of Cu and Zn by wheat from high transpiration rates, following irrigation, while Novák and Vidovic (2003) also found a direct relationship between N, P, and K uptake and leaf transpiration rates. Taken together, those findings indicate that soil mineral acquisition by plant roots is directly linked to leaf transpiration rates, stomatal conductance, and the water status of the rhizosphere. The parallel drawn here between the effect of elevated CO_2 and lumichrome on stomatal functioning is that a decrease in stomatal conductance from elevated CO_2 causes reduced transpiration rates and decreased nutrient uptake, while an increase in stomatal conductance from lumichrome supply elevates the transpirational pull and promotes mineral uptake and transport in the xylem stream. These findings clearly suggest that the uptake of mineral nutrients and their accumulation in plants is controlled by stomatal functioning, and hence by the factors that modulate stomatal opening and closure.

Plant water and nutrient relations are thus intimately linked to stomatal functioning, such that leaves close their stomata when the roots sense soil water deficit via organic molecules. Stomatal closure in response to water stress (be it drought or waterlogging) is signaled by simple metabolites such as lumichrome, riboflavin and ABA, which are produced more abundantly by symbiotic rhizobia than other bacterial endophytes (Kanu and Dakora, 2009, 2012). Because its accumulation in leaves has been associated with stomatal closure during waterlogging or drought (Jackson and Hall, 1987), ABA is perceived as the major molecule regulating stomatal function, a role confirmed in several experiments using ABA-deficient mutants and their wild types (Jackson and Hall, 1987).

However, recent studies have identified new players in stomatal functioning of symbiotic legumes. For example, applying lumichrome and infective rhizobial cells to plant roots increased stomatal conductance and transpiration rates in cowpea and lupin, which was similar to the ABA control treatment, but decreased them in soybean, Bambara groundnut and maize, as also found with ABA (Matiru and Dakora, 2005b). Stomatal functioning in pea and sorghum was however not affected by lumichrome and rhizobial application, or by ABA (Matiru and Dakora, 2005b). These changes in stomatal functioning in response to lumichrome, ABA, and infective rhizobial cells were so similar in all test plant species that lumichrome and ABA appeared to play an identical role in stomatal functioning. It therefore seems that lumichrome and ABA can act separately or collectively to achieve the same desired outcome in stomatal functioning, be it aperture closure or opening.

The identical effects of lumichrome and ABA on stomatal functioning therefore suggest molecular cross-talk by the two compounds in controlling stomatal closure and opening. As shown in **Figure 2B**, the transmission of root-to-shoot signals such as lumichrome, riboflavin, ABA, and possibly Nod factors, can individually or collectively cause an increase in stomatal conductance and greater transpirational pull, leading to enhanced water absorption and increased mineral uptake. Except for Nod factors, the presence of the other signals (lumichrome, riboflavin and ABA) in the xylem stream en route to photosynthetic leaves has already been confirmed in legumes (Jackson and Hall, 1987; Phillips et al., 1999; Matiru and Dakora, 2005b), and their accumulation in leaves of cowpea and soybean also established (Matiru and Dakora, 2005b). So far, however, no study has shown the presence of rhizobial Nod factors in the xylem sap of symbiotic legumes.

A recent report has revealed increased mineral accumulation in high N_2-fixing cowpea genotypes than their low-fixing counterparts (Belane et al., 2014). The concentration of P in leaves of high N_2-fixers was two-fold greater than the low-fixers. This increase in mineral accumulation could be attributed to a range of factors, which include (i) rhizobial exudation of metabolites (e.g., siderophores, IAA, ABA, and organic acids), (ii) host–plant secretion of root exudates that solubilize unavailable minerals (Dakora and Phillips, 2002), and (iii) plant/rhizobial release of growth-promoting molecules (Dakora, 2003) that increase root hair production and nutrient absorption. However, the increase in stomatal aperture induced by lumichrome, riboflavin and ABA followed by the concomitant increase in transpiration rates, which promoted mineral uptake (Novák and Vidovic, 2003; Tani and Barrington, 2005) suggests a direct role of these metabolites in the accumulation of nutrient elements by high N_2-fixing cowpea varieties.

As a working model, we propose that lumichrome, riboflavin, ABA and possibly Nod factors secreted by symbiotic rhizobia in the rhizosphere get taken up by plant roots and translocated to shoots (Carpenter, 1943; Jackson and Hall, 1987; Matiru and Dakora, 2005b) where they elicit stomatal opening via molecular cross-talk (**Figure 2B**) in a concentration-dependent manner. That way, water and mineral uptake is enhanced. However, because the N_2-fixing efficacy of rhizobial strains is directly linked to the quality and quantity of the secreted symbiotic signals, their molecular effect on stomatal functioning is also linked to the strains' symbiotic efficiency. In fact, we

have shown elsewhere that N_2-fixing efficacy of rhizobia is correlated to leaf stomatal conductance of the host plant, and hence mineral accumulation in the legume. This relationship between strain symbiotic efficiency and stomatal functioning of the host plant is believed to control the symbiosis-induced accumulation of mineral nutrients in nodulated legumes (Belane et al., 2014).

A recent study has shown increased accumulation of ABA and IAA in lotus plants treated to lumichrome (Gouws et al., 2012). While such an increase in the formation of phytohormones in lumichrome-fed plants could help to explain the developmental changes associated with lumichrome application to plant roots, it could however also imply that these molecules cross-talk in their regulation of stomatal functioning, which leads to increased water and mineral uptake by roots. It is therefore our view that the symbiosis-induced accumulation of mineral nutrients in legumes (Belane et al., 2014) is due to the rhizosphere effect of lumichrome, riboflavin, IAA, ABA, and possibly Nod factors secreted by rhizobial bacteria (**Figure 2B**). The stomata in plants consist of specialized guard cells that regulate photosynthetic CO_2 uptake and leaf transpiration (Chen et al., 2012; Hills et al., 2012; Liu et al., 2014). The guard cell slow anion channel (SLAC) gene is apparently the "master switch" for stomatal closure (Maierhofer et al., 2014; Zheng et al., 2014). But how lumichrome, riboflavin and ABA work together to induce stomatal opening or closure, and hence increase or decrease mineral uptake, is still unclear. However, the greater root proliferation caused by the application of lumichrome (5 nM) to sorghum and millet (Matiru and Dakora, 2005a), or to lotus and tomato (Gouws et al., 2012), can also increase nutrient uptake in plant species. Rhizobia and other rhizosphere diazotrophs probably play a much greater role in the mineral nutrition of legumes and non-legumes than previously imagined.

Rhizosphere Ecology of Lumichrome, Riboflavin, and IAA Secreted by Rhizobia

In both natural and agricultural ecosystems, low or high production of lumichrome, riboflavin and IAA can have ecological consequences in ecosystem functioning. For example, an increase in root respiration induced by lumichrome and riboflavin from root-colonizing rhizobia can lead to an elevated concentration of rhizosphere CO_2, which is needed for growth of rhizobial populations in soil (Lowe and Evans, 1962). Furthermore, the increase in rhizosphere CO_2 concentration from lumichrome and riboflavin can also stimulate growth of vesicular-arbuscular fungi (Bécard and Piché, 1989; Bécard et al., 1992) and therefore promote the incidence of mycorrhizal symbiosis. These indirect benefits of lumichrome and riboflavin to legume symbioses via their effects on the plant are essential for enhancing N and P nutrition.

Furthermore, rhizobia and nodule endophytes isolated from eight *Psoralea* species (namely, *Psoralea pinnata*, *P. aphylla*, *P. aculeata*, *P. monophylla*, *P. repens*, *P. laxa*, *P. asarina*, and *P. restioides*) growing naturally in different locations within the Cape fynbos of South Africa exhibited large variations in their exudation of lumichrome, riboflavin and IAA (Kanu and Dakora, 2009, 2012), possibly due to bacterial adaptation to the localities where they were sampled. For example, two *P. repens* strains isolated close to the Atlantic Ocean secreted large amounts of lumichrome and riboflavin at both low and high salinity (Kanu and Dakora, 2009). Similarly, *Psoralea* isolates adapted to the acidic soils of the Cape fynbos also produced greater amounts of IAA even under very low pH conditions (Kanu and Dakora, 2009). The ability of native rhizobia to secrete symbiotic signals such as lumichrome, riboflavin and IAA under harsh environmental conditions implies that, even with climate change, indigenous legumes and their associated microsymbionts are unlikely to be affected in their symbiotic functioning.

Additionally, while most root-colonizing bacteria produce and release lumichrome and riboflavin (Phillips et al., 1999), others can synthesize and release eight times more extracellular riboflavin relative to their internal cellular concentration (Yang et al., 2002). These findings suggest that the two molecules have evolved directly or indirectly as rhizosphere signals influencing the outcomes of plant–bacterial interactions. It is clear from these studies that natural changes in pH, salinity and temperature within plant rhizospheres can elevate the concentrations of lumichrome, riboflavin and IAA in soils, with consequences for ecosystem functioning. For example, the high lumichrome production at 10°C than 30°C temperature (Kanu and Dakora, 2009) can alter nodulation and N_2 fixation of legumes in the Mediterranean fynbos habitat, where winter rainfall supports plant growth, nodulation and N_2 fixation.

Conclusion

Bacterial exudation of the rhizosphere signals lumichrome, riboflavin and IAA can vary with rhizobial strain, salinity, soil temperature and pH. Lumichrome taken up by plant roots and transported to the shoots probably elicits the formation of morphogenic molecules that cause cell division, cell expansion and cell extensibility, leading to an increase in leaf expansion, and stem elongation. Rhizobial inoculation as well as lumichrome and ABA supply to plant roots induced identical effects on stomatal functioning in both monocots and dicots. The three treatments consistently increased, or decreased, stomatal conductance and transpiration rates depending on the plant species. Plant roots therefore seem capable of collecting environmental signals from soil in the form of simple organic molecules released by microbes, and using them to adapt to their niches. An increase in the concentration of ABA and IAA in organs of lotus plants supplied with lumichrome (Gouws et al., 2012) could suggest that the observed developmental changes caused by lumichrome application to roots of monocots and dicots (Matiru and Dakora, 2005a) was probably due to increased levels of phytohormones elicited by the applied lumichrome. This however remains speculative in the absence of any genetic studies on the molecular basis for plant responses to lumichrome and riboflavin. Future studies need to address many unanswered questions. For example, what are the mechanisms underlying plant growth stimulation by lumichrome and riboflavin? Will rhizobial inoculation elicit same response in both legume and non-legume species as observed

with lumichrome application to roots of monocots and dicots? Future experiments should quantify classical phytohormones such as ABA, IAA, cytokinins and gibberellins in lumichrome and riboflavin-treated plants in order to unravel the mechanisms underlying plant response to these bacterial metabolites, and in so doing, add to our current understanding of the functioning of bacterial metabolites in plant rhizospheres. The relationship between rhizobial inoculation, leaf stomatal functioning, and mineral accumulation also need to be further explored.

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Acknowledgments

The South African Research Chair in Agrochemurgy and Plant Symbioses, the National Research Foundation, and Tshwane University of Technology are duly acknowledged for their continued support of FD's research. We are grateful to the reviewers and the guest editor for their constructive comments, which have helped to significantly improve the quality of this paper.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

REVIEW
published: 01 July 2015
doi: 10.3389/fpls.2015.00491

Secretion systems and signal exchange between nitrogen-fixing rhizobia and legumes

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The formation of symbiotic nitrogen-fixing nodules on the roots and/or stem of leguminous plants involves a complex signal exchange between both partners. Since many microorganisms are present in the soil, legumes and rhizobia must recognize and initiate communication with each other to establish symbioses. This results in the formation of nodules. Rhizobia within nodules exchange fixed nitrogen for carbon from the legume. Symbiotic relationships can become non-beneficial if one partner ceases to provide support to the other. As a result, complex signal exchange mechanisms have evolved to ensure continued, beneficial symbioses. Proper recognition and signal exchange is also the basis for host specificity. Nodule formation always provides a fitness benefit to rhizobia, but does not always provide a fitness benefit to legumes. Therefore, legumes have evolved a mechanism to regulate the number of nodules that are formed, this is called autoregulation of nodulation. Sequencing of many different rhizobia have revealed the presence of several secretion systems - and the Type III, Type IV, and Type VI secretion systems are known to be used by pathogens to transport effector proteins. These secretion systems are also known to have an effect on host specificity and are a determinant of overall nodule number on legumes. This review focuses on signal exchange between rhizobia and legumes, particularly focusing on the role of secretion systems involved in nodule formation and host specificity.

Keywords: rhizobia, nodulation, symbiosis, signal exchange, type III secretion system, type IV secretion system, type VI secretion system, effector proteins

OPEN ACCESS

Edited by:
Etienne Yergeau, National Research Council, Canada

Reviewed by:
Katharina Pawlowski, Stockholm University, Sweden
Erik Limpens, Wageningen University, Netherlands

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Specialty section:
This article was submitted to Plant Biotic Interactions, a section of the journal Frontiers in Plant Science

Received: *02 March 2015*
Accepted: *19 June 2015*
Published: *01 July 2015*

Citation:
Nelson MS and Sadowsky MJ (2015) Secretion systems and signal exchange between nitrogen-fixing rhizobia and legumes. Front. Plant Sci. 6:491. doi: 10.3389/fpls.2015.00491

Introduction

Plants interact with many different types of microbes, and these associations can be pathogenic, mutualistic, or commensal in nature. The type of relationship between a specific microbe and plant can vary based on external factors, such as changes in environment, or due to intrinsic factors of both organisms. Both pathogenic and mutualistic interactions are dependent on communication between host and microbe and are primarily based on signal exchange (Tseng et al., 2009). The symbiotic relationship between rhizobia and legumes has long been a focus of study because of the nitrogen fixation that occurs during the symbiosis. This symbiosis requires the rhizobia to be in close physical proximity to the legume to allow for exchange of nutrients. Nitrogen is essential for all agricultural crops, but only legumes can access nitrogen from the atmosphere through symbiosis with rhizobia. Signal exchange between rhizobia and legumes has been studied as a potential process regulating symbiosis on non-legume plants and a mechanism by which to increase nitrogen fixation in legumes.

The symbiosis between legumes and rhizobia has evolved to incorporate many different levels of signal exchange, from initial contact to senescence. Two primary reasons for this signal exchange are to distinguish between symbionts and pathogens and to ensure mutualism through the exchange of carbon and fixed nitrogen. The line between symbiont and pathogen is not always clear, as both partners can have a fitness benefit to alter the relationship to their advantage. Symbiotic associations may shift from mutually beneficial to pathogenic or vice versa, such as in the case of the plant pathogen *Argobacterium*, having a common ancestral history with rhizobia. It has been suggested that rhizobia can be viewed as refined pathogens (Deakin and Broughton, 2009). The symbiotic relationship between rhizobia and legumes can easily turn pathogenic if the plants loses the ability to regulate the total number of nodules formed or the rhizobia form nodules that do not fix nitrogen – with the plant experiencing decreased fitness by providing too much carbon to the rhizobia (Herridge and Rose, 2000; Kiers et al., 2003). Co-evolution between rhizobia and legumes is more complex because of rhizobia selection can oscillate between pathogen and symbiont.

The evolutionary arms race between pathogens and plants has long been studied (Jones and Dangl, 2006). Pathogens develop new strategies for creating infections, such as evolving secretion systems to alter the host cell. In response, plants develop new strategies for detecting pathogens, such as microbe-associated molecular patterns (MAMPs), and R genes (Dodds and Rathjen, 2010). Sequencing of various rhizobial strains has shown the presence of secretion systems similar to those used by pathogens to transfer proteins into the hosts' cytosol. These secretion systems include the Type III (T3SS), Type IV (T4SS), and Type VI secretion systems (T6SS; Fauvart and Michiels, 2008). The evolutionary presence of these secretion systems suggests that while rhizobia and legumes co-evolved a system allowing establishment and maintenance of a symbiosis, a relationship similar to a pathogen/plant interaction also co-evolved. This review focuses on legume–rhizobia signal exchange that occurs during nodule formation, plant mechanisms for limiting nodule number, and potential strategies used by rhizobia to overcome the plants ability to limit nodule number using the T3SS, T4SS, or T6SS.

Signaling Exchange During Nodule Formation

Rhizobia are free-living, soil saprophytes, prior to symbiosis with plants in the family Leguminosae. Rhizobia, once inoculated into soil, can persist at low levels in the absence of a suitable host (Howieson, 1995). The plant initiates symbiosis by secreting flavonoids, which are detected by the rhizobia. Flavonoids vary by plant species and are only recognized by certain, yet specific, rhizobial species, offering the first level of symbiosis specificity (Hassan and Mathesius, 2012). The flavonoids diffuse across the membrane of the rhizobia and induce synthesis of the NodD protein to activate transcription of other genes involved in nodulation including nod factor (NF) production (Wang et al., 2012). NFs are a primary signal molecule produced by bacteria and detected by the plant to induce nodule organogenesis. Structurally NFs are lipochitooligosaccharides (LCOs) with a chitin oligomer backbone (Oldroyd and Downie, 2008). The *nodABC* genes encode for the proteins required to make the core NF structure and are conserved across all rhizobia species, except two *Aeschynomene*-infective species (Perret et al., 2000; Giraud et al., 2007). The NF core is then modified by species-specific proteins resulting in various substitutions on both the reducing and non-reducing end, including glycosylation and sulfation (Long, 1996). These substitutions are specific for each host legume and offer another level of symbiosis specificity (Dénarié et al., 1996; Long, 1996). Many surface polysaccharides are also involved in symbiosis specificity including lipopolysaccharides (LPSs), extracellular polysaccharides (EPSs), and capsular poylsaccharides (KPSs; Deakin and Broughton, 2009). The specific structure of LCOs is known to be important for recognition by host nod factor receptors (NFRs), which are receptor kinases containing lysin motifs (LysM; Radutoiu et al., 2007). Leucine rich repeat receptor-like kinases (LRR-RLKs) are also involved in NF perception and signaling, which results in nodule formation (Endre et al., 2002).

Root hair curling and crack entry are the two infection mechanisms used by rhizobia. Crack entry involves rhizobia entering through cracks at the lateral root bases or stems (Goormachtig et al., 2004). Root hair curling involves recognition of NFs, this recognition results in both calcium spiking and the curling of the root hair (Esseling et al., 2003). This is thought to involve a change in the plant cells' polarity, resulting in a new growing direction of the root hair tip (Gage, 2004). The infection chamber enlarges and changes into a globular apoplastic space. Next, root tip growth in switched from radial to polar tip elongation (Fournier et al., 2015). The continued growth of the infection thread is dependent on NF specificity as well as EPS (Jones et al., 2007). Both the epidermis and the cortex recognize NFs, the epidermis regulates rhizobia infection and the root cortex is responsible for nodule formation (Oldroyd and Downie, 2008). Cortical cells develop into a nodule primordium. When the infection thread reaches the nodule primordium, the rhizobia enter into the inner cells and become encapsulated within a peri-bacteroid membrane (Oldroyd and Downie, 2008).

There are two main types of nodules, indeterminate and determinate, and this is determined by the legumes. For indeterminate nodules, cell division typically begins in the inner cortex (Ferguson et al., 2010). Indeterminate nodules maintain a persistent meristem and form distinct zones, including rhizobia invasion, active nitrogen fixation and senescence (Udvardi and Poole, 2013). These zones contain rhizobia in various developmental states with the proximal zone losing the ability to reproduce (Mergaert et al., 2006). Legumes belonging to the inverted repeat-lacking clade manipulate bacterial differentiation through secretion of cysteine-rich peptides, which induce membrane permeabilization, endoreduplication, and loss of independent viability (Mergaert et al., 2006; Van de Velde et al., 2010; Oldroyd et al., 2011). In contrast, cell division begins in the outer cortex for determinate nodules (Ferguson et al., 2010). Determinate nodules do not have a persistent meristem and form

a homogenous group of rhizobia with full viability (Saeki, 2011). In mature nodules, plants exchange small carbon molecules for ammonia with the rhizobia. Another important aspect of symbiosis regulation is amino acid exchange and cycling between the plant and the rhizobia. During symbiosis some plants secrete branched chained amino acids, into the peribacteroid space, and in return the rhizobia secrete aspartate and, in some cases, alanine. Rhizobial biosynthesis of branched chained amino acids is shut down during symbiosis, preventing the use of ammonium by rhizobia and allowing the plant to incorporate ammonium into aspartate to produce asparagine (Lodwig et al., 2006; Prell et al., 2009).

After many weeks of plant growth, nodules begin to senescence, with a maximum lifespan well-short of that of the host plant (Puppo et al., 2005). Dark stress, water stress, defoliation, or addition of nitrate can initiate premature nodule senescence (González et al., 1998; Matamoros et al., 1999; Hernández-Jiménez et al., 2002). This suggest that the plant controls the duration of the symbiosis by being able to induce nodule senescence. These external factors are thought to lead to an increase in reactive oxygen species, which initiates senescence (Puppo et al., 2005). During nodule senescence, the host plant initiates plant cell death and some rhizobia not in the symbiosome survive this process and return to a saprotrophic state in the soil (Hernández-Jiménez et al., 2002).

Plant Signaling Limits Nodule Number

The symbiotic relationship between rhizobia and legumes has the potential to become pathogenic if the plant loses the ability to regulate the total number of nodules or perceives the rhizobia as a pathogen. Rhizobia will generally initiate nodule formation because a symbiotic relationship always has a fitness benefit for the rhizobia. However, if the plant forms too many nodules then there is a negative effect on vegetative growth and yield (Herridge and Rose, 2000; Takahashi et al., 2003; Matsunami et al., 2004). Legumes use a process called autoregulation of nodulation (AON) to control nodule number by preventing new nodule formation (Mortier et al., 2012). The AON is thought to involve a root-derived signal being transported to the shoot, which induces a shoot-derived signal to be transported to the root – this inhibits nodule formation (Suzaki et al., 2015).

After nodule formation, the plant cell begins to produce CLV3/ESR-related (CLE) peptides. CLE peptides are thought to be the signal molecule transported from the roots to the shoot as part of the signaling pathway involved in AON (Reid et al., 2011, 2013). The CLE-RS2 is a post-translationally arabinosylated glycopeptide derived from the CLE domain, and if externally added CLE-RS2 sufficient to inhibit nodule formation (Okamoto et al., 2013). The CLE-peptides are recognized by LRR-RLKs (Krusell et al., 2002; Nishimura et al., 2002; Sasaki et al., 2014). These receptors then cause a signal cascade which results in cytokinins being transported from the shoot to the root, which could act as the shoot-derived signal to suppress nodule formation (Sasaki et al., 2014). In the *Lotus japonicas tml* mutant, shoot-applied cytokinin does not suppress nodule formation (Sasaki et al., 2014). This implies that TML acts downstream of cytokinins, and may act directly in the root cells to suppress nodulation. TML encodes a Klech repeat-containing F-box protein and has been hypothesized to target a protein for degradation which has a positive role in nodule formation (Takahara et al., 2013; Suzaki et al., 2015).

Autoregulation of nodulation signaling is a complex process involving numerous steps, some of which are still unknown. Disruption of AON at many different steps has been shown to results in a hyper-nodulation phenotype. This suggests that the AON signaling process could be potential targets for rhizobia to disrupt, in order to increase nodule formation. Inhibition of AON, could result in the symbiotic relationship between rhizobia and legumes becoming a pathogenic one (Herridge and Rose, 2000).

Bacterial Secretion Systems

Bacteria use a wide variety of secretion systems to export proteins and other compounds across their membranes and cell walls. Interaction with the external environment is vital to bacterial survival, and many different transmembrane channels have evolved independently to fulfill this need (Wooldridge, 2009). There have been reports of up to many different secretion systems, but only the first seven have been significantly investigated (Tseng et al., 2009). These secretion systems have evolved independently, each containing a different set of core proteins. Each secretion system itself diverged into unique subfamilies based on different functions. The T1SS, T2SS, and T5SSs are thought to simply transport proteins and compounds outside of the cell. The T3SS, T4SS, and T6SSs contain subfamilies with the ability to transport effector proteins into the cytosol of eukaryotic cells (Wooldridge, 2009). This is important because it allows for the direct communication with, and modification of, the eukaryotic cytosol. These three secretion systems are well-understood for their role in pathogenesis as key factors in virulence and, in some cases, symbiosis.

Rhizobia Secretion Systems

As discussed above, rhizobia enter into unique symbioses with eukaryotic cells, through the formation of relationship with legumes. Sequencing of rhizobia strains has shown that they typically contain multiple secretion systems. However, the presence of these systems in the bacterial genome does not mean they have a role in symbiosis. Rhizobia surface polysaccharides (LPS) have been known to suppress plant immune responses, but the T3SS and T4SS have also been speculated to have a role in suppressing the plant immune system (Masson-Boivin et al., 2009).

The T3SS and T4SS are each sub-divided into seven families based on function and protein homology (Wooldridge, 2009;

Sugawara et al., 2013). The T3SS, T4SS, and T6SSs have been identified throughout various rhizobial genera and sequence homology shows similarity between known secretion systems used by bacterial pathogens. Specifically, sequence analysis of *Sinorhizobium* has shown that they can contain either the T3SS, T4SS or the T6SS, but typically only have one involved in symbiosis per strain (Sugawara et al., 2013). The T3SS, T4SS, and T6SS have all been shown to be involved in symbiosis and translocate effector proteins during symbiosis. These effector proteins could potentially have a function by promoting nodule formation, disrupting AON, or suppressing the plant's immune response during invasion. In plant pathogens, the T3SS effectors have been shown to target and suppress the plant immune response (Macho and Zipfel, 2015). Deletion of a specific subfamily of the T3SS or the T4SS has been shown to reduce nodule number and affect host range specificity (Sugawara et al., 2013; Tampakaki, 2014). However, their role in symbiosis is still not very well-understood.

Type III Secretion System

The T3SS is a structure composed of 20–27 different proteins, and this transporter is responsible for secretion of type III effector proteins (T3Es; Ghosh, 2004; Tampakaki, 2014). Approximately 50% of proteins involved in secretion system channel formation are conserved in most T3SSs (Ghosh, 2004). These proteins are generally found clustered in a 22–50 kb pathogenicity island (Tampakaki, 2014). The T3SS complex spans the bacterial inner and outer membrane as well as the hosts' membranes and allows protein transport into the host. Regions flanking the pathogenicity island can contain genes that encode for effector proteins, but most effector genes are scattered throughout the genome (Lindeberg et al., 2008).

Many different variations of T3SS, with varying functions, are found throughout the kingdom of bacteria. In the literature, the T3SS is first grouped by species, and then grouped by homology. The genes encoding the rhizobial T3SSs are called rhc (*Rhizobium* conserved). The rhc are further subdivided into four families based on phylogenetic analyses, Rhc-1 to Rhc-4 (Gazi et al., 2012). Of these four families, only Rhc-I has been showed to be involved in symbiosis (Tampakaki, 2014). The functions of the other families are still unknown. The T3SS is among the best studied secretion systems in rhizobia due to the wide species distribution of Rhc-1 and its role in symbiosis.

T3SS – Rhc-I Effect on Symbiosis

Early studies of the T3SS – Rhc-1 focused on knocking out the entire system through deletions or disruption of core genes. A diverse range of rhizobial species are known to contain a functional T3SS – Rhc-1 and are listed in **Table 1**. The influence of

TABLE 1 | Symbiotic effect of the T3SS – Rch-1 in rhizobia.

