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Review article.

Global gene expression in the rhizobial-legume symbiosis

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Abstract

With the sequencing of entire genomes it has become technically feasible to study transcription on a global scale. Accessing an organism's transcriptional profile provides a glimpse into its inner workings. Transcriptional studies help determine how an organism adapts to diverse environments and how it interacts with other organisms. In the symbiosis between rhizobial bacteria and legume plants, the two organisms must be able to adapt to various environmental stresses and communicate to form a mutually beneficial relationship. The study of global gene expression during this nitrogen-fixing symbiosis has confirmed results of earlier studies and has shed new light on the molecular players involved in this complex, highly choreographed interaction.

Keywords: Symbiosis, nitrogen fixation, transcription, transcriptome, proteome, metabolome, legume, *Sinorhizobium, Mesorhizobium, Rhizobium, Bradyrhizobium, Medicago, Lotus*

Contents

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This world is divided roughly into three kinds of nations: Those that spend lots of money to keep their weight down; those whose people eat to live; and those whose people don't know where their next meal is coming from.

David S. Landes
Author, Professor of economics and history (1924–)

1. Introduction

Ours is a world of exploding populations. The human population has grown exponentially in the last 50 years, and is predicted to reach 9 billion by the year 2050 (Ahmad, 2001). Consequently, continued production of sufficient food and energy for the world's burgeoning population is a basic challenge facing us this century (Tilman et al., 2001). One critical factor in determining food productivity and arable field fertility is the amount of "fixed," or reduced, nitrogen in the soil. For growth and development, plants require fixed nitrogen, which is typically absorbed by roots from the soil. Dinitrogen is "fixed" when it is converted into a reduced form such as ammonium; nitrogen fixation is the process of converting dinitrogen to ammonium. Last century's development of industrial fertilizer production

now accounts for a substantial fraction of available fixed nitrogen, fueling the present green revolution. About half of soil fixed nitrogen comes from biological nitrogen fixation, a process carried out only by prokaryotes (Zahran, 1999). Since biological nitrogen fixation cannot currently sustain world agriculture, continued use of chemical fertilizers is presently necessary. However, there are many down sides to industrial production of fertilizers (Cordovilla et al., 1994; Newton, 2004; Yousef and Sprent, 1983), not the least of which is the expense of its manufacture, which requires an enormous energy input: approximately 4% of all the natural gas extracted annually goes toward the production of fertilizers (Energy Information Administration, 2005; Smith, 2002), making this process ultimately non-sustainable. In contrast, biological nitrogen fixation is free of the economic, environmental and geopolitical consequences of fossil fuel-dependent nitrogen fixation.

About half of all biological nitrogen fixation is carried out by rhizobial bacteria (Madigan et al., 1997; Werner and Newton, 2005), which colonize roots of compatible leguminous plants to engage in a nitrogen-fixing symbiosis (James et al., 2006; Spaink et al., 1998). Most of these bacteria belong to the α-proteobacteria class, although recent reports show that some β-proteobacteria also carry out symbiotic nitrogen fixation (Chen et al., 2003b; Moulin et al., 2001). The α-proteobacteria, broadly dispersed throughout the environment and inhabiting diverse ecological niches, are evolutionarily related organisms of great metabolic, morphologic and reproductive variety. Strict aerobes and obligate and facultative anaerobes, often rod-shaped, are all found in the α-proteobacteria class. Members of the α-proteobacteria include the symbiotic nitrogen-fixing rhizobia, plant pathogenic agrobacteria, dimorphic stalked *Caulobacter*, purple non-sulfur photosynthesizers such as *Rhodobacter*, mammalian pathogens such as *Bartonella, Rickettsia* and *Brucella*, chemoorganotrophs such as *Pelagibacter ubique* that use dissolved organic carbon from the sea, and methanotrophs, aerobic single-carbon compound utilizers (Batut et al., 2004; Giovannoni et al., 2005; Sällström and Andersson, 2005). Thus the α-proteobacteria display such wide diversity that their Gram-negative cell wall is one of the few features shared by all members.

2. Genomes

Bacterial genomes

In recent years, complete genome sequences of several rhizobia have been determined, including *Sinorhizobium meliloti* (Barnett et al., 2001; Capela et al., 2001; Finan et al., 2001; Galibert et al., 2001), *Mesorhizobium loti* (Guerrero et al., 2005; Kaneko et al., 2000; Sullivan et al., 2002), *Bradyrhizobium japonicum* (Göttfert et al., 2001; Kaneko et al., 2002a; Kaneko et al., 2002b), *Rhizobium leguminosarum* (Young et al., 2006), *Rhizobium etli* (González et al., 2006) and NGR234 (Freiberg et al., 1997; Streit et al., 2004; Viprey et al., 2000). Substantial sequence data have been gathered for some of their symbiotic hosts, particularly *Medicago truncatula* and *Lotus japonicus* (Cannon et al., 2005; Choi et al., 2004; Town, 2006; Udvardi et al., 2005; Zhu et al., 2005). Moreover, both bacterial and plant mutants have been isolated and characterized for many stages of the symbiosis: these are useful tools for further dissection of regulatory and developmental pathways (Geurts et al., 2005; Madsen et al., 2003; Mao et al., 2005; Oldroyd et al., 2005; Radutoiu et al., 2003; Saeki and Kouchi, 2000; Sato and Tabata, 2006). Together these genomic and genetic advances constitute a virtual gold mine of available new information, from which researchers can extract valuable understanding of the basic biology of these important organisms and of how they establish a functioning nitrogen-fixing symbiosis. We have learned much from work on leguminous crop plants such as alfalfa (*Medicago sativa*), soybean (*Glycine max*), clovers (*Trifolium repens* and *T. pratense*), pea (*Pisum sativum*) and bean (*Phaseolus vulgaris*) as molecular-genetic tools have been steadily brought to bear. But over the past dozen years two symbioses, the *Medicago truncatula-Sinorhizobium meliloti* and the *Lotus japonicus-Mesorhizobium loti* pairs, have emerged as model systems because of the small, relatively simple genomes of the plant partners and their utility in genetic and genomic studies. The proven ability to extrapolate model system results to agronomically important crops makes such work highly significant (Udvardi et al., 2005; Zhu et al., 2005).

Rhizobia possess large and often multipartite genomes. For example, the model bacterium *S. meliloti*'s genome is distributed among three replicons (Barnett et al., 2001; Capela et al., 2001; Finan et al., 2001; Galibert et al., 2001): a chromosome of 3.65 Mb where most housekeeping and essential genes are found, and two symbiotic megaplasmids (pSymA, 1.4 Mb, bearing the nodule formation (*nod*) and most nitrogen fixation, but no essential genes; and pSymB, 1.7 Mb, encoding exopolysaccharide (EPS) biosynthesis, dicarboxylate transport/utilization, and a few essential genes). *M. loti*, on the other hand, has a single large (7 Mb) chromosome and two small plasmids (Kaneko et al., 2000), but virtually all symbiotic genes are localized to a ~0.6 Mb "symbiosis island" on the chromosome (Sullivan and Ronson, 1998). It has been speculated that a large part of these genomes is involved in free-living growth in soil and is evolutionarily maintained because of the heterogeneity of the soil and the need to be able to survive in such a diverse environment (Barnett et al., 2001).

Plant genomes

While no legume genome sequence has been completely determined, projects are underway for several including bean, pea, soybean, alfalfa, and the two model legumes, *M. truncatula* and *L. japonicus*. Most such projects involve sequencing cDNAs, usually referred to as Expressed Sequence Tags (ESTs), while the gene space of both model legumes is being sequenced using genomic clones (Alkharouf and Matthews, 2004; Cannon et al., 2005; Cronk et al., 2006; Gonzales et al., 2005; Town, 2006; Udvardi et al., 2005). ESTs have been used to construct arrays (see below) to jump start analysis of the plant transcriptome. Arrays are available for soybean and pea and a small pilot array was used to examine gene expression in soybean during nodulation (Lee et al., 2004). However, the only comprehensive published studies (as of this writing) on plant symbiosis have been conducted on the two model legumes.

Stability of eukaryotic RNAs and polyadenylation makes the study of gene expression in plants technically simpler than in bacteria. In the pre-microarray era, this made it possible to construct cDNA libraries and perform Northern analysis and differential hybridization experiments. As a result, many plant genes important for nodule formation were identified over the past two decades using these techniques and laborious approaches involving mutation and positional (map-based) cloning (Sato and Tabata, 2006; Stacey et al., 2006).

Overview of arrays

An array is a physical substrate to which is fixed a large set of DNA sequences at known positions. Arrays may consist of synthesized oligonucleotides or larger gene segments produced by PCR. In this review we will use "microarray" to refer to both. As described below, microarrays can be used to assess the RNA population contained in a cell at a particular time in development or in a specific environmental situation. It follows, though, that the completeness of such assessments and the depth of interpretations will depend on both the quality and quantity of the sequences present on the microarray itself. Current research using microarrays to study symbiosis utilizes genetic sequences in differing stages of completeness and knowledge.

Interpretation of plant arrays is particularly complicated by the absence of complete genome sequences. Because EST sequencing can miss genes expressed transiently or at low levels, these genes will likely be omitted from EST-based microarrays. Furthermore, without complete genome sequence, it is difficult to determine whether an EST belongs to a single gene or a multi gene family, and gene family members often are expressed in different patterns. Also, about half the predicted genes induced in nodules have no predicted function. As described above, the choice of genes that ultimately are arranged on an array necessarily introduces bias into what can be deduced from use of that array.

