Molecular basis of plant growth promotion and biocontrol by rhizobacteria

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Plant-growth-promoting rhizobacteria (PGPRs) are used as inoculants for biofertilization, phytostimulation and biocontrol. The interactions of PGPRs with their biotic environment, for example with plants and microorganisms, are often complex. Substantial advances in elucidating the genetic basis of the beneficial effects of PGPRs on plants have been made, some from whole-genome sequencing projects. This progress will lead to a more efficient use of these strains and possibly to their improvement by genetic modification.

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Abbreviations

AFM	anti-fungal metabolite
AHL	*N*-acyl homoserine lactone
DAPG	2,4-diacetylphloroglucinol
GFP	green fluorescent protein
IAA	indole acetic acid
ISR	induced systemic resistance
IVET	*in vivo* expression technology
PGPR	plant-growth-promoting rhizobacterium
SAR	systemic acquired resistance

Introduction

In the rhizosphere, that is on the plant root or its close vicinity, bacteria are abundantly present, most often organized in microcolonies. Some of these rhizobacteria not only benefit from the nutrients secreted by the plant root but also beneficially influence the plant in a direct or indirect way, resulting in a stimulation of its growth. These plant-growth-promoting rhizobacteria (PGPRs) can be classified according to their beneficial effects. For instance, biofertilizers can fix nitrogen, which can subsequently be used by the plant, thereby improving plant growth when the amount of nitrogen in the soil is limiting. Phytostimulators can directly promote the growth of plants, usually by the production of hormones. Biocontrol agents are able to protect plants from infection by phyto-pathogenic organisms.

The large-scale application of PGPRs to crops as inoculants would be attractive as it would substantially reduce the use of chemical fertilizers and pesticides, which often pollute the environment. In addition, the application of PGPRs would increase crop yield, thereby helping to feed the growing world population. A growing number of PGPRs are being marketed. These strains can only be used optimally, however, if the molecular basis of their beneficial effects, and the way these traits are influenced by biotic and abiotic factors, are understood. This brief review focuses on the most recently published results on the molecular basis of plant growth promotion by rhizobacteria. It emphasizes developments in the field of microbial control of phytopathogenic fungi.

Biofertilization

At present, biofertilization accounts for approximately 65% of the nitrogen supply to crops worldwide. Legumes are often used as green fertilizers. The most efficient nitrogen fixers are bacterial strains belonging to the genera *Rhizobium*, *Sinorhizobium*, *Mesorhizobium*, *Bradyrhizobium*, *Azorhizobium* and *Allorhizobium*, and it is these strains that have been studied in most detail. All of these bacteria form a host-specific symbiosis with leguminous plants. The symbiosis is initiated by the formation of root or stem nodules in response to the presence of the bacterium. Lipooligosacharide signal molecules that are secreted by the bacterium play a crucial role in this process (for recent reviews see [1–3]). The bacteria penetrate the cortex, induce root nodules, multiply and subsequently differentiate into bacteroids, which produce the nitrogenase enzyme complex. Within the root nodules, the plant creates a low oxygen concentration, which allows bacterial nitrogenase to convert atmospheric nitrogen into ammonia. In return, the plant supplies the bacteria with a carbon source.

The gene products of both the *nif* and the *fix* genes, which are involved in nitrogen fixation, have been characterized and described in detail. A major challenge in rhizobial research is to understand how the bacterial signals, which trigger the formation of the nodule, are perceived by the plant. Genome analyses of model legumes such as *Lotus* and *Medicago* are in progress, and comparative genomics with other non-legumes may indicate which genes make leguminous plants susceptible to the establishment of the symbiosis. A practical challenge is to widen the host range of the symbiosis towards major non-leguminous food crops such as rice. An exciting new finding is that rhizobial bacteria contain genes on the Sym (Symbiosis) plasmid [4] or chromosomally in a symbiotic island [5] that are homologous to those encoding type-III secretion systems, which are used by pathogenic bacteria to deliver virulence factors into host cells. Recent results show that rhizobial type-III secretion systems secrete specific proteins and are involved in the establishment of the symbiosis ([6]; M Bladergroen, K Badelt, O Stronk, B Lugtenberg, H Spaink, unpublished data), suggesting that type-III secretion systems also function in symbiosis. Remarkably, genes encoding type-III secretion systems have also been identified in a plant-beneficial *Pseudomonas fluorescens* strain [7].

