Transcription Profiling of Soybean Nodulation by *Bradyrhizobium japonicum*

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Legumes interact with nodulating bacteria that convert atmospheric nitrogen into ammonia for plant use. This nitrogen fixation takes place within root nodules that form after infection of root hairs by compatible rhizobia. Using cDNA microarrays, we monitored gene expression in soybean (*Glycine max*) inoculated with the nodulating bacterium *Bradyrhizobium japonicum* 4, 8, and 16 days after inoculation, timepoints that coincide with nodule development and the onset of nitrogen fixation. This experiment identified several thousand genes that were differentially expressed in response to *B. japonicum* inoculation. Expression of 27 genes was analyzed by quantitative reverse transcriptase-polymerase chain reaction, and their expression patterns mimicked the microarray results, confirming integrity of analyses. The microarray results suggest that *B. japonicum* reduces plant defense responses during nodule development. In addition, the data revealed a high level of regulatory complexity (transcriptional, post-transcriptional, translational, post-translational) that is likely essential for development of the symbiosis and adjustment to an altered nutritional status.

Additional keyword: rhizobium.

Air is largely composed of nitrogen, and yet, nitrogen is often a limiting nutrient for plants since, they are unable to directly process or ‘fix’ dinitrogen into a form that can be readily assimilated. Some members of the Fabaceae (legumes) plant family establish a symbiotic relationship with nitrogen-fixing bacteria to directly capture dinitrogen to support plant growth.

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Expression data is publicly available at the NCBI Gene Expression Omnibus (GEO) database as GSE10340 as well as on our Soybean Gene Expression Database (SGED).

*The e-Xtra logo stands for “electronic extra” and indicates that one supplemental figure and four supplemental tables are published online.

Nitrogen conversion takes place in specialized bacteria-induced organs, root nodules, and involves the tight symbiotic association between a plant and a bacterium of the Rhizobiaceae family. In the case of soybean, the most-researched bacterial symbiont is *Bradyrhizobium japonicum*.

Initiation of nodulation requires the mutual secretion and recognition of signal molecules by both plant host and bacterial symbiont (Day et al. 2000a; Limpens and Bisseling 2003). In the case of *B. japonicum*, the bacterium recognizes isoflavones excreted by the plant and, in turn, produces a lipo-chito-oligosaccharide nodulation signal (Nod factor). This Nod factor is recognized by the plant and induces many of the early, committed steps in nodulation, including root hair curling and root cortical-cell division. Bacterial attachment to the root hair occurs within 1 min after inoculation and induces a calcium influx and membrane depolarization that can be detected within the same timeframe (Day and associates 2000a). These initial cellular signaling events subsequently lead to deformation and curling of the root hair within 12 h, followed by invasion of the root hair by the bacterium enclosed in a host-produced infection thread within 24 h. Cortical-cell division beneath the epidermis follows (within 24 to 96 h), with the infection thread ramifying into these tissues, with subsequent release of the bacterium into plant cells. A visible, determinate, spherical nodule, in which meristematic activity is transient, is usually apparent within 4 to 10 days after infection, depending on plant species and growth conditions.

Considering the number of fascinating cellular events that occur within the first few days of rhizobial infection of legumes, considerable attention has been focused on both bacterial and plant genes expressed during this period. Plant genes that show enhanced expression during nodulation are termed “nodulins” (Bladergroen and Spaik 1998), and those expressed early during infection (e.g., within 48 h of inoculation) are called “early” nodulins, such as ENOD2, ENOD12, and ENOD40 (Kouchi and Hata 1995; Pichon et al. 1992; Scheres et al. 1990). Among the most rapidly induced early nodulin genes are *RIP1* (rhizobium induced peroxidase), which is induced in *Medicago truncatula* within 3 h of inoculation with the compatible symbiont *Sinorhizobium meliloti* (Cook et al. 1995), and *G552* apyrase, which is induced in soybean within 3 h of inoculation by *B. japonicum* (Day et al. 2000b). Although nodulins were originally considered to be nodule-specific, it is now recognized that their products can be found in other plant organs. For example, while *ENOD40* is expressed during nodulation, it is also found in

flowers (Crespi et al. 1994). Furthermore, nodulin homologs were also identified in nonlegume species (Kouchi et al. 1999). Therefore, it appears that many nodulins are normal plant proteins that have been recruited to carry out specific functions during the nodulation process.

In the past, analysis of early gene expression in rhizobial symbioses focused on a single gene or only a few genes. However, more recently, investigators have begun to use the tools of functional genomics to analyze gene expression in both the symbiont and host. Microarray approaches enable the identification of large numbers of genes differentially regulated during the interaction between different leguminous roots and their corresponding symbiotic bacteria (Stacey et al. 2006). Genes coding for proteins associated with carbon and nitrogen metabolism as well as different types of transporters were found to be upregulated in *Lotus japonicus*, *M. truncatula*, and *Glycine max* nodules (Asamizu et al. 2005; Barnett et al. 2004; Colebatch et al. 2002; 2004; El Yahyaoui et al. 2004; Kouchi et al. 2004; Lee et al. 2004; Starker et al. 2006). Increased expression of these genes implies the exchange of nutrients between the two symbiotic partners. Colonization of roots by the bacteria also affects plant-defense reactions that may be characterized by mRNA accumulation coding for proteins associated with the phytoalexin biosynthesis pathway, cell-wall modifications, and pathogenesis-related (PR) proteins. Many defense-associated transcripts were more abundant during the early stages of nodulation and then decreased as the interaction advanced in *L. japonicus* and *M. truncatula* (Colebatch et al. 2002; El Yahyaoui et al. 2004; Kouchi et al. 2004; Lohar et al. 2006; Mitra et al. 2004).

Genes encoding transcription factors and genes involved in signal transduction, including various receptor kinases, calmodulins, kinases, and phosphatases, were reported to be upregulated in *L. japonicus* and in *M. truncatula* nodules (Colebatch et al. 2002; 2004; El Yahyaoui et al. 2004; Lohar et al. 2006; Starker et al. 2006). The characterization of the latter genes will help unravel genetic pathways leading to an efficient nitrogen-fixing symbiosis, since some proteins belonging to the same class are essential to the development of a successful symbiosis (Endre et al. 2002; Levy et al. 2004).

Microarrays of spotted cDNA are a powerful and cost-effective tool for the identification of differentially expressed genes specific to a given biological process or treatment. In this study, we employed cDNA microarrays that were derived from a diverse set of soybean cDNA libraries to identify genes that are differentially expressed in soybean roots after inoculation with *B. japonicum*. The experiment used two large arrays (totaling 36,760 different cDNA clones) and focused on differential gene expression at 4, 8, and 16 days after inoculation. This experiment identified 6,555 genes that were significantly differentially expressed during nodulation. These analyses provide many new leads for further study of the *B. japonicum*–soybean symbiotic interaction. Select genes from the cDNA microarray results were verified by real-time quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR).

RESULTS AND DISCUSSION

Previous research showed that only a specific region of the root in an area of rapid root-hair elongation was susceptible to infection (Bhuvaneswari et al. 1981). Nodules that form in this zone normally inhibit subsequent nodule formation via a process termed autoregulation (Caetano-Anolles and Gresshoff 1990; Carroll et al. 1985). Therefore, under normal conditions, isolation of RNA from the entire root after rhizobial inoculation would result in significant tissue dilution due to the nonresponsive root regions.

In an attempt to overcome this problem, we used a super-nodulating soybean mutated in the *GmNARK* gene (nodule autoregulation receptor kinase) in an initial microarray experiment to analyze gene expression in roots 0 to 72 h after inoculation by *B. japonicum* or mock-inoculated. Presumably, the highly localized infection process leads to little concurrent systemic signaling throughout the root tissue during this early stage of nodulation, as we were unable to convincingly identify many differentially expressed genes beyond the well-known *ENOD40* (data not shown).

