Enhancement of Plant-Microbe Interactions Using a Rhizosphere Metabolomics-Driven Approach and Its Application in the Removal of Polychlorinated Biphenyls

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Persistent organic pollutants, such as polychlorinated biphenyls (PCBs), are a global problem. We demonstrate enhanced depletion of PCBs using root-associated microbes, which can use plant secondary metabolites, such as phenylpropanoids. Using a “rhizosphere metabolomics” approach, we show that phenylpropanoids constitute 84% of the secondary metabolites exuded from Arabidopsis roots. Phenylpropanoid-utilizing microbes are more competitive and are able to grow at least 100-fold better than their auxotrophic mutants on roots of plants that are able to synthesize or overproduce phenylpropanoids, such as flavonoids. Better colonization of the phenylpropanoid-utilizing strain in a gnotobiotic system on the roots of flavonoid-producing plants leads to almost 90% removal of PCBs in a 28-d period. Our work complements previous approaches to engineer soil microbial populations based on opines produced by transgenic plants and used by microbes carrying opine metabolism genes. The current approach based on plant natural products can be applied to contaminated soils with pre-existing vegetation. This strategy is also likely to be applicable to improving the competitive abilities of biocontrol and biofertilization strains.

The establishment of large numbers of metabolically active populations of beneficial soil microbes is critical for the success of several environmental remediation and agricultural practices (Metting, 1992). These microorganisms are successful in getting established in the soil ecosystem due to their high adaptability in a wide variety of environments, their faster growth rate, and their biochemical versatility to metabolize a variety of natural and xenobiotic chemicals. Majority of the microbial population found in the soil is associated with the plant roots, where their numbers can reach up to $10^7$ to $10^{12}$ per gram of soil (Whipps, 1990), leading to a biomass equivalent to 500 kg ha$^{-1}$ (Metting, 1992). This abundance in vegetated soils is due to the availability of nutrients via plant root exudation (Brimecombe, 2001), which can stimulate microbial growth in the immediate vicinity of the roots (a region also known as the “rhizosphere”). Hence, the rhizosphere has been promoted as the ideal site to modify microbial populations (“rhizoengineering”) to suite various applications in the soil (O’Connell, 1996).

Rhizoengineering has been demonstrated successfully by devising strategies that favored the growth of the targeted microbes that possessed the ability to metabolize exotic nutrients exuded by plants (Lugtenberg, 2001). It was thus possible to create a nutritional bias that can be especially successful in identifying microbial populations due to the general nutrient-limiting conditions in the rhizosphere. One of the earliest successes in rhizoengineering was based on favorably partitioning the exotic nutrient, opines, which were produced by the transgenic plants (Oger, 1997; Savka and Farrand, 1997). This led to the improved and competitive growth of the metabolizing strains in comparison with the microbes unable to metabolize opines. Success of the above approach is contingent upon the general nutrient-limiting conditions that prevail in the rhizosphere. Studies have shown that such conditions are prevalent in the rhizosphere of many plant species, which leads to starvation of root-associated bacterial (“rhizobacterial”) populations (Normander, 1999).

We reasoned that it might be possible to rhizoengineer by partitioning the nutrients with complex structures produced naturally by the wild-type plants without having to rely on transgenic plants, whose establishment in contaminated soils may pose practical limitations. We have therefore investigated the role of the natural products with highly complex structures produced naturally by the wild-type plants without having to rely on transgenic plants, whose establishment in contaminated soils may pose practical limitations.
structures, the secondary metabolites, and we have carried out profiling of the root exudates to identify targeted compounds for creating the nutritional bias. For convenience, we have referred to this as “rhizosphere metabolomics.” We have studied the utility of these exotic natural products for rhizoengineering purposes by establishing an Arabidopsis-Pseudomonas spp. rhizosphere model based upon the following considerations: (a) the identification of 125 secondary metabolites, including phenylpropanoids, in the root exudates of Arabidopsis; (b) the availability of near-isogenic lines of Arabidopsis mutants in secondary metabolism; (c) a Pseudomonas spp. (plant growth promoting rhizobacteria) strain (designated Pp-wt) that can efficiently colonize Arabidopsis roots, use phenylpropanoids, and independently degrade a major class of persistent organic pollutants, namely, the polychlorinated biphenyls (PCBs); and (d) the availability of a phenylpropanoid auxotrophic mutant (designated Pp-mut) of the above strain whose colonization and PCB-degradation traits are unaffected with respect to the Pp-wt. The three properties of the Pp-wt strain were critical in establishing this experimental model.

