Transcript Analysis of Early Nodulation Events in *Medicago truncatula*[1,2[W]]

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Within the first 72 h of the interaction between rhizobia and their host plants, nodule primordium induction and infection occur. We predicted that transcription profiling of early stages of the symbiosis between *Medicago truncatula* roots and *Sinorhizobium meliloti* would identify regulated plant genes that likely condition key events in nodule initiation. Therefore, using a microarray with about 6,000 cDNAs, we compared transcripts from inoculated and uninoculated roots corresponding to defined stages between 1 and 72 h post inoculation (hpi). Hundreds of genes of both known and unknown function were significantly regulated at these time points. Four stages of the interaction were recognized based on gene expression profiles, and potential marker genes for these stages were identified. Some genes that were regulated differentially during stages I (1 hpi) and II (6–12 hpi) of the interaction belong to families encoding proteins involved in calcium transport and binding, reactive oxygen metabolism, and cytoskeleton and cell wall functions. Genes involved in cell proliferation were found to be up-regulated during stages III (24–48 hpi) and IV (72 hpi). Many genes that are homologs of defense response genes were up-regulated during stage I but down-regulated later, likely facilitating infection thread progression into the root cortex. Additionally, genes putatively involved in signal transduction and transcriptional regulation were found to be differentially regulated in the inoculated roots at each time point. The findings shed light on the complexity of coordinated gene regulation and will be useful for continued dissection of the early steps in symbiosis.

The symbiotic interaction between legume roots and bacteria in the Rhizobiaceae (collectively called rhizobia) leads to the formation of root nodules, where rhizobia fix atmospheric dinitrogen into ammonia for use by the plant. The process of nitrogen fixation has economic importance, plus biological significance for understanding the interaction between plants and bacteria. Use of the model legumes *Lotus japonicus* and *Medicago truncatula* (Cook, 1999; Stougaard, 2001; Gage, 2004) has aided definition of early events in nodulation. In the symbiosis between *M. truncatula* and *Sinorhizobium meliloti*, after the plant has perceived the bacterial signal molecule Nod factor, likely through LysM domain receptor kinases (Riley et al., 2004), root hairs quickly respond with calcium spiking and calcium and ionic fluxes (Cárdenas et al., 2000; Shaw and Long, 2003a). Swelling of the root hair tip occurs within 1 h of Nod factor treatment, followed by new tip outgrowth after 2 to 3 h and root hair branching by 16 h (Catoira et al., 2000). Inner cortical cells become activated for cell division 18 to 24 h post inoculation (hpi) with rhizobia, and a nodule primordium is initiated 24 to 48 hpi (Timmers et al., 1999). Infection threads that enclose rhizobia start to form in curled root hairs by 48 hpi (Timmers et al., 1999). A large proportion of these initial infections are arrested in an ethylene-dependent manner (Penmetsa and Cook, 1997). Nodule primordia that have been successfully invaded by rhizobia are visible as bumps on the root surface 72 hpi. Thus, in the first 3 d of the interaction, important events occur that determine the success of the symbiosis.

Understanding of the plant's genetic control of early events in nodulation is coming into focus. Several key genes, first identified by phenotype, have been cloned that encode proteins required for these early stages, including several protein kinases, putative transcriptional regulators, and proteins that may regulate

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and/or respond to ion fluxes (Endre et al., 2002; Limpens et al., 2003; Ané et al., 2004; Mitra et al., 2004a; Kaló et al., 2005; Smit et al., 2005). Events leading to induction of nodules likely require regulated expression of a large number of plant genes. Elevated expression of several genes during early nodule development has been reported. Well-characterized markers of early responses include the early nodulin genes *ENOD11*, *ENOD12*, and *RIP1* that are induced in the root epidermis within 2 h of treatment with Nod factor (Journet et al., 1994, 2001; Cook et al., 1995). *ENOD20* and *ENOD40* are induced later in the cortex and pericycle cells, respectively (Asad et al., 1994; Vernoud et al., 1999). However, comparatively few transcriptionally regulated genes that participate in the determinative events initiating nodule formation have been characterized.

DNA arrays can be used to quantify expression of many genes simultaneously (Schena et al., 1995; Liu et al., 2003; Meyers et al., 2004). Colebatch et al. (2002) used a cDNA macroarray to compare gene expression in nitrogen-fixing nodules and uninfected roots of *L. japonicus*. They identified 83 genes to be more abundant in nodules than in roots. Liu et al. (2003) used a cDNA array to examine transcript profiles in *M. truncatula* roots during symbiosis with *Glomus versiforme*. They found that 3% of the genes evaluated showed significant changes in transcript levels during the development of arbuscular mycorrhizal infections. Similarly, Mitra et al. (2004b) used an oligonucleotide chip representing about 10,000 genes to identify those that were differentially regulated in response to *S. meliloti* and its Nod factors. At 24 h, the single time point evaluated, 46 plant genes were differentially regulated in wild-type *M. truncatula* in response to *S. meliloti*, most of which required Nod factor perception for their correct expression. Küster et al. (2004) used a *M. truncatula* cDNA microarray containing about 6,000 genes to identify genes expressed in 10-d-old nodules and in *Glomus intraradices*-colonized roots. Similarly, Yahyaoui et al. (2004) identified more than 750 differentially expressed genes among 6,000 probes in wild-type and mutant *M. truncatula* and *S. meliloti* interactions at 3 and 10 d post inoculation (dpi). However, a transcript profiling experiment involving multiple time points during early, determinative events (e.g. before 3 dpi) in legume/rhizobia interactions has not been reported to our knowledge.

Here, we describe results from transcript profiling experiments examining transcript abundance in *M. truncatula* roots from 1 to 72 hpi with *S. meliloti*. The cDNA microarray consisted of more than 6,000 cDNA clones from a broad diversity of *M. truncatula* libraries. A large number of genes were significantly differentially regulated at each time point in inoculated roots compared to uninoculated roots. The root response to *S. meliloti* was divided into four stages based on transcript abundance profiles. The first two stages are characterized by an apparent induction, then suppression, of defense and disease response genes, and by differential expression of many genes related to signaling and infection induction. Marked induction of genes encoding proteins related to cell proliferation characterized the third and fourth stages. Potential new markers for each of the stages are presented.

RESULTS AND DISCUSSION

Stages of the *M. truncatula*/*S. meliloti* Symbiosis Selected for Transcript Profiling

To evaluate early events in response to rhizobia and nodule induction, *M. truncatula* roots were harvested for microscopic observation at the same time points as for transcript profiling. Swelling of root hair tips was observed 1 hpi, followed by resumption of polar growth, producing an asymmetrical root hair tip by 6 hpi and hair branching by 12 hpi (Fig. 1). Curled root hairs were visible by 24 hpi, and inner cortical cells started dividing between 24 and 48 hpi. The initiation of infection threads in the tightly curled root hairs was also observed at 48 hpi. At 72 hpi, nodule primordia were observed with infection threads penetrating the cortical cells and entering the primordia. The morphological changes observed in the inoculated roots were comparable to published reports of events in *M. truncatula* and *S. meliloti* interactions (Timmers et al., 1999; Catoira et al., 2000). Therefore, the transcript profiles from cDNAs produced from these tissues should serve as a good baseline for interpreting infection responses generally.

Hundreds of Genes Are Differentially Regulated in Response to *S. meliloti* Inoculation

Data for all time points for all genes on the array are listed in Supplemental Table I, which contains mean normalized intensities for inoculated and uninoculated roots, as well as expression ratios of inoculated to uninoculated root intensities. Genes that show statistically significant differences between inoculated and uninoculated roots (herein referred to as significant

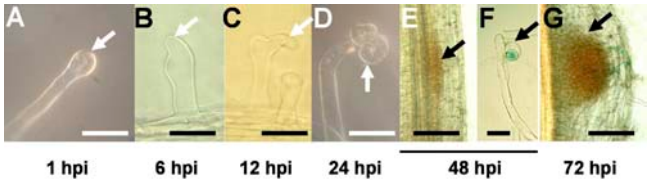

Figure 1. *M. truncatula* root responses to *S. meliloti*. Time points correspond to those used for harvesting RNA from inoculated roots for production of cDNAs for probing microarrays. A, A root hair with swollen tip (arrow) at 1 hpi. B, A root hair with an asymmetrically growing tip (arrow) at 6 hpi. C, Root hair deformation and branching (arrow) at 12 hpi. D, A curled root hair at 24 hpi. E, Cell divisions in the root inner cortex forming a nodule primordium (arrow) at 48 hpi. F, A curled root hair with a nascent infection thread (arrow, blue) at 48 hpi. G, A nodule primordium (arrow) with infection threads (blue) penetrating the dividing cells at 72 hpi. Bars in A, B, C, D, and F are 25 μm, and in E and G are 100 μm.

genes) are flagged. Genes are annotated with the GenBank accession number for an expressed sequence tag (EST) corresponding to the cDNA clone and a gene description based on BLAST analysis of the EST and The Institute for Genomic Research (TIGR) tentative consensus sequence (TC; version 7) containing the EST corresponding to the spotted cDNAs. Additionally, significant genes with fold changes of ≥1.5 at each time point are presented in Supplemental Tables II to VII. The entire data set has been deposited in Gene Expression Omnibus with a GEO accession number GSE3441.

