Chapter 13
Arabidopsis as a Model System to Decipher the Diversity and Complexity of Plant Responses to Plant-Growth-Promoting Rhizobacteria

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13.1 Introduction

Plant-growth-promoting rhizobacteria (PGPR) are naturally occurring soil microorganisms that colonize the rhizosphere of many plant species and confer beneficial effects, including plant growth stimulation and reduced susceptibility to pathogens (Van Loon et al. 1998; Bloemberg and Lugtenberg 2001; Dobbelaere et al. 2003; Bashan et al. 2004; van Loon 2007; Babalola 2010). PGPR have been applied to a wide range of crops and agricultural conditions for the purpose of enhancing plant growth and health, hence improving crop yields (Kloepper et al. 1989, 1991, 2004; Zhuang et al. 2007). While the mechanisms involved in biocontrol, especially the induced systemic resistance (ISR) elicited by PGPR, have been investigated in details (Van Loon et al. 1998; Kloepper et al. 2004; van Loon 2007), the mechanisms involved in plant growth promotion are still elusive. The main reason for this lack of knowledge is that the mechanisms involved in plant nutritional and developmental responses underlying plant growth promotion have received little or no attention, in comparison with the ISR mechanism that has been investigated in details. Indeed, until recently, all the studies on the elicitation of plant growth promotion by PGPR focused on the bacterial partner without consideration of plant's physiology.

One difficulty in studying plant–PGPR interactions is the manifold species of PGPR that can elicit growth promotion and the manifold plant species that respond positively to PGPR. The second difficulty lies in the weak host specificity of PGPR, though these bacteria exhibit differences in their metabolism, their localization in roots and rhizosphere, and their potential modes of action, whereas third difficulty

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is that growth promotion is an integrative phenotype that does not correspond to a specific function but is the reflection of many developmental and nutritional processes, all of which being potential targets for PGPR action. Until recently, studies on plant growth promotion by beneficial rhizobacteria used the plant as a broad phenotype to screen bacterial strains in their ability to stimulate plant growth and to identify bacterial compounds or genes. However, to understand how plants respond to PGPR requires also focusing on the plant partner to identify the plant's targets and determine how signaling pathways are modified by PGPR interaction. Obviously, a plant approach of plant–PGPR interaction needs to use a model plant species, so that to avoid diluting research efforts and to provide genetic and genomic tools. In addition, to determine how PGPR modulate plant nutritional and developmental processes, it is necessary to use a model plant in which there is a deep knowledge of the molecular and cellular bases of these processes. *Arabidopsis thaliana* is unequivocally the best plant model, and in the last decade, a few groups have begun to use it to investigate the signaling pathways involved in the plant growth promotion response to rhizospheric bacteria. These investigations, although not numerous and very recent, already revealed unexpected effects of PGPR and propose new paradigms to explain plant responses. This chapter presents new insights of plant responses to PGPR that are responsible for the stimulation of plant growth, obtained in *Arabidopsis*, excluding other aspects of the plant–PGPR interaction, such as recruitment of beneficial soil bacteria by root exudates, rhizosphere colonization, indirect effects on plant nutrition like solubilization of nutrients in the soil, and biocontrol processes including ISR.

13.2 PGPR Emit Volatile Organic Chemicals that Elicit Plant Developmental Responses

Plants are powerful producers of low-molecular-weight volatile organic chemicals (VOCs) of diverse nature in response to either internal clues (e.g., developmental stages) or external stimuli. Historically, the first gaseous plant signal discovered was the hormone ethylene, which is involved in both plant development and defense (Abeles et al. 1992; Bleecker and Kende 2000). A blend of chemically diverse compounds, including fatty acid derivatives, terpenes, indole, and molecules from other chemical families, have been shown to have important roles in the interaction between the plant and its immediate environment, neighboring plants, and attackers, including pathogenic microorganisms and herbivores (Paré and Tumlinson 1999; Farmer 2001; Piechulla and Pott 2003). Although it was known that microorganisms release VOCs (Stotzky and Schenck 1976) and the fact that similar compounds have been identified as signal molecules for plants, the role of volatiles emitted by bacteria in plant development was barely discerned until recently. In their pioneering work on the effects of bacterial VOCs on plant growth promotion, Ryu et al. (2003) showed that some PGPR strains, including *Bacillus subtilis* GB03 and

Bacillus amyloliquefaciens IN937a, stimulate the growth of *A. thaliana* seedlings cultivated in one part of divided Petri dishes when these bacteria were cultivated in the other part of the plates. The partition of Petri dishes forming a tight seal between the bacterial and the plant media, only airborne signals can be transmitted from one side to the other side as evidenced by plant growth promotion. Since growth promotion was not obtained with the *Escherichia coli* DH5α used as a nongrowth-promoting control strain, this simple experiment elegantly demonstrates that some PGPR release VOCs that support plant growth. Furthermore, only three of the seven PGPR strains tested by Ryu et al. (2003) led to increased growth rate of *Arabidopsis* in the divided Petri dishes system indicating that the synthesis of bioactive VOCs is a strain-specific phenomenon. Collecting and analyzing VOCs emitted by the two PGPR strains *B. subtilis* GB03 and *B. amyloliquefaciens* IN937a and by the non-growth-promoting strains *E. coli* DH5α and *Pseudomonas fluorescens* 89B61, the authors identified 2,3-butanediol and acetoin as volatile components released by the two growth-promoting strains but not from the nongrowth-promoting ones; pharmacological application of 2,3-butanediol and inoculation with bacterial mutants deficient in 2,3-butanediol and acetoin synthesis indicated that these VOCs were responsible for the plant-growth-promoting effect.

To investigate the effects of VOC emission from different rhizobacterial strains, Gutiérrez-Luna et al. (2010) cocultivated *Arabidopsis* with 12 bacterial strains isolated from the rhizosphere of lemon plants (*Citrus aurantifolia*) in partitioned Petri dishes and analyzed plant fresh weight and root system architecture. Differences in plant growth promotion were related to differential modulation of root system architecture. Emitted VOC analysis identified both common and specific compounds, and comparison with the phenotypic data suggests that differential VOC emission can modulate plant growth promotion and root system architecture in response to the PGPR strains.