Free-living nitrogen-fixing rhizobacteria such as *Azospirillum*, *Herbaspirillum*, *Acetobacter*, *Azotobacter* and *Azoarcus* are also able to fix atmospheric nitrogen (for a recent review see [8]). They use a nitrogenase complex that functions under low oxygen conditions and that is not as specific in its interaction with the plant as are rhizobia. *Azospirillum* predominantly colonizes the rhizosphere, whereas the other bacteria are predominantly found as endophytes inside roots, stems and leaves. The genes involved in nitrogen fixation, nitrogen assimilation and nitrogen regulation have been described for *Azospirillum*. Several of the *nif* genes have also been described for the other free-living nitrogen fixers, which all have similar nitrogenase complexes, except for *Azoarcus* which possesses three differently encoded nitrogenase complexes.

Phytostimulation

Phytostimulators enhance plant growth in a direct way. The mechanisms behind the stimulatory effect on root development and crop yield caused by *Azospirillum* spp. are beginning to be understood. Besides having nitrogen-fixing ability, *Azospirillum* spp. secrete phytohormones such as auxins, cytokinins and gibberellins [8]. Auxins are quantitatively the most abundant phytohormones secreted by *Azospirillum*, and it is generally agreed that auxin production, rather than nitrogen fixation, is the major factor responsible for the stimulation of rooting and, hence, enhanced plant growth. The construction of *Azospirillum* mutants that completely lack auxin production is complicated because three pathways for indole acetic acid (IAA) biosynthesis are present in *Azospirillum*. Recently, it was indicated that the pathways are differently regulated by catabolite repression [9], and that IAA synthesis is regulated by the autoinduction of IAA [10]. *Azospirillum* also enhances the growth of the freshwater microalga *Chlorella vulgaris*, and therefore could potentially be used to improve water cleaning [11•].

Biocontrol agents

'Suppressive soils' contain rhizobacteria that are able to control plant diseases that are caused by fungi or bacteria. The mechanisms responsible for this biocontrol activity include competition for nutrients, niche exclusion, induced systemic resistance (ISR), and the production of anti-fungal metabolites (AFMs). The biocontrol agents that are best-characterized at the molecular level belong to the genus *Pseudomonas*.

Most of the identified *Pseudomonas* biocontrol strains produce AFMs, of which phenazines, pyrrolnitrin, 2,4-diacetylphloroglucinol (DAPG) and pyoluteorin are the most frequently detected classes. However, new AFMs belonging to the class of cyclic lipopeptides, such as viscosinamide [12] and tensin [13], have been discovered. Viscosinamide prevents the infection of sugarbeet by *Pythium ultimum* [14]. The genetic basis of the biosynthesis of the more frequently detected AFMs in *Pseudomonas* has been elucidated. More recently, new information has become available on the biosynthesis of pyoluteorin in *P. fluorescens* Pf-5 [15] and of 2,4-diacetylphloroglucinol (DAPG) in *P. fluorescens* Q2-87 [16,17]. Individual genes have been discovered that are responsible for the presence of functional groups on phenazine compounds, such as *phzO*, which is required for the 2-hydroxy group [18] and *phzH*, which is responsible for the 1-carboxamide group [19•]. On the basis of homology and mutant studies, functions have been proposed for many of the identified biosynthetic genes. Biochemical studies are required, however, to prove these functions.

Streptomyces and *Bacillus* species can also exert biocontrol, which is being characterized at the molecular level. For example, the biosynthetic gene cluster responsible for the production of the antibiotic zwittermicin A in *Bacillus cereus* has been identified [20].

Regulation of anti-fungal metabolite production in *Pseudomonas* biocontrol strains

The production of AFMs in *Pseudomonas* is subject to complex regulation. Key factors in the regulation of the biosynthesis of most AFMs are global regulation and quorum sensing. Global regulation is directed by the *gacS*/*gacA* genes, which encode a two-component regulatory system that senses an as yet unknown signal(s). Quorum sensing involves the production of *N*-acyl homoserine lactone (AHL) signal molecules by an AHL synthase such as LuxI (for a recent review see [21]). At a threshold concentration of AHL, which is reached only when a certain density of bacterial cells is present, the AHL will sufficiently bind to and activate a transcriptional regulator, such as LuxR. The activated form of the transcriptional regulator then stimulates gene expression.