Therefore, we conducted a second experiment switching our focus to later timepoints, when it was expected that more of the root would be responding to inoculation. This second experiment examined gene expression levels at 0, 4, 8, and 16 dai, across four biological replicates, according to the loop design shown in Figure 1, and identified 6,555 significant differentially expressed genes. Results are summarized by percent of differentially expressed genes that fit a specific functional

Fig. 1. Loop design of the microarray experiment to identify genes differentially regulated 0, 4, 8, and 16 days after inoculation (T0, T4, T8, T16) with *Bradyrhizobium japonicum*. The loop design was performed with four different biological replicates.

Table 1. Classification of the 6,555 differentially regulated genes into different functional categories

Functional categories	Number of genes	% in each category
Cell development	51	0.78
Cell wall	182	2.78
Cytoskeleton	75	1.14
Defense	126	1.92
DNA / RNA	676	10.31
Energy	174	2.65
Lipids	72	1.10
Membrane	352	5.37
Miscellaneous	921	14.05
No Hits	913	13.93
Oxidation	160	2.44
Primary metabolism	504	7.69
Protein	554	8.45
Secondary metabolism	165	2.52
Senescence	13	0.20
Signaling	555	8.47
Stress	62	0.95
Unknown	1,000	15.26

category (Table 1) as well as a false discovery rate (fdr)-corrected *P* values, fold changes, and annotations (Supplemental Table 1). The power of statistical analysis of variance (ANOVA) allowed the identification of genes that had strong differential expression, including genes that had very slight fold-change differences (e.g., 17 of the 6,555 genes are identified as statistically significant even though their fold-change values were less than 1.2-fold up or down).

The sequence of the soybean genome is currently being determined but is not yet available. Therefore, we cannot guarantee that our microarrays harbored cDNAs of all genes or all the isoforms of the same gene family. Several genes belonging to the same multigenic family or having the same annotation, or both, were identified that did not show the same expression pattern during nodulation. Whether this was due to diverse expression patterns of closely related family members from different promoters or interference by cross hybridization cannot be determined. Therefore, for our microarray data analysis, we focused on the genes belonging to the same functional category or to the same multigenic family (same annotation) for which the majority of the corresponding representatives presented the same expression pattern with a strong expression ratio. Percentages of isoforms given below are calculated based on the number of differentially regulated genes. Annotation calls for a given cDNA within this study was made by

analyzing both 5' and 3' expressed sequence tag (EST) annotations provided by multiple public databases.

Validation of the microarray data.

Microarray data was validated by three approaches: qRT-PCR, expression analysis of known nodulins, and expression of genes that have been implicated in the nitrogen assimilation pathway in legumes. For qRT-PCR analysis, 27 genes were selected based on their strong relevance to nodulation and spot quality, as determined by visual inspection of spotted replicates and expression data in our GeneTraffic database (Table 2). For positive nitrogen fixation-related controls, the previously characterized *ENOD40* and *NOD35* genes (Minami et al. 1996; Takane et al. 1997) were included with the 27 test genes. The expression profiles of the 29 genes were determined by qRT-PCR in roots at 0, 4, 8, and 16 dai, as in the microarray experiment, but also in an extended timecourse including 24- and 32-dai samples. Comparisons were made between *B. japonicum* and mock-inoculated supernodulating mutant (SS2-2) or the wild-type Sinpaldalkong 2. The RNA for the qRT-PCR study originated from four new biological replicates and did not utilize RNA used for the microarray experiments. A gene was considered differentially expressed when its relative fold change was >2.0 or <0.5 in at least three out of the four biological replicates.

Table 2. Selected genes for quantitative reverse transcriptase-polymerase chain reaction analysis^a

ID	Similarity	4 dai		8-dai		16-dai	
		<i>P</i> value	Fold change	<i>P</i> value	Fold change	<i>P</i> value	Fold change
Cell wall							
AW568835	Cellulose synthase-like	0.1375	1.21	2.7E-10	4.61	1.0E-11	12.86
AI443160	Extensin-like protein	0.1263	1.17	9.0E-06	0.58	1.0E-11	0.18
AI495459	Proline-rich protein	0.0034	1.30	9.0E-06	0.65	1.0E-11	0.08
Cytoskeleton							
AI973913	Kinesin homolog	0.6242	0.88	3.1E-01	0.84	7.3E-08	2.97
Defense							
AW164459	Bax inhibitor	0.4372	0.82	3.4E-07	3.61	2.9E-07	2.86
AW279105	Chitinase-like protein	0.3864	1.08	2.3E-01	1.10	1.0E-11	5.18
BG550771	Hypersensitive induced protein	0.8462	1.04	2.6E-03	0.70	1.0E-11	10.34
Signaling Hormone							
AW830743	Cytokinin oxidase	0.4160	1.09	1.7E-01	1.14	1.0E-11	5.87
AW201351	IAA hydrolase	0.9261	1.01	6.9E-01	0.97	1.0E-11	5.69
Membrane transport							
BE021552	Peptide transporter	0.0059	1.50	3.2E-07	2.69	3.6E-10	3.30
Metabolism							
AW233919	Apyrase GS52	0.3545	1.11	2.2E-04	1.54	1.7E-12	3.72
Protein folding							
BF424564	Heat shock protein	0.4914	0.86	2.5E-08	3.96	6.7E-02	1.26
Protein proteolysis							
AW509055	Carboxypeptidase	0.6473	0.92	4.1E-05	2.10	1.4E-06	2.22
AW307312	F-box-like protein	0.7544	0.94	9.8E-01	1.00	7.1E-11	5.04
BE021322	Subtilisin-like protein	0.0057	1.56	2.2E-05	2.10	1.0E-11	11.65
Signaling							
BI786974	Calcineurin B	0.6560	0.86	1.3E-06	4.11	8.7E-01	0.97
BG043180	Calmodulin-like protein	0.9759	0.99	1.2E-02	1.28	1.5E-12	3.29
AW100648	Lectin	0.7409	0.97	2.8E-11	3.60	1.0E-11	5.02
AW185772	Protein phosphatase 2C	0.0865	1.21	1.9E-10	3.80	5.2E-12	3.54
AI988174	Receptor-like kinase SYMRK	0.3743	1.23	4.0E-01	1.17	1.8E-07	2.77
Gene regulation							
BQ629088	MYBST1	0.0006	1.88	1.8E-09	4.86	1.6E-12	5.56
BE021550	Transcription factor WRKY4	0.0155	0.57	1.2E-01	0.71	5.5E-09	0.19
AW318109	Transfactor-like	0.6000	1.08	8.1E-01	0.96	1.4E-11	4.89
AI440555	Zinc finger protein	0.0057	2.08	4.4E-06	4.05	2.4E-07	4.08
Miscellaneous and unknown							
AW278145	Acid phosphatase	0.7995	1.04	2.2E-07	3.00	1.0E-11	10.62
AI988526	FW2.2	0.0016	3.22	8.1E-08	10.18	1.8E-12	34.74
AW459252	Unknown	0.9231	0.98	8.7E-06	2.22	3.9E-09	3.64

^a Microarray fold changes (inoculated versus uninoculated; average of four biological replicates) in roots of the supernodulating mutant 4, 8, and 16 days after inoculation (dai) by *Bradyrhizobium japonicum* and *P* value.

A significant increase in *ENOD40* mRNA was detected 4 dai in both the wild type and SS2-2 mutant. In contrast, an increase of *NOD35* was not seen until 8 dai in the mutant and 16 dai in the wild type (Table 3; Fig. 2). This profile of gene expression is as expected and testifies to the quality of the biological material. Except for the extensin-like and the proline-rich proteins that were reduced in abundance, transcripts of all the selected genes were shown to be significantly increased at one or both the early or late stages of the nodulation. The genes coding for a cytokinin oxidase, a subtilisin-like protein, a MYBST1 transcription factor, and unknown function FW2.2 (fruit weight 2.2) were found to be significantly increased from the early stage (4 dai) to the late stages of nodulation (32 dai) in both genotypes (Table 3; Fig. 2). The increase of some other genes started at 8 dai and continued at later timepoints and included homologs to kinesin, hypersensitive-induced protein, apyrase, carboxypeptidase, calmodulin-like protein, and lectin (Table 3; Fig. 2). Gene transcripts shown to increase at 16 dai and later, included homologs to Bax inhibitor, indole 3-acetic acid (IAA) hydrolase, heat shock protein, F-box protein, protein phosphatase 2C, SymRK,

and transfactor-like protein (Table 3). Other genes were differentially regulated at periodic timepoints.

