Microbial Ecology

Exploiting New Systems-Based Strategies to Elucidate Plant-Bacterial Interactions in the Rhizosphere

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Abstract

The rhizosphere is the site of intense interactions between plant, bacterial, and fungal partners. In plant-bacterial interactions, signal molecules exuded by the plant affect both primary initiation and subsequent behavior of the bacteria in complex beneficial associations such as biocontrol. However, despite this general acceptance that plant-root exudates have an effect on the resident bacterial populations, very little is still known about the influence of these signals on bacterial gene expression and the roles of genes found to have altered expression in plant-microbial interactions. Analysis of the rhizospheric communities incorporating both established techniques, and recently developed "omic technologies" can now facilitate investigations into the molecular basis underpinning the establishment of beneficial plant-microbial interactomes in the rhizosphere. The understanding of these signaling processes, and the functions they regulate, is fundamental to understanding the basis of beneficial microbial–plant interactions, to overcoming existing limitations, and to designing improved strategies for the development of novel *Pseudomonas* biocontrol strains.

Introduction

Modern agriculture is heavily dependent on the application of chemical inputs, including fertilizers and pesticides. Because of concerns regarding both human health and environmental protection, viable alternatives to these chemicals are being sought. It has been long recognized that many naturally occurring rhizospheric bacteria and fungi are antagonistic toward crop pathogens and, as a result, may offer a viable substitute to the use of these chemicals [10]. Of particular interest are the soil-borne Pseudomonads, which can be utilized in low-input sustainable agriculture applications such as biocontrol (reviewed in [66]). Indeed, *Pseudomonas* strains that produce 2,4-diacetylphloroglucinol (PHL) have been shown to induce systemic disease resistance in plants [63]. Production of PHL is governed at the transcriptional and posttranscriptional level and analysis of these complex regulatory networks is ongoing [1, 2, 11]. Despite advances, there are still limitations in the development of biocontrol technology for widespread use in agriculture. Although many strains show good performance in specific trials, this is often not translated into consistent, effective biocontrol in diverse field situations. These microbes do not live in isolation, and very little is known about the complex interactions that occur between plant, fungal, and bacterial communities in the rhizosphere. An understanding of these interactions and subsequent exchange of signals between these communities could offer information for the development of new strategies to combat disease or to promote those interactions of benefit to the eukaryotic partner [34]. It is now generally accepted that extracellular signals from the plant can influence the behavior and structure of bacterial communities in the rhizosphere. Classically established molecular methods such as denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) offered valuable insights into the genetic diversity of these rhizospheric microbial populations [36]. However, it is now recognized that the presence of a bacterial strain does not necessarily indicate that it is metabolically active in the rhizosphere. Analysis of populations that are metabolically or functionally active can now be performed using stable isotope probing (SIP), and this is facilitating the task of elucidating the key functions performed by microorganisms in the rhizosphere.

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Despite these advances, little is still known about the influence of plant signals on bacterial gene expression.

In relation to gene function associated with plant–microbe interactions, established genetic tools such as *In Vivo* Expression Technology (IVET) permit the identification of genes specifically up-regulated in the rhizosphere. However, based on some limitations associated with this technique, it is complemented by more powerful genetic tools and strategies. The recent publication of complete genome sequences for several *Pseudomonas* strains including: *P. aeruginosa* PAO1 [56], http://www.pseudomonas.com, *P. putida* KT2440 [39], http://www.tigr.org, and *P. syringae* pv tomato DC3000 [9], http://www.tigr.org, as well as recent advances in the application of "omic technologies" such as genomics, proteomics, and metabolomics, offers new approaches that could provide new insights into the molecular basis underpinning the establishment of beneficial plant-microbial "interactomes" in the rhizosphere. Indeed, these techniques, each offering their own level of complexity, combined into an integrated approach will allow the study of the rhizosphere as a system in a more complete and holistic manner. Figure 1 shows how the incorporation of these technologies in an integrated approach can offer a greater understanding of these plant-bacterial interactions.