The growth of *Arabidopsis* seedlings was drastically inhibited by cocultivation with *Serratia odorifera* 4Rx13 in partitioned Petri dishes in one set of experiment (Vespermann et al. 2007) while it was promoted in a second set (Kai and Piechulla 2009). This strong difference in *Arabidopsis* pattern responses to 4Rx13 was attributed to sealed or nonsealed Petri dishes (Kai and Piechulla 2009): plant growth promotion was obtained when Petri dishes were sealed with parafilm, while seedlings did not develop in the nonsealed setup (the seedlings stopped growing at very early stage after germination and they were albino). Using tripartite Petri dishes to trap CO_2 with barium hydroxide, Kai and Piechulla (2009) showed that there was a significant rise of CO_2 levels in sealed Petri dishes due to bacterial growth and that elevated CO_2 level was responsible for the plant growth promotion by 4Rx13. The deleterious effect of bacterial cells in nonsealed Petri dishes would be due to VOCs emitted at ambient CO_2 concentration. The 4Rx13 bacteria release more than 100 volatile compounds, among which dimethyl disulfide (DMDS) and ammonia have growth-inhibiting effect on *Arabidopsis* (Kai et al. 2010). The other volatile substances extracted had no effect on *Arabidopsis* growth. However, the bioassay used to test the effects of these VOCs could have been insufficient, and the extraction method may have failed to isolate all bioactive molecules; it cannot

be excluded that some 4Rx13 emitted VOCs have antagonistic or synergistic effects with DMDS and NH_3 on plant growth.

The possibility that bacterial CO_2 production affects the plant growth in plant–PGPR interaction studies has to be considered. However, the application of purified VOCs or genetic approaches like in the study reported by Ryu et al. (2003) demonstrated that PGPR-emitted VOCs have beneficial effects on plant growth besides having the role of possible increase in CO_2 level. In addition, investigating the targets of these VOCs in plants by using *Arabidopsis* mutants further identified specific effects that cannot be attributed to the provision of supra optimal level of CO_2. Using *Arabidopsis* mutant lines defective in hormonal pathways, Ryu et al. (2003) showed that the ethylene, gibberellin, and brassinosteroid-signaling pathways were not involved in the promotion of growth by *Bacillus* spp. GB03 and IN937a strains-emitted VOCs. Although an *eir1*/*pin2* mutant deficient in one IAA efflux transporter retained the growth promotion response to both PGPR strains, the authors could not exclude the possibility that the auxin signaling pathway be implicated in the response of *Arabidopsis* seedlings to VOCs. Indeed, because of the great number of auxin efflux transporters acting in the various tissues and organs, the IAA transport pattern required for the GB03 response could still operate normally in the mutant. Moreover, because no mutant in the IAA transduction pathway has been included in this study, the implication of the auxin signaling pathway itself was not directly tested. A cytokinin receptor *cre1* mutant did not exhibit growth promotion when exposed to GB03, suggesting a role for the cytokinin signaling pathway in plant response to VOC emission by this strain. By contrast, the other *Bacillus* spp. strain tested in this study, IN937a, still promoted the growth of *cre1* mutant seedlings, which suggests that (1) several plant signaling pathways are targets of PGPR-originating VOCs responsible for developmental changes and growth stimulation, and (2) volatile blends released by GB03 and IN937a differ in their composition.

Farag et al. (2006) further characterized 38 volatile metabolites from the GB03 and IN937a strains, most of these compounds being branched-chain alcohols not identified in the previous study by Ryu et al. (2003). Comparison of the GB03 and IN397a VOCs profiles showed apparent differences, with IN397a producing higher amounts of 3-methyl-1-butanol, 2-methyl-1-butanol, and butane-1-methoxy-3-methyl. It can be speculated that the release of these alcohols only in IN937a volatile blend is responsible for plant growth promotion through a cytokinin-independent pathway. In any case, the fact that both *Bacillus* spp. strains similarly promote the *Arabidopsis* growth using VOCs as elicitors of plant signaling pathways, but differ in the composition of their VOCs bouquets and in the regulatory pathways targeted, is extremely interesting to understand the diversity and specificity of mechanisms responsible for plant growth promotion by rhizobacteria. Because of the availability of a large number of mutant lines altered in the different steps of hormones synthesis, transport, sensing, and transduction, the model plant *Arabidopsis* provides the tools to investigate further the specific targets of VOCs emitted by the two *Bacillus* spp. strains.

13.3 PGPR Elicit Plant Developmental Responses by Modulation of Plant Hormonal Pathways

The plant hormones have roles in both plant physiology and development, thus determining plant growth. Most of the PGPR genera secrete auxins, gibberellins, etc., and it has been considered that plant hormones produced by PGPR strains are essential in plant growth promotion. However, the results of recent investigations using Arabidopsis have questioned this hypothesis, showing that PGPR can elicit plant hormonal pathways without a hormone of bacterial origin be involved, as detailed below.

13.3.1 The Auxin Signaling Pathway Is Elicited by IAA-Nonproducing PGPR

Probably, the most widely accepted mechanism for plant growth promotion by PGPR remains the synthesis of auxin by bacterial cells and its release in the rhizosphere (Loper and Schroth 1986; Dobbelaere et al. 1999; Spaepen et al. 2007). This hypothesis has been supported by studies that used auxin-deficient bacterial mutants especially with the PGPR strains *Azospirillum brasilense* sp245 (Barbieri and Galli 1993) and *Pseudomonas putida* GR12-2 (Patten and Glick 2002), two high auxin producers. Furthermore, some studies reported a correlation between the growth parameters (root and shoot elongation, root and shoot dry weight) of inoculated seedlings and the in vitro auxin production by several PGPR strains (e.g., Khalid et al. 2004). Other studies, however, failed to find such a correlation (e.g., Kishore et al. 2005). This discrepancy indicates that bacterial production and release of indole-3-acetic acid (IAA) is likely to be one mechanism that can affect positively plant growth by some rhizobacteria strains but that this mechanism cannot be generalized among all the PGPR. In addition, until very recently, no investigation has been made in the plant partner to confirm the implication of auxin in plant responses and to characterize the role of the plant auxin signaling pathway in specific plant developmental or metabolic responses.

The only studies that actually investigated the implication of auxin signaling pathway in plant responses to PGPR took advantage of the model plant *Arabidopsis* to perform genetic or reverse genetic approach. In the earliest of these very few studies (Persello-Cartieaux et al. 2001), a screen for *Arabidopsis* mutants insensitive to the inoculation with a *Pseudomonas thivervalensis* strain was developed based on the reduction of primary root length upon inoculation. The screen resulted in the isolation of two *aux1* mutants impaired in the major transporter for the influx component of auxin polar transport. This finding thus reinforces the conclusion of rhizobacterial studies that auxin is involved in plant responses to PGPR. More precisely, genetic evidence obtained by Persello-Cartieaux et al. (2001) demonstrates that IAA influx is required for the rhizobacteria-induced root

morphology change, but there is no direct evidence that the auxin signal transduction pathway is implicated in this response.