Data from the microarray and qRT-PCR analyses on the SS2-2 mutant were compared in order to estimate the reliability of the microarray results. Comparison of both approaches was consistent, since 78% of the microarray data were confirmed by qRT-PCR when data obtained from 0 to 16 dai were used to compare expression from at least one timepoint. Furthermore, 100% of the results obtained by microarray analysis were confirmed by qRT-PCR, if one compares results from at least one timepoint from 4 to 32 dai. This difference in timing of expression changes can be explained by the fact that different batches of RNA were used to perform the microarray and qRT-PCR experiments and that the kinetics of nodulation apparently differed slightly between the two experiments. The expression profiles of the 29 selected genes were 81% similar in Sinpaldakong 2 and the SS2-2 mutant genotypes, indicating that the supernodulating phenotype did not grossly alter the gene-expression profile. The differences observed between the two genotypes

Table 3. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) relative fold changes (FC)^a

Gene	0 dai		4 dai		8 dai	
	WT	SS2-2	WT	SS2-2	WT	SS2-2
Cell wall						
Cellulose synthase-like AW568835	1.1 ± 0.3	0.6 ± 0.1	1.6 ± 0.6	1.1 ± 0.2	1.9 ± 0.7	1.2 ± 0.4
Extensin-like protein AI443160	0.9 ± 0.2	1.1 ± 0.2	0.8 ± 0.3	0.7 ± 0.2	0.9 ± 0.1	0.3 ± 0.1
Proline-rich protein AI495459	0.8 ± 0.1	1.1 ± 0.3	0.9 ± 0.2	0.4 ± 0.1	1.9 ± 0.8	0.7 ± 0.1
Cytoskeleton						
Kinesin homolog AI973913	1.4 ± 0.4	1.8 ± 0.4	0.6 ± 0.1	0.5 ± 0.1	11.3 ± 2.7	1.9 ± 0.6
Defense						
Bax inhibitor AW164459	1.2 ± 0.3	1.4 ± 0.1	1.0 ± 0.2	0.7 ± 0.2	1.4 ± 0.3	1.2 ± 0.1
Chitinase-like protein AW279105	0.8 ± 0.3	1.4 ± 0.7	0.9 ± 0.4	0.7 ± 0.1	1.7 ± 0.6	1.4 ± 0.2
HS induced protein BG550771	1.1 ± 0.5	0.9 ± 0.3	1.0 ± 0.1	0.3 ± 0.1	108.0 ± 35	1.1 ± 0.4
Signaling hormone						
Cytokinin oxidase AW830743	0.9 ± 0.3	1.1 ± 0.2	6.1 ± 1.9	4.9 ± 1.8	109.0 ± 60	54.2 ± 24.2
IAA hydrolase AW201351	1.3 ± 0.3	1.0 ± 0.1	1.1 ± 0.1	0.7 ± 0.1	1.8 ± 0.3	0.9 ± 0.2
Membrane transport						
Peptide transporter BE021552	1.3 ± 0.4	1.6 ± 0.1	0.9 ± 0.3	0.7 ± 0.1	0.6 ± 0.1	0.8 ± 0.2
Metabolism						
Apyrase GS52 AW233919	ND	ND	1.9 ± 0.8	0.9 ± 0.3	1.8 ± 0.9	5.4 ± 1.9
Protein folding						
Heat shock protein BF424564	1.3 ± 0.2	1.5 ± 0.3	1.2 ± 0.4	0.5 ± 0.2	0.4 ± 0.2	1.0 ± 0.2
Protein proteolysis						
Carboxypeptidase AW509055	1.1 ± 0.1	1.6 ± 0.5	0.8 ± 0.3	0.8 ± 0.1	2.4 ± 0.5	2.8 ± 0.7
F-box-like protein AW307312	1.6 ± 0.3	1.4 ± 0.2	1.5 ± 0.2	0.7 ± 0.3	2.2 ± 0.5	0.8 ± 0.2
Subtilisin-like protein BE021322	1.4 ± 0.2	1.0 ± 0.3	154.0 ± 81.1	179.0 ± 141	213.6 ± 24.3	739.2 ± 220.2
Signaling						
Calcineurin B BI786974	1.3 ± 0.4	1.2 ± 0.2	0.5 ± 0.2	0.5 ± 0.1	3.9 ± 1.2	1.5 ± 0.4
Calmodulin-like protein BG043180	1.4 ± 0.4	1.6 ± 0.2	1.6 ± 0.5	1.6 ± 0.4	6.4 ± 3.2	2.1 ± 0.3
Lectin AW100648	1.5 ± 0.5	1.4 ± 0.7	0.5 ± 0.2	1.6 ± 0.7	8.8 ± 3.4	11.3 ± 3.2
Protein phosphatase 2C AW185772	1.8 ± 0.5	1.3 ± 0.0	1.0 ± 0.4	1.0 ± 0.2	1.2 ± 0.2	0.4 ± 0.1
SYMRK AI988174	0.8 ± 0.3	1.2 ± 0.4	1.0 ± 0.2	0.7 ± 0.1	3.3 ± 1.4	1.6 ± 0.2
Gene regulation						
MYBST1 BQ629088	ND	ND	5.1 ± 1.2	4.6 ± 1.0	9.6 ± 1.7	13.9 ± 5.8
TF WRKY4 BE021550	2.0 ± 0.3	1.7 ± 0.3	1.2 ± 0.3	0.8 ± 0.3	2.3 ± 0.6	0.3 ± 0.1
Transfactor-like AW318109	1.4 ± 0.2	1.5 ± 0.2	0.8 ± 0.3	0.9 ± 0.2	1.3 ± 0.1	1.9 ± 0.3
Zinc finger protein AI440555	1.1 ± 0.2	1.3 ± 0.4	0.8 ± 0.3	0.6 ± 0.1	10.6 ± 3.5	1.0 ± 0.5
Miscellaneous and unknown						
Acid phosphatase AW278145	1.5 ± 0.4	1.7 ± 0.4	0.7 ± 0.1	0.6 ± 0.1	1.0 ± 0.1	0.3 ± 0.1
Fw2.2 AI988526	ND	ND	98.3 ± 22.8	47.2 ± 10.3	999.9 ± 205.	1,617.0 ± 424.2
Unknown AW459252	1.9 ± 0.7	1.2 ± 0.1	0.9 ± 0.3	0.7 ± 0.2	9.0 ± 7.8	1.2 ± 0.2
Control						
ENOD40 nodulin AI442251	1.1 ± 0.2	1.5 ± 0.5	16.4 ± 4.0	12.7 ± 3.8	26.2 ± 10.5	12.2 ± 3.0
NOD35 (Uricase) AB002810	1.7 ± 0.4	1.4 ± 0.1	1.4 ± 0.3	0.9 ± 0.2	2.2 ± 0.9	3.1 ± 0.4

Continued on the following page

^a Fold changes obtained by qRT-PCR of selected genes in roots of wild type (WT) and supernodulating mutant (SS2-2) at different days after inoculation (dai) with *Bradyrhizobium japonicum*. Values given represent the fold change as the average of four biological replicates ± the standard error. Significant changes are indicated in bold. A gene is significantly regulated when its relative fold change >2 or <0.5 in at least three of the four biological replicates.

were mainly due to the different kinetics of gene expression, since no genes were found to have a qualitative difference in expression pattern between the two genotypes.

