Plant and Bacterial Symbiotic Mutants Define Three Transcriptionally Distinct Stages in the Development of the Medicago truncatula/Sinorhizobium meliloti Symbiosis

Raka Mustaphi Mitra and Sharon Rugel Long*
Department of Biological Sciences, Stanford University, Stanford, California 94305

In the Medicago truncatula/Sinorhizobium meliloti symbiosis, the plant undergoes a series of developmental changes simultaneously, creating a root nodule and allowing bacterial entry and differentiation. Our studies of plant genes reveal novel transcriptional regulation during the establishment of the symbiosis and identify molecular markers that distinguish classes of plant and bacterial symbiotic mutants. We have identified three symbiotically regulated plant genes encoding a β1,3 endoglucanase (MtGLUL1), a lectin (MtLEC4), and a cysteine-containing protein (MtN31). MtGLUL1 is down-regulated in the plant 24 h after exposure to the bacterial signal, Nod factor. The non-nodulating plant mutant dmi1 is defective in the ability to down-regulate MtGLUL1. MtLEC4 and MtN31 are induced 1 and 2 weeks after bacterial inoculation, respectively. We examined the regulation of these two genes and three previously identified genes (MtCAM1, ENOD2, and MtLB1) in plant symbiotic mutants and wild-type plants inoculated with bacterial symbiotic mutants. Plant (bit1, rit1, and Misym1) and bacterial (exoA and exoH) mutants with defects in the initial stages of invasion are unable to induce MtLEC4, MtN31, MtCAM1, ENOD2, and MtLB1. Bacterial mutants (fix) and nifD) and a subset of plant mutants (dnf2, dnf5, dnf6, and dnf7) defective for nitrogen fixation induce the above genes. The bacA bacterial mutant, which senesces upon deposition into plant cells, and two plant mutants with defects in nitrogen fixation (dnf1 and dnf5) induce MtLEC4 and ENOD2 but not MtN31, MtCAM1, or MtLB1. These data suggest the presence of at least three transcriptionally distinct developmental stages during invasion of M. truncatula by S. meliloti.

The two partners in the legume-Rhizobium symbiosis navigate a complex developmental pathway, resulting in the formation of a plant-derived root nodule in which bacteria reside and reduce molecular nitrogen for use by the plant. Nodulation initiates with chemical signaling between the plant and the bacteria; the plant secretes flavonoid molecules into the rhizosphere, and the bacteria respond with lipochitooligosaccharide signaling molecules termed Nod factors (Long, 1996). The plant responds to bacterial Nod factors by initiating cortical cell divisions to build the nodule. Rhizobia enter the plant through a tubular plant-derived structure constructed in the root hair termed the infection thread (Bauer, 1981). Once the infection thread reaches the inner cortical cells of the plant, the bacteria are released into the plant cell, encapsulated in plant membranes. Within these symbiosomes, the bacteria differentiate into bacteroids that in the presence of low oxygen tension express the proteins necessary to reduce molecular nitrogen into ammonia for transport to the plant (Oke and Long, 1999b).

Bacterial mutants have revealed distinct developmental signals and stages in the progression of the symbiosis. Bacterial mutants that cannot provoke the cell divisions necessary for nodule formation (Nod−) have defects in the genes required for Nod factor synthesis (Spaink, 2000). Bacterial mutants with defects in cell surface polysaccharides are unable to successfully infect the plant (Gonzalez et al., 1996). The bacA bacterial mutant, which has a defect in a putative peptide antibiotic transporter and compromised membrane integrity, senesces upon deposition into plant cells, suggesting an environmental change from infection thread to symbiosome (Glazebrook et al., 1993; Ichige and Walker, 1997). In addition, bacterial mutants that can successfully invade the plant, begin bacteroid development, but cannot fix nitrogen have defects in the signaling or enzymatic components necessary for the reduction of molecular nitrogen into ammonia (Ruvkun et al., 1982; David et al., 1988).