Strain of rhizobia with T3SS – Rch-1	Secreted proteins	Positive effect on symbiosis	Negative effect on symbiosis	Reference
Rhizobium etli CNPAF512	2	*Phaseolus vulgaris*	Unknown	Michiels et al. (1995), Fauvart and Michiels (2008)
Bradyrhizobium elkanii USDA61	8	*Macroptilium atropurpureum, Glycine max* ev. Clark, *G. max* cv. Enrei	*Vigna radiata* cv. KPS1, *G. max* cv. Hill	Okazaki et al. (2009), Okazaki et al. (2013)
Mesorhizobium loti MAFF303099	8	*Lotus glaber, Lotus japonicus, Lotus corniculatus* subsp. *frondsus, Lotus filicaulis*	*Leucaena leucocephala, Lotus halophilus, Lotus peregrinus* var. *carmeli, Lotus subbiflorus*	Hubber et al. (2004), Sánchez et al. (2009), Sánchez et al. (2012), Okazaki et al. (2010)
Sinorhizobium fredii NGR234	15	*Tephrosia vogelii, Flemingia congesta, Lablab purpureus*	*L. leucocoephala, Pachyrhizus tuberosus, Crotalaria juncea*	Viprey et al. (1998), Skorpil et al. (2005), Kambara et al. (2009), Kimbrel et al. (2013)
S. fredii HH103	8	*G. max* cv. Peking, Heinong 33, Kochi, and Williams, *Glycyrrhiza uralensis*	*Erythrina variegata*	Rodrigues et al. (2007), López-Baena et al. (2008)
S. fredii USDA207	13	Unknown	Unknown	Kimbrel et al. (2013)
S. fredii USDA257	13	*G. max* cv. Peking and Williams, *M. atropurpeum*	*G. max* cv. McCall, *E. variegata*	Krishnan et al. (2003), De Lyra et al. (2006), Kimbrel et al. (2013)
Bradyrhizobium japonicum USDA6	33	Unknown	Unknown	Kimbrel et al. (2013)
B. japonicum USDA110	36	*M. atropurpureum G. max* cv. Williams	*V. radiata* cv. KPS2	Krause et al. (2002), Wenzel et al. (2010), Kimbrel et al. (2013)
B. japonicum USDA122	31	Unknown	Unknown	Kimbrel et al. (2013)
B. japonicum USDA123	32	Unknown	Unknown	Kimbrel et al. (2013)
B. japonicum USDA124	33	Unknown	Unknown	Kimbrel et al. (2013)
Cupriavidus taiwanensis LMG19424	Unknown	Unknown	*L. leucocephala*	Saad et al. (2012)

**Only strains with functional T3SS – Rch-1 with a known effect on symbiosis are listed. More strains have been sequenced that contain the T3SS – Rch-1, but these have not been experimentally tested for function (de Souza et al., 2012). The number of secreted proteins includes proteins identified through analysis of proteins found externally after induction of the T3SS, and proteins shown to be transported into the cytosol of Arabidopsis.*

T3SSs on nodulation can vary from positive, in which nodulation is increased, to negative, in which nodulation is reduced. In *Sinorhizobium fredii* strain NGR234, the T3SS has both a positive and negative affect on multiple different legume species, but may also have a neutral phenotype, where nodulation is not affected, for example on *Vigna unguiculata* (Viprey et al., 1998; Skorpil et al., 2005; Kambara et al., 2009). Similarly, rhizobia with the T3SS – Rch-1 show host-dependent phenotypes in regard to nodulation efficiency. This could explain why the T3SS – Rch-1 is found in many genera of rhizobia, but is not ubiquitous at the strain level.

The horizontal transfer of the T3SS could be an important evolutionary driver toward symbiosis or pathogenesis between bacteria and plants. The pathogen *Ralstonia solanacearum* was shown to be unable to nodulate *Mimosa pudica* when the symbiotic plasmid of *Cupriavidus taiwanensis* was added, but was able to nodulate *M. pudica* if the T3SS was also deleted (Marchetti et al., 2010). This shows that the T3SS can prevent symbiosis. However, deleting the T3SS effector protein GALA7 prevented pathogenic infection of *Medicago truncatula* (Angot et al., 2006). This shows that the T3SS in *R. solanacearum* is required for pathogensis. In addition, *C. taiwanensis* was able to nodulate *Leucaena leucocephala* when the T3SS in *C. taiwanensis* was deleted (Saad et al., 2012). These examples show how the presence of the T3SS can restrict host range by preventing symbiosis, and could have a role in bacteria transitioning from a symbiont to a pathogen.

Regulation of the T3SS – Rhc-1

Expression of the T3SS is induced by plant flavonoid recognition through production of the transcriptional activator TtsI (Viprey et al., 1998; Krause et al., 2002; Kobayashi et al., 2004). TtsI initiates transcription of the T3SS genes and effector proteins by binding to specific *cis*-elements, known as *tts* boxes (Wassem et al., 2008). The number and location of *tts* boxes varies between species and *Bradyrhizobium japonicum* USDA110 is known to have 52 different *tts* boxes. Proteins secreted by the T3SS are found downstream of *tts* boxes.

There is not a consensus motif for proteins secreted through the T3SS. However, the signal sequence is typically found in the first ∼15 amino acids, on the N-terminus, of translocated proteins (Ghosh, 2004). In addition not all gene transcription activated by *tts* boxes, are effector proteins translocated through the T3SS; some can have other roles in symbiosis such as the production of rhamnose-rich polysaccharides (Marie et al., 2004). These rhamnose-rich polysaccharides were shown to be surface LPSs, important in nodule formation, independent of the T3SS (Broughton et al., 2006). This suggests an interesting link between secretion systems and surface polysaccharides involved in nodule formation specificity.

Proteins Secreted by the T3SS – Rhc-1

Early studies to identify proteins secreted through the T3SS focused on using flavonoids to induce expression in culture and compared the external proteins to those found in a T3SS mutant. However, these experiments did not show translocation into the host cytosol. This led to uncertainty as to whether an identified protein was an effector protein, acting inside the plant cell. A new, high-throughput technique was used to properly identify proteins that translocate through the T3SS as well as to identify effector proteins (Kimbrel et al., 2013). However, this technique did not test for effector translocation into legumes, but rather the proxy of translocation through *Pseudomonas syringae* pv. *tomato* DC3000 into *Arabidopsis* Col-O. The T3E candidates are fused to Δ79AvrRpt2, which induces a hypersensitive response (HR) in *Arabidopsis*. Using this technique on three different strains of *S. fredii* and *B. japonicum,* between 13 and 36 T3Es per strain were identified (Kimbrel et al., 2013). The T3Es can vary between species and strains, but members of the same species tend to use very similar effector proteins.

Proteins secreted by the T3SS can be separated into two categories – pilus forming and effectors. Proteins involved in pilus formation are secreted through the channel to assist in forming a channel through the plants cell wall or plasma membrane. NopA, NopB, and NopX are thought to be involved in the terminal formation of the T3SS, forming a pilus that penetrates the plant's cell wall and plasma membrane (Lorio et al., 2004; Deakin et al., 2005; Saad et al., 2005, 2008). The other secreted proteins are thought to be effector proteins, but few of these proteins have a predicted function *in planta* (**Table 2**).

As shown in **Table 1**, deleting the T3SS can have a positive or negative effect on symbiosis. The T3SS is simply the means of transport for effector proteins. Deleting the T3SS prevents effector protein transport. These effector proteins play key roles in symbiosis. Despite having a known effect on symbiosis, none of these effector proteins has been expressed in legumes. Only the effectors NopL, NopT, and NopM have all been expressed in eukaryotic cells. NopL was first shown to be phosphorylated by plant kinases (Bartsev et al., 2003). Next, NopL was shown to interfere with mitogen-activated protein kinase (MAPK) signaling in *Nicotiana tabacum*. MAPK signaling is involved pathogen recognition in both basal plant defense and R-mediated resistance (Pedley and Martin, 2005). Part of the plant defensive response is the induction of HR. The plant pathogen *P. syringae* uses effector proteins AvrPto and AvrPtoB to interrupt MAPK signaling by degrading the plant protein FLS2 (Göhre et al., 2008; Shan et al., 2008). Overexpression of MAPK signaling in plants induces HR to prevent pathogen infections. NopL was shown to suppress cell death induced by the overexpression of MAPK signaling (Zhang et al., 2011). NopT when expressed in *N. tabacum* or *Arabidopsis thaliana* elicited a strong HR response and necrotic symptoms. The authors did suggest that it could function as a protease and had similarity to the effector family YopT – AvrPphB (Dai et al., 2008). AvrPphB is an effector in *P. syringae* and functions as an autoprotease, cleaving itself to expose a myristolation site (Puri et al., 1997; Shao et al., 2002). The addition of myristoyl groups after cleavage, target AvrPphB to the cell membrane (Nimchuk et al., 2000). NopT has been shown to have cysteine protease activity and may use autoproteolysis for target to cell membranes, but its role is still uncertain (Fotiadis et al., 2012). NopM was shown to possess E3 ubiquitin ligase activity. Furthermore, when this ability was lost through a point mutation,

TABLE 2 | Predicted functions of T3SS secreted proteins.

T3SS – Rch-1 secreted proteins	Strains containing homolog	Predicted function	Reference
NopA	*B. japonicum* USDA110, *M. loti* MAFF303099, *S. fredii* NGR234, *S. fredii* HH103, *S. fredii* USDA257	Part of the T3SS extracellular pilus which spans the plants cell wall	Deakin et al. (2005), Saad et al. (2008)
NopB	*B. japonicum* USDA110, *M. loti* MAFF303099, *S. fredii* NGR234, *S. fredii* HH103, *S. fredii* USDA257	Part of the T3SS extracellular pilus which spans the plants cell wall	Saad et al. (2005), Saad et al. (2008)
NopD	*S. fredii* HH103	Homology to a predicted C48 cysteine peptidase	Hubber et al. (2004), Rodrigues et al. (2007)
NopL	*B. japonicum* USDA110, *S. fredii* NGR234, *S. fredii* HH103, *S. fredii* USDA257	Suppresses cell death induced by mitogen-activated protein kinase (MAPK)	Zhang et al. (2011)
NopM	*B. japonicum* USDA110, *S. fredii* NGR234, *S. fredii* HH103	E3 ubiquitin ligase, thought to be involved in protein–protein interactions	Rodrigues et al. (2007), Xin et al. (2012)
NopP	*S. fredii* NGR234, *R. etli* CNPAF512, *S. fredii* HH103, *S. fredii* USDA257	Phosphorylated by plant kinases	Skorpil et al. (2005)
NopT	*S. fredii* NGR234	Cysteine protease	Fotiadis et al. (2012)
NopX	*M. loti* MAFF303099, *S. fredii* NGR234, *S. fredii* HH103, *S. fredii* USDA257	Terminal part of the T3SS extracellular pilus which spans the plants cell wall	Saad et al. (2008)
Mlr6361	*M. loti* MAFF303099	Shikimate kinase	Sánchez et al. (2009)

**Subset of known proteins secreted by the T3SS – Rch-1. Only proteins with a predicted function or that have been experimentally tested are listed. None of the proteins have been tested in legumes, but some have been tested in planta in Nicotiana tabacum.*

the positive effects on nodule formation were also lost (Xin et al., 2012).

Even though the function of many specific proteins has not been determined, the accumulated effect of the T3SS effector proteins can be determined through deletion of the entire secretion system. *Bradyrhizobium elkanii,* containing the T3SS, but not the T3SS mutant, was shown to increase the transcription of two genes in the roots of a soybean line deficient in NF recognition (Okazaki et al., 2013). These genes, *ENOD40* and *NIN*, are involved in early nodulation regulation. This suggests that the T3SS effector proteins may be involved in up-regulating host genes involved in nodule formation. Further research is needed to more completely understand how these individual effectors are functioning *in planta*.

Type IV Secretion System

The T4SS-b is functionally similar to the T3SS-Rch-1 and is also involved in protein translocation, but has a separate evolutionary origin. The T4SS is generally sub-divided into three families based on function, including conjugation, DNA uptake and release, and protein translocation (Cascales and Christie, 2003). These three families can use similar core proteins to form the main channel and may share sequence similarity. Properly identifying which sub-family is present in a specific strain is key. In rhizobia, the T4SS-b shares strong homology to the VirB/VirD4 subunits found in *Agrobacterium*. The core structure consists of 12 proteins, VirB1-B11 and VirD4. The T4SS-b, in *Agrobacterium tumefaciens*, is used for translocation of both T-DNA and effector proteins (Kuldau et al., 1990; Zupan and Zambryski, 1995). The function of the T4SS-b is well-understood because of its role in plant transformation. *Agrobacterium* and rhizobia are closely related, and understanding of the T4SS-b in *Agrobacterium* has been leveraged to better understand the T4SS-b in rhizobia.

T4SS-b Effect on Symbiosis

Unlike the T3SS, there is a paucity of information regarding the role of the T4SS in symbiosis. A functional T4SS-b has only been identified in three different species (**Table 3**). Similar to the T3SS, the T4SS-b can have both a positive or negative effect on symbiosis. In *Mesorhizobium loti* R7A, nodulation on *Lotus corniculatus* reduced, but not completely lost, when the T4SS-b was partially deleted. This same deletion allowed *M. loti* R7A to gain the ability to form nodules on *L. leucocephala* (Hubber et al., 2004). Deleting the T4SS-b in *Sinorhizobium meliloti* KH46c resulted in approximately a 50% decrease in nodule number on *M. truncatula* A17, but did not have a significant effect on *M. truncatula* F83005-5 (Sugawara et al., 2013). This dual positive and negative selection could explain why only 9 of 33 *S. meliloti* and 11 of 13 *S. medicae* strains were found to contain the T4SS-b (Sugawara et al., 2013).

Regulation of the T4SS-b

Transcription of the T4SS is controlled by a two-component response regulator VirA/VirG (Stachel and Zambryski, 1986). VirA is a membrane bound kinase that phosphorylates VirG in response to external factors (Hansen et al., 1994). In contrast, VirG is a transcriptional activator that binds to *vir* boxes. In *Rhizobium* these regulators are induced by flavonoids that activate VirG (Hubber et al., 2007). Unlike the T3SS effectors, which can be present throughout the genome, T4SS tend to be near VirG (Vergunst et al., 2000; Tampakaki, 2014). Research in *A. tumefaciens* has identified a sequence motif, a positive charged C-terminus, present on effector proteins needed for translocation (Vergunst et al., 2005). This same sequence motif is also present on the only two effector proteins identified, Msi059 and Msi061,

TABLE 3 | Symbiotic effect of the T4SS-b.

Strain of rhizobia with T4SS – B	Secreted proteins	Positive effect on symbiosis	Negative effect on symbiosis	Reference
M. loti R7A	2	*L. corniculatus*	*L. leucocephala*	Hubber et al. (2007)
S. meliloti KH35c	Unknown	*M. truncatula* A17, *M. tricycla*	Unknown	Sugawara et al. (2013)
S. medicae M2	Unknown	*M. truncatula* A17	Unknown	Sugawara et al. (2013)

**Only strains with a functional T4SS-b are listed. More strains containing a T4SS-b have been sequenced, but not experimentally tested for function (Sugawara et al., 2013). Both secreted proteins in M. loti R7A, have been shown to be translocated into Arabidopsis (Hubber et al., 2004).*

both in *M. loti* R7A (Hubber et al., 2004). VirD4 interacts with the positive charge signal sequence to transport the protein through the channel (Vergunst et al., 2005). VirD4, and the requirement of a more specific signal sequence, could result in more specificity in protein transport.

Proteins Secreted by the T4SS-b

Thus far, only two proteins have been shown to transport through the T4SS-b, Msi059, and Msi061 in *M. loti* R7A. The Msi059 showed partial protein sequence similarity to a C48 cysteine peptidase. Interestingly, the NopD T3E in *S. fredii* HH103 also was a predicted C48 cysteine peptidase (Rodrigues et al., 2007). The C48 cysteine peptidase family contains the protein XopD, a T3E from the plant pathogen *Xanthomonas campestris* (Hotson et al., 2003). XopD encodes an active cysteine protease, and functions *in planta* to target SUMO-conjugated proteins (Hotson et al., 2003). This interferes with the plant's ability to regulate the expression of specific proteins. Msi061 has shared protein similarity with *A. tumefaciens* effector VirF. The VirF interacts with the host Skp1 to facilitate protein degradation of effector proteins VirE2 and Vip1 to unbind the T-DNA after into the host cell (Schrammeijer et al., 2001; Tzfira et al., 2004). Skp1 is a core component of the E3 ubiquitin ligase, which mediates protein degradation (Schrammeijer et al., 2001). The precise activity of Msi059 and Msi061 are still unknown, but current evidence suggests a role in changing protein expression levels *in planta*.

Type VI Secretion System

The T6SS is among the least researched secretion system involved in protein translocation. The T6SS is known to contain different subfamilies, but the sub-families and their functions have yet to be clearly defined. The number of proteins involved in forming the core structure seem to vary and there is no known secretion signal for protein transport (Bingle et al., 2008). Additionally, how T6SS expression is regulated is unknown. Still, the T6SS is thought to play an important role in the virulence of multiple pathogens, like *Burkholderia mallei* (Schell et al., 2007).

T6SS Effect on Symbiosis

The sequence for the T6SS has been found in five different species of rhizobia, *R. leguminosarum*, *B. japonicum*, *M. loti, S. saheli,* and *S. fredii* (Bladergroen et al., 2003; Bingle et al., 2008; Sugawara et al., 2013). However, a functional T6SS, with an effect on symbiosis, has only been shown in *R. leguminosarum.* In this bacterium a negative effect on symbiosis was observed, where the T6SS prevented nodulation on *Pisum sativum* cv. Rondo (Bladergroen et al., 2003). A single protein was identified that is secreted through the T6SS. Sequencing of the first 50 amino acids suggested a role in ribose transport (Bladergroen et al., 2003). The effect that ribose transport has on symbiosis is unclear. More strains containing the T6SS have been identified, but not experimentally tested for function (Bingle et al., 2008; Sugawara et al., 2013).

Example of Effector Involvement in Symbiosis

Most studies have focused on deleting specific genes in the core structure, instead of the effector proteins, and observing the overall phenotypic change. This is likely due to the fact that the core genes, unlike effectors, do not vary between species. Additionally, the phenotypic effect(s) of a single effector knockout might be small, again with some strains containing 36 different T3Es. One of the most well-characterized examples of the how the T3SS functions is in *S. fredii* strain USDA257. In this case *S. fredii* USDA257 is both a pathogen and a symbiont.

Legumes limit nodule number, and one mechanism used is to abort nodule formation, through a process similar to HR (Vasse et al., 1993). The *S. fredii* USDA257 strain contains NopL, which suppresses cell death through preventing MAPK signaling from inducing HR and cell death (Bartsev et al., 2004; Zhang et al., 2011). This would, in theory, increase the total number of nodules formed. Soybeans have evolved an R gene, Rfg1, capable of detecting T3Es from *S. fredii* USDA257 (Yang et al., 2010). Rfg1 encodes a TIR-NBS-LRR disease resistance protein, which are known to recognize pathogen effectors to induce disease resistant (Belkhadir et al., 2004). In soybean lines expressing Rfg1, the plant prevents nodulation by *S. fredii* USDA257, but not in the T3SS knockout mutant (Trese, 1995; Yang et al., 2010). In addition *S. fredii* USDA257 formed almost twice as many nodules on the soybean lines without the Rfg1 and the recessive rj2 genes as did the T3SS knockout mutant, on three different soybean lines (Yang et al., 2010). Taken together, the T3SS, including NopL, can increase nodulation in soybean. Recognition of the T3Es, by Rfg1, results in complete prevention of nodulation. NopL restricts the plant's ability to prevent infection and nodule formation, and rhizobia become partially pathogenic through using this strategy. The specific protein which is recognized by Rfg1, either directly or indirectly, is still not known. Though this is just one example, it is consistent

with observations from other studies showing both the positive and negative effects of the T3SS as listed in **Table 1**. This dual selection also explains why the T3SS is not found in all strains of Rhizobia.

Proposed Model

Most of these studies were done by deleting the entire secretion system, versus knocking out only specific effector proteins. Secretion systems are not found in all strains for any species of rhizobia. Typically, if the T3SS or T4SS has a positive effect on nodulation, then deletion of the T3SS results in ~40–60% reduction in nodule number. This shows that secretion systems are not essential for effective nodulation. If the T3SS has a negative effect on nodulation, then knocking out the T3SS or T4SS results in a gain of function phenotype, where the strain is now able for form nodules on a host genotype that it was previously unable to nodulate effectively. This shows that secretion systems restrict host range. Taken together, the evidence suggests that effector proteins may act in a pathogenic manner. The function of most effector proteins are not known. Many are predicted to modify *in planta* protein levels, and NopL was shown to suppress defense responses. This suggest that rhizobial effector proteins act in a pathogenic manner, similar to the function of other known bacterial effector proteins (Shames and Finlay, 2012).

The model we propose here (**Figure 1**), is to demonstrate three points regarding effector proteins: (1) the role of effector proteins is strictly pathogenic, and not involved in symbiosis communication between the rhizobia and host; (2) the role of effector proteins may lead to an increase nodule number. AON is the plants system for regulating nodule number. The mechanism of action for individual effector proteins will differ, but the unifying aspect is the increase in nodule number. This increase could be achieved through forming additional nodules or the prevention of nodule senescence; and (3) plants use R genes to recognize effector proteins. This recognition results in host defense responses, which can prevent nodulation. This serves to establish a host range for rhizobial strains possessing effector proteins which are recognized by the host.

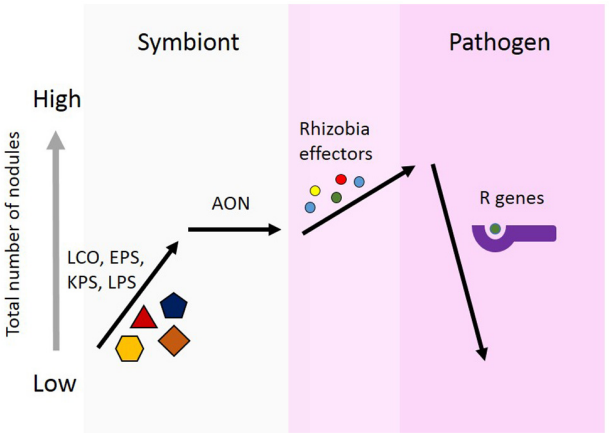

FIGURE 1 | Proposed model for the role of effector proteins in symbiosis. Rhizobia secrete nod factors, which are lipochitooligosaccharides (LCOs), and are important for nodule formation and host specificity. Surface polysaccharides are also known to be involved in determining specificity for nodule formation. These include, extracellular polysaccharides (EPSs), capsular polysaccharides (KPSs), and lipopolysaccharides (LPSs). Legumes limit the total number of nodules formed using autoregulation of nodulation (AON). Rhizobia use effector proteins, similar to pathogens, to alter plant cells to facilitate increased nodule formation. Effectors alter the symbiotic state toward pathogenesis. In response, plants can develop R genes capable of recognizing the presence of these effector protein, either directly or indirectly. Effector recognition results in the plant initiating a defense response and preventing nodule formation.

Conclusion

The T3SS, T4SS, and T6SS all play an important role in nodule formation in the symbiosis between rhizobia and legumes. Many studies have shown that these secretion systems have an effect on host range. NFs and surface polysaccharides are also known to effect symbiotic host range. These factors are important for host recognition of a symbiont versus a pathogen and facilitate infection for nodule formation. However, pathogens use effector proteins during invasion to promote virulence, and these effectors have an effect on the pathogens host range. Thus, other factors besides host range have to be used to determine the role of secretion systems in rhizobia/plant interaction. The T3SS, T4SS, and T6SS are all known to transport effector proteins. The predicted function of these proteins *in planta*, plus identifying R genes which respond to the T3SS or its effectors, strongly suggest that these secretion systems are acting in a pathogenic manner.

These secretion systems function to transport proteins from rhizobia into the plant cytosol. Once in the cytosol, they act to either increase nodulation or result in decreased nodulation through plant defense recognition. Specific changes *in planta* are not yet known. Identifying how rhizobia use effector protein could have an important agricultural application. Rhizobia may be using these proteins to suppress or prevent AON, and manipulation of this regulation may lead to the development of new strategies for increasing nodule formation. These effector proteins still have not been expressed *in planta*, in legumes, and thus their functions remain unclear. Although several hypotheses have been postulated, the role of T3SS and T4SS are still not fully understood and warrant further research.

Acknowledgments

We thank Chris Staley for helpful comments. The work was supported, in part, by grant 1237993 from the National Science Foundation.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

MINI REVIEW
published: 02 June 2015
doi: 10.3389/fpls.2015.00401

Does plant immunity play a critical role during initiation of the legume-rhizobium symbiosis?

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Plants are exposed to many different microbes in their habitats. These microbes may be benign or pathogenic, but in some cases they are beneficial for the host. The rhizosphere provides an especially rich palette for colonization by beneficial (associative and symbiotic) microorganisms, which raises the question as to how roots can distinguish such 'friends' from possible 'foes' (i.e., pathogens). Plants possess an innate immune system that can recognize pathogens, through an arsenal of protein receptors, including receptor-like kinases (RLKs) and receptor-like proteins (RLPs) located at the plasma membrane. In addition, the plant host has intracellular receptors (so called NBS-LRR proteins or R proteins) that directly or indirectly recognize molecules released by microbes into the plant cell. A successful cooperation between legume plants and rhizobia leads to beneficial symbiotic interaction. The key rhizobial, symbiotic signaling molecules [lipo-chitooligosaccharide Nod factors (NF)] are perceived by the host legume plant using lysin motif-domain containing RLKs. Perception of the symbiotic NFs trigger signaling cascades leading to bacterial infection and accommodation of the symbiont in a newly formed root organ, the nodule, resulting in a nitrogen-fixing root nodule symbiosis. The net result of this symbiosis is the intracellular colonization of the plant with thousands of bacteria; a process that seems to occur in spite of the immune ability of plants to prevent pathogen infection. In this review, we discuss the potential of the invading rhizobial symbiont to actively avoid this innate immune response, as well as specific examples of where the plant immune response may modulate rhizobial infection and host range.

Keywords: legume, root nodule symbiosis, plant immunity, receptor-like kinase, nod factor, lipo-polysaccharides

OPEN ACCESS

Edited by:
Etienne Yergeau, National Research Council Canada, Canada

Reviewed by:
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Christian Staehelin, Sun Yat-sen University, China

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Specialty section:
This article was submitted to Plant-Microbe Interaction, a section of the journal Frontiers in Plant Science

Received: *03 April 2015*
Accepted: *19 May 2015*
Published: *02 June 2015*

Citation:
Tóth K and Stacey G (2015) Does plant immunity play a critical role during initiation of the legume-rhizobium symbiosis? Front. Plant Sci. 6:401. doi: 10.3389/fpls.2015.00401

Introduction

The root nodule symbiosis (RNS) is one of the most fascinating, yet not completely understood beneficial host–microbe interactions. RNS is limited to the FaFaCuRo (Fabales, Fagales, Cucurbitales, and Rosales) clade that belongs to Eurosid I plants (Kistner and Parniske, 2002). Under nitrogen limiting conditions, many legume plants are infected by nitrogen-fixing soil bacteria, termed rhizobia. Subsequent to an initial signal exchange between host and symbiont, the bacteria enter the host root usually through epidermal root hair cells. An infection thread (IT) of plant origin is formed that extends and eventually delivers the rhizobia into newly dividing cortical cells. These cells give rise to a nodule primordium that develops into the nodule, a new root organ. In the

nodule, bacteria differentiate into bacteroids, the nitrogen-fixing form of rhizobia, which reduces atmospheric dinitrogen into ammonia that is used by the host plant. In exchange, the bacteria receive a steady carbon source provided by plant photosynthesis.

Although the first observation of legume nodulation was reported a few 100 years ago, we still do not fully understand the underlying mechanisms that maintain a perfect balance between host and symbiont to allow such an intimate symbiosis to develop. Among the exciting new findings is a growing recognition that the plant immune system is active during RNS. In this review, we will point out recent observations to indicate when and how the host plant immune system acts to control nodule formation and host range.

Rhizobia are Part of a Diverse and Active Rhizosphere Microbiota

In the soil, there is an extremely large population of microorganisms that keep the soil ecosystem functioning. For instance, a metagenomics study of the *Arabidopsis thaliana* rhizosphere revealed 43 bacterial phyla and divisions (Bulgarelli et al., 2012). Microorganisms of the rhizosphere (part of the soil directly surrounding and impacted by the root) interact with the roots, providing nutrients and protection against biotic and abiotic stress. Specific rhizosphere microbes also have the ability to enter the root and become inter- or intracellular inhabitants, sometimes contributing to plant growth and development (Bulgarelli et al., 2012; Lundberg et al., 2012). Given the diversity of rhizosphere microbes and the potential threat for the plant, it is not surprising that plants have the ability to distinguish threatening intruders (i.e., pathogens) from beneficial microbiota.

Hundreds of different microorganisms are attached to the surface of a root. Leguminous plants under nitrogen limiting conditions secrete secondary metabolites (e.g., flavonoids) that can signal to and recruit compatible, symbiotic rhizobia (Oldroyd et al., 2011). Specific flavonoids act as inducers of the rhizobial nodulation genes, which encode the enzymes needed for synthesis of the lipo-chitooligosaccharide (LCO) nodulation factor [Nod factors (NF)], the key rhizobial signaling molecule that elicits the first plant responses in establishing RNS (Fisher and Long, 1992).

Parallels between Symbiont- and Pathogen-Triggered Responses

The term 'microbe-associated molecular pattern' (MAMP) is used for specific recognition signatures found in conserved molecules [e.g., bacterial flagellin, cell wall components like lipopolysaccharide, chitin and peptidoglycan (PGN)] derived from microbes, usually pathogens that infect both plants and animals (Ausubel, 2005). MAMPs are characterized by their ability to induce an innate immune response in the host. Therefore, NF is usually not considered a MAMP since it induces nodule formation on the host, as opposed to inducing immunity. However, NF can induce some responses that are normally associated with plant innate immunity (Day et al., 2001; Ramu et al., 2002; Pauly et al., 2006). This is perhaps not surprising since longer chain chitin oligomers (degree of polymerization > 6) are strong inducers of plant innate immunity (Liang et al., 2014). Unlike simple chitin, NF is a LCO molecule comprised of an *N*-acetylglucosamine backbone with site-specific decorations and an *N*-acyl chain (D'Haeze and Holsters, 2002). The addition of very low concentrations of NF (<10 nM) was shown to induce a variety of responses on the compatible legume hosts. These include plasma membrane depolarization, perinuclear calcium spiking, cytoskeletal changes, root hair deformation, induction and repression of gene expression and, in a few plant species, induction of nodule primordia (D'Haeze and Holsters, 2002; Oldroyd and Downie, 2008).