Transcribed RNA is isolated, converted to a labeled form, and hybridized to an array of the genes from the organism. When the array is scanned the correspondingly labeled gene can be detected, allowing a determination of which RNAs were present in the cells and thus which genes were being expressed at the time of harvest. Relative abundance can be determined for each transcript by comparing these global snapshots. The term "transcriptome" describes all the transcripts in the cell under a set of environmental conditions; in other words, the trancriptome is the pattern of global gene expression.

Global gene expression

Many "genes" sequenced in these genomic projects have no biosystematically identified function; homologs – with or without a defined function – of some sequences (ESTs) are found in other organisms, but many are "orphans" with no counterparts in any other sequenced organism. Availability of complete bacterial genome sequences, and to a lesser extent the incomplete legume genome sequences, has informed researchers as to the catalog of genes that can direct function in each organism, and has led to the construction and use of genomic scale arrays to study different stages in the nitrogen-fixing symbiosis. These global arrays permit the researcher to assess gene expression at chosen points in time, producing snapshots of the organism's transcriptional state (Clarke and Zhu, 2006; Conway and Schoolnik, 2003; Hinton et al., 2004; Rensink and Buell, 2005; Rhodius and LaRossa, 2003). Transcriptome studies using genome scale arrays have confirmed the involvement of previously known genes, and have identified previously undiscovered genes involved in particular genetic and metabolic pathways and stages of the symbiosis. Thus, even for orphan genes, the association of sequence expression with a particular developmental stage will provide valuable clues to function. This approach holds great promise in identification of candidate genes involved in both adaptation and symbiosis.

The purpose of this review is to assess the emerging biological picture that is being painted from studies using global microarrays. Some experimental questions have been broad in nature, for example, asking basic questions about overall bacterial metabolic changes during a shift from rich to minimal medium, or asking what changes occur during the transition from the free-living to the symbiotic state. Other questions have been more narrowly focused on specific aspects of physiology or interaction,

such as stress responses, the role of cell surface components, effects of iron or phosphate limitation, microaerobiosis, etc. On the plant side, microarrays have been used to address questions about the plant response to the invading microbe that takes up residence inside the plant cell. What changes occur in the plant to make it receptive to the invaders in its midst? Must bacteria circumvent plant defenses as they slip behind the plant's outer perimeter?

It is important to keep in mind that with global analyses, the largest source of variability is biological (Hinton et al., 2004; Rhodius and LaRossa, 2003). Small differences in growth conditions, nodule age, host plant, etc., can greatly alter results. The controls used will also affect outcome; e.g., different results are obtained when arrays from stationary phase cultures, rather than log phase cultures, are used as a baseline for comparisons of nodule bacteria (Capela et al., 2006; Clarke and Zhu, 2006). In addition, because this is an ongoing effort by many active laboratories, this review must be viewed as a snapshot of the field as it currently stands.

The question of primary interest is what are the changes that occur during symbiosis as a result of the back-and-forth signaling between the two partners? From the bacterial perspective, the transition from the free-living to the symbiotic state is dramatic: many adjustments have to be made when shifting from free-living, dividing cells to factories streamlined for ammonium production. From the plant perspective, allowing a microbe inside requires careful tailoring of the defense response and construction of a tube that pierces the plant interior. Several groups have published studies relevant to these questions, and their findings will be summarized in this review.

In addition to use of arrays for defining transcription in the context of development and differentiation, another exciting recent application of array technology has been its use to identify and clone genes corresponding to phenotypic mutants based on transcript abundance. This approach, pioneered by Mitra et al. (2004a), identified DMI3, a Ca^{2+}/calmodulin-dependent protein kinase required for nodule formation. A 14-bp deletion in *dmi3* results in a truncated protein in the non-nodule-forming *M. truncatula dmi3* mutant. This Transcript-Based Cloning (TBC) method was also employed in the identification of *nsp2* and *dmi2*, two mutant alleles first identified by positional cloning (Endre et al., 2002; Kaló et al., 2005; Mitra et al., 2004a). TBC was further used to identify and clone the transcription factor BIT1, required for Nod factor signaling, and new alleles of DMI2 and DMI3 (Giles Oldroyd, personal communication). This approach holds special promise for legume crop species with their large and complex genomes. This potential has already been realized in soybean where the molecular basis of a seed coat pigment mutant has been determined using TBC (Vodkin et al., 2004).

3. Early Symbiosis

Initial signaling

Rhizobia's ability to invade, colonize and differentiate into nitrogen-fixing bacteroids requires constant communication between the bacterial and plant partners, a communication mediated by chemical signals (Fig. 1). These signals determine the "host range," i.e., which plant and bacterial partners will engage in a successful symbiosis (Fisher and Long, 1992; Schultze and Kondorosi, 1998). For example, *S. meliloti* only forms nodules on legume species in the genera *Medicago*, *Melilotus* and *Trigonella*. At the earliest stages of the symbiosis, bacteria in the rhizosphere sense plant signal molecules such as flavonoids and betaines exuded from the seeds and roots of the plant; these signals serve as inducers of gene expression in the bacteria. Many of these induced bacterial genes encode enzymes that synthesize a responsive signal molecule, a modified lipochitooligosaccharide known as Nod factor. Nod factor is a plant morphogen that stimulates the formation of a new meristem in the plant root (Stougaard, 2000). These meristematic cell divisions give rise to the root nodule, a novel organ that the bacteria will colonize and the site of symbiotic nitrogen fixation (Long, 1989). In root epidermal cells Nod factor induces depolarization of the plasma membrane, oscillations in cytosolic Ca^{+2} known as calcium spiking, and distortion of polar tip growth in root hairs (Ehrhardt et al., 1992; Ehrhardt et al., 1996; Harris et al., 2003; Shaw et al., 2000; Sieberer et al., 2005). In root inner cortical cells Nod factor induces mitotic activation that leads to formation of the nodule primordium. These reciprocal signal-response interactions between the plant and bacterial partners in the establishment phase of the symbiosis are among the best-characterized aspects of the symbiosis (Capoen et al., 2005; Cullimore and Dénarié, 2003; Parniske and Downie, 2003; Spaink, 2002; Udvardi and Scheible, 2005). Subsequent stages in the symbiotic process are even more complex, requiring a tight metabolic coordination between the two partners, and making the symbiotic interactions increasingly more difficult to unravel from a technical perspective.

Infection

Successful early communication between the two partners leads to invasion of the plant host (Fig. 2). The bacteria grow and penetrate plant tissue within a tube, the infection thread, that is synthesized from plant components: topologically the bacteria remain outside the root hair in the infection thread (Brewin, 1998; Gage, 2004; Gage and Margolin, 2000; Yano et al., 2006). Bacterial cell surface components such as EPS, lipopolysaccharide (LPS), and cyclic beta-glucans are essential for successful invasion

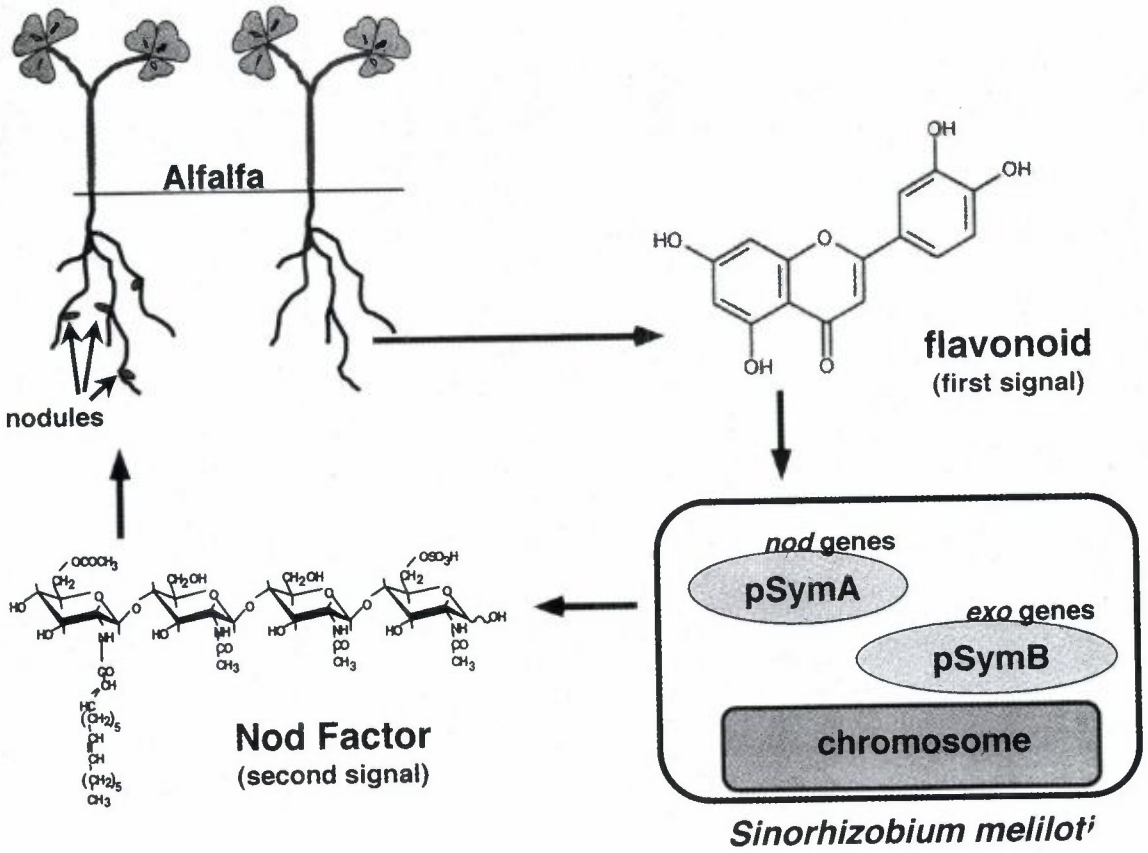

Figure 1. Early signal exchange between alfalfa (*Medicago sativa*) and *Sinorhizobium meliloti*.