More recently, two studies, one with a VOC producing PGPR (Zhang et al. 2007) and the other with a VOC nonproducing PGPR (Contesto et al. 2010) showed the implication of the plant auxin pathway in growth promotion response without elicitation by bacterial auxin. The modifications of *Arabidopsis* transcriptome by *B. subtilis* GB03-emitted VOCs (plants and rhizobacteria positioned on separate sides of partitioned Petri dishes, see Sect. 13.2) identified several auxin biosynthesis genes as upregulated by GB03 VOCs (Zhang et al. 2007). The authors further showed that these genes were specifically upregulated in the shoots of GB03-exposed plants. Using a transgenic *DR5::GUS* auxin marker line (Ulmasov et al. 1997) revealed that auxin accumulation decreased in leaves while increased in roots with GB03 exposure. These opposite changes in organ auxin contents suggest that GB03 VOCs activate basipetal auxin transport (Zhang et al. 2007). Application of the auxin transport inhibitor 1-naphthylphthalamic acid both prevented GB03-mediated decrease in shoot auxin level and thwarted GB03-mediated growth promotion. All together, the discovery by Zhang et al. (2007) stated that bacterial VOCs devoid of auxin or other known plant hormones regulate auxin homeostasis and cell expansion (histochemical analysis of leaves section and induction of a group of genes involved in cell-wall loosening) provides a new paradigm as to how PGPR promote plant growth.

We showed that this behavior is not restricted to VOCs-emitting rhizobacteria and gave further indications on the implication of the auxin signaling pathway by investigating root architecture modifications in *Arabidopsis* seedlings inoculated with the PGPR strain *Phyllobacterium brassicacearum* STM196 (Contesto et al. 2010). This strain (former isolate 29-15) was isolated from the roots of canola plants grown on a canola field soil of the Burgundy region (France) (Bertrand et al. 2001; Mantelin et al. 2006b). This particular strain was selected among the canola-associated rhizobacteria isolated in this study for its higher efficiency to promote canola growth in a bioassay. A detailed analysis of the effect of STM196 on canola seedlings grown in vertical Petri dishes showed that plant growth promotion is accompanied by an increase in the individual lateral root growth rate (Larcher et al. 2003). Similar growth promotion and root architecture changes are recorded in the model plant *A. thaliana* (Mantelin et al. 2006a), making this PGPR a good model to decipher the signaling pathways involved in plant responses to beneficial rhizobacteria. Using a mutant severely altered in IAA transduction (*axr1*, Estelle and Somerville 1987; del Pozo et al. 2002), we demonstrated for the first time that not only the IAA molecule is involved in the beneficial effect of some PGPR but also that the IAA transduction pathway per se is actually implicated in this response (Fig. 13.1). The free IAA level was significantly lower in the roots of STM196-inoculated wild-type plants than in the roots of noninoculated ones, which would be difficult to reconcile with IAA release by the PGPR. Consistent with the lack of a significant provision of bacterial auxin to the plant roots, STM196 appears to be a very low-IAA producer: its capacity to synthesize and release IAA is dramatically

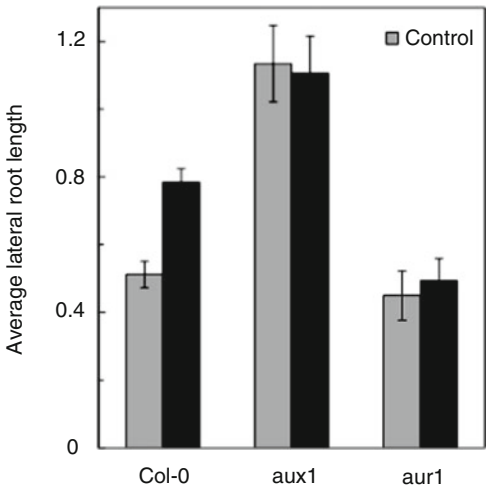

Fig. 13.1 The stimulation of lateral root growth by the PGPR strain *Phyllobacterium brassicacearum* STM196 involves the auxin signaling pathway. This rhizobacterium increases the average lateral root length of *Arabidopsis thaliana* wild-type plants (Col-0 ecotype) grown on a solid medium in Petri dishes. Both mutations in the major IAA influx transporter (aux1) and in the IAA receptor complex (axr1) block this response. Data drawn from Contesto et al. (2010)

lower than that of the well-characterized *A. brasilense* sp245 strain often used in studies that support a role for bacterial auxin in plant growth promotion (Barbieri and Galli 1993; Spaepen et al. 2008), and equal to that of its IAA-low-producing *ipdc* mutant (Costacurta et al. 1994). In support to the hypothesis that bacterial IAA is not involved in root development changes of STM196-inoculated *Arabidopsis* seedlings, the root system phenotype of these plants—longer lateral roots—differs markedly from the root phenotype of sp245-inoculated plants—increased number of lateral roots, strong shortening of primary root, shorter lateral roots—whereas this latter phenotype resembles the root phenotype obtained by addition of IAA to the medium supplied to noninoculated seedlings (Contesto et al. 2010). Transgenic *DR5::GUS* plants indicated higher IAA accumulation levels in the tissues where it normally accumulates (within primary and lateral root meristems) and an extension in size of these regions toward older tissues along the central cylinder. This change in the distribution of IAA within the root system explains the apparent contradiction between the decreased total root IAA content in STM196-inoculated plants and the implication of IAA transduction pathway in lateral root response. Therefore, STM196 must specifically affect the IAA transport without either providing supra-auxin to the plant or increasing total IAA production by the plant.

This new paradigm of a modification of IAA distribution within plant organs and tissues upon inoculation with PGPR raises several questions. Firstly, the strains *B. subtilis* GB03 and *P. brassicacearum* STM196 used in the studies that led to this discovery are very unlikely to elicit IAA redistribution in plant via the same bacterial molecules: while in the experiments by Zhang et al. (2007) the elicitors were necessarily VOCs, in fact STM196 fails to elicit the root development changes reported by Contesto et al. (2010) when the roots are separated from

the inoculums (unpublished data). Identifying these different bacterial molecules is certainly an important aspect for further research issue. Secondly, with regard to the plant response, this shows that besides its key role in plant organs specialization, organization, and development and in plant adaptation to abiotic constraints (e.g., unidirectional light or gravity force, nutrient availability at the root surface), the polar auxin transport is also a major integrator of plant responses to biotic interactions. The polarity of auxin transport at the tissue and organ levels is determined by the subcellular localization of auxin carriers, especially PIN efflux transporters, which is regulated by specific kinases, phosphatases, and GTPases (Benjamins and Scheres 2008). The details of this polarity loop are far from being completely deciphered yet, but it is likely that some of its elements are, directly or indirectly, the targets of PGPR components. Identifying these targets is another important perspective of future research. The PGPR–*Arabidopsis* interaction is a good model to help understanding how the polarity loop can integrate possibly antagonistic or synergistic effects from internal cues, abiotic constraints, and biotic interactions.