The second validation of the microarray experiment was the observation that numerous, known nodulin-like genes were significantly increased during the symbiosis (Supplemental Table 2). Out of the 54 significant genes annotated as a putative nodulin, 38 (70%) increased in abundance during our timecourse, including leghemoglobin, nodulins 19, 22, 24, 35, 36a, 40, 44, 51, 55, and 61. These nodulin genes or their homologs were shown to be upregulated in other studies in *G. max*, *M. truncatula*, and *L. japonicus* (Barnett et al. 2004; Colebatch et al. 2002; El Yahyaoui et al. 2004; Kouchi et al. 2004; Lee et al. 2004; Lohar et al. 2006). Several genes annotated as nodulins were reduced in abundance in nodulated roots; an observation that may be explained by cross hybridization of homologous sequences, incomplete sequence identities giving misleading annotations, or by the timepoints used in our experiment (some nodulins might be upregulated before 4 dai). An example was nodulin 26, a member of the large aquaporin gene family. Of the 39 significantly changing genes annotated as aquaporins, 37 were significantly down in abundance. Therefore, cross hybridization with other mem-

bers of this family might have occurred leading to nodulin 26 transcripts appearing to be reduced.

The last approach used to validate the microarray data was to assess the expression pattern of known genes involved in the nitrogen-assimilation pathway. Looking at the timepoint at which nodules were presumably functional and fixing nitrogen (16 dai); most of the genes in this pathway were found to be more abundant in inoculated samples (Fig. 3; Supplemental Table 3). Out of nine enzymes involved in the nitrogen-assimilation pathway, transcripts of five genes encoding these enzymes were increased. For the four other enzymes, 67% of the malate dehydrogenase isoforms, 80% of the aspartate aminotransferase isoforms, 75% of the glutamine synthetase isoforms, and 43% of the IMP dehydrogenase isoforms were found to be more abundant in nitrogen-fixing nodules (Fig. 3). These data are consistent with the activation of nitrogen fixation within 16 dai by *B. japonicum* infection.

Nodulation profoundly affects metabolism.

Our microarray experiment utilized a large cDNA collection and four biological replicates and focused on mid-to-late timepoints during nodule development. We identified 6,555 significantly differentially expressed genes in response to *B. japoni-*

Table 3. Continued from preceding page

Gene	16 dai		24 dai		32 dai	
	WT	SS2-2	WT	SS2-2	WT	SS2-2
Cell wall						
Cellulose synthase-like AW568835	1.2 ± 0.2	1.3 ± 0.5	4.5 ± 1.9	5.2 ± 1.7	1.1 ± 0.1	0.9 ± 0.1
Extensin-like protein AI443160	0.9 ± 0.2	0.3 ± 0.1	2.0 ± 1.2	2.7 ± 1.1	2.6 ± 0.5	1.1 ± 0.3
Proline-rich protein AI495459	0.4 ± 0.1	0.1 ± 0.1	1.4 ± 0.8	2.1 ± 0.5	1.3 ± 0.3	0.5 ± 0.2
Cytoskeleton						
Kinesin homolog AI973913	26.4 ± 6.0	51.9 ± 18.5	13.8 ± 4.2	38.4 ± 10.3	10.2 ± 2.0	17.6 ± 4.9
Defense						
Bax inhibitor AW164459	8.4 ± 3.0	4.8 ± 0.7	9.7 ± 4.3	5.4 ± 1.4	9.3 ± 3.6	11.5 ± 3.9
Chitinase-like protein AW279105	2.7 ± 0.7	1.0 ± 0.3	3.0 ± 1.4	11.8 ± 8.9	36.7 ± 20.8	31.2 ± 29.2
HS induced protein BG550771	32.0 ± 7.9	29.8 ± 8.9	25.7 ± 11.7	38.9 ± 10.4	49.1 ± 20.1	41.2 ± 18.7
Signaling hormone						
Cytokinin oxidase AW830743	537.0 ± 186	937.0 ± 525	152.0 ± 130	226 ± 175	44.2 ± 32.1	114 ± 46.9
IAA hydrolase AW201351	7.2 ± 2.7	15.1 ± 4.1	8.4 ± 4.5	34.4 ± 14.0	6.4 ± 5.0	7.9 ± 3.7
Membrane transport						
Peptide transporter BE021552	0.9 ± 0.1	0.8 ± 0.2	3.4 ± 1.5	4.4 ± 1.8	1.8 ± 0.7	1.3 ± 0.6
Metabolism						
Apyrase GS52 AW233919	94.6 ± 34.1	76.3 ± 33.5	46.1 ± 29.3	145.1 ± 84.1	28.3 ± 5.2	32.5 ± 23.5
Protein folding						
Heat shock protein BF424564	3.7 ± 1.2	4.6 ± 0.3	13.2 ± 7.0	11.2 ± 5.8	5.9 ± 2.4	12.2 ± 7.1
Protein proteolysis						
Carboxypeptidase AW509055	6.4 ± 2.1	8.2 ± 2.6	8.4 ± 4.5	35.6 ± 25.3	9.8 ± 0.4	10.2 ± 7.8
F-box-like protein AW307312	3.8 ± 0.7	3.4 ± 0.8	18.9 ± 15.6	9.7 ± 4.8	5.5 ± 4.1	3.9 ± 2.0
Subtilisin-like protein BE021322	1,672.0 ± 487.9	2,123.0 ± 1220	244.6 ± 229.5	565.4 ± 515.1	294.1 ± 122.9	600.8 ± 337.1
Signaling						
Calcineurin B BI786974	1.4 ± 0.5	1.5 ± 0.6	1.3 ± 0.6	4.1 ± 1.6	7.2 ± 4.8	5.4 ± 3.6
Calmodulin-like protein BG043180	39.1 ± 15.0	55.8 ± 17.8	32.1 ± 15.8	50.6 ± 15.8	9.8 ± 1.5	23.0 ± 9.9
Lectin AW100648	64.4 ± 18.4	241.7 ± 95.7	56.9 ± 45.7	151.8 ± 51.7	15.3 ± 2.7	25.2 ± 17.1
Protein phosphatase 2C AW185772	4.6 ± 1.3	2.4 ± 0.3	1.5 ± 0.5	9.1 ± 4.7	2.1 ± 0.4	3.0 ± 0.9
SYMRK AI988174	5.7 ± 2.2	5.9 ± 1.7	6.9 ± 2.9	9.6 ± 2.9	9.7 ± 7.4	8.7 ± 2.8
Gene regulation						
MYBST1 BQ629088	39.4 ± 18.5	83.6 ± 31.5	41.1 ± 31.8	187.9 ± 82.2	23.6 ± 10.3	179.4 ± 75.9
TF WRKY4 BE021550	1.1 ± 0.5	1.6 ± 0.4	4.0 ± 3.0	5.9 ± 2.9	1.1 ± 0.3	3.1 ± 2.2
Transfactor-like AW318109	3.9 ± 1.0	4.8 ± 1.3	7.5 ± 4.8	22.8 ± 9.1	3.7 ± 1.0	5.4 ± 1.8
Zinc finger protein AI440555	0.7 ± 0.2	0.7 ± 0.2	2.2 ± 0.9	3.4 ± 1.0	2.0 ± 0.6	1.5 ± 0.2
Miscellaneous and unknown						
Acid phosphatase AW278145	1.8 ± 0.6	1.5 ± 0.3	3.5 ± 2.1	4.8 ± 1.7	3.8 ± 1.5	2.4 ± 0.7
Fw2.2 AI988526	2,680.0 ± 1,083	5,157.0 ± 4958	104.1 ± 87.2	466.8 ± 272.0	1,344.0 ± 761.2	2,468.0 ± 1,528.0
Unknown AW459252	0.1 ± 0.0	3.1 ± 0.8	4.7 ± 4.0	12.5 ± 7.7	3.1 ± 1.3	11.6 ± 7.4
Control						
ENOD40 nodulin AI442251	87.2 ± 32.7	23.8 ± 11.1	228.9 ± 212.4	559.1 ± 417.1	61.3 ± 15.6	96.5 ± 46.3
NOD35 (Uricase) AB002810	164.6 ± 66.1	136.0 ± 80.8	261.7 ± 218.2	356.5 ± 133.3	133.6 ± 86.8	755.8 ± 696.2

cum inoculation. This alone testifies to the profound effects on the plant during the nodulation process. During the nitrogen-fixing symbiosis, the plant supplies bacteria with carbohydrates and the plant receives nitrogen compounds in return. Therefore, as expected, among the genes that increased in transcript abundance by 16 dai were several that would support nutrient exchanges between the two symbionts, including sugar, amino acid, peptide, nitrate, sulfate, potassium, and phosphate transporters.

