Transcriptomic Analysis of *Sinorhizobium meliloti* and *Medicago truncatula* Symbiosis Using Nitrogen Fixation–Deficient Nodules

Claus Lang and Sharon R. Long

Department of Biology, Stanford University, Stanford, CA 94305-5020, U.S.A.

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The bacterium *Sinorhizobium meliloti* interacts symbiotically with legume plant hosts such as *Medicago truncatula* to form nitrogen-fixing root nodules. During symbiosis, plant and bacterial cells differentiate in a coordinated manner, resulting in specialized plant cells that contain nitrogen-fixing bacteroids. Both plant and bacterial genes are required at each developmental stage of symbiosis. We analyzed gene expression in nodules formed by wild-type bacteria on six plant mutants with defects in nitrogen fixation. We observed differential expression of 482 *S. meliloti* genes with functions in cell envelope homeostasis, cell division, stress response, energy metabolism, and nitrogen fixation. We simultaneously analyzed gene expression in *M. truncatula* and observed differential regulation of host processes that may trigger bacteroid differentiation and control bacterial infection. Our analyses of developmentally arrested plant mutants indicate that plants use distinct means to control bacterial infection during early and late symbiotic stages.

Nitrogen fixation by rhizobial bacteria in symbiosis with legumes is an important component of the global nitrogen cycle (Vance 2001). Rhizobium-legume symbioses involve the formation of specialized plant organs called root nodules, in which bacteria receive plant-derived carbohydrates in exchange for reducing dinitrogen to plant-usable ammonia. Nodulation is initiated when plant root compounds such as flavonoids trigger production of a bacterial signal, Nod factor. Perception of this lipochitooligosaccharide by specific plant receptors alters root hair growth and stimulates dedifferentiation of cortical root cells to establish the nodule primordium (Xiao et al. 2014). Bacteria invade the developing nodule via plant-produced infection threads. Invasion requires live bacterial cells that produce one or more extracellular polysaccharides (EPS), such as succinoglycan (EPS-I) or galactoglucan (EPS-II) (Cheng and Walker 1998; Pellock et al. 2000). At the tips of infection threads, bacteria are ultimately released into invasion-competent host cells by an endocytosis-like mechanism to form organelle-like structures called symbiosomes. In *M. truncatula* and some other legumes, host cells

and bacteria within symbiosomes undergo a differentiation process involving genome endoreduplication and morphological changes (Kondorosi et al. 2013). Nitrogen fixation is carried out by endosymbiotic bacteroids, not by free-living cells.

M. truncatula produces indeterminate nodules with a distal tip meristem. Continuous division of plant meristem cells, infection, and differentiation result in a spatial zonation of developmental stages within the nodule organ along a distal to proximal axis. Based on plant cell morphology, five zones can be distinguished in *Medicago* nodules (Fig. 1A): the meristematic zone I; infection zone II; interzone II-III; nitrogen-fixation zone III; and senescence zone IV (Vasse et al. 1990). Each zone contains characteristic bacterial developmental stages, ranging from free-living-like bacteria within infection threads in the infection zone to elongated, Y-shaped bacteroids (type 4) in the nitrogen-fixation zone and senescing bacteroids (type 5) in the senescence zone (Fig. 1A) (Vasse et al. 1990).

Genome-wide transcription profiling can identify symbiotically regulated plant and bacterial genes. Initial comparisons of free-living bacteria to whole root nodules revealed that the expression levels of 10 to 20% of all *S. meliloti* genes change during symbiosis (Barnett et al. 2004; Becker et al. 2004; Capela et al. 2006). Several different approaches have been used to study transcriptomic changes during nodule development. Nodulation time-course experiments were carried out to identify early and late symbiosis genes in *S. meliloti* (Capela et al. 2006) and *M. truncatula* (Maunoury et al. 2010). More recently, laser-capture microdissection (LCM) has been used to study plant (Limpens et al. 2013; Roux et al. 2014) and bacterial (Roux et al. 2014) gene expression in different nodule zones. In addition, LCM was used to compare *M. truncatula* gene expression during rhizobial and root knot–nematode infection (Damiani et al. 2012). Bacterial mutants have been used to characterize bacterial symbiotic regulators (Barnett et al. 2004; Bobik et al. 2006; Capela et al. 2006), and several *M. truncatula* and *S. meliloti* symbiosis mutants have been used to characterize plant gene expression changes during early symbiotic stages (Maunoury et al. 2010; Mitra et al. 2004; Moreau et al. 2011). To date, plant mutants have not been used for genome-wide studies of bacterial gene expression during nodule development.

Plant mutants that develop nodules and support bacterial infection but are defective in nitrogen-fixation (*dnf*) mutants may be particularly useful to study bacterial gene expression at intermediate and late symbiotic stages (Starker et al. 2006). In previous studies on seven plant mutants, expression assays of the nitrogenase gene *nifH* revealed that mutants *dnf4* and *dnf7* are blocked at an earlier symbiotic stage than *dnf3* and *dnf6* but at a later stage than *dnf1*, *dnf2*, and *dnf5* (Starker et al. 2006). *dnf1* is deficient in one component of a nodule-specific signal

Nucleotide sequence data is available in the National Center for Biotechnology Information Gene Expression Omnibus database under accession number GSE53705.

Corresponding author: S. R. Long; Telephone: +1.650.723.3153; Fax: +1.650.725.8309; E-mail: srl@stanford.edu

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peptidase complex of the secretory pathway that is required for the targeting of nodule-specific cysteine-rich (NCR) peptides (Wang et al. 2010). NCR peptides, encoded by a family of approximately 600 genes, are implicated in terminal bacteroid differentiation, as bacteria fail to differentiate into nitrogen-fixing bacteroids in *dnf1* plants (Van de Velde et al. 2010). *dnf2* is deficient in a putative phosphatidylinositol-specific phospholipase C; bacteria are released from infection threads in *dnf2* plants, but fail to differentiate further (Bourcy et al. 2013; D. Wang and S. R. Long, *unpublished*). Genetic and functional characterization of other *dnf* mutants is ongoing.

In this study, we used Affymetrix GeneChips containing probe sets for both *S. meliloti* and *M. truncatula* genes (Barnett et al. 2004) to analyze global gene expression of both organisms in

developmentally arrested root nodules from six *dnf* plant mutants. By using plant mutants arrested at different developmental stages and by detailed comparisons with previous *S. meliloti* microarray experiments, we identified symbiotically regulated processes in *S. meliloti*, placed them in the nodule developmental context, and related them to processes that take place in the plant.

RESULTS AND DISCUSSION

Bacterial gene expression differs most from wild-type in early-arrest plant mutants.