Responses elicited by MAMP perception have been well-studied in many plants (De Coninck et al., 2015). These include generation of reactive oxygen species (ROS), cytosolic Ca^{2+} elevations, activation of mitogen-activated protein kinase (MAPK) and calcium-dependent kinases, callose deposition and defense-related gene expression (Boller and Felix, 2009; Greeff et al., 2012). However, in comparison with leaves, less attention has been paid to MAMP responses in roots even though many pathogens do invade via roots. MAMP-triggered immune signaling in *Arabidopsis* roots occurs in a similar fashion to leaves (Millet et al., 2010; De Coninck et al., 2015). Roots of seedlings responded by callose deposition to MAMPs like flg22 (a peptide molecule originating from bacterial flagellin), PGN and chitin. Callose deposition was observed in the root elongation zone in response to flg22 and PGN, while chitin elicited callose deposition in the root maturation zone (Millet et al., 2010), indicating the ability of different root tissues to distinguish between these MAMPs (De Coninck et al., 2015).

Receptor-Like Kinases Involved in Symbiotic and/or Immune Signaling

Microbe-associated molecular patterns are recognized by pattern recognition receptors (PRRs) localized at the cell surface, including receptor-like kinases (RLK) and receptor-like proteins (Zipfel, 2014). The extracellular region of RLKs can be composed of lysin motif (LysM)-domains (LysM-RLK) and/or leucine-rich repeats (LRR-RLK), both of which are involved in microbe detection (Greeff et al., 2012).

NF is perceived by RLKs with an extracellular domain of 2–3 LysM domains, a single membrane-spanning region and an active or inactive intracellular kinase domain. These LysM-RLKs were identified in model legume species such as LjNFR1/LjNFR5 (NF Receptor 1 and 5) in *Lotus japonicus*, GmNFR1/GmNFR5 in soybean (*Glycine max*), and LYK3/NFP [Lysin motif receptor-like kinase 3 and NF Perception (NFP)] in *Medicago truncatula* (Amor et al., 2003; Limpens et al., 2003; Madsen et al., 2003; Radutoiu et al., 2003; Indrasumunar et al., 2010, 2011). Mutations in these genes significantly alter nodulation capability of the legume host.

The data suggest that the NF receptor is composed of a heterodimer or, perhaps, heterotetramer. LjNFR5 binds NF with higher affinity than LjNFR1 (Broghammer et al., 2012). However, LjNFR5 or MtNFP lack kinase activity (Arrighi et al., 2006; Madsen et al., 2011) and, therefore, likely signal by activation of the NFR1 or LYK3, respectively, kinase domain. Co-expression

of LjNFR1 and LjNFR5, as well as MtNFP and MtLYK3, in a heterologous *in planta* tobacco system induced strong defense responses in the absence of NF (Madsen et al., 2011; Pietraszewska-Bogiel et al., 2013). These responses were similar to those elicited by over-expression of CERK1 in *Arabidopsis*, another LysM-RLK. AtCERK1 has an active intracellular kinase domain and functions as a heterotetramer with AtLYK5, which lacks a functional kinase domain, to recognize long-chain chitooligosaccharides (dp > 6) to induce plant immune responses (Cao et al., 2014; Liang et al., 2014).

Recently, it was shown that the rice MAMP receptor OsCERK1, is also required for establishment of symbiosis with mycorrhizal fungi (Miyata et al., 2014; Zhang et al., 2015). Similar to rhizobia, establishment of this symbiosis also involves a LCO signal, called Myc factor, as well as short-chain chitooligosaccharides (dp < 6; Maillet et al., 2011; Genre et al., 2013). OsCERK1 displays the highest homology with LjNFR1. Therefore, a possible role of LjNFR1 and MtLYK3 in mycorrhization was tested with the results implicating both in the establishment of this symbiosis (Zhang et al., 2015). In *M. truncatula*, MtNFP was shown to be involved in the response to root oomycete pathogen *Aphanomyces euteiches*, *nfp* mutant plants were more susceptible to the oomycete than wild type plants (Rey et al., 2013). Indeed, recently, mutations in a number of *M. truncatula* symbiotic genes were shown to affect the ability of *Phytophtora palmivora* to infect roots; again emphasizing the overlap between symbiont and pathogen response (Rey et al., 2015).

Taken together, the data support the hypothesis that chitin and LCO reception are functionally related with the latter likely evolving from the more wide-spread and ancient chitin recognition system (Liang et al., 2014). The fact that, in some species, CERK1 and its orthologs function both in pathogen and symbiont recognition argue that this step may not be involved in discerning the beneficial or detrimental nature of the infecting microbe. This is a rather heretical view given the dogma from earlier studies that argued that LCO reception plays a key role in host range determination (Oldroyd et al., 2011).

Do Rhizobia Suppress the Plant Immune System?

The question whether the plant immune system might be involved in RNS is an obvious one considering the intimacy of the RNS (Fisher and Long, 1992). Unfortunately, this question has not received a great deal of direct, experimental examination. However, there are a number of observations that are consistent with a rapid, defense-like response occurring in legumes when infected by rhizobia (**Figure 1**). For instance, strong production of ROS was observed on alfalfa roots in response to the compatible symbiont *Sinorhizobium meliloti* (Santos et al., 2001). Transient and rapidly elevated ROS levels were observed on common bean *Phaseolus vulgaris* root hairs upon NF addition at physiological concentration (10^{-9}M; Cardenas et al., 2008). Silencing of NADPH oxidase, required for ROS production, resulted in aborted IT formation and reduced nodule numbers on common bean roots (Montiel et al., 2012). The results suggest that ROS production is necessary for infection initiation but prolonged, elevated levels could be detrimental to nodulation.

A hypersensitive, cell death response was also reported on alfalfa roots in response to *S. meliloti* (Vasse et al., 1993). These results are consistent with recent large-scale transcriptomic and phosphoproteomic studies, performed on soybean and *M. truncatula* in response to their symbiotic rhizobia or purified NF, that revealed rapid induction of defense-related gene expression, as well as phosphorylation of proteins known to be involved in plant immune responses (Libault et al., 2010; Nguyen et al., 2012; Rose et al., 2012).

The levels of salicylic acid (SA), a key secondary signal involved in plant innate immunity (An and Mou, 2011), were found to increase in alfalfa roots upon inoculation with NF-defective (*nodC* mutant) rhizobia (Martínez-Abarca et al., 1998). Indeed, transgenic roots in which SA levels were reduced by expression of NahG, showed increased rhizobial infection (Stacey et al., 2006). Similarly, a number of other phytohormones, also involved in plant innate immunity, can affect the RNS (e.g., jasmonic acid; Ding and Oldroyd, 2009).

If the plant does mount a defense response to invading rhizobia, then, by analogy to bacterial pathogens, it is possible that rhizobia also have the ability to actively suppress this response. Indeed, suppression of immune responses, such as ROS production and SA accumulation, was demonstrated in *M. truncatula* and *M. sativa* roots upon addition of NF (Martínez-Abarca et al., 1998; Shaw and Long, 2003). In addition, down-regulation of a *PR2* (pathogenesis-related protein) homolog in *M. truncatula* was reported in response to *S. meliloti* inoculation, while a *S. meliloti* mutant defective in NF synthesis failed to induce the same response (Mitra and Long, 2004). Surprisingly, NF application can suppress defense responses not only in legumes but also non-legumes, such as *Arabidopsis*, tomato, and corn. For example, *Arabidopsis* leaves pre-treated with flg22 elicit a strong innate immune response that was suppressed by addition of NF (Liang et al., 2013). These findings suggest that LCO/NF might have a dual role in actively inducing RNS development while also actively suppressing plant immunity, which could inhibit RNS (**Figure 1**).

Nodulation without Nod Factor Signaling Reveals a Key Role for Plant Innate Immunity in RNS

The dogma that existed for many years in the field of RNS research is that nodulation cannot occur in the absence of NF signaling. Thus, it was quite surprising when some rhizobia were found to nodulate specific *Aeschynomene* species in the complete absence of the nodulation genes, required for NF synthesis (Giraud et al., 2007). More recently, Okazaki et al. (2013) showed that a nodulation defective, *nfr1* mutant of the soybean cultivar Enrei could be nodulated by a *Bradyrhizobium elkanii* mutant unable to produce NF. Even more surprising was the finding that nodulation by this mutant was dependent on an active type III secretion system (T3SS). Microarray analysis revealed that symbiosis marker genes such as *ENOD40* and *NIN* were induced in the *nfr1* mutant suggesting T3SS-induced signaling

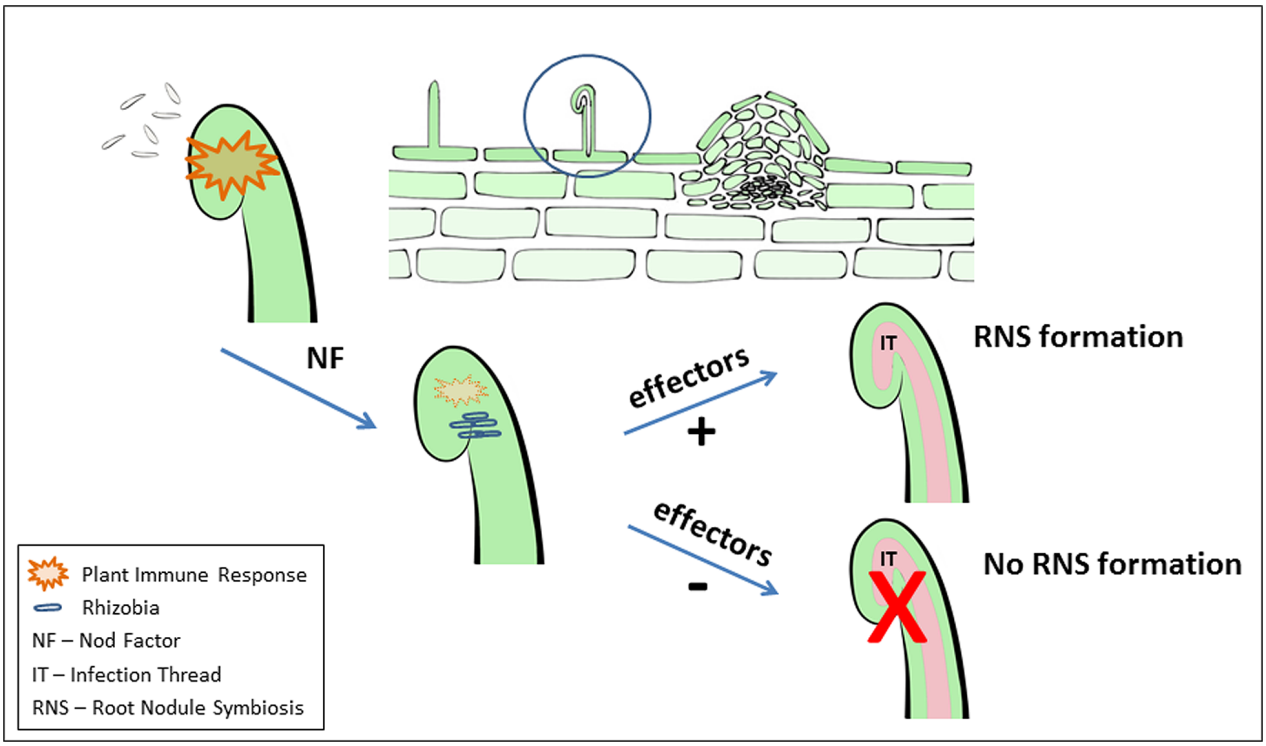

FIGURE 1 | Schematic illustration of elicitation and suppression of immune responses by Rhizobia during root nodule symbiosis (RNS) formation. As discussed in the manuscript, during the early events of initiation of the legume-rhizobia symbiosis, a plant immune response is induced (illustrated by orange). In addition to acting as a signal to induce RNS development the Nod factor signal also acts to suppress this immune response. Bacterial effector proteins (e.g., delivered through a T3SS) can act to either negatively or positively modulate RNS. Strictly for the purposes of illustration, these events are shown as acting on a single root hair. However, clearly the situation is much more complex and it is likely that the plant immune response can impact RNS at various steps during its development.

(Okazaki et al., 2013). In plant pathogens, the T3SS secretes effector proteins directly into the plant cell that can enhance infection or, when the appropriate R protein is present, induce effector-triggered immunity (ETI; Boller and Felix, 2009).

Effectors are directly or indirectly perceived by nucleotide-binding site-LRR (NBS-LRR) receptors encoded by R (resistance)-genes (Boller and Felix, 2009). In soybean, *Rj2* and *Rfg1* alleles were found to restrict nodulation in a strain-specific manner; that is, while *Rj2* prevents nodulation with certain *B. japonicum* strains, *Rfg1* restricts the symbiosis with at least one *S. fredii* strain (i.e., USDA257; Yang et al., 2010). Tsukui et al. (2013) showed that the incompatibility of *B. japonicum* (USDA122) with *Rj2* soybean genotypes is mediated by the T3SS. This type of strain-specificity seems very analogous to the race-specificity of plant pathogens that is known to be determined by ETI. Kimbrel et al. (2013) examined Type III effector genes in *S. fredii* and *B. japonicum* and found that these genes exhibit a high degree of conservation in comparison to those secreted by pathogens.

The results of Okazaki et al. (2013) stand out since, for the first time, they suggest that the T3SS and associated effector proteins play a central role in RNS establishment. However, it remains to be determined which of the various *B. elkanii* effectors are required for nodule formation on soybean cv. Enrei. There is a wealth of earlier literature that supports a role for rhizobial effectors in modulating host range. Much of this work was done using *Rhizobium sp*. NGR234, which exhibits a very extended host range providing a variety of host species on which to examine nodulation (Perret et al., 2000). For example, the effector NopL from *Rhizobium* sp NGR234, when expressed in tobacco and *L. japonicus* was shown to suppress pathogen induction of PR protein expression and to interfere with MAPK signaling (Bartsev et al., 2004; Zhang et al., 2011). The dominant *Rj4* allele in soybean encodes a PR protein that was found to restrict soybean nodulation with certain *B. elkanii* and *B. japonicum* strains. These strains were restricted in infection of the epidermal cell layers of wild soybean (*G. soja*) roots (Hayashi et al., 2014; Tang et al., 2014). Perhaps relevant to the work on soybean, the *S. fredii* effector NopP and the *B. japonicum* effectors NopE1 and NopE2 were shown to be directly transported into the host plant cells of *Vigna* roots (Schechter et al., 2010; Wenzel et al., 2010). Both NopE and NopT exhibit protease activity. *B. japonicum* effector NopT1 triggered cell death response when expressed in tobacco, while the NopT2 did not induce the same response (Dai et al., 2008; Kambara et al., 2009; Fotiadis et al., 2012). As mentioned earlier, strong ROS production was observed in response to NF application (Cardenas et al., 2008). NGR234 NopM (an E3 ubiquitin ligase) effector expressed in tobacco inhibited ROS

production, while inducing defense-related gene expression (Xin et al., 2012).

Published data suggest that the need for an active effector secretion system (e.g., T3SS) is widespread in legumes. For example, wild type *M. loti* (MAFF303099) is not able to infect *Leucaena leucocephala* (a mimosoid tree), while the T3SS mutant was able to efficiently nodulate this same species (Hubber et al., 2004; Sánchez et al., 2009). Not all rhizobia possess a T3SS but in these cases other systems may operate. For example, *M. loti* strain R7A, *S. meliloti* and *R. etli* possess a type IV secretion system (T4SS; Soto et al., 2006). Deletion of T4SS in *M. loti* strain R7A extended the nodulation host range to include *L. leucocephala*, which is not nodulated by the wild type strain (Hubber et al., 2004). On the other hand, mutation of the T4SS in *S. meliloti* did not seem to impact formation of a functional symbiosis on alfalfa roots (Jones et al., 2007).

Conclusion and Future Perspectives

Some 29 years ago, our laboratory published a review that sought to compare and contrast rhizobium, agrobacterium and pathogen infection of plants (Halverson and Stacey, 1986). Therefore, it is satisfying to now see how many interesting parallels have been documented between rhizobial–plant, mycorrhizal–plant, and pathogen–plant interactions. For example, MAMP signaling and the associated receptors are clearly relevant to these associations. It is now well accepted that LCO and chitin signaling share similar receptors, reflecting an evolutionary connection. Indeed, in some cases, the chitin receptor plays a dual role in recognizing plant fungal pathogens, while also promoting symbiotic development.

When well established dogma in any field gets overturned, it means that research progress is being made. An example in the rhizobial field is the realization that nodulation does not *sensu stricto* require NF production. In the case of soybean, nodulation can occur without NF but this requires an active T3SS. Although unidentified, the assumption is that rhizobial effector proteins are exported to the soybean host that is allowing nodulation to occur (**Figure 1**). The parallels to plant–pathogen interactions are clear, where effectors can either promote virulence or resistance. R proteins are clearly important in the rhizobial symbiosis, at least in modulating host range. At this point, the role of effectors and R proteins in RNS cannot be refuted. However, perhaps the more interesting question is whether these components are necessary, perhaps essential, for nodule formation either mediated by NF or not. The case in soybean cv. Enrei clearly argues for an essential role but could the research focus on NF signaling be hiding a general, essential role in RNS in other plant species?

Regardless of what form it may take, the available data clearly point to the need for more research that directly addresses the possibility of an important role for plant innate immunity in RNS. This aspect has been understudied for some time and sufficient evidence has now accumulated to strongly suggest that important information would come from such research. Using plant pathogen–host research as an example, one would expect that knowledge would emerge that could enhance the use of RNS in agriculture. For example: efforts to avoid inoculant competition with indigenous soil rhizobia that currently limits effectiveness or information that would increase nodulation under stressful environments or allow greater levels of biological nitrogen fixation.

Acknowledgment

We thank Johann Oliver Zinamon for helping with the Figure preparation. Research of the authors is supported by National Science Foundation (IOS/1025752)

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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MINI REVIEW
published: 14 September 2015
doi: 10.3389/fpls.2015.00722

Inter-organismal signaling and management of the phytomicrobiome

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The organisms of the phytomicrobiome use signal compounds to regulate aspects of each other's behavior. Legumes use signals (flavonoids) to regulate rhizobial *nod* gene expression during establishment of the legume-rhizobia N_2-fixation symbiosis. Lipochitooligosaccharides (LCOs) produced by rhizobia act as return signals to the host plant and are recognized by specific lysine motif receptor like kinases, which triggers a signal cascade leading to nodulation of legume roots. LCOs also enhance plant growth, particularly when plants are stressed. Chitooligosaccharides activate plant immune responses, providing enhanced resistance against diseases. Co-inoculation of rhizobia with other plant growth promoting rhizobacteria (PGPR) can improve nodulation and crop growth. PGPR also alleviate plant stress by secreting signal compounds including phytohormones and antibiotics. Thuricin 17, a small bacteriocin produced by a phytomicrobiome member promotes plant growth. Lumichrome synthesized by soil rhizobacteria function as stress-sensing cues. Inter-organismal signaling can be used to manage/engineer the phytomicrobiome to enhance crop productivity, particularly in the face of stress. Stressful conditions are likely to become more frequent and more severe because of climate change.

Keywords: inter-organismal signals, phytomicrobiome, plant agriculture, crop productivity, plant stress

OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Plant Biotic Interactions, a section of the journal Frontiers in Plant Science

Received: *30 April 2015*
Accepted: *28 August 2015*
Published: *14 September 2015*

Citation:
Smith DL, Praslickova D and Ilangumaran G (2015) Inter-organismal signaling and management of the phytomicrobiome. Front. Plant Sci. 6:722. doi: 10.3389/fpls.2015.00722

Background

The perspectives provided in this theme volume illustrate that members of the phytomicrobiome utilize inter-organismal signal compounds to affect the behavior of the plants they associate with, and signal compounds from the plants regulate the behavior of the phytomicrobiome. Presumably, one organism alters the behavior of another for its own benefit, but often to the benefit of the other organism as well, leading to mutualistic symbiosis. An example of this is improved stress tolerance in a plant by a signal compound from an associated microbe, where the resulting enhanced plant growth means expanded niche space and more reduced carbon for the specific phytomicrobiome member.

Signaling in the Legume-rhizobia Symbiosis

Plants must allow beneficial microorganisms to colonize near them or in their tissues in order to establish mutualistic relationships. This kind of close association (for example, the legume-rhizobia symbiosis, where rhizobia reside inside the legume roots) necessitates a filtering system in the plants, disallowing unsuitable microorganisms, perhaps pathogens that could harm their tissues. On the other side, a microbe entering a disadvantageous plant would risk being recognized

as unacceptable and killed. Signal/recognition compounds facilitate communication between mutually beneficial organisms and ensure continuum of their relationship until senescence. Flavonoids (examples: luteolin, 7,4′ dihydroxyflavone, quercetin, kaempferol, myricetin, genistin, etc.,) in the rhizosphere are constituents of root exudates and well studied for their function as legume-to-rhizobia signal compounds (Nelson and Sadowsky, 2015). Their structural diversity and substitutions in the carbon skeleton determines their characteristic function (Weston and Mathesius, 2013). The release of specific flavonoids (or mixtures) from a legume host is only recognized by certain rhizobial species, which partially determines the host-symbiont specificity. The flavonoids diffuse through the rhizobial membrane and bind to NodD proteins in rhizobia, which then activate transcription of *Nod* genes involved in synthesis of nodulation factors (NF; Hassan and Mathesius, 2012). Altered flavonoid profiles at different symbiosis stages regulate Nod factor synthesis (Dakora et al., 1993). Flavonoids also cause auxin accumulation in root tissues that initiates nodule formation and differentiation (Hassan and Mathesius, 2012). Flavonoids regulate development of nodules and phytoalexin resistance in rhizobia (Cooper, 2004). Thus, these signal compounds regulate the behavior of appropriate partner organisms down to the gene expression level.

A range of very diverse non-flavonoid compounds present in the root exudates also induces *Nod* genes in some rhizobia (Mabood et al., 2014): betaines (stachydrine and trigonelline; Cooper, 2007), aldonic acids (erythronic and tetronic acids), and jasmonates (jasmonate and methyl jasmonate; Mabood et al., 2006). The jasmonates have been commercialized and products are now available (http://agproducts.basf.us/products/vault-hp-plus-integral-for-soybeans-inoculant.html).

Activated rhizobial *Nod*-genes secrete signals (Nod factors) back to the plant: lipochitooligosaccharides (LCOs) and exopolysaccharides (EPS). LCOs are conserved at the core but are diverse due to degree of saturation and the substitutions (glycosylation or sulfation) in the N-Acetyl chain at both reducing ends and vary widely between different rhizobial species, which are essential for host plant specificity (Oldroyd, 2013). Genes at the loci of Nod factors perception encode receptor like kinases with N-Acetyl glucosamine binding lysine motifs (LsyM RLK), which include Nod factor receptors (NFR1), NFR5, LysM receptor kinase 3 (LYK3), Nod factor perception (NFP). NFR/NFP binds to NF and are essential in determining NF specificity of rhizobial symbionts and activation of nodulation signaling (Oldroyd, 2013). Signaling from the receptor complex generates calcium oscillations in the nucleus of cortical cells, which activate a localized protein, calcium and calmodulin (CaM) dependent serine/threonine protein kinase (CCamK), and phosphorylates CYCLOPS, which is required for rhizobial colonization and nodule development (Oldroyd, 2013). The rhizobial specific gene expression is regulated by the Nodulation signaling pathway (*NSP1* and *NSP2*) and encodes GRAS domain transcription factors involved in nodulation specific functions. They are associated with promoters of Nodulation inception genes (*NIN*) and early nodulation genes (*ENOD*) and ensure that nodulation is stimulated under appropriate circumstances (Kalo et al., 2005; Smit et al., 2005).

In some rhizobia-legume systems (for example, *Bradyrhizobium*, and *Glycine soja*) application of correct Nod factors (LCOs isolated from *B. elkanii*) trigger formation of complete and anatomically precise, albeit, empty nodules (Stokkermans and Peters, 1994). It is impressive that the external application of a signal compound can lead to complete organogenesis.

Although many parallels are observed in the signaling mechanisms, plants exhibit subtle regulatory pathways to establish mutualistic associations and protect from pathogenesis (Toth and Stacey, 2015). During rhizobial infection, legume defense responses are elicited in the early stages but suppressed soon after (Libault et al., 2010). Increased activation of mitogen activated protein kinase (MAPK) and production of reactive oxygen species were observed in legumes when inoculated with rhizobia (Jamet et al., 2007; Lopez-Gomez et al., 2012). Chitooligosaccharides, chitosan, lipopolysaccharides, and peptidoglycan associated with fungal and bacterial pathogens are recognized as microbe-associated molecular patterns (MAMPs) by pattern recognition receptors (PRRs) in the plant cell membrane (Dangl and Jones, 2001; Zipfel, 2014). Recognition of MAMPs is crucial for the activation of MAMP triggered immunity (MTI) in plants, which triggers expression of defense related genes, leading to structural hardening (callose formation) of plant tissues, accumulation of phytoalexins and antimicrobial peptides (Ahuja et al., 2012). NF remain active even after nodulation, suggesting a role in suppression of MTI (Liang et al., 2013). Exopolysaccharide of rhizobia (example succinoglycan from *Sinorhizobium meliloti*) is known to supress plant immunity (Aslam et al., 2008). LCO recognition has been evolved from a pathogenic role to symbiosis. Even though LCOs are structurally similar to chitin oligomers (MAMPs) and their recognition is mediated by LysM RLK, modifications in amino acid sequences of LysM RLK which confer specificity to recognition of LCO or chitin oligosaccharides (Nakagawa et al., 2011). For example, chimeric proteins in the ectodomain of chitin elicitor receptor kinase (CERK1) for chitin perception are replaced with ectodomain of NFR involved in NF recognition (Zhang et al., 2007).

Effector proteins secreted by pathogens trigger effector mediated immunity (ETI) in plants due to activation of resistance (R) genes encoding nucleotide-binding site—leucine rich repeat proteins (Jones and Dangl, 2006). Leucine rich repeats receptor like kinases (LRR—RLK) are involved in NF perception and nodule formation (Endre et al., 2002). Effectors are transported and injected into the host cytoplasm through type III (T3SS) and type IV (T4SS) secretion systems. Effector proteins of rhizobia (NopM of *S. fredii* NGR234, NopL from *S. fredii* USDA247) have been shown to facilitate colonization of rhizobia in roots, prevent MAPK signaling, supress the plant immune system, affect formation of nitrogen-fixing nodules, timing of nodule establishment and final number of nodules formed (Zhang et al., 2011). Interestingly, rhizobial NF, T3SS and T4SS depend on a common regulator activated by legume secreted flavonoids (Gourion et al., 2015).

Bacteroid differentiation inside the nodule is regulated by antimicrobial peptides (nodule cysteine rich peptides), which functions similar to plant defensins (de Velde et al., 2010).

The bacteroids are separated from the host by a symbiosome membrane and immune activity is modulated inside the nodules and the expression of defense related genes is relatively low (Limpens et al., 2013). The plant controls the duration of symbiosis and regulates the senescence of nodules and the suppression of plant immunity reverses during nodule senescence (Puppo et al., 2005). The number of nodules is controlled by the legumes through a process called autoregulation of nodules (AON; Mortier et al., 2012). Shoot derived signals involve production of cytokinins and downstream signaling to the roots regulates AON (Sasaki et al., 2014).

Rhizobia signaling and associations can be affected by other members of the phytomicrobiome, this is because they function together as a consortia exerting synergism, playing a vital role in plant growth, nutrient uptake, alleviation of abiotic stress, and protecting from disease. The more frequently studied co-inoculation partners of rhizobia are *Bacillus* species. Inoculation of *Rhizobium* with *Bacillus* strains improved root structure and nodule formation in bean, pigeon pea and soybean (Halverson and Handelsman, 1991; Petersen et al., 1996; Srinivasan et al., 1997; Rajendran et al., 2008). Inoculation of pea with *Bacillus simplex* 30N-5 and *Rhizobium leguminosarum* bv. *viciae* 128C53 increased root nodulation and plant growth (Schwartz et al., 2013). When pea plants carrying *DR5*::*GUS* promoter are co-inoculated with *B. simplex* 30N-5 and *R. leguminosarum* bv. *viciae* expression of GUS was higher in nodule meristems and young vascular bundles of developing nodules (Schwartz et al., 2013). *Azospirillum brasilense* co-inoculated with *R. tropici* on bean relieved negative effects of salt stress on *nod* genes transcription (Dardanelli et al., 2008). Co-inoculation of rhizobia and arbuscular mycorrhizal fungi (AMF) promoted growth of soybean under low phosphorous and nitrogen conditions, indicated by increase in shoot dry weight (Wang et al., 2011).