To detect significant genes with regulated expression and to eliminate those that have inconsistent expression data among replicated experiments, we employed a statistical method adapted specifically for microarrays, which allows estimation of the false discovery rate (FDR) for multiple testing (Tusher et al., 2001). A delta criterion that allowed a FDR < 0.5% was applied. Genes that satisfied the statistical threshold were identified as significantly up- or down-regulated in inoculated roots. The number of genes identified as significantly regulated differs depending on the fold change threshold used, with fewer significant genes identified with a stricter change criterion. A large number of important genes with low transcript abundance levels may be overlooked if a ≥2.0-fold criterion is used for interpreting microarray results. For example, Yahyaoui et al. (2004) reported that the induction ratios of three early nodulin genes (*MtENOD40*, *MtN3*, and *MtN13*) in response to *S. meliloti* 3 dpi were below 2.0 in wild-type *M. truncatula*. A reason to avoid a high fold-change threshold is lack of knowledge about thresholds of expression that would be required to induce downstream effects. Thus, the penalty for not reporting a differentially regulated gene may be greater than reporting falsely a gene as differentially regulated under strict statistical conditions. Yang et al. (2002) suggested that changes in gene expression smaller than 2.0-fold could be reliably identified as differentially expressed if well optimized laboratory and analytical techniques were used. Therefore, we have utilized evaluation of statistical significance in combination with a FDR of <0.5% as criteria for determining regulated genes for our experiments, with an emphasis on those genes with a fold change of ≥1.5. Table I presents numbers of genes meeting these criteria at each of the time points evaluated.

Table I. *Numbers of genes significantly up- or down-regulated in inoculated roots compared to uninoculated roots*

FC indicates fold change in transcript abundance, equal to the ratio of transcripts in inoculated roots to transcripts in uninoculated roots.

Regulation	1 hpi	6 hpi	12 hpi	24 hpi	48 hpi	72 hpi
Up-regulated (≥1.5 FC)	195	108	49	240	41	54
Down-regulated (≤0.67 FC)	56	191	62	241	58	58

Multiple Approaches Validate the Results from Microarray Analysis

Several approaches were utilized to validate the microarray results obtained. First, the expression patterns for known markers of early nodulation responses were evaluated (Table II). *ENOD40* is an early nodulin that encodes an unusually small peptide and has been reported to be involved in controlling Suc use in nodules (Charon et al., 1997; Rohrig et al., 2002). *ENOD40* induction was detectable by 6 hpi, and it was strongly up-regulated by 48 hpi. *ENOD12*, which encodes a (hydroxy)Pro-rich protein that is induced during nodule formation (Scheres et al., 1990; Pichon et al., 1992), was up-regulated significantly in inoculated roots by 12 hpi. *MtN1* has been shown to be associated with the infection process (Gamas et al., 1996, 1998). Here, *MtN1* was found to be significantly induced by 12 hpi and at later time points with the strongest fold change at 24 hpi. An ortholog of Arabidopsis (*Arabidopsis thaliana*) *Response Regulator 4* (*ARR4*; TC78129) was significantly up-regulated at 1, 6, 12, and 48 hpi in the inoculated roots. Lohar et al. (2004), using a *β-glucuronidase* (*GUS*) reporter gene behind the *ARR5* (a close homolog of *ARR4*; D'Agostino et al., 2000) promoter, showed that *ARR5* is transcriptionally induced in curled root hairs, cortical cells, and nodule primordia in inoculated transgenic hairy roots of *L. japonicus*.

We also compared transcript abundance of genes that were reported as significantly up-regulated at 24 h after treatment with Nod factor or *S. meliloti* Rm1021 (Mitra et al., 2004b). Mitra et al. (2004b) identified 40 genes up-regulated (with a fold change of ≥2) and six genes down-regulated in response to a 24-h Nod factor treatment. In this study, at the same time point, 66 and 85 genes were found to be significantly up- and down-regulated, respectively, using a similar criterion of ≥2.0-fold change. As presented in Table II, out of nine TCs common to both our array and that of Mitra et al. (2004b), this study had data for six TCs at 24 hpi, and all of them had significantly up-regulated expression in inoculated tissue. The similar fold change for these genes between our microarray results and those of Mitra et al. (2004b) further demonstrates the reproducibility of these results.

As a second approach to validate the microarray results and to demonstrate the validity of pooling RNA samples from three independent biological replicates for microarray hybridizations, we selected several significant genes with different fold changes for evaluation of transcript abundance by quantitative real-time reverse transcription (RT)-PCR (qRT-PCR) on RNA samples from three independent biological replicates (Table III). The results showed the same direction of fold change of transcript abundance in all three biological replicates for all tested genes, thus confirming the validity of the microarray results.

A semiquantitative approach (SQRT-PCR) was used on pooled RNA samples to verify the expression of additional significant genes in the microarray

Table II. *Transcript abundance changes (mean fold change between inoculated and uninoculated roots) for nodulins and some other reported genes at different time points after inoculation*

NS (superscript), Fold change not significant; MD, missing data; NA, no hit in GenBank.

TIGR TC[a]	Gene Description	Fold Change					
		1 hpi	6 hpi	12 hpi	24 hpi	48 hpi	72 hpi
TC87327	*ENOD12*	1.2^{NS}	MD	1.5	MD	3.7	8.9
TC85858	*ENOD40*	0.9^{NS}	1.3	1.3	1.3	2.4	2.1
TC8633	*MtN1*	0.9^{NS}	MD	2.2	6.6	1.9	3.3
TC78129	ARR4	1.2	1.6	1.3	1.2^{NS}	1.2	0.9^{NS}
TC86110[b]	Aquaporin	1.0^{NS}	2.1	1.6	3.8	1.5	1.4
TC77604[b]	SAR DNA-binding protein	1.2	0.9^{NS}	1.3	1.9	1.6	MD
TC76514[b]	NuM1 protein	MD	0.9^{NS}	1.2	1.8	1.4	1.1
TC78657[b]	Acyl-activating enzyme	1.9	1.5	1.7	3.9	1.0^{NS}	0.8^{NS}
TC90606[b]	NA	1.2	0.7	0.9	1.8	0.8	1.2^{NS}
TC80571[b]	Nucleolar protein like	0.8^{NS}	0.8	MD	1.7	MD	1.0^{NS}

[a]Corresponding to TCs in MtGI 7.0. [b]TCs corresponding to genes found to be significantly up-regulated at 24 hpi by Mitra et al. (2004b).

experiments. Figure 2 shows the results from SQRT-PCR for five different genes representing 17 pairs of data points from microarray experiments. Genes showing significant changes in expression on the microarrays displayed visible differences in the quantities of the amplified products in SQRT-PCR for all pairs of data points. We were able to validate the direction of change of significant genes with fold changes as low as 1.15 in microarray experiments (TC80422, 12 hpi). Overall, the RT-PCR results corresponded to the microarray results in terms of direction of change and again indicated the reproducibility of our microarray results.