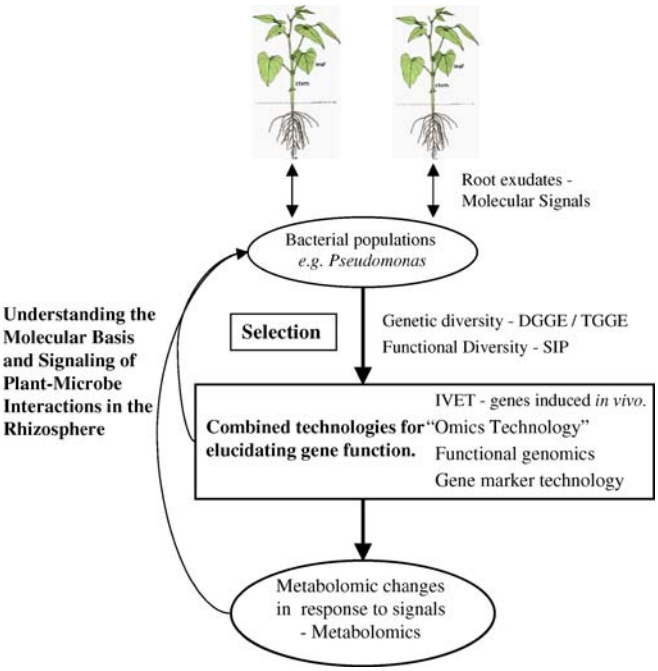

Figure 1. Integrated approach for the analysis of the microbial-host interactions in the rhizosphere. Integration of techniques for the analysis of plant–microbe interactions can give a more accurate account of what is occurring at the gene level in plant–microbe associations.

Importance of Culture-independent Techniques for Studying Plant–Microbe Interactions

It is well established that different plant species can select for genetically distinct microbial populations in the rhizosphere [24, 55]. We have also established that different plant varieties can select for genetically distinct populations of resident culturable fluorescent pseudomonads in the rhizosphere [57].

Classical studies and analysis of plant-bacterial interactions focused primarily on how plants and their derived signals influence the genetic diversity of culturable microbial populations in the rhizosphere. This analysis has been hampered because only 1% of the rhizosphere population is readily culturable. The advent of culture-independent methods such as DGGE and TGGE has offered scientists the ability to study the genetic diversity of the microbial community in the rhizosphere. For the first time, a comprehensive picture of how plant hosts influenced the genetic diversity of a microbial community could be assessed. It has become apparent, however, that limitations still remain with some of these technologies. There are issues regarding sensitivity as well as a tendency for more abundant groups of microbes to bias the outcome of the detection analysis. Ribosomal RNA-targeted oligonucleotide microarrays (PhyloChips) provide a hybridization format that allows for the detection of identified microbes of interest with identical levels of specificity. Once created, a PhyloChip allows for a greater sample throughput and comparable analysis of complex microbial communities [25]. The value of utilizing the PhyloChip however, is dependent on the suitability of the available probe set for any given environmental niche. The application of these techniques in rhizospheric microbiology along with SIP should offer increased information regarding the population dynamics of differing rhizospheric environments.

Assessing the Influence of Plant-Host on Microbial Functional Diversity Using SIP Technology. Several new methodologies and approaches have emerged that allow this key question to be addressed. A very promising approach for the analysis of bacterial functional diversity is SIP. This developing technology can now permit the measurement of metabolic activity *in situ*. It has radically changed the focus of study from that of analyzing the effects of plant signals on general microbial diversity in the rhizosphere toward understanding the effects these signals may have on the metabolically active and potential function of specific genetically diverse microbial populations.

SIP is based on the principle that microbes fed substrates labeled with [^{13}C] incorporate this "heavy" C into DNA that can then be separated from normal [^{12}C] DNA by density gradient centrifugation [46]. Thus, by