Four broad classes of plant symbiotic mutants have been identified: mutants that cannot make nodules (Nod−), mutants with defects in infection, mutants that make nodules that cannot fix nitrogen (Fix−), and mutants that make an excessive number of nodules (supernodulator; Bénaben et al., 1995; Penmetsa and Cook, 1997; Catania et al., 2000, 2001; Penmetsa et al., 2003; C.C. Starker, L. Smith, G.E.D. Oldroyd, J. Doll, and S.R. Long, unpublished data). The Nod− mutants of Medicago truncatula have been characterized for cell signaling responses, cell growth, and...
transcriptional responses to bacterial signals. For example, the Nod⁻ plant mutant dmil perceives Nod factor, showing a calcium flux and root hair swelling, but is unable to mount a calcium spiking response or initiate the expression of genes normally induced in the symbiosis (Catoira et al., 2000; Wais et al., 2000; Shaw and Long, 2003a). In contrast to the Nod⁻ mutants, few infection or Fix⁻ mutants of M. truncatula have been characterized. The MtSYM1 mutant forms two classes of nodules when infected with Sinorhizobium meliloti: small, round nodules in which the infection aborts in the outer cortical cells of the root and elongated nodules in which bacteria are released but are unable to differentiate into nitrogen fixing bacteroids (Bénaben et al., 1995). The ratio of nodule classes is dependent on the bacterial strain with which the plants are inoculated; 70% of the nodules induced by Rm2011 versus 30% of those induced by CC169 are small and round. A recent genetic screen in M. truncatula has identified two new infection mutants and a panel of seven Fix⁻ mutants (C.G. Starker, L. Smith, G.E.D. Oldroyd, J. Dal, and S.L. Long, unpublished data). When inoculated, the mutants with infection defects, rII1 and bit1, induce small bumps on the root surface and infection threads that abort in the epidermis or outer cortex of the root. Unlike the infection mutants, the Fix⁻ mutants (dnf [defective in nitrogen fixation] mutants) form infection threads that proceed through the cortical cells and produce limited nodule-like structures. Based on light microscopy, the infection threads of these Fix⁻ mutants are indistinguishable from those of wild-type plants. Although broad categories of infection and fixation mutants have been established, in general, the nature of the mutations responsible for the defects is unclear.

The characterization of symbiotically required and regulated genes provides insight into the molecular events in the symbiosis. In some cases, plant genes responsible for symbiotic defects have been shown to be symbiotically regulated (Schauer et al., 1999; Kruzell et al., 2002; Nishimura et al., 2002). In M. truncatula, several genes induced during nodulation, termed “nodulins” or “enods” (early nodulins), have been identified. RIP1 encodes a peroxidase that is induced preceding infection of the plant (Cook et al., 1995) and may have a role in protection of the plant from oxidative damage (Ramu et al., 2002). ENOD2, an early nodulin, is induced during infection (Dickstein et al., 1988) and has been successfully used as a marker for nodule organogenesis. Leghemoglobin, a protein that is necessary to maintain low oxygen levels within the nodule, is induced in mature nodules (Gallusci et al., 1991). More recently, with the accumulation of M. truncatula expressed sequence tags (ESTs), bioinformatics approaches have allowed for screening of regulated genes based on expression levels in different libraries (Fedorova et al., 2002; Journet et al., 2002) and the identification of novel nodulins, such as a nodule-specific calmodulin-like protein. To date, genes that are suppressed early in the symbiosis have not been described.

We have studied symbiotically regulated genes to gain clues about the initial physiological events of the plant in the establishment of the symbiosis and to distinguish Fix⁻ plant mutants that appeared phenotypically indistinguishable. We report the identification of one gene suppressed early in the symbiosis, encoding a β, 1–3 endoglucanase, and two genes induced later in the symbiosis: a lectin and a novel gene. Using the expression of these two genes and three previously identified nodulins, we were able to distinguish between infection and fixation mutants of both the plant and bacteria, defining three transcriptionally distinct developmental stages in the symbiosis.

RESULTS

Identification of Three Symbiotically Regulated M. truncatula Genes

We used two approaches to identify plant genes that are differentially regulated in the symbiosis and, thus, act as markers for distinct developmental stages. First, we used subtractive hybridization (Diatchenko et al., 1999) in an attempt to produce a library enriched for symbiotically induced plant genes (see “Materials and Methods”). This screen did not yield any reliably up-regulated genes; however, northern-blot analysis of 45 library clones identified a clone (no. 108) that was weakly suppressed 24 to 48 h after plants were flood inoculated with S. meliloti (Fig. 1A). Although subtracted libraries enrich for differentially regulated genes, factors such as transcript abundance result in the representation of randomly amplified genes in the library (Ji et al., 2002). Clone 108 was suppressed 2.9–1.1-fold at 24 h and 3.2–1.2-fold at 48 h postinoculation with S. meliloti (n ≥ 5, Fig. 1A). Under the same conditions, the positive control RIP1 was induced 4.5–2.7-fold at 24 h and 6.4–2.6-fold at 48 h postinoculation (n ≥ 4, Fig. 1A). We used the BLASTX algorithm to determine homology of newly identified genes to previously described proteins (http://www.ncbi.nlm.nih.gov/BLAST/). The full-length sequence containing the identified clone (TC78899) shows homology to the glucan endo-1,3-β-glucosidases GL153 (E value = e-101) and PR2 (E value = 5e-98) from tobacco (Nicotiana tabacum), which are members of the pathogenesis-related protein 2 (PR2) family of genes. These genes are rapidly induced in leaves upon exposure to pathogens (Ward et al., 1991). Based on homology to β-glucosidases, we refer to this gene as MtBGLU1.