The expression of one gene was further analyzed using a promoter-reporter fusion. A cytokinin receptor-like kinase (TC80422) that is similar to *CRE1b* of Arabidopsis was significantly up-regulated between 6 and 48 hpi, although the fold change by microarray evaluation was never more than 1.37. We fused the putative promoter region of this gene to a *GUS* reporter gene, and followed *GUS* activity after *S. meliloti* inoculation in transgenic hairy roots of *M. truncatula* (Fig. 3). Without inoculation, staining was visible in root tips, and faint staining was occasionally seen in cortical cells (Fig. 3A). After inoculation, strong *GUS* expression was observed in the zone above the root tip (Fig. 3B) and in patches of cortical cells in mature roots (Fig. 3C); however, no staining was observed in root hairs in the presence or absence of rhizobia (data not shown). Strong expression was observed in lateral root primordia (Fig. 3, D–H), which became restricted to the root tip once the lateral roots grew out of the parent root. Strong *GUS* expression also occurred in nodule primordia, which became restricted to nodule meristems in mature nodules (Fig. 3, I–N). Overall, the patterns of *CRE1b* promoter activity in nodule and lateral root primordia indicated that transcript of the *CRE1b* homolog might accumulate most strongly in mitotically activated cells. Whether *CRE1b* is induced by cytokinins needs further investigation.

Potential New Markers for Early Stages of Symbiosis

Gene expression profiles at all time points were clustered to identify transcriptional stages of early interactions between *M. truncatula* and *S. meliloti*. Figure 4A presents clustering of all significant genes

Table III. *Microarray fold change in pooled samples and qRT-PCR fold change in biological replicate samples*

rep1, rep2, and rep3, Biological replicates.

TC No.	Annotation	Time Point	Microarray Fold Change in Inoculated Roots	qRT-PCR Fold Change			
				rep1	rep2	rep3	Mean ± SD
		hpi					
TC90246	Receptor kinase	1	2.07	1.31	1.30	1.28	1.30 ± 0.02
TC78560	NA	1	4.4	1.93	1.69	2.31	1.98 ± 0.31
TC78984	Protease inhibitor	1	4.81	1.59	1.44	1.84	1.62 ± 0.20
TC89068	Receptor kinase	1	3.56	2.42	8.04	7.73	6.06 ± 3.16
TC80693	Ran GTPase	1	2.22	1.31	1.25	2.13	1.56 ± 0.49
TC78236	bHLH transcription factor	12	1.21	1.22	1.47	1.21	1.30 ± 0.15
TC77186	Phosphate transporter	12	1.56	3.37	3.91	1.63	2.97 ± 1.19
TC78189	NAC domain protein	48	1.72	1.16	1.24	1.36	1.25 ± 0.10
TC88112	RNA-binding protein	48	1.74	1.24	1.16	1.29	1.23 ± 0.07
TC78627	Pathogenesis-related protein	72	1.69	5.49	1.98	1.63	3.30 ± 2.13
TC59582	3-Hydroxy-3-methylglutaryl CoA reductase	72	1.78	2.76	2.6	4.00	3.12 ± 0.77

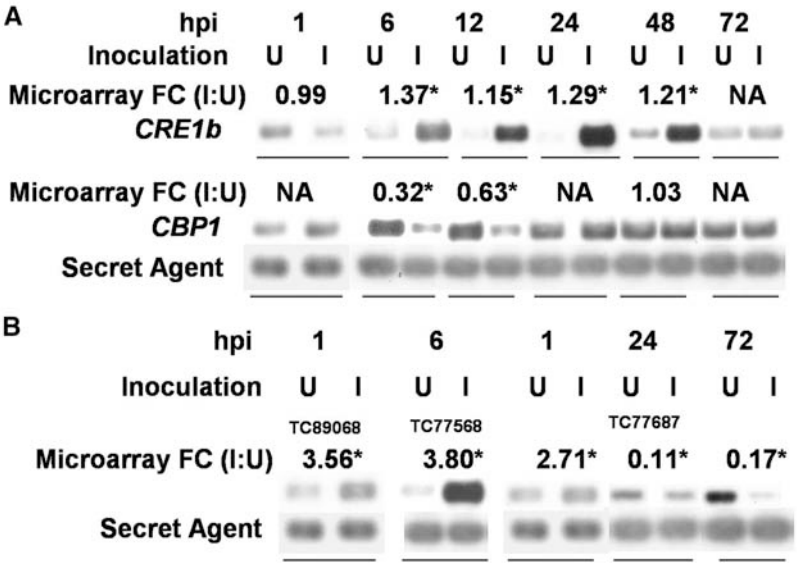

Figure 2. SQRT-PCR validation of microarray results for selected cDNAs. The TC numbers correspond to the TCs that contain the ESTs from the clones used on the microarrays. Probable functions are deduced from top BLAST hits of the TCs mentioned. A, TC80422, a putative cytokinin receptor-like His kinase (*CRE1b*); TC80225, a putative calcium-binding protein (*CBP1*); and TC77416, the Medicago homolog of the Arabidopsis gene Secret Agent, as a loading control. B, TC89068, a probable receptor-like kinase; TC77568, a transcript encoding a protein of unknown function; TC77687, a probable zinc finger transcription factor, with Secret Agent shown as a loading control. Microarray FC (I:U) is the ratio of expression between root tissue inoculated with *S. meliloti* and uninoculated root tissue in microarray experiments. FC, Fold change; U, uninoculated; I, inoculated; NA, data not available. Asterisk indicates a significant fold change.

at different time points. Results of cluster analysis of the experiments identified close similarities in transcriptional responses between roots at 6 and 12 hpi, and similarly between roots at 24 and 48 hpi. Therefore, we defined four stages corresponding to transcriptional changes in response to rhizobia: stage I represented gene expression changes observed at 1 hpi; stage II, at 6 to 12 hpi; stage III, at 24 to 48 hpi; and stage IV, at 72 hpi. Interestingly, gene expression changes between 1 and 12 hpi were more dissimilar than those between 24 and 72 hpi. This may reflect more diverse physiological and regulatory changes in the root in the first hours after inoculation than during primordium formation.

Fifty genes were identified as new markers for early stages of symbiosis (Fig. 4B). Because experiments corresponding to 6 and 12 hpi, and 24 and 48 hpi, clustered together (Fig. 4A), common marker genes were identified for these two pairs of time points, designated as stage II and stage III, respectively. The genes selected as markers for a particular stage were selected based on ≥1.5-fold up-regulation in the inoculated roots, statistical significance, and the lack of high fold induction earlier in the time series for stages II, III, and IV. For stage I, significant up-regulation of ≥1.5-fold at 1 hpi and no significant up-regulation at later time points were chosen as criteria. By these criteria, among the previous markers identified in Table II, one gene (TC78657, an acyl-activating enzyme homolog) would be a marker for stage I, three (*ENOD12*, *MtN1*, and an aquaporin [TC86110]) would be markers for stage II, and the remainder would be markers for stage III. Twenty-six new potential markers were identified for stage I (1 hpi). Similarly, 13 genes were identified as potential markers for stage II (6 and 12 hpi), seven genes for stage III (24 and 48 hpi), and four genes for stage IV (72 hpi; Fig. 4B). The *CRE1b* homolog was included as a marker even though it had a lower fold induction because it was confirmed as an up-regulated gene by SQRT-PCR and reporter gene expression (Figs. 2 and 3). An RNA-binding protein (TC88112), which has been shown to be exported to the cytoplasm from the nucleus in *ENOD40*-expressing cells during nodule development (Campalans et al., 2004), was identified as a marker for 24 to 48 hpi. *ENOD40* is also significantly induced at 6 hpi and later (Table II), indicating a possible coregulation of expression of these genes at early time points. Eleven of these marker genes were among the genes used to validate the microarray results by qRT-PCR. As presented in Table III, the expression changes of all 11 genes were confirmed for all three biological replicates.