separation of community DNA following a pulse with labeled substrate, the DNA of actively dividing cells can be recovered and analyzed by a range of molecular methods. The scope of the method was extended further to allow analysis of rRNA as well as DNA [31]. This is significant as rRNA is more dynamic, incorporates more [^{13}C] than DNA, and is probably a more accurate reflection of metabolic status than DNA. The potential is also there to develop the technology for mRNA and other dynamic components of the metabolome. To date, SIP has been successfully applied to explore biodegradation of pollutants as well as to dissect aspects of biogeochemical cycles. Although still a relatively new technique that is in the developmental stage, some interesting and novel data have already emerged from such SIP studies. In one study assessing the degradation of the organic pollutant naphthalene by a natural microbial community, a novel bacterium related to *Polaromonas vacuolata* was identified as the dominant active species [21]. This was unexpected as most prior work on naphthalene degradation focused on bacteria related to *P. putida*. The basic method employed in that particular study was to add [^{13}C]-labeled naphthalene to natural soil at physiological concentrations. Heavy rRNA was then purified by density centrifugation, and a library of enriched rDNA clones was created and analyzed. A clone from the novel bacterium was identified in this library and used to facilitate identification of the bacterial species. This study illustrates one of the strengths of SIP technology—namely, the ability to probe microbial function in a natural ecosystem under relevant physiological conditions. Several studies have also probed biogeochemical cycles using this technology. Friedrich and colleagues have explored aspects of methanogenesis in complex communities [25–28]. By feeding labeled substrates such as [^{13}C]methanol and [^{13}C]propionate to natural anoxic communities, it was possible to identify microbes actively involved in the methanogenic cycle. One noteworthy outcome was the incorporation of label into fungi and protozoa, indicating the potential of the technique for analyzing complex food webs [26]. This also illustrates the potential of using indirect labeling techniques to explore ecosystem function. Most studies have relied on the direct addition of labeled substrate to the microbial community, but several methods of indirect addition are also possible. Some work, for example, has been performed on allowing plants to incorporate labeled $^{13}CO_2$, which is incorporated into photosynthate, some of which is released to the soil microbial community as organic root exudates. Tracking the incorporation of the labeled exudates into microbes is a very exciting new approach to address intimate plant–microbe associations in the rhizosphere, although some technical challenges still need to be addressed [17]. We speculate that combining more efficient heavy RNA soil extraction techniques with PhyloChips will increase the accuracy of identification and analysis of active rhizospheric communities. In summary, it is evident that the continued development of SIP-RNA and related techniques will provide new insights to help resolve the function/activity conundrum in microbial ecology studies.

Discovering the Role of Novel Genes Involved in Plant-Bacterial Interactions

Use of In Vivo Expression Technology As a Genetic Tool. Advances in gene fusion technology such as IVET can provide a useful approach to study bacterial gene expression in the rhizosphere [47]. IVET is a promoter-trapping technology that allows selection for environmentally induced promoters, through the use of genetic complementation of a conditional mutation, mediated by the fusion of an *in vivo*-expressed promoter to the coding sequence complementing the mutation [54]. The major advantage of IVET is that it allows the study of ecologically significant microbes in their natural environment, where the full repertoire of genes and associated physiological conditions necessary for survival *in vivo*, are expressed. Another advantage of this technique in comparison to other methods is that the genes can be selected for further study irrespective of whether a mutation in those genes could be lethal [54]. Limitations with IVET include the fact that only those genes found to be activated in the rhizosphere can be selected. Genes that are down-regulated or not expressed are not amenable to identification with this technology.

Examples of IVET application have been demonstrated in the work completed by Rainey [47], in which 20 *P. fluorescens* SBW25 genes were shown to display elevated levels of expression during rhizosphere colonization. Genes identified included those that code for proteins involved in Type III secretion, oxidative stress, and transport. Recently, Silby and Levy [54] also used IVET to identify genes that may play a role in the survival of *P. fluorescens* PF0-1 in the soil. This study showed that genes involved in nutrient utilization, transport, detoxification, and metabolism were up-regulated in the bacterium exposed to the soil environment. Functional genomic analysis using targeted mutagenesis was carried out on three of those genes predicted to be involved in the survival of *P. fluorescens* PF0-1 in the soil. Mutant strains were found to be significantly affected in the ability to grow after 1 day; however, by day 3 mutant populations were similar to wild-type levels. It is suggested that these genes may be involved in the early establishment phase of the population in soil. With the rapid increase in sequenced genomes and the reduced cost of microarrays, many researchers are now conducting transcriptomic studies for the global changes in bacterial gene expression.