The legume-rhizobia symbiotic relationship tends to be less specific in tropical agriculture, involving much wider sets of rhizobial partners, while it is often quite specific in the temperate zones (Dakora, 2000). A wider range of rhizobia forming relationships with any given legume, and the more diverse signaling involved, may alter the effect of environmental conditions on the nitrogen-fixing symbiosis for that particular legume species. Exploitation of the rhizobia-legume symbiosis has occurred for over a century yet, there is considerable scope for improved understanding of this complex relationship in tropical zones.

Other Phytomicrobiome Signaling Systems

While the legume-*Rhizobium* symbiosis is well understood of signaling interactions, given its significance of biological nitrogen fixation, extensive research in other phytomicrobiome signaling systems has been conducted. Mycorrhizal symbiosis uses a signaling system similar to that of the legume-rhizobia symbiosis (Harrison, 2005; Oldroyd, 2013) and it plays a critical role in solubilisation of minerals and plant protection. In this association plants emit strigolactones, triggering production of Myc factors including LCOs by the fungus and stimulate hyphal branching (Bonfante and Requena, 2011). AMF have a broad host range and hence they produce diverse array of LCOs for recognition by the host plants. LysM RLK are also associated with mycorrhizal colonization (Young et al., 2011). It would be interesting to study the mechanisms employed by the plants to differentially recognize mycorrhizal and rhizobial LCOs.

Nitrogen fixing symbiotic association occurs between *Frankia* and actinorhizal plants. *Frankia* sp. colonizes roots of actinorhizal plants and induces root hair curling and nodule formation similar to those observed in legumes suggesting common symbiotic mechanisms but with important structural differences, particularly the signaling compounds produced by *Frankia* differ from rhizobia (Pawlowski and Bisseling, 1996; Gherbi et al., 2008).

Many plant growth promoting rhizobacteria (PGPR; example, *Bacillus*, *Pseudomonas*, *Serratia*, *Azospirillum*, *Acetobacter*, etc.,) secrete phytohormones, such as cytokinins, gibberellins, auxin, and ACC deaminase and influence plant growth and functions (Vessey, 2003). They are also capable of alleviating drought stress by promoting root growth and hampering stomatal conductance (Vessey, 2003; Gray and Smith, 2005). The phytomicrobiome also improves the uptake of nutrients by forming siderophores or solubilizing phosphates and other minerals (Vessey, 2003). Phytomicrobiome members synthesize and excrete a range of inter-organismal signal compounds that defend their host plant against pathogens and abiotic stresses: broad-spectrum antibiotics, lytic enzymes, organic acids and other metabolites, proteinaceous exotoxins and antimicrobial peptides (bacteriocins).

Several products of PGPR have been commercialized as biofertilizers and biocontrol agents owing to their diverse modes of action. There is considerable scope for application of phytomicrobiome signals in agriculture. For instance, *Bacillus thuringiensis* NEB17 produces the bacteriocin thuricin 17. Intriguingly, this peptide is also a bacteria-to-plant signal that stimulates the growth of many plants (Lee et al., 2009). Thuricin 17 (10^{-9} to 10^{-11} M) changes the hormone levels of *Arabidopsis* and soybean (increased IAA and SA) and causes profound alterations in the proteome (major increases in energy related proteins; Subramanian, 2014). Thuricin 17 almost completely overcomes the negative plant growth effects of salt stress (250 mM NaCl). For the producer bacterium *B. thuringiensis* NEB 17, thuricin 17 is a dual function peptide, acting both as a bacteriocin that reduces competition from closely related bacteria, and to enlarge the available niche space by promoting plant growth. *Bacillus subtilis* OKB105 contains genes (*yecA*, *speB*, *ACO1*) involved in synthesis of spermidine, a plant growth stimulating polyamine (Xie et al., 2014).

Bacterially produced lumichrome (breakdown product of riboflavin) accelerates leaf production, onset of stem elongation, and leaf area development (at a concentration of 5×10^{-9} M), leading to greater production of biomass in many plants (maize, sorghum, tomato, lotus), related to enhanced starch and ethylene metabolism. Adversely, 10-fold greater concentrations can retard plant growth and development (Matiru and Dakora, 2005; Gouws et al., 2012). Similar effects were observed in legumes (soybean, cowpea) in response to the signal compounds (lipopolysaccharides and lumichrome), suggesting their role in the nitrogen-fixing symbiosis. Lumichrome promotes nodulation and mycorrhizal establishment in legumes

(Dakora and Phillips, 2002). Lumichrome also helps plants deal with drought and salinity stress (Kanu and Dakora, 2009).

Quorum sensing signals including those of beneficial bacterial such as rhizobia (Zarkani et al., 2013) can elicit immune responses (Schenk et al., 2012; Hartmann et al., 2014), and change hormone profiles in plants, inducing those regulating growth responses and disease resistance (Hartmann and Schikora, 2012). Quorum sensing regulates mobility, virulence and biofilm formation in bacteria. Biofilm formation (bacteria embedded in a thick matrix of EPS, proteins and water) enables bacteria to adhere to host tissues. Biofilm improves plant growth, root proliferation (*Azospirillum* in wheat) and function in as biocontrol (*B. subtilis*, Farrar et al., 2014). In the case of *N*-acyl-homoserine lactones (AHL), the length of the lipid side chain dictates characteristics of the signal compound's activity (Schikora et al., 2011). Quorum sensing in the phytomicrobiome will be the subject of an upcoming Frontiers in Plant Science theme volume (Plant responses to bacterial quorum sensing signal molecules, topic editors Schikora A and Hartmann A).

Engineering the Phytomicrobiome

Given our intense reliance on higher plants for food and other resources, our expanding understanding of the phytomicrobiome associated with these plants, advances in genetic engineering and synthetic biology, it seems reasonable to consider "engineering" the phytomicrobiome to improve crop productivity, including enhancement of photosynthesis and growth, nutrient assimilation, disease and insect resistance and improved ability to resist increases in abiotic stresses likely to be associated with environmental disturbances, or even mitigating the impact of climate change through CO_2 sequestration. The host plant with its phytomicrobiome constitutes a holobiont (Hartmann et al., 2014), a collective community with broader genomic, proteomic, metabolomics and physiologic capacity, making it better able to adjust to environmental (biotic and abiotic) challenges. The potential to alter the composition of the microbial consortia residing near, on or in plant tissues has been explored through inoculation processes to some extent. The inoculation strategy to manipulate the microbiome focuses on co-inoculation of several strains of PGPR, arbuscular mycorrhizal fungi and other endosymbionts. Increase in the abundance of beneficial microbes in the rhizosphere (for example biofertilizers) has resulted in less disease incidence and high levels of microbial activity (Bunemann et al., 2006).

Understanding plant microbe interactions requires a holistic approach to analyze this complex and dynamic system. However, the difficulty to readily culture many members of phytomicrobiome (for example, obligate endosymbionts) in the laboratory can be overcome by culture independent techniques such as metagenomics, metaproteomics, and metabolomics and usage of next generation sequencing tools to understand the complexity of the phytomicrobiome (Bulgarelli et al., 2012; Quiza et al., 2015). Our ability to implement large-scale manipulations of the microbial populations is currently limited. Plant microbiome engineering facilitates modulation of nutrient cycling, synthesis of phytohormones, production of antibiotics (biocontrol agents), leading to improved plant growth and resistance to disease, insects, drought, salinity stress, etc. (Quiza et al., 2015). Introducing recombinant strains in the rhizosphere could improve the persistence of endogenous microbial population by horizontal gene transfer (Taghavi et al., 2005) and community level microbiome engineering could result in higher resilience across disruptive environments (Loreau et al., 2001). The ability to engineer the phytomicrobiome will be pivotal in furthering long-term sustainability of agricultural crop production and affecting related issues such as climate change, human health and global food security (Quiza et al., 2015). While we are progressing in our understanding of mechanisms involved in the interspecies interactions, nature of the complex relationships within the phytomicrobiome, role of the host plants and its microbiome as a holobiont (Lakshmanan et al., 2014), engineering the whole metaorganism is a promising strategy that finds application in nitrogen fixation, disease control, nutrient cycling and phytoremediation (Bakker et al., 2013; Bell et al., 2014).

It is clear that members of the phytomicrobiome exchange signal compounds that are effective at hormonal concentrations, so that inter-organismal, indeed, inter-kingdom exohormones are now understood to play a crucial role in controlling the growth, composition and development of plants, including the crop plants that we depend on as food sources. The commercial deployment of LCOs in non-legume crop plants (Souleimanov and Prithiviraj, 2002; Prithiviraj et al., 2003) indicates that there is enormous scope for application of these compounds, to help crop plants be more productive, and to remain productive under the more challenging environmental conditions of climate change. Indeed, many of the positive effects of phytomicrobiome signals on plant growth seem to involve activation of stress response systems. Understanding the mechanisms and consequences of signal interactions occurring between the phytomicrobiome and host plants and development of methods to manipulate these interactions for increased plant growth, is an important challenge for this century.

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Conflict of Interest Statement: The DS conducts research in collaboration with Inocucor Technologies, which manufactures and sells microbial consortia for inoculation on plants, where the research is funded through a Canadian Federal Government program (Mitacs) which levers industrial funding. The other authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 24 September 2015
doi: 10.3389/fpls.2015.00784

Mining the phytomicrobiome to understand how bacterial coinoculations enhance plant growth

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In previous work, we showed that coinoculating *Rhizobium leguminosarum* bv. *viciae* 128C53 and *Bacillus simplex* 30N-5 onto *Pisum sativum* L. roots resulted in better nodulation and increased plant growth. We now expand this research to include another alpha-rhizobial species as well as a beta-rhizobium, *Burkholderia tuberum* STM678. We first determined whether the rhizobia were compatible with *B. simplex* 30N-5 by cross-streaking experiments, and then *Medicago truncatula* and *Melilotus alba* were coinoculated with *B. simplex* 30N-5 and *Sinorhizobium* (*Ensifer*) *meliloti* to determine the effects on plant growth. Similarly, *B. simplex* 30N-5 and *Bu. tuberum* STM678 were coinoculated onto *Macroptilium atropurpureum*. The exact mechanisms whereby coinoculation results in increased plant growth are incompletely understood, but the synthesis of phytohormones and siderophores, the improved solubilization of inorganic nutrients, and the production of antimicrobial compounds are likely possibilities. Because *B. simplex* 30N-5 is not widely recognized as a Plant Growth Promoting Bacterial (PGPB) species, after sequencing its genome, we searched for genes proposed to promote plant growth, and then compared these sequences with those from several well studied PGPB species. In addition to genes involved in phytohormone synthesis, we detected genes important for the production of volatiles, polyamines, and antimicrobial peptides as well as genes for such plant growth-promoting traits as phosphate solubilization and siderophore production. Experimental evidence is presented to show that some of these traits, such as polyamine synthesis, are functional in *B. simplex* 30N-5, whereas others, e.g., auxin production, are not.

Keywords: coinoculations, *Bacillus simplex*, genome studies, rhizosphere, legumes

OPEN ACCESS

Edited by:
Donald Lawrence Smith, McGill University, Canada

Reviewed by:
Nai-Chun Lin, National Taiwan University, Taiwan
Balakrishnan Prithiviraj, Dalhousie University, Canada

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Specialty section:
This article was submitted to Plant Biotic Interactions, a section of the journal Frontiers in Plant Science

Received: *01 April 2015*
Accepted: *10 September 2015*
Published: *24 September 2015*

Citation:
Maymon M, Martínez-Hidalgo P, Tran SS, Ice T, Craemer K, Anbarchian T, Sung T, Hwang LH, Chou M, Fujishige NA, Villella W, Ventosa J, Sikorski J, Sanders ER, Faull KF and Hirsch AM (2015) Mining the phytomicrobiome to understand how bacterial coinoculations enhance plant growth. Front. Plant Sci. 6:784. doi: 10.3389/fpls.2015.00784

Introduction

Rhizosphere bacteria function as a consortium, synergistically protecting plants from disease (Kloepper et al., 2004), providing plants with essential nutrients (Pradhan and Sukla, 2005; Martínez-Hidalgo et al., 2014), and stimulating plant growth by producing growth-promoting factors (El-Tarabily et al., 2008; Merzaeva and Shirokikh, 2010). Rhizosphere bacteria are analogous to gut bacteria in mammals, which perform similar functions, and like gut bacteria, the microbes that live on and within plant tissues are indispensable for plant survival. Although the microbial composition of the root microbiomes for many plants is known (Schlaeppi et al., 2014), defining the mechanisms driving the microbe/plant synergism in the soil is challenging. This is because soil is complex and the experiments are difficult to perform. Thus, simpler models have been employed, such as using microcosms or rhizotrons and also limiting the number of plant and microbial species to be studied. This is especially true for specific interactions such as those involved in nitrogen fixation, where investigations of the interactions between nitrogen-fixing bacteria and other soil bacteria or fungi consist of coinoculating a legume plant with a rhizobium and a single plant growth promoting bacterial (PGPB) species. Such interactions usually result in an enhancement of plant growth over inoculation solely with rhizobia (see references in Schwartz et al., 2013).

The most frequent bacterial partners in coinoculation studies involving rhizobia are *Bacillus* species, including among others, *B. subtilis*, *B. amyloliquefaciens*, *B. licheniformis*, and *B. pumilus*. Earlier, we showed that coinoculating *Pisum sativum* L. with *Rhizobium leguminosarum* bv. *viciae* 128C53 and *B. simplex* 30N-5 resulted in better nodulation and an overall increase in plant dry weight (Schwartz et al., 2013). *B. simplex* 30N-5 is a relatively new player in the panoply of bacteria that positively influence plant growth. This species is mainly known for its phenotypic adaptations with respect to growing on the sun compared to shade walls of "Evolution Canyon" in Israel (Koeppel et al., 2008). However, a number of publications, including our own, have reported that *B. simplex* also functions as a PGPB species (Ertruk et al., 2010; Hassen and Labuschagne, 2010). Recently, the sequenced genomes of several *B. simplex* strains became available and allowed prediction of possible molecular mechanisms for the observed interactions. The essential extension of such genome comparisons include the identification of the expressed proteins, and perhaps most importantly, the identification of the small molecule products of their activity.

In this study, we coinoculated *B. simplex* 30N-5 with either *Sinorhizobium* (*Ensifer*) *meliloti* 1021 (alpha-rhizobium) or *Burkholderia tuberum* STM678 (beta-rhizobium), on their respective hosts. To our knowledge, *Bu. tuberum* STM678 (Moulin et al., 2001; Vandamme et al., 2002) has not been previously employed in coinoculation studies. To obtain a better understanding of the traits that are important for the plant responses in the coinoculation experiments, we analyzed the *B. simplex* 30N-5 genome for genes known to encode PGPB traits. To do this, we compared *B. simplex* 30N-5 with the well-established PGP *Bacillus* strains, namely *B. subtilis* GB03, *B. amyloliquefaciens* subsp. *plantarum* FZB42, and others. In this report, we also demonstrate that several of these PGPB traits are functional in *B. simplex* 30N-5.

Materials and Methods

Phylogenetic Analysis

Nucleotide sequences were obtained from the Joint Genome Institute (IMG/ER) database for microbial genomes (Markowitz et al., 2012). Five housekeeping genes *atpD*, *urvA*, *rpoB*, *lepA*, and *recA* were used to construct concatenated sequences (Table S1). The concatenated gene sequences were aligned with Clustal X (Thompson, 1997), and phylogenetic distances were calculated according to the Kimura two-parameter model (Kimura, 1980). The phylogenetic tree topology was inferred from the maximum-likelihood method employing MEGA5 (Tamura et al., 2011). Confidence levels on each node are the product of 1000 bootstrap replicates.

Growth of Bacteria

Bacillus strains were grown on LB (Luria-Bertani; Miller, 1972), Tryptic Soy Agar (TSA; Difco®, Becton Dickenson) or Tryptone Yeast Extract (TY; Beringer, 1974) medium at 30°C or 37°C. Rhizobial strains were cultured at 30°C on either Yeast Mannitol Agar (YMA; Somasegaran and Hoben, 1994) or on TY medium with or without 10 μg/mL tetracycline. *Bu. tuberum* STM678 was grown on LB minus salt or on BSE medium (Caballero-Mellado et al., 2007) with or without antibiotics. Cell density was determined from the OD_{600} nm of the cultures. The bacterial strains studied in this report are listed in **Table 1**.

To introduce fluorescent markers into *Sinorhizobium* (*Ensifer*) *meliloti* 1021, the plasmid pHC60 (Cheng and Walker, 1998) carrying a green fluorescent protein (GFP) construct was mobilized into *S. meliloti* using a triparental mating procedure (Figurski and Helinski, 1979) as adapted by Schwartz et al. (2013). The *Bu. tuberum* STM678 GFP+ strain was a gift from Dr. J. Peter Young (University of York).

The Voges-Proskauer test (Voges and Proskauer, 1898) was performed as modified by Werkman (1930) and Barritt (1936). Each strain was tested three times.

Chemical Analysis

Cell pellets (1.8×10^9 cells/sample) from *B. simplex* 30N-5 were lysed in 5 ml of either methanol or aqueous trifluoroacetic acid (TFA, 10%) or aqueous trichloroacetic acid (TCA, 8.3%). The homogenates were centrifuged (16,000 × g, 5 min, room temperature) and the supernatants were taken to dryness in a vacuum centrifuge. The dried residue was resuspended in water (500 μL), centrifuged (16,000 × g, 5 min, room temperature) and the supernatant transferred to LC injector vials. For the polyamines, aliquots of the supernatant were injected (8 μl) onto a reverse phase HPLC column (Phenomenex Kinetex C18 100 × 2.1 mm, 1.7 μ particle size and 100 Å) equilibrated in 80% solvent A (0.1 mM perfluoro-octanoic acid in water) and 20% solvent B (0.1 mM perfluoro-octanoic acid in methanol), and eluted (100 μL/min) with an increasing concentration of solvent B (min/%B; 0/20, 5/20, 15/75, 20/75,

TABLE 1 | Strains and plasmids used in this study.

Strain number	Species name and relevant characteristics	Source or reference
30N-5	*Bacillus simplex*	Schwartz et al., 2013
237	*Bacillus simplex*	Kaplan et al., 2013
11	*Bacillus simplex*	Kaplan et al., 2013
FZB42	*Bacillus amyloliquefaciens* subsp. *plantarum*	*Bacillus* Stock Center
DSM13 Goettingen/ATCC 14580	*Bacillus licheniformis*	*Bacillus* Stock Center
GB03	*Bacillus subtilis*	*Bacillus* Stock Center
NRRL B-4317	*Paenibacillus polymyxa*	*Bacillus* Stock Center
60b4	*Bacillus subtilis*	Flora Pule-Meulenberg
26a1	*Bacillus cereus*	Flora Pule-Meulenberg
HB101	*E. coli*	Cathy C. Webb
Rm1021	Wild-type *Sinorhizobium meliloti*	Lab strain
Rm1021/pHC60	GFP+, Tet^r derivative of wild-type *S. meliloti*	This study
STM678	Wild-type *Burkholderia tuberum*	Moulin et al., 2001; Vandamme et al., 2002
STM678/TnGFP	Tet^r derivative of wild-type *Bu. tuberum*	Elliott et al., 2007
Plasmids	**Relevant characteristics**	**Source or Reference**
pHC60	GFP plasmid, Tet^r	Cheng and Walker, 1998

22/20, 30/20). The effluent from the column was directed to an electrospray ion source connected to a triple quadrupole mass spectrometer (Agilent 6460) operating in the positive ion tandem mass spectrometric (MS/MS) mode, and the time-dependent intensity of multiple reaction monitoring (MRM) transitions were recorded at previously optimized settings [spermine, m/z (MH^+) 203➔129, 112, 84, fragmentor 55, collision energy 16; spermidine, m/z (MH^+) 146➔129, 112, and 72, fragmentor 55, collision energy 12; putrescine, m/z (MH^+) 89➔72, fragmentor 40, collision energy 4]. Peak areas for each compound at the corresponding retention times (spermine, spermidine, and putrescine at 16.4, 16.0, and 15.6 min, respectively) were computed with instrument manufacturer-supplied software (Agilent MassHunter). A standard curve was prepared with each experiment from samples containing known concentrations of all three compounds using the signals for the most intense MRM transitions (203➔112, 146➔112, and 89➔72 for spermine, spermidine, and putrescine, respectively), and the amount of each amine in each biological sample was calculated by interpolation from the standard curves. Under the prescribed conditions, the limit of detection for the amines was about 1 pmol injected for spermine and spermidine and 10 pmol injected for putrescine.

For indole acetic acid (IAA, auxin), aliquots of the supernatants were injected (8 µl) onto a mixed cationic/anionic/reverse phase HPLC column (Imtakt Scherzo SS-C18, 100 × 2 mm, 3 µ particle size and 130 Å pore size) equilibrated in 40% solvent C (water/acetonitrile/formic acid, 97/3/0.1, all by vol) and 60% solvent D (45 mM aqueous ammonium formate/acetonitrile, 65/35, v/v), and eluted (200 µL/min) with an increasing concentration of solvent D (min/%D; 0/60, 5/60, 20/100, 22/60, 30/60). The effluent from the column was directed to the same ESI mass spectrometer as described above, and the time-dependent intensity of the IAA MRM transition was recorded at previously optimized settings [m/z (MH^+) 176➔130, fragmentor 45, collision energy 12]. Peak areas for the transition response at the corresponding retention time (10.1 min) were computed as described above. A standard curve was prepared with each experiment from samples containing known concentrations of IAA. Under the prescribed conditions, the limit of detection (LOD) for IAA was about 5 pmol injected, which was about four-fold lower than what could be achieved in the negative ion mode also under previously optimized conditions by monitoring the transition of the $(M\text{-}H)^-$ ion at m/z 174➔130. Also, the LOD using combined liquid chromatography-MS/MS-MRM (LC/MS/MS-MRM) in either the positive or negative ion mode was significantly lower than what could be achieved by combined gas chromatography/mass spectrometry (GC/MS) in the selected ion-monitoring (SIM) mode (Waters GCT) of the trimethylsilyl derivative.

Cross-streaking Experiments

Fresh samples of each bacterial strain were taken from frozen cultures and grown on either LB minus NaCl, LB, or TY agar for cross streaking (Lertcanawanichakul and Sawangnop, 2008). A single colony from one strain was first streaked vertically down the middle of the plate and 24 h later the second strain was streaked perpendicularly to the first. The order of microbes was changed in each experiment, which was repeated 4 times with 3 or 4 biological replicates. Qualitative data were obtained by photographing the plates daily for 7 days. For the pairs of *S. meliloti* and *B. simplex*, we also performed parallel streaking and overlapping streaking experiments. As before, the order of microbes was changed in each experiment, and the plates were followed for 10 days.

Plant Coinoculation Experiments

Macroptilium atropurpureum (siratro), *Medicago truncatula* A17, and *Melilotus alba* L. U389 (white sweetclover) seeds were planted in black polyethylene boxes (Really Useful Boxes®) or Magenta® jars (Magenta Corp.). The substrate used for the black boxes was Seramis® (Mars GmbH) and perlite, and for the Magenta jars, a 2:1 mixture of vermiculite and perlite. The substrates were autoclaved and then watered with 1/4 strength Hoagland's medium minus nitrogen (Machlis and Torrey, 1956). Prior to planting seeds were scarified for 1 min, soaked in 95% ethanol for 5 min, and then in full-strength commercial bleach for 30–45 min. The conditions for siratro seed sterilization and inoculation with *Bu. tuberum* are detailed in Angus et al. (2013). The imbibed seeds were transferred to the boxes or Magenta jars using sterile tools, and inoculated singly with *Bu. tuberum* (siratro) or *S. meliloti* (*Medicago* and *Melilotus*) and *B. simplex* 30N-5, together and alone. The bacteria were diluted with

sterile water to a final OD_{600} nm of 0.1–0.2. The siratro seeds were coinoculated with a 1:1 mixture of *Bu. tuberum* and *B. simplex* 30N-5 whereas the *Melilotus* and *Medicago* seeds were coinoculated with a 1:1 mixture of *S. meliloti* 1021 and *B. simplex* 30N-5. Each Magenta jar or black box was inoculated with 4 mL of the inoculum. Controls were included for all experiments and were used as a phenotypic reference for −N, +N, and no nutrient conditions (water). The control sample size was smaller than the experimental due to space limitation, but the controls consistently gave the same phenotype (see Angus et al., 2013, 2014). The siratro plants were grown in a temperature controlled Conviron growth chamber at 24°C and the *S. meliloti* hosts in a Percival growth cabinet at 21°C. The *Medicago* and *Melilotus* species were harvested 5 weeks after inoculation and the siratro plants 5–6 weeks after inoculation. Each experiment was repeated three times. The plants were photographed, their shoot height and nodule numbers were recorded, and they were then dried (48 h, 65°C) before dry weight measurements were made. Statistical significance of the data was validated using One-way ANOVA with Tukey's post hoc test (**Figure 3A**) and multiple comparison procedure. Jittered boxplot and family-wise error rates (**Figure 3B** and Supplementary Figure 2) were used for assessment (Herberich et al., 2010).

Genome Analysis

Selection of Strains

Draft and finished genome sequences of several PGPB from the Joint Genome Institute IMG/ER (Markowitz et al., 2012) or from NCBI (http://www.ncbi.nlm.nih.gov) were queried by BLAST (Altschul et al., 1990) using sequences of genes encoding known PGPB traits. Thirteen *bona fide* PGP *Bacillus* and two *Paenibacillus* strains were chosen for comparison against *B. simplex* 30N-5 (**Figure 1**). The JGI genomes queried included *B. simplex* 30N-5 (permanent draft), *B. simplex* II3b11 (permanent draft), *B. firmus* DS1 (permanent draft), *B. licheniformis* DSM 13^T/ATCC 14580 (finished), *B. kribbensis* DSM 17871 (permanent draft), *B. megaterium* DSM 319 (finished), *B. amyloliquefaciens* subsp. *plantarum* $FZB42^T$ (finished), *B. subtilis* GB03 (permanent draft), *B. subtilis subtilis* 168 (finished), *B. cereus* JM-Mgvxx-63 (permanent draft), *B. thuringiensis* sv. *israelensis* (permanent draft), *Paenibacillus polymyxa* ATCC 12321 (permanent draft), *Paenibacillus pini* JCM16418 (permanent draft), *Pseudomonas fluorescens* strains A506 and CHAO (finished), and *Azospirillum brasilense* FP2 (permanent draft) and *Azospirillum* sp. B510 (permanent draft). *B. simplex* 30N-5 was isolated from the Mildred E. Mathias Botanical Garden at UCLA and strain II3b11 belongs to the Putative Ecotype 9 (Koeppel et al., 2008). It originates from the south facing, hot or "African savannah-like" slope of "Evolution Canyon II" in Nahal Keziv, Israel (Sikorski and Nevo, 2005). In addition, the genome of *B. simplex* strains P558 (Croce et al., 2014) and BA2H3 (Khayi et al., 2015), both from NCBI, were queried when a gene from one or both IMG/ER *B. simplex* strains was missing. Details of the *Bacillus* strains studied for the genomic analysis are found in **Table 2**.