We used plant *dnf* mutants (*dnf1*, *dnf2*, *dnf3*, *dnf4*, *dnf5*, *dnf7*) to enrich for bacterial cells arrested at different stages of

Fig. 1. Root-nodule structure and *dnf* plant phenotypes. **A**, Overview of nodule zones and bacteroid differentiation stages in indeterminate nodules. The image shows a longitudinal section (10-μm thickness) of a 21-day-old *Medicago truncatula*–*Sinorhizobium meliloti* nodule. Nodule zones I to III are indicated on the right and the corresponding bacterial differentiation stages and their characteristic features are listed in the table. The nodule was embedded in Technovit 7100 without prior fixation. Sectioning and staining was carried out as described previously (Wang et al. 2010). IT = infection thread. **B**, Developmentally arrested plant mutants. Images of shoots (upper row) and root nodules (lower row) of wild type (A17 and Jemalong) and *dnf* mutants. Plants were photographed three weeks after inoculation with *S. meliloti* CL150. **C**, Principal component analysis of bacterial gene-expression data. The first two principal components and their contribution to the overall variability of the data (%) are shown on the *x* axis and on the *y* axis. A 68% confidence ellipse is shown for each nodule type.

bacteroid development. Previous characterization of *dnf* plants showed that root-nodule development is arrested at distinct stages: development in *dnf5* and *dnf1* plants is arrested earlier than in *dnf2*, *dnf7*, *dnf4*, and *dnf3* plants (Domonkos et al. 2013; Mitra and Long 2004; Pislariu and Dickstein 2007; Starker et al. 2006; Wang, et al. 2010). In our experiments, we found that *dnf* plants were smaller than wild-type plants at 21 days postinoculation (dpi), and leaves of mutants with an early (*dnf1*, *dnf5*) or intermediate (*dnf2*, *dnf4*, *dnf7*) arrest showed chlorosis (Fig. 1B) consistent with nitrogen limitation. Nodules of early- and intermediate-arrest plant mutants were white and spherical, indicative of deficient function (Fig. 1B). While early-arrest mutant nodules were typically smaller than wild-type nodules, some nodules from intermediate-arrest plants were as large as those of wild-type plants. One mutant, *dnf3*, was similar to the wild-type in both plant and nodule appearance. The here-observed phenotype of the *dnf3* mutant is consistent with the original description by Starker et al. (2006). Acetylene reduction assays demonstrated that the *dnf3* mutant, albeit phenotypically more similar to the wild-type than other *dnf* mutants, is significantly impaired in nitrogen fixation (Starker et al. 2006). To identify genes that are regulated during bacteroid differentiation, we harvested nodules from roots of six *dnf* mutants and two wild-type plant lines and defined their transcription profiles. Principal component analysis (PCA) of bacterial gene expression profiles in the different lines (Fig. 1C) is consistent with inference from morphology, i.e., bacterial gene expression profiles of the early-arrest mutants *dnf1* and *dnf5* are most different from wild-type and fairly similar to each other. Bacterial expression profiles of nodules from intermediate-arrest plants *dnf2*, *dnf4*, and *dnf7* are closer to that of wild type. Among these, we noted that *dnf4* and *dnf7* profiles are similar to each other but the *dnf2* nodule profiles are distinct. The expression profiles of *dnf3* nodules are most similar to wild-type.

Another comparison metric is differential expression of bacterial genes in *dnf* mutants versus Jemalong wild type. We identified 482 differentially expressed genes (significance criteria: adjusted *P* value < 0.05, fold change >2) (Supplementary Table S1). Most differential changes in comparison with wild type were identified in *dnf1* and *dnf5* nodules, followed by *dnf2*, *dnf7*, and *dnf4* nodules (Fig. 2A). No significant bacterial gene expression differences were found when *dnf3* and A17 nodules were compared with Jemalong nodules. Overall, these pairwise comparisons and the PCA show that our *dnf* nodule analysis resolves four developmental stages of bacteroid development. The earliest developmental stage is represented by *dnf1* and *dnf5* nodules. Development of *dnf2* nodules is arrested slightly later than *dnf1* and *dnf5* but distinctly before the intermediate-arrest nodules *dnf4* and *dnf7*. The *dnf3* mutant is arrested later than the other *dnf* mutants, and we do not detect significant changes in bacterial gene expression in comparison with wild-type nodules.

We derived six main clusters of bacterial genes based on hierarchical clustering of differential expression patterns (Fig. 2B and C; Table 1; Supplementary Table S2). The largest cluster, SmA, consists of 197 genes expressed at higher levels both in early- and intermediate-arrest nodules than in *dnf3* or wild-type nodules. The second largest cluster, SmE, consists of 122 genes that are expressed at lower levels in early- and intermediate-arrest nodules than in wild-type. Cluster SmF consists of 102 genes that display lower expression levels in early-arrest nodules but are closer to normal in intermediate-stage lines. The other clusters are fairly small, totaling 61 genes. Genes in clusters SmB and SmD are expressed at reduced levels in most mutants but are expressed at wild-type levels in *dnf1* or *dnf2* nodules, respectively. The 28 genes of Cluster SmC are expressed at their highest levels in *dnf2* nodules.

To validate microarray results, we tested 12 genes from four different clusters by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) (Fig. 3). The RT-qPCR profiles of all tested genes were in good agreement with the microarray hybridization results. For instance, RT-qPCR results confirmed that three genes from cluster SmA (*rsiA1*, *smc00795*, *smc00885*) were expressed at the highest levels in early-arrest mutants. They were >20 times more abundant in *dnf5* nodules and approximately 10 times more abundant in *dnf1* nodules than in wild-type nodules. Similarly, RT-qPCR confirmed that the *sitA* gene from cluster SmC is expressed highly in *dnf2* nodules and that transcripts of genes from cluster SmE and SmF were expressed most abundantly in wild-type nodules (Fig. 3).

Genes with increased expression in free-living cells in comparison with root nodules and known early symbiotic genes show increased expression in early-arrest nodules.

Comparison of our results with previous *S. meliloti* transcriptome studies revealed that many genes that are differentially expressed in *dnf* mutant nodules were also identified during previous root-nodule gene-expression studies. Our primary tool for analysis was gene set enrichment analysis. We used Fisher's exact test to calculate if a gene set was enriched in a cluster and we used parametric gene-set enrichment analysis (PGSEA) to determine if a gene set showed increased or decreased expression in *dnf* mutant nodules in comparison with wild type. PGSEA revealed that genes previously shown to be increased in free-living cells in comparison with root nodules show higher expression levels in early- and intermediate-arrest nodules. These genes are enriched in cluster SmA (Supplementary Tables S3 and S4). Conversely, genes previously found to be increased in nodules in comparison with free-living cells (Barnett et al. 2004; Becker et al. 2004; Capela et al. 2006) were depleted from transcript populations of early- and intermediate-arrest nodules but enriched in late-arrest and wild-type nodules. These genes are significantly ($P < 0.001$) enriched in clusters SmE and SmF. Comparison of the here-presented microarray data with previously published free-living and root-nodule samples (Barnett et al. 2004) by PCA also suggest that bacterial expression profiles in early-arrest (*dnf1*, *dnf5*) and *dnf2* nodules are more similar to those of free-living cells, whereas the profiles of late-arrest and wild-type nodules are more similar to those of previously published wild-type root-nodule samples (Supplementary Fig. S1).