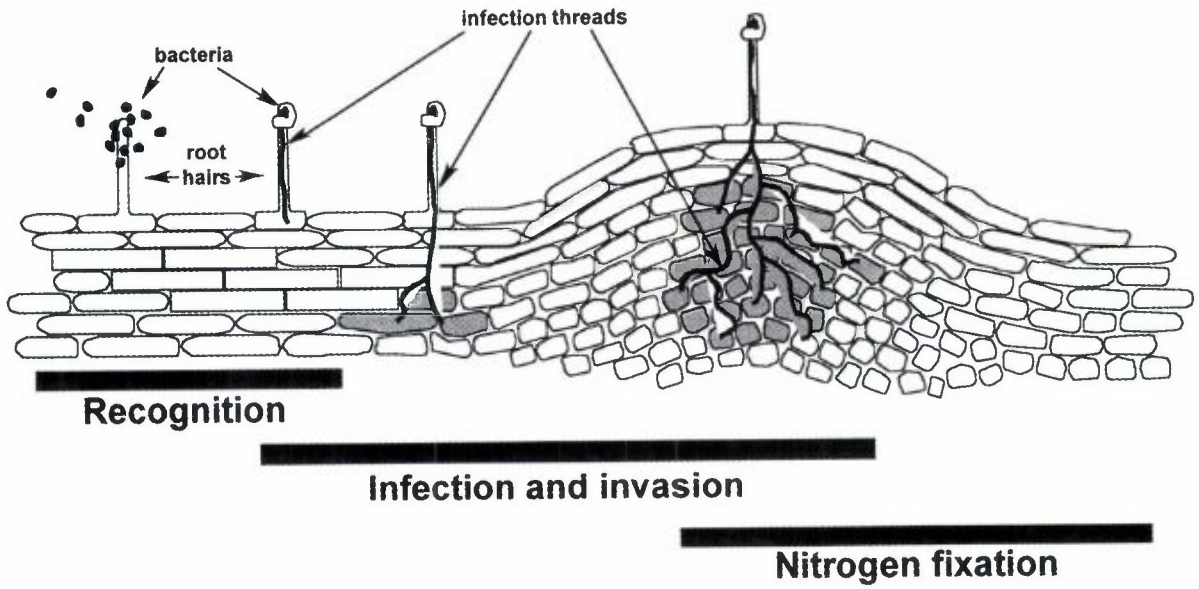

Figure 2. Infection of plant roots by *S. meliloti*. Bacterial Nod factor induces cell division in the root cortex to form the nodule meristem. The infection thread ramifies to deliver bacteria to the plant cells (gray cells).

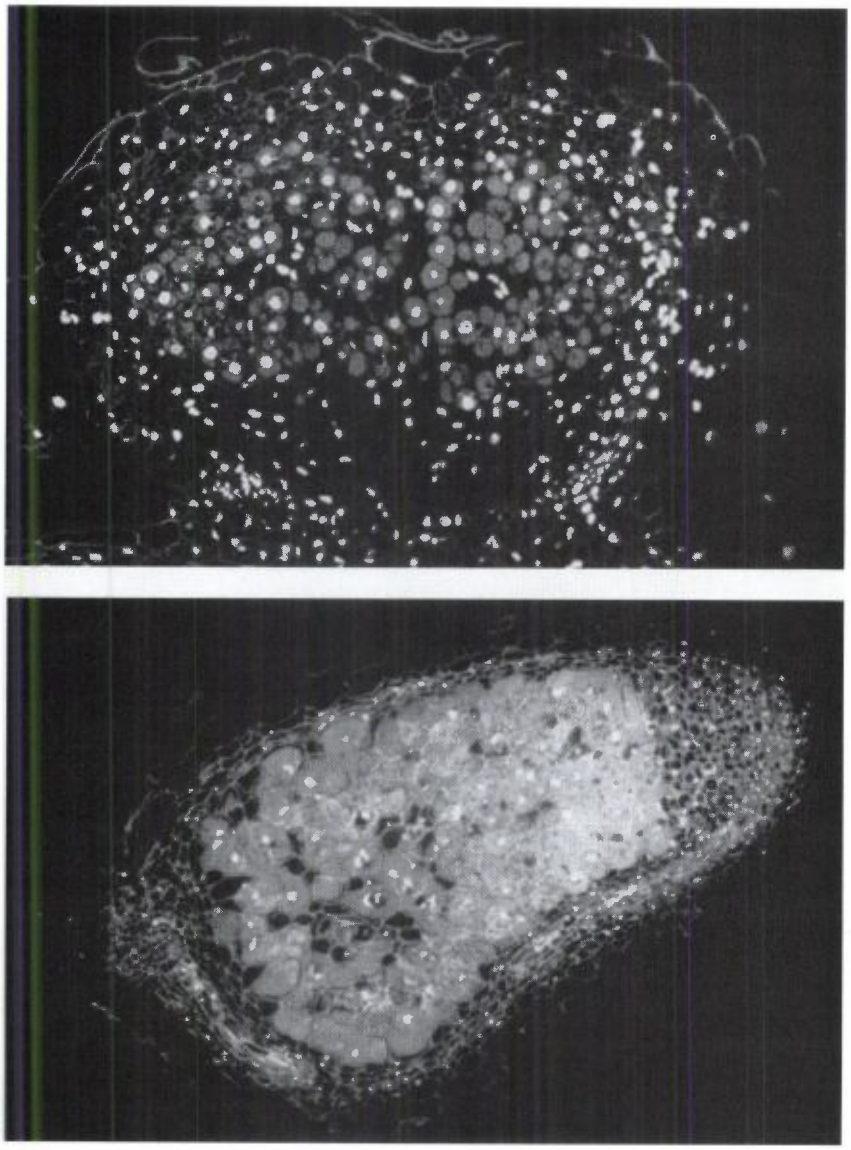

Figure 3. Cross sections of determinate (upper) and indeterminate (lower) nodules. For orientation, the root axis is at the bottom of the upper image, and at the lower left corner in the lower image. Note the uniform appearance of infected cells in the DAPI-stained determinate nodule (upper) in contrast to the multiple zones in the DAPI/Acridine Orange-stained indeterminate nodule (lower). The meristematic zone in the indeterminate (lower) nodule is at the right and abuts the infection zone where the plant cells are being filled with bacteria. The slightly darker zone to the left of the infection zone is where symbiotic nitrogen fixation occurs. The less dense zone on the left is where senescence has initiated. The upper image was kindly provided by Makoto Hayashi (University of Munich) and the lower image by Mark Dudley and Sharon Long (Stanford University). *See cover illustration.*

(Becker et al., 2004; Brewin, 1998; López-Lara et al., 2003; Pellock et al., 2000). The infection thread can be viewed as a composite structure in that it is made up of both symbiotic partners in intimate association, the dividing bacteria and the elongating plant tube, which grow synchronously (Gage, 2004). An extension of the plant plasma membrane surrounds the growing infection thread, which ultimately extends into the developing root nodule. The tip of the infection thread has no cell wall, and bacteria, surrounded by sacs of plant-derived membrane, are released from the progressively growing thread (Goodchild and Bergersen, 1966; Newcomb, 1981). These membrane-surrounded bacteria that are released into the cytoplasm of the plant cell make up the "symbiosome" (Brewin, 1998; Capoen et al., 2005; Udvardi and Day, 1997). As infected plant cells continue to develop, the plant makes additional symbiosome membrane that envelops the newly formed bacteria, so that topologically the bacteria remain outside the plant cytoplasm, arguing that the host plant retains control of invasion and infection. The bacteria then differentiate into the nitrogen-fixing form by undergoing a series of changes: individual cells enlarge, often becoming branched or club-shaped, and their cell walls grow more fragile. The intracellular and nitrogen-fixing rhizobia within infected cells are called bacteroids.

Determinate nodules such as in soybean (*Glycine max*) and *Lotus* species lack a persistent meristem since the initial cell division activity required for nodule primordium formation is finite (Mergaert et al., 2006). Mature determinate nodules contain a homogeneous population of

nitrogen-fixing bacteroids since differentiation of infected cells occurs synchronously, followed by senescence (Fig. 3, upper). In contrast, cell division activity is long lived in indeterminate nodules (Fig. 3, lower) such as in *Medicago* species and pea (*P. sativum*), giving rise to a persistent apical meristem (Cermola et al., 2000; Provorov et al., 2002; Timmers et al., 1998; Vasse et al., 1990). Mature indeterminate nodules contain a heterogeneous population of endosymbionts because of continued cell division activity, giving rise to a gradient of nodule development as the nodule continues to elongate and sometimes branch (Soupene et al., 1995): the apical meristematic zone, infection zone, bacteroid differentiation zone, nitrogen fixation zone, and basal senescence zone (Fig. 3, lower). Thus, all the bacteria in a determinate nodule (Fig. 3, upper) exhibit a uniform physiology; however, in an indeterminate nodule (Fig. 3, lower) there are multiple physiological states since the bacteria in the different zones display something akin to an ecological succession of cell types, progressing from the recently delivered bacteria in the early infection zone to the degenerating bacteroids in the late senescence zone. The bacteria likely receive and respond to different cues in these different zones of the nodule.