Second, we employed a bioinformatics approach, taking advantage of the publicly available M. truncatula EST database to identify induced genes (see “Materials and Methods”). At The Institute of Genomic
Research, *M. truncatula* ESTs are compiled into longer tentative consensus sequences (TCs) based on regions of overlap (Quackenbush et al., 2001). By comparing the representation of TCs in libraries from uninoculated and *S. meliloti*-inoculated roots, we identified nine candidates for further analysis. Using northern blotting to assess transcript abundance, we identified two transcripts strongly expressed in nodules but not roots: TC77066 and TC86036. TC77066 is induced 7 d postinoculation, and TC86036 is induced 2 weeks postinoculation with *S. meliloti* (Fig. 1B). In addition, we examined the expression of a recently identified nodule specific calmodulin-like protein (herein referred to as MtCAM1, Gen Bank accession no. AF494212; Fedorova et al., 2002), which shows induction at 2 weeks postinoculation. Under these conditions, ENOD2 and Leghemoglobin 1 (*MtLB1*), two previously characterized nodulins, are induced at 1 and 2 weeks postinoculation, respectively. TC77066 encodes a legume-specific lectin with closest homology (E value/GenBank accession no. H11005) to bark lectins of *Robinia pseudoacacia* (Van Damme et al., 1995). Lectins are carbohydrate-binding proteins that have been suggested to have roles in storage of nutrients, defense against pathogen attack, and rhizobial recognition (Van Damme et al., 1998). Although TC77066 does not have significant homology with previously identified nodule-specific *M. truncatula* lectins (Bauchrowitz et al., 1992), we refer to it as *MtLEC4* using the previously established naming system for *M. truncatula*. TC86036 has weak homology (E value/GenBank accession no. H11005) to a nodule-specific protein from *Galega orientalis* (GenBank accession no. CAB51773; Kaijalainen et al., 2002) and putatively encodes a Cys-containing protein (CCP) with a conserved signal peptide and nodule-enhanced expression (Fedorova et al., 2002; Mergaert et al., 2003). We refer to this transcript as *MtN31* (*M. truncatula* nodulin 31) according to previously established nomenclature (Gamas et al., 1996). This transcript is synonymous with the gene product designated as NCR 158 (for Nodule-specific Cys-rich protein) by Mergaert et al. (2003).
consequence, the mutant does not elicit early signaling responses (Wais et al., 2002) or induce nodule formation on the plant (S.R. Long., unpublished data). The SL44 bacterial mutant did not suppress the expression of MtBGLU1 (Fig. 1A), indicating the importance of Nod factor for the regulation of this gene. To determine whether Nod factor was sufficient to down-regulate MtBGLU1 expression, we treated plants with 100 pm Nod factor (Fig. 2). Expression of MtBGLU1 was suppressed at both 24 (2.5- ± 1.0-fold) and 48 (2.1- ± 0.6-fold) h posttreatment. Under these conditions, RIP1 was induced 2.2- ± 0.9-fold at 24 h and 2.5- ± 0.5-fold at 48 h postinoculation. These results suggest that Nod factor is the bacterial signal that triggers suppression of MtBGLU1.

The Nod^−^ Plant Mutant, dmi1, Is Defective for MtBGLU1 Suppression

To further validate the importance of Nod factor for suppression of MtBGLU1, we tested the expression of this gene in a plant mutant defective in Nod factor signaling. The dmi1 mutant responds to Nod factor but cannot initiate calcium spiking, gene expression, or nodule morphogenesis (Catoira et al., 2000; Wais et al., 2000; Shaw and Long, 2003a). In the mutant, MtBGLU1 transcript levels differed from the wild type (1.3- ± 0.8-fold), and RIP1 transcript levels were unchanged (0.7- ± 0.4-fold) 48 h postinoculation (n = 5, Fig. 3). This result suggests that dmi1 is defective in the ability to completely suppress MtBGLU1. Previous studies showed a slight induction of RIP1 in dmi1 mutants indicating possible leakiness of the mutation or an inability to sustain early gene expression changes in the mutant (Catoira et al., 2000). Our data are consistent with the theory that down-regulation of MtBGLU1 expression is part of a pathway leading to successful bacterial invasion and nodulation.