PGSEA showed that genes previously shown to be expressed specifically during early symbiotic stages in a nodulation time-course experiment (Capela et al. 2006, cluster 2) were also expressed at significantly higher levels in *dnf1*, *dnf5*, and *dnf2* nodules. These genes are also highly enriched in cluster SmA ($P = 2 \times 10^{-15}$). Moreover, genes expressed specifically at late symbiotic stages (Capela et al. 2006, clusters 3 and 4) are scarce in early-arrest and intermediate-arrest mutant nodules and are enriched in clusters SmE and SmF. Thus, consistent with previous time-course experiments, many of the genes that are characteristic of early symbiotic stages are, indeed, expressed at higher levels in *dnf1*, *dnf5*, and *dnf2* nodules, while genes that are upregulated during bacteroid differentiation and nitrogen fixation in wild-type nodules are not as highly expressed in *dnf* mutant nodules.

We also compared our results with an analysis of gene expression in laser-dissected root-nodule zones (Roux et al. 2014). Zone analysis provided information on development and differentiation, since *Medicago* nodules are organized in structurally distinct tissue zones that represent the various

stages of bacterial and plant differentiation. We find that genes highly expressed in the infection zone (Roux et al. 2014, clusters 3, 4 and 5) are enriched in cluster SmA and are expressed at significantly higher levels in *dnf1*, *dnf5*, *dnf2*, *dnf4*, and *dnf7* nodules than in wild-type nodules. Genes highly expressed in the infection zone (Roux et al. 2014, clusters 3, 4 and 5) display the highest expression levels in *dnf1* and *dnf5*, followed by *dnf2*, and then, by *dnf4* and *dnf7* nodules. Since most bacteria in the infection zone are localized in infection threads or have just been released into plant cells, our data indicate that *dnf1* and *dnf5* nodules are highly enriched for infection thread bacteria. Nodules from *dnf2*, *dnf4*, and *dnf7* nodules also contain a higher proportion of infection thread bacteria than wild-type nodules, but the differences are not as strong, indicating that at least some bacteria in *dnf2* and a higher proportion in *dnf4* and *dnf7* nodules start to differentiate into bacteroids.

Genes expressed in the interzone II-III and in the nitrogen-fixation zone (Roux et al. 2014, cluster 6) are most highly enriched in cluster SmF ($P = 2.66 \times 10^{-42}$), and genes specifically expressed in the nitrogen-fixation zone are most highly enriched in cluster SmE ($P = 2.60 \times 10^{-20}$).

A small cluster, SmC, contains genes that are most highly expressed in *dnf2* nodules and includes 15 genes previously found to be expressed in interzone II-III and in the proximal infection zone (Roux et al. 2014, clusters 9 and 11). Since this is the region in which bacteria differentiate into bacteroids, we believe that *dnf2* nodules are enriched for differentiating bacteroids and that genes in cluster SmC are expressed during bacteroid differentiation. The 28 SmC cluster genes include four tRNA genes, a *sitABCD*-encoded manganese transport system, and five protease genes (*clpP1*, *clpS1*, *clpX*, *degP1*, *ftsH*). The *sitABCD* manganese transport system is required for protection against oxidative stress and for symbiosis (Davies and Walker 2007). Strong expression of *sitABCD* in *dnf2* nodules implies that bacteria also encounter oxidative stress after being released from infection threads into plant host cells, consistent with observations that reactive oxygen species (ROS) are prominent in the plant environment (Cárdenas et al. 2008; Jamet et al. 2007). The role of the protease genes is not known; it is possible that they are involved in defense against nodule-specific plant-derived antimicrobial peptides that trigger bacteroid differentiation or their prominence may reflect extensive protein turnover associated with cellular remodeling. Thus functional tests of protease and other genes in cluster SmC may reveal whether they play a role in early bacteroid differentiation.

Nitrogen-fixation genes are expressed in intermediate-arrest nodules but not in early-arrest nodules.

A signature feature in N_2 -fixing bacteria is expression of nitrogenase (*nif*) structural genes. In *Sinorhizobium* spp., *nif* gene expression is regulated by the FixL/J-oxygen sensing system and the NifA transcriptional activator under microoxic conditions (David et al. 1988; Soupène et al. 1995). The FixL/J two-component system also regulates expression of the *fix* genes required for microoxic respiration and other functions (Batut et al. 1989; Bobik et al. 2006). We saw significant changes in expression in 28 of the 41 *nif* and *fix* genes during nodule development; 27 of the 28 genes fell into cluster SmF. Their expression profiles (Fig. 4A) show that most *nif* and *fix* genes (34 of 41) are highly expressed in wild type, *dnf3*, *dnf4*, and *dnf7* nodules, but have medium levels in *dnf2* and low levels in *dnf1* and *dnf5* nodules. The only exceptions are six colocalized chromosomal genes (*fixI2*, *fixN3*, *fixO3*, *fixP3*, *fixQ3*, *fixS2*) (Fig. 4A), which did not match our criteria for differential expression and are probably not strongly regulated

during symbiosis. Cluster SmF also contains several ferredoxins (*fdx*, *fdxN*, *fdxB*, *mocE*, *mocF*) and iron-sulfur cluster assembly proteins (*nifS*, *sufA*, *sufD*) that are probably involved in building the nitrogen-fixation apparatus. Expression of *nif* and *fix* genes in intermediate- and late-arrest and wild-type nodules indicates that the FixJ/FixL two component system is activated. By inference, bacteria are exposed to microoxic conditions. Functional enrichment and gen-set enrichment analyses are consistent with this conclusion. Genes that were upregulated under microoxic conditions and genes that were upregulated in nodules in a *fixJ*- or *nifA*-dependent manner at 14 days after inoculation (Bobik et al. 2006) were, likewise, typically expressed at lower levels in *dnf1*, *dnf5*, and *dn2* versus wild-type nodules and were significantly enriched in cluster SmF.

Sinorhizobium cell biology and physiology during symbiotic transitions.

While nitrogen-fixation regulation is a well-studied aspect of *Sinorhizobium* differentiation, other critical aspects of cell function are not as well understood. We assigned genes to functional classes according to Riley (1993; Serres and Riley 2000) as a way of exploring how these correspond to bacterial fates during normal versus mutant symbiosis development. As expected and consistent with the analysis above, we found that the functional class of nitrogen-fixation genes was among the groups with the lowest expression in early-arrest nodules in comparison with wild-type nodules.

Cell-division genes are expressed at significantly higher levels in early-arrest nodules (Supplementary Table S5). This group prominently includes genes with a binding motif for the cell cycle regulator CtrA (De Nisco et al. 2014) (Fig. 4B), and genes that are strongly expressed during G1 (De Nisco et al. 2014, cluster 1) or G2/M phases of the cell cycle (De Nisco et al. 2014, cluster 5). By these criteria, we infer that the plant gene products corresponding to *dnf1* and *dnf5* are required for the transition of bacteria from active division to a state in which they remain in S phase, undergoing multiple rounds of endoreduplication without division (Mergaert et al. 2006). These transcription results are consistent with the microscopic observation that bacteria in *dnf1* and *dnf5* nodules do not display the characteristic elongated bacteroid shape but resemble free-living rod-shaped cells (Domonkos et al. 2013; Wang et al. 2010).