The bacterial partner undergoes drastic metabolic changes in response to its new intracellular environment. Conversion of dinitrogen to ammonium in mature bacteroids is catalyzed by bacterial nitrogenase under microoxic conditions. Plant leghemoglobin, an oxygen-sequestering protein, is one of many nodule proteins required for nodule function: it helps to create the microoxic environment that nitrogenase needs to function (Downie, 2005). Low oxygen tension is also the signal that turns on the regulatory circuit cascade leading to expression of bacterial genes required for nitrogen fixation, the *nif* and *fix* genes (Fischer, 1996; Kaminski et al., 1998). In the case of the *S. meliloti-Medicago* symbiosis, the classic model for root nodule metabolism is that bacteroids provide fixed nitrogen in the form of ammonium to its host plant in exchange for photosynthate carbon in the form of C-4 dicarboxylates such as succinate, fumarate and malate (Yurgel and Kahn, 2004). In pea it has recently been shown that plant amino acids are also essential for symbiosis (Lodwig et al., 2004; Prell and Poole, 2006). More work is sure to follow that will shed light on the mechanism of nutrient exchange between bacteroid and host.

Reciprocal signaling and transcription

The initial signaling event is the detection of plant compounds that turn on sets of genes in the appropriate bacterial partner (Schultze and Kondorosi, 1998). This gene induction is mediated by NodDs, members of the LysR family of transcription activators, and the *nod* genes that are induced encode enzymes that make a responding signal (Fisher and Long, 1992). This regulatory circuit has received much attention, particularly in the establishment of the *S. meliloti-Medicago* symbiosis, where many of the details were worked out through a combination of forward genetics and molecular homology and regulatory approaches. *S. meliloti* has three NodDs (NodD1, NodD2 and NodD3), the last of which is inducer-independent. NodD3 makes up a regulatory circuit with SyrM (Symbiotic regulator of Mucoidy), another member of the LysR family (Mulligan and Long, 1989): SyrM activates expression of *nodD3* (and other genes, some regulatory), and NodD3 activates the expression of *syrM*. Microarray experiments looking for other targets of activation by NodD showed that this earlier work was quite thorough (Capela et al., 2005). Aside from the known inducible *nod* genes, few genes were up-regulated when *nodD1* was overexpressed in the presence of luteolin, its flavonoid inducer (Barnett et al., 2004; Capela et al., 2005). Many more genes were turned on when *nodD3* was overexpressed, likely an indirect result of the *nodD3-syrM* positive regulatory circuit (Barnett et al., 2004). One of the hallmarks of *syrM* expression is the mucoid phenotype resulting from the activation of expression of genes involved in EPS biosynthesis (Mulligan and Long, 1989), and presumably many of the *nodD3*-induced genes are involved in this phenotype.

As mentioned earlier, mutations in either the bacterial or the plant partner can set up metabolic or developmental blocks which can be exploited to study individual stages in the symbiosis. For example, *bacA* mutant bacteria elicit normal infection threads to colonize plant cells, but upon release into plant cells undergo rapid degeneration without differentiating into nitrogen-fixing bacteroids (Ferguson et al., 2002; Glazebrook et al., 1993). The net effect of this aborted infection is that nodule bacteria induced by the *bacA* mutant are found in infection threads, and these bacteria show strong repression of overall gene expression at this early stage (Capela et al., 2006). This is consistent with studies on bacterial growth within infection threads, where it was shown that active growth is restricted to those cells at the tip of the infection thread (Gage, 2004).

Global expression studies of model legume hosts engaged in symbiosis have been carried out at time points ranging from early (1 hour (h) post inoculation) to late (7 week old nodules). Hundreds of genes show altered expression in these studies. While use of different plants, nodule ages and growth conditions each confer a different "transcriptome", their overlap and the collective data set reveal unifying themes.

At the earliest time point in *M. truncatula* (1 h), calcium spiking has begun, preceding root hair deformation and development of nodule primordia (Lohar et al., 2006). Transcripts relating to disease and stress resistance are

induced and those encoding translation proteins are transiently down-regulated, all consistent with response to stress. Cell wall-related transcripts transiently increase in abundance: for example, α-fucosidases, enzymes that cleave fucose from xyloglucan, increase early on and return to pre-infection levels by 24 h. This could signal the gearing-up of processes involved in cell wall rearrangements that will result in infection thread initiation and growth or new cell division resulting in establishment of the nodule meristem. Interestingly, a novel transcription factor is transiently induced, returning to normal levels by 6 h. This transcription factor thus becomes an obvious target for future studies to determine if it is involved in control of induction of these early genes.

At these early time points, there are many genes whose expression is changing. The change in gene expression pattern is much greater between 1 h and 12 h than it is between 24 h and 72 h (Lohar et al., 2006). These changes are mainly due to gene induction rather than suppression (Lohar et al., 2006; Mitra et al., 2004b). Many genes encoding functions related to cytoskeletal structure and cell walls are induced by 6–12 h. This is also the time when root hair deformations are first visible. At 24–48 hours, transcripts related to ribosome biogenesis, rRNA processing, cell proliferation, defense/stress response and embryogenesis are induced (Lohar et al., 2006; Mitra et al., 2004b). These genes are all consistent with new activity in the root inner cortex, where rapid cell divisions are helping to initiate the nodule meristem that will result in forming the structure of the nodule (Fig. 2).

Purified Nod factor induces a transcriptional response at 24 h very similar to that generated in response to wild type bacterial cells, arguing that Nod factor is the primary signal controlling the early plant response (Mitra et al., 2004b). Moreover, none of the 46 genes whose expression changes in response to Nod factor show such change in six Nod$^-$ (i.e., unable to form nodules) *M. truncatula* mutants. Similarly, none of 27 genes induced in wild type *M. truncatula* at 3 d are induced in a non-nodule-forming *nfp* (Nod factor perception) mutant (El Yahyaoui et al., 2004).

A common feature in both model legumes at time points earlier than 4 d is an induction of genes related to those for cell growth, cell division, and response to stress and to pathogens (El Yahyaoui et al., 2004; Kouchi et al., 2004; Lohar et al., 2006; Mitra et al., 2004b). This window is the time period when plant cortical cells are undergoing rapid cell division as the nodule structure is being established, and fully functioning synthesis machinery is required for this to move forward. Moreover, the plant may need to lower its defensive shields to allow a foreign organism (the bacterium) to take up residence within the plant host. Induction of many of these early stress/pathogenesis genes is transient, but some pathogenesis-related transcripts remain strongly induced in mature nodules, perhaps to allow a level of control over infection progression. Consistent with this idea is the observation that six defense response-related genes are induced to lower levels in a hypernodulating mutant of *M. truncatula* (i.e., one that forms an excessive number of nodules): a limited defensive response coincides with excessive nodule formation, and suggests a correlation with mounting a defense response and controlling nodule number (Küster et al., 2004). Alternatively, up-regulated pathogenesis- and defense-related gene products could be involved in protecting nodules from pests or pathogen attack (El Yahyaoui et al., 2004): nodules constitute the diet of some insect larvae, and many of these pathogenesis- and defense-related gene products are toxic to insects (Gerard, 2001). Similarly, transcripts encoding proteins predicted to interfere with defense responses and cell death are up-regulated in mature *L. japonicus* nodules (Colebatch et al., 2004).

Transcripts related to cell division and growth may remain high during much of symbiosis (El Yahyaoui et al., 2004; Kouchi et al., 2004), or eventually be down-regulated as expected in mature determinate nodules (Colebatch et al., 2004). While plant hormone-related genes (for auxin, cytokinin, gibberellin, ethylene) change expression during nodule formation, their roles are unclear (Stougaard, 2000). Several gibberellin-related transcripts are down-regulated in *M. truncatula* nodules, leading to speculation that this represents a redirection from lateral root formation toward nodule development (El Yahyaoui et al., 2004). Nodulins (plant-encoded, nodule-specific proteins) and leghemoglobins are induced in both model legumes once the nodule primordium is established (El Yahyaoui et al., 2004; Kouchi et al., 2004; Starker et al., 2006). Many of these nodulins have been previously described (and some have been assigned functions), and new candidate nodulins have been discovered by microarray experiments.

4. Later Symbiosis

Transition to the bacteroid state

The journey from the soil to the plant interior is likely stressful for the bacteria. It has long been assumed that rhizobia must have mechanisms to evade or suppress host defense responses. The situation appears to be complex: physiological studies show that purified rhizobial Nod factor transiently slows the generation of reactive oxygen species by roots (Shaw and Long, 2003). In response to purified Nod factor at later times, however, *Medicago* species induce an oxidative burst, generating superoxide anions and hydrogen peroxide, postulated to ward off unwanted invaders (Ramu et al., 2002; Santos et al., 2001). *S. meliloti* is hypothesized to combat these reactive oxygen species by up-regulating expression of catalases and

superoxide dismutases and, indeed, genes whose products deal with reactive oxygen species are up-regulated during infection (Barloy-Hubler et al., 2004). Induction of bacterial heat shock and other genes suggests a general stress is induced during the transition. Microarray studies confirm that expression of genes encoding nitrogenase and those required for microoxic respiration, such as the genes encoding the terminal electron acceptor, is induced. The nitrogenase genes are induced before the nodules are fixing nitrogen, suggesting that bacteroid differentiation has already initiated (Capela et al., 2006).