Transcriptomics and Its Application in the Analysis of Plant-Bacterial Interactions. The transcriptome is a dynamic system that encompasses all mRNA transcripts in the cell. Transcriptomics is a global method of analyzing the transcripts expressed in a cell and thus provides a means of studying bacterial gene expression under various environmental conditions. The most common tools used in transcriptomic analysis, which was first introduced in the mid-1990s, are oligonucleotide microarrays and complementary DNA (cDNA) microarrays [51]. This technology is now a high-throughput, robust method of simultaneously quantifying thousands of defined mRNA species in a miniaturized, automated format. When using microarray technology, mRNA is extracted from experimental samples and controls, and is reverse-transcribed to cDNA. The cDNA is fluorescently labeled and hybridized to a microarray on which known oligonucleotide or cDNA probes are present to allow analysis of gene expression. The major advantage of microarrays is that the expression of thousands of genes can be analyzed in a single experiment. Information on the expression profiles of genes in response to a variety of external signals can then be studied in-depth so as to indicate gene function. Initial research focused on single aspects of plant–host interactions in artificial systems such as iron regulated genes and those involved in quorum sensing in *P. aeruginosa* PAO1 [42, 53]. Recently, we used *P. aeruginosa* PAO1 as a model to study the differences in gene expression in response to plant root exudates. These transcriptome profiles were generated using root exudates from two varieties of sugar beet that select for genetically distinct Pseudomonad populations. A number of genes were identified in this study as being either similarly regulated by both sugar beet varieties, but more interestingly 140 genes were differentially expressed in response to the two different sugar beet varieties. It is speculated these genes may be involved in host-specific responses and have provided targets for further functional genomic analysis [32].

A relevant application of this technology has been reported for pathogen–host interactions by Schenk *et al.* [52]. In that study, the expression of genes in *Arabidopsis* infected by an incompatible fungal pathogen or treated with defense-related signaling molecules, was analyzed. The obtained results indicate the existence of a considerable network of regulatory interactions and coordination of signaling pathways, illustrating that this approach facilitates a view of the global system rather than individual parts of it. Another example of the use of microarrays in plant–microbe interactions, was revealed by the study of Scheideler *et al.* [50], in which shifts in the metabolism of *Arabidopsis* during infection with *Pseudomonas syringae* pv. *tomato* were observed. Maleck *et al.* demonstrated another application of microarray technology, by studying changes in gene expression induced during the systemic acquired resistance response (SAR) in *Arabidopsis*. Subsets of genes were identified with common regulatory patterns [30].

DNA microarrays are becoming increasingly popular as a tool to study symbiotic and parasitic interactions between microbes and their host plants [44]. The construction of a dual genome chip has allowed for the simultaneous examination of bacterial and plant gene expression during root nodule development in *Medicago truncatula* by *Sinorhizobium meliloti* [6]. The development of dual chips provides an opportunity to study changes in the transcriptome of both the bacterial and host in unison. Simultaneous analysis of the transcriptomes of both the bacterial and plant partners will allow for a more holistic view of rhizospheric interactions.

As our knowledge on genes that are believed to play a role in plant-microbial interactions accumulates, the use of dedicated arrays, consisting of specific subsets of genes, will become more widespread. One limitation with transcriptomics is that the level of mRNA may not always be an accurate indicator of protein levels in the cell. This is especially true for genes that are regulated in a post-transcriptional manner [19]. In contrast, proteomic technology can be used to study the influence of plant signals on the levels of active protein found in the bacterial cell.

Proteomics and its Application in Analysis of Plant–microbe Interactions. Originally coined in 1995 by Marc Wilkins, the term "proteome" describes the "protein complement of the genome" [68]. Proteomics is thus the large-scale analysis of proteins. We are now in a postgenomic era, with recently annotated genomes simply offering us lists of genes with little insight into their expression or functional significance. Proteomics can be exploited as a powerful tool to understand the complex patterns of expression of genomes with respect to different environmental niches in which bacteria adapt and survive. It also has the ability to complement genomics by characterizing gene products and their response to a variety of changing biological and environmental factors.