Nodulins Show Altered Expression in Response to Bacterial Mutants with Defects in Alfalfa (Medicago sativa) Infection or Nitrogen Fixation

The timing of MtLEC4 and MtN31 expression suggests that they are induced later in the symbiosis, perhaps in response to bacterial invasion or nitrogen fixation. To test this hypothesis, we inoculated wild-type plants with S. meliloti mutants with known defects in infection of alfalfa or nitrogen fixation. Two mutants, exoA (Rm7031) and exoH (DW223), have defects in infection of alfalfa and exopolysaccharide biosynthesis (Finan et al., 1985; Leigh et al., 1985, 1987). We expect exoA mutants have a similar invasion defect as exoY mutants, which are blocked for the exopolysaccharide biosynthetic step preceding that which requires exoA (Reuber and Walker, 1993). exoY and exoH bacterial mutants induce infection threads that abort in the root hair cell (Fig. 4; Cheng and Walker, 1998). The bacA bacterial mutant can invade alfalfa, is deposited into plant cells, but senescence before developing into mature bacteroids (Fig. 4; Glazebrook et al., 1993). Both fixJ and nifD mutant bacteria, which cannot make nitrogenase, are released into alfalfa cells and can differentiate into elongate bacteroids, but the bacteria typically degenerate and the nodule prematurely senescence (Fig. 4; Ruvkun and Ausubel, 1981; Hirsch et al., 1982; Ruv
Both MtLEC4 and MtN31 showed altered expression in aberrant nodules formed by the bacterial mutants tested. The exoA and exoH mutants failed to induce expression of either MtLEC4 or MtN31 (Fig. 5A). The bacA mutant induced MtLEC4 but not MtN31. Both the fixJ and nifD mutants induced MtLEC4 to normal levels and MtN31 to reduced levels when compared with wild-type bacteria (Fig. 5A). The previously identified nodulins MtCAM1, ENOD2, and MtLB1 also exhibited altered expression in response to bacterial mutants (Fig. 5A). ENOD2 and MtLEC4 show similar expression patterns with no induction by exoA and exoH and induction by bacA, fixJ, and nifD mutants. This result contrasts with previous studies of alfalfa in which ENOD2 expression was induced by bacteria with defects in exopolysaccharide production (Dickstein et al., 1988, 1991). MtLB1 and MtCAM1 showed expression patterns similar to MtN31 (Fig. 5A). Both were induced by fixJ and nifD bacteria to reduced levels as compared with the wild type, and neither was induced by any other mutant strains tested. Acetylene reduction of fixJ and nifD mutants showed that all were unable to fix nitrogen, confirming the mutant phenotype of the bacteria throughout nodulation (n > 30 for each mutant; data not shown).

Photographs of M. truncatula nodules at the developmental stage used for transcript analysis illustrate the morphology of nodules induced by bacterial mutants (Fig. 5B). Although alfalfa makes large nodule-like structures in response to bacterial mutants with exopolysaccharide defects (Yang et al., 1992), we noted that M. truncatula inoculated with exoA or exoH mutants produces bumps that are much smaller than the nodules induced by the other bacterial mutants tested (Fig. 5B). It is not known whether bacterial infection arrests in the same manner in both M. truncatula and alfalfa, nor whether bacA, fixJ, or nifD bacterial mutants exhibit the same infection defects in M. truncatula as in alfalfa. However, if the developmental blocks are similar in both organisms, then these data suggest that the induction of MtLEC4 and

---

**Figure 4.** Model of infection and fixation defects. Bacterial (blue lettering) and plant mutants (black lettering) define distinct developmental blocks in the symbiosis. Bacterial invasion of plants (blue) can be arrested at one of three points: before inner cortical cell penetration (upper), after release into plant cells (upper middle), and after differentiation into bacteroids (lower middle). These phases can be transcriptionally separated using the expression patterns of MtLEC4, ENOD2, MtN31, MtCAM1, and MtLB1.

**Figure 5.** MtLEC4, MtN31, MtCAM1, ENOD2, and MtLB1 exhibit altered expression in response to bacterial mutants. a, Representative northern blots of RNA from M. truncatula plants exposed to bacterial mutants: exoA (Rm7031), exoH (DW223), bacA (VO2119), fixJ (VO2683), and nifD (VO2746) or to wild-type bacteria (Rm1021) for 4 weeks. Experiments were repeated at least three times. The upper blot was serially hybridized to an MtLEC4, actin probe. The lower blot was serially hybridized to an MtCAM1, MsENOD2, Actin, or MtLB1 probe. b, Representative photographs of M. truncatula root sections inoculated with each bacterial mutant. All photographs are at the same magnification. Scale bar = 0.5 mm.
ENOD2 in *M. truncatula* correlates with sustained plant infection, cell divisions, or cell expansion, whereas the initial induction of *MtN31, MtCAM1*, and *MtLB1* correlates with the presence of elongate bacteroids within the plant cells of the nodule (Fig. 4).