The cell surface is a crucially important interface for plant interaction with the bacterium (Downie 2010; Gibson et al. 2008; Jones et al. 2007). It has been shown that the lipopolysaccharide structure differs between free-living and nodule bacteria (Kannenberg and Carlson 2001), but most cell-envelope functions have not been studied in invading rhizobia. Our data show that genes for structural functions such as cell envelope, outer membrane, cell exterior, and cell surface polysaccharides are expressed at higher levels in early-arrest nodules than in wild-type nodules. This implies that bacterial cell surface architecture changes between infection and bacteroid differentiation.

We explicitly looked at previously identified regulators of ESP, outer membrane proteins and exported polysaccharides. For instance, the FeuP/Q two-component system induces the production of cyclic β -glucans in response to low osmolarity and is essential for symbiosis (Griffitts et al. 2008). FeuP itself and 11 of the 14 known FeuP-induced genes are found in cluster SmA. In addition, PGSEA showed that FeuP-induced genes are expressed at significantly higher levels in *dnf1*, *dnf2*, and *dnf5* nodules than in wild-type nodules. Considering that a *feuP* mutant is unable to form infection threads (Griffitts et al. 2008),

Fig. 2. Transcriptomic analysis of *Sinorhizobium meliloti* gene expression in nodules from *dnf* plants. **A**, The number of differentially regulated bacterial genes in the indicated plant mutants compared with those in wild-type *Medicago truncatula* cv. Jemalong root nodules (adjusted P value = 0.05, fold change >2). The black columns represent genes with increased expression levels compared with wild type and the white columns represent genes with reduced expression levels. **B**, Heatmap and hierarchical clustering of bacterial genes that are differentially expressed in comparison with *M. truncatula* cv. Jemalong wild-type nodules. The color scale bar indicates the fold change of gene expression in comparison with wild-type nodules; the color sidebar highlights six bacterial gene clusters (SmA to SmF). **C**, Boxplot representations of gene expression profiles of each cluster. The box indicates the upper and the lower quartile, the bar indicates the median and the whiskers indicate the highest and lowest values excluding outliers. Outliers were defined as values that are higher than 1.5 $\times$ the interquartile range above the upper quartile or lower than 1.5 $\times$ the interquartile range below the lower quartile.

Fig. 3. Validation of Affymetrix GeneChip data by quantitative reverse transcription-polymerase chain reaction (qRT-PCR). The relative expression levels of 12 *Sinorhizobium meliloti* genes from four clusters were assayed by qRT-PCR; expression levels were normalized to an internal control (*nuoL*). The data are expressed as the expression ratios of different plant lines to *Medicago truncatula* cv. Jemalong. Each data point is an average of three replicates; error bars indicate standard deviation of the fold change.

this elevated expression level of FeuP and its target genes in early-arrest mutants suggests that FeuP/Q-mediated cyclic β -glucan production is relevant to bacterial survival in infection threads. Conversely, lower *feuP* expression in intermediate-arrest and wild-type nodules indicates that cyclic β -glucan

production is downregulated after bacteria are released from the infection thread to form symbiosomes.

Regulatory mutations that lead to over- or under-production of EPS-I (succinoglycan) cause parallel expression changes for a canonical group of EPS-associated genes. These same genes

Fig. 4. Several processes are regulated during symbiosis. **A**, Expression profiles of *nif* and *fix* genes, **B**, *ctrA*-controlled genes (De Nisco et al. 2014), and **C**, tricarboxylic acid cycle genes. The signal intensities are scaled for each gene, as indicated by the color scale bar. **D**, expression profiles and hierarchical clustering of bacterial sRNA probe sets that are significantly changed (adjusted *P* value < 0.05, fold change > 2) relative to wild-type nodules. The color scale bar indicates the log₂ fold change of gene expression; a color sidebar highlights two main clusters.

are expressed at higher levels in early-arrest nodules than in wild-type nodules (Chen et al. 2009; Gibson et al. 2007; Wells et al. 2007). EPS such as succinoglycan (or the related galactoglucan) is essential for symbiosis in *S. meliloti* strains (Cheng and Walker 1998; Pellock et al. 2000). Our results support the proposal that succinoglycan production is required during the infection thread stage and, then, is repressed when the bacteria differentiate into bacteroids in symbiosomes.

Genes regulated by the polarity determinant *podJ* (Fields et al. 2012) are significantly enriched in early-arrest nodules. One of the main transcriptomic changes in a *podJ* mutant is the elevated expression of succinoglycan biosynthesis genes (Fields et al. 2012). Therefore, it is likely that *podJ*-regulated genes are enriched in early-arrest nodules because succinoglycan biosynthesis is activated both in the *podJ* mutant and in early-arrest nodules.

Stress response.

During symbiosis, bacteria are exposed to different environmental stresses including ROS and reactive nitrogen species (Downie 2010; Gibson et al. 2008). We note that the cluster typifying early-arrest mutants (SmA) includes general stress response sigma factor *rpoE2* and its antisigma factor *rsiA1* and is highly enriched for RpoE2 target genes (e.g., 48 of 193 genes that are upregulated upon RpoE2 overexpression, $P = 1.19 \times 10^{-30}$ [Schlüter et al. 2013]). PGSEA also shows that RpoE2 target genes are expressed strongly in early-arrest nodules. In addition, transcripts for genes that are induced in response to environmental stresses such as nitric oxide stress, zinc stress, acidic stress, osmotic stress, and heat shock are also enriched in early-arrest nodules (Barnett et al. 2012; Domínguez-Ferreras et al. 2006; Hellweg et al. 2009; Meilhoc et al. 2010; Rossbach et al. 2008; Sauviac et al. 2007). We see two, not mutually exclusive interpretations: i) the increased expression of stress genes in early-arrest nodules suggests that the general stress response is active in infection threads due to inducers such as oxidative stress; ii) it is possible that the general stress response is activated due to premature senescence of early-arrest nodules. The hypothesis that the general stress response is activated in infection threads is in agreement with the observation that many *rpoE2* regulated genes are also highly expressed in young wild-type nodules in which bacteria are predominantly found in infection threads (Capela et al. 2006; Sauviac et al. 2007).

Our analyses also implicated the heat-shock sigma factor RpoH in symbiosis. *S. meliloti* encodes two RpoH-like sigma factors. RpoH1 is required for successful symbiosis; RpoH2, the other heat-shock sigma factor in *S. meliloti*, is part of the *rpoE2* regulon (Sauviac et al. 2007; Schlüter et al. 2013). RpoH2 is not required on its own for efficient symbiosis but an *rpoH1 rpoH2* double mutant is inhibited during earlier symbiotic stages than an *rpoH1* single mutant (Barnett et al. 2012; Mitsui et al. 2004; Oke et al. 2001). The *rpoH2* gene and four of its 41 known target genes fell into cluster SmA (Barnett et al. 2012). The RpoH2 target gene set was enriched in *dnf1* and *dnf5* nodules. Increased expression of *rpoH2* in early-arrest mutants and the severe phenotype of the *rpoH1 rpoH2* mutant argue that RpoH2 is active during early symbiotic stages. RpoH1 mediates expression of 282 genes under heat-shock conditions (Barnett et al. 2012), and these targets were enriched in clusters containing genes highly expressed in *dnf2* nodules (clusters SmC, SmD) (Fig. 2C). Genes regulated by RpoH1 during acid shock (de Lucena et al. 2010) are similarly overexpressed in *dnf2* nodules. These data indicate that RpoH1 is highly active in *dnf2* nodules. Since *dnf2* nodules contain elongated bacteroids (Bourcy et al. 2013) and nodule development is arrested later in *dnf2* nodules than in early-arrest nodules but earlier than in *dnf4* and *dnf7* nodules, we suggest

that RpoH1 is active shortly after the bacteria are released into symbiosomes and does not remain active during later symbiotic stages.