Bacteroid development may involve a general decrease in bacterial metabolism. This is supported by microarray studies. The transcription profile of bacteria from intermediate-stage 5-day old nodules resembles that of rapidly growing log-phase bacteria with respect to the large number of genes expressed (Capela et al., 2006). However, in later-stage nodules (8-days and older) the transcription pattern is reminiscent of the slow growth pattern observed in stationary phase cultures. In the transition to the bacteroid state, cells stop dividing and there is a corresponding decrease in the expression of genes coding for cell division machinery, DNA replication, transcription, translation, chemotaxis, and motility. Bacterial metabolism, and the expression of the relevant genes, changes as cells adapt to a more confined symbiotic lifestyle in which acquisition of nitrogen-fixing capability is of prime importance. Generally, in mature nodules (Barnett et al., 2004; Becker et al., 2004; Capela et al., 2006; Uchiumi et al., 2004), the repressed genes far outnumber the induced genes.

As alluded to above, there appears to exist a replicon-based division of function in some rhizobial genomes. This is further reinforced in the transcriptome of bacteroids where induced genes are overrepresented on the symbiotic megaplasmids and a symbiotic island, whereas the majority of the repressed genes are chromosomal (Barnett et al., 2004; Becker et al., 2004; Uchiumi et al., 2004). Likewise, LPS and EPS biosynthesis genes, so important at the early stages of interaction, are also repressed. Genes involved in DNA repair are turned on in nodules, indicating that maintenance of genomic integrity is still important in bacteroids. Previous work demonstrated bacterial synthesis of a novel EPS by some strains of *B. japonicum* in soybean nodules (Streeter et al., 1992). This indicates that there are differences in EPS regulation in the different rhizobia, an issue that may be resolved by further transcriptomic analyses.

Repression of glycolysis is consistent with transition to the usage of C-4 dicarboxylates such as succinate, fumarate or malate as carbon sources. Recent work in the pea symbiosis has found that two rhizobial amino acid transporters, *aap* and *bra*, are required for nitrogen fixation. These transporters may play roles in amino acid cycling (Lodwig et al., 2004; Prell and Poole, 2006). The *aap* and *bra* genes are not induced in *S. meliloti* bacteroids. However, lack of induction in microarray experiments need not necessarily preclude an important role for a gene. As mentioned below, for example, *fixJ* is constitutively expressed (i.e., is not induced) in nodules. Alternatively, another bacterial amino acid transporter with sequence similarity to transporters of aspartate and glutamate is induced in the *Medicago* nodules (Barnett et al., 2004; Becker et al., 2004); this transporter may be a candidate for amino acid metabolism in bacteroids.

Earlier genetic and biochemical studies defined many bacterial genes involved in nitrogen fixation (Fischer, 1996; Kaminski et al., 1998). The *nif* genes encode enzymes, primarily the nitrogenase complex (NifHDK), and the regulatory NifA protein that controls fixation of atmospheric dinitrogen. The *fix* genes were defined by mutants that were defective in fixation; *fix* genes include those encoding the terminal electron acceptor, and regulatory proteins controlling their expression (Kaminski et al., 1998). As mentioned above, the nodule is microoxic, under control of plant leghemoglobin, making an environment appropriate for nitrogenase function. In *S. meliloti*, the two-component regulatory system FixL/FixJ coordinates expression of genes required both for microoxic respiration within nodules and for nitrogen fixation. The sensor FixL histidine kinase autophosphorylates under microoxic conditions and transfers the phosphate to the response regulator FixJ. FixJ is a transcription factor master regulator that, upon phosphorylation, turns on expression of two other regulatory genes, encoding NifA and FixK, which activate expression of the *nif* and *fix* genes whose products carry out nitrogen fixation and respiration. A few dozen direct and indirect FixJ targets were described before the FixJ regulon was re-assessed using microarrays, which resulted in the identification of numerous other targets. Currently, FixJ controls a regulon of 122 genes when considering both microoxia and symbiosis; these include genes involved in nitrogen metabolism, stress responses, transport and 41 genes annotated as hypothetical (Bobik et al., 2006), making the FixJ regulon one of the largest bacterial regulons known. In mature nodules, as many as 60% of the induced genes are FixJ-dependent, compared with only about 2% in very young nodules (Capela et al., 2006). FixJ-dependent genes constitute the majority of induced genes common to all *S. meliloti-M. truncatula* symbiotic experiments (Barnett et al., 2004; Becker et al., 2004; Capela et al., 2006). Not surprisingly, nearly all of the FixJ-activated genes reside on pSymA, the symbiotic megaplasmid. Identifying new members of the FixJ regulon generates provocative questions of regulatory circuitry. For example, why do the sets of FixJ-dependent genes induced during symbiosis and microaerobiosis in culture only partially overlap? For example, the denitrification genes are

under FixJ/FixK control during microaerobiosis in culture but not in mature bacteroids. Oxides of nitrogen are widely used in bacteria as terminal electron acceptors under anaerobic conditions, so perhaps denitrification serves an energetic function (Bobik et al., 2006). Alternatively, denitrification could protect against nitric oxide generated during infection. Before we can completely understand bacterial physiology under these two growth conditions, we will have to understand the roles that these regulatory genes and their targets play in either symbiosis or during growth under low oxygen (Becker et al., 2004; Bobik et al., 2006; Hauser et al., 2006).

Nodule metabolism: plant genes

With onset of nitrogen fixation, observed changes in patterns of plant gene expression coincide with observed changes in nodule metabolism. Transcriptome data here are consistent with earlier intensive biochemical and genetic studies on nodule metabolism. For example, mature nitrogen-fixing nodules act as a sink: sucrose is transported from the shoot and catabolized in the nodule. Consequently, it is no surprise that host genes encoding sucrose synthase, β-amylase, a hexose transporter, glycolytic enzymes, and enzymes involved in carbon fixation such as malate dehydrogenase, show increased expression in nodules (Colebatch et al., 2004; El Yahyaoui et al., 2004; Kouchi et al., 2004). Similarly, since the plant assimilates bacteroid-produced ammonium, it is no surprise that genes for ammonium assimilation (aspartate aminotransferase, asparagine synthase, glutamine synthetase) are induced. Most genes induced in effective (i.e., nitrogen-fixing) and ineffective (i.e., non-fixing) nodules were not induced when the roots were fed nitrate, and none were induced (some were weakly to strongly down-regulated) when roots were fed ammonium (Tesfaye et al., 2006). Moreover, transcripts for proline and polyamine synthesis are induced in nodules, perhaps a sign of osmotic stress in the nodule. Highlighting the important role of iron-containing proteins in nodule metabolism, genes encoding ferritin and proteins involved in heme metabolism are induced.

The transcriptome data complement previous biochemical data from other species. For example, transcripts for *L. japonicus* phosphoenolpyruvate carboxylase (PEPC) increase in abundance, consistent with experiments showing post-translational increase in PEPC activity in pea, soybean and alfalfa nodules (see references in Colebatch et al., 2004). There are indications of new symbiotic interactions in nodules. Phosphate may be limiting in 7-wk *L. japonicus* nodules based on increased expression of genes encoding PEPC and malate dehydrogenase (seen in roots of many plants in response to phosphate limitation); a high affinity phosphate transporter; and intracellular phosphatases that break down nucleic acids, nucleotides and phospholipids, replacing the last of these in the membrane with galactolipids and sulfolipids. Yet whole nodule measurements indicate that free phosphate levels are similar to those of uninfected roots. Why the paradox? One clue may be that excretion of organic acids such as malate and citrate facilitates uptake of inorganic phosphate from the rhizosphere by plant high affinity transporters. Moreover, a bacterial high affinity phosphate transporter is essential for effective fixation in *S. meliloti* (Bardin et al., 1996). Therefore, perhaps bacteroids take up phosphate as a way to induce the plant to synthesize more dicarboxylate, the bacteroids' preferred fuel for symbiotic nitrogen fixation (Colebatch et al., 2004). Additional work is clearly needed to test this hypothesis, yet this example demonstrates how a global transcriptional snapshot can lead to interesting models.

Because transcription factors are often expressed at low levels, and because minor changes in their expression can lead to amplified downstream effects, modifications in microarray data analysis had to be developed and applied to identify nodule-enhanced transcription factors (Colebatch et al., 2004; El Yahyaoui et al., 2004). This is an exciting development because transcription factors have been somewhat recalcitrant to identification. Most of these transcription factors remain uncharacterized, so this provides a great opportunity to identify the targets of these regulators. Other classes of regulatory proteins have been identified: for example, a set of six calmodulin-related genes induced 1–3 d after inoculation (Lohar et al., 2006) is particularly interesting in light of calcium's role as a second messenger in plant cells and calmodulin's role in mediating calcium signaling.

The expression profiles of three *M. truncatula* Nod^+ Fix^- (i.e., able to form nodules but unable to fix nitrogen) mutants were compared to wild type plants (Starker et al., 2006). Most of the genes expressed in wild type plants were also normally expressed in the mutants: only 8% to 19% were misregulated in the mutants. Moreover, only three of the genes showed altered expression in all three of the mutants. Similarly, very few expression differences were observed between the ineffective *sen1* mutant and wild type *L. japonicus* (Suganuma et al., 2004). A similar result is seen in nodules formed by a bacterial Fix^- mutant: most genes up-regulated in nodules formed by wild type *S. meliloti* are also induced in Fix^- nodules (Barnett et al., 2004; Tesfaye et al., 2006). These studies argue that many of the plant transcriptional differences between nodules and roots result from nodule morphogenesis and bacterial occupancy, rather than the nitrogen-fixing status of bacteria within the nodule.