Homologous Gene Identification

A curated set of more than 50 PGP genes (47 are displayed in **Figure 1**), all manually annotated in the *B. simplex* 30N-5 genome, were selected for use as the reference query genes for

IMG Gene ID	2517086222	2517082171	2517083001	2517084988	2517087511	2517086498	2517082191	2517084874	2517086977	2517084319	2517085602	2517086486	2517082448	2517083200	2517083201	2517084218	2517085369	2517085791	2517087099	2517084403	2517084404	2517084405	2517082809	2517083675	2517083677	2517085644	2517085649	2517086235	2517086236	2517086237	2517086410	2517086411	2517086412	2517086589	2517086590	2517086591	2517086592	2517086843	2517086844	2517086845	2517086846	2517084639	2517084640	2517084641	2517084642	2517084643	2517084644
Category	P solubiliz'n	hormones						antimicrobial peptides (serine proteases)						polyketide-related						antibiotics			siderophores; related to iron acquisition																		volatiles					
Gene	*gcd*	*aofH*	*yhcX*	*dhoS*	*ipdC*	*miaA*	*acdS*	*lactocepin*	*VprR*	*ser. protease*	*ser. protease*	*WprA*	*ser. protease*	*ABC transporter*	*ATPase comp.*	*disulfide isomer.*	*disulfide isomer.*	*squal.-hop.cycl.*	*disulfide isomer.*	*SagA-domain*	*bacteriocin syn.*	*SagB-domain*	*yusV**	*permease*	*periplasmic*	*sid. Synthetase*	*sid. Synthetase*	*ATPase*	*permease*	*periplasmic*	*permease*	*permease*	*periplasmic*	*yfmC**	*yfmD**	*yfmE**	*yfmF**	*permease*	*permease*	*Periplasmic*	*ATPase*	*acoA*	*acoB*	*acoC*	*acoL*	*butA, budC*	*acoR*
Bacillus amyloliquefaciens plantarum FZB42	nd	nd	79	75	33	60	nd	50	44	42	42	43	44	45	36	54	32	42	64	nd	28	31	73	31	nd	nd	nd	37	62	54	45	42	30	37	49	39	57	45	44	34	64	74	80	54	54	43	46
Bacillus subtilis subtilis 168	nd	54	80	74	31	60	nd	47	63	36	42	47	42	45	37	53	33	41	61	nd	nd	nd	73	28	nd	nd	nd	41	64	57	59	58	57	57	64	61	69	46	42	37	63	74	80	57	53	41	52
Bacillus licheniformis DSM 13 Goettingen	51	57	80	75	24	60	nd	44	43	46	41	48	46	44	40	51	33	44	59	45	62	nd	70	30	nd	63	58	50	63	61	60	59	62	42	49	48	57	49	46	37	63	76	81	56	57	45	53
Bacillus kribbensis DSM 17871	nd	50	84	84	62	66	nd	54	53	52	45	47	39	55	35	55	50	61	nd	nd	nd	nd	49	33	nd	nd	nd	40	56	52	40	37	29	28	41	40	50	45	39	52	58	37	43	32	40	39	44
Paenibacillus polymyxa ATCC 12321	nd	nd	79	75	33	59	nd	50	44	37	42	43	41	46	36	54	32	43	63	nd	27	31	73	31	nd	nd	nd	37	62	53	45	42	30	34	49	39	57	45	44	34	64	74	80	54	54	43	47
Bacillus subtilis GB03	nd	nd	79	75	34	59	nd	47	44	42	42	44	41	46	36	54	32	42	63	nd	nd	nd	73	30	nd	nd	nd	37	62	54	45	42	31	38	49	39	57	45	44	34	63	74	80	54	53	43	47
Bacillus pumilus S-1	nd	nd	78	75	25	61	27	51	49	41	42	43	44	42	38	47	47	39	24	nd	nd	nd	69	34	nd	62	56	61	61	53	47	50	44	30	49	41	58	48	46	66	63	79	81	58	59	43	52
Bacillus thuringiensis sv. israelensis	nd	52	27	77	32	64	80	44	59	40	39	38	39	25	39	46	47	42	76	47	61	57	67	31	29	nd	47	36	37	29	61	62	58	32	47	43	57	75	70	73	63	36	44	33	39	38	44
Bacillus cereus JM-Mgvxx-63	nd	50	28	77	38	nd	80	44	39	40	36	38	39	nd	40	47	32	32	75	nd	nd	nd	67	32	28	nd	nd	38	38	29	61	61	59	33	47	41	57	74	70	73	63	74	85	60	58	38	53
Bacillus megaterium DSM 319	nd	49	79	75	24	65	66	41	45	39	55	50	48	47	37	62	49	48	40	nd	nd	nd	71	34	nd	56	47	44	55	48	61	57	59	43	53	48	55	73	76	73	86	78	86	56	62	37	55
Bacillus panaciterrae DSM 19096	nd	57	80	80	24	68	88	30	52	54	41	42	40	47	39	48	32	57	51	65	79	72	71	34	27	nd	nd	64	70	65	65	61	61	33	45	41	57	43	42	35	50	38	44	33	41	37	57
Paenibacillus pini JCM 16418	40	nd	63	44	66	50	nd	30	40	35	39	42	36	29	40	nd	58	nd	29	nd	nd	nd	69	34	23	nd	nd	54	44	34	55	55	29	49	50	58	79	84	69	87	36	45	30	40	37	nd
Bacillus firmus DS1	58	71	86	83	24	66	30	59	55	41	42	41	40	43	76	68	54	58	nd	nd	nd	nd	73	39	26	62	55	64	69	61	74	73	61	30	43	45	55	79	77	69	84	38	45	32	39	41	53
Bacillus simplex 30N-5	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
Bacillus simplex II3b11	97	57	99	100	99	98	nd	99	98	40	98	99	43	99	97	nd	98	nd	97	95	99	97	99	98	99	nd	nd	99	99	98	99	98	98	97	98	99	99	99	99	98	98	99	100	97	97	97	98

FIGURE 1 | Homologs of reference set of Plant Growth Promoting (PGP) genes identified in *Bacillus* and *Paenibacillus*. Top row, IMG gene ID number; second row, general categories of PGP genes; third row, PGP genes identified in each *Bacillus* (or *Paenibacillus)* strain (left column) following comparison with the *B. simplex* 30N-5 gene (always 100%) and clustered using the K-means algorithm. The highest sequence identity alignment from blastp searches (% values in cells), sequence identities passing the 50% cutoff (cells highlighted in purple) show 3 clusters: cluster 1 (blue), cluster 2 (orange), and cluster 3 (purple). Genes not detected (nd). *Gene names from *B. megaterium*, *B. licheniformis*, and *B. amyloliquefaciens* (see text).

TABLE 2 | Genomic features of the *B. simplex* 30N-5 genome and comparison with the genomes of other *Bacillus* spp.

	B. simplex 30N-5	*B. simplex II3b11*	*B. thuringiensis sv. israelensis*	*B. pumilus S-1*	*B. kribbensis DSM 17871*	*B. firmus DS1*	*B. amyloliquifaciens pl. FZB42*	*B. licheniformis DSM 13/ATCC 14580*	*B. subtilis GB03*	*B. megaterium DSM 319*
Genome size (bp)	5459036	5582948	5643051	3692073	5054217	4971242	3918589	4222645	3849547	5097447
G+C content (mol%)	40.43	40.27	35.18	41.26	42.93	41.46	46.4	46.19	46.55	38.13
Protein-coding sequences	5288	4841	5349	3786	4914	4922	3693	4196	3705	5100
% of coding region	81.54	70.30	81.27	88.92	81.66	85.02	88.0	88.13	89.61	83.04

blastp homolog searches. The genes represented a wide variety of PGP functions. Because several of the genomes investigated have permanent draft status, our analyses of gene homologies occasionally found no matching gene; these "missing" genes are indicated by "n.d.". We used conservative criteria to compare the protein sequences. The blastp searches were filtered to include alignments with an $e < 10^{-5}$, and with a sequence identity of $\geq$50%, although homologs having smaller percentages and greater *e*-values were present in the other *Bacillus* genomes. *B. simplex* 30N-5 was the reference genome for the comparisons and the value of its gene identities for the comparison was set at 100%. Although both Gram-negative and Gram-positive bacteria were initially screened for PGP traits, the values for the Gram-negative bacteria as well as for the more distantly related Gram-positive species were generally low and hence deleted from the final data set.

We also used BAGEL3 (http://bagel2.molgenrug.nl/) to query the *B. simplex* 30N-5 genome for bacteriocins, and a stand-alone version of ANTIsmash (http://antismash.secondarymetabolites.org/) to search for non-ribosomal peptide synthetases (NRPSs).

K-means Clustering

The sequence identity matrix (**Figure 1**) contains 13 *Bacillus* strains and 2 *Paenibacillus* strains (rows) with the highest detected sequence identity for each reference PGP gene displayed in columns. Despite the 50% cutoff sequence identity limit, some sequence identity scores under 50% were also recorded because blastp sequence alignments with low sequence identity may still exhibit homology or contain conserved functional domains. This scheme enabled clustering, using the K-means clustering algorithm, to place *Bacillus* strains into groups that had overall similar profiles of PGP genes either as present or absent. The algorithm was implemented with an objective function to minimize the within-cluster Euclidean distance of the sequence identity vectors (rows) from their assigned clusters.

Our implementation of the K-means algorithm used a two-step iterative algorithm (Lloyd, 1982; Slonim et al., 2013). In the assignment step, the sequence identity vectors (rows) were assigned to the nearest cluster (measured by Euclidean distance between each sequence identity vector and centroid corresponding to each cluster). In the update step, the coordinates of each centroid were updated to the mean of the respectively assigned sequence identity vectors. The maximum number of iterations permitted was 10,000. Initial centroids were randomly assigned to the sequence identity vectors, and 100 random centroid initializations were run. Of the 100 K-means runs, the cluster arrangement minimizing the total Euclidean distance of the sequence identity vectors (rows) from their assigned clusters was retained for visualization (**Figure 1**).

Results

Phylogenies

A concatenated gene Maximum Likelihood phylogeny of the selected strains is shown in **Figure 2**. The housekeeping genes used for the tree are representative of the differences between the genomes of the species tested, as the most closely related species to *B. simplex* are also those with most similarities found in the PGPB genes studied (see colors in **Figure 1**). Although the topology within the clade was supported by high bootstrap values, the two subclades in the top part of **Figure 2** were supported by a low bootstrap value (52%). One subclade contained *B. amyloliquefaciens* subsp. *plantarum* FZB42, *B. subtilis* GB03, *B. subtilis subtilis* 168, *B. licheniformis* DSM13 Goettingen (ATCC 14580), and *B. pumilus* S-1, whereas the second subclade included the two *B. simplex* strains, and *B. firmus* DS1 and *B. kribbensis* DSM 17871. In this tree, *B. subtilis* GB03 and *B. amyloliquefaciens* subsp. *plantarum* FZB42 clustered together. A not-as-strongly supported branch of the top clade (54% bootstrap support) included *B. megaterium* DSM 319. The clade (bottom part of **Figure 2**) brought together with strong support, *B. panaciterrae* DSM 19096, *B. cereus* JM-Mgvxx-63, and *B. thuringiensis* sv. *israelensis*. *P. pini* JCM 16418 was the outgroup.

Pre-coinoculation (Cross-Streaking) Assays

Earlier we reported positive effects on pea growth when *B. simplex* 30N-5 was coinoculated with *R. leguminosarum* bv. *viciae* 128C53 (Schwartz et al., 2013). Before setting up coinoculation experiments with a different set of bacteria, cross-streaking assays were used to detect incompatibility or interference between the nodulating strains, *S. meliloti* 1021 (data not shown) and *Bu. tuberum* STM678 (Supplementary Figure 1A), to be used in the coinoculation study with *B. simplex* 30N-5. No inhibition

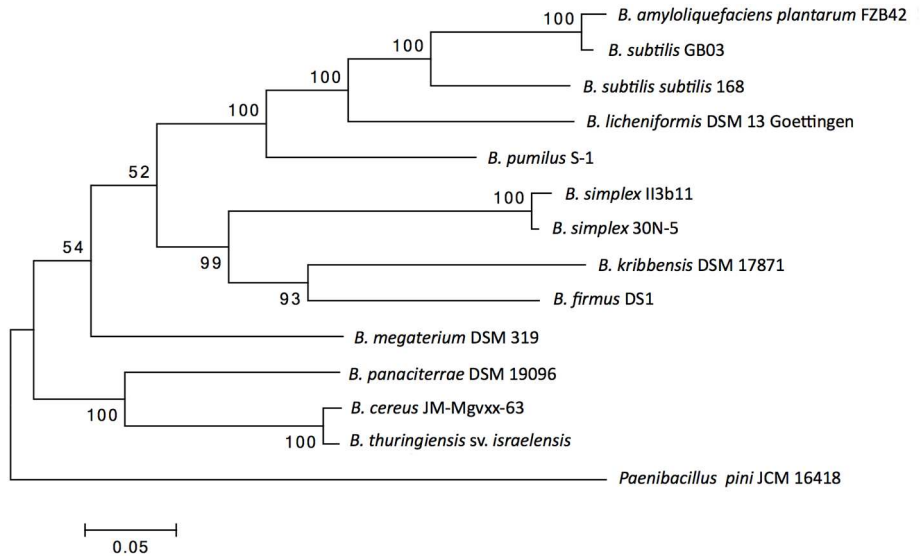

FIGURE 2 | Phylogenetic tree. Maximum-likelihood phylogenetic tree based on concatenated gene sequences of five housekeeping genes (*atpD*, *urvA*, *rpoB*, *lepA*, and *recA*). *Paenibacillus pini* JCM 16418 was used as the outgroup. Numbers at branch points indicate bootstrap values (based on 1000 replicates); only those above 50% are indicated. Bar, 0.05 substitutions per nucleotide position.

of growth was found. An additional *Bacillus* strain previously isolated and studied (Schwartz et al., 2013), *B. subtilis* 30VD-1, was also tested in these experiments. *B. subtilis* 30VD-1 inhibited *B. simplex* 30N-5 growth and *vice versa*, suggesting that one or both synthesized bacteriocins or other antimicrobial agents (Supplementary Figure 1A and see later section).

For *S. meliloti*, the results from the initial cross-streak experiments were less clear because although the *S. meliloti* streak was not touching the *B. simplex* one, it was closer to it than the distance observed for the *B. subtilis* and *B. simplex* cross-streaks (Supplementary Figure 1A). When we repeated the experiments by either doing a side-by-side streak or inoculating one strain over the other in a cross pattern, we observed no incompatibility between the two strains (data not shown).

Coinoculation Studies

Because *B. simplex* 30N-5 demonstrated a positive effect on both plant growth and rhizobial nodulation on pea (Schwartz et al., 2013), we tested whether or not this was a general phenomenon by coinoculating *B. simplex* 30N-5 and *S. meliloti* Rm1021 onto roots of *M. truncatula* and *M. alba*. In contrast to our previous results with pea, *M. alba* exhibited no significant growth enhancement when inoculated with *B. simplex* alone over the uninoculated control (**Figure 3A**). Although shoot height and nodule number were measured for all the conditions examined, no statistical significance was observed when the experimental treatments were compared with their respected controls (data not shown). Moreover, when single inoculations with *S. meliloti* and coinoculations with both strains were compared, the treatments (measured as dry weight increase) did not differ from each other although both were statistically different from the uninoculated and *B. simplex* alone-inoculated plants (**Figure 3A**). *M. truncatula* exhibited a similar response (data not shown). Overall, we found that dry weight increases were a more reliable measurement of plant biomass accumulation than any other parameters (see next section).

Siratro plants were coinoculated with *B. simplex* and *Bu. tuberum*; the latter nodulates siratro effectively (Angus et al., 2013). In contrast to the *S. meliloti* host plants, simultaneous coinoculation with *B. simplex* and *Bu. tuberum*, or coinoculation with *B. simplex* first and then *Bu. tuberum* 5 days later resulted in significant changes over the controls and were comparable to or better than the +N control. The siratro plants inoculated with *B. simplex* alone also exhibited an increase in dry weight over the −N control and were comparable to the +N control (**Figure 3B**, Supplementary Figure 2).

Nutrient Acquisition

Although *B. simplex* was isolated on a solidified N-free medium, it is not a diazotroph because it lacks *nifH,* a structural gene essential for nitrogenase function (Schwartz et al., 2013). In an N-free liquid medium, *B. simplex* 30N-5 ceased growing unless the medium was supplemented with 1-aminocyclopropane-1-carboxylate (ACC), which is broken down into 2-oxobutanoate and ammonia; the latter sustained bacterial growth for a short time. This finding suggested that *B. simplex* had *acdS* activity (see later section).

Phosphate Solubilization

B. simplex 30N-5 effectively solubilized mineralized phosphate as measured by activity on PVK plates (Schwartz et al., 2013). Although we detected a gene encoding a soluble quinoprotein glucose/sorbosone dehydrogenase, which is important for

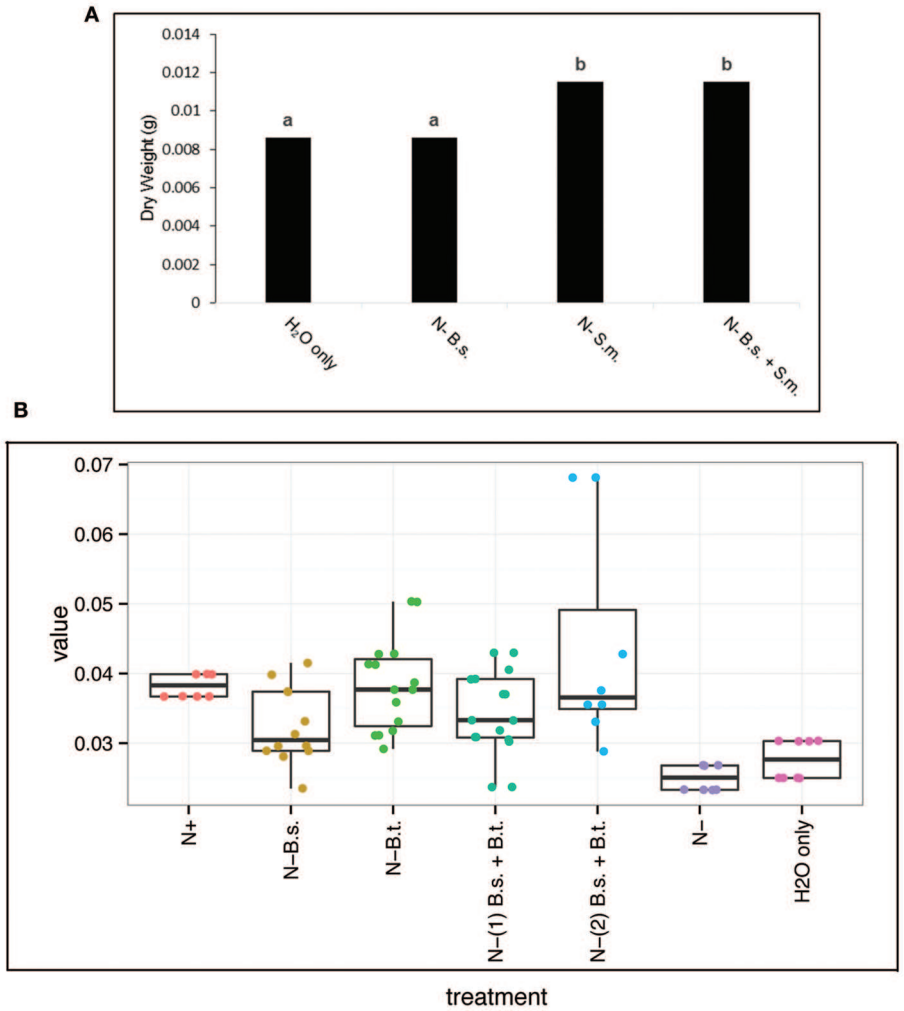

FIGURE 3 | Biomass measurements of *Melilotus alba* and *Macroptilium atropurpureum* 35 days post inoculation. (A) *Melilotus alba* plants were singly or coinoculated with *Bacilus simplex* (*B.s.*) and *Sinorhizobium meliloti* (*S.m.*); Different letters represent values that differ significantly, $p < 0.01$. **(B)** Jittered boxplot. *Macroptilium atropurpureum* plants were singly or coinoculated with *Bacillus simplex* (*B.s.*) and *Burkholderia tuberum* (*B.t*). The first coinoculation (1) introduced both bacteria species at the same time, whereas the second (2) was inoculated with *B.s.* first followed by *B.t.* inoculation 5 days later. Harvesting was performed as described in Methods. Boxes indicate minimum, maximum, 1st and 3rd quartiles and the median value.

gluconic acid production (de Werra et al., 2009) (**Figure 1**, column 1), no additional genes involved in the breakdown of either inorganic or organic phosphates were found. However, the putative *B. simplex* (*gcd*) gene was not overly similar to the comparable genes in either *B. licheniformis* (51%) or *B. firmus* (58%) (**Figure 1**). We were unable to detect an equivalent gene in the other PGPB strains. Species of *B. amyloliquefaciens* (Kim et al., 1998), *B. licheniformis* (Tye et al., 2002), and *B. subtilis* (Kerovuo et al., 1998) have been reported to produce phytase (myo-inositol-hexaphosphate 3-phosphohydrolase), which degrades organic phosphates. A phytase gene was not detected in the *B. simplex* strains.

Siderophores

Siderophores secreted by bacteria also support the development and growth of plants by helping them sequester iron from the environment. Previously, we showed that *B. simplex* 30N-5 exhibited a positive reaction in a CAS assay, which detects siderophore activity (Schwartz et al., 2013). We identified several siderophore operons in *B. simplex* 30N-5 (**Figure 1**). The iron-dicitrate transporter genes were similar to *yfmCDEF* of *B. megaterium* and several other bacilli, whereas the iron-compound transport system had genes conserved with *yfiZ* and *yfiY* of *B. licheniformis* as well as with *B. amyloliquefaciens* genes.

Production of Volatiles

Many PGPB emit volatiles that positively enhance growth, e.g., *B. amyloliquefaciens* subsp. *plantarum* FZB42 and *B. subtilis* GB03. The latter was reported to acidify the rhizosphere of Arabidopsis in response to volatiles (Zhang et al., 2007), which may help in phosphate solubilization. The most commonly studied volatiles are acetoin (Xiao and Xu, 2007), and 2,3-butanediol, which is

known to be involved in Arabidopsis defense induction (Ryu et al., 2003). The Voges-Proskauer test was used to demonstrate acetoin synthesis as well as the potential for production of 2,3-butanediol (Xiao and Xu, 2007) by means of a colorimetric reaction. We used this test on a number of *Bacillus* strains known to produce volatiles and included *B. simplex* 30N-5 along with two additional strains of *B. simplex* (Kaplan et al., 2013) for the analysis. *Escherichia coli* was the negative control. Although the known PGPB strains tested positive for acetoin, including *P. polymyxa* NRRL B-4317, the three *B. simplex* strains were negative even after a long incubation period (**Table 3**).

Genes from *B. amyloliquefaciens* subsp. *plantarum* FZB42 were used to search for sequences in the *B. simplex* genome that could encode proteins for acetoin synthesis. We detected five genes, several *aco* genes, as well as one encoding *alsS*, which is important for acetolactate synthesis (Xiao and Xu, 2007). However, no gene for *alsD*, which encodes an alpha-acetolactate decarboxylase, was detected although it was present in the reference PGPB genomes. Also, a *butA*/*budC* gene was detected in the *aco* operon and showed greater than 95% identity to the other *B. simplex* strain (II3b11) (**Figure 1**). The *butA*/*budC* gene encodes meso-butanediol dehydrogenase.

Root Colonization and Growth Promotion Factors

Motility

In response to root exudates, many bacteria migrate toward root surfaces and colonize roots. In *B. amyloliquefaciens* subsp. *plantarum* FZB42 and other *Bacillus* species, a number of genes are expressed (Chen et al., 2007), including flagellar genes (De Weger et al., 1987; Croes et al., 1993). In the *B. simplex* genome, flagellar and chemotaxis genes are located in two apparently unlinked areas (**Figure 4**). A similar arrangement exists for other PGPB *Bacillus* strains, such as *B. firmus* DS1, *B. kribbensis* DSM 17871, *B. megaterium* DSM 319 (Supplementary Figure 2), and the two *Paenibacillus* species included in our analysis (data not shown). However, a major difference in arrangement was observed in *B. cereus*, which contains strains that can be either beneficial or pathogenic (Bottone, 2010). For example, five chemotaxis-associated genes (*cheABCD* and *W*) within the flagellar gene operon are conserved among *B. simplex* and related PGP strains (**Figure 4**, Supplementary Figure 3). However, the chemotaxis genes, *cheB* and *cheD,* were not detected within the flagellar gene region of *B. cereus* JM-Mgvxx-63, which differs in organization (**Figure 4**). In addition, the flagellar genes are not highly related. For example, the *flhF* genes of *B. simplex* 30N-5 and *B. cereus* JM-Mgvxx-63 are 36% identical, but only 24% DNA identity is observed when the two *fliS* genes were compared.

Many PGPB, e.g., alpha-rhizobia (Amaya-Gómez et al., 2015) and *Bacillus* spp. (Dietel et al., 2013), swarm as well as swim prior to colonization. *B. simplex* 30N-5 cells swarm on 0.8% agar suggesting that genes for this behavior are present and expressed. The *swrC* gene shares 100% identity among the *B. simplex* strains. Although originally annotated as a cation/multidrug efflux pump, it is orthologous and 61 and 57% identical to genes annotated as *swrC* in *B. thuringiensis* and *B. amyloliquefaciens*, respectively.

TABLE 3 | Voges-Proskauer test results for selected strains.

Strain	30′	1 h
E. coli HB101*	−	−
B. amyloliquefaciens plantarum FZB42	+	+
B. cereus 26a1	−	−
B. licheniformis DSM 13/ATCC 14580	+	+
B. simplex 237	−	−
B. simplex 11	−	−
B. simplex 30N-5	−	−
B. subtilis GB03	+	+
B. subtilis 60b4**	+	+
P. polymyxa NRRL B-4317	+	+

*Negative control **Positive control.
Measurements were taken 30 min and 1 h after the addition of the colorimetric reagents.

Plant Growth-promoting Traits: Hormones

One of the most prominent features of PGPB is their ability to produce compounds that directly influence plant growth, e.g., the phytohormones. PGPB also synthesize gene products that affect plant growth in a more indirect way.

Auxin

Many bacteria are known to synthesize auxin (involved in lateral root proliferation), and at least five tryptophan-dependent or tryptophan-independent biosynthetic pathways are employed by bacteria to synthesize auxin (Patten and Glick, 1996; Spaepen and Vanderleyden, 2011). Because we obtained positive results with the Salkowski test following the addition of tryptophan (Schwartz et al., 2013), we hypothesized that the genes for auxin synthesis might be present in the genome of this species.

One of the most studied of the auxin biosynthetic pathways includes the genes encoding IAA monooxygenase (*iaaM*) and indole acetamide hydrolyase (*iaaH*), found in the gall-forming *Agrobacterium tumefaciens* and *Pseudomonas savastonoi*, but these genes were not detected in the *B. simplex* 30N-5 genome. However, putative *ipdC* genes were identified in the *B. simplex* strains with greater than 97% DNA identity among them, following a query with an indole pyruvate carboxylase gene from *Enterobacter cloacae* (Koga, 1995). Although <50% DNA identity to *ipdC* from several other PGPB was observed (**Figure 1**), *ipdC* genes in *B. kribbensis* and *P. pini* were 62 and 66% identical in DNA sequence, respectively, and orthologous to the *B. simplex* gene. A gene encoding a putative indole-3-acetaldehyde dehydrogenase, which is involved in tryptophan-dependent IAA synthesis and is the last step in the pathway, was also detected in *B. simplex* 30N-5. The gene is ca. 75% identical to *dhaS* of several bacilli, and has >84% identity to genes in *B. firmus* DS1 and *B. kribbensis* DSM 17,871.

Other auxin-related genes were also found in *B. simplex* 30N-5. A gene orthologous to *aofH*, which codes for an indole-3-acetic oxidase [suggesting that tryptamine, rather than tryptophan, is converted to indole-3-acetaldehyde (IAAld)], was uncovered using AZL.b03560 from *Azospirillum* sp. B510 (Wisniewski-Dyé et al., 2011) to query the *B. simplex* genome. Although the different *B. simplex* strains have almost identical *aofH* gene

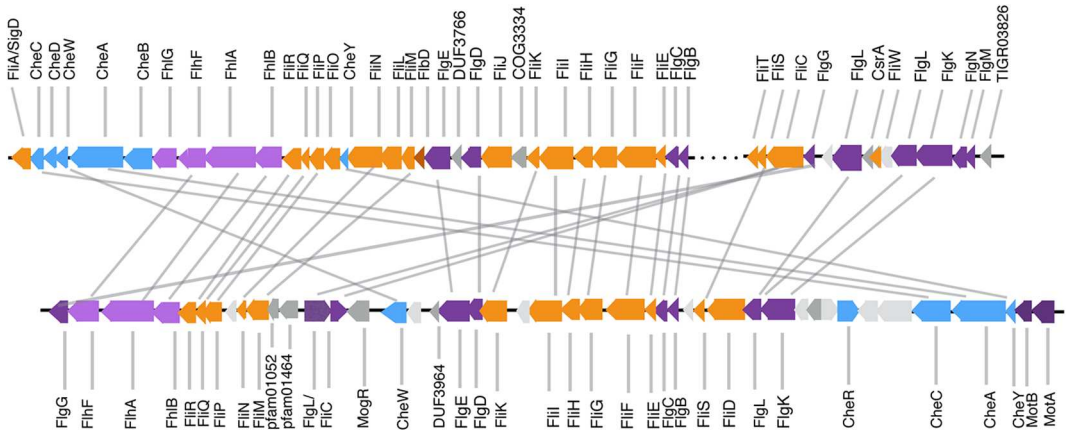

FIGURE 4 | Comparison of flagellar open reading frame (ORF) clusters. The top cluster is from *B. simplex* 30N-5, which consists of two unlinked regions. The bottom cluster is from *B. cereus* JM-Mgvxx-63 where most of the genes related to *B. simplex* are within a single region on the chromosome.

sequences, this gene is not well conserved with genes from other bacilli with the exception of *B. firmus* DS1 (71% identity) (**Figure 1**). Lastly, a *B. simplex* gene with 79% DNA identity and orthologous to a predicted nitrilase, the *yhcX* gene in *B. amyloliquefaciens*, was detected in the *B. simplex* 30N-5 genome. A similar gene identity was observed for many of the other PGP bacilli (**Figure 1**).

The lack of a complete pathway and the low identity for *ipdC* made us question whether IAA is actually synthesized by *B. simplex*. To address this question, we performed a chemical analysis. No signals for IAA were found in the LC/MS/MS-MRM assay for this compound. With a limit of detection of about 5 pmol injected, it is concluded the concentration of this hormone, if present, is less than 174 pmol/10^9 cells.