Genes with known function or that were homologs of genes of known function were divided into functional categories for further data mining. Assignments were based on Gene Ontology categories and suspected or known roles in early interactions between *M. truncatula* and *S. meliloti*, based on published accounts. As a caveat, it should be noted that because the assignment to categories for many of the genes is based only upon automated annotation of ESTs, it is therefore subject to error. Nevertheless, this approach is useful for observing patterns in gene expression and advancing hypotheses that may be evaluated by later experimentation. We evaluated the *Z* score, a standardized difference between observed and expected values, to determine whether up- or down-regulated genes in a functional gene group were found in numbers greater than would be expected by chance. *Z* scores for various gene groups, evaluating the occurrence of up-regulated genes among group members, are shown in Table IV. TCs included in gene groups used to calculate *Z* scores in Table IV are given in Supplemental Table VIII. Evaluation of transcriptional profiles for these functional groups revealed many insights about the time course of symbiotic responses. Three prominent

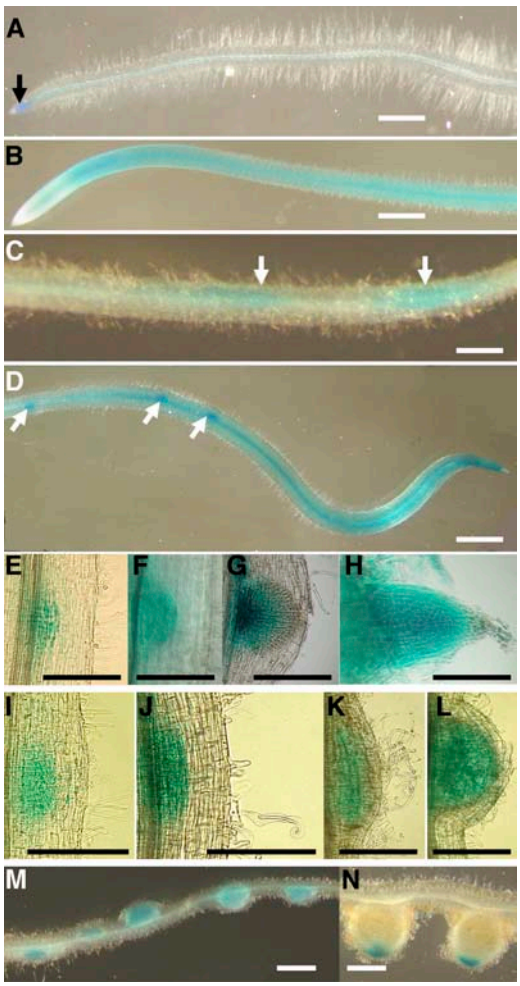

Figure 3. *M. truncatula* roots transgenic for a putative cytokinin receptor-like kinase promoter:*GUS* fusion. Blue staining indicates *GUS* expression and, hence, a transcriptional activation of the cytokinin receptor-like kinase gene. A, An uninoculated root showing *GUS* expression in the root tip (arrow). B, An inoculated root showing *GUS* induction in the root zone above the root tip. C, *GUS* expression in patches of a mature part of inoculated root (arrows). D, An inoculated root showing dark blue-stained lateral root primordia (arrows). E to H, *GUS* expression at different stages of lateral root formation. I to L, *GUS* expression at different stages of nodule formation. M, *GUS* induction in developing nodules in a segment of root. N, Persistent *GUS* expression in the tip of mature nodules. Bars in A to D and M to N are 3 mm, and in E to L are 100 μm.

patterns were observed, including up-regulation of defense- and stress-related genes during stage I, up-regulation of genes with presumed functions in cytomorphogenesis in stage II, and up-regulation of genes related to cell proliferation and protein synthesis in the later stages, as described below.

Induction of Many Putative Defense, Disease, and Stress Response Genes Demarcates Stage I of Transcriptional Responses to Rhizobia at 1 hpi

A successful symbiosis is thought to require inhibition of defense responses by the host plant (Mithöfer, 2002). Therefore, we examined expression of putative defense and disease response genes in the inoculated roots relative to uninoculated roots. Overall, up-regulated genes were significantly overrepresented within the disease and stress response gene group during stage I (Table IV). However, at later stages down-regulated genes were significantly overrepresented in this group, as indicated by significant *Z* scores. The maximum suppression of pathogen and defense response genes as a group occurred during stage III, coincident with infection thread penetration into root hairs.

Supplemental Figures 1 and 2 show the fold changes of transcripts in inoculated versus uninoculated roots for homologs of putative disease resistance and stress response genes, respectively. Different subgroups had notable patterns of expression. Chitinase genes were largely up-regulated between 1 and 24 hpi, though expression thereafter was variable. Salzer et al. (2004) reported the induction of class IV and V of chitinase genes in response to rhizobia as dependent on the *M. truncatula* genotype. Glutathione *S*-transferases, which play an important role in the detoxification and metabolism of many xenobiotic and endobiotic compounds (Edwards et al., 2000), were mostly down-regulated at 6 and 12 hpi, but some members were up-regulated at 24 hpi. Among stress response genes, two mannitol dehydrogenase genes were up-regulated at 1 hpi. An increase in mannitol dehydrogenase transcript abundance has been shown to reflect pathogen attack and other environmental stresses (Williamson et al., 1995).

The end products of the isoflavonoid pathway, pterocarpan phytoalexins, have antimicrobial activity, while certain pathway intermediates are potent elicitors of Nod factor biosynthesis in rhizobia (Dixon and Sumner, 2003). A comparison of *Z* scores of disease response genes and flavonoid biosynthesis genes reveals opposite expression profiles of these two groups (Table IV), and suggests that the flavonoid pathway may serve functions in addition to defense during symbiosis. Supplemental Figure 3 presents the expression profiles of genes known to be involved in flavonoid biosynthesis and homologs of known pathway genes, such as putative cytochrome P450s of unknown function. Many genes in this group were significantly regulated at each time point in the inoculated roots, and a significant *Z* score indicates overrepresentation of up-regulated genes in this group in stages II to IV. Members of the chalcone synthase and chalcone reductase families were all expressed more strongly in *S. meliloti*-inoculated roots than in uninoculated roots, especially at time points through 24 hpi. Isoliquiritigenin 2′-*O*-methyltransferase is specifically involved in the biosynthesis of a potent inducer of *S. meliloti* nodulation genes (Maxwell et al., 1993). Notably, two such genes were generally up-regulated, especially after 1 hpi. In addition to acting as inducers of Nod factor biosynthesis, accumulation of isoflavonoid pathway intermediates is thought to affect auxin accumulation, thus contributing to primordium formation