Current methods of proteomic analysis most commonly used involve the solubilization of protein samples, and the subsequent separation by 2D gel electrophoresis. Proteins are stained using Coomassie brilliant blue, and spots can be excised and identified using mass spectrometry. The procedure for proteomic analysis is constantly being refined and improved so as to minimize recognized limitations associated with the process and gel-free proteomic systems are also under development.

Proteomic analysis has now been used to identify proteins specifically expressed in plant–microbe interactions. Proteomics has also been used to investigate bacterial-plant symbiosis, and has specifically been used as a tool to analyze proteins involved in nitrogen fixing

symbiosis. The proteomic comparison of *Rhizobium leguminosarum* bv. *trifoli* strains ANU843, which forms nitrogen-fixing nodules, and ANU794, which forms aberrant nodules, has identified proteins that may be involved in the early stages of nodulation [33]. Proteins found to be differentially expressed include those involved in symbiosis and stress-related functions. Proteomic analysis has also been used in the analysis of both ectomycorrhizal and endomycorrhizal symbiosis [7]. Proteomic work has been particularly fruitful due to the availability of sequence information of genomes such as *Arabidopsis thaliana* (http://www.arabidopsis.org/agi.html) and *Oryza sativa* [69]. Examples of applications include work carried out by Peck *et al.* [43], who used a "directed proteomics" strategy to identify proteins that are rapidly phosphorylated in the response of *Arabidopsis* cells to microbial elicitors. Directed proteomics involves focusing on a particular subset of proteins (phosphorylated proteins), and the subsequent radioisotope labeling, 2D gel electrophoresis, and MS allowed differentially expressed proteins to be identified; Ndimba *et al.* [38] also studied the response of *Arabidopsis* to a variety of fungal elicitors. This study identified cell wall and extracellular proteins from an *Arabidopsis* suspension culture that were divergently expressed in the presence of both chitosan and *Fusarium* elicitor. Kim *et al.* [22] utilized adaptions to the general 2D gel electrophoresis protocol with the development of polyethylene glycol (PEG)-mediated prefractionation technique. This allowed the enrichment of rare proteins in the sample, thus increasing the sensitivity of the experiment. Proteomic analysis of the rice plant *O. sativa*, innoculated with blast fungus *Magnaporte grisea*, treated with jasmonic acid or subjected to various stresses, identified a number of new pathogen- and elicitor-responsive proteins in suspension cultured rice cells [23]. Additional recent advances have allowed for the parallel analysis of plant–microbe interactions, using both transcriptomics and proteomics [12]. Up to now, functional genomic analysis has been mainly analyzed at transcriptional and protein levels separately, with little correspondence between the two components [18]. This new protocol allows proteins to be analyzed from the same root sample as an mRNA population, thus enabling proteomic and transcriptomic data to be linked for harmonized analysis.

The continued progress with sequencing both plant and microbial genomes coupled to the recent and ongoing advances in this field will greatly project proteomic analysis as a valuable tool for future dissection of plant-bacterial interactions.

Metabolomics and its Applications in Plant-bacterial Interactions. Metabolomic studies have been shown to provide certain advantages over other "omic-type" data mining technologies such as transcriptomics and proteomics. The first and most obvious is that metabolomic analysis is carried out further down the line from gene to function than that of the other technologies. From this, we can assume that changes in the metabolome more closely reflect the activities of the cell at a functional level, and that these changes in the metabolome are expected to be more pronounced when compared to changes at a transcript level [62]. It is clear therefore, that metabolomics can be used in parallel with the other "omic" technologies in offering a clearer and more comprehensive picture of cellular activity. Indeed, examining metabolites or changes in the metabolome can play an important role in the integrative approach for assessing gene function and relationships to phenotypes.