Using the above model, we show that plant secondary metabolites are exuded in sufficient amounts to establish a rhizosphere biased to a rhizobacterial strain that is capable of metabolizing phenylpropanoids. In this regard, we also apply the rhizoengineering method for significantly enhanced removal of PCBs, which rank sixth in the list of hazardous substances (Agency for Toxic Substances and Disease Registry, 2001), and globally, PCBs are second only to benzene among the organic pollutants. The world production of PCB until 1988 was estimated to be 1.2 million tons. Of this, 31% is thought to be already in the environment, 4% has been destroyed, and 65% is still in use or in storage. Thus, more than twice the amount now in the environment is still available for future contamination (National Environment Indicators, Series, 1996). PCBs are additionally hazardous due to their rapid movement in the ecosystem, their high persistence, their ability to accumulate in the food chain, and their toxicity to various organisms. Hence, approaches to enhance removal of PCBs and other contaminants are going to be critical for alleviating this problem in the near future. Using the proposed approach here, already vegetated soils can be targeted for rhizoengineering. This approach, therefore, complements the previous ones requiring establishment of transgenic plants (Oger, 1997; Savka and Farrand, 1997).

RESULTS AND DISCUSSION

Rhizosphere Metabolomics

Identification and quantitation of the array of phenylpropanoid compounds present in the root exudates is a prerequisite for choosing target compounds that can be used for creating a nutritional bias in the rhizosphere. We have, therefore, analyzed the proportions of phenolic compounds in the root exudates of Arabidopsis to focus on the secondary metabolites. Phenylpropanoid compounds including lignins, coumarins, flavonoids, aurones, sinapates, and anthocyanins were identified as the most abundant class, which includes what we have used for creating a nutritional bias. Quantitative analyses of root exudates showed the total organic carbon content to be in the range of 14 ng mL$^{-1}$ root exudate. Total phenolics constitute 7.0 ± 0.1 ng mg$^{-1}$ root fresh weight, which in turn constitutes 50% of the total organic carbon content. Rhizosphere metabolomics using a combination of reverse phase (RP)-HPLC and electron spray ionization mass spectrometry (ESI/MS) analyses showed flavonoids as the major class (37%), followed by lignins (22%) in the root exudates (Fig. 1a). We have identified 149 hydrophobic compounds consisting of 125 secondary metabolites in the root exudates; this is the most comprehensive analysis of the rhizosphere metabolites for a single plant species to date. Of the 125 secondary metabolites, 76% belonged to the phenylpropanoid class of compounds (Fig. 1a). To have an experimental set up of Arabidopsis lines capable of accumulating phenylpropanoids to different levels, we have chosen mutants affected in the flavonoid biosynthetic pathway because these compounds formed the largest proportions (64%) of the phenylpropanoids. The mutants chosen were tt4 (Pelletier, 1999), ttg (Walker, 1999), and tt8 (Nesi, 2000) in the isogenic Landsberg erecta (Ler) ecotype background. Metabolic profiles of root exudates from the wild type and the three mutants showed a widespread effect of the mutations on secondary metabolism. The mutant tt4 lacks the structural gene for chalcone synthase and does not accumulate flavonoids (Pelletier, 1999; Fig. 1, b and d). However, the exudates from tt4 roots had an abundance of several other phenylpropanoid compounds. Interestingly, the mutant ttg (ttg gene encodes a WD40-repeat protein; Walker, 1999) accumulates both flavonoids and their conjugates to higher amounts in the roots. The mutant tt8 (tt8 gene encodes a basic helix-loop-helix type regulator; Nesi, 2000) largely accumulates aglycones of flavonoids (Pelletier, 1999; this study). Although, the three mutants are known to be affected in flavonoid metabolism, rhizosphere metabolomics revealed that several other phenylpropanoids are additionally affected (see supplementary information, which can be viewed at www.plantphysiol.org). For example, the following seven compounds were higher in tt8 with respect to the levels in ttg; numbers in parenthesis represents X-fold higher levels: dihydromyricetin (9.25), 3-O acetyl-4’-S’, 7 tri O-methyl kaempferol (8.36), quercetin 3-rhamnosylglucoside (7.41), coumaric acid (7.78), sinapoyl tetra acetyl Glc (16.44), leucocyanidin (5.10), and leucodelphinidin (8.67).
Similarly, there were another seven phenylpropanoid compounds that were present at higher levels in \textit{ttg} than in \textit{tt8} plants: naringenin rhamnoside (22.30), pyro-Glu (6.16), 3,4-dihydroxybenzoic acid (8.22), ferulic acid (6.46), syringic acid (12.35), cyanidin (6.16), and methyl IAA-Glc (10.29).

