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REVIEW ARTICLE

Rhizosphere engineering and management for sustainable agriculture

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Abstract This paper reviews strategies for manipulating plants and their root-associated microorganisms to improve plant health and productivity. Some strategies directly target plant processes that impact on growth, while others are based on our knowledge of interactions among the components of the rhizosphere (roots, microorganisms and soil). For instance, plants can be engineered to modify the rhizosphere pH or to release compounds that improve nutrient availability, protect against biotic and abiotic stresses, or encourage the proliferation of beneficial microorganisms. Rhizobacteria that promote plant growth have been engineered to interfere with the synthesis of stress-induced hormones such as ethylene, which retards root growth, and to produce antibiotics and lytic enzymes active against soilborne root pathogens. Rhizosphere engineering also can involve the selection by plants of beneficial microbial populations. For example, some crop species or cultivars select for and support populations of antibiotic-producing strains that play a major role in soils naturally suppressive to soil-borne fungal pathogens. The fitness of root-associated bacterial communities also can be enhanced by soil amendment, a process that has allowed the selection of bacterial consortia that can interfere with bacterial pathogens. Plants also can be engineered specifically to influence their associated bacteria, as exemplified by quorum quenching strategies that suppress the virulence of pathogens of the genus *Pectobacterium*. New molecular tools and powerful biotechnological advances will continue to provide a more complete knowledge of the complex chemical and biological interactions that occur in the rhizosphere, ensuring that strategies to engineer the rhizosphere are safe, beneficial to productivity, and substantially improve the sustainability of agricultural systems.

Keywords Plant engineering · Proton efflux · Organic cation efflux · Microbe engineering · Biological control · PGPR · Plant genotype selection · Take-all decline · Soil amendment · Biostimulation

Abbreviations

ACC 1-aminocyclopropane-1-carboxylate
ALMT Al^{3+}-activated malate transporter
DAPG 2,4-diacetylphloroglucinol
EST expressed sequence tags

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Ggt	*Gaeumannomyces graminis* var. *tritici*
MATE	multidrug and toxic compound extrusion
NAHL	N-acyl homoserine lactone
PCA	phenazine-1-carboxylic acid
PGPR	plant growth-promoting rhizobacteria
QS	quorum-sensing
QTL	quantitative trait locus(i)
TAD	take-all decline

Why engineer the rhizosphere?

The rhizosphere is the zone of soil around roots that is influenced by root activity. The intimacy of this interface between plants and their environment is essential for the acquisition of water and nutrients and for beneficial interactions with soil-borne microorganisms. Yet, this same intimacy increases the vulnerability of plants to a range of biotic and abiotic stresses. Plants have evolved a variety of strategies to modify the rhizosphere to lessen the impact of these environmental stresses, and an understanding of the processes involved will suggest ways in which the rhizosphere can be manipulated to improve plant health and productivity. Rhizosphere engineering may ultimately reduce our reliance on agrochemicals by replacing their functions with beneficial microbes, biodegradable biostimulants or transgenic plants. Some of these materials and techniques are still being developed, while others are being tested in the field. This paper reviews aspects of rhizosphere engineering and management with the view of developing novel approaches for sustainable agricultural production.

How to engineer the rhizosphere?

The physical and chemical milieu of the rhizosphere is the culmination of many competing and interacting processes that depend on the soil type and water content, the composition and biological activities of root-associated microbial communities and the physiology of the plant itself (Pinto et al. 2007). The rhizosphere can be modified over short periods of a plant's growth cycle by agronomic management (Bowen and Rovira 1999). Farmers influence the environment around the roots of their crops and pasture species every time they irrigate their fields or apply fertilizer. Ammonium-based fertilizers applied to plants tend to acidify the rhizosphere whereas nitrate-based fertilizers result in a more alkaline rhizosphere. Shifts in pH can alter soil chemistry around roots and influence the growth and composition of microbial communities. More prolonged changes in the rhizosphere that persist through the growth cycle can be generated with plant breeding and biotechnology. Selecting plants with favourable rhizosphere characteristics and introgressing these traits into elite breeding lines can lead to more permanent changes. This approach relies on being able to identify characters that are useful, heritable and easily detected: all of which are difficult when dealing with below-ground traits. Nevertheless, plant breeders have unintentionally engineered beneficial rhizosphere traits into crops by simply selecting for the best performing lines in field trials and recurrently crossing them to other breeding lines. A good example of this is the breeding for aluminium (Al^{3+}) resistance in wheat, which relies on the release of malate anions from the root apices—a rhizosphere process. The physiology of this mechanism has been understood for 15 years (Delhaize et al. 1993; Ryan et al. 1995) and the gene controlling it was isolated relatively recently (Sasaki et al. 2004), yet breeders have inadvertently been selecting for it for over a century by selecting the best performing lines on acid soils (de Sousa 1998). Microorganisms are a vital component of the rhizosphere, and the total biomass and composition of rhizosphere microbial populations markedly affects interactions between plants and the soil environment. There is considerable interest in developing methods for encouraging the proliferation of beneficial introduced or indigenous microbial populations that facilitate nutrient uptake (e.g., rhizobia and mycorrhiza), promote plant growth directly, or suppress plant pathogens. As the complexity of the rhizosphere is unraveled we can attempt to create conditions most beneficial to plant growth by amending the soil, breeding or engineering better plants, and manipulating plant/microorganism interactions. A summary of the above approaches is given in Fig. 1.

Engineering << individual >> rhizosphere components

Engineering plants

One of the main ways plants modify the rhizosphere is through the release of inorganic and organic

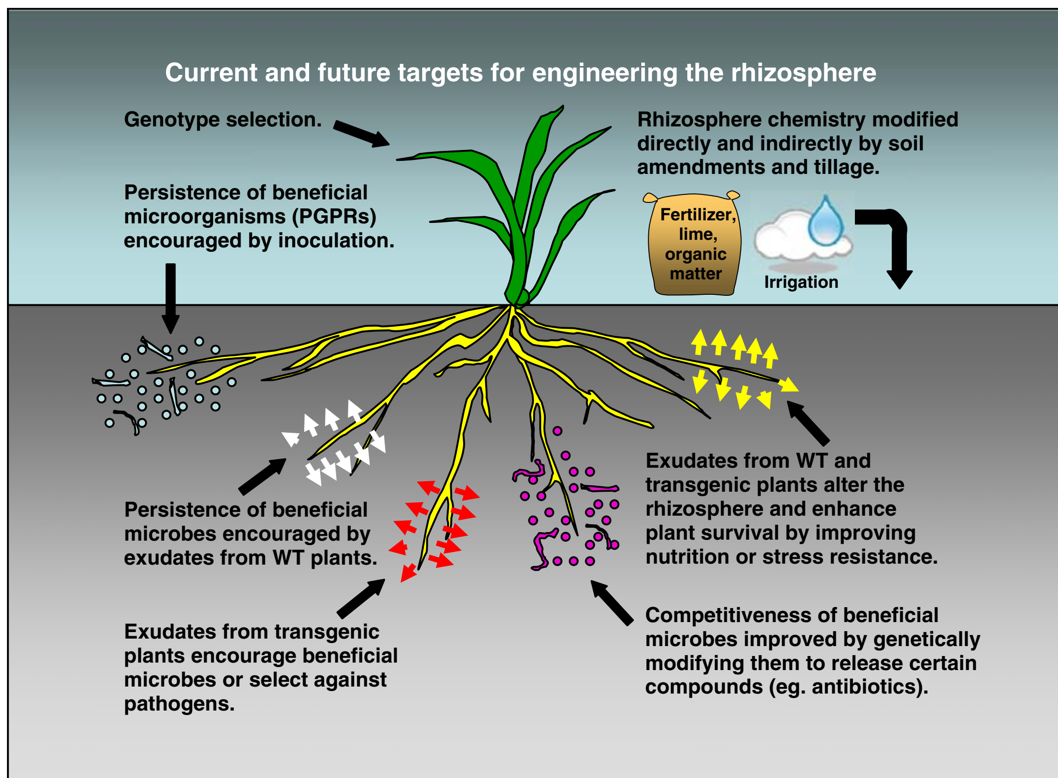

Fig. 1 Current and future targets for rhizosphere engineering The rhizosphere can be manipulated or engineered with agronomic practices, plant selection, soil inoculation or with biotechnology. Common practices such as soil tillage, fertilizer application or even irrigation can alter the chemistry of the plant-sol interface by changing aeration, root function or microbial communities. Plants with favorable roots traits that improve performance can be selected for by breeders. These traits could include exudates that increase nutrient accessibility, minimize stress or that encourage the persistence of beneficial micro-organisms. Biotechnology can be used to accentuate these useful traits or generate plants and micro-organisms with novel phenotypes that help plant survival. Transgenic plants and micro-organisms can be engineered to exude exogenous compounds that improve plant nutrition, repress pathogenic microbes and minimize the consequences of biotic or abiotic stresses