**Nodulins Show Altered Expression in Plants**

**Mutant for Bacterial Entry or Nitrogen Fixation**

Because the late nodulins distinguish three transcriptionally distinct classes of bacterial mutants, we tested whether they also distinguish classes of plant mutants that appear otherwise phenotypically similar. We examined the expression of these genes in 11 plant mutants with defects in infection or nitrogen fixation. Three infection mutants, *rit1, bit1*, and *Mtsym1*, have defects in the ability of the infection thread to penetrate past the outer cortical cells of the plant (Fig. 4; Bénaben et al., 1995; C.G. Starker, L. Smith, G.E.D. Oldroyd, J. Doll, and S.L. Long, unpublished data). Although *Mtsym1* can induce either small or elongate nodules, in our conditions, with the bacterial strain Rm1021, elongate nodules were never observed (data not shown). Seven *M. truncatula* mutants, *dnf1* to *dnf7*, have defects in nitrogen fixation. Although infection threads in these mutants are indistinguishable from the wild type based on light microscopy, it is unknown whether bacteria can differentiate into bacteroids (Starker et al., unpublished data).

As observed with bacterial infection mutants, the plant mutant *bit1*, which has defects in early infection, never induced *MtLEC4, MtN31, MtCAM1, ENOD2*, or *MtLB1* (Fig. 6). In most cases, these genes were not induced in *Mtsym1* and *rit1*, although for each mutant in one of four northern blots, weak expression of *MtLEC4 (Mtsym1)* or *MtLEC4 and MtN31 (rit1)* was observed. This result may indicate that the mutations responsible for the infection defects are leaky. Two of the Fix– plant mutants, *dnf1* and *dnf5*, showed gene expression similar to that observed when wild-type plants were inoculated with *bacA* bacterial mutant; only *MtLEC4* and *ENOD2* were induced in these mutants. In contrast, the remaining Fix– mutants induced all the genes tested (Fig. 6). These data suggest the presence of at least three distinct transcriptional phases in the symbiosis that can be correlated with phenotypes of both plant and bacterial mutants (Fig. 4).

**DISCUSSION**

We report the identification of three genes regulated in the legume-Rhizobium symbiosis and use them in concert with three previously identified nodulins to study plant and bacterial symbiotic mutants.

Although several symbiotically induced plant genes have been identified previously (Cook et al., 1995; Gamas et al., 1996; De Carvalho-Niebel et al., 1998), this study contains the first report, to our knowledge, of a gene, *MtBGLU1*, which is suppressed early in the symbiosis. Our results suggest that the same signal that induces expression of early nodulins, the bacterial signal Nod factor, is also sufficient to suppress *MtBGLU1*. This suppression is part of a Nod factor signaling pathway downstream of Nod factor recognition because the Nod factor signaling plant mutant *dni1* is defective in the ability to down-regulate *MtBGLU1*. This finding adds evidence to the theory that bacterial recognition can trigger suppression of plant pathways (Shaw and Long, 2003b), although the mechanism for concurrent induction and suppression is unclear. Perhaps the nod factor signal transduction machinery can function both in an inhibitory and activating manner toward different promoters. Alternatively, a branch point in the transduction of the Nod factor signal may allow the activation of different sets of molecular machines to either induce or suppress genes.

Based on homology, *MtBGLU1* encodes a β,1-3 endoglucanase with high homology to pathogenesis-induced proteins, GL153 and PR2. Although it is unclear whether these proteins exhibit antifungal activity (Sela-Buurlage et al., 1993), they are markers indicating the initiation of a plant defense response to pathogens (Ward et al., 1991). Because the PR2 class of genes is induced as part of plant defense to pathogens, the suppression of *MtBGLU1* we observe in response to a symbiont may indicate a suppression of plant defenses. Preliminary studies indicate that *MtBGLU1* is not induced by a benzothiadiazole de-
rivative (data not shown), which can normally induce plant defense responses in the salicylic acid pathway (Friedrich et al., 1996); however, recent work using fungal elicitors provides evidence for the suppression of defenses early in nodulation. Fungal oligosaccharide elicitors trigger a rapid oxidative burst in *M. truncatula* roots (Shaw and Long, 2003b). Nod factor suppresses this oxidative burst in wild-type plants; however, it is unable to do so in the Nod^-^ plant mutant *nfp*. These data suggest a direct link between Nod factor suppression of defenses and induction of nodulation.