Comprehensive profiling of secondary metabolites in the rhizosphere showed an abundance of phenyl-
propanoids in the exuded compounds and significant qualitative differences of several compounds in the four lines studied (Fig. 1c; supplementary information). Interestingly, in tt8 and ttg, the overaccumulation of quercetin, which is the major flavonoid of Arabidopsis, is not significantly different in the root exudates as it is within the roots (Fig. 1d). Quercetin is found in nanomole amounts in the root tissue, whereas it is present in almost picomole amounts in the root exudates. It is noteworthy that although a minor fraction of the flavonoids is exuded (Fig. 1d), it forms a high proportion (37%) of the secondary metabolites in the root exudate (Fig. 1a). On the basis of the above results, phenylpropanoids meet several criteria outlined earlier for serving as target nutrients for rhizoengineering: They are exuded by all plants, they are abundant in the rhizosphere, and they have complicated structures to qualify as being “unusual” nutrients for the rhizobacteria so as not to be easily metabolized by most rhizobacteria.

Rhizoengineering Based on Phenylpropanoid Compounds

To design a suitable plant-microbe pair for testing the efficiency of rhizoengineering, we set up an experimental model using the Arabidopsis wild-type and flavonoid metabolic mutant lines exuding different amounts of phenylpropanoid compounds, together with an efficient rhizocolonizing strain of Pseudomonas spp. (utilizer and nonutilizer strains for phenylpropanoids). The Pseudomonas putida PML2 (designated as Pp-wt here; Pillai and Swarup, 2002) was specially chosen for its ability to utilize various phenylpropanoid compounds, which form the majority of the exuded secondary metabolites (Fig. 1a). To study the effect of phenylpropanoid utilization on colonization and competition abilities of rhizobacterial strain, an auxotrophic mutant (Pp-mut) derived from Pp-wt was used. Over a 28-d period, Pp-wt consistently better colonized the roots of the flavonoid-producing Arabidopsis genotypes (Ler, tt8, and ttg) than the roots of flavonoid null mutant Pp-mut (Fig. 2a). On the roots of the three flavonoid-producing plants, higher colonization levels were clearly visible with green fluorescent protein (gfp)-tagged Pp-wt cells (data not shown). Occurrence of cells adjacent to each other in groups of two or more indicated that active cell division was taking place on the root (Bloemberg, 1997), validating a successful colonization. As previously reported, colonizing bacteria were present mainly along the furrows of the root epidermal cells surface (Bloemberg, 1997). On the basis of plate counts, there were no significant differences in the colonizing ability of Pp-wt on roots of the three flavonoid-producing Arabidopsis lines. However, slightly higher colonization levels by the gfp-tagged Pp-wt were seen on the flavonoid-overproducing strains ttg and tt8. This was more evident on the upper portions of the roots, from where higher exudation has been reported (Lugtenberg, 2001). Although the population of Pp-wt increased by almost two orders of magnitude on these flavonoid-producing lines (Ler, ttg, and tt8), there was no significant colonization by Pp-wt on the roots of tt4 plants, which do not produce flavonoids (Fig. 2a). In agreement with results based on plate counts, confocal microscopy also confirmed lower colonization levels of gfp-tagged Pp-wt on the roots of tt4 plants (data not shown). In comparison, the phenylpropanoid-auxotrophic mutant strain (Pp-mut) was unable to colonize any of the four Arabidopsis lines used in this study (Fig. 2b). These differences in the colonization ability of the wild-type and auxotrophic mutant Pseudomonas spp. strains on the four Arabidopsis lines therefore showed that the
presence of higher levels of phenylpropanoids led to higher levels of bacterial populations. The phenylpropanoid-utilizer strain, therefore clearly showed a nutritional advantage on the flavonoid-exuding plants.