substances from their roots (rhizodeposition). These exudates can enhance nutrient acquisition, help to avoid mineral stresses or encourage the growth of favourable microorganisms. For instance, most transport processes across the plasma membrane in plant cells, including the uptake of essential nutrients, rely on the efflux of H^+ to generate a membrane potential difference and an electrochemical gradient for H^+. In addition to generating a driving force for membrane transport, H^+ efflux can contribute to nutrient acquisition by acidifying the rhizosphere (Hinsinger et al. 2003). Local acidification can increase the availability of Fe^{3+} and phosphorus, which are often fixed to the surface of minerals or present as insoluble complexes. Iron uptake in dicots and non-graminaceous monocots also involves the release of organic compounds that chelate Fe^{3+} and facilitate its reduction to Fe^{2+} prior to uptake. Grass species, by contrast, rely on the release of compounds called phytosiderophores that help mine the poorly soluble Fe^{3+} and deliver it to the root surface for uptake (Neumann and Römheld 2007). The release of organic anions such as citrate and malate, as well as phytases and phosphatases, helps some species to access poorly-soluble organic and inorganic phosphorus in a similar way (Dinkelaker et al. 1995; Richardson et al. 2001; Ryan et al. 2001; Vance et al. 2003). Organic anion efflux also protects some crop species from Al^{3+} toxicity in acid soils by chelating harmful Al^{3+} ions and preventing their damaging interactions with root apices (Ma et al. 2001; Ryan et al. 2001). Therefore the release of organic anions from roots can have an important

influence on plant growth and nutrition. Since many of the genes controlling these exudates have now been identified, it is possible to manipulate conditions in the rhizosphere by modifying their expression via genetic engineering.

Plant genetic engineering is a relatively new science and several hurdles need to be crossed to achieve a successful outcome. Transgenic plants can exhibit phenotypes that are subtle, unexpected or even totally absent. The activity of exogenously-expressed proteins can be limited by metabolic feedback, substrate supply or post-translational regulation. Even when a desired trait, such as a root exudate, has been engineered successfully into a plant, the compounds might be rapidly degraded or otherwise inactivated in the soil, or the rate of exudation might simply be too small to influence the rhizosphere as predicted. The constitutive high or low expression of some genes can have pleiotropic effects on plant development, fertility or function that are detrimental to production. Also, transgenic plants can also display unexpected phenotypes that have nothing to do with the activity of the engineered protein (loss of function due to random insertion of transgene, somaclonal variation, etc.). Although many of these problems occur infrequently, they emphasise the need for careful analysis of the transgenic material and the use of suitable controls.

Once transgenic plants have been generated it is essential to analyze several independent transgenic lines and to compare them with control lines. There is no simple answer for how many transgenic and control lines should be analyzed. This will depend on how subtle or strong the phenotype is but in general, “more is better.” The analysis of several independently transformed lines provides confidence that the measured phenotype is caused by transgene expression. It also is reassuring to show that the magnitude of the phenotype is correlated with the level of transgene expression in independent lines.

Wild-type plants commonly are compared with transgenic lines, but other types of controls can be used as well. When transgenic lines are developed from tissue culture, many workers include control lines that are transformed with an empty vector or non-transformed plants that have passed through tissue culture. This practice improves the chances of detecting real differences between the transgenic and control lines because it simulates more of the variability that may be introduced into transgenic lines during tissue culture. However, the preferred controls are the null segregants generated by selfing the primary transformants and analyzing the resulting lines. Null plants are excellent controls because they have experienced the same treatments as the transgenic plants except that they no longer contain the transgene. The null segregants also are useful when transgenic lines are generated without tissue culture, such as by the flower-dipping method for transforming Arabidopsis (Clough and Bent 1998).

The following case studies describe attempts to engineer the rhizosphere by manipulating the efflux of H^+ and organic anions from the roots in transgenic plants.

Case Study 1: Manipulating rhizosphere pH Proton efflux from plant cells is largely controlled by a large family of H^+-ATPase proteins that use energy from ATP catalysis to pump H^+ across the plasma membrane against a steep electrochemical gradient. The genes encoding these proteins have been isolated and studied for many years (Palgren 1991; Gaxiola et al. 2007). Therefore the objective of manipulating rhizosphere pH by over-expressing these genes would appear relatively straightforward. Yet, this has not proved to be the case and few studies have successfully increased H^+-efflux enough to change root function. For instance, when *Nicotiana tabacum* and Arabidopsis plants were transformed with cDNA encoding H^+-ATPase genes, they showed no symptoms despite significant increases in protein levels (Gévaudent et al. 2007; Young et al. 1998; Zhao et al. 2000). It became clear that activity of the H^+-ATPase protein was being regulated by an auto-inhibitory domain on its C-terminal tail. This domain suppresses enzyme activity by preventing protein phosphorylation and subsequent interactions with 14-3-3 regulatory proteins (Palgren 1991). It was only when plants were transformed with a modified H^+-ATPase protein in which the auto-inhibitory region was removed that phenotypes such as increased H^+-efflux from roots, more acidic rhizosphere (Gévaudent et al. 2007; Yang et al. 2007), or improved growth at low pH (Young et al. 1998) appeared in the transgenic lines. Constitutive expression of a modified H^+-ATPase gene, *PMA4*, in tobacco was also associated with minor developmental phenotypes and better growth on 200 mM NaCl than wild-type controls (Gévaudent et al. 2007). The improved salinity resistance was

attributed to a greater capacity for Na^+/H^+ exchange across the plasma membrane.

Proton pumps also occur in subcellular compartments, and the transport of H^+ across the tonoplast membrane in plant cells is catalyzed by a vacuolar H^+-ATPase and a H^+-pyrophosphatase. Since these enzymes pump H^+ into the vacuole, and not across the plasma membrane, there was no *a priori* reason to suspect that modulating their activity would affect rhizosphere pH. Nevertheless, over-expression of the *AVP1* pyrophosphatase in Arabidopsis did induce a more acidic rhizosphere, apparently by up-regulating the activity of the plasma membrane H^+-ATPase (Yang et al. 2007). These transgenic Arabidopsis lines displayed an array of other phenotypes that appeared to be related to auxin transport (Li et al. 2005), since the changes in apoplasmic pH and the concomitant up-regulation of auxin transport proteins altered the distribution of auxin along the roots.

Case Study 2: Enhancing organic anion efflux from roots Once it was established that organic anions play a pivotal role in many rhizosphere processes, several groups began to investigate how efflux could be manipulated (see reviews by de la Fuente-Martinez and Herrera-Estrella 1999; López-Bucio et al. 2000b; Ryan et al. 2001). Two approaches have been tried to increase organic anion release from roots: (1) engineering plants with a greater capacity to synthesise organic anions, and (2) engineering plants with a greater capacity to transport organic anions out of the cell. These studies have mostly targeted malate and citrate anions because they occur in most living cells and are known to be released from roots in response to nutrient deficiency and mineral stress. In view of the large outwardly-directed electrochemical gradient for anions across the plasma membrane, some workers have argued that efflux from roots is more likely to be limited by transport across the plasma membrane than by synthesis (Ryan et al. 2001). If so, strategies aimed at enhancing transport capacity will be more successful than those attempting to modify metabolism. However both approaches rely on important assumptions as explained below.

