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Identification of New Potential Regulators of the *Medicago truncatula*–*Sinorhizobium meliloti* Symbiosis Using a Large-Scale Suppression Subtractive Hybridization Approach

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We set up a large-scale suppression subtractive hybridization (SSH) approach to identify *Medicago truncatula* genes differentially expressed at different stages of the symbiotic interaction with *Sinorhizobium meliloti*, with a particular interest for regulatory genes. We constructed 7 SSH libraries covering successive stages from Nod factor signal transduction to *S. meliloti* infection, nodule organogenesis, and functioning. Over 26,000 clones were differentially screened by two rounds of macroarray hybridizations. In all, 3,340 clones, corresponding to genes whose expression was potentially affected, were selected, sequenced, and ordered into 2,107 tentative gene clusters, including 767 MTS clusters corresponding to new *M. truncatula* genes. In total, 52 genes encoding potential regulatory proteins, including transcription factors (TFs) and other elements of signal transduction cascades, were identified. The expression pattern of some of them was analyzed by quantitative reverse-transcription polymerase chain reaction in wild-type and in Nod−*M. truncatula* mutants blocked before or after *S. meliloti* infection. Three genes, coding for TFs of the bHLH and WRKY families and a C2H2 zinc-finger protein, respectively, were found to be upregulated, following *S. meliloti* inoculation, in the infection-defective mutant *lin*, whereas the bHLH gene also was expressed in the root-hair-curling mutant *hel*. The potential role of these genes in early symbiotic steps is discussed.

Additional keywords: EST, macroarrays, nodulin, supernodulating mutants.

The symbiotic interaction of plants belonging to the legume family and a group of soil bacteria named rhizobia results in the production, on host plants, of newly formed organs called root nodules, inside which rhizobia fix atmospheric nitrogen for the benefit of the plant. This interaction is initiated by an exchange of signals between both partners. A key player in this dialogue are lipochitooligosaccharides produced by the bacteria, called Nod factors (NFs), which are responsible for the host specificity and are able, in the absence of bacteria, to trigger many early responses of legumes to rhizobia such as ion fluxes, calcium spiking, and specific gene expression (Geurts and Bisseling 2002). Infection is initiated in root hairs, through which the bacteria progress by initiating tubular structures, called infection threads, that lead them towards cortical cell layers (Brewin 2004; Gage 2004). Concomitantly, cells within the inner cortex reenter mitosis and generate a nodule primordium in which an apical meristem is formed that allows the subsequent nodule organogenetic process.

In recent years, several forward and reverse genetic approaches have led to the discovery of genes playing essential roles in initial steps of the *Rhizobium* sp.–legume symbiosis, reviewed by Stacey and associates (2006). Mutants blocked in successive steps of NF signal perception and transduction represent very useful tools for the dissection of early symbiotic events. Supernodulating mutants also are helpful to unravel the complex mechanisms by which legumes control the number of root nodules and infections (Searle et al. 2003).

In addition, for more than two decades, many legume genes, called nodulin genes, which are upregulated during different stages of nodulation, have been identified, using large-scale transcriptomic approaches in recent years (Barnett et al. 2004; Colebatch et al. 2002, 2004; El Yahyaoui et al. 2004a; Fedorova et al. 2002; Gamas et al. 1996; Journet et al. 2002; Kouchi et al. 2004; Lohar et al. 2001; Mergaert et al. 2003). These were based primarily upon large-scale expressed sequence tag (EST) programs in the two model legumes, *Medicago truncatula* (currently 226,923 ESTs, with approximately 30,000 nodule or *Sinorhizobium meliloti*-inoculated root cDNA libraries) and *Lotus japonicus* (currently 111,600 ESTs, including 28,000 nodule or nodulating root ESTs).