5. Bacterial Adaptation

As soil bacteria, rhizobia must be well adapted to survive in an often harsh environment where nutrients and oxygen may be limiting. Bacteria may face osmotic, desiccation, and temperature stresses. Heavy metals and other toxins may be present. It has been postulated that the large rhizobial genomes better equip them to deal with such stresses (Barnett et al., 2001). Besides providing insights into symbiosis, transcription profiling can help us better to understand how rhizobia adapt to diverse environments.

To determine how bacteria adapt to changes in nutritional availability, transcriptional profiling was carried out on *S. meliloti* grown on minimal medium containing glucose or succinate, a C-4 dicarboxylate (and a preferred bacteroid substrate) (Barnett et al., 2004). Once again, a replicon bias was observed: genes with altered expression were frequently found on pSymB, which seems to be specialized in sugar and polysaccharide metabolism (Finan et al., 2001). Similar results were seen when the glucose utilization pathway was markedly inhibited upon addition of glutamate (Davalos et al., 2004). Succinate-grown cells induce gluconeogenesis, essentially a reversal of glycolysis, an important pathway for synthesis of glucose when citric acid cycle intermediates are the sole carbon source (Barnett et al., 2004; Davalos et al., 2004). These microarray analyses were consistent with earlier metabolic studies that measured catabolic enzyme activities of cells grown on glucose or succinate and that helped to refine the metabolic pathways (Finan et al., 1988).

It is inarguable that *Escherichia coli* is the most intensively studied bacterium, and thus serves as a model organism to which all other bacteria are compared. But while serving as a paradigm, it must be remembered that what's true for *E. coli* is not necessarily true for other bacteria, including rhizobia. When *E. coli* grows in minimal medium, it slows its growth rate and induces expression of biosynthetic genes that will allow it to make the essential compounds that are missing from the minimal medium. *S. meliloti* also slows its growth rate but does not globally respond this way, suggesting that transcription is regulated differently when confronted with a change in nutritional status (Barnett et al., 2004). Furthermore, the *S. meliloti* genome does not encode a regulatory gene, *rpoS*, which plays a primary role in the transition from log phase to stationary phase growth in *E. coli* (Conter et al., 2001; Galibert et al., 2001; Gerard et al., 1999; Mandel and Silhavy, 2005). Thus, an entire set of assumptions on growth and starvation need to be re-evaluated and regulatory mechanisms must be established empirically.

Iron availability is limited in soil due to insolubility. Many proteins important in the nitrogen-fixing symbiosis, such as bacterial nitrogenase, contain iron. Free iron is scarce in plants during symbiosis, so intracellular bacteria must adapt to secure sufficient iron for these enzymes to function. Thus it is not surprising that expression of bacterial genes involved in iron metabolism is altered in the nodule (Ampe et al., 2003; Barnett et al., 2004; Becker et al., 2004). While important for proper function, too much iron can also cause problems such as formation of reactive free radicals.

In *S. meliloti*, the rhizobial iron regulator, RirA, controls iron uptake and many other iron-responsive genes (Chao et al., 2005). Putative binding sites upstream of RirA-regulated genes in *Rhizobium leguminosarum* are not found upstream of similar genes in *S. meliloti* (Chao et al., 2005). In contrast, the iron response regulator Irr binds to a 21-bp Iron Control Element (ICE) found upstream of the transcription start sites of iron-regulated genes in *B. japonicum* (Rudolph et al., 2006). Irr can act as either a positive or negative regulator as it functions in iron homeostasis. Studies in both organisms indicate that iron availability may influence the path of the metabolic respiratory chain. Neither *rirA* nor *irr* mutants have symbiotic phenotypes so future experiments are required to further unravel iron metabolism in symbiosis.

Phosphate, one of the most important nutrients for living organisms, is often limiting in the environment. It is important for symbiosis: *S. meliloti* mutants in phosphate transport form non-fixing nodules (Bardin et al., 1996). Not surprisingly, most genes of the Pho regulon are up-regulated when phosphate is limiting (Krol and Becker, 2004). Of the nearly 100 genes up-regulated in a PhoB-dependent manner upon phosphate limitation, many have a conserved sequence (the PHO box) upstream of their putative transcription start sites. The PHO box is predicted to be the binding site for PhoB, the regulatory protein controlling the regulon (Krol and Becker, 2004). A complementary computational search for PHO boxes identified 96 candidates in *S. meliloti* (Yuan et al., 2006b), one-third of which show phosphate- and PhoB-dependent regulation. In addition, some induced and most repressed genes are independent of PhoB. Some of the genes that were identified by these studies have no obvious connection to phosphate metabolism. Phosphate starvation causes oxidative stress: genes encoding catalase, peroxidases, and bacterioferritin, which protect against oxidative stress and detoxification of iron are induced (Krol and Becker, 2004; Yuan et al., 2005). Moreover, many iron uptake-related genes are repressed, as are many genes associated with a slower growth rate, encoding processes such as amino acid uptake, metabolism, and protein synthesis. This suggests that there is cross-talk between the Pho regulon and other cellular circuits that regulate oxidative stress and iron homeostasis (Yuan et al., 2006b).

Osmoadaptation is likely an important response in both soil and nodule environments. Genes whose expression changes under high salt (NaCl) have been identified

(Rüberg et al., 2003), and almost one-fifth of the repressed genes are related to iron uptake. Unpublished work shows that osmotic stress induced by either high salt or sucrose elicits a similar transcriptional response, and that many of the genes induced during osmoadaptation are localized on pSymB (Anke Becker, personal communication). In addition, expression of flagellar and chemotaxis genes is reduced during osmotic stress, as is found in *E. coli*, while expression of *exo* genes increases, presumably leading to increased production of succinoglycan (EPSI).

Bacterial cell density-dependent gene regulation, or "quorum sensing" (QS), is important in a variety of bacterium-host interactions such as bioluminescence, cell division, stress response, conjugal transfer, and production of colonization and virulence factors. QS likely plays roles in both symbiosis and plant pathogenesis (González and Marketon, 2003; Von Bodman et al., 2003). QS depends on synthesis and perception of small signal molecules, typically N-acyl homoserine lactones (AHLs) or modified oligopeptides (Camilli and Bassler, 2006), between bacteria. Legume hosts such as *M. truncatula* make compounds that mimic activity of AHLs, possibly affecting QS regulation and downstream behavior (Gao et al., 2003; Keshavan et al., 2005; Mathesius et al., 2003). The *sinR-sinI* system, which makes long-chain AHLs that control production of the symbiotically active EPS galactoglucan (EPS II), a product of the *exp* genes, has been most intensively studied in *S. meliloti* (Marketon et al., 2003; Marketon et al., 2002). *exp* gene expression requires *sin*-specified AHLs and the regulator ExpR: ExpR is a LuxR homolog that activates EPS II production in the presence of *sin*-AHLs. In the absence of succinoglycan (EPS I), the *sinR-sinI* system is essential for an effective symbiosis. Transcriptomic and proteomic analyses identified additional targets of the SinR-SinI circuit, such as motility, chemotaxis, nitrogen fixation, and metal and small molecule transport; most of these targets require the regulator ExpR (Gao et al., 2005; Hoang et al., 2004; Pellock et al., 2002). Surprisingly, *sinR-sinI* QS is also linked to production of the invasion-proficient low molecular weight form of succinoglycan via control of two endoglycanase genes, *exsH* and *eglC* (Hoang et al., 2004). The mechanism by which bacterial AHLs regulate expression of these targets is an area of active investigation. Plants synthesize compounds that mimic AHLs; how these compounds interfere with or contribute to QS, and to effective symbiosis, motility, chemotaxis, and transport, is the current focus of ongoing research. A second QS system is found in only certain strains of *S. meliloti*: the *tra* system controls conjugal plasmid transfer via short-chain AHLs in strain Rm41 and derivatives (Marketon and González, 2002).

These transcriptomic studies provide insight into the bacterial partner and highlight the apparent interconnections underlying adaptive responses. As mentioned above, either osmotic stress or phosphate starvation may remodel iron metabolism; salt stress induces genes for phosphate uptake; phosphate limitation induces genes involved in oxidative stress; iron availability may influence the respiratory pathway. Perhaps the most intriguing comparative finding is the sensitivity of the bacterial cell surface and motility to environmental perturbations. When cells are starved for iron or phosphate, or placed under osmotic stress, or grown in minimal medium, genes encoding chemotaxis and motility functions are down-regulated. Such down-regulation appears to be inversely related to increased expression of the *exo* genes, which encode the proteins involved in production of EPSI/succinoglycan (González et al., 1996; Skorupska et al., 2006). This is consistent with the defects in motility and flagellation previously seen in starved *S. meliloti* cultures (Wei and Bauer, 1998). When succinoglycan biosynthesis increases under nutrient-replete conditions, such as when *nodD3* or *syrM* are overexpressed (Barnett et al., 2004; unpublished for *syrM*), or in *exoR/S* mutants (Yao et al., 2004) (Wells et al., MS in preparation), motility genes are again down-regulated. Moreover, the reverse scenario is observed in QS studies of a *sinI* mutant: flagellar gene expression is induced when *exo* gene expression is suppressed (Hoang et al., 2004). How galactoglucan/EPSII fits into this picture is unknown, in part because *S. meliloti* strain Rm1021, the sequenced strain used for most of these studies, carries a null mutation in *expR*. Consistent with all of this is previous work on *S. meliloti* mutants that have longer flagella and are more densely flagellated, which translates into faster swarming cells; these mutants also show decreased EPS synthesis (Wei and Bauer, 1999). This inverse correlation between motility and EPS synthesis is proposed to make up part of a complex multitrait adaptation responsive to environmental cues (Barnett et al., 2004).