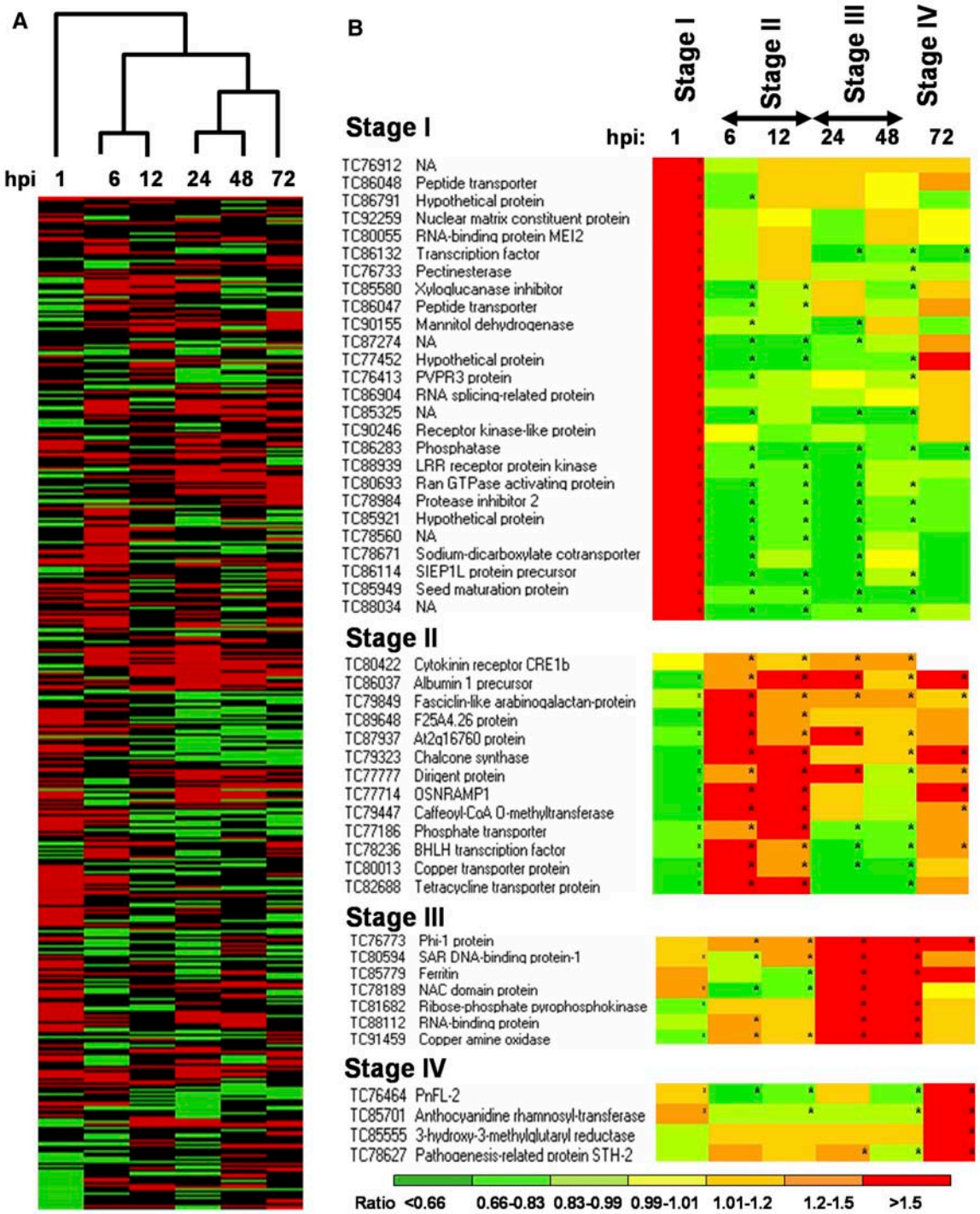

Figure 4. Clustering of time points based on gene expression profiles, and profiles of potential marker genes for different stages of nodulation. A, Two-dimensional clustering of significant genes and experimental time points. Distances for experiments were calculated using positive correlation and the Complete Linkage method. B, Potential marker genes for early interactions between *M. truncatula* and *S. meliloti*. Stage I, 1 hpi; stage II, 6 and 12 hpi; stage III, 24 and 48 hpi; stage IV, 72 hpi. A ratio higher than one indicates up-regulation and less than one indicates down-regulation of the transcript in inoculated roots compared to uninoculated control roots. Asterisks mark those ratios where expression in inoculated roots was significantly different than in uninoculated roots. NA, No hit in GenBank.

Table IV. *Z scores for groups of genes selected based on putative function*

A *Z* score of ≥2.0 or ≤−2.0 indicates that the gene group is represented at levels significantly above what would be expected by chance among up-regulated or down-regulated genes in inoculated roots, respectively.

Gene Groups	Stage I	Stage II		Stage III		Stage IV
	1 hpi	6 hpi	12 hpi	24 hpi	48 hpi	72 hpi
Defense and disease response	3.0	−3.7	−3.2	0.6	−6.0	−1.9
Stress response	2.0	0.9	−0.3	−0.5	−1.7	−2.4
Flavonoid biosynthesis	0.9	4.0	3.2	2.3	0.7	3.8
Cytoskeleton structure	−0.8	3.2	1.7	1.0	−0.5	1.4
Ca^{2+} binding and storage	3.5	−3.9	−1.7	0.6	−0.8	0.7
Peroxidases	−1.9	0.9	1.8	1.1	0.2	1.0
Cell wall related	2.2	1.5	1.6	0.8	0.7	−1.0
Protein kinases	−0.7	0.3	0.7	−0.2	0.5	0.3
Cell cycle	0.8	1.0	2.1	1.1	1.1	1.5
Translation	−5.0	−0.9	1.2	6.0	6.0	2.7
Chromosome organization	−0.0	−1.6	1.1	4.0	2.8	4.1

(Mathesius et al., 1998; Mathesius, 2001). The observed gene expression profiles are consistent with this theory and indicate that additional metabolomic analysis of inoculated roots should be fruitful.

Up-Regulation of Genes Governing Cytoskeleton Structure and Cell Wall Composition Correlates with Root Hair Deformation in Stage II

Nod factor treatment and rhizobial inoculation trigger depolymerization, repolymerization, reorganization, and proliferation of cytoskeletal elements in both root hairs and the root cortex (Takemoto and Hardham, 2004). Therefore, transcriptional regulation of proteins associated with the cytoskeleton might occur in concert with these events. In the cytoskeleton structure gene group, up-regulated genes were significantly overrepresented at 6 hpi, which is coincident with the onset of root hair deformation Table IV). Supplemental Figure 4 presents expression profiles of genes involved in cytoskeleton formation and function that had significant changes in expression following *S. meliloti* inoculation. At 24 hpi, some individual cytoskeletal genes are strongly up-regulated, most notably β-tubulin homologs, coincident with infection thread development and the onset of cell proliferation during nodule primordium formation. Increase in α-tubulin expression has been observed previously at a similar time point in pea (*Pisum sativum*) nodule formation (Stotz and Long, 1999).

Many new cell walls must be synthesized during cell divisions resulting in nodule primordia and during infection thread formation, corresponding to stages III and IV here. From our data, the up-regulation of cell wall genes in the group occurs most prominently at 1 hpi (Table IV), thus preceding infection or primordium formation. Supplemental Figure 5 presents expression profiles of genes related to cell wall organization and modification.

Several groups of genes encoding likely cell wall enzymes were also modulated early during the symbiosis, beginning at stage I. α-Fucosidases were generally up-regulated in stages I and II, and down-regulated thereafter in the inoculated root. α-Fucosidases cleave the terminal Fuc residue from xyloglucans. Liu et al. (2004) also reported up-regulation of an α-fucosidase gene in *M. truncatula* during symbiosis with an arbuscular mycorrhizal fungus. Some polygalacturonases were up-regulated in inoculated roots in stages II and III. This is in agreement with Muñoz et al. (1998), who reported the induction of a polygalacturonase gene in *M. sativa* 24 h after spot inoculation with *S. meliloti*.

Induction of Gene Families Involved in Cell Proliferation and Gene Expression Characterizes Stages III and IV

Induction of nodule primordia requires cell cycle activation in the pericycle and root cortex (Kondorosi et al., 2005). We examined expression profile of genes with functions related to cell proliferation, chromosome replication, transcription, and translation to determine if these groups could be used as markers during nodulation. Evaluation of *Z* scores of these groups indicated that cell cycle-related genes were significantly induced in stage II (12 hpi), presaging the prominent induction of genes related to chromosome organization and translation throughout stages III and IV (Table IV). Supplemental Figures 6, 7, and 8 depict gene expression ratios as heat maps in inoculated versus uninoculated roots of genes in these groups. Among genes involved in chromosome organization, many histones show a pattern of induction starting at 24 hpi (Table IV; Supplemental Fig. 6). By contrast, one centromere protein homolog was more strongly induced at 1 hpi in inoculated roots compared to uninoculated roots.

Mitra et al. (2004b) also reported an elevated expression of several genes involved in ribosome construction, ribosomal RNA processing, and cell proliferation at 24 hpi. Here, we show that this trend was most prominent at that time point (Supplemental Figs. 7 and 8). Pericycle cells start to be activated for cell division between 16 to 18 hpi as evidenced by increased microtubular cytoskeleton immunolabeling, and an initial

nodule primordium with dividing cells is formed between 24 to 48 hpi (Timmers et al., 1999). Thus, the generally significant up-regulation of these groups of genes observed in our microarray experiment corresponds well with the need of the plant cells for new proteins.

Interestingly, the changes in transcript abundance observed in stages I and II do not appear to involve a wholesale up-regulation of machinery for protein biosynthesis. By contrast, among genes encoding proteins involved in translation, down-regulated genes were significantly overrepresented in inoculated roots at stage I (Table IV; Supplemental Fig. 7). Inhibition of protein biosynthesis is a metabolic response of plants under stress (Rhodes and Nadolska-Orczyk, 2001). For example, overexpression of the disease resistance gene *Pto* in tomato (*Lycopersicon esculentum*) activates defense responses in the absence of pathogen inoculation (Mysore et al., 2003). Such plants show suppressed expression of genes involved in translation initiation as well as elongation factor and chromatin-associated protein genes. Here, the noticeable down-regulation of ribosomal protein genes at 1 hpi could be due to the recognition of rhizobia by plant roots as a biotic stress. The up-regulation of genes involved in defense responses at 1 hpi in the inoculated roots, as presented earlier, further supports this hypothesis.