Transgenic plants generated via the first approach typically exhibit enhanced expression of an enzyme involved in synthesis of an organic anion or reduced expression of an enzyme involved its consumption, the assumption being that these changes will increase anion accumulation in the cytosol and greater efflux from the cell. This approach will have a better chance of success if the target enzyme is rate-limiting in a pathway so that changes in protein concentration correspond to changes in flux through that pathway. Doubling or tripling the amount of an enzyme that catalyses a near-equilibrium reaction is unlikely to affect metabolism enough to generate a phenotype. Similarly, it is best to avoid enzymes whose activity is closely regulated by secondary modifications such as phosphorylation or other restrictive feedback mechanisms. Unwanted regulation may be minimized if the transgene is expressed in a sub-cellular compartment different from where it usually is located (i.e. expressing a mitochondrial gene in the cytosol) or by choosing a gene from a disparate source (expressing a bacterial gene in a plant) or a different species (expression of a carrot gene in Arabidopsis). Finally, this strategy assumes that an endogenous transport system is present for moving the organic anions across the plasma membrane and into the rhizosphere.

The second approach for increasing organic anion efflux transforms plants with genes encoding proteins which facilitate organic anion movement across the plasma membrane. This approach assumes that activity of the transport protein will not be regulated by the cell and that efflux will not be limited by the plant's capacity to synthesize organic anions.

Approach 1 Engineering metabolic pathways for greater organic anion efflux

One of the first attempts to manipulate organic anion release from plants was reported by de la Fuente et al. (1997). They increased citrate efflux from tobacco roots by transforming plants with a citrate synthase gene from *Pseudomonas aeruginosa* under the control of a constitutive promoter. The two- to three-fold increases in citrate synthase activity measured in the transgenic lines were associated with three-to ten-fold higher concentrations of citrate in the root tissue and a four-fold greater efflux of citrate from seedlings suspended in sterile water. Compared to control plants transformed with an empty vector, the transgenic plants reportedly showed enhanced resistance to Al^{3+} stress and a greater ability to acquire phosphorus from poorly soluble forms present in an alkaline soil (Lòpez-Bucio et al. 2000a).

Similarly, Koyama et al. (1999) transformed carrot (*Daucus carota*) suspension cells with a mitochondri-

al citrate synthase gene from Arabidopsis, and the four-fold greater release of citrate enhanced cell growth on media supplemented with $AlPO_4$. Koyama et al. (2000) later transformed Arabidopsis with a mitochondrial citrate synthase gene from carrot and although the increase in citrate release was relatively small at 60%, it was sufficient to confer a marginal increase in Al^{3+} resistance and an improved capacity to utilize sparingly soluble P compared to wild-type plants and null segregants derived from the transgenic lines. Other studies using similar approaches have reported some success in increasing the organic anion efflux from *Medicago sativa* (Tesfaye et al. 2001) and *Brassica napus* (Anoop et al. 2003).

More recently, rice and tomato plants were transformed with the Arabidopsis vacuolar H^{+}-pyrophosphatase gene *AVP1*. The transgenic lines showed approximately 50% greater citrate and malate efflux than wild-type plants when treated with $AlPO_4$. The authors argued that this, combined with greater rhizosphere acidification, was sufficient to enhance resistance to Al^{3+} stress and improve the ability to utilize insoluble phosphorus (Yang et al. 2007). The transgenic lines also exhibited larger roots, longer root hairs and greater shoot biomass, which appear to be caused by perturbations in auxin transport (Li et al. 2005). Since the transgenic plants exhibited many strong phenotypes, the claims of nutrient efficiency and Al^{3+} resistance need to be carefully reconciled with the differences in plant size under control conditions.

Other studies using this strategy have been less successful in manipulating organic anion efflux from roots (Delhaize et al. 2001; Delhaize et al. 2003). For example, attempts by independent groups to repeat the findings of de la Fuente et al. (1997) were unsuccessful despite using identical constructs and increasing the citrate synthase activity in transgenic tobacco lines to similar, or even greater levels, than those published originally (Delhaize et al. 2001; Jian Feng Ma, pers. comm.). These results demonstrate that increases in enzyme activity do not necessarily lead to anion accumulation and enhanced efflux, and suggest that metabolic or environmental factors can influence the effectiveness of this approach.

Approach 2 Engineering transport proteins for greater organic anion efflux

Until recently, there was no prospect of manipulating organic anion efflux from roots by targeting the transport step because the genes encoding the transport proteins had not been identified. Fortunately, over the last few years, the potential for engineering the rhizosphere has been revolutionized by the identification of candidate genes in two different families: the Al^{3+}-activated malate transporter (*ALMT*) and the multidrug and toxic compound extrusion (*MATE*) gene families. Members of these families encode membrane-bound proteins that facilitate the efflux of malate and citrate from plant cells. These recent developments have provided new possibilities for manipulating organic anion efflux from roots.

(i) *ALMT family*

The first gene identified to encode a transport protein that facilitates organic anion efflux from plant cells is *TaALMT1* from wheat (*Triticum aestivum*) (Sasaki et al. 2004). *TaALMT1* encodes the first member of a novel membrane protein family that functions as an anion channel to mediate malate efflux from roots (Ryan et al 1997; Zhang et al. 2008). Constitutive expression of *TaALMT1* in plant and animal cells including tobacco suspension cells, rice (*Oryza sativa*) and *Xenopus* oocytes (Sasaki et al. 2004), barley (*Hordeum vulgare*) (Delhaize et al. 2004) as well as Arabidopsis (Peter Ryan, unpublished data) all generate an Al^{3+}-activated efflux of malate anions. In some cases (tobacco suspension cells, barley and Arabidopsis), malate efflux also increased resistance to Al^{3+} stress. The finding that TaALMT1 confers the same phenotype to a diverse range of plant and animal systems demonstrates that a sustained efflux of organic anions from all these cell types and tissues will occur once a transport pathway across the membrane is provided.

TaALMT1 is a valuable tool for manipulating malate release into the rhizosphere but it has an important limitation that restricts its wider application to plant nutrition—it needs to be activated by Al^{3+}. Even when TaALMT1 is constitutively expressed in a heterologous system, malate efflux will not occur unless Al^{3+} is added to the bathing solution. The most likely explanation for this response is that TaALMT1 is a ligand-gated anion channel that requires direct interaction with Al^{3+} to shift the protein into an active configuration. While this property is ideal for conferring Al^{3+} resistance to transgenic plants, the practical application of TaALMT1 is limited to acid soils

where the concentration of Al^{3+} is sufficient to activate the protein. TaALMT1 is one member of a large protein family distributed widely among plant species (Delhaize et al. 2007), of which four additional members have been characterized in detail. Three of these from Arabidopsis and *Brassica napus* are involved in Al^{3+} resistance and are similarly activated by Al^{3+} (Hoekenga et al. 2006; Ligaba et al. 2006). The fifth member of the ALMT family, also from Arabidopsis, functions differently from the others. This protein, AtALMT9, localises to the tonoplast of shoot cells and transports malate out of the cytoplasm and into the vacuole with no requirement for Al^{3+} (Kovermann et al. 2007). AtALMT9 and other proteins that function similarly to it may be more useful members of the ALMT family for manipulating malate release from roots.