Spontaneous *gacS* or *gacA* mutants of *P. fluorescens* strain CHA0 have a substantial selective advantage over the wild-type strain when growing in a liquid medium (as has been demonstrated in a nutrient broth medium that contained yeast extract). This can present a severe problem to the production of inoculants. This difficulty can be reduced, however, by mineral amendments or by simply diluting the medium [22•], which could at least partially explain the observed stimulation of AFM production in *P. fluorescens* strain CHA0 by various minerals and carbon sources [23].

The involvement of GacS/GacA in the regulation of extracellular products, such as protease, hydrogen cyanide and other AFMs has been established firmly. Only very recently, it was shown that GacA indirectly controls the hydrogen cyanide synthase genes (*hcnABC*) and the protease gene *aprA* in *P. fluorescens* CHA0 by a posttranscriptional mechanism involving a distinct recognition site that overlaps the ribosomal binding site [24]. It has also been shown that the global translational repressor RsmA acts downstream of the GacA-dependent pathway [24], but that some products of the *infC* operon compete with RsmA and stimulate

production [25]. At the transcriptional level, the *hcnABC* genes are regulated by the anaerobic regulator ANR. In *P. aeruginosa*, the activity of ANR has also been indicated to respond to iron availability [26] and to AHL signal molecules [27].

The GacS/GacA regulatory system also controls quorum sensing, as was shown in *P. aureofaciens* 30–84 [28], illustrating the enormous complexity of the regulation of secondary metabolite production in *Pseudomonas*. In *Pseudomonas* biocontrol agents, various quorum systems have been identified that are involved in the regulation of AFMs. A complex regulation by quorum sensing has also been identified in *Rhizobium leguminosarum*, which contains multiple quorum-sensing systems that form a regulatory cascade [29•]. Recently, an AHL from biocontrol strain *P. fluorescens* F113 was elucidated and surprisingly identified as the rhizobial small bacteriocin *N*-(3-hydroxy-7-cis-tetradecanoyl) homoserine lactone [30]. The production of this bacteriocin and two more common AHLs is directed by the *hdtS* gene product, which belongs to a novel class of acyl synthases [31]. It should be noted that the involvement of *hdtS* in the production of 2,4-DAPG has not yet been shown. Mutant strains that have reduced levels of AHL produce reduced or even non-detectable amounts of AFMs. It was shown very recently that a mutation in the *lexA* gene of *Pseudomonas chlororaphis* PCL1391 resulted in a ten-fold increase in phenazine-1-carboxamide production, which can be explained by the fact that the *lexA* mutant produces elevated levels of AHL [32].

An exciting report on transgenic plants that produce various AHLs [33] presented novel perspectives on biocontrol and on optimizing the application of biocontrol strains. Recently, it was suggested that plants can produce and secrete substances that mimic AHL activity, and could therefore influence the density-dependent behavior of rhizobacteria [34]. Other forms of regulation of AFM production have been described. These include a novel form of global regulation of AFM production by the Lon protease in *P. fluorescens* Pf-5 [35•], regulation of DAPG production by the transcriptional repressor PhlF [17,18], and the positive effect of PrrB RNA on secondary metabolite production in *P. fluorescens* F113 [36•]. A striking new finding in *P. fluorescens* CHA0 is the regulation of DAPG production by autoinduction and its repression by salicylate and pyoluteorin produced by the same cells, as is the repression of DAPG production by the fungal metabolite fusaric acid [37••]. In contrast, phenazine-1-carboxamide production in strain PCL1391 is not subjected to autoinduction [38].

Induced systemic resistance

Various non-pathogenic *Pseudomonas* rhizobacteria have the ability to induce a state of systemic resistance in plants, which provides protection against a broad spectrum of phytopathogenic organisms including fungi, bacteria and viruses [39]. ISR acts through a different signaling pathway to that regulating systemic acquired resistance (SAR), the ISR pathway is induced when the plant is challenged by pathogenic organisms. The list of rhizobacterial *Pseudomonas* species that are known to induce an ISR is growing rapidly. A degree of dependence on plant genotype is observed in the generation of these ISRs. Bacterial determinants that are claimed to produce ISRs include siderophores, the *O*-antigen of lipopolysacharide and salicylic acid. The latter compound has even been indicated to cause an ISR when present in nanogram amounts [40]. Elucidation of the plant factors involved in the pathways leading to ISR and SAR has shown that induced disease resistance can be enhanced by the simultaneous activation of ISR and SAR pathways [41•].