Metabolic changes during nodule development.

Metabolic remodeling is a characteristic of bacterial differentiation, and underlying gene expression changes may occur due to the novel plant environments that the bacteria traverse during infection. While free-living bacteria utilize diverse carbon compounds, bacteroids appear to use only plant-provided dicarboxylic acids as carbon and energy sources. These dicarboxylic acids are taken up via DctA and metabolized via the tricarboxylic acid (TCA) cycle and provide energy for nitrogen fixation (Udvardi and Poole 2013). We found that wild-type and intermediate-arrest *dnf* mutant nodules had abundant TCA-cycle gene transcripts, but such transcripts were scarce in early-arrest nodules (Fig. 4C). Furthermore, TCA-cycle genes are significantly enriched in cluster SmE (Supplementary Table S6). Several gene-function groups related to small-molecule metabolism or energy metabolism (such as the pyruvate dehydrogenase, energy transfer, electron transport subgroups) are well-represented in cluster SmF, which contains genes that display high expression levels in intermediate-arrest and wild-type nodules but not in early-arrest nodules. Genes known to be induced during starvation (Capela et al. 2006; Krol and Becker 2011; Sauviac et al. 2007) or during growth on succinate instead of glucose (Barnett et al. 2004) are more highly expressed in early-arrest and *dnf2* nodules than in wild-type nodules. Taken together these findings are consistent with the hypothesis that carbon supply to bacteria is limited in early-arrest nodules but, once the nodules develop to intermediate stages and beyond, availability of plant-derived dicarboxylic acids increases. Thus, bacterial carbon metabolism switches to the utilization of dicarboxylic acids once the bacteria are released into plant cells and bacteroid differentiation starts. High expression levels of TCA-cycle and energy-metabolism genes in nitrogen-fixation defective intermediate-arrest nodules suggest that the bacteria have access to plant-derived carbon sources even though they do not fix nitrogen efficiently.

Processes specifically expressed during later symbiotic stages.

We looked at the difference between intermediate-arrest nodules and fully mature wild-type nodules for clues about bacteroid function in the final stages of endosymbiosis. Few genes were expressed at significantly higher levels ($P < 0.05$, fold change >1) in the intermediate-arrest nodules from *dnf4* and *dnf7* plants than in wild-type nodules. Genes that were expressed at lower levels in the intermediate-arrest nodules mostly corresponded to cluster SmE, which contains 122 genes that are specifically expressed in wild-type nodules, and are, therefore, characteristic of mature nodules. Cluster SmE includes several genes (*smoE*, *agpA*, *agpD*, *smb21302*) involved in sugar transport and metabolism; this may indicate that bacteria in mature nodules do not use solely dicarboxylates but, also, plant derived sugars such as melibiose and fucose. Other late-function genes that may be used to degrade alternative carbon sources are *nerA* (glycerol trinitrate reductase), *fdsG* (formate dehydrogenase), *gabD1* (succinate semialdehyde dehydrogenase), and *dppA1* (peptide transport).

A particularly prominent class of genes in cluster SmE is “elements of external origin” with transposon-related functions. In addition, several regulators, such as the motility regulator *visN*, succinoglycan regulator *exoX*, and the alternative sigma factors *rpoN*, *rpoE3* and *rpoE8*, are found in cluster SmE. We speculate that some of these functions might relate to

functions for surviving one or both nodule maturation and senescence.

sRNA expression.

To identify symbiotically regulated sRNAs, we matched probe sets for intergenic sequences by BLAST search to 389 previously identified sRNAs (del Val et al. 2007; Schlüter et al. 2010, 2013). Of these 389 sRNAs, 43 are differentially expressed in *dnf* mutant nodules (Supplementary Table S7). Clustering shows that different sRNAs are expressed during different symbiotic stages (Fig. 4D). Approximately half of the sRNAs (22) are expressed at higher levels in early- and intermediate-arrest mutant nodules than in wild-type nodules, which suggests that they are predominantly expressed in infection threads and during bacteroid differentiation. In contrast, the other 21 sRNAs are most highly expressed in wild-type nodules, suggesting that they are expressed after the onset of nitrogen fixation in mature nodules (Fig. 4D).

Differential expression of plant genes in *dnf* mutant nodules.

Our custom Symbiosis Chip allows us to analyze plant and bacterial gene expression simultaneously (discussed below). A total of 1,255 plant gene probe sets showed significant changes between mutant and wild-type nodules (Supplementary Table S8) and cluster analysis yielded nine groups (MtA to MtI) of similar expression profiles (Fig. 5; Supplementary Table S9). We compared our results on plant gene expression in developmentally arrested nodules with the results of studies on plant gene expression in different root-nodule zones (Limpens et al. 2013; Roux et al. 2014). These comparisons show that genes that were previously detected in the meristem and infection zone are expressed at significantly higher levels in early-arrest nodules than in wild-type nodules; conversely, genes that are expressed in the interzone II-III or nitrogen-fixation zone are expressed at significantly lower levels in early-arrest nodules than in wild-type nodules (Supplementary Table S10).

Clusters MtA, MtB, MtC, and MtD, comprising genes expressed predominantly in late-arrest and wild-type nodules, contain many genes that are most highly expressed in the nitrogen-fixation zone, the interzone II-III, or in infected cells of the nitrogen-fixation zone. The clusters are distinguishable in that genes of MtA, MtB, and MtC, which encode, for example, leghemoglobins and many NCR peptides, are expressed to varied extents in intermediate-arrest nodules, while those genes in MtD (e.g., late nodulins) are exclusively expressed in wild-type but not in the relatively normal *dnf3* mutant nodules. Clusters MtE, MtF, MtG, and MtH consist of genes that are generally expressed at higher levels in early- and intermediate-arrest nodules, such as genes encoding peroxidases, calmodulins, and chalcone synthases. Many genes in clusters MtE, MtF, MtG, and MtH were previously found to be highly expressed in the meristem, the infection zone, and in uninfected cells of the nitrogen-fixation zone (Limpens et al. 2013; Roux et al. 2014). The similarities between plant gene expression in *dnf* mutants and different root-nodule zones provide additional evidence that nodules from *dnf* mutants are enriched for plant and bacterial cells at different developmental stages.