Many genes involved in primary metabolism were increased by the symbiosis, starting at 8 dai and increasing further by 16 dai. Several aspects of metabolism appeared to be activated during nodulation, including amino acid (71% genes induced), carbohydrate (68% of genes induced), glycolytic (82% genes induced), nucleic acid (82% genes induced), and TCA cycle-related (75% genes induced). A total of 69% of the significantly expressed genes involved in the glyoxylate cycle decreased during nodulation.

Nodulation downregulates the plant defense response.

Many genes involved in anthocyanin, flavonoid, lignin, and terpenoid synthesis (secondary metabolism) were reduced during the later stages of our study. A total of 86% of soybean genes involved in flavonoid biosynthesis were reduced in transcript abundance in response to *B. japonicum* inoculation; whereas these genes increased when soybean were infected by *Pseudomonas syringae* (Zou et al. 2005), supporting the idea that these are defense-associated genes that are repressed to enhance symbiosis (Fig. 4). Transcript comparisons of root symbiosis and pathogen-infected leaves need to be interpreted carefully. However, the *P. syringae* dataset is the most comparable microarray set (i.e., 100% of the cDNA clones used for the *P. syringae* study were used in this nodulation study) available at this time, and paralogs of most defense genes would be expected to be expressed from any tissue of a plant. Preliminary studies support that most of these same defense-associated cDNA are regulated in *Fusarium*-infected roots in a compara-

Fig. 2. Relative fold changes determined by quantitative reverse transcriptase-polymerase chain reaction for the *ENOD40*, nodulin 35, cytokinin oxidase, apyrase, subtilisin-like protein, lectin, *MYBST1*, and *FW2.2* genes 0, 4, 8, 16, 24, and 32 days after inoculation (dai) with *Bradyrhizobium japonicum* in wild type (WT) and SS2-2 mutant soybean roots. No gene expression was detected for the apyrase, the *MYBST1*, and *FW2.2* genes for the 0 dai timepoint. Fold changes from the microarray (array) were also indicated when available.

ble way, as seen in *P. syringae*-infected leaves (S. J. Clough, unpublished data).

Of the soybean genes classified as defense-related, 65% were reduced after *B. japonicum* inoculation. This expression change was weak initially at 4 and 8 dai, but repression was much stronger by 16 dai. All the genes belonging to the elicitor-induced group and 83% of the putative resistance genes were reduced in response to *B. japonicum* inoculation but increased during compatible and incompatible interactions of soybean with *P. syringae* (Fig. 4), supporting the theory that *B. japonicum* has the ability to repress defense reactions, thereby permitting the establishment of nitrogen-fixing symbiosis.

Nodulation involves complex regulation.

The microarray data suggest that different levels of regulation, including transcriptional, post-transcriptional, translational, and post-translational, may occur during the development and functioning of the nitrogen-fixing symbiosis. We identified transcripts of 328 putative transcription factors (*MYB*, *WRKY*, *BEL1*, homeodomain, *MADS*, and *ZINC* finger families) differentially expressed at different stages of the nitrogen-fixing symbiosis (55% up; 45% down). Interestingly, all the *G. max* ethylene responsive factors (*ERF*) and more than 80% of the *WRKY* transcription factors were reduced during the nitrogen-fixing symbiosis. The *WRKY* transcription factor genes increased during the compatible and incompatible interactions between soybean and *P. syringae* (Zou et al. 2005), suggesting an important role for these genes in controlling defense (Fig. 4). Furthermore, a *MYB* transcription factor was also shown to be strongly increased by qRT-PCR after *B. japonicum* inoculation (Table 3; Fig. 2).

The response of various transcription factors to *B. japonicum* infection clearly implicates transcriptional regulation as an important controlling element during nodulation. Indeed, the role of transcription factors in the nodulation process was recently reviewed (Udvardi et al. 2007). A *MYBST1* gene was shown to be strongly regulated by qRT-PCR in *B. japonicum*-colonized soybean roots from 4 to 32 dai. MYB family gene transcription factors contain a MYB-DNA binding domain (Stracke et al. 2001) and have been implicated in a variety of plant processes (e.g., control of cell cycle [Ito 2005; Ito et al. 2001], the circadian cycle [Carre and Kim 2002], regulation of secondary metabolism [Jin and Martin 1999], in response to nutrient starvation [Miyake et al. 2003], or regulation of plant defense against pathogens [Lee et al. 2001; Mengiste et al. 2003; Raffaele et al. 2006; Vailleau et al. 2002]). A MYB-like protein was also suggested to be an early marker of the nodulation of *M. truncatula* in response to *S. meliloti* inoculation (Lohar et al. 2006).

WRKY transcription factors are generally involved in the response of plants to stress, including pathogen invasion (e.g., Chen and Chen 2000; Dong et al. 2003; Marchive et al. 2007; Ryu et al. 2006; Zheng et al. 2006). Consistent with our general finding of a suppression of plant defense-related gene expression by *B. japonicum* inoculation, transcripts of many WRKY transcription factors were significantly reduced during nodulation. More than 10 different WRKY genes were reduced during the symbiosis but increased during the interaction between soybean and *P. syringae* (Zou et al. 2005).

Posttranscriptional modifications may play a role in the regulation of nodulation, since several genes involved in RNA splicing or RNA silencing increased in abundance in our study, starting at 8 dai, with even more genes at 16 dai. Interestingly, this set of genes involved in RNA splicing was not differentially regulated in soybean inoculated by *P. syringae* (Zou et al. 2005), suggesting that these genes may be specific to symbiosis (Fig. 4).

Previously, alternative splicing during nodulation was suggested for only one gene, *LjNOD70* (Szczyglowski et al. 1998). Our results suggest that alternative splicing may play a much larger role during soybean nodulation, since more than 40 genes involved in this process were induced during our study.

The translation of proteins appears to be more important in nodulated roots than in mock-inoculated roots, since 155 genes (out of 180) encoding either ribosomal proteins or proteins involved in translation were up-regulated at different stages of the nodulation. This increase of translation-associated genes may also be linked to an increase in mRNA levels of genes encoding chaperonin-like proteins, since 84% of the genes in this class were more abundant in nodulated roots. Various post-translational modifications may also be required for a protein to be active. Protein phosphorylation is known to be critical to the nodulation process. For example, a nodulation receptor kinase (NORK), a Ca^{2+} and calmodulin-dependent protein kinase (DMI3), and a symbiosis receptor-like kinase (SYMRK) were described as essential factors in the signaling leading to a functional symbiosis (Endre et al. 2002; Levy et al. 2004; Stracke et al. 2002). The microarray study identified 179 putative kinases (69% up; 31% down) and 42 phosphatases (66% up, 34% down) differentially regulated after *B. japonicum* inoculation at different timepoints, emphasizing the complexity of protein phosphorylation and dephosphorylation regulation occurring during nodulation.

We identified 230 genes (67% up; 33% down) involved in protein degradation that were differentially expressed starting at 4 dai, and their expression increased continuously during nodule development. For example, a subtilisin-like protease was strongly increased (more than 1,000-fold) in nodulated roots as determined by qRT-PCR (Fig. 2; Table 3). Subtilisins are serine endopeptidases involved in many biological processes in plants, including germination of seeds (Beilinson et al. 2002; Fontanini and Jones 2002), exposure to light (Barnaby et al. 2004), formation of the cuticle (Tanaka et al. 2001), regulation of stomate density (Berger and Altmann 2000), or formation of lateral roots (Neuteboom et al. 1999). Subtilisin protease genes were also induced during pathogen-plant interactions in *L. esculentum* and *A. thaliana* (Jorda and Vera 2000; Jorda et al. 1999; Tornero et al. 1996, 1997) as well as during the early stages of actinorhizal nodule development (Laplaze et al. 2000; Ribeiro et al. 1995; Svistoonoff et al. 2003). Furthermore, the promoter of a subtilisin protease identified during actinorhizal nodulation was active in *M. truncatula* roots colonized by *S. meliloti* (Svistoonoff et al. 2004).