By contrast with STM196 (Contesto et al. 2010), *Bacillus megaterium* UMCV1 caused the same effects on *Arabidopsis* mutants altered in the auxin and ethylene pathways than in the wild-type plants, suggesting that this PGPR promotes growth and alters root system architecture through an auxin- and ethylene-independent mechanism (Lopez-Bucio et al. 2007). With regard to the auxin signaling pathway, the mutants used were *aux1* and *axr4*, thus blocking the same step (the AXR4 protein is specifically involved in AUX1 trafficking, Dharmasiri et al. 2006). Because the polar auxin transport depends much more on efflux transporters than on influx transporter, the fact that *aux1* and *axr4* mutations did not thwart the UMCV1 effect cannot be considered as a definite evidence for the nonimplication of the auxin signaling pathway in the growth-promoting effect of this PGPR strain. To demonstrate that UMCV1 alters root system architecture and promotes plant growth through an auxin-independent pathway would require using a mutant altered in the auxin transduction pathway. Lopez-Bucio et al. (2007) also reported concomitant GUS staining decrease in primary root tips and increase in lateral root primordia of transgenic *DR5::GUS* plants inoculated with UMCV1. These observations indicate that UMCV1 modifies auxin transport within roots, though with a different profile than GB03 or STM196 do.

Since published studies that investigated the implication of auxin signaling pathway in plant responses to PGPR used IAA-low-producer strains, their conclusions do not dismiss the possibility that high-IAA-producing PGPR can promote plant growth by releasing auxin in the rhizosphere as proposed earlier. To investigate this process would require characterizing the cellular and molecular responses of *Arabidopsis* to high-IAA-producing bacterial strains. Unfortunately, the literature provides no report that describes in detail the effect of such PGPR on *Arabidopsis* development parameters and/or investigates the signaling pathways elicited in the plant.

13.3.2 The Relationship Between PGPR and the Ethylene Signaling Pathway Is Complex

One hypothesis for the effect of PGPR on root system architecture is based on the reduction of plant ethylene production by bacterial ACC deaminase activity (Glick 2005). Indeed, the enzyme ACC deaminase (AcdS) that catalyzes the cleavage of the plant ethylene precursor, 1-amino cyclopropane-1-carboxylic acid (ACC), in α-ketobutyrate and ammonia is found in many plant-beneficial as well as pathogenic bacteria (Blaha et al. 2006). Rhizobacterial AcdS activity could divert ACC from ethylene biosynthesis and thereby lower the level of ethylene in plant root (Glick et al. 1998). Bacterial AcdS enzyme can effectively compete with plant ACC oxidase (ACO) despite a higher affinity of ACO than AcdS for ACC, provided that the AcdS level is 100- to 1,000-fold greater than the ACO level. Glick et al. (1998) argue that such a situation is likely to occur because ACO is an enzyme normally present at very low levels in plant cells, so that PGPR AcdS activity could actually lower ethylene levels in plant roots except when ACO is induced by environmental conditions or developmental stage. The best evidence in favor of Glick's hypothesis is that AcdS-deficient mutants of the *P. putida* GR12-2 and UW4 PGPR strains were found unable to promote root elongation of canola seedlings as the wild-type strains did (Glick et al. 1994; Li et al. 2000). However, the lack of studies on the plant partner did not permit to investigate the real impact of bacterial AcdS activity and the possible involvement of the ethylene signaling pathway in plant response to PGPR until studies have recently been conducted with the model plant *Arabidopsis*.

Seedlings from *Arabidopsis*-ethylene-insensitive mutant lines *etr1* and *ein2* displayed enhanced total leaf area upon exposure to GB03 VOCs, indicating that ethylene sensing and signaling is not involved in growth promotion by this PGPR (Ryu et al. 2003). Similarly, *etr1* and *ein2* mutants are not impaired in growth promotion responses to *B. megaterium* UMCV1 (Lopez-Bucio et al. 2007). Consistent with these two reports, we found no difference between the root system architecture of *Arabidopsis* seedlings inoculated with $AcdS^-$ mutants of *P. brassicacearum* STM196, *P. putida* UW4, *Rhizobium leguminosarum* bv. *viciae* 128C53K, and *Mesorhizobium loti* MAFF303099, and the root system architecture of seedlings inoculated with their respective wild-type counterparts (Contesto et al. 2008; Desbrosses et al. 2009). All together, these studies negate an essential role of bacterial AcdS activity and plant ethylene signaling in the activation of root development and plant growth by PGPR. One possible explanation for the discrepancy with Glick's reports is that the bacterial AcdS activity would be insufficient to compete with the plant ACO activity in the growth conditions used in these three studies. The capacity of PGPR to lower ethylene concentration in plant's roots and thereby affect plant development and growth, therefore, cannot be dismissed. In the reports of differential plant response patterns to $AcdS^-$ and

wild-type PGPR cells, however, the implication of the ethylene signaling pathway in plant responses is yet to be demonstrated.

Besides root system architecture, another useful phenotypic response to investigate the role of ethylene in plant–PGPR interaction is root hair elongation (Contesto et al. 2008; Desbrosses et al. 2009). Indeed, inoculation of *Arabidopsis* seedlings with PGPR strains led to a dramatic stimulation of root hair elongation: their length was increased by two- to threefold upon inoculation with the STM196, UW4, 128C53K, and MAFF303099 strains (Contesto et al. 2008). The AcdS$^-$ mutant cells of all four strains led to a slightly but significantly stronger stimulation of root hair elongation. Genetic studies in *Arabidopsis* have identified the ethylene and auxin signaling pathways as the main regulators of root hair elongation (Pitts et al. 1998), suggesting that these two hormonal pathways are the main regulators of root hair development. The differential root hair pattern response to AcdS$^-$ and wild-type STM196, therefore, is consistent with a lowering of plant ethylene level by AcdS activity of wild-type bacterial cells. On the other hand, AcdS activity does not explain the root hair elongating effect of PGPR, as it actually antagonizes this effect, indicating that PGPR elicit an ethylene-independent pathway to elongate root hairs. Consistent with this conclusion, we found that STM196 is able to stimulate root hair elongation in all the *Arabidopsis* mutant lines altered in the ethylene signaling pathway we tested (Desbrosses et al. 2009). Remarkably, mutants altered in auxin signaling also maintain full capacity to elongate root hairs when inoculated with STM196. These findings are much unexpected since, as mentioned above, auxin and ethylene are considered as the main regulating factors of root hair elongation. Nevertheless, this shows that PGPR must elicit some ethylene- and auxin-independent pathway(s) in the plant, not excluding demonstrated implication of the auxin signaling pathway and possible implication of the ethylene signaling pathway in some plant responses. Among other, this is strong evidence that multiple signaling pathways are elicited by PGPR and that the multiple plant responses are the result of a complex combination of these various regulations (so-called additive hypothesis, see Bashan et al. 2004). In addition, the fact that PGPR can induce a strong root hair elongation independently to auxin and ethylene pathways raises the question of what other plant factor can overcome these regulations.