Metabolomics has been defined by Oliver *et al.* [41] as "the quantitative complement of all low molecular weight molecules present in cells in a particular physiological or developmental state." Analysis of the metabolome can be carried out using one of four different approaches. (1) Target compound analysis is used to study the primary effect of a genetic alteration. Analysis is exclusively focused on the substrate and/or the direct product of the corresponding encoded protein. Subsequent analysis of any other metabolites is not required and because of this, extensive sample cleanup can be performed, thus avoiding inference from other compounds. (2) Metabolite profiling is used to elucidate the function of whole pathways or intersecting pathways. The analytical procedure is restricted to a select number of predefined metabolites, which allows the sample cleanup to focus on the chemical properties of these compounds. (3) Metabolomics is a comprehensive analysis of the metabolites of a biological system. Such analysis must be both qualitative and quantitative and, by definition, must include all metabolites produced. As yet, this complete analysis is not technically possible, given the lack of a simple automated analytical strategy that can carry out the procedure in a reproducible manner. In an attempt to gain a more complete metabolic snapshot, a variety of analytical techniques are used, each offering different advantages, in particular with respect to the dynamic range of metabolite that can be analyzed. (4) Metabolic fingerprinting is a rapid, global analysis of samples. This technique does not require the ability to identify different compounds, but rather is based on the ability to give reproducible profiles and have a high throughput.

For a comprehensive analysis of the metabolome, it is essential to use analytical techniques that have as wide a range as possible for analysis of the different metabolites produced by cells. Combinations of techniques—for example, parallel liquid chromatography (LC) separations coupled to mass spectrometry (MS) and/or nuclear magnetic resonance (NMR)-based detection methods.

The main problem with metabolomics to date is the bias introduced when choosing particular separation techniques that miss out on some of the metabolites, and result in the modeling of only a fraction of the metabolome and is therefore a metabolic profile rather than true metabolomics. The current popular method for global metabolite analysis in plants is gas chromatography-mass spectrometry (GC-MS). Disadvantages associated with this technique include its limitation with the molecular mass of targets and that nonvolatile polar metabolites need to be derivatized to convert them to less-polar, volatile, thermally stable forms, which can then be separated on a GC column. There is recent interest in GC-GC-MS, which offers increased throughput of sample [64]. Methods involving flow injection analysis using direct infusion into electrospray ionization (ESI) coupled to time of flight (TOF), or Fourier transform-ion cyclotron resonance (FT-ICR) MS analysis are also becoming popular [4, 20]. FT-ICR MS is of particular interest because of its sensitivity and its high mass resolution.

Examples of the use of metabolomics in the analysis of plant–microbe interactions include work by Narasimhan *et al.*, in which they coined the term "rhizosphere metabolomics" for the study of secondary metabolites secreted by resident microbial populations. This technique was used to show the depletion of persistent organic pollutants, such as polychlorinated biphenyls (PCBs) from the rhizosphere over a 28-day period. The metabolites secreted by the *Arabidopsis* roots were characterized so as to identify targeted compounds for creating a nutritional bias for microbes involved in plant–microbe interactions. This technique was developed for the subsequent degradation of polychlorinated biphenyls by the rhizospheric population. However, it could also be applied for strategies aimed at improving competitive abilities of biocontrol and biofertilization strains [37]. Maier *et al.* [29] have also studied secondary metabolites produced from mycorrhizal roots of both tobacco and tomato plants. Results indicate the presence of numerous glycosylated C_{13} cyclohexanone derivatives due to the presence of the arbuscular mycorrhizal fungi. Metabolic analysis will continue to grow as a tool used in a "systems biology" approach to studying plant–microbe systems as a whole [67], and in functional genomic analysis. Indeed, Raamsdonk *et al.* [45] showed that by linking functional genomic analysis with metabolomic analysis, it is possible to reveal and study the effects of silent mutations [45]. Differing applications of metabolic analysis have been shown; for example, increasing metabolic fluxes into valuable biochemical pathways using metabolic engineering or pathways needed for the production of pharmaceuticals in plants [16].

The application of these "omic technologies" toward the analysis of plant-bacterial interactions is only now possible due to the availability of genome sequences for both plants and rhizospheric bacteria. Although each of the "omic technologies" described have advantages and disadvantages, it is only by collectively exploiting them that we will be able to attain a more detailed understanding of the highly complex interactions that exist between bacteria and plants. Significant challenges will remain in processing and integrating the biological relevance of the results these new high-throughput technologies are capable of generating.