Protein levels during nodulation may also be differentially regulated by degradation, since 93 genes (out of 118) involved in protein degradation mediated by the proteasome (including, for example, different subunits of proteasome, cyclin-like F-box, ubiquitin protein ligase, F-box protein, and ubiquitin-conjugating enzyme) were significantly increased during nodulation. Given the possible role of ubiquitination in hormone signaling, senescence, and pathogen-host interactions (Angot et al. 2007; Moon et al. 2004), our microarray results suggest that more attention should be paid to the importance of the proteasome during nodule development.

Cell-wall modifications.

More than 70% of the genes involved in the cell-wall category were significantly decreased in response to *B. japonicum* inoculation starting 4 dai, with even more genes decreased by the 8- and 16-dai timepoints. The repressed genes primarily belong to gene families of β -1,4-endoglucanase (90% genes repressed), extensin (85% genes repressed), xyloglucan endotransglucosylase (85% genes repressed), pectinesterase (90% genes repressed), and proline-rich protein (92% repressed),

suggesting that cell-wall modifications occur during nodulation initiation. Interestingly, the cluster of proline-rich protein genes was reduced during this nodulation study but increased when soybean was inoculated with the pathogenic bacterium *P. syringae* (Fig. 4).

Cell-wall modifications probably occur during nodulation, since different early nodulin genes (*ENOD11*, *ENOD12*) encoding predicted cell-wall proteins were found to increase during the early stages of the symbiotic interaction between *M. truncatula* and *L. japonicus* and their corresponding rhizobia (Colebatch et al. 2002; El Yahyaoui et al. 2004). Cell-wall strengthening is a mechanism by which plants defend against pathogen attack (Hückelhoven 2007). Proline-rich protein genes are induced in common bean in response to infection and wounding (Corbin et al. 1987; Sheng et al. 1991) and in soybean infected by *P. syringae*. However, these genes were found to be repressed during soybean nodulation.

Regulation of secondary metabolites and phytohormones.

Enzymes of the phenylpropanoid pathway are involved in lignin, plant pigment and phytoalexin synthesis (Dixon et al. 2002). Phytoalexins accumulate in plants during pathogen infection (Grayer and Kokubun 2001; Hammerschmidt 1999; Jeandet et al. 2002). In addition to their well-documented involvement in defense, genes of the phenylpropanoid pathway were reported to be differentially regulated during nodulation (Samac and Graham 2007). For example, phenylalanine ammonia-lyase and chalcone synthase genes were shown to be induced during the early stage of soybean nodulation (Estabrook

and Sengupta-Gopalan 1991; Lohar et al. 2006). Flavonoids are essential for early nodulation due to their role as inducers of the rhizobial nodulation genes, and they also play a role in modulating polar auxin transport (Subramanian et al. 2007). However, our data suggest that many genes involved in the phenylpropanoid pathway are repressed during the later stages of soybean nodulation, which is a similar result to nodulation and mycorrhization of *L. japonicus* (Deguchi et al. 2007).

Plant hormones including cytokinin, auxin, gibberellin, ethylene, and jasmonic acid (JA) play critical roles during nodule development (Gonzalez-Rizzo et al. 2006; Lievens et al. 2005; Pii et al. 2007; Sun et al. 2006). JA and ethylene were shown to act as negative regulators of the nitrogen-fixing symbiosis (Ma et al. 2002; Nakagawa and Kawaguchi 2006; Sun et al. 2006). Addition of methyl jasmonate to *L. japonicus* shoots inhibited nodulation (Nakagawa and Kawaguchi 2006). In contrast, many plant responses to biotic and abiotic stresses involve activation of JA- and ethylene-signaling pathways (Delker et al. 2006; Dong 1998; Halim et al. 2006).

Many genes involved in auxin, cytokinin, ethylene, gibberellin, and JA synthesis or degradation, as well as proteins known to be regulated by those hormones, were differentially regulated throughout the development of the nitrogen-fixing symbiosis. Transcripts for enzyme involved in ethylene (ACC synthase, ACC oxidase) and JA (12-oxophytodienoate reductase, oxo-phytyldienoic acid reductase) biosynthesis were reduced during nodulation. Interestingly, the genes involved in JA biosynthesis increased during the interaction between soybean and *P. syringae*, suggesting again the ability of *B. japonicum* to repress the

Fig. 3. Nitrogen assimilation and export in soybean nodules at 16 days after inoculation (based on drawings by Buchanan et al. 2000). Enzymes within red boxes (phosphoenolpyruvate carboxylase, asparagine synthetase, GOGAT, uricase, allantoinase) had mRNA levels higher in inoculated roots versus mock inoculation. Enzymes within red/green hatched boxes (malate dehydrogenase, aspartate aminotransferase, glutamine synthetase, IMP dehydrogenase) had multiple representatives on the slides that showed either increased or decreased mRNA levels in inoculated versus mock. For each box of enzymes, the surface percentage of red indicates the percentage of upregulated isoforms and the green the percentage of downregulated isoforms.

plant defense reaction enabling the establishment of the symbiosis (Fig. 4).

Hormone levels may be modulated by catabolism since a cytokinin oxidase gene encoding an enzyme that degrades cytokinin was induced from 4 to 32 dai (Fig. 2; Table 3). The silencing of a cytokinin receptor homolog in *M. truncatula* and the overexpression of a cytokinin oxidase gene in *L. japonicus* were both shown to reduce nodulation (Gonzalez-Rizzo et al. 2006; Lohar et al. 2004). Furthermore, exciting recent data

showed that a cytokinin receptor is essential for nodulation and response to the Nod signal. A mutation in a *L. japonicus* cytokinin receptor gene did not affect the entry of the symbiotic bacteria into the root hair, but it did inhibit nodule organogenesis (Murray et al. 2007; Oldroyd 2007; Tirichine et al. 2007). Fine tuning of cytokinin levels is undoubtedly important during nodule development.

Auxin is also important to nodulation. *M. truncatula* auxin import carrier genes were strongly expressed in nodule primor-

Fig. 4. Clustering of soybean genes in nodulated roots after inoculation with *Bradyrhizobium japonicum* and infiltrated unifoliates with *Pseudomonas syringae* (HR = incompatible, VIR = compatible [Zou et al 2005]). These genes might be involved in plant defense regulation (resistance genes, elicitor-induced genes, proline-rich proteins, jasmonic acid synthesis, WRKY transcription factors, flavonoid metabolism) and RNA splicing. 1, Nodulated roots 4 days after inoculation (dai) with *Bradyrhizobium japonicum*. 2, Nodulated roots 8 dai with *B. japonicum*. 3, Nodulated roots 16 dai with *B. japonicum*. 4, *P. syringae*-infiltrated unifoliates harvested after 2 h (HR). 5, *P. syringae*-infiltrated unifoliates harvested after 2 h (VIR). 6, *P. syringae*-infiltrated unifoliates harvested after 8 h (HR). 7, *P. syringae*-infiltrated unifoliates harvested after 8 h (VIR). 8, *P. syringae*-infiltrated unifoliates harvested after 24 h (HR). 9, *P. syringae*-infiltrated unifoliates harvested after 24 h (VIR).

dia during the initial stages of nodulation and in the peripheral region of the nodules at later stages (de Billy et al. 2001). Expression of the auxin response promoter *GH3* was used to detect significant auxin activity in the dividing nodule cortical cells in *L. japonicus* roots (Pacios-Bras et al. 2003). However, at later stages, our microarray data suggest that auxin levels may be reduced due to the induction of an IAA hydrolase gene by 16 to 32 dai.

Other notable responses.

A predicted BAX inhibitor gene was induced in nodulated soybean roots. BAX proteins are mitochondrial membrane-localized proteins involved in programmed cell death (Kirkland and Franklin 2003). BAX-induced cell death was suggested to be comparable to the hypersensitive response in tobacco (Lacomme and Santa Cruz 1999). BAX inhibitor proteins were shown to have antiapoptotic activity in *A. thaliana* (Kawai-Yamada et al. 2001) and to decrease cell death triggered by biotic and abiotic stresses (Watanabe and Lam 2006). The soybean BAX inhibitor was significantly induced from 16 to 32 dai (Table 3). The induction of this gene may be part of the active suppression of plant defense pathways by *B. japonicum*.