6. Arbuscular Mycorrhizal Symbiosis

Availability of legume arrays has accelerated comparative studies of nodule formation and the arbuscular mycorrhizal (AM) symbiosis (Hohnjec et al., 2005; Liu et al., 2003). AM fungi are obligate symbionts that engage in mutually beneficial endosymbiotic associations with root cortical cells (for a review, see Harrison (2005)). AM fungi aid the plant in acquisition of minerals, especially phosphorous; in return, the fungi obtain their carbon from the plant. Unlike rhizobial symbioses, early signals exchanged in AM interactions remain undiscovered. However, AM and rhizobial symbioses must share some signaling components, because plant mutants that neither form nodules nor engage in AM have been isolated and

characterized (Ané et al., 2004; Endre et al., 2002; Imaizumi-Anraku et al., 2005; Kanamori et al., 2006; Lévy et al., 2004; Stracke et al., 2002). One locus identified by mutation encodes a putative leucine-rich repeat-type receptor in the common signaling pathway (Endre et al., 2002; Limpens et al., 2005; Stracke et al., 2002), but the signal molecule that triggers this receptor is unknown. Arrays have been used to identify 26 *M. truncatula* genes, verified by qPCR, that are common to both nitrogen fixation and AM symbioses (Manthey et al., 2004). This surprisingly small set contains genes with diverse predicted functions.

7. New Insights from Global Analyses

Earlier studies on how external nitrogen sources affect symbiotic nitrogen fixation in *S. meliloti* resulted in isolation of a transposon insertion in *ntrR*, the second gene of the *ntrPR* operon. Since this mutation increased expression of the *nod* and *nif* genes, it was postulated to be a nitrogen regulatory gene. However, when this gene was subjected to microarray analysis, the mutation in *ntrR* was shown to affect the expression of hundreds of genes with roles in a wide range of cellular processes, including transcription and translation, symbiotic nitrogen fixation, transport, and basic metabolic functions (Puskás et al., 2004). These findings led to a re-assessment of *ntrR*'s role from a nitrogen regulatory gene to a modulator of a common mechanism such as phosphorylation/ dephosphorylation or stability or degradation of proteins or RNA. This is an example of how using microarrays can resolve questions about the role of a putative regulatory gene. Recent work shows that NtrP and NtrR constitute an antitoxin-toxin module (Bodogai et al., 2006) that may contribute to adjustment of metabolic processes under symbiotic and other stressful conditions. The antitoxin NtrP counteracts the lethal or bacteriostatic effects of the toxin, NtrR. When NtrR is expressed in *E.coli* in the absence of NtrP, cell growth and viability both decrease. Microarray data on fixing wild type nodules and ineffective *fixJ*-induced nodules show differential expression of *ntrP* (Barnett et al., 2004). *ntrP* expression is higher in wild type nodules and in free-living bacteria than in ineffective nodules. This means that in *fixJ*-induced nodules there may be insufficient NtrP antitoxin to counteract the deleterious effects of NtrR toxin and may contribute to earlier nodule senescence. Alternatively, onset of senescence could lead to premature degradation of NtrP and contribute to an acceleration of senescence. Further experiments will be required to clarify the nature of NtrP's involvement in senescence, if any.

Transcriptome studies on iron metabolism in *S. meliloti* and *B. japonicum* (Chao et al., 2004; Rudolph et al., 2006) show that regulation in rhizobia differs from the *E. coli* Fur (ferric uptake regulator) repressor system, and the two rhizobia have different regulatory circuits. In *S. meliloti*, Fur strongly represses in a Mn(II)-dependent manner, and moderately represses in a Fe(II)-dependent manner, expression of the *sitABCD* metal transporter, leading to the suggestion that it be renamed Mur (Manganese uptake regulator; (Chao et al., 2004)). Surprisingly, *sitABCD* is the only locus relating to metal metabolism induced in the *fur* mutant. *sitABCD* is also induced in nitrogen-fixing nodules, and *sitABCD* mutants show decreased nitrogen fixation, perhaps indicating a role in iron rather than manganese uptake in this situation.

Transcription analysis methods such as SAGE (Serial Analysis of Gene Expression, a way to quantify expression using short unique sequence tags) and cDNA-AFLP (Amplified Fragment Length Polymorphism of cDNA, a PCR-based technique for fingerprinting genomic DNA) do not have the bias limitations referred to above and can be used to determine relative abundance among groups of transcripts (Asamizu et al., 2005). Neither technique requires array construction, the cost of which can be prohibitive. SAGE has been used to compare transcript populations of a hypernodulating *L. japonicus* mutant to wild type plants: leghemoglobin mRNA was the most abundant nodule transcript, and metallothionein mRNA the most abundant root transcript (Asamizu et al., 2005). cDNA-AFLP has been used to identify *L. japonicus* transcripts enhanced in both rhizobial and AM symbioses: six "symbiosins" common to both symbioses were identified from a set of 344 differentially expressed genes (Kistner et al., 2005). Moreover, AM-induced transcriptional activation was abolished, but AM-induced repression was not, in seven Nod^- AM^- *L. japonicus* mutants. These results are consistent with those of array experiments.

8. More Omics: Proteomics and Metabolomics

While this review is focused on transcriptomics, there are other avenues of analysis of global gene expression in this post-genomic era. "Proteome" is the term that describes all the proteins that are found in a cell under a given set of conditions. Proteomic studies have examined luteolin-induction (Chen et al., 2005; Chen et al., 2000a; Chen et al., 2000b), regulatory circuitry (Djordjevic, 2004; Kajiwara et al., 2003; Rolfe et al., 2003; Sarma and Emerich, 2006), protein secretion (Rosander et al., 2003; Sarma and Emerich, 2005), nutrient limitation (Bestel-Corre et al., 2002; Encarnación et al., 2003), quorum sensing (Cantero et al., 2006; Chen et al., 2003a; Gao et al., 2005), salt tolerance (Shamseldin et al., 2006), pH response (Reeve et al., 2004), root hair infection (Morris and

Djordjevic, 2001; Wan et al., 2005), and nodule occupancy (Djordjevic et al., 2003; Natera et al., 2000; Saalbach et al., 2002). Unlike transcriptomics, proteomics provides a measure of relative protein abundance. Proteomic data have confirmed many translated, annotated ORFs, and have identified new un-annotated proteins encoded in the *S. meliloti* genome. Proteomic data have also revealed post-transcriptional regulation and post-translational modifications. It has been noted that the transcriptome is not necessarily congruent with the proteome: when transcriptomes have been compared with proteomes, proteins have been found present even when their encoding genes are not detectably expressed (Becker et al., 2004; Gao et al., 2005). Conversely, protein products of many transcriptionally highly expressed genes are not detected proteomically. However, parallel comparisons on the same species grown under the same conditions (i.e., identical source material), have yet to be carried out, a necessity for such analyses to be meaningful. Such direct comparisons face major hurdles because the techniques applied to attaining a transcriptome and a proteome are so different. Take the example of comparing the bacteroid transcriptome and proteome. For the transcriptome, entire nodules from plant roots can be rapidly harvested, ground in liquid nitrogen, and RNA extracted and purified. The labeled nucleic acid product can specifically hybridize to its cognate spot on the microarray. For the proteome, bacteroids are purified from plant material in an hours-long process that provides an opportunity for changes in the bacteroid protein profile, whether through normal turnover based on protein stability or from induction of proteolysis as a stress response, during bacteroid isolation. One must always be mindful of where the bacteria are coming from in both types of analyses, so that one knows what contributes to the RNA or protein being analyzed: bacteroids, or bacteria in infection threads, or both? The proteome has other inherent limitations of a technical nature: the proteins are separated on 2D gels, which only selectively accommodate proteins depending on their ionic charge and solubility. Proteins with a pI at either extreme, especially highly basic ones, are poorly displayed so that even abundant proteins may be excluded from analysis. Thus, since the two types of studies, transcriptomics and proteomics, are technically very different and have different limitations, the two approaches should be considered complementary, providing researchers with distinct rather than duplicative sets of information.

One of the more recent post-genomic approaches is metabolomics, the global analysis of small-molecule metabolite profiles. In this method, metabolites are extracted and analyzed. For example, *S. meliloti* grown in different carbon sources is distinguishable by its metabolite profile (Barsch et al., 2004). The metabolomes of model legume root nodules reveal not only expected but also novel compounds (Barsch et al., 2006; Desbrosses et al., 2005). Moreover, nodules inhabited by bacterial mutants with defects in EPS biosynthesis or the bacterial nitrogenase protein exhibit metabolomics profiles distinct from that of nodules induced by wild type bacteria (Barsch et al., 2006). While not as comprehensive as transcriptomics or proteomics, metabolomics can closely relate to a phenotype.

Transcriptomics is widely regarded as the most comprehensive of the three major global techniques being applied to post-genomic analysis, but it is also the "farthest" from the actual phenotype of the cell, in that post-transcriptional processes play major roles in determining what physiological changes occur in the cell. Transcriptomics provides a picture of the state of the cell and accurately depicts the immediate transcriptional response of the cell to changes in its environment. But this is only an early step in the adaptive response: transcripts must be translated, and the translation products (only part of which can be detected in the proteome) must function to effect adaptation, reflected in changes in the metabolic profile (the metabolome). Thus there is no direct correlation between gene expression and a given output. Metabolomics is "closest" to the actual phenotype since it provides a snapshot of the metabolic contents of the cell, but has limitations due to the nature of metabolite extraction. A combination of all three techniques, transcriptomics, proteomics and metabolomics, will provide the most information and, combined with reverse genetics studies targeting specific pathways in a cell, provide the best opportunity for understanding the biology of the cell.