Transcriptional Responses Identify New Plant Candidate Genes That May Regulate Signal Transduction and Development in Response to Rhizobia

Gene Products Affecting Second Messenger Production and Perception

Ca^{2+} flux and spiking occur in root hairs in response to Nod factors in legume plants (Ehrhardt et al., 1996; Cárdenas et al., 1999; Shaw and Long, 2003a). Several *M. truncatula* mutants that fail to establish a symbiosis with *S. meliloti* have defective root hair Ca^{2+} responses, and the affected genes appear to be involved in calcium sensing or responses (Wais et al., 2000; Walker et al., 2000; Shaw and Long, 2003a; Oldroyd et al., 2005). Expression profiles of significantly regulated genes that are putatively involved in Ca^{2+} transport, storage, or binding are presented in Supplemental Figure 9. As seen in Table IV, 1 hpi is the time point at which

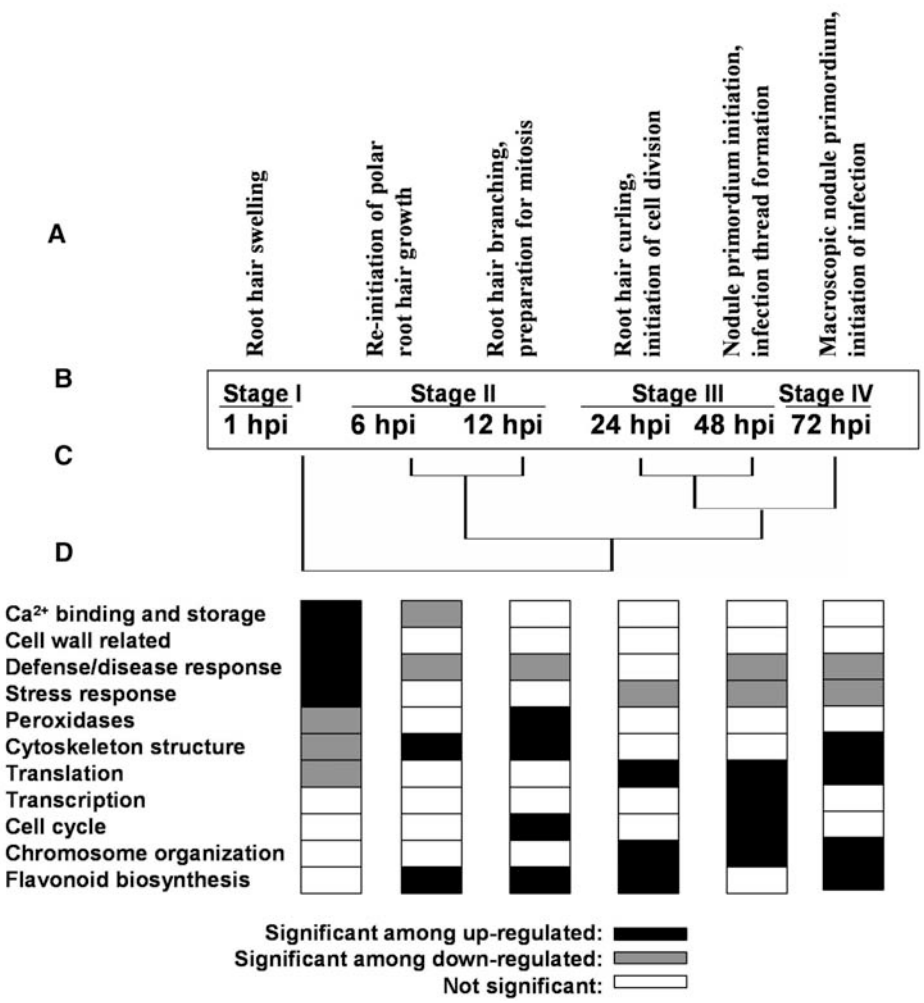

Figure 5. Expression changes of some gene groups in roots of *M. truncatula* inoculated with *S. meliloti*, and accompanying morphological changes from 1 hpi to 72 hpi. A, Root responses observed at different time points in B. C, Clustering of time points based on gene expression changes as presented in Figure 4. D, Representation of some gene groups among up- or down-regulated genes at different time points based on *Z* scores.

up-regulated genes showed a significant overrepresentation in the Ca^{2+} storage and binding gene group, although only one gene, a putative Ca^{2+}-ATPase (TC90329), was induced at high levels at this time point. Several calmodulin-related genes showed significant up-regulation in inoculated roots at 24 and 72 hpi. This transcriptional response occurs much earlier than the accumulation of transcripts from novel calmodulin-related genes in *M. truncatula* nodules (Fedorova et al., 2002). Interestingly, some other members of this group showed significant down-regulation at 6 hpi, contributing to a significant pattern for the group (Table IV). Clearly, differential accumulation of transcripts encoding likely calcium-binding proteins has the potential to impact signaling in early time points after inoculation.

Another important signaling event happening during early stages of symbiosis is the modulation of the production/accumulation of reactive oxygen species (ROS; Ramu et al., 2002; Shaw and Long, 2003b). Peroxidases are involved in the metabolism of ROS (Lambeth, 2004), and, therefore, the expression of these genes may affect or respond to the level of ROS in roots after rhizobia inoculation. As a group, peroxidases on the array did not show a prominent pattern of regulation (Table IV), although eight genes showed marked induction at one or more time points between 2 and 24 hpi (Supplemental Fig. 10). Nitroblue tetrazolium staining of whole plants 12 to 24 hpi suggested an increase in ROS concentration in the root that was implicated in the induction of a rhizobium-induced peroxidase gene, *RIP1* (Ramu et al., 2002). Though *RIP1* was not included in our microarray, an up-regulation of peroxidase genes at 6 hpi and later is in agreement with results from Ramu et al. (2002) and Cook et al. (1995). In addition to their effect on ROS accumulation, extracellular peroxidases may mediate oxidative cross-linking of cell wall components via aromatic residues (Passardi et al., 2004).

Several protein kinases have been found to regulate very early steps of nodule formation (Oldroyd et al., 2005). As a group, protein kinases on the array did not show significant patterns of differential expression in response to inoculation (Table IV), though subsets of these genes were noteworthy exceptions. Supplemental Figure 11 presents the six protein kinases on the array that showed significant changes of greater than 1.5-fold. Both patterns of up- and down-regulation were observed. The nodulation receptor kinase NORK (TC77448) that is required for both mycorrhizal and rhizobial symbiosis in *M. truncatula* (Endre et al., 2002) is significantly up-regulated at low but reproducible levels at all time points after 1 hpi (Supplemental Table I).

Transcription Factors during Early Symbiotic Interactions

Reports on transcription factors regulating nodulation are relatively rare in the literature (Schauser et al., 1999; Zucchero et al., 2001), although two putative transcriptional regulators in the GRAS family have recently been shown to be required for nodulation in *M. truncatula* (Kaló et al., 2005; Smit et al., 2005) as has one transcription factor in *L. japonicus* (Schauser et al., 1999). We examined the differential expression of putative transcription factors during early stages of interaction between *M. truncatula* roots and *S. meliloti*. Several numbered among genes proposed above as new markers for early stages of symbiotic interactions (Fig. 4B), including homologs of a Myb-like protein (TC86132), a bHLH transcription factor (TC782236), and a NAC domain protein (TC78189).

Supplemental Figure 12 presents expression patterns of transcription factors at different time points in the inoculated roots as compared to uninoculated roots. One particularly interesting group of genes was made up WRKY genes. One WRKY homolog (TC86532) was significantly up-regulated in the inoculated roots in stage I (1 hpi), whereas this and many other WRKY members were significantly down-regulated at 6 hpi and later (Supplemental Fig. 12). Many WRKY proteins