Next, we studied the abilities of the phenylpropanoid-utilizer strain and its auxotrophic mutant to compete for colonization on the four Arabidopsis lines. In the presence of Pp-mut, the Pp-wt strain was consistently able to better colonize the three flavonoid-producing Arabidopsis lines wild type, tt8, and ttg. On these Arabidopsis lines, Pp-wt showed higher growth levels than Pp-mut by log_{10}1 to 1.5 units, clearly indicating a better competitive ability of Pp-wt over Pp-mut. This higher colonization ability of Pp-wt in a competitive situation diminished by 28 d after germination in the wild-type Arabidopsis plants (Fig. 3a), whereas such an advantage persisted on tt8 and ttg plants (Fig. 3, c and d), which exuded double the amount of flavonoids (Fig. 1d). The decrease in colonization of wild-type Arabidopsis roots after 21 d could be due to exhausted carbon source once the bacterial population reached its maximal size, whereas the growth of bacteria in the rhizosphere of the plant mutants, which secrete double amount of root exudates (Fig. 1d), could be maintained for a longer time. Alternatively, the decline after 21 d of the bacterial growth on wild-type roots could be due to a rapid decrease in the phenylpropanoid exudation from the roots of wild-type plants in comparison with that from the phenylpropanoid-overproducing mutants.

Comparison of the growth of Pp-mut strain in colonization (Fig. 2b) and competition (Fig. 3b) showed that it was better able to colonize roots of flavonoid-producing plants after mixed inoculations. The presence of Pp-wt cells somehow improved the growth of Pp-mut strain slightly albeit for a short period. These observations suggested some form of cross feeding, perhaps of metabolic intermediates, as reported in several other cases of mixed inoculations (Dekkers, 2000).

**Rhizoengineering-Based Improvement of PCB Removal**

The last set of experiments address the question of whether rhizoengineering based on plant secondary metabolites can lead to an improvement in the removal of persistent organic pollutants, such as PCBs, from soils. Although PCB-degrading bacteria are found ubiquitously in the environment, a majority of them are inefficient in degrading PCBs (Donnelly, 1994). Major cause for this seems to be the lack of sustaining nutrients in the near-starvation conditions found in the soils, including the rhizosphere (Normander, 1999). Hence, the primary challenge for successful bioremediation of PCB-contaminated soil is to devise methods to encourage the growth (leading to more efficient PCB-removal) of a select species of microbes, which either are indigenous to PCB-contaminated sites or are introduced to these sites. Hence, for our present studies, the dual properties of the Pp-wt strain to use phenylpropanoids as nutritional sources (Pillai and Swarup, 2002) and its ability to degrade PCBs (Fig. 4) were a key element for testing our hypothesis. Liquid cultures of Pp-wt containing three PCBs, Aroclor, 4Cl-PCB, and 2Cl-PCB (53 μM each), allowed maximal microbial growth to be achieved in 96 to 120 h. Both Pp-wt and Pp-mut