Phenylacetic Acid

In *Azospirillum brasilense*, IpdC is also involved in the production of phenylacetic acid (PAA), which has weak auxin activity and is also antimicrobial against both bacteria and fungi (Somers et al., 2005). As in *Azospirillum*, the *B. simplex* genome has the *paa* operon (data not shown), which is important for the degradation of PAA.

Cytokinin

Many PGP bacilli have been reported to produce cytokinins, but few cytokinin biosynthetic genes have been detected (see Vacheron et al., 2013). Querying various *Bacillus* genomes with *tzs* (trans-zeatin synthase) from *A. tumefaciens*, where it is required for tumor formation, yielded no hits. On the other hand, *miaA*, which encodes tRNA dimethylallyltransferase that removes a zeatin precursor from tRNA, is common among the PGP bacilli, including *B. simplex*.

Polyamines

Many bacteria produce polyamines such as spermine, spermidine, and putrescine, which in *B. subtilis* OKB105 have PGP properties (Xie et al., 2014). A number of genes involved in polyamine synthesis (Sekowska et al., 1998) were detected in the *B. simplex* 30N-5 genome including *speA*, which results in agmatine synthesis; *speB*, putrescine synthesis; and *speE* and *speD*, which encode the stages for spermidine synthesis. Also, *metK*, responsible for the conversion of methionine to S-adenosyl-methionine, was detected (data not shown). Many of these were ≤80% identical to genes in the PGP bacilli (*speD*), whereas others, e.g., *speE*, are less similar (<50%) albeit orthologous. Genes for various binding proteins, permeases, and transporters for polyamines are also present in the genome of *B. simplex* 30N-5.

Clear strong signals were obtained for cell lysates prepared in either TCA or TFA showing the presence of significant quantities of spermine, spermidine, and putrescine in all samples examined (**Figure 5**). Verification of the assignments was made with co-chromatography experiments in which known amounts of authentic standards were added to cell lysate samples prior to LC/MS/MS-MRM. In these experiments, single peaks for each amine were obtained for the spiked samples, with appropriate area and intensity enhancement of the signals. The signals were slightly more intense for spermine and spermidine when TFA was used compared to TCA during cell homogenization (1.6- and 1.2-fold, respectively), and slightly more intense for putrescine when TCA compared to TFA was used (2.8-fold) (**Table 4**). The MRM chromatograms from methanol extracts were less clear with peaks at other retention times and significantly less intense peaks for the polyamines. Quantitation based on external standards shows spermine, spermidine and putrescine concentrations in the range of 333, 222, and 2.2 nmol/10^9 cells, respectively, although for more precise measurements, the work requires repeating using an internal standard to correct for losses during extraction. To this end, it was noted that the cell extracts did not contain any detectable amount of hexamethylediamine (MRM transition (MH^+) m/z 117➔100) that could be used for this purpose.

AcdS

Ethylene is inhibitory to root development and also induces plant defense response pathways. We detected a sequence in *B. simplex* 30N-5 that is similar to genes annotated as *acdS* in

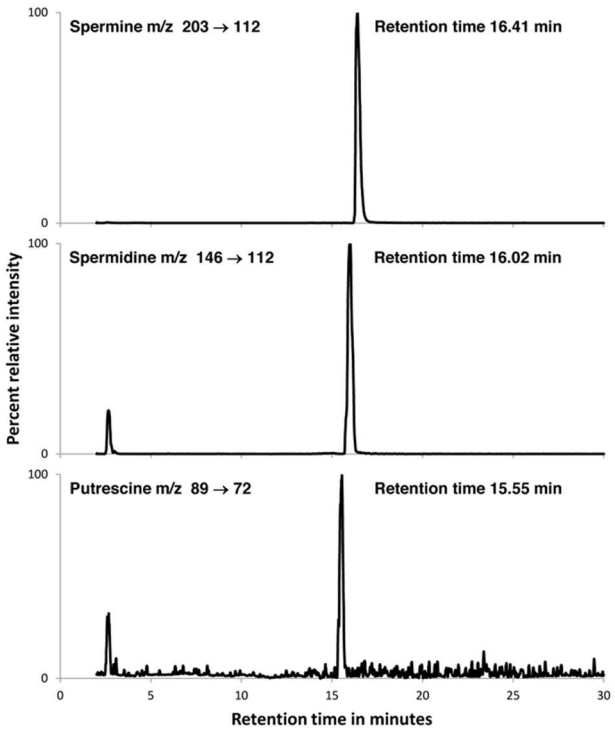

FIGURE 5 | LC/MS/MS-MRM traces for the TCA extract of *B. simplex* 30N-5. Peaks for spermine (top), spermidine (middle) and putrescine (bottom) are shown. Samples were prepared and analyzed as described in Methods. Co-chromatography experiments in which the authentic compounds were added to the bacterial extract showed single peaks for each trace with appropriate augmentation of the peak areas. A quantitative summary of the results is presented in **Table 4**.

TABLE 4 | The concentrations of spermine, spermidine, and putrescine in methanol, TFA, and TCA extracts of *B. simplex* 30N-5 measured by LC/MS/MS-MRM using external standards.

Sample	nmol/sample	nmol/sample	nmol/sample
30N-5 methanol extract 1	0.92	1.46	1.09
30N-5 methanol extract 2	1.03	5.38	1.18
30N-5 TFA extract 1	613.75	355.15	2.02
30N-5 TFA extract 2	622.45	462.71	1.18
30N-5 TCA extract 1	400.68	388.64	5.28
30N-5 TCA extract 2	380.60	312.82	3.53

B. thuringiensis and in *B. cereus* JM-Mgvxx-63 (80%) and as D-cysteine desulfhydrase in *B. panaciterrae* DSM 19096 (88%) (**Figure 1**). The comparable genes for the two additional *B. simplex* strains available at NCBI were annotated as a cytochrome C biogenesis protein/D-cysteine desulfhydrase. These proteins are part of the PLP-dependent ACC family. However, genes homologous to this sequence were not detected in the typical PGPB group (blue group; **Figure 1**).

Antibiotics and Related Compounds

Bacteria synthesize a number of compounds that contribute to their survival in the rhizosphere. *B. subtilis* has been a paradigm for studying these antimicrobial compounds, which fall into two major classes: those synthesized on ribosomes (e.g., bacteriocins and lantibiotics) and post-translationally modified and those produced on large multienzymes known as Nonribosomal Peptide Synthetases (NRPSs), e.g., iturin and fengycin (Stein, 2005).

Bacteriocins

Many nonpathogenic bacteria produce bacteriocins, molecules used to compete with closely related bacteria, and many classes of these antimicrobial peptides are known. We scanned the *B. simplex* genome for genes potentially encoding bacteriocins and found three candidates, but genes comparable to those in *B. simplex* were not detected (n.d.) in any of the other PGP bacilli (**Figure 1**). The highest DNA sequence identity of genes encoding proteins for bacteriocin synthesis (**Figure 1**) was to the sequences found in *B. panaciterrae*. The highest DNA sequence identity of the bacteriocin biosynthesis gene, based on amino acid sequence, was 79% (**Figure 1**). These same gene sequences were picked up using the BAGEL3 website and a gene map is depicted in Supplementary Figure 1B.

Another protein with 99 and 100% identity to the two *B. simplex* strains in NCBI (*B. simplex* P558, CEG34010.l; *B. simplex* BA243, WP 034090.1, respectively) was also found. It matched to a protein described as a colicin V production protein. Although the gene neighborhoods were well conserved among the *Bacillus* species in **Figure 2**, the percentage DNA identity was 70% or lower (data not shown).

Additional Secondary Metabolites

Many PGPB synthesize diverse secondary metabolites, which have antibiotic activity, including lantibiotics, nonribosomally synthesized peptides, and polyketides. For example, subtilin is a 32-amino acid pentacyclic lantibiotic produced by *B. subtilis* (Stein, 2005). Although several subtilisin-like serine protease (AprE-like) genes are present in the *B. simplex* genome as well as proteins involved in subtilin processing (WprA and Vpr-like), no evidence was found in the *B. simplex* genome for the presence of genes similar to *spaS* and *spaBTC*, the subtilin structural genes and the genes promoting subtilin expression, respectively, nor to the genes *spaIFEG*, which confer immunity. Similarly, we saw no matches to genes encoding lantibiotic-like peptides such as sublanchin or subtilisin A produced by *B. subtilis*.

We looked for, but did not find, genes for the synthesis of nonribosomally synthesized peptide antibiotics in *B. simplex*, such as surfactin (see next section), iturin, or bacillomycin or for antimicrobial polyketides such as macrolactin, bacillaene, or difficidin, which are found in many PGPB *Bacillus* strains.

Other Nonribosomal Peptide Synthetase (NRPS) Products

Genes were found for the synthesis of koranimine, a cyclic imine. The genes involved are: *korA*, *korB*, *korC*, *korD*, as well as genes encoding a phosphopantetheinyl transferase (*kfp*) and a type II thioesterase (*korTE*) (Supplementary Figure 4). These genes had been detected earlier in an environmental *Bacillus* strain (NK2003) using a proteomics-based approach (Evans

et al., 2011). We found them using a *B. amyloliquefaciens* subsp. *plantarum* FZB42 gene sequence (*srfAA*) in a blastp search, and although only 34% sequence identity was found between *korA* and *srfAA,* their amino acid adenylation domains were highly conserved (89.7%). Genome analysis led to the discovery of an orthologous gene in *Bacillus* sp. NK2003, which was annotated as a nonribosomal peptide synthetase. Koranimine synthetic genes were also found in the *B. simplex* II3b11 genome, each gene having greater than 95% DNA sequence identity to the *kor* genes of *B. simplex* 30N-5. An amino acid adenylation domain sequence that lines up with the middle part of *korC* was found in another part of the *B. simplex* genome (data not shown).

Although we utilized a gene encoding a surfactin to uncover the *kor* genes, we could not find any genes for surfactin production itself or any other NRPS-produced metabolites in *B. simplex*. Moreover, using a modification of a published procedure of the drop-collapsing assay (Kuiper et al., 2004) to determine surfactant activity, we found none of the three *B. simplex* strains tested exhibited a change in the diameter of the drops due to decrease in surface tension of the droplet (data not shown), suggesting that *B. simplex* 30N-5 lacks surfactant activity.

We also detected the polyketides described above using the ANTIsmash webserver. In addition, evidence for a gene encoding squalene synthetase and highly conserved with the genes of other *B. simplex* strains, but not found in the PGPB bacilli. An orthologous gene with 60 and 57.5% DNA identity was detected in *B. panaciterrae* and *B. megaterium*, respectively (data not shown). Similarly, a gene encoding chalcone synthase that is orthologous and 63–61% identical to genes in *B. kribbensis* and *B. firmus*, respectively, was found using ANTIsmash. The genes of the PGPB bacilli are orthologous and 51% identical to the *B. simplex* gene (data not shown).

Other Pathways

Vitamins

More than one-third of the bacteria that have been sequenced possess genes for cobalamin (vitamin B12) synthesis (Raux et al., 2000), including *B. simplex* 30N-5. The *B. simplex* 30N-5 and the other *B. simplex* genomes also contain genes for riboflavin (vitamin B2) synthesis as previously described for *B. subtilis* (Stahmann et al., 2000). Riboflavin subunit alpha (*ribF*) was also found in all genomes. Similarly, the menaquinone (vitamin K2) pathway genes found in *B. subtilis* (Sato et al., 2001) were detected in the genomes of the *B. simplex* strains (chorismate synthase, isochorismate synthase, demethylmenaquinone methyltransferase, and 2-heptaprenyl-1,4-naphthoquinone methyltransferase).

Protein Secretion Systems

Gram-positive bacteria secrete proteins usually by translocation across the single membrane by the Sec pathway or via the two-arginine (Tat) pathway. *B. simplex* also possesses the genes, with a 99% identity, *tatA*, *tatC* and a third gene from the same family. In addition, a specialized secretion system, which is responsible for protein translocation across both the membrane and the cell wall, called a type VII secretion system (Tseng et al., 2009), was detected in the *B. simplex* 30N-5 genome.

Discussion

B. simplex has been shown in a number of reports to be an effective PGPB (Ertruk et al., 2010; Hassen and Labuschagne, 2010; Schwartz et al., 2013). In this report, we show that *B. simplex* strains 30N-5 and II3b11 are phylogenetically and genetically different from the known PGPB bacilli. Based on an analysis of 5 different housekeeping genes, they cluster in a separate subclade from most PGPB bacilli and their PGP-related genes. We thus placed them into a group separate from other PGPB (purple, **Figure 1**). Because interest in this species as a PGPB species and producer of novel enzymes has been increasing (Velivelli et al., 2015; Venkatachalam et al., 2015), we undertook a detailed investigation of the potential of *B. simplex* 30N-5 to act as a PGPB.

A survey of the three legume hosts used in the coinoculation studies suggests that *B. simplex* may behave differently among plant species. In our study on pea, we observed that simultaneous inoculation of *B. simplex* and *R. leguminosarum* bv. *viciae* resulted in an enhancement of nodulation and plant dry weights (Schwartz et al., 2013). In contrast, coinoculation of *S. meliloti* Rm1021 and *B. simplex* did not produce a significantly different dry weight measurement for either *Melilotus alba* or *Medicago truncatula* compared to the single inoculation. Moreover, *B. simplex* alone did not enhance the growth of the *S. meliloti* hosts compared to pea (**Figure 3A**).

In contrast, siratro responded positively to single inoculation with *B. simplex* and showed an increase in dry weight, as we had observed for pea (Schwartz et al., 2013). Whether or not this difference between the *S. meliloti* hosts and siratro and pea in response is a consequence of having smaller seeds vs. larger seeds is not known. The larger-seeded legumes contain more stored N, which results in a protracted growth response under N-deficient conditions. In addition, some PGPB strains appear to exhibit host specificity toward different plants (Kloepper, 1996). We are investigating these possibilities further.

Flagella are important for root colonization via cell motility, swarming, and biofilm formation. We found that *B. simplex* and the PGPB bacilli of the blue group (**Figure 1**) have an identical flagellar gene arrangement (**Figure 4**, Supplementary Figure 3). In contrast, members of the orange group (**Figure 1**), *B. thuringiensis* and *B. cereus*, have a different flagellar arrangement (**Figure 4**). It is not known if this difference is significant. In our previous studies of plant-associated *Burkholderia* species, the arrangement of the flagella genes between the plant-associated *Burkholderia* species and the mammalian and opportunistic pathogens was also dissimilar (Angus et al., 2014). In *Burkholderia*, the flagellar genes were linked together on a chromosome in the plant-associated species whereas they were located in different parts of the genome in the pathogen-clade. Flagella from pathogenic species are well known for triggering induced systemic resistance (ISR) in numerous plant species, but information about whether flagella from beneficial bacteria, especially from PGPB *Bacillus* spp., affect host responses is not available.

Volatiles are also important for inducing a systemic response. Although several genes associated with the acetoin pathway

are present in *B. simplex* 30N-5, this strain did not produce detectable quantities of acetoin based on the Voges-Proskauer test. We observed that none of the *B. simplex* genomes that have been sequenced contain the *alsD* gene, whereas the genomes of the typical PGPB bacilli do. Because AlsD is missing, alpha-acetolactate, which is unstable following synthesis by AlsS via the condensation of two pyruvate molecules, cannot be converted to 3-hydroxy-2-butanone (acetoin), the precursor of 2,3-butanediol (Xiao and Xu, 2007). *B. simplex* M3-4, which has a positive effect on potato tuber yields, was also found to be negative for the Voges-Proskauer test (Velivelli et al., 2015). Nonetheless, this strain produces volatiles, such as 2-hexen-1-ol, 2,5-dimethylpyrazine, and several others, which inhibit *Rhizoctonia solani* growth. Thus, *B. simplex* strains are effective PGPB even though they do not emit 2,3-butanediol (this work; Velivelli et al., 2015). Future studies will investigate whether *B. simplex* 30N-5 produces similar volatiles.

Plant growth promotion frequently results from the action of hormones, and auxin synthesis is a common trait that is associated with PGPB bacilli. In it, tryptophan is converted to IPA by L-tryptophan aminotransferase (Patten and Glick, 1996). Although several aminotransferases were detected in the *B. simplex* genome, none could be specifically designated as this enzyme or as tryptophan transferase. In addition, even though genes were found for *ipdC* (indole pyruvic acid carboxylase), *dhaS* (indole-3-acetaldehyde dehydrogenase), *aofH* (indole-3-acetaldehyde oxidase), and *yhcX* (a nitrilase) based on similarities to other *Bacillus* spp., the gene identities were low except for *dhaS* and *yhx* (**Figure 1**). Using a sensitive and specific LC/MS/MS-MRM assay, we could not detect IAA in cell culture homogenates, further supporting the absence of an active biosynthetic pathway for this hormone in *B. simplex* 30N-5. These results lead us to propose that the commonly used Salkowski test is not definitive for the synthesis of auxin by bacteria.

Polyamines are also PGP compounds. Studies by Xie et al. (2014) showed that mutations in *yecA* (a permease) and *speB* (encoding one of the first steps in the conversion of agmatine to putrescine and then spermidine) eliminated the PGP activities of *B. subtilis* OKB105, e.g., root elongation. Reverse phase HPLC with UV detection of chemically-derivatized samples detected spermidine in the OKB105 culture filtrate (Xie et al., 2014). Our data using a more specific and sensitive assay show that all three polyamines are present in the culture medium of *B. simplex* 30N-5, and indeed based on the genome information, the entire pathway for polyamine production is present.

The enzyme *acdS* is thought to improve plant growth by interfering with ethylene formation. It does this by deaminating 1-aminocyclopropane-1-carboxylic acid (ACC), a direct precursor to ethylene production. Previously, we cloned a potential ACC deaminase gene from *B. simplex* 30N-5 by using *acdS* primers and found a sequence that was closely related to a gene encoding a pyridoxal phosphate-dependent enzyme. Experimental evidence will be needed to determine if this gene product has AcdS activity, but based on the fact that ACC deaminase is a member of the above protein family, it might be *acdS*. Nevertheless, because this gene in the *B. simplex* genomes (P558 and BA2H3) available at NCBI was annotated as a cytochrome C biogenesis protein/cysteine desulfhydrase, we cannot be completely certain that it encodes AcdS. However, ethylene synthesis is inhibited in plant root cells in response to *B. subtilis* OKB105 in response to polyamine synthesis (Xie et al., 2014), suggesting that polyamines may be an additional or alternative mechanism used by certain bacilli for reducing ethylene content in plants.

Genes encoding surfactin or related polyketides were not detected in *B. simplex* 30N-5, but the entire pathway for the synthesis of koranimine, a newly identified NRPS-synthesized peptide (Evans et al., 2011) was found. Although the function of this compound is unknown, cyclic imines are well known marine-based bacterial compounds that accumulate in crustaceans and fish possibly for defense purposes because of their toxicity to predators when ingested (Otero et al., 2011). Hence, the possibility exists that koranimine may play an antibiotic role in its interactions with other microbes and in protecting the plant. Again, additional studies are needed.

In summary, *B. simplex* 30N-5 exhibits potentially novel PGPB properties that are shared, but also dissimilar from some of the more commonly studied PGP bacilli. To be certain that this microbe has no deleterious effects on plants, we are testing it and related strains on both legumes and nonlegumes as well as on *Caenorhabditis elegans* to determine its lack of virulence. Published data showed that *B. simplex* 237 did not have a detrimental effect on *C. elegans* (Angus et al., 2014), and our preliminary results with *B. simplex* 30N-5 demonstrate no toxic effects on nematodes or onions (M. Arrabit and A.M. Hirsch, unpubl.). Also, based on our investigations of the genome, no obvious virulence genes are observed. Thus, *B. simplex* 30N-5 may be an excellent candidate to be added to the group of beneficial bacilli that help plants grow and survive under sustainable agriculture conditions.

Author Contributions

MM, PM, KF, and AH designed and conducted experiments, and wrote the manuscript. ES and AH conceived the work, and ES made critical revisions to the manuscript. ES, WV, PM, KC, TA, JS, and AH acquired the genomics data, and ST and AH interpreted it. MM, PM, KF, TI, LH, MC, TS, NF, and JV acquired experimental data. MM and JS did the statistical analyses. All authors read and approved the final manuscript.

Acknowledgments

We thank Dr. Kris Reddi (UCLA) for isolating the *B. simplex* 30N-5 DNA for genome sequencing and Dr. Flora Pule-Meulenberg (Botswana College of Agriculture) for additional *Bacillus* strains. The following UCLA undergraduates, Leah Briscoe, Fiorella Candamo, Ariga Gharibian, Ethan Humm, Sofianne Kajouke, Suzie Martikyan, and Darren Shum, are acknowledged for their contributions. This research was supported in part by NSF Award IOS 1201735, and a grant from the Shanbrom Family Foundation to AH. Much of the genomics

work was performed through the IMG/ER website of the U.S. Department of Energy Joint Genome Institute, a DOE Office of Science User Facility, which is supported under Contract No. DE-AC02-05CH11231. Additional funding came from a postdoctoral fellowship awarded to PM from "Fundación Ramón Areces" (Spain) and an award to MC from the State Foundation for Studying Abroad of China. WV was funded in part by NSF DUE grant 1022918. Funding to sequence the *B. simplex* 30N-5 genome was provided by the Howard Hughes Medical Institute (Award No. 52006944).

Supplementary Material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2015.00784

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

REVIEW
published: 14 July 2015
doi: 10.3389/fpls.2015.00507

Harnessing phytomicrobiome signaling for rhizosphere microbiome engineering

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The goal of microbiome engineering is to manipulate the microbiome toward a certain type of community that will optimize plant functions of interest. For instance, in crop production the goal is to reduce disease susceptibility, increase nutrient availability increase abiotic stress tolerance and increase crop yields. Various approaches can be devised to engineer the plant–microbiome, but one particularly promising approach is to take advantage of naturally evolved plant–microbiome communication channels. This is, however, very challenging as the understanding of the plant–microbiome communication is still mostly rudimentary and plant–microbiome interactions varies between crops species (and even cultivars), between individual members of the microbiome and with environmental conditions. In each individual case, many aspects of the plant–microorganisms relationship should be thoroughly scrutinized. In this article we summarize some of the existing plant–microbiome engineering studies and point out potential avenues for further research.

Keywords: rhizosphere, signaling, beneficial microorganisms, agriculture, plant–microbe interactions

OPEN ACCESS

Edited by:
Jean-Michel Ané, University of Wisconsin–Madison, USA

Reviewed by:
Oswaldo Valdes-Lopez, National Autonomous University of Mexico, Mexico
Thomas Robert Rey, De Sangosse, France

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Specialty section:
This article was submitted to Plant Biotic Interactions, a section of the journal Frontiers in Plant Science

Received: *13 May 2015*
Accepted: *25 June 2015*
Published: *14 July 2015*

Citation:
Quiza L, St-Arnaud M and Yergeau E (2015) Harnessing phytomicrobiome signaling for rhizosphere microbiome engineering. Front. Plant Sci. 6:507. doi: 10.3389/fpls.2015.00507

Introduction

Virtually every plant part is colonized by microorganisms, including bacteria, archaea, fungi, collectively designated as the plant–microbiome or phytomicrobiome. Depending on the plant part it colonizes, the phytomicrobiome is often referred to as endophytic (inside plant parts), epiphytic (on aboveground plant parts), or rhizospheric (in the soil closely associated to the roots) (Kowalchuk et al., 2010; Lakshmanan et al., 2014). Microorganisms are a key component of the plant, often inextricable from their host and the plant–microbiome is thought to function as a metaorganism or holobiont (Bosch and McFall-Ngai, 2011; Vandenkoornhuyse et al., 2015). The biomass and composition of the microbiome strongly affects the interactions between plants and their environments (Ryan et al., 2009). The rhizosphere harbors a largely increased bacterial abundance and activity, not only as compared to other plant compartments (Smalla et al., 2001; Kowalchuk et al., 2002), but also when compared to bulk soil. However, bacterial diversity in the rhizosphere is generally lower than in the bulk soil (Marilley and Aragno, 1999) and microbial community composition is very different (Smalla et al., 2001; Kowalchuk et al., 2002; Griffiths et al., 2006; Kielak et al., 2008; Bulgarelli et al., 2012; Peiffer et al., 2013), suggesting a strongly selective environment. As the microbial density, diversity, and activity in the endosphere (the microbial habitat inside both above- and belowground plant organs) and phyllosphere (the aboveground plant surfaces) are generally lower than in the rhizosphere, the focus of this contribution will be mainly on the rhizosphere.

There is ample evidence that shows that the plant–microbe relationship is critical to health, productivity and the overall condition of the plant (Baudoin et al., 2003; Chaparro et al., 2012, 2014; Marasco et al., 2012; Adesemoye and Egamberdieva, 2013; Ziegler et al., 2013; Glick, 2014). There are different kinds of interactions between plants and microbes, spanning the whole spectrum from beneficial to pathogenic, and the outcome of the interaction between a plant and a microbe can vary among this spectrum depending on plant species, nutrient conditions, etc. (**Figure 1**). The goal of plant–microbiome engineering is to push this interaction toward enhanced beneficial outcomes for the plant. Many microbially mediated functions are important to enhance beneficial outcome, including nutrient cycling, mineralization of soil organic matter, induction of disease resistance and response to abiotic stresses such as drought and salinity (Marasco et al., 2012; Zolla et al., 2013). The plant–microbiome interactions are complex and often depend on plant species/cultivar, soil type and environmental conditions such as biotic/abiotic stress, climatic conditions, and anthropogenic effects. Different soils as well as different environmental stresses (e.g., nutrient deficiencies, metal toxicity, pathogen attack, etc.) have been shown to trigger plant-species-dependent physiological responses and consequently different exudation patterns (Bais et al., 2006; Oburger et al., 2013). Microbes in the rhizosphere can also influence the plant exudation, as for example, when antimicrobial-resistant *Pseudomonas* block the production of plant antimicrobial compounds (Hartmann et al., 2009). One interesting avenue for rhizosphere microbiome engineering is to harness these variations in exudation patterns to enhance the beneficial rhizosphere microbiome.

Signaling in the Rhizosphere

A variety of direct and indirect interactions take place in the rhizosphere such as plant–plant, microbe–microbe, and plant–microbe, as well as the interaction with the other eukaryotic micro-, meso-, and macro-soil inhabitants (Tarkka et al., 2008; De-la-Peña et al., 2012). In view of the complexity of these interactions, knowledge of the chemical communication between all members is essential to unravel how microbial populations coordinate their behavior and interact with the plant roots. Numerous literature reviews have addressed the many different molecules and mechanisms that coordinates the establishment of specific symbiotic interactions in the rhizosphere with the potential to enhance plant growth and productivity (Mabood et al., 2008; Pieterse et al., 2009; Berendsen et al., 2012; Miller and Oldroyd, 2012; Sugiyama and Yazaki, 2012; Bakker et al., 2013; Morel and Castro-Sowinski, 2013; Oldroyd, 2013). However, the understanding of the interactions between the plants and the microbiome as a whole is still rudimentary as the diversity of organisms, molecules, and mechanisms of interaction involved is staggering. Nevertheless, the signaling compounds that make part of this complex rhizosphere interaction have the potential to improve plant functions of interest if understood and harnessed.

Plants have been found to release 5–20% of net photosynthetically fixed C into the rhizosphere (Marschner, 1995). These rhizodeposits include inorganic (CO_2 from cell respiration and H+ efflux) and a variety of complex organic compounds like sloughed-off cells and tissue, intact root border cells, mucilage (polysaccharides) and proteins, all of them classified as high molecular weight compounds. Also, part of the rhizodeposits are the insoluble and soluble low molecular weight (LMW) organic compounds, collectively known as root exudates, which are actively or passively released by growing roots. Root exudates can be classified in different classes such as sugars, amino acids, and amides, organic acids, as well as aromatic and phenolic acids (Bais et al., 2006, 2008; De-la-Peña et al., 2012; Oburger et al., 2013; Zhang et al., 2015). This complex cocktail of root-secreted molecules mediate the interactions occurring in the rhizosphere (Bakker et al., 2012; Berendsen

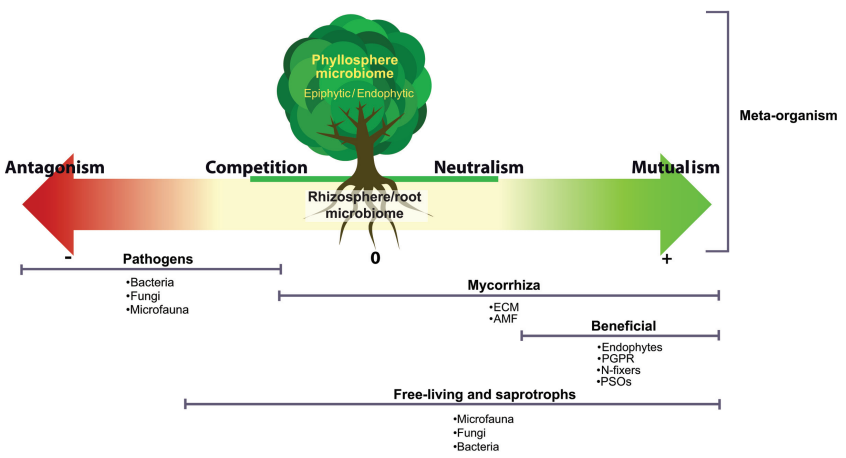

FIGURE 1 | The different interactions taking place within the plant–microbiome meta-organism. A vast spectrum of microorganisms are involved in these interactions: ectomycorrhiza (ECM), arbuscular mycorrhizal fungi (AMF), plant growth promoting rhizobacteria (PGPR), phosphate-solubilizing organisms (PSOs), endophytes, epiphytes, and microfaunal organisms.

et al., 2012; Lakshmanan et al., 2014; Qiu et al., 2014) and acts as chemical attractants and repellants to shape the root microbiome (Walker et al., 2003; Berendsen et al., 2012; Ellouze et al., 2012). From the plant point of view, the goal of shaping the rhizosphere microbiome is to attract preferred partners like plant growth promoting microorganisms through the exudation of specific carbon compounds that can be used as feed and to deter pathogens or unwanted competitors for nutrients through the exudation of antimicrobial compounds such as volatiles or proteins (Bais et al., 2006; Lioussanne et al., 2008; Badri et al., 2009; Hartmann et al., 2009; De-la-Peña et al., 2012; Dangl et al., 2013). Plant exudates are also involved in coping with herbivores, encouraging beneficial symbioses, changing the chemical and physical properties of the soil, and inhibiting the growth of competing plant species (Ping and Boland, 2004; Badri et al., 2009; Morel and Castro-Sowinski, 2013).