Rhizosphere competence

Inoculant bacteria are often applied in seed coatings. After sowing, the inoculant bacteria must be able to establish themselves in the rhizosphere at population densities sufficient to produce a beneficial effect. Therefore, efficient inoculant bacteria should survive in the rhizosphere, make use of nutrients exuded by the plant root, proliferate, be able to efficiently colonize the entire root system and be able to compete with endigenous microorganisms. Inadequate biocontrol in field experiments has often been correlated to poor root colonization. Identification of the genes and traits involved in the processes of inoculation and root colonization will give a more detailed insight into plant–bacterial interactions and lead to the more efficient application of inoculant strains.

The first step in the inoculation process is the attachment of the bacterial cells to the seed. A screen for mutants of the rhizobacterial strain *Pseudomonas putida* KT2440 resulted in the identification of a set of putative surface and membrane proteins involved in attachment to corn seeds. Among these proteins are homologs of a calcium-binding protein, of hemolysin and of a potential multi-drug efflux pump [42]. *P. fluorescens* genes that are specifically expressed in the rhizosphere (i.e. *rhi* genes) have been identified using *in vivo* expression technology (IVET) [7]. More then twenty *rhi* genes have been identified, of which fourteen showed significant homology to genes that are involved in nutrient acquisition, stress response, or secretion. Another 6 *rhi* genes show no homology to genes with identified functions. Many root colonization genes and traits from *Pseudomonas* biocontrol species have been identified (for recent reviews see [43,44]) and have even been used to improve colonization by *Pseudomonas* wild-type strains [45•]. Recent studies have shown that organic acids form the nutritional basis of rhizosphere colonization. A defect in the utilization of organic acids, which form the major group of tomato exudate components, results in decreased competitive colonization of the tomato rhizosphere (A Wijfjes *et al.* unpublished data), whereas a defect in the use of sugars does not result in a colonization defect [46]. Efficient scavenging for iron using siderophores makes *Pseudomonas*

strains more competitive. On the basis of identified colonization genes and traits, colonization mutants of *P. chlororaphis* strain PCL1391 have been constructed. These mutants have lost their ability to control tomato foot and root rot, showing for the first time that root colonization is an essential trait for biocontrol [47•].

To come to a better understanding of how bacteria function in the rhizosphere, the plant should also be taken into account. Therefore, the effect of the rhizosphere of different recombinant inbred tomato lines on the ability of *B. cereus* to control *Pythium torulosum* was analyzed [48]. The results indicated a genetic basis from the plant side for the efficient growth and performance of biocontrol agents in the rhizosphere. Interestingly, a follow-up study showed a clear and consistent growth difference for *B. cereus* in the spermosphere of the different inbred tomato lines, whereas this difference was not observed for two *Pseudomonas* biocontrol species [49].

In the rhizosphere, inoculant bacteria compete for nutrients and niches with endogenous microorganisms, such as other bacteria and fungi. Recently, genes of *Pseudomonas* biocontrol strains have been identified that can be induced or repressed by the presence of phytopathogenic fungi. IVET technology has been used to show that the presence of *Phytophtora parasitica* can induce various genes in *P. putida*, including genes encoding diacylglycerol kinase, ABC transporters and outer membrane porins [50•]. In contrast, two ribosomal RNA operons of *P. fluorescens* were found to be repressed by *Phytium ultimum* [51].

Genetic modification to improve PGPRs

The identification of genes involved in the ability of rhizobacterial strains to improve plant growth creates the potential to improve the performance of biocontrol strains or to construct novel biocontrol strains by genetic modification. Complete operons, as well as single genes under the control of their own regulatory genes or regulated by the constitutive expression of the *tac* or *lac* promoters, have been transferred to rhizobacterial strains. *P. fluorescens* strains carrying a mini-Tn5 vector that included the complete biosynthetic operon for the antifungal metabolite phenazine-1-carboxylic acid (PCA) were enhanced in their rhizosphere competence, as well as in their ability to suppress fungal diseases [52•]. Similarly, the biocontrol ability of *Pseudomonas* strains producing PCA was extended after the introduction of the *phzH* gene from *P. chlororaphis* PCL1391. The introduction of this gene resulted in the production of phenazine-1-carboxamide (PCN) by these strains and in their ability to biocontrol tomato root and foot rot [19•]. There have been other studies in which the introduction of genes into rhizobacterial strains has enhanced biocontrol and/or plant growth promotion. These genes include the Cry-toxin-encoding *cry1Ac7* gene of *Bacillus thuringiensis* [53], the chitinase-encoding *chiA* gene of *Serratia marcescens* [53,54], and the 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase gene from *Enterobacter cloacae* [55]. In addition, Dekkers *et al.* [45•] showed that the transfer of the *sss* gene of *P. fluorescens* WCS365 can enhance the competitive colonization ability of other *P. fluorescens* strains.