Table V. *Primers used in quantitative and SQRT-PCR*

TC No.	Forward Primer	Reverse Primer
TC80422	5′-GCCAAATTGGACAGCACTTT-3′	5′-CCCAGGATCTCCC ATAACAA-3′
TC80225	5′-TACCTTGGTGACATGGACGA-3′	5′-AGGCATATGACAGGAGCAGA-3′
TC77277	5′-GCACCTGTTTCAACACCACA-3′	5′-CATTGTCCATTCCACTCTGC-3′
TC77687	5′-TCCCTGAGTGGAACACAACA-3′	5′-ACAAATGATTCCGGTGAAGC-3′
TC89068	5′-CATGGAGAATGGGAATTTGG-3′	5′-GATTCTGAGAGCCCTGTTGG-3′
TC77416	5′-GGCAGGTCTGCCTATGGTTA-3′	5′-GGTCAGACGCACAGATTTGA-3′
TC90246	5′-CGAGTAATTCGGTTAGGGATTG-3′	5′-ATTCCCAATCTTACCACCCTCT-3′
TC80693	5′-AGTAAGGCGCTGGAAGGTCT-3′	5′-AGGACCCAGAATGTCAGGTG-3′
TC78236	5′-TGAGAGGCTCAAGGAAAGGA-3′	5′-GCATCAGCTTGTGGTTAGCA-3′
TC77186	5′-GGCGACAAACTTGGTAGGAA-3′	5′-AAACCTTGCATGGCAAAAAC-3′
TC78189	5′-TTCCCTTCCAATTGCTGTTC-3′	5′-AGGCTTTCCAATCGGCTTAT-3′
TC88112	5′-CACAACAGCAAACCACCATC-3′	5′-ATGCATCTCATTGCGTGAAG-3′
TC78627	5′-CATTAGGGGACAAGCTGGAA-3′	5′-AATGGTGCTTTCCCTTCCTT-3′
TC85555	5′-AAGAAGAACCAAAGGCAGCA-3′	5′-GCGATGAGGGAAACTACAGC-3′
TC78560	5′-CAGGTGCAAAGGATGCAGTA-3′	5′-CAGCATTTTTAGCACCAGCA-3′
TC78984	5′-GTTCATGGGCAGAGTTGGTT-3′	5′-TCCAATTGTTGGAACCTGGTA-3′
TC89068	5′-TGGTTATGATTTTCCCATTGTG-3′	5′-ATTCGAGCTGTCCCAAAATC-3′

have a regulatory function in response to pathogen infection and other stresses (Eulgem et al., 2000). Expression of WRKY genes has been reported to be induced by infection with viruses, bacteria, and fungal elicitors (Eulgem et al., 2000). The expression pattern observed here is consistent with expression of defense response genes (Table IV). Possible targets for WRKY proteins include pathogenesis-related protein genes (Rushton and Somssich, 1998). Whether up-regulation of some pathogenesis related proteins (e.g. TC77138 and TC77137; Supplemental Fig. 1) at 1 hpi is regulated by WRKY genes is a subject for further investigation.

CONCLUSION

We identified hundreds of genes that are differentially expressed in *M. truncatula* roots from 1 h to 72 h following inoculation with *S. meliloti*. A clustering of gene expression profiles indicated four identifiable stages of interaction between these two symbiotic partners during this period (Fig. 5). These stages correspond to root hair swelling (stage I); root hair branching/deformation (stage II); root hair curling, cortical cell division, infection thread formation, and nodule primordium initiation (stage III); and the development of an infected macroscopic nodule primordium (stage IV). Genes from some groups are preferentially suppressed at some time points while induced at some other time points. Our results indicate an induction of putative defense response genes at 1 hpi but a strong suppression later on, particularly at 48 hpi. If a role in defense is verified for these genes, it may indicate that *M. truncatula* roots may initially recognize *S. meliloti* more as a biotic stress than as beneficial symbiont. Similarly, an overall down-regulation of genes involved in translation at 1 hpi may be an indication of plant roots being under stress (such as pathogen attack) at this time point. The induction of a defense response during early interaction between plant roots and beneficial microsymbionts such as arbuscular mycorrhizal fungi has been reported (Volpin et al., 1995). Mycorrhizal symbiosis in land plants is more ancient than is the legume-rhizobia symbiosis (Lum and Hirsch, 2003). It is possible that the legume-rhizobia symbiosis arose from the more widespread mycorrhizal symbiosis, and, therefore, plants employ similar strategies for both. Several mutant plants that fail to establish symbiosis with rhizobia are also defective for mycorrhizal symbiosis (Catoira et al., 2000; Endre et al., 2002). It further strengthens the hypothesis of shared genetic pathways for both symbioses.

Not surprisingly, putative signal transduction and regulatory genes do not show a consistent pattern of transcriptional regulation during early rhizobial responses. However, genes in these groups that do show significant modulation in expression may be components of signaling cascades in response to Nod factor or other bacterial signals. Ivashuta et al. (2005) successfully used transcriptome data to select a calcium-dependent protein kinase for functional evaluation via a gene silencing approach. These authors found that the calcium-dependent protein kinase was implicated in both root and symbiotic function. Further study of candidate genes, selected from among genes shown here to be transcriptionally regulated, holds promise for future investigation of the cause and effect relationship between molecular and morphological changes in roots in response to *S. meliloti* inoculation and to rhizobial signal molecules.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Medicago truncatula genotype A17 was germinated and grown as described previously (Penmetsa and Cook, 2000). In brief, seed was treated with concentrated sulfuric acid for 5 to 7 min with gentle agitation, and washed with sterile water. The seeds were then vernalized at 4.0°C for about 24 h with five to six changes of chilled water, prior to being placed in the dark at room temperature for germination. After about 24 h, germinated seedlings were transplanted into aeroponic tanks (caissons) and grown in a nutrient solution without fixed nitrogen. The composition of the nutrient solution was as follows: 0.52 mM K_2SO_4, 0.25 mM $MgSO_4$, 1 mM $CaCl_2$, 50 μM Na_2EDTA, 30 μM H_3BO_3, 10 μM $MnSO_4$, 0.7 μM $ZnSO_4$, 0.2 μM $CuSO_4$, 1 μM Na_2MoO_4, 0.04 μM $CoCl_2$, 50 μM $FeSO_4$, and 5.5 mM KPO_4 in sterile deionized water. For inoculation treatments, plants were grown for 5 d before inoculating with *Sinorhizobium meliloti* ABS7M containing pXLGD4 (Penmetsa and Cook, 1997). The bacterial culture was grown in TY medium (Sambrook et al., 1989) with 6 mM calcium chloride and 10 μg mL^{-1} tetracycline at 30°C for 48 h. The culture as washed three times with sterile distilled water (dH_2O) and finally resuspended in 10 mL sterile dH_20 to an OD600 of 1.0, which was used to inoculate aeroponic caissons with 10 L of plant culture medium. The control caissons received 10 mL of sterile dH_2O.

To verify developmental stages of roots harvested for RNA isolation, several roots were harvested at each time point for microscopic observation. Rhizobia in the root were detected using X-GAL staining as described (Boivin et al., 1999) with a modified fixation procedure. Roots at different time points after inoculation with *S. meliloti* were fixed in 0.1 M HEPES, pH 7.5, with 0.25 M glutaraldehyde, under three cycles of vacuum and vent for 30 s each and for 1 h further at atmospheric pressure. The roots were washed three times for 10 min each with 0.1 M HEPES. Washed roots were mounted on glass slides in 50% glycerol and observed under a Nikon microscope (DIAPHOT 200), and photographed using a Nikon E4500 digital camera.

Construction of cDNA Microarray

Clones selected for the array were obtained from a wide variety of cDNA libraries that were previously utilized for EST sequencing, and were obtained from the University of Minnesota and the Noble Foundation. Supplemental Table IX contains the percentage representation of various cDNA libraries from which the clones were drawn for the microarray. We utilized the publicly available TCs from the TIGR Medicago Gene Index (http://www.tigr.org/tdb/mtgi/; Quackenbush et al., 2001) and Minnesota Contigs from the Center for Computational Genomics and Bioinformatics of the University of Minnesota (Lamblin et al., 2003) to select 6,144 clones to be included on the cDNA microarray. All selected clones represented the 5′-most clone in a contig with maximal base call identity to the contig consensus. Since all clones were poly-dT primed, it was assumed that the 5′-most clone was most likely to include a full-length cDNA (i.e. the left-most clone was chosen from contigs having a majority of forward and reverse reads in the correct [sense] orientation). Clones selected for the array included those from the same contig as those present on the kiloclone array (S. Peñuela, G. Endre, N. Sharopova, N. Young, K. VandenBosch, and D. Samac, unpublished data), clones from contigs that were verified to be free of chimerism and assembled consistently in both the TC and the corresponding Minnesota Contig, and clones from the same contig as those present in a sample long oligo set marketed by Operon, to enable comparisons with experiments using that data.

The clones selected as described above for this microarray were resequenced to verify clone identity, and new sequences were deposited in GenBank. All cDNA clones used for the array were previously prepared by directionally ligating poly(A^+) enriched RNA into a pBluescript SK− vector (Stratagene), using the *Eco*RI and *Xho*I cloning sites, according to the manufacturer's directions. Primers used for sequencing were SKmod (CTAGAACTAGTGGATCC), used for sequencing the 5′ end of the insert, and T7 (GTAATACGACTCACTATAGGGC), used for sequencing the 3′ end.