In summary, the results published for PGPR–*Arabidopsis* interactions suggest that the impact of bacterial AcdS activity is rather modest and probably affects specifically local processes such as root hair elongation (Desbrosses et al. 2009). By contrast, more integrated processes that depend upon systemic regulation by shoot-derived compounds, such as root development and root system architecture, are unlikely be affected by AcdS activity of PGPR. Another difficulty with the Glick's model is the existence of a negative feedback loop affecting ethylene biosynthesis (Guzman and Ecker 1990), so that stimulated ethylene emission can be induced indirectly by PGPR while typical ethylene responses are not displayed.

13.4 PGPR Thwart the N-Dependent Regulation of Lateral Root Development

From the observation that the inoculation with *P. brassicacearum* STM196 induced increased lateral root growth in *Arabidopsis* whereas increasing NO_3^- has the opposite effect (Zhang et al. 1999; Tranbarger et al. 2003), we investigated how these two processes interfere with each other (Mantelin et al. 2006a). Remarkably, STM196 countervails the inhibitory effect of high NO_3^- on lateral root development: the negative correlation observed between NO_3^- concentration and lateral root length was not observed in STM196-inoculated *Arabidopsis* (Fig. 13.2). Considering that regulatory pathways in plants are highly interconnected as illustrated by hormonal signaling cross talks, it is not so surprising that plant development integrates the antagonistic effects of a biotic agent and an abiotic constraint via some process. However, to our knowledge, the alteration of nitrate-dependent control of root architecture by STM196 (Fig. 13.2) is the first example of such interference between metabolic-dependent and PGPR-induced developmental controls. Inhibition of lateral root development by high NO_3^- is a systemic mechanism by which the high N status in leaves control the root system architecture, and it is usually considered that the leaf NO_3^- is the sensing pool (Scheible et al. 1997; Forde and Lorenzo 2001). Because no systematic correlation was found between leaf NO_3^- and lateral root length in STM196-inoculated seedlings, we concluded that the inoculation with STM196 alleviates the N-dependent regulation of root development downstream the sensing of N status. The mechanism by which NO_3^- accumulation in leaf regulates lateral root development is not known yet, but some pieces of evidence suggest that auxin

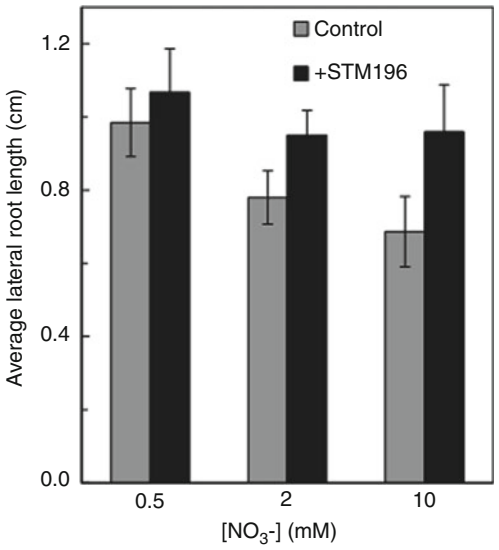

Fig. 13.2 Nitrate-dependent regulation of lateral root development and PGPR-elicited lateral root growth are antagonistic processes. High external NO_3^- concentrations reduce root development, but the *Phyllobacterium brassicacearum* STM196 PGPR strain thwarts this effect. Indeed, STM196-inoculated *Arabidopsis* seedling displayed a NO_3^--independent lateral root development. Data drawn from Mantelin et al. (2006a)

be involved in the shoot-to-root signaling. For instance, transferring *Arabidopsis* seedlings from 50 to 1 mM NO_3^- led to concomitant decrease in shoot IAA concentration and increase in root IAA concentration within 24 h, followed by a restoration of lateral root development (Walch-Liu et al. 2006). Since changes in polar IAA transport are implicated in the lateral root development response to STM196 (Contesto et al. 2010), it is likely that integration of bacterial-originating signals and N-status-sensing to control root system architecture involves regulation of IAA homeostasis.

13.5 PGPR Modify Nutrient Uptake Both Indirectly and Directly

A common hypothesis to explain plant growth stimulation by beneficial rhizobacteria considers that PGPR primarily increase root surface area due to lateral root proliferation induced by bacterial auxin, and increased plant growth is a consequence of increased nutrient and water acquisition. However, this model suffers from several problems. First, because hormones have systemic effects in plant and their impact is never restricted to a unique organ, it would be very unlikely that PGPR affect root development through plant hormonal pathway without altering shoot development. Second, root ion transporters are known to be regulated by internal cues related to the nutritional demand (e.g., see Imsande and Touraine 1994; Lappartient and Touraine 1996; Lappartient et al. 1999; Nazoa et al. 2003), so that the regulations of root development and ion transporter activities are antagonistically coordinated to maintain acquisition rate (Touraine 2004). If PGPR specifically exerted an effect on root development, therefore, nutritional demand controls should downregulate the ion uptake systems, so that the whole acquisition rate of nutrient by the plant would not change and shoot growth would not increase if drawn solely by higher acquisition rate as hypothesized. In other words, to elicit both increased nutrient acquisition rate and plant growth promotion, PGPR must interfere with the development–nutrition normal coordination. To achieve this, they needs to promote shoot like root growth, so that the whole plant growth rate is higher and the increased nutritional demand draws nutrient uptake to sustain this supra biomass production. In addition, as discussed below, PGPR can also more directly stimulate the ion transport systems in root, thus modifying the coordination of developmental and nutritional processes. Thirdly, a model that would consider local effects as the main plant responses to PGPR is not consistent with transcriptome studies of PGPR-inoculated *Arabidopsis* plants, which show larger modifications of the gene expression profiles in shoots than roots (Cartieaux et al. 2003). Finally, the *B. subtilis* GB03 strain has been shown to augment photosynthetic efficiency through the modulation of endogenous sugar/ABA signaling (Zhang et al. 2008b, see Sect. 13.6). Such a regulatory role in plant acquisition of energy provides the PGPR with a

supplementary mechanism to affect leaf metabolism and development not only as a consequence of the enhanced nutrient acquisition that would be drawn by increased root surface area.