The quality and amount of root exudates are highly dynamic in time and space and they depend on the plant species/cultivars, the physiological stage of the plant (Chaparro et al., 2013, 2014), presence or absence of neighbors, plant nutritional status, mechanical impedance (Bengough and Mullins, 1990), sorption characteristics of the soil, and the microbial activity in the rhizosphere (De-la-Peña et al., 2012; Oburger et al., 2013). Plant productivity, nutrient allocation, and tissue chemistry can also vary significantly depending on the identity of neighboring individuals, suggesting that the effects of a given plant host on the soil microbiome may be substantially mediated by the community context of that host (Bakker et al., 2012, 2013). Although very complex and still not well understood, exudation has therefore the potential to highly influence plant performance, health, and competiveness. Some studies have started analyzing the composition of plant root exudates (Phillips et al., 2008; Oburger et al., 2013; Ziegler et al., 2013), but the diversity of the compounds involved and the complexity of the soil matrix makes comprehensive analysis difficult to perform.

Many microorganisms also secrete signaling compounds in the rhizosphere. According to their functions and characteristics, these compounds have been categorized into: phytohormones, extracellular enzymes, organic acids, surface factors [compounds recognized by the host plant that activate an immune response via high-affinity cell surface pattern-recognition receptors (PRR), e.g., flagellins and lipopolysaccharides in *Pseudomonas* (Ping and Boland, 2004; Dangl et al., 2013)], antibiotics and volatile signals. Plant-associated bacteria produce and utilize diffusible quorum sensing (QS) molecules (e.g., *N*-acyl-homoserine lactones, AHLs) to signal to each other and to regulate their gene expression (Berendsen et al., 2012). Bacterially produced AHLs have been shown to affect root development of *Arabidopsis* (Ortiz-Castro et al., 2008) and have been suggested to elicit a phenomenon known as induced systemic resistance (ISR) which allows the plants to endure pathogen attacks that could be lethal without the presence of these bacterial factors. The effect of this mechanism is systemic, e.g., root inoculation with many different plant growth promoting rhizobacteria (PGPR) such as *Pseudomonas*, *Burkholderia,* and *Bacillus* sp. results in the entire plant being non-susceptible to pathogens (Schuhegger et al., 2006; Choudhary et al., 2007; Tarkka et al., 2008), further highlighting the importance of AHLs in cross-kingdom signaling in the rhizosphere. Plant can also exploit this microbial communication system to manipulate gene expression in their associated microbial communities. For instance, some plant-associated bacteria have LuxR-like proteins that are stimulated by plant signals (Soto et al., 2006; Ferluga and Venturi, 2009). Certain bacteria have the capacity to quench signals by degrading various plant- and microbial-produced compounds in the rhizosphere such as quorum sensing signals (Tarkka et al., 2008) and other compounds, like ethylene, that might have negatively affected plants (Bais et al., 2006).

Many of the plant response implicate the intervention of the plant immune (system systemic acquired resistance or SAR) consisting of two interconnected levels of receptors, one outside and one inside the plant cell, that govern recognition of microbes and response to infections. The first level of the plant immune system is governed by extracellular surface PRRs that are activated by recognition of evolutionarily conserved pathogen or microbial-associated molecular patterns (PAMPs or MAMPs). Activation of PRRs leads to intracellular signaling, transcriptional reprogramming, and biosynthesis of a complex output response. This response limits microbial colonization (Dangl et al., 2013) and shapes the soil microbial community in the rhizosphere by selective feeding of beneficial microorganisms and by excreting substances with antimicrobial potential such as root volatiles or root proteins which acts as the primer barriers of plant defense (Bais et al., 2006; Badri et al., 2009; Hartmann et al., 2009; De-la-Peña et al., 2012; Dangl et al., 2013).

How Can we Engineer the Rhizosphere Microbiome?

The efforts to elucidate rhizosphere interactions have often been directed to the potential of single plant root exudates to affect single bacteria or fungi. The clear limitation of this type of approach is the removal of the organism from any context that would give relevance to interspecies interactions. The high diversity of the root- and microbe-secreted molecules involved in rhizosphere interactions suggests that studying the direct influence of a single compound on the microbiome might be impossible or not realistic in nature (Ziegler et al., 2013). Rhizosphere engineering in the environment is still a major challenge even though some studies showed some promising results (**Tables 1–3**). A vast diversity of approaches based on inter-kingdom communication has been utilized in laboratory, greenhouse, and field experiments in order to favor beneficial services to the plants while minimizing inputs requirements. Three potential routes are suggested below in **Table 1**, the microbiome approach, **Table 2**, the plant approach, and **Table 3**, the meta-organism approach. In these tables, we review more generally rhizosphere engineering efforts, but below we will more specifically focus on rhizosphere microbiome engineering studies that took advantage of signalisation channels.

TABLE 1 | Selected microbiome-based methods used to engineer the rhizosphere microbiome.

Method	Mechanisms/examples	Advantages	Disadvantages	Reference
Application of microbial inoculants (biofertilizers).	Plant growth promoting rhizobacteria (PGPR), Nitrogen fixing Rhizobia Arbuscular Mycorrhizal Fungi (AMF), Ectomycorrhiza (ECM) Endophytes	Enhance plant disease control and plant performance. Phytohormone production. Increase plant immunity inducing defense mechanisms system systemic acquired resistance – induced systemic resistance (SAR – ISR) in the plant. Improve soil fertility by granting access to nutrients. Promote nodulation and nitrogen fixation. Fill empty niche spaces increasing community evenness. Induction of suppressive soils.	Establishment of very high population densities immediately after inoculation, but densities decline over time and distance from the inoculum source. Potential risks associated with the release into the environment. Unknown effect over native microbial communities.	Bünemann et al. (2006), Mabood et al. (2008), Ryan et al. (2009), Taghavi et al. (2009), Friesen et al. (2011), Bakker et al. (2012), Chaparro et al. (2012), Morel and Castro-Sowinski (2013)
	Recombinant strains.	Transfer of specific genes by horizontal gene transfer (HGT) inducing the expression of beneficial functions. Adaptation and competence development (resistance, resilience, stability).	Loss of the gene of interest within the time. Potential risks associated with the release into the environment (recombinant strains). Unpredictable or undesired results related to the HGT.	Lynch et al. (2004), Mercier et al. (2006), Ryan et al. (2009)
Disruption of microbial communities to facilitate introduction of beneficial microorganisms	Imposition of mechanical or chemical disturbances: tillage, fungicides, antibiotics, etc.	Easier to establish exogenous communities.	Induce soil vulnerability.	O'Connell et al. (1996), Brussaard et al. (2007), Bakker et al. (2012)

The Microbiome Route

Many of the bacteria in the rhizosphere are currently unable to grow in the laboratory and culture-based methods are often inadequate for qualitative analysis of the rhizosphere microbiome. As a consequence, culture-independent approaches such as metagenomics, metatranscriptomics, metaproteomics, and metabolomics have been the approaches of choice when investigating the rhizosphere microbiome (De-la-Peña et al., 2012; Bell et al., 2014a,b; Yergeau et al., 2014; Zhang et al., 2015). However, many rhizosphere microbiome engineering approaches require having microbial isolates at hand, and further efforts to increase the cultivability of rhizosphere microorganisms will be needed. Even though cultured microorganisms show certain functional capacity of their own, it is not clear yet how they behave once they are introduced in a new environmental niche as in some cases they have been shown to be out-competed by the indigenous microbial population (Ryan et al., 2009). The persistence and functionality of these isolates after inoculation need to be further assessed in order to ascertain positive impacts when used as a strategy to manipulate the rhizosphere microbiome (Stefani et al., 2015). Colonization and dominance of specific microbial species in the rhizosphere is critical for both pathogenic and beneficial soil microbes and will have an impact on disease incidence. Although a general increase in the abundance of microbes is always noted in the rhizosphere as compared to bulk soil, the community structure and functional consequences associated to this increase are poorly understood (Bais et al., 2006; Bakker et al., 2012). An increase in the abundance, activity, or diversity of soil organisms is generally viewed as positive (Bünemann et al., 2006), maximizing overall microbial activity or niche saturation which results in competitive exclusion of pathogens, higher levels of nutrient cycling and increased community stability (**Figure 2**). In that regard, the main microbial strategy to enhance the rhizosphere microbiome include the direct inoculation of microorganisms, focussing on co-inoculation with several strains or mixed cultures of arbuscular mycorrhizal fungi (AMF), ectomycorrhizal fungi (ECM), PGPR and endophytes, enabling combined niche exploitation, cross-feeding, enhancement of one organism's colonization ability, modulating plant growth, achieving niche saturation and competitive exclusion of pathogens (Ping and Boland, 2004; Bünemann et al., 2006; Ryan et al., 2009). Equally important as the recruitment of the adequate microbiome for the plant, is the activation of its specific functions. Quorum sensing (QS) is the mechanism used to regulate distinct microbial activities (biofilm formation, virulence, symbiosis, antibiotic production, conjugation) and is essential for within-species communication as well as for the crosstalk between species which defines if the relationship with the host plant is synergic or antagonist (Soto et al., 2006; Hartmann et al., 2009; Straight and Kolter, 2009).

Enhancing the rhizosphere and root endosphere microbiome often leads to an improvement of beneficial plant functional traits as the microbes are able to expand the plant biochemical capabilities or alter existing pathways (**Table 1**). For instance, PGPR promote plant growth by acting as biofertilizer and entering in symbiosis with their host plants, endosymbiotic rhizobia (*Bradirhizobium, Mesorhizobium*, *Rhizobium*, *Sinorhizobium,* etc) and free-living diazotrophs (*Azospirillum*,

TABLE 2 | Selected plant based methods used to engineer the rhizosphere microbiome.

Method	Mechanisms/examples	Advantages	Disadvantages	Reference
Plant breeding and cultivar selection.	Enhancing exudates production of stimulatory or inhibitory factors.	Influence microbial populations by inhibiting or enhancing the growth of selected microbial members of the rhizosphere community. It does not require change in infrastructure or management in the field.	Need for deeper knowledge on the impact of diversity, quantity, and consistency of exudation shaping the microbiome. There is no control over the variability across environments, soil types, and microbial communities. There is no breeding program that evaluates plant lines for interactions with the soil microbiome.	Lemanceau et al. (1995), Hartmann et al. (2009), Ryan et al. (2009), An et al. (2011), Bakker et al. (2012)
	Alteration of plant resistance to disease and environmental factors.	Improved ability to resist to adverse environmental conditions (climatic, edaphic, and biological).	May produce unexpected or undesirable outcomes.	O'Connell et al. (1996), Lynch et al. (2004)
	Selection of mutants with enhanced capacity to form mutual symbiosis	Improved access to nutrient	Could be deleterious under high nutrient conditions Higher percentage of carbon allocated to symbionts	Solaiman et al. (2000)
Genetic modification: change in the amount and/or quality of the organic exudates, signal molecules, and residues entering the soil.	Engineering plants to produce exudates to favor specific diversity or beneficial services.	Plant induction of microbiome beneficial functional traits such as nodulation, siderophore, anti-microbial, anti-fungal, or biological control compounds. Improve resistance to adverse environments. Use in bioremediation of toxic compounds.	Inter-species plant-microbe gene transfers. When a desired trait has been engineered successfully into a plant, the compounds might be rapidly degraded, inactivated in the soil, or the rate of exudation might be too small to influence the rhizosphere as predicted.	Truchet et al. (1991), Downie (1994), O'Connell et al. (1996), Zupan et al. (2000), Brussaard et al. (2007), Broeckling et al. (2008), Bakker et al. (2012), Sharma et al. (2013)
	Engineering plants to produce exudates to modify soil properties (acidic pH, anion efflux from roots).	Improve plant growth at low pH, salinity resistance, and water deficit. Enhance plant Al^{3+} resistance. Improve ability to acquire insoluble P. Larger roots, longer root hairs, and greater shoot biomass.	Enzyme activities do not necessarily lead to anion accumulation and enhanced efflux, and suggest that metabolic or environmental factors can influence the effectiveness of this approach. The gene TaALMT1 (malate release in the rhizosphere) needs to be activated by Al^{3+}.	Koyama et al. (1999), Koyama et al. (2000), Tesfaye et al. (2001), Anoop et al. (2003), Li et al. (2005), Brussaard et al. (2007), Delhaize et al. (2007), Gévaudant et al. (2007), Yang et al. (2007), Ryan et al. (2009)
	Generation of transgenic plants producing quorum sensing signal molecules *N*-acyl-homoserine lactone (AHL).	May lead to an increase in plant disease resistance by blocking communication among members of the plant-associated bacterial community.	Blocking communication among members of the beneficial plant associated bacterial community.	Teplitski et al. (2000), Savka et al. (2002), Bakker et al. (2012)
	Engineering plants to produce an enzyme responsible for degradation of the quorum sensing signal (lactonases, acylases).	Prevention of bacterial infection.	Rhizosphere populations would be able to capture and stably integrate transgenic plant DNA, in particular antibiotic resistance genes used in the selection of successful transgenic plants.	Dong et al. (2001), Braeken et al. (2008), Zhang et al. (2015)

Acetobacter, *Herbaspirillum*, *Azoarcus,* and *Azotobacte*r, etc) fix atmospheric nitrogen, mycorrhiza recover N from NH_4 and NO_3 (Toussaint et al., 2004; Adesemoye et al., 2008; Mabood et al., 2008; Ryan et al., 2009), and phosphate solubilizing bacteria, AMF, ECM, and siderophore producers increase availability of many nutrients such as phosphorous (P), iron (Fe), cooper (Cu), Cadmiun (Cd), and zinc (Zn) (Savka et al., 2002; Mabood et al., 2008). Rhizobacteria also act as biocontrol agents, for instance, Pseudomonads, *Bacillus,* and *Streptomyces* produce antibiotics (DAPG, phenazine, hydrogene cyanide, oligomycin, etc), bacteriocines (Nisin) and antifungal compounds (pyoluteorin, phenazines, and phoroglucinols; Ping and Boland, 2004; Mabood et al., 2008; Paulin et al., 2009; Ryan et al., 2009).

Another strategy is to induce plant metabolic activities by modulating phytohormone synthesis by microbes (phytostimulation). As we mention before, microorganisms are capable of altering plant physiological pathways since they are able to produce all plant hormone identified to date (Friesen et al., 2011) or synthetize compounds that can mimic their actions (**Table 1**). Using microbes to exploit the plant hormonal system could improve plant growth and

TABLE 3 | Selected meta-organism-based methods and other complementary methods used to engineer the rhizosphere microbiome.

Method	Mechanisms/examples	Advantages	Disadvantages	Reference
Meta-organism-based				
Selecting and managing complementary plants and microbiomes	Crop Rotation	Induction of suppressive soils by managing soil diversity. Higher level of nutrients cycling and increase of organic carbon. Improvement of physico-chemical soil characteristics.	Mechanisms are not fully understood	Mazzola (2002, 2007), Ryan et al. (2009)
Engineering plants to produce one or more compounds and engineering the inoculated bacteria to degrade these compounds.	Opine producing plants co-inoculated with opine utilizing bacteria	Establishing a direct trophic link between the two partners of the interaction.		Savka and Farrand (1997), Dessaux et al. (1998), Savka et al. (2002)
Other methods				
Agricultural Inputs	Mineral fertilizers: urea, ammonium nitrate, sulfates, and phosphates.	Indirectly enhance soil biological activity via increases in system productivity, crop residue return, and soil organic matter.	N fertilization generates soil acidification and P fertilization affect root colonization of AMF.	Savka et al. (2002), Bünemann et al. (2006), Mazzola (2007)
	Organic fertilizers: animal manures, composts, and biosolids.	Increase in soil organic matter increase soil biological activity (organic fertilizers).	Biosolids: possible presence of toxic substances for the soil microflora. Inability to predictably reproduce compost composition.	

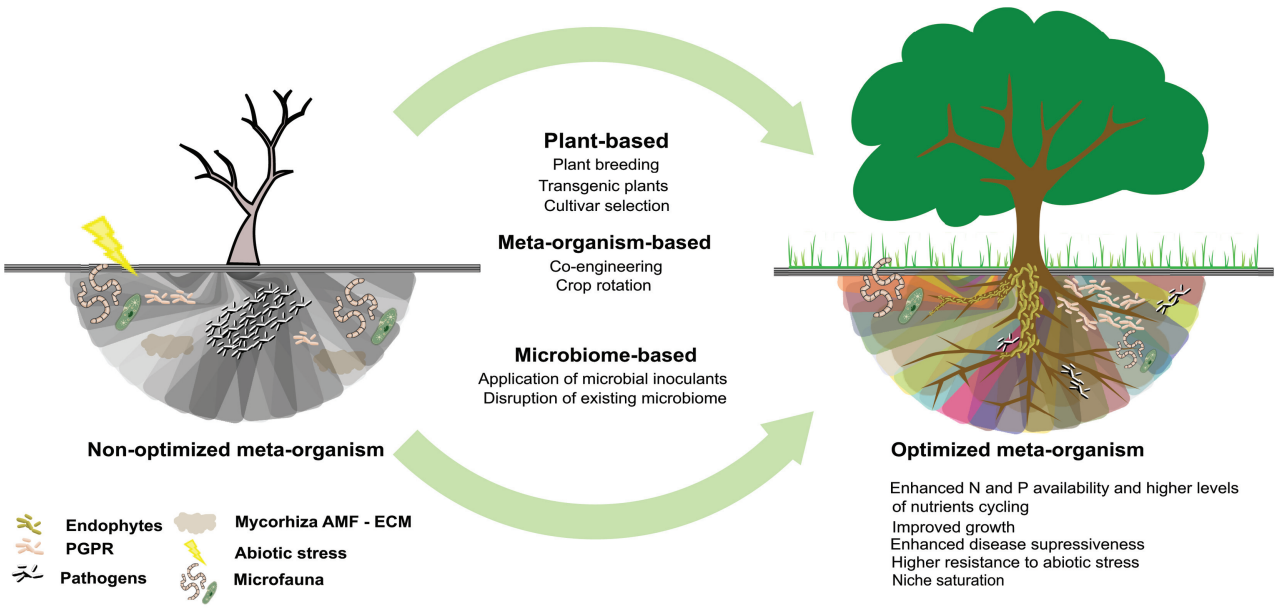

FIGURE 2 | Different approaches to rhizosphere microbiome engineering used to bring the microbiome from a low diversity and vulnerable state, with limited functions and productivity to a diverse and resilient state with high functional redundancy and consistent functioning across variable environments and increased resistance to pathogen invasion.

root development, leading to higher yields. Phytohormones produced by microorganisms such as auxins (indole-3-acetic acid), gibberelins and citokinins mirror the action of jasmonic acid (JA) which is critical for plant defense against herbivory, plant responses to poor environmental conditions (abiotic and biotic stress tolerance), regulation of signals exchange and nodulation (Hause and Schaarschmidt, 2009) and is involved in the signaling pathway against necrotrophic pathogens (Stein et al., 2008). Crosstalk mediated by salicylic acid, JA and ethylene activate plant SAR and induce systemic resistance (ISR) reducing phytotoxic microbial communities (Ping and Boland, 2004; van Loon et al., 2006; Mabood et al., 2008). Production of 1-aminocyclopropane-1-carboxylate (ACC) deaminase by rhizosphere microorganism is another characteristic that can

have a high impact on plant health as this enzyme degrades the ethylene precursor ACC, thereby reducing ethylene levels in the plant. When present in high concentrations ethylene can lead to plant growth inhibition or even death, but in lower amounts ethylene can also help the plant respond to a wide range of environmental stresses (Ryan et al., 2009; Glick, 2014).

Inoculation of recombinant strains is another strategy to enhance plant performance. In some cases, recombinant strains can resolve problems related to rapid decrease in population density and short persistence (Ryan et al., 2009), and as reported by Taghavi et al. (2005), could result in the enhancement of many members of the endogenous population by the transmission of genetic information via horizontal gene transfer (HGT). Even though very promising, the release of recombinant strains to the environment needs to be thoroughly assessed in order to evaluate the potential risks associated. To maximize the effects of inoculations, the disruption of existing microbial communities by fungicide application, crop rotation or tilling can be used to favor the selection of the appropriate microbiome for specific crops in order to establish biological functions in the rhizosphere (O'Connell et al., 1996; Savka et al., 2002; Bakker et al., 2012; **Table 1**). It is also essential to understand the evolution, organization, and structure of the rhizosphere microbial community throughout plant developmental stages and the way they naturally manipulate the composition of the rhizosphere microbiome, promoting, for instance, suppressive soils (Baker and Cook, 1974; Ryan et al., 2009; Mendes et al., 2011) or particular microbial functions in the rhizosphere like nutrient cycling or resistance to abiotic stress. In addition to specific microbial taxa or functions, community-wide characteristics can also be the target of microbiome engineering efforts as rhizosphere microbiome richness and evenness is linked to higher resilience to disruption, stress, and diseases. Increased microbial richness often results in greater community-level trait diversity and/or functional redundancy, which leads to more consistent functioning across variable environments (Loreau et al., 2001) Because rare members of the microbiome may be unable to effectively perform important functions, high evenness of the microbiome is also very important (**Figure 2**; Bünemann et al., 2006; Badri and Vivanco, 2009; Bakker et al., 2012; Qiu et al., 2014).

The Plant Route

Plant-based strategies to improve plant productivity through the selection of a more adapted microbiome include the manipulation of plants characteristics of interest mainly by two different approaches: plant breeding (cultivar selection) and specific genetic modifications (**Table 2**). Using plant breeding to select for a specific microbiome is an interesting avenue, as the technique has mainly focused on improving yields, plant resistance to pests/diseases and other plant physiological traits (Ryan et al., 2009). When microbiome selection was included in plant breeding programs, very specific functions or taxa were targeted. For example, Neal et al. (1970, 1973) used chromosomal substitution between two lines of wheat to improve resistance to root-rot while preserving beneficial populations of rhizosphere bacteria, and Mazzola (2002) compared wheat cultivars for their capacity to stimulate disease suppression by enhancing populations of specific antagonist (Pseudomonads) against *Rhizoctonia solani*.

Choosing a naturally occurring plant species or cultivar with a high capacity to recruit a beneficial microbiome or to promote the "suppressiveness" of soils is an alternative option that has been explored (Neal et al., 1973; Mazzola, 2002; Bakker et al., 2012). For instance, in the rhizosphere of plants growing in contaminated soils, the host plant exudes specialized antimicrobials and signaling molecules (i.e., flavonoids, salicylic acid, and phytoalexins), carbon and nitrogen compounds that promotes the expression of hydrocarbon degradation genes such as the genes coding for alkane hydroxylases (responsible for the aerobic degradation of aliphatic hydrocarbons) and several genes coding for enzymes implicated in the metabolism of aromatic compounds (Yergeau et al., 2014; Pagé et al., 2015). This recruitment was shown to be cultivar-specific, with native cultivar having an increased capacity to recruit beneficial ECM when growing in highly contaminated soils (Bell et al., 2014a), suggesting that native cultivars can better communicate with indigenous soil microorganisms. Accumulating evidence supports the feasibility of creating biotic soil environments that promote root health using selected plant genotypes. Ellouze et al. (2013) showed in the semiarid grasslands of North America, certain chickpea cultivars can select a more beneficial microbiome for the subsequent wheat plants and were associated with the antagonist species *Penicillium canescens*.

Using plants as selective agents to enrich beneficial microbial functions in soil implies the inclusion of other variables such as soil type and properties: different soil types not only shape the microbial communities, but also impact plant physiology, which in turn could alter interactions with soil microbes. The creation of genetically modified plants with enhanced ability to harbor particular exudation patterns to change soil properties have already been investigated (**Table 2**). Li et al. (2005); Gévaudant et al. (2007), and Yang et al. (2007) have worked to manipulate rhizosphere pH using transgenic lines of *Nicotiana tabacum* and *Arabidopsis* plants, transformed to over express a modified H^+ATP-ase protein (*PMA4* in tobacco and *AVP1 pyrophosphatase* in *Arabidopsis*) generating phenotypes such as increased H+-efflux from roots, more acidic rhizosphere, improved growth at low pH, improved salinity resistance (tobacco lines), plant mineral nutrition (P mineralization), and exhibiting enhanced resistance to water deficits (*AVP1*).

Plants can also be genetically modified to alter soil organic anion efflux and transportation from roots by (1) engineering plants with a greater capacity to synthesize organic anions or (2) engineering plants with a greater capacity to transport organic anions out of the cell. The organic anions malate and citrate have been studied as they are commonly release in response to nutrient deficiency and mineral stress. Koyama et al. (1999) and Koyama et al. (2000) reported that transgenic

plants with higher ability to excrete citrate from the roots grew better on P-limited soil than the wild type, suggesting crop plants with an enhanced ability to use Al-phosphate and therefore an enhanced ability to grow in acid soils and superior Al tolerance. In order to address the toxicity of the Al^{3+} in acidic soils, a very common problem in agriculture, Tesfaye et al. (2001), Anoop et al. (2003), and Delhaize et al. (2007) have also reported the use of transgenic plants *(Medicago sativa, Brassica napus,* and *Hordeum vulgare*) expressing genes coding for ALMT (Al^{3+}-activated malate transporter) and MATE (multi-drug and toxin extrusion) membrane proteins, a strategy to improve the P efficiency of plants and Al^{3+} resistance (**Table 2**).

Quorum sensing has been targeted by creating transgenic plants that would be able to mimic or interrupt bacterial QS signals by producing enzymes responsible for their degradation (acylases and lactonases). These modifications allowed these plants to defend themselves more efficiently against some pathogenic bacteria (Savka et al., 2002; Braeken et al., 2008; Ryan et al., 2009; Bakker et al., 2012). Many other studies have focused on the genetic manipulation of plants in order to modify the key exudates to favor the establishment of the desired plant–microbiome (**Table 2**). Genetic engineering provides unique opportunities to modulate plant–microbe signaling, to diversify exudation, to encourage diverse microbiomes or to stimulate beneficial microbial functions in the rhizosphere. However, despite these efforts, large-scale breeding/genetic improvement programs rarely take into account the plant–microbiome signaling channels during the development of new plant lines.

The Meta-Organism Route

The microbiome and the plant are highly dependent on each other as the microbiome contribute a significant portion of the secondary genome of the host plant, highlighting that the plant an its microbiome might function as a meta-organism or holobiont (Lakshmanan et al., 2014). Taking into account the meta-organism and trying to optimize the whole system instead of each of the part separately is a promising avenue for rhizosphere microbiome engineering. This is the case of the "opine concept" that combined engineering plants to produce specific exudates together with the inoculation of engineered microbes that are able to degrade this substrate, resulting in the colonization of the rhizosphere by a specific population (**Table 3**). It was also observed that opine production by transgenic plants led, in the long term, to the selection of bacterial populations adapted to the rhizosphere that can maintain themselves at high concentrations, even after removal of the transgenic plants (O'Connell et al., 1996; Savka et al., 2002; Ryan et al., 2009). These strategies (using specific metabolic resources) are highly specific, focusing on interactions between, for example, opine-producing plants, and members of the microbiome responsible for functions such as nodulation and *N*-fixation. However, this "opine" approach does not take into account others species that could have important functions or fill niche to reduce pathogen vulnerability.