It is clear from microscopic observations that root-hair curling, formation of the infection thread, and nodule formation involves extensive rearrangement of the cytoskeleton (Takemoto and Hardham 2004). A kinesin gene identified in this study was significantly increased by qRT-PCR from 8 to 32 dai (Table 3). Kinesins are motor proteins that are associated with microtubules and are involved in the movement along the microtubule. According to its expression profile, the kinesin gene identified in this study is probably involved in the spatial organization of the symbiosome and may function in the localization of organelles (mitochondria and plastid) at the periphery of the colonized cells, as described for soybean nodules by Whitehead and associates (1998).

Apyrase is an enzyme catalyzing the hydrolysis of ATP to AMP and pyrophosphate. It was described as having several plant functions, including phosphate nutrition (Thomas et al. 1999), light stimulation (Ghosh et al. 1998), resistance to herbicide (Windsor et al. 2003), growth (Wu et al. 2007), and enhancement of nodulation (McAlvin and Stacey 2005). Etzler and associates (1999) proposed that an ectoapypase found on the root-hair membrane may play a role in Nod signal recognition. Navarro-Gochicoa and associates (2003) failed to see induction of the apyrase gene in *M. truncatula* upon rhizobial inoculation. However, other authors reported rapid induction of apyrase transcription in both soybean (Day et al. 2000b) and *M. truncatula* (Cohn et al. 2001). Our cDNA microarray results show a significant induction of the soybean *GS52* apyrase gene during the later stages of nodule development. Given the variety of roles attributed to these

proteins, it will be interesting to better define their roles during the entire nodulation process.

Comparison of gene expression during the nodulation of *G. max*, *L. japonicus*, and *M. truncatula*.

Genes significantly regulated during the nodulation of *M. truncatula* (El Yahyaoui et al 2004; Starker et al 2006) and *L. japonicus* (Asamizu et al 2005; Colebatch et al 2004; Kouchi et al 2004) were compared with those identified during the nodulation of *G. max* (this study). EST found significantly regulated after rhizobium inoculation in *M. truncatula* and *L. japonicus* were aligned against a soybean EST database of the cDNA printed on the soybean microarray slides leading to the identification of 1,631 soybean orthologous EST from *L. japonicus* and 386 from *M. truncatula*. EST of the six nodulation experiments (two *M. truncatula*, three *L. japonicus*, and one *G. max*) were compared. Analysis of the data indicated that the majority of the EST regulated during nodulation were identified in only one experiment (Fig. 5).

Hierarchical cluster analysis was also performed to compare gene regulation during soybean nodulation to gene regulation during nodulation of *L. japonicus* and *M. truncatula* (Supplemental Fig. 1). Relatively few *L. japonicus* or *M. truncatula* genes showed the same expression pattern as soybean after inoculation with the appropriate rhizobium.

Many of the genes identified as being differentially expressed in our study and annotated as proteins involved in nutrient exchange between the two symbionts (transporters), in nitrogen and carbohydrate metabolism, in signaling (kinases, phosphatases), in cell-wall modifications, and defense reaction were also found regulated during nodulation of *M. truncatula* and *L. japonicus* (Asamizu et al 2005; Colebatch et al 2004; El Yahyaoui et al 2004; Kouchi et al 2004; Starker et al 2006). However, a high degree of correspondence was not seen in our analysis when experimental results were directly compared across projects by identifying conserved genes based on the highest BLAST BIT scores. Variability in microarray experiments, which occur by the use of different protocols, different statistical analysis, and different platforms, has already been described (Draghici et al 2006; Suárez-Fariñas and Magnasco 2007; van der Spek et al 2003; Yauk and Berndt 2007). Whether the different gene-expression patterns during nodulation of *G. max*, *L. japonicus*, and *M. truncatula* is due to DNA microarray methodology, to the construction of the microarrays (best ortholog EST were not spotted for the three plant species), to the identification of EST corresponding to different protein isoforms in different plants, or to different gene regulation during nodulation of the three plants remains to be determined. A complete annotation of the genomes (genes and promoters) as well as the identification of orthologous genes of *M. truncatula*, *L. japonicus*, and *G. max* is required to be able to compare accurately gene expression during the nodulation of the three plants. Additionally, one would have to examine all gene-family members for expression across different treatments to identify the best functional homolog.

MATERIALS AND METHODS

Plant growth.

Seeds from the ethylmethane sulphonate-derived, super-nodulating soybean lines SS2-2, which has a point mutation in the *GmNARK* gene identical to that found in the nts246 mutant derived from cultivar Bragg (Kim et al. 2005), and the corresponding wild-type parental line Sinpaldalkong 2 were surface-sterilized by soaking for 10 min in 20% commercial bleach, followed by five sterile water rinses, a 10-min treatment in 0.1 M HCl, and an additional five rinses in sterile water. Sterile

Fig. 5. Comparison of expressed sequence tags (EST) corresponding to genes significantly regulated during nodulation of *Glycine max* (this study), *Lotus japonicus* (Asamizu et al 2005; Colebatch et al 2004; Kouchi et al 2004), and *Medicago truncatula* (El Yahyaoui et al 2004; Starker et al 2006). This chart represents the number of EST found commonly to be regulated in comparisons of an increasing number of independent studies (using the *G. max* data as the starting point).

seeds were germinated in petri dishes containing sterile, moist paper towels, in a 30°C dark growth chamber. Two-day-old seedlings were transferred into Leonard's jars containing 25% perlite 75% vermiculite on the upper part and B and D solution in the lower part (Broughton and Dilworth 1971). Plants were inoculated by *B. japonicum* USDA110 and were grown at 60% humidity and 30°C with a 16-h light photoperiod. Roots and nodulated roots were harvested 0, 4, 8, and 16 dai for microarrays and 0, 4, 8, 16, 24, and 32 dai for qRT-PCR, were rinsed in water, and were frozen in liquid nitrogen for RNA extraction after excising the root-tip meristem. Four biological replicates were performed for each timepoint used in the microarray experiment as well as for the qRT-PCR experiment.

Inoculation.

B. japonicum USDA110 was grown at 30°C in liquid minimal medium (1 ml of RDY trace elements [So et al. 1987], 4 ml of glycerol, 0.6 g of K₂HPO₄, 0.6 g of KH₂PO₄, 0.1 g of MgSO₄ × 7 H₂O, 0.05 g of NaCl, and 0.5 g of NH₄NO₃, adjusted to 1 liter with water) to an optical density at 600 nm (OD₆₀₀) of approximately 0.5 (3 days). Cells were spun down, and the pellets were washed twice with sterile water and were diluted to OD₆₀₀ = 0.1 in sterile water, and 1 ml of bacterial solution was used to inoculate the SS2-2 and Sinpal-dalkong 2 plants. Control plants were mock-inoculated with sterile water.

RNA extraction, DNase treatment, and cDNA synthesis.

For each timepoint, RNA extraction from nodulated roots or mock-inoculated roots was performed using Trizol following the manufacturer's protocol (Invitrogen, Carlsbad, CA, U.S.A.). RNA from each extraction was analyzed for quality, quantity, and purity, using a spectrophotometer and gel electrophoresis. RNA was treated with DNase according to the instruction of the manufacturer (Turbo DNA-free, Ambion, Austin, TX, U.S.A.). cDNA were synthesized in a final volume of 25 µl from 2 µg of DNA-free RNA, using M-MLV reverse transcriptase (Promega, Madison, WI, U.S.A.). RNA and 1 µg of oligodT₁₅ (Promega) were added to sterilized water to a final volume of 13.5 µl. After denaturing at 70°C for 5 min, the mixture was placed on ice for at least 2 min and dNTPs (final concentration of 500 µM dATP, dGTP, dTTP, and dCTP), 5 µl 5× M-MLV reaction buffer, and 300 U M-MLV RT were added. First-strand cDNA was synthesized at 25°C for 15 min, followed by 1 h at 42°C, and the enzyme was then inactivated 2 min at 95°C.