(ii) *MATE family*

MATE genes are widely distributed among all kingdoms of living organisms, where they function to export a wide range of small organic compounds including secondary metabolites such as flavanoids and alkaloids, as well as exogenous antibiotics (Eckardt 2001; Omote et al. 2006). More recently, members of the *MATE* family have been shown to facilitate citrate efflux from plant cells. Recent studies have demonstrated that *MATE* genes in sorghum (*Sorghum bicolor*) and barley (*Hordeum vulgare*) control Al^{3+} resistance in these species by facilitating citrate efflux from the roots (Magalhaes et al. 2007; Furukawa et al. 2007; Wang et al. 2007). The citrate binds with the toxic Al^{3+} ions in the rhizosphere and protects the root apices much like malate efflux protects wheat from Al^{3+} stress. The heterologous expression of *SbMATE1* in Arabidopsis, and *HvMATE* in tobacco, conferred Al^{3+}-activated efflux of citrate and increased resistance to Al^{3+} stress (Magalhaes et al. 2007; Furukawa et al. 2007).

Two additional *MATE* genes, *Frd3* and *OsFRDL1* from Arabidopsis and rice (Oryza sativa), respectively, also facilitate citrate efflux from cells but not as part of an Al^{3+}-resistance mechanism. Instead, they control the release of citrate from vascular cells into the xylem, where it forms a complex with iron and moves to the shoot (Durrett et al. 2007; Yokosho et al. 2009). Expression of *Frd3* and *OsFRDL1* in *Xenopus laevis* oocytes revealed a fundamentally different mode of action from MATEs involved in Al^{3+} resistance because both facilitated citrate efflux in the absence of Al^{3+}. Over-expression of *Frd3* in Arabidopsis under the control of the CaMV 35S promoter also conferred greater Al^{3+} resistance because the plants constitutively released citrate from their roots. That FRD3 is active without exposure to Al^{3+} highlights a real advantage over the ALMT1 genes examined so far. FRD3 provides an opportunity to enhance citrate efflux into the rhizosphere over a wider range of soil types. This type of efflux engineering should take in account the metabolic and energy costs associated with both the production and exudation of organic acids anions in the rhizosphere.

This section has described how biotechnology can influence the chemical properties of the rhizosphere. The examples presented provide proof of principle that manipulation of root exudates has the potential to improve plant nutrition and protect plants from stress. It seems likely that increased exudation of organic acids by transgenic plants also will influence the composition and density of rhizosphere microbial populations capable of metabolizing these carbon sources, but the magnitude and potential consequences of increased nutrient availability have yet to be determined.

Engineering microbes

In addition to their direct influence on soil chemistry, root exudates support large microbial populations that afford a basal level of protection to roots against pathogens simply through their metabolic activity (see Section "Natural engineering of microbial populations" below). Some isolates which have additional beneficial properties are plant growth-promoting rhizobacteria (PGPR)—free living strains able to colonize roots and stimulate plant growth. Growth stimulation can be mediated directly, as through enhanced nutrient acquisition or modulation of phytohormone synthesis, or indirectly, through induction of the plant's own defense responses or antagonism of soil-borne pathogens, and several mechanisms of promotion can operate simultaneously in a single strain. Hundreds, if not thousands, of PGPR representing diverse genera have been described over the past 50 years. Despite their appeal as a "natural" means of plant protection few strains have been developed commercially. This is partly because

uneconomically large doses often must be applied and performance can be inconsistent in the field. Genetically engineered strains have been valuable in identifying some of the causes of variable strain performance and they offer a means to develop PGPR that are effective at low inoculum doses and under a variety of environmental conditions.

Strategic issues for strain development To be effective, introduced PGPR must establish and maintain biologically active populations in competition with the already-adapted resident microflora. A variety of cell surface molecules contribute to the colonization process (Lugtenberg et al. 2001), as does efficient utilization of the major carbon and nitrogen sources available in root exudates (Kamilova et al. 2005, 2006), but genetic engineering of individual fitness determinants generally has not led to improved strain performance. In contrast, genes involved in growth promotion have proven effective as targets for strain improvement, either by modifying the timing or level of their expression or by transferring and expressing them in alternate hosts with other desirable attributes. Important considerations for field application of such strains include not only whether plant growth is enhanced, but also whether the engineered trait is stably maintained and expressed, its effects on the fitness of the modified strain, and the effects of the modified strain on nontarget organisms in the environment.

PGPR activity is enhanced in engineered strains The *chiA* gene, encoding a chitinase targeted to chitin in the cell walls of fungal root pathogens, was among the first to be transferred to heterologous bacteria with the goal of enhancing PGPR activity via pathogen suppression. *Escherichia coli* (which itself lacks the ability to control plant pathogens) expressing *chiA* caused rapid and extensive bursting of the hyphal tips of *Sclerotium rolfsii* and effectively reduced its ability to cause disease on bean (Shapira et al. 1989). An engineered strain of *Pseudomonas* expressing a *chiA* gene from *Serratia marcescens* more effectively controlled *Fusarium oxysporum* f. sp. *redolens* and *Gaeumannomyces graminis* var. *tritici* (Sundheim et al. 1988), and *P. fluorescens* expressing the introduced gene constitutively, either on a plasmid or integrated into the genome (Downing and Thomson 2000; Koby et al. 1994), provided improved control of fungal pathogens in the growth chamber or greenhouse.

Many rhizobacteria produce phytohormones that undoubtedly affect root growth, but efforts to engineer the rhizosphere through hormone manipulation have focused mainly on degradation of so-called “stress” ethylene, which is synthesized by plants upon exposure to stresses such as flooding, drought, salt, and the presence of metals, organic contaminants and pathogens. Ethylene is required for proper plant development, growth and survival, including the induction of systemic defense mechanisms against pathogens, but the elevated levels associated with stress can trigger damaging physiological processes that exacerbate environmental pressures and exaggerate disease symptoms (reviewed by Glick et al. 2007). Rhizobacteria that produce 1-aminocyclopropane-1-carboxylate (ACC) deaminase, which catalyzes the cleavage of ACC, the immediate precursor of ethylene, are a metabolic sink for ACC, diminishing ethylene concentrations to levels too low to initiate the physiological cascade leading to reduced growth or plant tissue damage. *P. fluorescens* CHA0 transformed with the ACC deaminase gene *acdS* from *P. putida* UW4 (formerly classified as *Enterobacter cloacae*) increased root length in canola seedlings and provided improved protection of cucumber against *Pythium,* demonstrating the involvement of ethylene in this plant-pathogen interaction (Wang et al. 2000). Canola, tomato and tobacco plants grown in the presence of ACC deaminase-producing bacteria or transformed directly with the bacterial *acdS* gene generally exhibited improved growth upon exposure to abiotic stresses in the greenhouse (Glick et al. 2007). Ethylene’s central regulatory role in plant growth and survival, and the ability of PGPR to modulate stress ethylene levels, make it an attractive target for rhizosphere engineering, particularly when plant growth is limited due to abiotic stress. A more complex situation requiring empirical evaluation may prevail with pathogen-induced stresses where ethylene is needed to trigger the systemic defense cascade but stress ethylene levels can exacerbate pathogen-induced apoptosis and tissue damage.

Successful disease control by PGPR that produce antibiotics requires that such strains produce active compounds in sufficient quantity and within a window of opportunity dictated by the disease cycle of the target pathogen. This can be particularly challenging for the suppression of pre-and post-emergence damping-off diseases caused by *Pythium*

and *Rhizoctonia* spp., which can attack seeds and seedlings before introduced strains are able to synthesize inhibitory levels of metabolites. Ligon et al. (2000) addressed this problem by engineering derivatives of *P. fluorescens* BL915, which synthesizes the antifungal compound pyrrolnitrin. In one derivative the regulatory gene *gacA*, which controls the synthesis of pyrrolnitrin in *Pseudomonas*, was constitutively expressed on a multicopy plasmid in BL915. This regulatory derivative produced about 2.5-fold more pyrrolnitrin than the parental strain. A second derivative in which the entire four-gene *prnABCD* operon was constitutively expressed from a plasmid produced 4-fold more pyrrolnitrin, and production was increased to levels 10-fold over those of the parental strain when both plasmids were expressed in the same cells. In greenhouse trials, the derivative strains were as protective of cucumber and impatiens in soil infested with *R. solani* as BL915 applied at 10-fold higher doses. In the field, control of *R. solani* and *P. ultimum* on cotton was better than that provided by BL915 and not significantly different from chemically-treated and healthy controls. Other experiments showed that control of *R. solani* on cotton was proportional to the amount of pyrrolnitrin synthesized in vitro (Ligon et al. 2000).