However, random sampling of cDNA clones gives preferential access to strongly expressed genes, which can be represented by numerous ESTs, whereas weakly expressed genes (or genes expressed in a few cells in a given sample) can be very difficult to identify. The suppression subtractive hybridization (SSH) approach was developed in order to both normalize libraries (and thus minimize clone redundancy) and enrich them in sequences differentially represented between two samples (and thereby have a more direct access to genes of interest) (Diatchenko et al. 1999). This method was successful in identifying various differentially expressed genes, especially in plant species, to study, for example, developmental processes.
(Bouton et al. 2005; Le et al. 2005) and response to water stress (Zheng et al. 2004) or plant pathogens (Schulze Gronover et al. 2004). In *M. truncatula*, it was used to identify novel gene markers of interactions with symbiotic arbuscular mycorrhizal fungi (Brehenmacher et al. 2004; Weidmann et al. 2004; Wulf et al. 2003), endophytic *Pseudomonas fluorescens* (Sanchez et al. 2005), and a pathogenic oomycete, *Aphanomyces euteiches* (Nyamsure et al. 2003).

We decided to use SSH as a complementary approach to our previous characterization of a standard nodule cDNA library (El Yahyaoui et al. 2004; Journet et al. 2002) to extend our knowledge of the *M. truncatula* symbiotic program and, in particular, regulatory genes that potentially are weakly expressed and, thus, difficult to identify.

RESULTS

Generation of a series of SSH libraries covering various symbiotic stages.

The SSH technique allows two populations of cDNAs to be compared and the differentially expressed ones to be preferentially polymerase chain reaction (PCR) amplified and cloned. The principle of this method is to subtract, by hybridization, sequences that are shared between a control (or “driver”) sample and the sample of interest (or “tester”). The subtraction can be done in two reciprocal ways, designated “forward” and “reverse,” in order to enrich in sequences up- or downregulated, respectively, between two conditions (Diatchenko et al. 1999). We constructed seven SSH libraries covering various stages of the *M. truncatula–S. meliloti* symbiotic interaction (Table 1). The first library (MtSC4) was constructed to better understand the effects of nitrogen starvation as a prerequisite for nodulation. This library was obtained by the subtraction of cDNA populations deriving from nitrogen-starved roots harvested at 8, 24, 48, and 96 h by the cDNA representing unstarved roots. To study early responses to purified NFs, we also examined the cDNA representation of whole-root systems of a supernodulating *M. truncatula* mutant, *sun-2* (originally TR122) (Sagan et al. 1995, Schnabel et al., 2005), inoculated with either a wild-type *S. meliloti* or a non-nodulating *nodA* *S. meliloti* mutant 3 and 6 days postinoculation (dpi). Finally, nodule organogenesis and functioning were analyzed using two libraries, MtSN0 and MtSN4, obtained by reciprocal subtractions between 4- and 10-day-old isolated wild-type nodules versus noninoculated root material (Table 1).

<table>
<thead>
<tr>
<th>SSH library</th>
<th>Subtraction</th>
<th>Tester cDNA populations*</th>
<th>Driver cDNA populations</th>
</tr>
</thead>
<tbody>
<tr>
<td>MtSC4</td>
<td>Forward Nitrogen starved roots (8, 24, 48, and 96 h)</td>
<td>Unstarved roots</td>
<td></td>
</tr>
<tr>
<td>MtSNF</td>
<td>Forward 10^{-8} M Nod factor-treated roots (1, 3, 6, 24, and 48 h)</td>
<td>Mock-treated roots (1, 3, 6, 24, and 48 h)</td>
<td></td>
</tr>
<tr>
<td>MtSCF</td>
<td>Reverse Mock-treated roots (1, 3, 6, 24, and 48 h)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MtSTW</td>
<td>Forward Supernodulating TR122 roots inoculated with wild-type Sinorhizobium melloti (3 and 6 dpi)</td>
<td>Supernodulating TR122 roots inoculated with nodA <em>S. meliloti</em> (3 and 6 dpi)</td>
<td></td>
</tr>
<tr>
<td>MtSTA</td>
<td>Reverse Supernodulating TR122 roots inoculated with nodA <em>S. meliloti</em> (3 and 6 dpi)</td>
<td>Supernodulating TR122 roots inoculated with wild-type <em>S. meliloti</em> (3 and 6 dpi)</td>
<td></td>
</tr>
<tr>
<td>MtSN4</td>
<td>Forward A17 excised root nodules (4 and 10 dpi)</td>
<td>Noninoculated A17 roots</td>
<td></td>
</tr>
<tr>
<td>MtSN0</td>
<td>Reverse Noninoculated A17 roots</td>
<td>A17 excised root nodules (4 and 10 dpi)</td>
<td></td>
</tr>
</tbody>
</table>

* Days postinoculation = dpi.