Although our in silico examination of nodulins identified two nodulins, *MILEC4* and *MtN31*, not all candidates tested showed differential expression. Previous studies validating expression patterns of 91 predicted nodule-enhanced TCs using macroarray hybridizations showed that TCs specified by five or more ESTs were reliably induced in nodules (Fedorova et al., 2002). Under our conditions, this was not the case. For example, the expression pattern, TC32071, comprised of 32 ESTs from inoculated and nodule libraries and no ESTs from uninoculated root libraries, was validated using macroarrays (Fedorova et al., 2002). Using northern blotting, we were unable to confirm differential expression of this TC. The discrepancy in the validation of in silico predictions may be explained by the technique used, differences in the growth conditions, nodulation kinetics, or the bacterial strain used.

*MtN31* belongs to a large family of nodule-specific CCPs (Fedorova et al., 2002; Mergaert et al., 2003). CCP gene family members have only been identified to date in galegoide legumes forming indeterminate nodules (Mergaert et al., 2003). These genes are transcriptionally up-regulated in nodules, although the initiation of gene induction can vary between 7 and 13 d after inoculation (Mergaert et al., 2003). Previous macroarray hybridization studies of gene expression of 14 CCPs under a series of conditions yielded parallel observations as those we report for *MtN31* (Mergaert et al., 2003). As observed with *MtN31*, the 14 CCPs are induced by a bacterial mutant defective in nitrogen fixation (*fixG*; Mergaert et al., 2003). Although induction of the 14 CCPs is greatly reduced in plants inoculated with bacterial mutants defective in exopolysaccharide production (*exoB*) or with a mutation in *bacA*, the authors note a limited plant transcriptional response to these mutants (Mergaert et al., 2003). We never observed induction of *MtN31* in response to *exoA* or *bacA* bacterial mutants; this difference can be attributed to the sensitivity or fidelity of the assay used.

The developmental blocks defined by five bacterial mutants and 11 plant mutants fell into three classes (Fig. 4). The first class, including bacterial (*exoA* and *exoH*) and plant (*bit1*, *rit1*, and *Misym1*) mutants defective in infection, was unable to induce any of the nodulins tested, and inoculation resulted in the formation of small bumps on the surface of the root. Previous studies using alfalfa (Dickstein et al., 1988) showed that bacterial mutants with defects in exopolysaccharide biosynthesis or nitrogen fixation could induce *ENOD2* expression and large nodule-like structures that lack a persistent meristem (Yang et al., 1992). Under our conditions, alfalfa constructs larger nodule-like structures than *M. truncatula* when inoculated with *exoA* or *exoH* bacterial mutants (data not shown). In addition, alfalfa, unlike *M. truncatula*, can induce nodule formation in the absence of Rhizobia (Truchet et al., 1989) and allow effective nodulation by *ipsB* bacterial mutants (Niehaus et al., 1998). These data suggest that the development of the symbiosis is more tightly controlled in *M. truncatula* than in alfalfa and that the induction of *ENOD2* and *MILEC4* requires that the symbiosis progress past the developmental block induced by early infection mutants.

The second class of bacterial (*bacA*) and plant (*dnf1* and *dnf5*) mutants, which showed infection of the inner cortex, induced *ENOD2* and *MILEC4* and created larger nodule-like structures than those induced by early infection mutants (Fig. 4). *MILEC4* has homology to bark lectins. The role of lectins in the legume-Rhizobium symbiosis has been well studied (Hirsch, 1999). Most research has focused on the role of lectins in nod factor recognition or bacterial attachment. The heterologous expression of a pea (*Pisum sativum*) lectin in alfalfa enhances nodule formation by non-host bacteria that generate host-specific nod factor (van Rhijn et al., 2001). In *Dolichos biflorus*, a nod factor-binding lectin with apyrase activity is localized to root hairs (Etzler et al., 1999; Kalsi and Etzler, 2000). Lectins have been found in nodules of several species (VandenBosch et al., 1994; Bauchrowitz et al., 1996; Kardailsky et al., 1996), and alfalfa plants expressing antisense versions of nodule-enhanced lectins show severe developmental defects (Brill et al., 2001). Previously studied lectins should be assessed for their induction pattern in plant mutants and in wild-type plants inoculated with bacterial mutants to determine whether they show similar expression patterns to *MILEC4*. Although the role of *MILEC4* is unknown, its induction is correlated with bacterial penetration of the root cortex and sustained cell divisions; thus, *MILEC4* may have a role in the formation of a persistent nodule meristem or in the release of bacteria into plant cells. Future studies that biochemically define the lectin substrate may offer clues as to the role of the lectin in the recognition of plant or bacterial carbohydrates.