The results of Ligon et al. (2000) suggest that constitutive production or overproduction of antibiotics can improve strain effectiveness and are supported by other works in which biosynthesis operons for the antibiotics phenazine-1-carboxylic acid (PCA) and 2,4-diacetylphloroglucinol (DAPG) were transferred to heterologous strains to enhance control of damping-off diseases. In one case, the constitutively expressed seven-gene PCA operon was introduced into random sites in the genome of *P. fluorescens* SBW25. Chromosomal insertion provides genetic stability and effective gene containment, which are important in minimizing the potential for horizontal gene transfer in the rhizosphere. The PCA-producing derivatives reduced damping-off disease on pea seedlings more effectively than did wild-type SBW25 and the level of PCA produced was correlated directly with the efficacy of the bacteria. Moreover, pretreatment of the soil with the engineered strain effectively decontaminated it, reducing disease incidence (Timms-Wilson et al. 2000). In another study, engineered derivatives of *P. fluorescens* 5-2/4 expressing an integrated cassette carrying the DAPG biosynthesis operon from *P. fluorescens* Q2-87 (a B genotype strain; see Section "Take-all decline in Washington State: a case study") provided increased control of *P. ultimum* (Alsanius et al. 2002).

The PCA integrative cassette has been exploited to extend the range of diseases controlled by *P. fluorescens* Q8r1-96 (a D-genotype strain; see Section "Take-all decline in Washington State: a case study"), which produces DAPG and is highly effective against the take-all pathogen *Gaeumannomyces graminis* var. *tritici* but less effective against root rot caused by *Rhizoctonia solani*. Expression of the phenazine biosynthesis operon normally is regulated by quorum sensing, and as expected, a constitutively engineered derivative in Q8r1-96 produced more PCA, both in vitro and in the wheat rhizosphere (Huang et al. 2004), than did *P. fluorescens* 2-79, from which the operon had been cloned. Surprisingly, however, the PCA-producing derivatives also synthesized more DAPG than did the parental strain, suggesting that a PCA biosynthesis intermediate might have derepressed expression of the DAPG operon. Levels of antibiotic synthesis also differed among individual recombinants, presumably due to positional effects resulting from random insertion of the PCA cassette within the Q8r1-96 genome. As in transgenic plant lines, it is necessary to compare several independent recombinant clones with the parental and plasmid-transformed control strains. Vectors targeting introduced genes to a neutral chromosomal site (Craig 2002) also can be used. In the greenhouse, PCA-producing strains suppressed rhizoctonia root rot at inoculum doses 10-to 100-fold lower than the dose of Q8r1-96 required for comparable disease suppression (Huang et al. 2004) and in the field, wheat treated with the engineered strains consistently had yields 8–20% greater than those treated with Q8r1-96 alone (D. M. Weller et al., unpublished), supporting the feasibility of pyramiding antibiotic pathways to control mixed populations of yield-limiting soil-borne pathogens that typically exist in the field.

Recombinant strains and rhizosphere competence The ecological fitness of PGPR, whether engineered or not, is an important factor in evaluating the potential risks associated with their release into the environment. Using isogenic derivatives of *P. fluorescens* SBW25 tagged with marker genes, De Leij et al. (1998) detected no effect of metabolic burden on the

bacteria in the rhizosphere of pea or wheat. The question of fitness also has been addressed in strain Q8r1-96 and its PCA-producing derivatives. Because PCA contributes to the competitiveness of *Pseudomonas* strains (Mazzola et al. 1992), it is conceivable that Q8r1-96 constitutively producing PCA would be more competitive than the wild type. On the other hand, PCA synthesis is energetically costly, and constitutive production, combined with the upregulation of DAPG synthesis in the recombinant strains, likely would create a metabolic load that would reduce the fitness of the recombinants. Indeed, this could explain why wild-type isolates producing both PCA and DAPG are seldom if ever found in nature. To distinguish between these possibilities, the persistence on wheat of Q8r1-96 and its PCA-producing derivative Z30-97 was monitored under greenhouse conditions (Bankhead et al. 2004a) and in a 3-year field study (Bankhead et al. 2004b). No consistent strain-specific differences in rhizosphere competence were observed when Q8r1-96 and Z30-97 colonized separate rhizospheres. In contrast, when the strains were co-inoculated on the same plants to provide more intense competitive pressure, Q8r1-96 displaced Z30-97 in the wheat rhizosphere (Bankhead et al. 2004a). Collectively, the data suggest that on wheat, any benefit of PCA synthesis ultimately was overridden by its metabolic cost to the engineered strain, but apparently that cost was not enough to adversely affect competitiveness against indigenous rhizosphere microorganisms, even over 3 field seasons. In related studies in which these same strains were co-inoculated on barley, pea and navy bean under controlled conditions to evaluate the effect of the plant host, wild-type Q8r1-96 outcompeted Z30-97 on barley, both strains maintained similar population densities on navy bean, and surprisingly, the engineered strain displaced the wild type on pea (S.B. Bankhead, L.S. Thomashow and D.M. Weller, unpublished). The results indicate that the crop species modulates strain competitiveness and must be considered when assessing the potential fate of and risk posed by the release of recombinant strains into the environment.

Non-target effects of wild-type and genetically engineered PGPR Studies of the non-target effects of antibiotic-producing and non-producing PGPR introduced into the rhizosphere have considered the abundance and community structure of microorganisms that are closely related or unrelated to the introduced rhizobacteria, soil enzyme activities and available nutrients, microbial indicators such as rhizobia, protozoa and nematodes, and effects on the plant (review: Winding et al. 2004). Some of the best studies to date of the population dynamics and non-target effects of recombinant rhizobacteria have been conducted with *P. putida* WCS358r, modified to produce either PCA or DAPG (Glandorf et al. 2001; Leeflang et al. 2002; Viebahn et al. 2003). This work is especially notable because it was conducted in the field, PCA was shown to be produced in the rhizosphere by the recombinant strain, and both cultivation-dependent and cultivation-independent methods were employed to quantify non-target effects. The results indicated that the wild-type and recombinant strains both had transient effects on the composition of the rhizosphere fungal and bacterial microflora of wheat, and the effects of the recombinant strains sometimes were longer-lasting. Perhaps more importantly, the impact of the recombinant strains differed from year to year and study to study. These results, which mirror those of most other studies conducted under controlled and field conditions, are not so surprising given that WCS358r and other PGPR typically establish very high population densities immediately after inoculation, and then the densities decline (sometimes precipitously) over time and distance from the inoculum source. In addition, introduced rhizobacteria do not become uniformly distributed throughout the rhizosphere or among roots of the same or different plants. Collectively, studies of the non-target effects of wild-type and transgenic rhizobacteria indicate that while the bacteria have definite impacts on non-target bacterial, fungal and protozoan populations, the effects vary from study to study and are transient.

Engineering microbial populations and plant-microbe interactions

The influence of plant species

The plant genotype profoundly influences both the quantity and composition of indigenous microorganisms and the population dynamics of introduced rhizobacteria. This occurs because rhizosphere micro-

organisms are dependent for their growth on substrates liberated from the root and because rhizodeposition is largely under the genetic control of the plant. Numerous studies demonstrating this concept occur in the earlier literature (Miller et al. 1989). The classic studies conducted by Neal, Atkinson and Larson (Neal et al. 1970, 1973; Atkinson et al. 1975) utilizing chromosomal substitution lines of wheat demonstrated beautifully the impact of plant genotype on rhizosphere microflora. The wheat lines S-615 and Rescue, both of which were susceptible to common root rot, harbored larger populations of rhizosphere bacteria than did the root rot-resistant line Apex. Substitution of the chromosome pair 5B from Apex for its homologue in S-615 yielded the chromosomal substitution line S-A5B that was as resistant as Apex to root rot. Furthermore, the bacterial population in the rhizosphere of S-A5B was similar to that of Apex. In addition, the resistant lines had greater percentages of bacteria antagonistic to the common root rot pathogen than did the susceptible lines.