In order to amplify the spectrum of diversity and ecological services to the crops, another strategy to shift rhizosphere microbiome is crop rotation. This strategy could be optimized by taking into account the whole plant–microbiome metaorganism. Indeed, plants are cultured in turns bringing their associated microbiome and generating beneficial allopathy. Crop history and cultivar selection stimulate specific rhizobacterial populations that complements each other developing a beneficial synergy between the cultures. Sturz et al. (1998) reported evidence for the role of bacterial endophytes resulting from the intercrop alternation of red clover and potato promoting plant growth and yield in the potato crops. Mazzola (2007) stated that most common and effective scheme to modify the rhizosphere has been the use of crop rotation. As increased plant diversity can enhance microbial community biomass, mixed cropping systems will generate a more diverse microbial community and thus should be more resilient to pathogen invasion. Disease control is achieved as the plant host for certain pathogen is absent resulting in the diminished viability of this pathogen. Some other advantages of these rhizosphere microbiome engineering approaches are an increase of soil organic carbon, a higher level of nutrients cycling and an improvement of physico-chemical soil characteristics (O'Connell et al., 1996; Sturz et al., 1998; Bakker et al., 2012).

Phytoremediation is another application where harnessing the plant–microbiome holobiont could significantly improve processes (Bell et al., 2014b). In the rhizosphere of contaminated soils, microbes increase the recycling and dissolving of mineral nutrients and the synthesis of amino acids, vitamins, auxins, and gibberellins that stimulate plant growth. These highly competitive populations seem to be selected by the host plant via exudation of specialized antimicrobials and signaling molecules (e.g., flavonoids, salicylic acid, and phytoalexins), carbon and nitrogen compounds, resulting in the degradation or transformation of contaminants due to both increased microbial activity and plant intervention (Marihal and Jagadeesh, 2013; Yergeau et al., 2014). Microorganisms also facilitate the uptake of contaminants and plant resistance to pollutant stress (Taghavi et al., 2005; Nadeem et al., 2013; Bell et al., 2015).

Finally, we should briefly mention organic agricultural inputs as a complementary strategy not included in the abovementioned categories (microbe, plant, or meta-organisms), but that also results in the modification of the rhizosphere microbiome. Organic agriculture aims at limiting or preventing the exposure of plants, microbes, and humans to unnecessary hazards such as pesticides, herbicides, insecticides, and fungicides. Organic fertilizer such as animal manure, biosolids, and compost has been proposed as a resource to amend crops but some disadvantage should be taken into account, namely, increase salinity, presence of active therapeutic agents (manure and sludge waste), heavy metals such as zinc, cooper, and cadmium (industrial biosolids) and residues of synthetic molecules like pesticides, herbicides etc, (green wastes or compost; **Table 3**). An important aspect to consider when applying any agricultural inputs is to target the increase in the levels of soil organic matter that in turn, will also increase soil biological activity (Savka et al., 2002; Bünemann et al., 2006; Mazzola, 2007).

Conclusion and Outlook

The microbiome is emerging as a fundamental plant trait, resulting in beneficial or detrimental effects on plant growth, health, productivity, and functions. This delicate balance is controlled by complex chemical signals interplay between the plant and its microbiome. Further research aiming at understanding this interplay at the community level is needed to fully understand the factors controlling microbiome assemblage and its feedback to the plant host. New 'omics tools will undoubtedly help attaining that goal, but at the same time further efforts to cultivate the rhizosphere microbiome will also be needed to reach a deeper mechanistic understanding of it. Based on the engineering efforts detailed in this contribution, further research will hopefully result in methods to purposefully, reliably, and sustainably engineer plant–microbiomes. A full optimization of the plant–microbiome meta-organism should result, among others, in a more sustainable agriculture, reduced greenhouse gas emissions, and increased rates of soil decontamination.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

REVIEW
published: 30 October 2015
doi: 10.3389/fpls.2015.00909

Bacteriocins from the rhizosphere microbiome – from an agriculture perspective

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Bacteria produce and excrete a versatile and dynamic suit of compounds to defend against microbial competitors and mediate local population dynamics. These include a wide range of broad-spectrum non-ribosomally synthesized antibiotics, lytic enzymes, metabolic by-products, proteinaceous exotoxins, and ribosomally produced antimicrobial peptides (bacteriocins). Most bacteria produce at least one bacteriocin. Bacteriocins are of interest in the food industry as natural preservatives and in the probiotics industry, leading to extensive studies on lactic acid bacteria (colicin produced by *Escherichia coli* is a model bacteriocin). Recent studies have projected use of bacteriocins in veterinary medicine and in agriculture, as biostimulants of plant growth and development and as biocontrol agents. For example, bacteriocins such as Cerein 8A, Bac-GM17, putidacin, Bac 14B, amylocyclicin have been studied for their mechanisms of anti-microbial activity. Bac IH7 promotes tomato and musk melon plant growth. Thuricin 17 (Th17) is the only bacteriocin studied extensively for plant growth promotion, including at the molecular level. Th17 functions as a bacterial signal compound, promoting plant growth in legumes and non-legumes. In *Arabidopsis thaliana* and *Glycine max* Th17 increased phytohormones IAA and SA at 24 h post treatment. At the proteome level Th17 treatment of 3-week-old *A. thaliana* rosettes led to >2-fold changes in activation of the carbon and energy metabolism pathway proteins, 24 h post treatment. At 250 mM NaCl stress, the control plants under osmotic-shock shut down most of carbon-metabolism and activated energy-metabolism and antioxidant pathways. Th17 treated plants, at 250 mM NaCl, retained meaningful levels of the light harvesting complex, photosystems I and II proteins and energy and antioxidant pathways were activated, so that rosettes could better withstand the salt stress. In *Glycine max*, Th17 helped seeds germinate in the presence of NaCl stress, and was most effective at 100 mM NaCl. The 48 h post germination proteome suggested efficient and speedier partitioning of storage proteins, activation of carbon, nitrogen and energy metabolisms in Th17 treated seeds both under optimal and 100 mM NaCl. This review focuses on the bacteriocins produced by plant-rhizosphere colonizers and plant-pathogenic bacteria, that might have uses in agriculture, veterinary, and human medicine.

Keywords: *Bacillus* species, *Bacillus thuringiensis*, bacteriocins, anti-microbial peptides

OPEN ACCESS

Edited by:
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University of Maryland, Baltimore County, USA

Reviewed by:
Yuanyuan Song,
Fujian Agriculture and Forestry University, China
Zhilong Bao,
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Specialty section:
This article was submitted to Plant Biotic Interactions, a section of the journal Frontiers in Plant Science

Received: *30 April 2015*
Accepted: *12 October 2015*
Published: *30 October 2015*

Citation:
Subramanian S and Smith DL (2015) Bacteriocins from the rhizosphere microbiome – from an agriculture perspective. Front. Plant Sci. 6:909. doi: 10.3389/fpls.2015.00909

INTRODUCTION TO BACTERIOCINS

Microbial population dynamics are primarily controlled by the products bacteria produce and excrete into their environs. This versatile and a dynamic suit of compounds order the defense mechanisms, rightly described as "a never ending arms race" (Riley, 1998) against microbial competitors and also as signaling compounds for plant colonization in a given soil. The excreted bacterial compounds we recognize now include a wide range of broad-spectrum non-ribosomally synthesized antibiotics, lytic enzymes (lysozymes), metabolic by-products such as organic acids, proteinaceous exotoxins, and chromosomally and/or ribosomally produced antimicrobial peptides, referred to as bacteriocins that are of particular importance in bacterial defense. It is supposed that most bacteria produce at least one bacteriocin.

Bacteriocins are extracellular substances produced by bacteria having distinctive morphological and biochemical characteristics, ranging from a very low to high molecular weight complexes, wherein the activity is predominantly associated with a protein. They are mostly synthesized from plasmids, but many are of chromosomal origin as well and are synthesized at various stages of bacterial growth and under various environmental conditions (Daw and Falkiner, 1996); they affect the growth of related bacterial species. Bacteriocins are grouped into four distinct classes based on peptide characteristics such as post translational modifications, side chains, heat stability, N-terminal sequence homology, and molecular weight (Klaenhammer, 1993). *Bacillus* species were first reported to produce bacteriocins in 1976 and the diversity of these is well described in the review by Abriouel et al. (2011). The low-molecular-weight bacteriocins of the Gram-positive bacteria were reported to demonstrate bactericidal activity, mainly against certain other Gram-positive bacteria (Tagg et al., 1976). The most studied bacteriocin is colicin of the Enterobacteriaceae (Pugsley, 1984). Due to their commercial importance as natural preservatives, and as therapeutic agents against pathogenic bacteria, these antimicrobial peptides have been a major area of scientific research (Tagg et al., 1976; Jack et al., 1995; de la Fuente-Salcido et al., 2013). Nisin, synthesized by *Lactococcus lactis*, is the only bacteriocin generally regarded as safe for human consumption (GRAS) but has limited usage since it is ineffective against Gram-negative bacteria (Olasupo et al., 2003) necessitating exploration of newer bacteriocins. Hence, this review is an update regarding the bacteriocins produced by plant-rhizosphere colonizers and those from plant-pathogenic bacteria that might have uses in agriculture and veterinary or human medicine.

BACTERIOCINS FROM RHIZOSPHERE BACTERIA

The bacteriocin cerein7, from *Bacillus cereus* with a mass of 3.94 kDa was the first to be isolated from this species (Oscáriz et al., 1999) although other bacillus species such as *B. thuringiensis*, *B. subtilis*, *B. stearothermophilus*, *B. licheniformis*, *B. megaterium*, and *B. cereus* were reported earlier to produce bacteriocin like products, of which subtilin from *B. subtilis* has been studied widely. The earliest studies of bacteriocin mode of action focused on *Rhizobium lupini* isolated from root nodules of lupines that harbor two strains of the species 16-2 and 16-3, the latter of which produces a bacteriocin to inhibit the growth of its closely associated 16-2 strain (Lotz and Mayer, 1972). A comparison of these two strains revealed that bacteriocin activity of one bacteria can be neutralized by lipopolysaccharides of other associated bacteria, by micellar modulation of LPS for bacteriocin adsorption. This bacteriocin neutralizing activity, as seen in bacteriocin sensitive *R. lupini 16-2* (Pfister and Lodderstaedt, 1977), leads one to wonder why certain bacteria are able to adapt and colonize plant roots more effectively, despite their sensitivity to bacteriocins. Now we are aware that bacteriocin resistance can be innate or acquired and this varies across strains of the same bacterial species. In general changes to the bacterial cell wall resulting in loss of bacteriocin insertion or binding regions, sequestration of bacteriocins, export or degradation of bacteriocins have been adopted by Gram-positive bacteria (de Freire Bastos et al., 2015).

Cerein 8A isolated from *B. cereus* 8A, interferes with cell membrane integrity and causes cell wall damage (Bizani et al., 2005), which is seen to be the mode of action of many bacteriocins. (Please refer to **Table 1** for bacteriocins from *B. thuringiensis* and examples of other bacteria). Bacteriocin Bac-GM17 from the rhizosphere bacteria *Bacillus clausii* strain GM17 of *Ononis angustissima* Lam. is a 5.158 kDa monomer protein with a unique sequence and having bactericidal effect on *Agrobacterium tumefaciens* C58 and fungistatic effect on *Candida tropicalis* R2 CIP203 (Mouloud et al., 2013). The bacteriocin putidacin, produced by *Pseudomonas putida* strain BW11M1, isolated from banana root, is very similar to mannose-binding plant lectins (Parret et al., 2003). Amylocyclicin, a 6.381 kDa peptide from *Bacillus amyloliquefaciens* FZB42, is a novel circular, ribosomally synthesized bacteriocin with high antibacterial activity to closely related Gram-positive bacteria (Scholz et al., 2014). *B. subtilis* strain 14B produces Bac 14B, a 21 kDa bacteriocin that is effective against crown gall disease caused by *A. tumefaciens* (Hammami et al., 2009). *B. subtilis* strain IH7 produces a bacteriocin Bac IH7 which is reported to be a plant growth promotor. Tomato and muskmelon treated with Bac IH7 showed enhanced germination percentage and increased shoot weight and height and root lengths; it also served as a biocontrol for *Alternaria solani* and other seed borne pathogens (Hammami et al., 2011). A 25–35 kDa bacteriocin from *Lysinibacillus* jx416856, a bacteria isolated from fruit and vegetable waste, was observed to inhibit food borne pathogens such as *Staphylococcus aureus*, *Staphylococcus epidermidis*, and *B. cereus* (Ahmad et al., 2014).

Some plant pathogens have also been found to produce bacteriocins. Phytopathogenic strain *Erwinia carotovora* NA4, isolated from diseased fruits and vegetables, produces the bacteriocin erwiniocin NA4 and *Agrobacterium radiobacter* NA5 (pepper rhizosphere isolate) produces agrocin NA5 (Jabeen et al., 2004). Tomato pathogen *Clavibacter michiganensis* ssp. *michiganensis* produces a bacteriocin michiganin A, which inhibits the growth of another pathogen *C. michiganensis* subsp.

sepedonicus, which causes ring rot of potatoes. This bacteriocin also has similarity to a type B lantibiotic produced by the actinomycete *Actinoplanes liguriae* (Holtsmark et al., 2006). *Pseudomonas syringae* pv. *syringae* produces S-type pyocins (Feil et al., 2005), which are also produced by the opportunistic human pathogen *Pseudomonas aeruginosa*. Carocin S1, is a 55 kDa bacteriocin from *Pectobacterium carotovorum* (previously known as *E. carotovora* ssp. *carotovora*; Holtsmark et al., 2008).

Bacillus thuringiensis is the most studied *Bacillus* species due to its interesting array of excreted proteins. *B. thuringiensis* is a Gram-positive spore-forming bacterium characterized and distinguished from closely related *Bacillus* species by its ability to synthesize characteristic endotoxins that are active against diptera, coleoptera, and Lepidoptera larvae (Schnepf et al., 1998; Palma et al., 2014). Widely used as a bioinsecticide, it also accounts for about 90% of the commercially available biopesticides produced (Chattopadhyay et al., 2004), apart from the Bt genes incorporation in several commercial crops, the proteomics and genomics of which is well known (Ibrahim et al., 2010; de la Fuente-Salcido et al., 2013). *B. thuringiensis* was discovered as early as 1901 in Japan by bacteriologist S. Ishiwata as an isolate from diseased *Bombyx mori* (L.) larvae and was named Sottokin meaning "Sudden death bacillus". A similar study by Ernst Berliner, described *B. thuringiensis* as the causal organism of insect death isolated from *Anagasta kuehniella* (Zeller) larvae from Thuringia, Germany in 1915 [Beegle and Yamamoto, 1992 (a very good review for the history of *B. thuringiensis*)].

Bacillus thuringiensis strains have been found to produce bacteriocins such as thuricin (>950 kDa; Favret and Yousten, 1989), tochicin (10.5 kDa; Paik et al., 1997), thuricin 7 (11.6 kDa; Cherif et al., 2001), thuricin 439A and thuricin 439B (2.9 and 2.8 kDa, respectively; Ahern et al., 2003), entomocin 9 (Cherif et al., 2003), bacthuricin F4 (3.160 kDa; Kamoun et al., 2005), thuricin 17 (3.162 kDa; Gray et al., 2006b), etc. A comprehensive list of known bacteriocins is provided in **Table 1**. New bacteriocins are being discovered regularly. Raddadi et al., (2009) evaluated 16 strains of *B. thuringiensis* for their capability to protect plants from phytopathogens. Among them, Bt HD868 tochigiensis and Bt HD9 entomocidus strains were observed to be the least cytotoxic, and hence potentially acceptable for the food industry and field crop application for protection against deleterious bacteria. This compatibility was based on the levels of autolysins, bacteriocins and AHL-lactonases, and antibiotic Zwittermicin A activities. Further these strains were also active against fungal diseases caused by *Aspergillus niger*, *Aspergillus fumigatus*, *Aspergillus flavus*, *Cryphonectria parasitica*, *Fusarium oxysporum*, *Monilia sitophila*, *Monilia hiemalis*, *Penicillium digitatum*, and *Rhizopus* sp. (Raddadi et al., 2009). While many *B. thuringiensis* strains have been identified and bacteriocins were isolated and characterized to an extent, none of these bacteriocins have been studied for plant growth promotion as extensively as thuricin 17 from *B. thuringiensis* NEB17.

Bacillus thuringiensis NEB17 was isolated from soybean root nodules as putative endophytic bacteria in 1998 in Prof. Donald Smith's laboratory at McGill University. When co-inoculated

TABLE 1 | Bacteriocins identified from *Bacillus thuringiensis* and examples of other bacteriocin producers.

Bacillus thuringiensis strain	Name of Bacteriocin identified	Molecular weight	Reference
HD-2	Thuricin	>950 Da	Favret and Yousten, 1989
ssp. *tochigiensis* HD868	Tochicin	10.5 kDa	Paik et al., 1997
BMG1.7	Thuricin 7	11.6 kDa	Cherif et al., 2001
B439	Thuricin 439	3 kDa	Ahern et al., 2003
ssp. *entomocidus* HD9	Entomocin 9	12.4 kDa	Cherif et al., 2003
BUPM4	Bacthuricin F4	3.1 kDa	Kamoun et al., 2005
NEB17	Thuricin 17	3.16 kDa	Gray et al., 2006b
ssp. *entomocidus* HD110	Entomocin 110	4.8 kDa	Cherif et al., 2008
ssp. *entomocidus* HD198	Thuricin S	3.1 kDa	Chehimi et al., 2007
ssp. *morrisoni* (LBIT 269	Morricin 269	10 kDa	Barboza-Corona et al., 2007; de la Fuente-Salcido et al., 2008
ssp. *kurstaki* (LBIT 287)	Kurstacin 287	10 kDa	Barboza-Corona et al., 2007; de la Fuente-Salcido et al., 2008
ssp. *kenyae* (LBIT 404)	Kenyacin 404	10 kDa	Barboza-Corona et al., 2007; de la Fuente-Salcido et al., 2008
ssp. *entomocidus* (LBIT 420)	Entomocin 420	10 kDa	Barboza-Corona et al., 2007; de la Fuente-Salcido et al., 2008
ssp. *tolworthi* (LBIT 524)	Tolworthcin 524	10 kDa	Barboza-Corona et al., 2007; de la Fuente-Salcido et al., 2008
SF361 [isolated from honey]	Thuricin H	3.1 kDa	Lee et al., 2009a
DPC6431	Thuricin CD	2.763/ 2.861 kDa	Rea et al., 2010
BUPM103	Bacthuricin F103	11 kDa	Kamoun et al., 2011
ssp. *kurstaki* Bn1 [isolated from a Hazel nut pest]	Bn1	3.193 kDa	Ugras et al., 2013
Examples from *Bacillus* and other bacterial species			
Bacillus cereus	Cerein 7	3.94 kDa	Oscáriz et al., 1999
Pseudomonas putida BW11M1	Putidacin		Parret et al., 2003
Bacillus cereus 8A	Cerein 8A		Bizani et al., 2005
Clavibacter michiganensis ssp. *michiganensis*	Michiganin A		Holtsmark et al., 2006
Bacillus licheniformis	BL8	1.4 kDa	Smitha and Bhat, 2012
Bacillus clausii	Bac GM17	5.158 kDa	Mouloud et al., 2013

with *Bradyrhizobium japonicum* under nitrogen free conditions, this bacterium promoted soybean growth, nodulation, and grain yield (Bai et al., 2002b, 2003). Subsequently, the causative agent of plant growth promotion, a bacteriocin, was isolated from *B. thuringiensis* NEB17, and is now referred to as thuricin 17 (Gray et al., 2006b). Initially, its partial sequence was determined (Gray et al., 2006a), and its full sequence has since reported (Lee et al., 2009b). Thuricin 17 is a low molecular weight peptide of 3.162 kDa, stable across a pH range of 1.0–9.25, highly heat resistant and is inactivated by treatment with proteolytic enzymes. Based on its N-terminal sequence homology of Th17 and that of bacthuricin F4, a new class of bacteriocins, class IId was proposed (Gray et al., 2006b). The bacteriocins produced by *B. thuringiensis* strain NEB17 (Th17) and *B. thuringiensis* ssp. *kurstaki* BUPM4 (bacthuricin F4 – 3160.05 Da) have been reported to show functional similarities and anti-microbial activities (Jung et al., 2008a).

In addition, Th17, applied as leaf spray and root drench, has positive effects on soybean and corn and stimulated plant growth. The leaves of 2-week-old soybean leaves sprayed with Th17 showed increased activities of lignification-related and antioxidative enzymes and their isoforms (Jung et al., 2008a; Lee et al., 2009b); this constituted the first report of plant growth stimulation by a bacteriocin. Recent research on Th17 has highlighted its plant growth promotion and abiotic stress alleviation properties. It was found that at 24 h after exposure to Th17, *Arabidopsis thaliana* Col-0 rosettes showed decreased levels of cytokinins, gibberellins, JA, and ABA; and an increase in IAA (85.39%) and SA (42.21%) as compared to untreated control plants. *A. thaliana* responded positively to treatment with Th17 in the presence of salt stress (up to 250 mM NaCl). Shotgun proteomics of unstressed and 250 mM NaCl stressed *A. thaliana* rosettes (7 days post stress) in combination with Th17 revealed carbon and energy metabolic pathways being affected under both unstressed and salt stressed conditions. Chloroplast proteins and proteins of photosystem I and II that are generally strongly and negatively affected by salt stress and PEP carboxylase, Rubisco-oxygenase large subunit, and pyruvate kinase, were some of the noteworthy proteins enhanced (>2-fold changes in the activation of the carbon and energy metabolism pathway) by Th17 application, along with other stress related proteins. These findings suggest that the proteome of *A. thaliana* rosettes is altered by the bacterial signal, and more so under salt stress, thereby imparting a positive effect on plant growth under high salt stress (Subramanian, 2014).

Application of Th17, under water stress conditions, to 1 month-old soybean plants increased plant biomass by 17%, root biomass by 37% and root nodule biomass by 55%, and also the amount of abscisic acid in soybean roots by 30% (Prudent et al., 2014). Application of Th17 to soybean seeds (variety Absolute RR) caused accelerated seed germination under salt stress of up to 150 mM NaCl, with the best response seen at 100 mM NaCl. Shotgun proteomics of unstressed and 100 mM NaCl stressed seeds (48 h) in combination with Th17 revealed that carbon, nitrogen and energy metabolic pathways were affected under both unstressed and salt stressed conditions. PEP carboxylase, Rubisco oxygenase large subunit, pyruvate kinase, alcohol dehydrogenase, and isocitrate lyase were some of the noteworthy proteins enhanced (>2-fold changes), by the signal, along with antioxidant glutathione-*S*-transferase and other stress related proteins. The up-regulation of PEP carboxylase and a marked down-regulation of α- and β-subunits of conglycinin, glycinin, as compared to the control treatment, is indicative of efficient storage protein utilization in conjunction with thioredoxin. These findings suggest that the germinating seeds alter their proteome based on bacterial signals and on stress level; the specificity of this response plays a crucial role in organ maturation and transition from one stage to another in a plant's life cycle; understanding this response is of fundamental importance in agriculture and, as a result, global food security (Subramanian, 2014). As observed in our experiments, the effective concentration of bacteriocin for enhanced plant growth and production is in the order of nanomolar (10^{-9} M), which makes it economically viable as method to decrease the use of energy based fertilizers and chemicals used in agricultural crop production systems.

BACTERIOCINS IN THE VETERINARY INDUSTRY AND HUMAN MEDICINE

Bacteriocins from *B. thuringiensis* also have proven to be of importance in veterinary medicine. *S. aureus* causes clinical and subclinical bovine mastitis, which is difficult to treat due to increased frequency of resistance to antimicrobial agents. *S. aureus* isolates recovered from milk composite samples of Holstein lactating cows in Mexico were evaluated for susceptibility of the isolates to 12 antibiotics and five bacteriocins from *B. thuringiensis*. *S. aureus* isolates were resistant to penicillin (92%), dicloxacillin (86%), ampicillin (74%), and erythromycin (74%) and susceptible to gentamicin (92%), trimethoprim (88%) and tetracycline (72%). *S. aureus* isolates also showed susceptibility to the five bacteriocins synthesized by *B. thuringiensis*, morricin 269 and kurstacin 287 followed by kenyacin 404, entomocin 420 and tolworthcin 524 suggesting an alternate method of controlling bovine mastitis (Barboza-Corona et al., 2009).

Mexican strains of *B. thuringiensis*, *B. thuringiensis* ssp. *morrisoni* (LBIT 269), *B. thuringiensis* ssp. *kurstaki* (LBIT 287), *B. thuringiensis* ssp. *kenyae* (LBIT 404), *B. thuringiensis* ssp. *entomocidus* (LBIT 420) and *B. thuringiensis* ssp. *tolworthi* (LBIT 524) produce proteinaceous Bt-BLIS bactericidal activities against *B. cereus* and *Vibrio cholera* (Barboza-Corona et al., 2007) but had no effect against Gram-negative bacteria such as *Escherichia coli*, *Shigella* species, and *P. aeruginosa*, all of which are human pathogens. Entomocin 9 was found to be bactericidal to *Listeria monocytogenes*, pathogenic *P. aeruginosa* and several fungi causing cell lysis of growing cells and non-toxic to Vero cells (Cherif et al., 2003). Thuricin 439, however, is a narrow spectrum bacteriocin capable of affecting growth of *B. cereus* (Ahern et al., 2003), while thuricin S is anti-*Listeria* (Chehimi et al., 2007) and a pore-forming bacteriocin (Chehimi et al., 2010). *B. cereus* cells protect themselves from Enterocin AS-48 produced by *Enterococcus faecalis* S48 by up-regulating the

BC4207 membrane protein for probable membrane structure modulation (Burgos et al., 2009). While pyocins produced by *P. aeruginosa* strains have proven to be toxic by degrading DNA in sensitive bacterial cells (Parret and De Mot, 2002). *B. subtilis* strain LFB112 from Chinese herbs produces a 6.3 kDa bacteriocin that is effective against *E. coli*, *Salmonella pullorum*, *P. aeruginosa*, *Pasteurella multocida*, *Clostridium perfringens*, *Micrococcus luteus*, *Streptococcus bovis*, and *S. aureus* IVDC C56005, all of which are common domestic animal related pathogens (Xie et al., 2009).

Optimizing medium composition, incubation and agitation speed can result in enhancement of the production of some bacteriocins. For example, *B. thuringiensis* subsp. *kurstaki* strain, producing the bacteriocin Bacthuricin F4 when grown in TSB medium with an optimal carbon–nitrogen ratio of 9 increases the bacteriocin production fourfold (Kamoun et al., 2009). Bacteriocin *Bacillus* sp. YAS 1 could be increased 1.6-fold by this method. This bacteriocin had a wide pH range (1–13) as well as temperature (45–80°C) with antimicrobial activity to human pathogens such as *Clostridium*, *Staphylococcus*, *Enterococcus*, and *Salmonella*, and plant pathogens such as *E. amylovora*, and showing no effect on lactic acid bacteria (Embaby et al., 2014).

BACTERIOCINS FROM OTHER INTERESTING BACTERIAL SOURCES

While most bacteriocins we know today have been isolated from the rhizosphere bacteria, bacteria producing bacteriocins are wide spread. A *B. subtilis* strain isolated from a Chinese fermented seasoning produces a 3.4 kDa bacteriocin that is active against *B. cereus* and *L. monocytogenes* (Zheng and Slavik, 1999). Maari, an alkaline fermented food condiment made from baobab tree seeds, is comprised of several strains of *B. subtilis*, all of which are necessary for enhancing the flavor and texture of the product. Three *B. subtilis* strains (B3, B122, and B222) isolated from maari produced bacteriocins that had antibacterial activities against *B. cereus* NVH391-98, a common opportunist human pathogen that contaminates maari (Kaboré et al., 2013). People in Burkina Faso and neighboring countries consume a fermented product called bilakga, derived from the seeds of *Hibiscus sabdariffa*. The fermentation concoction is largely comprised of *B. subtilis* subsp. *subtilis* and *Bacillus licheniformis* isolates. PCR detection of genes coding for surfactins and plipastatins (fengycins) suggested the production of subtilosin, subtilin and lipopeptide, while a protein (a probable bacteriocin) with a mass of 3.347 kDa was also isolated (Compaoré et al., 2013). *B. thuringiensis* ssp. *kurstaki* Bn1 isolated from hazel nut pest *Balaninus nucum* L. produces a bacteriocin Bt-Bn1, the first of its kind of insect origin. Like its potential commercial counterparts, this bacteriocin has antibacterial activity against *B. cereus*, *P. syringae*, and *P. lemoignei*, and other *B. thuringiensis* strains (Ugras et al., 2013).

CONCLUSION

Excessive use of fertilizers and other chemicals in agriculture and multi-drug resistant microbes are two major challenges for scientists worldwide. Bacteriocins evaluated as plant growth promotors and those with disease suppression mechanisms are a viable option for efficient use in agriculture, to reduce the use of fertilizers and chemical inputs such as fungicides and insecticides. With respect to multi-drug resistance, bacteriocins can be interesting in commercial utilization as target proteins for replacing ineffective antibiotics or for combinatorial drug therapy both in veterinary and human medicine, apart from their use in food preservation.

FUNDING

The authors wish to acknowledge support from the NSERC strategic Green Crop Network, from the Eastern Canadian Oilseed Development Alliance ECODI network and from the NCE funded BioFuelNet Canada.

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