To prepare cDNAs for spotting on arrays, the cDNA inserts were PCR amplified using the SKmod and T7 primers. The amplified inserts were then purified and resuspended in 3× SSC or 50% dimethyl sulfoxide for spotting on glass slides coated with SuperAmine substrate (Telechem International). The printing solution was spotted once for each element (EST) on each slide using Gene Machine's Omnigrid Array Spotter in combination with SMP3 pins from Telechem International.

RNA Preparation, cDNA Synthesis, and Hybridization

Roots from inoculated plants and from uninoculated plants at comparable ages were collected 1, 6, 12, 24, 48, and 72 hpi. While still frozen, root tips (about 3–4 mm in length) and shoots (cut at the point where the hypocotyls showed green pigmentation) were removed, and the rest of the root was stored at −80°C before the isolation of RNA. Root samples from three independent biological replicates were collected for the experiment. RNA from frozen root samples was isolated using the RNeasy Plant Mini Kit (Qiagen) following the manufacturer's instructions. RNA concentrations were quantified using a Genova spectrophotometer and stored at −80°C before use. Eleven micrograms of RNA from each of three biological replicates was pooled to make a total of 33 μg for cDNA synthesis with a RT primer for labeling with either Cy3 or Cy5 dye molecules using a 3DNA Array 50 Expression Array Detection Kit for cDNA Microarrays (Genisphere). cDNA was synthesized following instructions from 3DNA Array 50 Expression Array Detection Kit Appendix A (Genisphere).

Microarrays for each time point were hybridized to cDNAs from both inoculated and uninoculated wild-type roots, with cDNAs from the two different treatments labeled with different dyes. Each hybridization was repeated a total of six times to sample the technical variability, with three repeats of each dye combination to control for dye effects. The hybridization and washing procedures of the 3DNA Array 50 Expression Array Detection Kit were followed. Briefly, the hybridization mix consisted of 2.5 μL of cDNAs from inoculated and uninoculated roots, 2.5 μL of Cy3 and Cy5 dyes, 2 μL of locked nucleic acids dT blocker, and 9 μL of 2× SDS hybridization buffer. Hybridization was carried out at 62.0°C for 20 to 23 h. Hybridized slides were washed in 2× SSC, 0.2% SDS for 10 min at 55°C, 2× SSC for 10 min at room temperature, and 0.2× SSC for 10 min at room temperature with gentle agitation.

Signal Detection and Data Analysis

Microarray slides were scanned using an Axon two-laser scanner, and image analysis was performed using GenePix (Axon) software. Background-subtracted mean intensities for both tissues were log transformed and normalized before further analysis. Normalization of the data was performed using a statistical module developed as part of Lab Information System, which includes several scripts and modules written in PERL and R languages. The Lab Information System is not a commercial product. Within-slide normalization was carried out using local linear regression (LOWESS function; Yang et al., 2000), followed by between-slide normalization using four-way ANOVA with replications for multislide dye-swap experiments (Kerr et al., 2000). Data were analyzed only for features with no missing data, or with one missing data point for each dye-swap replicate. Following data normalization, identification of differentially expressed genes was done using the Statistical Analysis of Microarrays method developed by Tusher et al. (2001).

A standardized difference score, or Z score, is a difference between observed and expected values expressed in terms of SDs of observed values (Doniger et al., 2003). Observed and expected values herein are the number of genes in a group having ratios higher than the threshold applied. Such standardized difference score could approximate normal Z score. Scores ≥ 2 or ≤ -2 (2 is threshold for Z score frequently used in statistics) could indicate greater amount of group members among up- or down-regulated genes than expected by chance, respectively. Z scores were calculated for each gene group using all the genes in the group. The lists of genes included in the groups for which Z scores were evaluated are presented in Supplemental Table VIII.

Two-dimensional hierarchical clustering was done on data sets that included genes significant at least at one time point. Complete-linkage algorithm was applied to the correlation matrix for both directions as implemented in GeneExpressionist (GeneData). The entire data set has been deposited in Gene Expression Omnibus with a GEO accession number GSE3441.

Quantitative RT-PCR and Product Detection

RNA was sampled as for microarrays, as described above. To prevent genomic DNA contamination, RNA samples were treated with DNA-*free* (Ambion). RNA was quantified after DNase treatment, and 4.5 μg of total RNA for each biological replicate of each treatment was used to synthesize cDNA separately. cDNA was synthesized with the First Strand cDNA Synthesis Kit for RT-PCR (AMV; Roche) following the manufacturer's protocol in a 20-μL reaction mix. For qRT-PCR, SYBR Green PCR and RT-PCR Reagents (part no. 4304886) from Applied Biosystems were used as per the manufacturer's instructions with 1 μL of cDNA as template per reaction. The PCR was performed in an ABI Prism 7000 Sequence Detection System from Applied Biosystems with the following regime: 10 min at 95.0°C, 45 cycles of denaturation at 95.0°C for 15 s, and annealing/extension at 60.0°C for 1 min. For each biological replicate, three qRT-PCR reactions were run from a cDNA synthesis. The means from three qRT-PCR reactions are presented for each biological replicate. Secret Agent (TC77416), a constitutively expressed gene, was used as an endogenous control (Kuppusamy et al., 2004). The data analysis and fold change calculation was done as per the manufacturer's instructions (SYBR Green PCR and RT-PCR Reagents, Protocol; Applied Biosystems). The $\Delta\Delta$CT (threshold cycle) method of comparing expression data was applied, and the relative quantitative value was expressed as $2^{-\Delta\Delta CT}$. The specificity of the amplification was confirmed by a single peak in a dissociation curve at the end of the PCR reaction, and a single product of expected size on an ethidium bromide-stained agarose gel.

For SQRT-PCR, 1.5 μg of RNA was pooled from each of three biological replicates after DNase treatment to make a total of 4.5 μg RNA for first-strand cDNA synthesis. cDNA was synthesized as above. Each reaction included 200 μM dNTPs, 500 nM of each primer, 1× Taq DNA polymerase buffer, 1.25 units of Taq DNA polymerase (Promega), and 2 μL of cDNA as template. The initial denaturation was for 2 min at 95°C, followed by annealing at 55°C for 30 s and extension for 1.5 min at 72°C. The subsequent cycles had denaturation at 95°C for only 30 s, and the PCR was carried on for 16 additional cycles. RT-PCR on each sample was carried out in duplicates from two independent cDNA syntheses. Secret Agent (TC77416), a constitutively expressed gene, was used as a loading control (Kuppusamy et al., 2004). PCR products were run on a 1% agarose gel and transferred to Hybond N+ nylon membrane (Amersham Biosciences). The membranes were then hybridized with the labeled clone-specific probe, washed, and exposed to Hyperfilm ECL following the instructions from ECL Direct Nucleic Acid Labeling and Detection Systems (Amersham Biosciences). Primers used for quantitative and SQRT-PCR amplification are listed in Table V.

Promoter-Reporter Construct and Transgenic Hairy Root Production

An approximately 2.5-kb fragment upstream of the predicted cytokinin receptor-like protein *CRE1b* (*M. truncatula* genomic clone AC142094.5) was amplified and cloned into the *Bam*HI site of the binary plasmid vector pBI101.1 (Jefferson et al., 1987). The primers used were 5′-aaggatccCCTAGAACCAATATAAAGAC-3′ and 5′-aaggatccTTCAAGAGAAGACCCATTAC-3′ (the lowercase letters are additions to the primers for adding *Bam*HI sites to the amplified fragment). A part of the vector and the cloned fragment was sequenced to confirm the presence and the orientation of the cloned fragment with respect to the *GUS* gene. The binary vector thus constructed was mobilized into *Agrobacterium rhizogenes* strain ARqua1, and transformed hairy roots were generated in *M. truncatula* A17 following the method reported by Boisson-Dernier et al. (2001). Transformed roots were stained with GUS assay buffer as described by Jefferson et al. (1987) at 37.0°C for 2 to 4 h. Stained roots were mounted on glass slides in 50% glycerol and observed microscopically, as described above. Roots from at least 15 plants were observed.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers listed in GEO submission with accession number GSE3441.

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