Microarray analysis.

The soybean cDNA slides consisted of libraries Gm-r1021, Gm-r1083, and Gm-r1070 (print set '18kA') as well as Gm-r1088 and Gm-r1089 (print set '18kB'). Together, these two slide sets consisted of 36,760 different PCR-amplified inserts, including 64 diverse controls (Vodkin et al. 2004, 2007). Printed slide sets Gm-r1021 and Gm-r1083 consisted primarily of PCR-amplified inserts from the following number of cDNA clones from the following libraries: 4,089 from library Gm-c1004 (roots of 8-day-old plants, *Bradyrhizobium* free), 3,055 from library Gm-c1028 (derived from root of nts382 seedlings, *B. japonicum*-inoculated), 1,117 from library Gm-c1009 (roots of 2-month-old plants), and 820 from library Gm-c1013 (2- to 3-week-old whole seedlings). Print library Gm-r1070 consisted of mainly developing green seed and floral cDNA (approximately 48 and 43%, respectively of 9,200 spots); print library Gm-r1088 consists of 9,200 cDNA from a variety of tissue sources, approximately 60% coming from germinating cotyledons, seed coat, and growing shoots, and approximately 40% coming from stress, pathogen-challenged, or hormone-

treated tissues; print library Gm-r1089 consists of 9,200 spotted cDNA from a wide variety of stressed tissues. Methods for aa-dUTP indirect RNA labeling with Cy3 and Cy5 dyes and microarray hybridization were as described by Zou et al. (2005). Protocol details can be viewed and downloaded from the University of Illinois Crop Science's Clough Lab website.

Data analysis.

Microarray hybridizations were performed according to a loop design (Fig. 1), with four biological replicates for each sample. Loop designs are desirable for microarray studies as they conserve resources and have a built-in dye-swap balance (Kerr and Churchill 2001). Images were obtained with a ScanArray Express confocal microarray scanner (Perkin-Elmer, Foster City, CA, U.S.A.), and data was extracted using GenePix v4.1 (Axon, Milpitas, CA, U.S.A.). Images and intensity data were uploaded to a server running GeneTraffic database software (StrataGene, San Diego, CA, U.S.A.) to facilitate troubleshooting and quality control checking. GenePix intensity data files were parsed and processed by in-house PERL programs to create a composite input data file of median spot intensity and to calculate average value of the negative control (X13988 human myosin heavy chain) for a single labeling (whether Cy3 or Cy5). Weak spots ($[Cy3+Cy5]/2 < X13988$ average intensity value) and spots that were flagged in GenePix as being an artifact or heavily influenced by background were converted to the X13988 average intensity for each slide to force their Cy5/Cy3 ratio to be 1.0, in order to minimize the effect of these poor quality spots on normalization. Data were log₂-transformed and normalized by the GLOWESS method of R/MA ANOVA (Wu et al 2003). After normalization, all weak and flagged spots that had been converted to the average X13988 value were replaced by a period, indicating missing values in subsequent SAS analysis. ANOVA was run on the data in SAS, using the PROC MIXED. These observations were analyzed using a linear mixed effects model, $Y = \mu + \text{Array} + \text{Dye} + \text{Treatment} + \text{Time}(\text{Treatment}) + \varepsilon$, where Y is the log signal intensity value for individual spot and channel, μ is the overall average log₂ fluorescent signal intensity, and ε the residual error that was normally distributed. The arrays were assumed to be normal and the Array effect was set to random. The 'Treatment' variable was nested within 'Time', as these variables were unbalanced (three timepoints with two treatments and one timepoint with one treatment). LSMEANS were computed, and a t -test was run in SAS on all data to measure the significance of the difference for each gene in all possible pair-wise treatment comparisons. The estimate was later used to compute intensity ratios and prepare a table of fold changes of all comparisons together with their multiple testing fdr-adjusted P values (Benjamini and Hochberg 1995). Gene selection was based on several factors. The spot representing a given gene had to have a valid signal in at least three of the four replicates, an overall treatment effect fdr-adjusted P value of at least 0.001, and the treatment fdr-adjusted P value had to be more significant than that gene's dye effect fdr-adjusted P value. Additionally, a gene had to be significant in at least one timepoint using fdr-adjusted P value cutoffs of 0.01, 0.001, and 0.0001 for timepoints 4, 8, and 16 dai, respectively. Because the amount of tissue response would be weaker and gene expression differences more subtle at 4 dai versus 16 dai, we staggered the stringency of the cutoffs to enable a more balanced identification of the most significant genes across the study. Combining these criteria resulted in a final gene list table of 6,555. Genes were classified into functional categories based on analysis of both 5' and 3' EST information off each cDNA in a method similar to that described (Zou et al, 2005). Step-by-step tutorials explaining our analysis methods

and access to the PERL scripts used to process prenormalized data are available at the University of Illinois Crop Science's Clough Lab website.

Hierarchical clustering analysis was performed using publicly available analysis tools integrated into our Soybean Gene Expression Database. The measurement of the Euclidean distance between genes was used. Gene expression data of soybean infected by *P. syringae* comes from Zou and associates (2005).

EST found significantly regulated during the nodulation of *M. truncatula* and *L. japonicus* (Asamizu et al 2005; Colebatch et al 2004; El Yahyaoui et al 2004; Kouchi et al 2004; Starker et al 2006) were compared against the soybean database of cDNA present on the microarray slides using BLASTn to identify the *G. max* ortholog EST. A bit score of 100 or higher was used as a cutoff, and all soybean EST identified by BLASTn with a score lower than 100 or with a *P* value in the *Medicago* and *Lotus* studies higher than 0.05 were removed. Hierarchical clustering was then performed using the Euclidean distance measure between genes and the Pearson correlation.

Real-time qRT-PCR.

Primers used for qRT-PCR were designed using Primegens (Xu et al. 2002), which enabled the identification of primers specific to each gene among the soybean unigene database. Primer sequences are summarized in Supplemental Table 4. Reactions were carried out in an ABI7500 real-time PCR system (Applied Biosystem, Foster City, CA, U.S.A.). PCR reactions were performed in a final volume of 10 µl containing 1× of SYBR green PCR master mix (Applied Biosystem), 900 nmol primers, 1 µl of cDNA template, and sterile water. PCR cycles were 50°C for 2 min, 95°C for 10 min, 40 cycles for 15 s at 95°C, and 1 min at 60°C, followed by the dissociation curve. The visualization of a single melting point on the dissociation curve and a single PCR product on a 2% agarose gel, as well as the sequence (DNA Core, University of Missouri-Columbia) identity between the PCR product and the EST used to define the primers, verified the accuracy and specificity of the primers used in this study.

Quantification was based on a cycle threshold (Ct) value (PCR cycle number when reaction exceeds a threshold value set in the exponential phase of reaction fluorescence; $R_n = 0.2$ for all PCR) and PCR efficiency, which is determined by using LinRegPCR software (Ramakers et al. 2003). The expression level of each gene normalized by the *SUB12* (ubiquitin) gene was calculated, and the relative fold change of genes during nodulation was determined according to the following formula.

$$RFC = \frac{(E_{\text{target}})^{-(Ct_{\text{target}} - Ct_{\text{SUB12}})}}{(E_{\text{targetUN}})^{-(Ct_{\text{targetUN}} - Ct_{\text{SUB12UN}})}}$$
where RFC = the relative fold change, E = PCR efficiency, Ct = the cycle threshold, I = inoculated, UN = Uninoculated.

No amplifications were obtained by using the template coming from cDNA synthesis without reverse transcriptase, testifying to the absence of genomic DNA in the samples.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

The Soybean Gene Expression Database (SGED):
aragon.ncsa.uiuc.edu/soybean-microbe; login: guest, password: guest
 University of Illinois Crop Science's Clough Lab website:
www.cropsci.uiuc.edu/faculty/clough/protocol.htm