The final class of bacterial (*fixJ*) and *nifD* and plant mutants (*dnf2*, 3, 4, 6, and 7) induced all the genes tested and created large nodule-like structures but still had defects in nitrogen fixation (Fig. 4). Interestingly, *fixJ* bacterial mutants could not induce *MtN31*, *MtCAM1*, and *MtLB1* to the same level as was induced by *nifD* bacterial mutants (Fig. 5A). Both mutants are unable to fix nitrogen, but the *FixJ* defect is
upstream of the NifD defect and may result in transcriptional misregulation of additional bacterial genes. The differential response of the plant to fix and nifD mutant bacteria may suggest that the plant monitors bacterial physiology, bacterial development, or the availability of reduced nitrogen closely after bacteroid differentiation.

The three classes of infection and fixation mutants may indicate the presence of three discrete checkpoints in the progression of nodulation. It will be interesting to determine what signals mediate these potential checkpoints. Our studies of the transcriptional profile of two groups of genes revealed similar developmental arrests induced in plant mutants and in wild-type plants inoculated with bacterial mutants. The identification of correlated phenotypes suggests a complex interplay between the two partners in which they coordinate symbiotic development. Future studies examining the mechanism of this coordination will be useful in determining whether environmental, physiological, metabolic, or signaling components are important for the development of a successful symbiosis.

MATERIALS AND METHODS

Plant Growth Conditions

Medicago truncatula (Gaertn.) cv Jemalong A-17 seeds were scarified using concentrated sulfuric acid for 10 to 15 min, rinsed with sterile water, and sterilized in commercial bleach for 3 min. After rinsing to remove residual bleach, seeds were imbibed in sterile water for 4 to 12 h while shaking and were stored under water at 4°C to be used within a week. Mutant seeds were prepared in the same manner except rit1 seeds, which were scarified for 3 min and sterilized for 3 min in a solution of 2% (v/v) commercial bleach for 10 to 15 min, rinsed with sterile water, and then diluted in 0.5 ml

Bacterial Strains and Nod Factor

Sinorhizobium meliloti strain Rm1021 is a streptomycin-resistant derivative of wild-type field isolate SU47 (Meade et al., 1982), cxva (Rm703; Leigh et al., 1985), cxvii (DW223; Wells and Long, 2002), bacA (VO219; Oke and Long, 1999a), fixJ (VO2683; Oke and Long, 1999a), and SL44 (Fisher et al., 1988) mutant strains were published previously. The nifD mutant (VO2746) was created by transduction of the mutation from Rm13125p (Barnett et al., 1998) into Rm1021 (V. Oke, unpublished data).

Bacteria were grown in liquid tryptone-yeast extract (Beringer, 1974) supplemented with appropriate antibiotics. Antibiotics were used at the following concentrations: 500 μg ml−1 streptomycin, 50 μg ml−1 spectinomycin, and 50 μg ml−1 neomycin. For inoculations, bacteria were pelleted, washed in 0.5× BNM, and then diluted in 0.5× BNM to an approximate OD600 of 0.03 (approximately 3×10^7 cfu ml−1). Plant roots were flooded inoculated 5 d after planting with 10 ml of inoculum per plate (245×245×20 mm, Naglene Nunc Intl, Naperville, IL). NodRm-IV (Ac and S) was purified previously (Ehrhardt et al., 1996) and diluted to 100 pm in 0.5× BNM and applied as above.

RNA Preparation and Northern Hybridizations

RNA was purified with Trizol (Invitrogen, Carlsbad, CA) using the manufacturer’s protocol for tissues with high lipid content. Roots were harvested and ground in liquid nitrogen using a mortar and pestle before Trizol addition. If necessary, further homogenization was performed using a Polytron homogenizer (PT 10/35, Brinkman, Westbury, NY). Poly(A⁺) RNA was purified using the Qiagen Oligoex mRNA Mini kit (Qiagen, Valencia, CA). To verify yield and quality of RNA, samples were subject to spectrophotometric examination (Sambrook et al., 1989) and gel electrophoresis. For each preparation, approximately 40 μg of total RNA was isolated from 30 roots. Five to 15 micrograms of total RNA was electrophoresed on a 1.2% (w/v) agarose gel with 6.2% (v/v) formaldehyde in MOPS buffer, and RNA was transferred to a nylon membrane (Hybond N⁺, Amersham Pharmacia Biotech, Buckinghamshire, UK). PCR or reverse transcriptase-PCR was used to generate DNA fragments for probing northern blots. Primer pairs and probe lengths used for newly identified genes are: MibGLU1 (CTCTTGATGCCAATATTAGACTGATA and TAAAGATTGCCAATCTCACCAC, 449 bp), MleLCE4 (TGAAGTGAAGACCATGAGTAGATG and CACAGTTATCTCAACCTTCCCAAG, 675 bp), and Min3 (ATCAACTTTGGAAATAATTAGGTG and CCTATTTCATGATAACCAAGCA, 505 bp). A 670-bp DNA fragment representing previously published probe sequences was used for PGVN-5510 (Fedorova et al., 2002). A 419-bp fragment of M. truncatula MtLBI1 (Galluscii et al., 1991), a 419-bp fragment of M. truncatula RIPI (Cook et al., 1995), and a 209-bp fragment of alfalfa (Medicago sativa) ENOD32 (Dickstein et al., 1998) were used to represent known nodulins. Actin was used as a loading control (Oldroyd et al., 2001). Radiolabeled DNA probes were generated using the Amplers Rediprime II Random Prime Labeling System. Radioactivity on northern blots was quantitated using a phosphor imager to give a calculated signal “volume” (pixel density × area of band) for each band on the blot (Bio-Rad GS-363 Molecular Imager, Bio-Rad, Hercules, CA). For calculations of fold suppression of MibGLU1, normalized for loading, the following equation was used:

Fold suppression of Mibglu1 = [volume MibGLU1actin/volume actinactin] / [volume MibGLU1trans/volume actintrans]

A reciprocal equation was used to calculate fold induction of RIPI. The average fold change and so of the fold change are calculated for each gene.

Subtractive Hybridization

Subtractive hybridization was performed on 8 μg of Poly(A⁺) RNA using the PCR-Select subtractive hybridization kit (CLONTECH, Palo Alto, CA). Plant roots were exposed to buffer or S. meliloti for 6 h. In an attempt to enrich for plant genes induced early in the symbiosis, RNA derived from buffer inoculated roots was subtracted from RNA derived from bacterial inoculated roots.

Database Analyses

Excel (Microsoft, Redmond, WA) was used to analyze the M. truncatula Gene Index (MtGI) Release 4.0 (http://www.tigr.org/tdb/mtgi) and identify TCS containing ESTs from Rhizobia-inoculated or nodule libraries. In an attempt to eliminate constitutively expressed genes, TCs that were represented in uninoculated root or leaf libraries were omitted from further analyses. To identify putative Rhizobium-induced genes, the number of ESTs from inoculated or nodule libraries was tallied for each TC. TCs were then sorted based on this factor normalized for library size. Nine genes with interesting homology or with the most extreme nodule-specific representation were examined by northern hybridization. TCs representing these genes were numbered as follows: 28734, 32071, 32103, 35875, 35941, 36232, 39290, 39747, and 39884 and have been renumbered in the current version of MtGI (4.0). MtLEC4 and MtN31 were represented by TCs 32103 and 35875, respectively. In the current release of MtGI (7.0), Min31 and Mibglu1 represented by TCs 77544, 77066, and 77544, and 85172 (TCs that were split into two are indicated by a slash). Four interesting homology or with the most extreme nodule-specific representation were examined by northern hybridization. TCs representing these genes were numbered as follows: 28734, 32071, 32103, 35875, 35941, 36232, 39290, 39747, and 39884 and have been renumbered in the current version of MtGI (4.0). MtLEC4 and MtN31 were represented by TCs 32103 and 35875, respectively. In the current release of MtGI (7.0), Min31 and Mibglu1 represented by TCs 77544, 77066, and 85172 (TCs that were split into two are indicated by a slash). Four

Distribution of Materials

Upon request, all novel materials described in this publication will be made available in a timely manner for noncommercial research purposes.
subject to the requisite permission from any third party owners of all or parts of the material. Obtaining any permissions will be the responsibility of the requestor.

ACKNOWLEDGMENTS

We would like to thank Valerie Oke (University of Pittsburgh) for the nifD strain V02746, Harita Veereshlingam and Rebecca Dickstein (University of North Texas, Denton) for providing the plasmid template for the Eno2 probe, and Susan Miller and Carroll Vance (University of Minnesota, St. Paul) for providing pGVN-5510. Colby Starker generously provided the seed for rit1, hit1, and the dnf1 mutants and probe for MILR1. Lucinda Smith (Stanford University, Stanford, CA) produced the seed for the dmi1 mutant. We thank former and current members of the lab, especially Colby Starker (Stanford University, Stanford, CA), Sidney Shaw (Stanford University, Stanford, CA), Giles Oldroyd (John Innes Centre, Norwich, UK), Melicent Peck (Stanford University, Stanford, CA), David Keating (Loyola University Chicago, Maywood, IL) and Robert Fisher (Stanford University, Stanford, CA) for critical reading of the manuscript before publication.

Received August 14, 2003; returned for revision August 27, 2003; accepted October 13, 2003.

LITERATURE CITED


Transcriptionally Distinct Symbiotic Stages of Medicago truncatula