Lemanceau et al. (1995) isolated fluorescent pseudomonads from a silty loam soil and from the rhizosphere, rhizoplane or root interior of flax and tomato grown in that soil. Isolates were characterized on the basis of enzyme activities and their ability to utilize organic substrates. Numerical analysis of these characteristics was used to cluster the isolates, and the results indicated that the plant species had a selective influence on the populations. Isolates from flax and tomato were grouped into nine clusters. Flax isolates were distributed in six clusters, three of which were specific for flax. Tomato isolates also formed six clusters, two of which were specific for tomato. Furthermore, a much greater proportion of tomato isolates than flax isolates could assimilate inositol, ribose, saccharose, trehalose, erythritol, m-hydroxybenzoate, and 5-cetogluconate.

More recently, studies targeting DAPG-producing *P. fluorescens* (*phlD*$^+$) strains known for their PGPR activity have demonstrated that plant species and varieties differentially enrich and support populations (Bergsma-Vlami et al. 2005; De La Fuente et al. 2006; Mazzola et al. 2004) and genotypes (see Section "Take-all decline in Washington State: a case study"; Landa et al. 2002; Landa et al. 2006; Mazzola et al. 2004; Picard et al. 2004) of this specific group of *P. fluorescens*, and that DAPG accumulation in the rhizosphere also differs among plant hosts (Bergsma-Vlami et al. 2005). For example, wheat, sugar beet and potato grown in a Dutch soil supported greater DAPG production and population densities of *phlD*$^+$ isolates than did lily (Bergsma-Vlami et al. 2005). Notz et al. (2001) showed that DAPG accumulation by *P. fluorescens* CHA0 is correlated with expression of the DAPG biosynthesis gene *phlA*, and expression was significantly greater in the rhizosphere of monocots (maize and wheat) than dicots (bean and cucumber). They also observed differences in gene expression on six maize cultivars. Wheat varieties also differentially support *phlD*$^+$ populations, genotypes of *phlD*$^+$ strains, and DAPG accumulation on roots. For example, Mazzola et al. (2004) showed that the population densities of *phlD*$^+$ isolates from wheat varieties grown in apple orchard soils differed significantly. For instance, variety 'Lewjain' supported densities >10^5 CFU g^{-1} root, but no *phlD*$^+$ isolates were detected on 'Eltan.' Okubara and Bonsall (2008) quantified DAPG on roots of wheat varieties 'Tara,' 'Buchanan,' and 'Finley' inoculated with *P. fluorescens* Q8r1-96 (D genotype) or Q2-87 (B genotype) and grown on moist filter paper. Both strains colonized roots equally, but DAPG accumulation differed significantly between strains and among wheat varieties.

Natural engineering of microbial populations

Disease-suppressive soils provide some of the best examples in which plants protect themselves against soil-borne pathogens by "naturally engineering" the composition of rhizosphere microbial populations. As defined by Baker and Cook (1974), suppressive soils are those "in which the pathogen does not establish or persist, establishes but causes little or no damage, or establishes and causes disease for awhile but thereafter the disease is less important, although the pathogen may persist in the soil." In contrast, conducive (non-suppressive) soils are soils in which disease readily occurs. "General suppression," the aforementioned ability of essentially all soils to suppress the growth or activity of soilborne pathogens to a limited extent, is due to the activity of the total microbial biomass in soil competing with the pathogen and is not transferable between soils. "Specific suppression" is superimposed over the background of general suppression, is highly effective, is due to the activity of individual or select groups of microorganisms, and is transferable between soils (Weller

et al. 2002). Suppressive soils owe their activity to both general and specific suppression.

Suppressive soils are known for many soil-borne pathogens (Weller et al. 2002) and have been further categorized as "long-standing" or "induced" (Hornby 1983, 1998). Long-standing suppression is naturally associated with soil and is of unknown origin whereas induced suppression develops as a result of a cropping practice such as monoculture. Two well-known examples of suppressive soils are take-all decline of wheat (Weller et al. 2002; Hornby 1983; 1998) and scab decline of potato (Menzies 1959).

Take-all decline in Washington State: a case study Take-all, caused by *G. graminis* var. *tritici* (*Ggt*), generally develops at a soil pH of 5.5 to 8.5 and is most severe where wheat is grown under moist conditions, but it also is common under dryland conditions. As a result, take-all occurs throughout the world and is considered the most important root disease of wheat (Cook 2003; Freeman and Ward 2004). The pathogen survives saprophytically in dead roots, crowns, and tiller bases, the inoculum source for the next crop. Primary infection occurs with the growth of dark runner hyphae on the root surface. Hyaline hyphae penetrate the cortex and colonize the vascular tissue, causing characteristic black lesions. Runner hyphae continue to grow over the root surface, to other roots, and upward to the crown and stem bases. Early infection ultimately causes yellowing of lower leaves, stunting, and premature death of plants in patches. Wheat is highly susceptible to take-all but other Poaceae (barley, rye and triticale) also are infected (Cook 2003; Freeman and Ward 2004). Crop rotation and tillage are widely used as controls for take-all, but trends in modern cereal-based production systems are toward less tillage and two or three consecutive wheat crops, and these practices exacerbate the disease. No resistant commercial varieties exist and chemical controls are limited (Cook 2003; Paulitz et al. 2002). Take-all decline (TAD), the spontaneous decrease in take-all incidence and severity induced by continuous wheat or barley monoculture after a severe outbreak of the disease (Hornby 1998, Weller et al. 2002, 2007), also is widely used to manage take-all.

TAD follows a similar pattern worldwide, but local biotic and abiotic factors modulate the speed of its development and its robustness. Amongst these factors, soil physico-chemical properties such as manganese (Heckman et al. 2003) or ammonium concentrations (Sarniguet et al. 1991) may play a significant role. The number of continuous crops of wheat or barley required to initiate TAD varies considerably, but often ranges from four to six (Weller et al. 2002). It has been known for decades that TAD involves microbiological changes in the soil or rhizosphere that suppress the pathogen (Weller et al. 2007). The specific suppression associated with TAD is transferable to a conducive soil and is eliminated by soil pasteurization (60°C, 30 min) or fumigation, and by rotation with non-cereal crops (Hornby 1998; Weller et al. 2002). TAD effectively suppresses severe disease, but yearly fluctuations in its robustness are common. Several types of microorganisms have been studied as potential mechanisms of TAD suppressiveness (Hornby 1998) and beginning in the 1970s, antagonistic *Pseudomonas* spp. were implicated (Smiley 1979; Weller et al. 1988).

A series of studies of soils from irrigated and non-irrigated, and conventionally cultivated and direct-seeded continuous monoculture wheat fields throughout Washington State, U.S.A. (dating back to the late 1960s) resulted in the demonstration of a key role for DAPG-producing *P. fluorescens* in TAD (reviewed in Weller et al. 2007). For example, it was shown that DAPG producers colonized wheat roots from TAD soils at densities above the threshold (10^5 CFU g^{-1} root) (Raaijmakers and Weller 1998) required for take-all control, but were below the threshold or not detected on roots from conducive soils (Raaijmakers et al. 1997). Suppressiveness was lost when DAPG producers were eliminated from TAD soils by soil pasteurization, and adding TAD soil to conducive soil transferred suppressiveness and established populations of DAPG producers above the threshold required for disease control (Raaijmakers and Weller 1998). Introduction of DAPG producers into conducive soils rendered the soils suppressive (Raaijmakers and Weller 1998). Finally, DAPG was detected on roots of wheat grown in TAD soil but not conducive soil (Raaijmakers et al. 1999). The role of DAPG producers in TAD is not restricted to Washington State. DAPG-producing strains of *P. fluorescens* occurred above the threshold needed for take-all suppression on wheat grown in monoculture soils collected from major wheat growing regions of the U.S.A. (Landa et al. 2006; Weller et al. 2007), and De Souza et al. (2003) demonstrated that they also play a role in Dutch TAD soils.