The impact of PGPR on nutrient uptake systems has been much less studied than their effects on root development. In canola, both NO_3^- and K^+ net influx rates per unit root surface area increased upon inoculation with *Achromobacter* sp. strain U80417 (Bertrand et al. 2000). The net H^+ efflux was also enhanced, so that increased NO_3^- and K^+ uptake rates may be part of a general increase in ions uptake rate as a consequence of root cells plasma membrane energization by enhanced proton pump activity. Supra acidification of the rhizosphere by plant roots has also been observed with *Arabidopsis* seedlings exposed to GB03 (Zhang et al. 2009), suggesting that the stimulation of root H^+ ATPase may well be a general response to PGPR. Alternatively, the increased nutrient demand associated to stimulated growth rate in inoculated plants could be responsible for increased NO_3^- and K^+ uptakes. Indeed, nutrient uptake is controlled by plant demand, so that it does not depend only on nutrient availability and local regulation exerted in roots but also systemic regulations that link ions transport activity in roots to the whole plant nutritional status (Imsande and Touraine 1994). Therefore, it is difficult to determine whether ions uptake increase is a consequence or a cause of the growth stimulation by PGPR.

The difficulty in determining whether PGPR primarily stimulate plant growth or enhance nutrient uptake lies in the fact that, as discussed by Mantelin and Touraine (2004) for nitrogen uptake, the consequences are about the same, since mineral nutrient are assimilated and diluted in the biomass produced in both cases. Such a chicken and egg question cannot be resolved by a combination of microbiology and whole plant physiology approaches. To decipher the links between PGPR, plant development and growth control, and uptake and nutrition processes, it is necessary to identify the plant signaling pathway elicited. Using the model plant *Arabidopsis* provided some pieces of evidence, but these indications are still very fragmented and do not give a clear picture of PGPR effects on nutrient uptake.

One indication in favor of "developmental" rather than "nutritional" primary effects is that PGPR do exert a direct effect on developmental processes such as root hair elongation and lateral root development independently to any change in nutrient uptake and assimilation (see above). The fact that STM196 thwarts the N-dependent regulation of lateral root development independently to an effect on NO_3^- uptake or distribution is also a strong support for the "developmental" hypothesis. However, it must be kept in mind that PGPR elicit a large array of responses in plants via multiple signaling pathways ("additive hypothesis," see above), and it is all the more feasible that the PGPR induce both developmental processes and nutrient transport and metabolism. Hereafter are summarized evidence recently published that demonstrate the capacity of PGPR to induce modifications of nitrate, sodium, and iron ion transports in *Arabidopsis*.

13.5.1 *The Effect of* Phyllobacterium brassicacearum *STM196 on Nitrate Uptake Can Be Direct or Indirect*

In *Arabidopsis*, measurement of NO_3^- uptake led to contradictory results: NO_3^- influx was increased in seedlings 24 h after transfer on STM196-inoculated medium while it was reduced 7 days later (Mantelin et al. 2006a). Moreover, it is difficult to draw conclusion from the results since the efflux component has not been measured, so that the pattern response of net NO_3^- uptake rate is not known. The accumulation of nitrate and ammonium transporters transcript was very slightly or not significantly changed upon STM196 inoculation, except for the *NRT2.5* and *NRT2.6* genes (Mantelin et al. 2006a). These two genes are likely to be involved in plant response to STM196 but, their function being unknown, their role is still elusive. In any case, they are mostly expressed in shoots (Mantelin et al. 2006a) and their mutations do not induce significant changes in NO_3^- uptake rate (unpublished data), and STM196 is unlikely to exert a transcriptional regulation on NO_3^- uptake. Nevertheless, STM196 must increase NO_3^- uptake rate per unit root surface area in *Arabidopsis* because total N content increased in STM196-inoculated plants (Mantelin et al. 2006a), and taking into account the relative root and shoot growth rate increases, N acquisition rate per unit root weight is higher in STM196-inoculated plants than in noninoculated plants. Although contributions of N_2 fixation by associated bacteria to the plant N budget have been reported for several plants, with higher levels in sugar cane, the impact of N_2 fixation by PGPR is still debated, and it is rarely credited for the stimulation of plant growth (for review see Dobbelaere et al. 2003; Vessey 2003). Specifically, STM196 is unlikely to fix N_2 nitrogen (Mantelin et al. 2006a) and to supply an alternative source of nitrogen to *Arabidopsis* since it does not restore growth to nitrate-reductase-deficient mutant grown in a NO_3^--free medium (unpublished data). Therefore, the increased N acquisition in STM196-inoculated *Arabidopsis* requires net NO_3^- uptake rate through the root cells plasma membrane be increased upon STM196 inoculation. As discussed above, the PGPR can stimulate NO_3^- uptake either as a consequence of increased N demand in PGPR-inoculated plants or due to a more direct effect on NO_3^- transporter activity concomitantly to its effect on plant growth (also see Mantelin and Touraine 2004). Also, increased H^+ ATPase activity recorded in canola (Bertrand et al. 2000) and *Arabidopsis* (Zhang et al. 2009) with other PGPR can be part of the explanation for increased NO_3^- uptake in STM196-inoculated *Arabidopsis*.

13.5.2 Bacillus subtilis *GB03 Induces Salt Tolerance by Manipulation of a Sodium Transporter Expression and Accumulation of Osmoprotectants*

Salt stress can damage plants by several mechanisms, including water deficit, ion toxicity, nutrient imbalance, and oxidative stress. Plant response mechanisms to salt

stress include, among others, movement of Na^+ and K^+ ions and the production of osmoprotectants such as proline, glycine betaine, and sugar polyols (Wang et al. 2003). These two mechanisms are induced by GB03 VOCs, as demonstrated by experiments performed on GB03-exposed *Arabidopsis* seedlings treated with high NaCl exogenous concentration (Zhang et al. 2008a) or exogenous mannitol (Zhang et al. 2010).