A hallmark of plant differentiation in mature nodules is the presence of specialized heme-containing proteins such as leghemoglobins, which give nodules their characteristic pink color. Leghemoglobins are required to establish microoxic conditions and are essential for nitrogenase activity and maximal *nif* gene expression (Ott et al. 2005, 2009). Analysis of clusters based on MapMan classification (version Mt_Mt3.5_0411) showed that 14 heme-containing redox

proteins are expressed at low levels in early-arrest and *dnf2* nodules (Supplementary Table S11). Eleven of these probe sets detect leghemoglobin transcripts. While leghemoglobin expression levels are similar in *dnf4*, *dnf7*, *dnf3*, A17, and Jemalong nodules, only *dnf3*, A17, and Jemalong nodules display the characteristic pinkish color, whereas *dnf4* and *dnf7* nodules are white. Thus, levels of the pink, reduced form of leghemoglobin protein appear to be lower in intermediate-arrest nodules despite similar transcript levels. This points to control beyond transcription, such as translation, maturation, or stability of leghemoglobin proteins. Since a reduction of functional leghemoglobin levels would be expected to cause nitrogen deficiency, future analyses of leghemoglobin proteins in *dnf4* and *dnf7* nodules may reveal how the mutations in *dnf4* and *dnf7* impact symbiosis.

Comparison of early-arrest with wild-type nodules might be useful, not only by showing what is missing in early nodules due to a genetic block in differentiation but by revealing functions that may be transient in nodule development. On the other hand, early-arrest nodules might, instead, be characterized by abnormal expression due to inappropriate developmental signals with consequent stress responses. We observed several functional groups to be expressed at higher levels in early-arrest mutants. The strongest differences are observed for biotic stress, ascorbate and glutathione redox proteins, and phenylpropanoid and flavonoid metabolism. Biotic stress genes are also expressed at higher levels in intermediate-arrest nodules, suggesting that biotic stress genes are active during the early stages of symbiosis and down-regulated with the onset of nitrogen fixation. Activation of the defense response during microbial infection causes the release of ROS. Thus, ascorbate and glutathione redox proteins and other proteins with a role in detoxification of ROS, including thioredoxins, glutaredoxins, and peroxidases (Becana et al. 2010), are probably expressed highly in early-arrest nodules to increase resistance toward elevated ROS levels. We cannot exclude the possibility that elevated biotic stress gene expression and elevated ROS levels are a result of premature senescence in early-arrest nodules. However, findings of high concentrations of ascorbate, glutathione, and antioxidant proteins in healthy nodules (Matamoros et al. 2003) suggest that antioxidant proteins are expressed during normal nodule development. In addition, cysteine proteases, which can be used as senescence markers (Van de Velde et al. 2006), were expressed at lower levels in early- than intermediate-arrest nodules and were assigned to clusters MtA (Medtr4g047610, Medtr4g107930, Medtr4g047610) and MtB (Medtr5g022560, Medtr4g079800). This suggests that senescence has either not been initiated or has not progressed very far in early-arrest nodules.

Flavonoids and phenylpropanoids are important signaling molecules during the early stages of the symbiosis as inducers of nodulation gene expression and play a role in the plant defense response (Stafford 1997). The differential expression of genes for NAD(P)H dependent 6' deoxychalcone synthase, isoliquiritigenin O-methyltransferase, and isoflavone reductase would be consistent with phytoalexin production occurring in early-arrest mutants. Flavonoid and phenylpropanoid metabolism are also increased in nodules formed by a bacterial *exoA* mutant (Maunoury et al. 2010; Moreau et al. 2011), and it has also been reported that some *dnf2* nodules exhibit a dark-brown pigmentation consistent with the accumulation of phenolic compounds (Bourcy et al. 2013). This raised the question whether flavonoid and phenylpropanoid biosynthesis in plant and bacterial mutants results from early senescence or if it reflects gene expression during early symbiotic stages. Since several probe sets for flavonoid biosynthetic proteins including

those for isoflavone reductase, isoliquiritigenin reductase, caffeoyl-CoA methyltransferase and 4 coumarate-CoA ligase were detected at higher levels in the nodule tip than in the interzone II-III (C. Lang and S. R. Long, *unpublished*; Roux et al. 2014), we speculate that there may be early deployment of plant flavonoid-mediated plant defenses that are subsequently downregulated during symbiosome formation.

Expression of NCR peptides in mutant nodules.

In *Medicago* spp. and other legumes that induce bacteroid differentiation, infected plant cells express genes for nodule-specific glycine-rich (NGR) and NCR secreted peptides. NCR peptides are targeted to symbiosomes, affect bacterial growth, and induce bacteroid-like morphological changes (Kondorosi et al. 2013). Treatment of *S. meliloti* cultures with the purified NCR247 peptide results in blockage of cell division but not DNA replication (Penterman et al. 2014). We observed higher NCR- and NGR-peptide expression levels in intermediate- and late-arrest than in early-arrest nodules. Correspondingly, most NCR- and NGR-probe sets were assigned to cluster MtA (131 NCR, two NGR probe sets) or MtC (38 NCR, six NGR probe sets). One NCR probe set (Medtr1g042895) fell into cluster MtD, indicating that it is expressed specifically in nitrogen-fixing nodules. Given their antimicrobial properties and expression profiles, NCR peptides may play a role in controlling bacterial infection during intermediate and late symbiotic stages after plant ROS- and flavonoid-mediated plant defense mechanisms are downregulated.

The maturation and subsequent transport of NCR peptides depends on the DNF1-signal peptidase (Van de Velde et al. 2010; Wang et al. 2010). In *dnf* mutant analyses, we found that the *dnf1* gene itself is expressed at similar levels in all plants

except the *dnf1* mutant, in which expression was not detected. Low expression of NCR peptide-encoding genes in the *dnf1* mutant indicates that NCR peptide-encoding genes are not fully expressed in the absence of a functional NCR maturation and secretion system. It is possible that failure to export NCR peptides results in intracellular accumulation of NCR peptides and subsequent downregulation of NCR biosynthesis by a negative-feedback mechanism. Alternatively, NCR peptide biosynthesis may be downregulated due to aberrant nodule development in *dnf1* nodules.

Transcriptomic studies demonstrated the effects of NCR peptides on *S. meliloti* in culture (Penterman et al. 2014; Tiricz et al. 2013). We asked whether genes that are regulated in response to NCR peptide treatment in culture are enriched in certain plant mutants. We were surprised to find that genes that are induced in culture by NCR peptides were expressed at significantly higher levels in early-arrest (*dnf1*, *dnf5*) and *dnf2* mutant nodules than in intermediate-arrest or wild-type nodules. Similarly, genes whose expression was decreased in response to NCR peptide treatment in cultures were expressed at lower levels in early-arrest than in wild-type nodules. This was unexpected. Since it had been proposed that NCR peptides are responsible for bacteroid differentiation (Kondorosi et al. 2013), one might thus expect higher NCR-induced gene expression in late-arrest and wild-type nodules. Bacterial cells in nodules with the lowest levels of NCR peptide expression, such as *dnf1*, *dnf2* and *dnf5*, show similar behaviors as NCR peptide-treated cells. One interpretation is that cultured cells respond to NCR peptide treatment by activation of stress-response genes and cell-envelope changes including activation of succinoglycan biosynthesis and cyclic β -glucan production (Penterman et al. 2014; Tiricz et al. 2013). These functions are indeed expressed at higher levels in early-arrest nodules than in wild-type nodules, but they are plausibly caused, not by NCR peptides, but by other environmental stress factors such as plant-produced ROS, characteristic of early symbiosis. In addition, we are aware that the *S. meliloti* response to a single NCR peptide in liquid cultures almost certainly differs from response of nodule bacteria that encounter additional stresses and multiple species of NCR peptides.