Functional Genomic Analysis of Plant-Bacterial Interactions

The generation of data from high-throughput technologies must cope with the practical reality that putative positive and negative results may not be biologically robust or relevant. Therefore, subsequent functional genomic analysis is required to verify the physiological significance of genes of interest that may be implicated from global profiling approaches. Functional genomic analysis has now become much more feasible as a result of the development of bacterial mutant gene libraries. Mutant libraries are available for *P. aeruginosa* PAO1 (http://www.pseudomonas.com), as well as various plant species including *A. thaliana* (http://www.biotech.wisc.edu/Arabidopsis). The subsequent use of these resources in the verification of the functions of specific target genes found to have altered expression in the rhizosphere will provide direct evidence on the roles of particular genes of interest in plant-bacterial interactions. Recently, we exploited the *P. aeruginosa* PAO1 mutant library from University of Washington Genome Centre (http://www.genome.washington.edu/uwgc/pseudomonas/index.cfm) to facilitate screening of genes with putative or unknown functions identified by transcriptome profiling. Of an initial seven strains, two had a reduction in growth compared to wild-type; of the remaining five, three were found to have a decrease in their competitive ability in the rhizosphere [32]. The use of genome libraries will allow for the screening of a large number of mutant strains without the time-consuming process of creating individual mutants.

Applications of Reporter Genes in Plant–Microbe Analysis. One of the challenges in microbial ecology is trying to correlate the behavior of microorganisms in the laboratory with that in their natural environment. Although many of the underpinning aspects of microbial gene regulation and physiology can be discerned in the laboratory, it is very difficult to reproduce the environmental conditions that pertain in natural ecosystems. This is an issue both for the basic understanding of microbial ecology, and for the exploitation of microbes for biotechnological applications such as biocontrol or bioremedia-

tion. Reporter gene technology has been used for some time to try to address this specific issue. By creating fusions of the promoters of genes of interest to reporter genes such as *lacZ*, *xylE*, or *gusA*, it is possible to explore whether expression is occurring *in situ*. These reporters are quite limited, however, with a requirement to culture cells or add external substrates, as well as potential problems with background signals from indigenous microbes, restricting the range of applications. One of the more powerful reporter genes is luciferase (*luc* or *luxAB*), which generates a light output that can be measured with luminometric devices. This overcomes the requirement to culture the cells, but crucially requires the addition of substrate as well as cellular energy (ATP). For many applications, this is a disadvantage but it can also be used positively, and luciferase systems are a very useful tool for monitoring the metabolic status of a microbe introduced into a particular environment. The advent of green fluorescent protein (GFP) as a molecular tool and reporter gene has overcome many of the limitations of earlier systems, and for many applications it has become the reporter of choice. The primary advantage of GFP for these applications is its autofluorescence: in response to exposure to light of an appropriate wavelength, GFP will emit light on a longer wavelength without the addition of external substrates and without the requirement for cellular energy.

GFP has gained widespread use in microbial ecology, with many examples of applications in biosensor studies, biofilm maturation, and plant–microbe interactions [35]. In its simplest form, a microbe can be tagged with a constitutively expressed *GFP* gene and monitored *in situ* in its natural environment. More complex approaches include linking *GFP* to particular promoters to monitor gene expression *in situ* and the use of unstable variants of GFP to explore short-term changes in gene expression. There are various detection methodologies, but the most applicable for studying plant–microbe interactions is confocal laser scanning microscopy (CLSM). The combination of CLSM with GFP allows analysis of the spatial interactions in real-time, without excessive sample processing. The potential of autofluorescent proteins (AFPs) as a tool for microbial ecologists has been further strengthened by the identification of novel AFPs, as well as the development of improved variants of existing AFPs. These new variants are designed with improved biochemical and spectral characteristics, increasing the range of applications available. Apart from these specific improvements, the other big advance is that because of different spectral characteristics, it is possible to conduct dual labeling experiments, for example, where one microbe is marked with GFP and another with a red fluorescent protein. Indeed, there are also examples of dual labeling within a single cell to monitor different physiological traits.