Table 2. Number of clones screened per suppression subtractive hybridization (SSH) library and analysis of known nodulin content

<table>
<thead>
<tr>
<th>Clones</th>
<th>Forward SSH libraries</th>
<th>Reverse SSH libraries</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MtSC4</td>
<td>MtSNF</td>
<td>MtSTW</td>
</tr>
<tr>
<td>Screened clones</td>
<td>4,992</td>
<td>5,376</td>
<td>3,680</td>
</tr>
<tr>
<td>M1ENOD11</td>
<td>n.d.</td>
<td>2</td>
<td>10</td>
</tr>
<tr>
<td>M1N6</td>
<td>n.d.</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>M1Lh1</td>
<td>n.d.</td>
<td>0</td>
<td>119</td>
</tr>
<tr>
<td>Other nodulin genes</td>
<td>n.d.</td>
<td>19</td>
<td>282</td>
</tr>
<tr>
<td>Total nodulin genes</td>
<td>n.d.</td>
<td>21</td>
<td>412</td>
</tr>
<tr>
<td>M1PR10-1</td>
<td>n.d.</td>
<td>n.d.</td>
<td>1</td>
</tr>
<tr>
<td>M1NRT2</td>
<td>132</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Spotted clones</td>
<td>843</td>
<td>768</td>
<td>480</td>
</tr>
</tbody>
</table>

and 19 with the 35 nodulin gene mix probe were found in the MtSNF library, compared with only 0 and 4, respectively, in the reverse MtSCF library (Table 2).

To assess the quality of the nitrogen-starved roots library (MtSC4), we identified an MtNRT2 cDNA homologous to AtNRT2.1, a high-affinity nitrate transporter gene known to be a good marker of nitrogen starvation in Arabidopsis thaliana (Gansel et al. 2001). The predicted MtNRT2-encoded protein (MENS [Medicago EST Navigation System] cluster MtC45479, GenBank ID AL369554) shows strong similarity (approximately 75% sequence identity) with AtNRT2.1, and MtNRT2 was checked by Northern analysis to be induced in M. truncatula roots upon nitrogen starvation (data not shown).

In all, 132 clones hybridizing with the MtSNF probe were identified in the MtSC4 library (2.6% of the total clones) compared with 0.07% in three nitrogen-starved nonsubtracted cDNA libraries (MENS database).

From this first analysis of forward and reverse libraries with marker gene probes, we could conclude that the subtractions worked properly and that the SSH libraries we produced were substantially enriched for genes known to be differentially expressed during the symbiotic M. truncatula–S. meliloti interaction. The colonies hybridizing with these control probes were not taken into consideration for the subsequent library analyses. The bacterial colony filters of each library then were used to perform a first round of differential screening, using radioactively labeled probes made from the respective subtracted cDNAs (tester and driver) and nonsubtracted PCR-amplified cDNA populations (using the SMART technique, Clontech, Le Pont de Claix, France). In total, we selected 3,941 clones (2,781 clones from the forward libraries and 1,160 from the reverse libraries) that gave hybridization signal ratios between respective tester and driver probes of at least 1.5, with a maximal P value of 0.05 (Table 2). The corresponding cDNA inserts then were PCR amplified for sequencing.