Under salt stress, exposure of *Arabidopsis* seedlings to GB03 VOCs concurrently down- and upregulates *HKT1* expression in roots and shoots, respectively (Zhang et al. 2008a). In *Arabidopsis*, the HKT1 transporter functions in the shoots' phloem tissues to retrieve Na^+ from the xylem, thus facilitating shoot-to-root Na^+ recirculation (Berthomieu et al. 2003). By removing large amounts of Na^+ from the leaves, and consequently maintaining a high K^+/Na^+ ratio in leaf tissues, this recirculation would play a crucial role in plant tolerance to salt. In addition to its role in Na^+ recirculation, however, HKT1 is also involved in Na^+ uptake by roots so that its activities in roots and shoots may have opposite effects on Na^+ accumulation in plants (Rus et al. 2001). Consistent with the dual role of HKT1 in shoots and roots, the differential regulation of *HKT1* expression in these two organs of plants exposed to GB03 resulted in reduced accumulation of Na^+ and increased accumulation of K^+ in both organs of salt-stressed seedlings (Zhang et al. 2008a). Consistent with the effect of GB03 on HKT1 and the role of this transporter in salt tolerance, GB03 increased shoot growth of salt-stressed *Arabidopsis* wild-type seedlings, but it failed to rescue salt-stressed *hkt1* mutant seedlings from elevated Na^+ accumulation and stunted foliar growth. These results demonstrate that a PGPR strain can regulate the expression of specific plant transporter and consequently control ion homeostasis in plant organs.

In addition to its effect on Na^+ transports, GB03 enhances the biosynthesis and accumulation of the osmoprotectants choline and glycine betaine in plants under mannitol- and drought-induced dehydration stress (Zhang et al. 2010). Upon 100 mM exogenous mannitol stress, *Arabidopsis* plants exposed to GB03 VOCs exhibited increased phosphoethanolamine *N*-methyltransferase gene (*PEAMT*) transcript level compared with stressed plants that were not exposed to the bacteria. The enzyme PEAMT catalyzes the three methylation steps to produce choline, the precursor of glycine betaine, from phosphoethanolamine. Consistent with *PEAMT* transcriptional regulation, endogenous choline and glycine betaine metabolite pools were strongly increased by GB03 treatment. The *xipotl* (*peamt*) mutant line failed to display GB03-induced tolerance to exogenous mannitol, which confirms a role for *PEAMT* in GB03-induced osmotic stress tolerance. Zhang et al. (2010) found similar levels of abscisic acid (ABA) in the shoots and roots of osmotic-stressed plants with or without GB03 exposure, suggesting that GB03-induced osmoprotection is ABA independent. The regulatory pathways responsible for GB03-dependent regulation of *HKT1* and *PEAMT* expression, lower Na^+ accumulation and higher K^+ accumulation, and increased accumulation of choline and glycine betaine remain to be identified.

13.5.3 Bacillus subtilis *GB03 Stimulates Iron Reduction and Uptake by Roots*

In their investigation on the effects of the GB03 strain on *A. thaliana*, the Paré's group has also demonstrated that GB03-emitted VOCs activate the plant's iron acquisition machinery leading to increased iron assimilation (Zhang et al. 2009). In dicots and nongraminaceous monocots, iron acquisition is performed through the strategy 1 process: under iron-deficient conditions, the plant acidifies the soil through activation of a plasma membrane H^+-ATPase of the root epidermal cells, leading to increased iron solubility, and Fe^{3+} chelates are reduced by a specific root reductase prior to transport of released Fe^{2+} ions across the root plasma membrane via Fe^{2+} transporters (Curie and Briat 2003). Exposure of *Arabidopsis* seedlings to GB03 led to a stimulation of all these activities (Zhang et al. 2009). Firstly, GB03 acidifies the rhizosphere, both directly due to chemical effects of some unidentified VOCs and indirectly through increased root proton efflux. Secondly, GB03 upregulates the expression levels of *FRO2* and *IRT1* genes, coding respectively for a Fe^{3+} chelate reductase and a Fe^{2+} transporter. As a result, GB03-exposed *Arabidopsis* has enhanced ferric chelate reductase activity and increased iron content. Microbial siderophores have been observed to facilitate iron uptake by plants (Vansuyt et al. 2007), but the partition that separates *Arabidopsis* from the bacteria and the fact that none of the VOCs characterized so far have known siderophore activity strongly suggest that bacterial siderophores are not implicated in the stimulation of iron uptake by GB03. Some volatiles compounds can be classified as organic acids which could participate at the rhizosphere acidification (Farag et al. 2006), but the main effect of GB03 is via the elicitation of plant activities, namely H^+-ATPase, ferric chelate reductase, and Fe^{2+} transporter.

In plants cultivated without PGPR, induction of *FRO2* and *IRT1* is observed under iron starvation conditions, and these transcriptional regulations have been shown to involve the Fe-deficiency-induced transcription factor FIT1 (Colangelo and Guerinot 2004). Consistent with a role for FIT1 in the GB03 induced increase in *FRO2* and *IRT1* expression, GB03 induced *FIT1* expression in wild-type *Arabidopsis* and it failed to increase root ferric reductase activity and plant iron content in *Arabidopsis fit1* mutants (Zhang et al. 2009). All together, the study by Zhang et al. (2009) again shows that a PGPR strain can modify plant activities by interfering with plant regulatory processes, thus activating plant transduction cascades. However, similar to the other activities elicited by PGPR described before, neither the bacterial chemicals nor the plant sensors and factors that interfere with downstream plant regulatory processes have been identified yet: how GB03 induces *FIT1* expression is not known. Reciprocally, characterization of plant factors involved in FIT1 induction by GB03 may reveal insights into regulatory steps in plant iron uptake and homeostasis.

13.6 Augmentation of Photosynthetic Activity by *Bacillus subtilis* GB03 Involves Modulation of ABA and Sugar Signaling

Again by exposure to the GB03 strain in partitioned Petri dishes, Zhang et al. (2008b) showed that strain augments the photosynthetic capacity by increasing photosynthetic efficiency and chlorophyll content in *Arabidopsis*. GB03 suppressed classic glucose signaling responses, including hypocotyl elongation and seed germination inhibitions by high exogenous glucose. Concurrently, GB03 led to higher hexose accumulation in shoots. GB03, therefore, attenuates glucose inhibitory effects through the repression of sugar signaling rather than by lowering sugar accumulation. Furthermore, GB03 failed to enhance the photosynthetic activity of two *Arabidopsis* mutants defective in hexokinase-dependent sugar signaling, indicating that it augments photosynthesis through repressing hexokinase-dependent, rather than hexokinase-independent, sugar signaling (Zhang et al. 2008b). In addition, GB03-exposed plants exhibited a reduction in ABA biosynthesis transcript levels and shoot ABA levels. Since sugar signaling is known to overlap with the ABA transduction pathway (Rolland et al. 2006), the reduction of ABA levels could explain the repressed glucose signaling in GB03-exposed plants. Consistent with this hypothesis, exogenous ABA thwarts GB03-induced increases in photosynthetic efficiency and chlorophyll content. Overall, this study demonstrates that some PGPR can affect photosynthesis through the modulation of endogenous ABA/sugar signaling regulatory pathways. Considering that PGPR modulate many plant hormonal pathways, as illustrated by the studies performed with *Arabidopsis* summarized herein, and that these pathways interfere with sugar signaling, directly like the ABA pathway or indirectly, this result is not so surprising. On the contrary, the modulation of sugar signaling is likely to be a general feature of PGPR pattern responses, though it remains very elusive.