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**Figure 3.** Competition experiments using Arabidopsis-Pseudomonas spp. model. Growth is shown for the phenylpropanoid utilizor (Pp-wt) and the auxotrophic mutant (Pp-mut) strains following mixed inoculations on seeds of Arabidopsis wild type and three flavonoid mutants (tt4, tt8, and ttg). Bacterial counts were determined as described in Figure 2 legend. Five randomly chosen plants were used as replicates for each sample. Vertical bars represent s.e. Experiments were repeated at least three times.
had comparable growth in the different PCBs used in the study (data not shown). Depletion was quantified for 2Cl-PCB and 4Cl-PCBs only because Aroclor is an undefined mixture of several constituents. Pp-wt strain was able to deplete 4Cl-PCB almost by 90%, whereas it was able to deplete 2Cl-PCB by almost 30% (Fig. 4). Higher depletion rate of 4Cl-PCB paralleled the higher growth of Pp-wt in this PCB compared with that of 2Cl-PCB. A gnotobiotic system (Simons, 1996) was set up, which consisted of four canonical plant-microbe combinations, all grown in the presence of 53 µM 2Cl-PCB. Sand was used instead of soil because PCBs are known to bind irreversibly to soil particles, thereby reducing the bioavailability. We established that the plant genotypes (data not shown) as well as the microbial strains (Fig. 4) are not adversely affected in growth at 53 µM PCB concentration used in these studies. Bacterial counts and PCB depletion studies were done (a) using sand adhering to the roots, and (b) using the remaining sand in the tube (Fig. 5). Uninoculated controls showed 98.6% and 98.1% of PCBs remaining at 7 and 14 d, respectively. This indicated negligible natural degradation of PCBs during the course of the experiment. Microbial populations showed growth between 1 and 2 log_{10} units in the four combinations of PCBs.
inoculations studied. As expected, microbial populations were higher near the plant roots (Fig. 5c). In all cases studied, significant amounts of PCB depletion were observed. The least amount of PCB depletion was seen in the case of the flavonoid null mutant \(tt4\) supporting the growth of either of the two *Pseudomonas* spp. strains (Fig. 5b), which is expected based on results from the colonization studies. In the total soil, an average depletion of almost 50% of PCB was observed after the microbial populations increased by 1 log\(_{10}\) unit. Hence, a small increase in microbial population could stimulate a significant amount of PCB depletion. Near the roots (Fig. 5c), the same plant genotype supported a higher increase of 2.1 log\(_{10}\) units in the two microbial populations. Concomitantly, there was almost a 70% depletion of the PCB. The most remarkable depletion was achieved by Pp-wt colonizing the wild-type Arabidopsis plant roots. In the region surrounding these roots, more than 90% of PCB was removed within 2 weeks. In the same period, microbial population increased by 2 log\(_{10}\) units. Pp-mut strain shows a slightly higher growth and PCB-depletion when adhering to the roots of wild-type plants as compared with those of \(tt4\) plants. This could perhaps be due to altered metabolism in both plants and microbes used in such a way as to provide a slight nutritional advantage to Pp-mut strain on the roots of wild-type plants.

In summary, a small increase in population (10- to 100-fold) could lead to a significant depletion of PCB present in the system. Pp-wt strain degraded 90% of PCBs on wild-type plants as compared with only 60% by the Pp-mut strain. Rhizoenhancement-based PCB depletion is, therefore, clearly evident in the gnotobiotic system used in our studies. Wider application of this rhizoenhancement approach is not limited by the strain used in the study. Microbes that process such dual properties of degradation of natural product and pollutants by soil microbes have been previously reported (Donnelly, 1994) and can be directly used for rhizoenhancement purposes. The approach shown here can be applied to enhance root colonization of those microbes, which may have additional pollutant degradation properties such as polyaromatic hydrocarbons (Kuiper, 2001). It is tempting to speculate that under field conditions, the strains targeted for rhizoenhancement may face competition by some resident microbes capable of phenylpropanoid utilization. However, such competition is likely to be offset by the number of introduced microbes to be used in rhizoenhancement. In addition, compared with Arabidopsis used in these studies, which has small root biomass and correspondingly lower exudate levels, plants in the field situation with higher root biomass are likely to have higher amounts of exudates leading to higher microbial colonization levels and thus to improved removal of pollutants. The strategy of enhancing the soil microbial populations using natural secondary metabolites exuded by wild-type plants could therefore be applied to the removal of many classes of pollutants in vegetated soils. In cases where the pollutant-degrading microbes are not known to use secondary metabolites (Donnelly, 1994), such property could be introduced into them using genetic-engineering methods. This strategy is also likely to be applicable for improving competitive abilities of biocontrol and biofertilization strains.

**MATERIALS AND METHODS**

**Bacteria and Plants**

Seeds of Arabidopsis plants were obtained from the Nottingham Arabidopsis Stock Center (UK). The *Pseudomonas putida* (Pillai and Swarup, 2002) PML2 strain (Pp-wt) is capable of using phenylpropanoids such as flavonoids (Pillai and Swarup, 2002), lignins, sinapates, coumarins, and indole compounds (this study), whereas *Flav1-1* (Pp-mut) is an auxotrophic mutant of PML2, which cannot use any of the above phenylpropanoids as a sole carbon source (Pillai and Swarup, 2002).