Among worldwide collections of DAPG-producing strains of *P. fluorescens*, 22 genotypes (A-T, Pfy, Pfz) currently have been recognized, with many genotypes showing substantial endemicity while others such as A, D and F are more widely distributed. Several genotypes occur in TAD soils in Washington State, but the D genotype comprises 50–90% of all *phlD*$^{+}$ isolates. Genotype D isolates play the major role in Washington TAD soils because of their unique ability to colonize wheat and barley roots and maintain densities necessary for take-all suppression throughout the growing season and from year to year of monoculture (Raaijmakers and Weller 2001).

Of the root pathogens infecting Pacific Northwest wheat fields, *Ggt* is the most sensitive to DAPG (Allende-Molar 2006). Mazzola et al. (1995) screened isolates of *Ggt* from different countries and regions of the U.S.A., and found differential sensitivity to the antibiotic. In addition, they reported that isolates not inhibited by 3 μg ml^{-1} of DAPG were not suppressed by the DAPG-producing strain Q2-87 on wheat roots. A common question about the role of DAPG in take-all suppression is whether isolates of the pathogen with tolerance to the antibiotic become enriched in TAD fields as a result of exposure during wheat monoculture. Kwak et al. (2009) addressed this question by determining the sensitivity of *Ggt* isolated from TAD and non-TAD fields. For isolates from all fields, the 90% effective dose (ED_{90}) of DAPG ranged from 3.1 to 11.1 μg ml^{-1}. Sensitivity of isolates to the antibiotic was normally distributed in all fields and was not correlated with geographic origin or cropping history of the field, indicating that tolerance to DAPG does not develop in the take-all pathogen during monoculture. This study was the first to address the question of emergence of tolerance in a soil-borne pathogen to introduced or indigenous PGPR.

Soil amendment as a means of modifying microbial populations

Modification of the soil or rhizosphere microbial component is often the unintentional consequence of human activity. For instance, soil pollutants may drastically affect the composition of soil and plant-associated microflora (e.g. Colores et al. 2000; Siciliano et al. 2001; reviewed by Lynch 2002), and repeated cultivation of certain crop species can lead to the emergence of disease-suppressive soils (see above).

The development of new techniques in microbiology and microbial ecology has provided opportunities to modify the soil microbiota in a manner paralleling the selective “rhizosphere engineering” that occurs in Nature. For instance, the plant species *Calystegia sepium* (hedge bindweed), *Convolvulus arvensis* (morning glory), *Scopolia japonica* (scopolia) and *Morus alba* (white mulberry) all produce alkaloid calystegyns (Asano et al. 1994, 1996), and numerous calystegin-degrading bacteria (eg. *Sinorhizobium sp.*) have been identified in the rhizosphere of these plants (Tepfer et al. 1988). The microflora around the roots of some legume species is similarly influenced by root exudates. *Leucanea sp.* and *Mimosa sp.* plants produce the amino acid mimosine, which is toxic to many bacteria (Hammond 1995). Nitrogen-fixing *Rhizobiaceae* that inhabit the nodules on these plants have acquired the ability to degrade mimosine, a feature not found in non-colonizing *Rhizobiaceae* (Soedarjo et al. 1994). This example of mimosine “engineering” appears advantageous for both the plant and symbionts: the plants select beneficial, well-adapted nitrogen-fixing symbionts, and the symbionts are able to out-compete other microbes in the root environment (Fox and Borthakur 2001; Soedarjo and Borthakur 1996).

Several examples of microbial engineering have involved the application of chemical and biological amendments to the soil. Devliegher et al. (1995) selected bacterial communities able to degrade Igepal and di-octyl sulfosuccinate from soils amended with these molecules. Amongst the selected bacteria, a detergent-habituated PGPR *Pseudomonas* strain survived better in amended bulk and rhizosphere soils than in control soils. Amendments have also been used to favor plant growth. Though the exact mechanism was not fully understood, a cocktail of Triton X100, EDTA and a strain of *Sinorhizobium sp.* altered the soil microflora enough to improve the growth of *Brassica juncea* plants (Di Gregorio et al. 2006). In earlier series of studies, a strain of *P. fluorescens* was engineered to utilize salicylic acid as a source of carbon (Colbert et al. 1993a, b). Growth of this strain was enhanced in salicylate-amended bulk and rhizosphere soils but the magnitude of the response was sensitive to inoculum level, field site,

and soil depth. When dusted onto leaves, salicylate stimulated the epiphytic growth of similarly engineered pseudomonads (Wilson and Lindow 1995) including a strain with biological control activity against bacterial speck disease of tomato (Ji and Wilson 2003).

Bacterial strains capable of interfering with the pathogenicity of *Pectobacterium carotovorum* (formerly *Erwinia carotovora*; Whitehead et al. 2001; Barnard et al. 2007; Faure and Dessaux 2007) were selected by an amendment/enrichment-based approach. The virulence of *Pectobacterium* depends upon the production and sensing of quorum sensing (QS) N-acyl-homoserine-lactone (NAHL) molecules, the concentration of which parallels cell density. When a certain cell density (quorum) of the pathogen is reached, a threshold NAHL concentration is detected by a sensor protein (reviewed in Whitehead et al. 2001; Miller and Bassler 2001; Williams et al. 2007), which activates the synthesis of maceration enzymes such as cellulase, pectate lyase, etc. (review Faure and Dessaux 2007). Bacteria that naturally degrade NAHL have the potential to break this cycle, quenching the pathogenicity of *Pectobacterium*. Several candidates, including promising strains of *Rhodococcus erythropolis*, have been selected from soils amended with NAHL or NAHL analogues (Leadbetter and Greenberg 2000; Dong et al. 2002; Uroz et al. 2003; D'Angelo-Picard et al. 2005). Another strategy developed by Cirou et al. (2007) identified gamma-caprolactone and 4-heptanolide as potent growth stimulators of bacterial communities able to degrade NAHL and quench *Pectobacterium* pathogenicity in potato tuber assays. This ecological engineering approach is both elegant and environmentally friendly because the NAHL analogues, which are used as flavoring agents by the food industry, are degraded by the same bacterial community selected to degrade NAHL.

Plant genetic engineering as a tool to shape plant-associated microbial populations

The strategies described above exploit either natural processes or exogenous soil amendments to manipulate microbial populations. The following section describes alternative approaches which engineer plants to produce compounds that either modulate the growth of defined bacterial rhizosphere populations or modify their biochemical functions.

The opine model Numerous studies have generated transgenic plants that release xenotopic compounds into the rhizosphere. Xenotopic compounds are those that are not naturally present in a particular environment. The compounds targeted in these studies, the opines, are low molecular weight amino acid derivatives that occur in tumors induced by *Agrobacterium tumefaciens* (see Dessaux et al. 1998). Opines can be synthesized and released into the rhizosphere by plants transformed with one to three biosynthesis genes (Savka et al. 1996).

In one such experiment, bacteria colonizing the rhizosphere of *Lotus corniculatus* plants engineered to produce opines were compared with those colonizing near-isogenic wild-type plants (Oger et al. 1997). The population densities of culturable bacteria, agrobacteria, pseudomonads, sporulating, and thermotolerant bacteria were identical in the rhizospheres of the transgenic and control plants. However, the density of the bacterial community able to degrade opines was two to three orders of magnitude greater in the rhizosphere of the opine-producing plants than in that of wild-type plants. Furthermore, the size of this community was related to the magnitude of opine production by the transgenic plants (Oger et al. 2004). More subtle modifications also were noted. For instance, while the total density of all pseudomonads was unaltered by opine production, the proportion of those capable of degrading opines was greater in the rhizosphere of opine-producing plants than in that of wild-type plants. However, this shift in community composition depended upon the type of opines produced (Oger et al. 1997). When opine-producing plants were removed from the soil and replaced by wild-type plants, the population density of the opine-degrading microorganisms declined over time but remained higher than that in control experiments involving only wild-type plants (Oger et al. 2000). The opine-induced bias was observed in two different soils (a clay-rich and a sandy-loamy soil), and for three different plant species (*Lotus corniculatus*, *L. japonicus*, and *Solanum nigrum*), indicating that it was not specific for a single soil type or plant system (Mansouri et al. 2002).