13.7 Concluding Remarks and Future Research Perspectives

Until very recently, the studies on plant growth promotion by PGPR exclusively focused on the bacterial partner without consideration of plant's physiology. This approach did not succeed in unraveling the mechanisms elicited by the rhizobacteria that operate to promote plant growth. Using *A. thaliana* to investigate how a PGPR can stimulate the growth of plants has demonstrated to being very efficient when considering the limited number of researchers involved in *Arabidopsis*–PGPR studies and the complexity of the biological system. This success lies in the availability of genetic and genomic tools; the deep molecular, cellular, and physiological knowledge; and the experimental convenience of this model species (Fig. 13.3). Indeed, several lessons already arise from this short story (less than a decade).

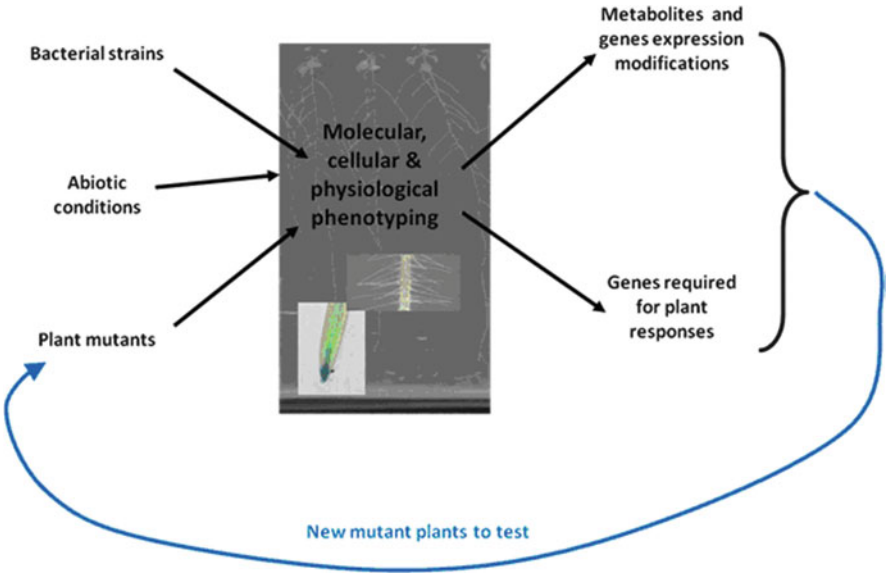

Fig. 13.3 The model plant *Arabidopsis thaliana* provides unique tools to investigate the common and strain-specific signaling pathways involved in plant responses to PGPR because of the largest availability in plant genetic tools, the deepest knowledge in plant biology, and its experimental convenience. Molecular, cellular, and physiological phenotypic responses to inoculation with a PGPR strain (illustrated by IAA accumulation pattern visualized in a DR5::GUS transgenic plant, root hairs, and root system architecture) allow identifying genes and metabolites that are affected by the rhizobacterium and, using mutant plants, the genes that are required for some of these responses. These data lead to question the role of other genes in plant response to PGPR, hence providing new mutants for testing molecular, cellular, and/or physiological response pattern to inoculation. This reverse genetic approach already revealed the occurrence of numerous, and often unexpected, plant response to PGPR, and it will unravel the underlying mechanisms of the complex regulatory network elicited in plants by beneficial rhizobacteria

The first lesson to be drawn from *Arabidopsis*–PGPR studies is that there is an enormous diversity of plant responses to PGPR: a single strain can elicit several hormonal pathways, modulate the activity of several transporters, modify photosynthetic activity and other physiological processes, etc. Furthermore, the list of plant responses is certainly far from being completed. In addition, these various responses are interconnected by plant regulatory pathways, making difficult the identification of PGPR targets.

The second lesson is that to classify a specific PGPR strain as a "phytostimulator" or a "biofertilizer" would not be very meaningful for two reasons: (1) the strains that have been the most extensively studied with *Arabidopsis*, GB03 and STM196, have been proved to affect both plant developmental and nutritional processes, and (2) regulations of nutrition and development are so tightly interconnected in plants that signaling pathways are not entirely distinguishable.

The third lesson is that PGPR not only can modulate plant hormonal pathway, as postulated before, by providing hormones or hormone-like molecules to plant roots, but they also modulate plant hormonal pathways per se. This ability to subtly modify plant endogenous regulatory pathways establishes a new paradigm that showed the complexity of plant responses to PGPR.

The fourth lesson relates to the plant biology knowledge: among the modifications of developmental or physiological traits induced by PGPR, some appeared to involve yet unidentified regulatory pathways. For instance, GB03 induces FIT1 via unknown mechanism (Zhang et al. 2009), so that characterizing the plant factors involved in FIT1 induction by GB03 may reveal to be a useful way to get insights into regulatory steps in plant iron uptake and homeostasis. Another example is the regulation of root hair elongation: while this process has been considered to be mainly dependent upon auxin and ethylene signaling pathways up to now, STM196 is able to dramatically elongate root hairs independently to both hormonal pathways (Contesto et al. 2008; Desbrosses et al. 2009), indicating that another key regulator of root hair development remains to be discovered. These two examples, and other that could be drawn from the studies summarized herein, show that PGPR–*Arabidopsis* interaction studies may be a good model to decipher new regulatory pathways, or new interactions between known pathways, in plants.

In conclusion, the PGPR–*Arabidopsis* interaction appears to be a very powerful model to decipher plant responses to PGPR, but also plant regulatory mechanisms and how a plant integrate endogenous signals with environmental, both biotic and abiotic, signals. Further research using this model will need to combine approaches from the molecular to the ecophysiological level to make a clear picture emerging of such a complex network.

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