Conclusion.

Earlier reports have identified bacterial exposure to plant-root exudates as a key transition toward the symbiotic state (Peters and Long 1988). Also, transcription profiles of bacteria in nodules (Barnett et al. 2004; Becker et al. 2004; Capela et al. 2006; Roux et al. 2014) show that hundreds of bacterial genes are up- or downregulated in response to the accumulated effects of nodule development, presumably reflecting activity of many plant genes over time. In this study, we used developmentally arrested *dnf* plant mutants to study transcriptional changes during root-nodule development in both symbiotic partners, *S. meliloti* and *M. truncatula*. Use of mutants allowed us to observe gene expression changes that are obscured in whole mature nodules and to identify four stages of bacterial differentiation within the nodule that correspond to the activity of individual plant genes.

We systematically compared our results to previous *S. meliloti* microarray studies and carried out functional enrichment analyses to identify processes that are characteristic of different symbiotic stages. During the infection thread stages, we observed expression of certain cell cycle- and envelope-related genes. Several genes whose products deal with stress-related functions were also expressed predominantly during early symbiotic stages, while other stress-related genes, such as the heat-shock sigma factor *rpoH1* are expressed slightly later and, thus, may play a role after bacteria are released into

Fig. 5. Heatmap diagram and hierarchical clustering of plant probe sets that are significantly changed (adjusted *P* value = 0.05, fold change >2) in comparison with *Medicago truncatula* cv. Jemalong wild-type nodules. The color scale bar for expression level is located at the figure bottom and indicates the log₂ fold change of gene expression relative to wild-type nodules. The color sidebar highlights nine plant gene clusters (MtA to MtI).

symbiosomes. Bacteroid differentiation and nitrogen fixation are characterized by increased transcript levels of genes with roles in the TCA cycle and nitrogen fixation. We analyzed *M. truncatula* gene expression with an emphasis on processes that may control bacterial infection. Our results indicate that general plant defense responses such as ROS production and phenylpropanoid metabolism play a role in the infection zone. During later symbiotic stages, transcription of genes involved in these processes is suppressed and nodule-specific peptides (NCR and NGR peptides) become the primary mechanism to control bacterial infection. A potential caveat of using plant mutants to study bacteroid differentiation is that senescence may be induced prematurely in mutants and affect plant and bacterial gene expression. However, strong correlations of plant and bacterial gene expression in *dnf* nodules with previous nodulation time-course studies and laser-capture microdissection-based studies of genes support future use of plant mutants for analysis of bacterial differentiation. A major advantage of the plant mutant approach is that, unlike transcriptomic analyses (which can be carried out after amplification of scarce material from laser microdissection samples), developmentally arrested nodules can be easily obtained in large quantities, an obvious asset for proteomic, metabolomic, and other studies.

MATERIALS AND METHODS

Plant cultivation, inoculation, and root-nodule harvest.

The wild-type strain *Sinorhizobium meliloti* CL150 was used throughout this study. CL150 is a *S. meliloti* 1021 derivative, corrected for point mutations in *pstC* and *ecfR1* (Schlüter et al. 2013). Bacterial cultures were prepared for plant inoculation as described previously (Mittra and Long 2004). *Medicago truncatula* [Gaertn.] cvs. Jemalong, A17, and *dnf* plant seeds were lightly scratched with sandpaper and were sterilized in undiluted bleach for 5 min. Seeds were rinsed with sterile water and were imbibed for 8 h at room temperature and 48 h at 4°C, were germinated overnight in inverted petri dishes in the dark, and were planted into presterilized vermiculite in cone-tainer tubes (Stuewe & Sons). Plants were watered every third day with 5 ml of 0.1× buffered nodulation medium (BNM) (Ehrhardt et al. 1992). Five-day-old seedlings were inoculated with 5 ml of a suspension (optical density at 600 nm = 0.1) of CL150 in 0.1× BNM.

RNA purification, amplification, and microarray hybridization.

Samples for transcriptomic analysis were obtained from root nodules harvested 21 days after inoculation, placed in liquid nitrogen, and stored at –80°C. For each of the three replicates,

9 to 17 plants were used. Root nodules from *dnf* mutants and from wild-type cultivars Jemalong and A17 were placed in 30 µl of RLT buffer (RNeasy mini kit; Qiagen) and frozen in liquid nitrogen. The nodules were ground with a microcentrifuge tube pestle as the buffer thawed. The lysate was brought to a volume of 450 µl with RLT buffer and processed through a Qias shredder column (Qiagen) and an RNeasy Mini spin column (Qiagen). The RNA was DNase-treated (DNase I; Thermo Fisher Scientific) in the presence of RNase inhibitor (SuperaseIN) (Barnett et al. 2004). The DNase-treated RNA was repurified using RNeasy MinElute columns (RNeasy MinElute kit). RNA quality and quantity were assayed using Bioanalyzer RNA Nano chips (Agilent). Template RNA (1 µg) was amplified and was biotin-labeled, using the MessageAmp Bacteria kit (Ambion). Amplified RNA (20 µg) was fragmented in 40 µl of 200 mM Tris-acetate, pH 8.1, 500 mM potassium acetate, 150 mM magnesium acetate at 94°C for 35 min. The reaction was brought to 78.5 µl with water and was hybridized to Affymetrix GeneChips as described previously (Barnett et al. 2004).

Microarray analysis.

Hybridization experiments used a custom Symbiosis Chip that contains probe sets for the complete *S. meliloti* genome (6,247 probe sets for open reading frames, 5,788 probe sets for intergenic regions) and 10,893 probe sets for *M. truncatula* tentative consensus (TC) sequences (Barnett et al. 2004). We updated the *M. truncatula* annotations of our GeneChip by finding sequence identity between the TC sequences used to design the microarray and the updated *M. truncatula* genome sequence, based on the annotation versions Mt3.5 and Mt4.0 (Young et al. 2011), using BLASTn (National Center for Biotechnology Information [NCBI] BLAST 2.2.27+). The latest annotations of *S. meliloti* genes were obtained from the EnsemblBacteria database release 22 (Kersey et al. 2014). To study bacterial sRNA expression, we matched AffyChip probe sets for bacterial intergenic regions to noncoding RNAs found by Schlüter et al. (2013) and experimentally verified sRNAs found by del Val et al. (2007) by BLASTn. We only considered probe sets for intergenic sequences that displayed a 100% match to a sRNA for at least 100 bp. In addition, we included sRNAs that were identified with our chip by Schlüter et al. 2010. Specifically, we included sRNAs encoded in intergenic regions (transencoded sRNA) and sRNAs encoded in antisense orientation of an annotated open reading frame (*cis*-encoded sRNA) (Schlüter et al. 2010).