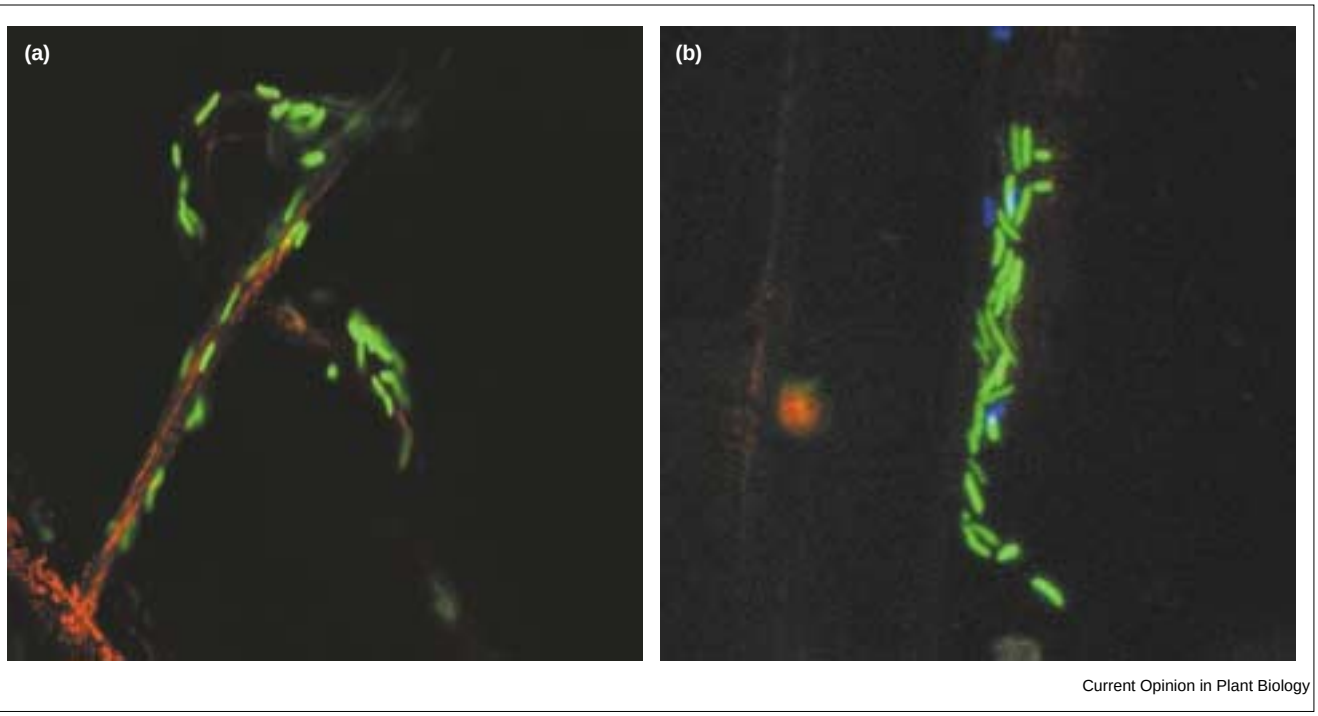
Whole-genome analysis

The revolutionary technological developments in high-throughput DNA sequencing have resulted in the publication of many whole-genome sequences. The sequencing of approximately 35 microbial genomes has been completed (URL http://www.tigr.org/tdb/mdb/mdbcomplete.html), and those of another 150 are in progress (URL http://www.tigr.org/tdb/mdb/mdbiprogress.html). Among these are several rhizosphere-inhabiting bacteria such as *P. aeruginosa* [56••], *P. putida*, *P. fluorescens*, *P. syringae* pathovar *tomato*, *Sinorhizobium meliloti*, *Mesorhizobium loti* [57], *Bacillus subtilus* [58] and *Streptomyces coelicolor*. In addition, an initiative to build a genomic encyclopedia of the rhizobacterial strain *P. fluorescens* SBW25 (PfSBW25) on the basis of short-run noncontiguous sequence data is underway at Oxford University (URL http://www.plants.ox.ac.uk/sbw25/) [59]. The obtained sequence data will, by comparative and functional genomics, facilitate the identification of genes that are specifically present in (plant beneficial) rhizosphere bacteria, that are specifically expressed on the seed or in the rhizosphere, that are involved in the regulation and production of secondary metabolites (e.g. anti-fungal metabolites) or whose expression is influenced by other rhizosphere organisms, such as fungi. The construction of bacterial artificial chromosome (BAC) ibraries for the study of gene expression and to identify genes of interest is of great value, especially in the study of bacteria whose genome has not been sequenced, as has been shown for *B. cereus* [60].