**Rhizosphere Metabolomics**

Surface-sterilized Arabidopsis seeds (300) were germinated on 0.8% (w/v) water agar plates that were kept in an inverted position to allow aeroponic-like growth of the roots. Root exudates were harvested by submerging the roots of 20-d-old plants in 2 mL of water for 1 h. RP-HPLC and ESI/MS were previously described (Pillai and Swarup, 2002). Quantitative analysis of the quercetin aglycone was carried out using SIM mode of the deprotonated molecule peak for acid-hydrolyzed root exudates. Intensity values obtained from the ESI/MS analysis for the individual compounds were plotted using function of the Mat Lab statistical package. The phenolic compounds in the root exudates were concentrated using disposable 3-mL LC-18 SPE tubes (Supelco Inc, Bellefonte, PA). Rhizosphere metabolomics are presented in Figure 1c in the same scale for wild type and the three mutants, where position on the horizontal axis represents different secondary metabolites, as listed in the supplementary information.

**Bacterization, Root Colonization, and Competition Assays**

Bacterization was carried out by treating 50 surface-sterilized seeds per milliliter of resuspended bacteria (8 × 10⁶ colony forming units mL⁻¹) for 15 to 20 min at 28°C, which led to an establishment of 100 colony forming units per strain for each bacterial strain per seed. For competition studies, cells of Pp-wt and Pp-mut strains were mixed in equal proportions. Plants were grown in Magenta GA-7 boxes in a growth chamber maintained at 25°C and 70% relative humidity with 16 h of daylight. Fifty bacterized seeds were planted in 45 g of sterile potting mix (peat:vermiculate:acid washed sand, 1:1:1) in Magenta GA-7 boxes consisting of one replicate, whereas five such replicates were used for each treatment. Samples consisting of eight plants per replication were taken under axenic conditions at weekly intervals after sowing. Loosely adherent soil from roots was gently removed, and roots were carefully separated from the plants and macerated in 0.5 mL of 0.9% (w/v) NaCl. Viable counts of the inoculated plants were determined by dilution plating on Luria-Bertani agar plates supplemented with appropriate antibiotics. For each data point, means were calculated after log\(_{10}\) transformation of the total bacterial count and the experiments were repeated three times.

**Confocal Microscopy**

Wild-type *Pseudomonas* spp. strain was tagged with a highly stable, broad host range vector pSMC2 that expresses gfp (Bloemberg, 1997). The growth characteristics of the gfp-tagged Pp-wt (Pp-wt/gfp) were identical to that of the parental Pp-wt strain. Secondary roots were viewed for the presence of Pp-wt/gfp cells using a laser scanning confocal microscope (LSM 410, Zeiss, Germany).
Welwyn Garden City, UK) with an excitation 488-nm wavelength and detection of the emission at 520 nm, respectively.

PCB Depletion Studies

Three PCB compounds, 2Cl-biphenyl, 4Cl-biphenyl (Riedel-de Haen, Germany), and Aroclor 1254 (Supelco, Bellefonte, PA), in 100 mg mL⁻¹ acetone were used at 53 µM final concentrations in liquid cultures or in a gnotobiotic system setup according to Simons et al. (1996) with minor modifications. Aroclor depletion was not quantified because it is an undefined mixture of PCBs. Acid-washed sand (10 g; GPR, BDH Laboratory Supplies, Poole, UK) was used in glass test tubes because PCBs are known to bind irreversibly/strongly to both soil and plastic. Fifteen to 20 seeds were sown per test tube, and five such replicates were set up per treatment for each time point. The experiments were repeated three times.

PCB Extraction and GC Analysis

PCBs from soil samples (Mohn, 1997) and cultures (Mackova, 1997) were extracted. Sample analysis (Gill, 1996) was carried out using GC-MS (QP5050 gas chromatograph-mass spectrometer, Shimadzu, Tokyo) equipped with DB-5 fused silica capillary column (30-m × 0.32-mm i.d.; film thickness, 0.25 µm; J&W Scientific, Folsom, CA). Standards and samples were analyzed in SIM mode for quantitative analysis. Total PCB removal was calculated using three PCB compounds as calibration standards.

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