The majority of studies using GFP to understand plant–microbe interactions have focused on model bacteria such as *Rhizobium* or *Pseudomonas*. Many details of the *Rhizobium*–legume symbiosis were known of course, but by using GFP and DsRED, it has been possible to visualize the events during the interaction [13, 14, 58, 67]. Particularly exciting images of *Rhizobia* growing within infection threads demonstrated very clearly that infection threads are colonized by different bacteria rather than a unique infecting cell [13]. A number of studies have explored the association of nonpathogenic or beneficial strains of *Pseudomonas* with plant roots [8, 40, 48, 49, 59–61, 65]. To date, the technology has mainly been applied to individual strains, but dual labeling approaches open up possibilities of looking at competition, for example, between wild-type and mutants, and to look at spatial distribution of microbes in the rhizosphere. One recent study along these lines explored the colonization patterns of phase (phenotypic) variants of *Pseudomonas brassicacearum* [3]. This study labeled phase I variants with GFP and phase II variants with DsRed, and found that each phase had a specific colonization pattern, suggesting that phase variation might be an evolutionary mechanism to extend habitat under adverse conditions. Moving beyond model systems, GFP has also been used as a marker to explore the function of new bacterial species identified in particular ecological settings. For example, one study exploring the endophytic flora of poplar trees isolated a novel strain of a *Pseudomonas*-like bacterium [15]. This bacterium was labeled with GFP and was demonstrated

Table 1. Techniques used for the analysis of plant–microbe interactions

Technique	*Function*
Stable isotope probing	Analysis of metabolically active microbial populations
In vivo expression technology	Identification of genes found to be up-regulated in the rhizosphere
Transcriptomics	Analysis of levels of mRNA transcript profiles
Proteomics	Analysis of protein profile expression
Metabolomics	Analysis of metabolite profiles produced
Functional genomic analysis	Verification of function/role of genes
Reporter gene technology	Analysis of *in situ* gene expression

Incorporated together, the various techniques highlighted in this review could be utilized in an integrated approach for the study of plant–microbe interactions. An integrated approach offers the ability to study plant–microbe interactions as a complex system, as opposed to looking at the functions of specific genes.

to grow endophytically within the vasculature of the tree root. A study of bacteria interacting with mycorrhizal fungi identified a novel isolate of *Bacillus cereus*, which was subsequently tagged with GFP and demonstrated to adhere specifically to mycorrhizal hyphae [5]. These studies establish the breadth of the technology and demonstrate that its use is not restricted to simple visualization of interactions, but can be used to test hypotheses and biological questions and to generate new knowledge of complex ecological interactions. As techniques improve and better AFP variants tailored for microbial ecology become available, it is likely that the use of AFPs will become an indispensable part of the repertoire of techniques used to understand any microbe–plant interaction *in situ*.

Conclusion

Exploitation of beneficial microbe–plant interactions in the rhizosphere can result in the promotion of plant health and has a significant importance for low-input sustainable agriculture applications such as biocontrol. However, as microbes do not live in isolation, the complex interactions that occur in the rhizosphere need to be further elucidated. It is established that plant species can define the bacterial communities present in the rhizosphere *via* selection of genetically diverse populations or by favoring specific dominant groups. Despite the acceptance that plant extracellular signals influence the microbial populations in the rhizosphere, little is known about the genes involved and the roles they may play in plant–microbe interactions. See Table 1 for the variety of techniques used in the identification of genes with a role in plant–microbe interactions. Indeed, the characterization of novel genes involved in plant–microbe interactions is vital in understanding the response of resident bacterial populations to plant signals at a molecular level. Incorporating established techniques such as *in vivo* expression technology (IVET), with the recently advanced "omic technologies," now offers the ability to search for genes on a global scale that are found to be induced or repressed due to the presence of plant signals. The subsequent linking of these techniques with functional genomics and reporter gene technology will prove invaluable in the characterization of plant–microbe "interactomes." The advantage of linking these techniques has been recently highlighted in the work of Mark et al. [32], whereby transcriptome profiles were validated and furthered by functional genomic analysis. Indeed, the exploitation of each of the systems-based strategies highlighted in this review in an integrated approach will lead to a systems-based understanding of these complex interactions in the rhizosphere. An understanding of the complex molecular signals between these communities could offer a solid foundation for the development of new strategies to combat disease or to promote those interactions of benefit to plant health.

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