Affymetrix CEL files were analyzed using R and BIOCONDUCTOR software packages (Gentleman et al. 2004) (R Foundation for Statistical Computing, Vienna, Austria). Probe

Table 1. Key characteristics of *Sinorhizobium meliloti* gene clusters

Cluster	No. of genes	Profile description	Characteristic genes and processes
SmA	197	Increased expression in <i>dnf</i> nodules, especially <i>dnf1</i> and <i>dnf5</i>	NADH dehydrogenase (<i>nuoA2</i> , <i>nuoK2</i> , <i>nuoJ2</i>), stress response (<i>rpoE2</i> , <i>rsiA1</i> , <i>rpoE5</i> , <i>rpoH2</i>), cyclic β-glucan biosynthesis (<i>feuP</i> , <i>ndvA</i>), cell cycle (<i>ctrA</i> , <i>dnaA</i> , <i>ftsZ1</i> , <i>minE</i>)
SmB	18	Decreased expression in <i>dnf</i> nodules, except for <i>dnf2</i> nodules	Regulators (<i>lsrB</i> , <i>exsB</i>), phenyl acetic acid degradation (<i>paaC</i>)
SmC	28	Increased expression in <i>dnf</i> nodules, especially <i>dnf2</i>	Manganese transport (<i>sitABCD</i>), proteases (<i>degP1</i> , <i>ftsH</i> , <i>clpP1</i> , <i>clpS1</i> , <i>clpX</i>)
SmD	15	Increased expression in <i>dnf1</i> and <i>dnf2</i> nodules	<i>ntrR2</i> , glutaredoxin (<i>grxC</i>)
SmE	122	Decreased expression in <i>dnf</i> nodules	Regulators (<i>rpoE3</i> , <i>rpoE8</i> , <i>rpoN</i>), sugar utilization (<i>fucU</i> , <i>smoE</i> , <i>agpA</i> , <i>agpU</i>)
SmF	102	Decreased expression in <i>dnf1</i> , <i>dnf5</i> , and (partially) <i>dnf2</i> nodules	Nitrogen fixation (<i>nif</i> and <i>fix</i>), dicarboxylic acid metabolism and tricarboxylic acid cycle (<i>dctA</i> , <i>pdhAa</i> , <i>pdhBb</i> , <i>pdhC</i> , <i>lpdA1</i> <i>gltA</i> , <i>mdh</i> , <i>sucBCD</i> , <i>sdhABCD</i>)

summarization and normalization were carried out with the AFFY package and the RMA algorithm (Irizarry et al. 2003). To account for varying plant to bacterial RNA ratios in different samples, normalization was conducted separately for *M. truncatula* and *S. meliloti* probe sets. PCA was carried out with the RMA normalized bacterial expression data using R with the “prcomp” function. The PCA plot was created using “ggbiplot” in R. The LIMMA software package was used to identify differentially expressed genes (Smyth 2005). We calculated fold changes for pairwise comparisons of mutants to the Jemalong wild-type. We used the Benjamini and Hochberg (1995) adjustment method for multiple testing to calculate adjusted *P* values. We considered probe sets to be differentially expressed if the expression levels changed more than two-fold ($\log_2$ fold change = 1) and the adjusted *P* value was below 0.05. Hierarchical clustering with average linkage was carried out using the “1-Pearson correlation” dissimilarity metric. Probe sets for bacterial open reading frames, bacterial sRNAs, and plant genes were analyzed separately. All microarray data in this study were deposited in the NCBI Gene Expression Omnibus database (Edgar et al. 2002) under accession number GSE53705.

qRT-PCR.

To validate microarray results, cDNA was prepared from the same RNA templates used for RNA amplification and microarray analysis, as described previously (Barnett et al. 2004). qRT-PCR was performed using a C1000 Thermal Cycler equipped with a CFX96 Real-Time system (Bio-Rad) and the DyNamo Flash SYBR green qPCR kit (Thermo Fisher Scientific). PCR reactions for each of the three biological replicates were performed in technical triplicate. A total of 3 ng of cDNA and 10 pmol primer (sequences are available upon request) were used for each 20- μ l reaction reaction. The PCR program was 95°C for 5 min, followed by 40 cycles of 95°C for 10 s, 60°C for 20 s, 72°C for 30 s, and 76°C for 10 s, during which the fluorescence was recorded. After a final extension step of 72°C for 1 min, a dissociation curve from 60 to 95°C was recorded to confirm the specificity of the amplification. Data were analyzed using CFX manager software (Bio-Rad) and normalized with the $2^{-\Delta\Delta C(t)}$ method (Livak and Schmittgen 2001). The variance and average of the microarray intensity values for each bacterial gene in all plant mutant samples were calculated to select an appropriate reference gene. From a list of the ten bacterial genes with the lowest signal variance, *nuoL* was chosen as an internal control gene.

Comparison with previous transcriptome experiments.

We compared our microarray results with the data of 28 previous studies investigating *S. meliloti* gene expression in root nodules, during growth on various nutrients, during adaptations to different environmental conditions, after exposure to different stresses, and after deletion of certain genes. From these previous studies, we defined 125 gene sets. Each gene set consisted either of a previously identified gene cluster of coregulated genes or of genes that were found to be increased or decreased by a treatment during earlier studies. To compare our results with earlier studies that used different microarray platforms, we included only genes that are shared in common between our Symbiosis Chip and the platform used in the previous study, e.g., if we compare our data with those of Becker et al. (2004), we only consider those 6,103 genes that can be detected by our Symbiosis Chip and the Sm6Koligo chip that was used by Becker et al. (2004). We used two methods for gene-set enrichment analysis. First, we calculated if genes belonging to a gene set from a previous experiment are over- or underrepresented in the *dnf* mutants in comparison with the

wild-type (Jemalong) using PGSEA (PGSEA software). We used the PGSEA and LIMMA (Smyth 2005) R packages as described in the PGSEA documentation to determine $\log_2$ fold changes and *P* values for each functional group found in the genome. Second, we tested whether a gene set from a previous experiment is significantly enriched in bacterial gene clusters. We calculated the *P* value for the case of finding the same or higher numbers of genes belonging to a functional group within a cluster, using Fisher's exact test, which is implemented in R with the “fisher.test” function.

Functional enrichment analysis.

To study if functional bacterial gene categories are enriched among the differentially expressed genes, we carried out functional enrichment analysis based on the Riley functional classification system and the annotation at the *S. meliloti* genome browser. For analysis of plant gene expression, we used the MapMan functional classification system (Mt_Mt3.5_0411 MapMan bins). To analyze whether a certain functional category is either positively or negatively enriched in nodules from *dnf* mutants, we calculated *P* values and $\log_2$ -fold changes using PGSEA and LIMMA (Smyth 2005) R packages. We used Fisher's exact test, as described for the enrichment analysis of functional categories, to identify if a functional group is over-represented in plant or bacterial gene clusters.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

MapMen Site of Analysis database:

<http://MapMan.gabipd.org/web/guest/MapManstore>

PGSEA software:

<http://www.bioconductor.org/packages/release/bioc/html/PGSEA.html>