9. High-throughput Genetics

While it is clear that global analyses, whether of the transcriptome, proteome or metabolome, provide much data and insight, one must keep in mind that these studies are not ends unto themselves, but rather an opportunity to use this newly gleaned information to develop new hypotheses and to design focused experiments to answer specific questions. The availability of complete genome sequences has greatly facilitated genetic analyses, whether those analyses are applied to individual genes or the entire genome, as with IVET-type promoter-trapping screens (Oke and Long, 1999; Zhang and Cheng, 2006). For *S. meliloti*, new sets of tools are available that promise much use in functional genomics. For example, a platform was developed in which all of the bacterium's annotated open reading frames (the ORFeome) have been cloned into a mobilizable plasmid (House et al., 2004; Schroeder et al., 2005). Using recombination, these ORFs can be transferred to other plasmids designed for specialized purposes, allowing the creation of protein expression plasmids,

deletion mutations, or operon fusions. Signature-tagged insertion and plasmid insertion mutants are available that cover 60–70% of the *S. meliloti* genome (Luo et al., 2005; Pobigaylo et al., 2006). These collections have been used to identify mutants with altered growth patterns (Pobigaylo et al., 2006) and to characterize mutants in regulatory genes, such as those encoding all 90 *lysR*-family transcriptional regulators (Luo et al., 2005). A reporter gene fusion library to nearly half of the *S. meliloti* genes is being used to screen compounds for induction of specific genes. (http://info.mcmaster.ca/fusionlibrary.html). These functional genetics platforms have the potential to serve as a valuable resource that can greatly accelerate investigations into specific biological questions on the *S. meliloti-Medicago* symbiosis, and on the basic biology of the bacterium itself.

Similarly, new genetic tools will aid understanding of the plant side of symbiosis (Buzas et al., 2005; Tadege et al., 2005). RNA interference (RNAi) technology recently experimentally demonstrated that specific symbiotic leghemoglobins buffer oxygen levels in *L. japonicus* nodules, creating an environment appropriate for nitrogenase activity (Ott et al., 2005). An RNAi-based screen for gene function in *M. truncatula* identified a calcium-dependent protein kinase gene involved in root development. When this gene is silenced, plants have shorter root hairs and root cell lengths, and are defective in both rhizobial and arbuscular mycorrhizal symbiotic colonization (Ivashuta et al., 2005). Microarray analysis also shows that silencing this gene alters expression of genes involved in cell wall formation and defense. RNAi techniques are currently being applied on a high-throughput basis to study 1,500 *M. truncatula* genes, primarily selected based on microarray expression studies, in an effort to understand the roles they may play in root development, nodule formation and function, and AM symbiosis (Colby Starker, personal communication).

10. Other Applications

Microarrays have also been used to address developmental questions, including one of the most controversial topics of rhizobial-legume symbiosis research: does DNA replication continue in developing bacteroids after cytokinesis ceases? In indeterminate nodules, DNA replication of the entire genome occurs in the absence of cell division, leading to genomes many times the size of those in free-living bacteria (Mergaert et al., 2006). Such is not the case for determinate nodules. That DNA endoreduplication is associated with bacteroid death upon nodule senescence raises interesting questions about the selective advantage of symbiosis for indeterminate nodule-forming bacterial species.

Microarrays are unlikely to replace traditional methods of studying genetic diversity such as those used to identify novel sequences in natural *S. meliloti* strains (Guo et al., 2005), but specialized arrays can be useful tools to support genetic analysis. For example, pools of mutants, each marked with a unique "signature-tag," can be screened for competitiveness. Their prevalence in a given population can be rapidly determined via hybridization to a tag-specific microarray (Pobigaylo et al., 2006). In addition, small, relatively inexpensive microarrays have also been used for genotyping rhizobial strains (Bailly et al., 2006; Bontemps et al., 2005). An advantage to this method is that no cultivation of the endosymbionts is required: typing can be directly carried out on bacterial DNA extracted from a single legume nodule. Recently, comparative genomic hybridization to a whole-genome *S. meliloti* array was applied to several previously uncharacterized *S. meliloti* strains. Interestingly, a higher proportion of the variable genes (mainly transposases and orphans) were located on pSymA (Giuntini et al., 2005). The natural strains, from northern Italy and near the Aral Sea, also appeared to contain the same large duplication found in the sequenced reference strain. Arrays have also identified differences in transcription profiles in different strains. A comparison of the two very closely related *S. meliloti* strains Rm1021 and Rm2011 were found to have 50 genes whose expression changed more than threefold. A substantial proportion of these genes represent phosphate starvation-induced genes that remain derepressed in Rm1021 under phosphate replete conditions, probably because of a 1-bp deletion in Rm1021 *pstC* (Krol and Becker, 2004; Yuan et al., 2006a).

11. Future Directions

Application of microarray analysis to understanding the nitrogen-fixing symbiosis has helped us to answer questions and to pose new ones. We now see that many genes are induced, in both partners, as the infecting bacterium seeks to evade the plant's defense response during the early stages of colonization. We do not yet have a full understanding of what each of these genes and their products are doing, particularly the many orphan genes of unknown function. Yet just knowing that they are involved is progress because researchers need to know who the players are that are in the game before they can assign them positions to play.

Discovery of the two initial signals, plant flavonoids/betaines and bacterial Nod factor, led to an explosion of new information about the interactions between the two partners. Yet we still do not know if there is a requirement for the *nod* genes, beyond their initial signaling role, for an effective symbiosis. Do they play a role aside from making Nod factor? Low molecular weight

EPSs are proposed to play a signaling role during invasion. Are Nod factor and EPS the only bacterial signals exchanged during symbiosis? It will be interesting to learn what other signals, if any, are exchanged between the two partners at each stage of the symbiosis. End-of-life issues are important as well: nodules eventually die (senesce) and molecular mechanisms driving the end of the interaction remain largely unexplored (Puppo et al., 2005). Recent transcriptome analysis (using cDNA-AFLP) in *M. truncatula* shows that nodule senescence is a developmental process where nodule tissue makes a transition from a carbon sink to a general nutrient source (Van de Velde et al., 2006). Interestingly, nodule and leaf senescence transcriptomes display considerable overlap, suggesting the use of similar regulatory circuitry and degradation pathways in both organs.

Continued exploitation of similarities between rhizobial, mycorrhizal and actinorhizal symbioses will stimulate progress in understanding these mutualistic interactions (Pawlowski and Newton, 2006). Similarities between rhizobia and pathogens can be exploited as well. Rhizobial core genomes are very similar to the core genomes of α-proteobacterial plant (e.g., *Agrobacterium*) and animal (e.g., *Brucella* and *Bartonella*) pathogens (Alsmark et al., 2004; DelVecchio et al., 2002; Goodner et al., 2001; Halling et al., 2005; Wood et al., 2001), and components common to parasitic symbioses and rhizobial mutualistic symbioses have already been identified. For example, *bacA* is required for effective infection of roots by *S. meliloti,* and of mammalian cells by *Brucella abortus* (Ferguson et al., 2002; Roop et al., 2002). Moreover, cyclic beta (1,2) glucans are required for infection in both of these and in *Agrobacterium tumefaciens* (Arellano-Reynoso et al., 2005; Breedveld and Miller, 1994). Surprisingly, the nodulation pathway may even have components in common with the response to root knot nematodes (Weerasinghe et al., 2005).

The degree of microbial diversity is simply breath-taking; new symbiotic interactions involving microbes are being described virtually every day. Research carried out on the agronomically vital rhizobial-legume symbioses can serve as road maps for study of other, less well understood interactions whether they be those of marine sponge symbionts, termite hindgut bacteria, or others.

Study of the rhizobial-legume symbiosis can aid us in addressing broad questions as well. One of the most vexing problems in evolutionary biology is how to explain maintenance of mutualistic cooperation between species. Host monitoring and "sanctions" on cheaters could favor cooperation: decreased oxygen supply may be one such possible sanction (Denison and Kiers, 2004; Kiers et al., 2003). Additional research should provide insights into the molecular and genetic bases for such sanctions whether they be direct or indirect. In addition, such research will aid our understanding of genome evolution. For example, among the α-proteobacteria, obligate intracellular pathogens appear to have undergone genome reductions whereas soil-dwelling plant symbionts appear to have greatly expanded their genomes (Sällström and Andersson, 2005). What did their common ancestor look like, and how does genome content correlate with niche and function?

A major ongoing mystery concerns the restriction of symbiotic nitrogen fixation, with the exception of *Parasponia*, to the legumes (Cronk et al., 2006; Hadri et al., 1998). It can be argued that engineering nitrogen-fixing non-legumes is a matter of national security in light of the role of petrochemicals in fertilizer manufacture in a deteriorating international situation. Recent efforts directed toward determining if such restriction constitutes an insurmountable barrier leave us with the tantalizing suggestion that this is a barrier which may be breached (Gleason et al., 2006). If this is indeed possible, only continued exploration and discovery can lead us toward this goal. Transcriptomic analyses, particularly the use of arrays, will continue to be important tools in achieving a fuller understanding of the rhizobial-legume symbiosis.

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