These results support three important conclusions. Firstly they demonstrate that plant exudates directly

affect the composition of the rhizosphere microflora. Secondly, they indicate that the rhizosphere "bias" can be extended beyond the period of opine production, an interesting feature in terms of ecological engineering. Thirdly, they provide a precautionary observation that the composition of the rhizosphere microbial community is influenced by root exudation that occurred previously, as well as that in progress at the time of the analysis.

Other studies have investigated whether the opine approach could be used to favor the multiplication of single bacterial strains. Growth of an epiphytic strain of *Pseudomonas syringae* capable of degrading mannityl opines (Wilson et al. 1995) showed a modest 2-to 3-fold increase on the leaves of opine-producing as compared with control plants. In similar studies, growth of a rhizosphere strain of *Pseudomonas* sp. engineered to utilize certain opines was moderately enhanced on the roots of opine-producing plants (Savka and Farrand 1997). Several reasons may account for the limited bacterial growth stimulation observed in these experiments. Firstly, the strains investigated were already well adapted to either the leaf or the root environment. Second, only a single strain was investigated in each study, and competition with other organisms was limited (on the leaf surface) or non-existant (in sterilized soil). In spite of these issues, the opine model is a promising strategy for favoring the growth of a bacterial strain in the plant environment, mindful that factors other than favorable carbon allocation are likely to contribute to strain fitness (Savka et al. 2002).

The opine system from *Agrobacterium* is not the only one that can be used to select for specific plant-bacteria interactions. Other studies have attempted to engineer interactions based on the synthesis (by plants) and the degradation (by bacteria) of rhizopine (i.e., 3-O-methyl-*scyllo*-inosamine), a compound found in nitrogen-fixing nodules of legumes (Murphy et al. 1987). To this end, genes involved in the synthesis and degradation of rhizopine have been isolated from *Sinorhizobium meliloti* (Saint et al. 1993; Murphy et al. 1993). Three rhizopine biosynthesis genes were transformed into *Arabidopsis* and while all were expressed, no rhizopine was detected in any of the transgenic plants (McSpadden-Gardener et al. 1998). The four genes involved in rhizopine degradation also were cloned and tentatively expressed in various bacteria. These genes enabled strains of the *Rhizobiaceae,* but not other bacterial hosts including members of the alpha-proteobacteria, to degrade rhizopine (Rossbach et al. 1994; Rossbach, personal communication). Engineering plant-microbe interactions based on rhizopine metabolism appears to be more complex than anticipated. Nevertheless, since rhizopine-degrading strains are favoured in a rhizopine-rich environment (Gordon et al. 1996), the strategy is worth pursuing, especially because rhizopine could favor the growth of beneficial nitrogen-fixing bacteria.

The quorum quenching model As indicated above, damage caused by the plant pathogen *Pectobacterium* can be reduced by microorganisms that interfere with the QS NAHL signal essential to the pathogen's life cycle. Some workers have taken this concept a step further by modifying plants to degrade or alternatively, to synthesize NAHL signals. In one approach, plants were transformed with a bacterial lactonase gene, *aiiA,* enabling them to degrade NAHL molecules. The *aiiA* gene from *Bacillus sp.* (Dong et al. 2000, 2002) was transferred to potato (*Solanum tuberosum*) to generate transgenic lines that indeed inactivated NAHL (Dong et al. 2001). These lines were more tolerant—not to say resistant—to *Pectobacterium*, as no maceration symptoms were visible on tubers inoculated with the pathogen. Tobacco (*Nicotiana tabacum*) is a non-host species for *Pectobacterium*, so the pathogen only induces a localized hypersensitive reaction in response to the QS-dependent production of the toxic peptide harpin. Transgenic tobacco lines expressing *aiiA* did not exhibit this hypersensitive reaction when inoculated with the pathogen. Therefore, expression of AiiA by these plant species completely abolished the pathogenicity of *Pectobacterium* (Dong et al. 2001).

Other studies have modified plants to constitutively synthesize and release NAHLs so that damaging maceration enzymes are released prematurely from *Pectobacterium*. This benefits plants by enabling them to defend themselves more efficiently against limited numbers of pathogenic bacteria. NAHL synthase genes used in this way include *yenI* from *Yersinia enterocolitica* (Fray et al. 1999) and *expI* from *Pectobacterium* (Mae et al. 2001). Tobacco plants transformed with *yenI* targeted for expression in the chloroplast were able to complement the biocontrol ability of *Pseudomonas aureofaciens*

strains defective in NAHL synthesis, indicating that crosstalk indeed occurred between the plant and bacteria (Fray et al. 1999). Reduced hypersensitivity to *Pectobacterium* was seen on non-host tobacco plants producing NAHL (Mae et al. 2001), but increased virulence was reported upon inoculation of potato, a host plant, with the pathogen (Toth et al. 2004).

Collectively, these studies suggest that while quorum quenching strategies incorporating genetically modified plants are conceivable (reviews: Fray 2002; Zhang and Dong 2004), strategies relying on signal degradation appear to be more efficient at present than those involving signal overproduction. Interestingly, the impact of signal overproduction on microbial populations and QS pathways appears limited or non-existent (D'Angelo-Picard et al. 2004). Whether this is true for the signal degradation approach remains to be determined, especially considering that several bacterial functions beneficial to plants (e.g. production of antifungal compounds, root colonisation) rely on QS regulation (e.g. Chin-A-Woeng et al. 2001; Maddula et al. 2006).

Future research orientations

Research to date has shown that the rhizosphere can be engineered through appropriate selection of crop species and varieties, by the introduction of microorganisms or soil amendments, and by genetic modification of plant and microbial biological activities. The emergence of molecular techniques now allows the direct manipulation of genes that influence rhizosphere functions, and continuing advances in biotechnology ensure more progress for the future. High throughput and "omics" techniques further make it possible to screen and analyse large and complex microbial communities in the soil. Genomics has given rise to metagenomics, an approach that will benefit from the remarkable development of mass sequencing procedures and which will enable us to explore the microbial diversity of the rhizosphere more rapidly and in greater detail.

While progress in a number of avenues has been encouraging, our ability to reliably and predictably engineer the rhizosphere remains a challenge. One major scientific obstacle impedes further progress: a detailed understanding of the complex chemical and biological interactions that occur in this zone. The complexity of rhizosphere chemistry and biology continues to present a multitude of "black boxes" to our understanding. Fundamental issues concerning microbial abundance and diversity in the soil remain unresolved. The role of predation and the phenomenon of resilience are poorly understood. The complex relationships between the structure of microbial communities and their function make attempts to predict and manipulate their ecology very difficult, and will certainly remain for several more years the touch stone of rhizosphere ecology.

Societal lack of acceptance of the emerging technology remains a stumbling block as well. Though relatively limited in Canada, China, and the USA, the controversy surrounding genetically modified organisms is strong in Europe and parts of South America, even amongst members of the scientific community. Indeed, trials of genetically-modified organisms, including plants that could benefit the environment, are extremely unlikely in Italy, Germany and France in the near future. The situation might improve if more restrictive regulations for the use of agrochemicals are implemented. However, a movement towards banning of agrochemicals exists. This should encourage the development of more ecologically friendly alternatives, such as non polluting amendments, novel natural biocontrol agents, and —possibly—genetically modified options. The demands of an ever-increasing world population conjugated to a risk of reduction of arable surface (for instance in fertile river deltas, or low lands) will only make these needs more pressing. It is imperative that scientists continue their work so that a more receptive public of the future can benefit from safe, sustainable and environmentally sound agricultural practices.

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