Visualization of bacteria and of gene expression in the rhizosphere

After inoculation, bacteria must become established in the rhizosphere where they interact with the root and with the endogenous population of microorganisms, which includes phytopathogenic fungi and mycorrhizal fungi. Progress has been made during the past year in using confocal laser scanning microscopy (CLSM) in combination with various fluorescent markers to visualize and monitor bacterial populations in the rhizosphere (Figure 1). The results of these studies indicate that *Pseudomonas* biocontrol strains colonize the seed and root surface at the same positions as do the phytopathogenic fungi that they control ([61,62]; A Lagopodi *et al.*, unpublished data). Using a combination of immunofluorescence and an rRNA-targeting probe that monitors the presence and metabolic activity of *P. fluorecens* DR54 inoculant cells in the sugar beet rhizosphere, Lübeck *et al.* [63] showed that bacteria at the root tip are metabolically most active and that endigenous bacteria enter the rhizosphere two days

Figure 1



Tomato root colonization by *Pseudomonas fluorescens* WCS365. Confocal laser scanning microscopy analyses of tomato roots colonized by *Pseudomonas fluorescens* WCS365. (a) The attachment of *P. fluorescens* WCS365 marked with GFP to a tomato root hair. (b) A microcolony of *P. fluorescens* WCS365 cells formed on the tomato root surface after the inoculation of tomato seedlings with a mixture of two WCS365 suspensions, one marked with GFP and the other with cyan fluorescent protein. Plant cell walls are red because of autofluorescence.

after inoculation. Visualization of interactions among the carrot roots, mycorrhizal mycelium and *P. fluorescens* CHA0 bacteria showed that mucoid mutant strains of CHA0 adhere much better to the root, indicating that acidic extracellular polysaccharides can enhance root colonization [64].

The study of microbial communities has been facilitated by the use of combinations of the green fluorescent protein (GFP), its color variants cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP), and DsRed as markers (Figure 1). Using rhizosphere stable vectors [65], broad-host-range plasmids expressing the *e-gfp*, *e-yfp*, *e-cfp* or the *rfp* (*red fluorescent protein*) have been constructed [66•,67]. Using these plasmids, up to three differently marked bacterial populations can be studied on the root. Such experiments have indicated that *Pseudomonas* microcolonies on the root surface are initiated by one bacterial cell and that bacteria from outside the growing colony can join, as indicated by the frequent presence of mixed-color colonies on the older part of the root [45•,66•]. The construction of unstable variants of the GFP [68] makes it possible to study transient gene expression in the rhizosphere, as has been shown for ribosomal activity in *P. putida* cells [69]. A gfp-based system for the detection of AHLs should allow the visualization of quorum sensing and cross-talk among bacteria in the rhizosphere [70•]. Visualization of gene expression in the rhizosphere will provide detailed information on the functioning of bacterial cells in a specific environment. Spatio–temporal analysis of gene expression in the rhizosphere will be made possible by visualizing bacterial cells that harbor an unstable *gfp* variant under control of the promoter to be analyzed and the constitutive expression of *rfp*.

Conclusions

Because PGPRs are an environmentally friendly alternative to chemical fertilizers and pesticides, the use of which is regulated and sometimes forbidden, the market for bioinoculants is still expanding. *Rhizobium* and *Bradyrhizobium* inoculants have been marketed with success for over a century. Approximately twenty bacterial biocontrol products based on *Pseudomonas*, *Bacillus*, *Streptomyces* and *Agrobacterium* strains have been marketed (for an inventory list see URL http://www.barc.usda.gov/psi/bpdl/bpdlprod/bioprod.html). There still is a need to optimize the efficacy of these products. The discovery of many traits and genes that are involved in the beneficial effects of PGPRs has resulted in a better understanding of the performance of bioinoculants in the field, and provides the opportunity to enhance the beneficial effects of PGPR strains by genetic modification.

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