**Sequence analysis.**

Among the clones selected from the seven SSH libraries, 3,340 sequences were determined from one end. Insert length varied from 100 to 800 bp, with a median value ranging from 325 to 515 bp depending on the library, with 95% being at least 200 bp long. After editing, these sequences were clustered with the 184,338 M. truncatula ESTs available in the MENS database (release January 2003), generating 2,107 clusters (Table 3). It could be noted that the redundancy within each sequenced library sample was low, with an average of 1.7 SSH ESTs per cluster, compared with 5 ESTs per cluster in standard cDNA libraries (MENS), underlining the importance of the normalization procedure during SSH approaches (even though some nodulin genes were highly represented). Importantly, the overlap between forward and reverse SSH libraries generated from the same biological material was extremely low (3% of the clusters shared between MtSNF and MtSCF, and 1% between MtSTW and MtSN4 and their respective reverse library), as expected from efficient SSH procedures. Overlap among forward libraries (MtSNF, MtSTW, and MtSN4) made from different symbiotic material also was very limited, because less than 10% of all clusters appeared to be in common between the three libraries.

A substantial fraction of SSH ESTs could not be incorporated into existing MtCD clusters, leading to the generation of 767 novel clusters designated MtS. SSH libraries comprised from 18 (MtSCF) to 61% (MtSTA) of such MtS clusters, indicating that this SSH approach had been successful in identifying novel genes (Table 3). The MtS clusters were semi-automatically annotated, using the tools and results available on MENS (partly described in Journet et al. 2002), especially with Interproscan searches, allowing functional motifs to be identified in predicted encoded proteins. In addition to most “classical” nodulin genes, we identified many of the more recently described ones, such as 9 clusters encoding calmodulin-like proteins (Table 4) and 59 encoding members of the nodule-specific cystein-rich (NCR) protein family (Fedorova et al. 2002; Mergaert et al. 2003). Interestingly, we identified ESTs for several nodulins that are not or very poorly represented in standard (non-SSH) EST collections, possibly because of cDNA cloning problems (e.g., highly expressed Pro-rich protein genes SvENOD2-like, MsENOD10-like, MtN8, and MtENOD11), or because of low gene expression levels (MtN18 and NCR protein genes).

As a first analysis of SSH clones, electronic Northern analysis was carried out for MtCD clusters represented in MtSNF, MtSTW, and MtSN4 libraries, using data provided in MENS (release January 2003, section “cluster content summary”). Approximately 70% of the MtCD clusters from the MtSN4 library corresponded to genes predicted to be upregulated in response to S. meliloti on the basis of their EST distribution (among which 112 were exclusively represented in S. meliloti–inoculated roots or nodule libraries). The same was true for 50% of MtCD clusters from the TW library sample (45 of them being specific for S. meliloti roots or nodule libraries). In contrast, no such enrichment was found for the NF library (data not shown).

**Identification of novel potential symbiotic regulatory genes.**

The 768 MtSNF, 480 MtSTW, and 690 MtSN4 cDNA inserts selected by the initial colony screening were PCR amplified and spotted onto nylon macroarrays, along with 30 nodulin cDNAs, including 5 known to be NF induced (discussed below). A secondary screening then was performed to further select for upregulated genes among the sequenced clones, using radioactively labeled probes representing induced versus control samples at different time points.

A global view of these hybridizations is given on the scatterplots shown on Figure 1. The result of two representative hybridizations, an earlier and a later time point sample plotted against a control experiment, are shown for each library. The nodulin gene controls were found to be upregulated, as ex-

**Table 3. Analysis of expressed sequence tag (EST) cluster and suppression subtractive hybridization (SSH)-specific EST cluster contents per SSH library**

<table>
<thead>
<tr>
<th>Roots</th>
<th>SSH library</th>
<th>Number of ESTs</th>
<th>EST clusters</th>
<th>MtS (SSH-specific) clusters</th>
<th>MtS clusters (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitrogen-starved roots</td>
<td>Forward library (MtSC4)</td>
<td>287</td>
<td>157</td>
<td>43</td>
<td>27</td>
</tr>
<tr>
<td>Nod factor-treated roots</td>
<td>Forward library (MtSNF)</td>
<td>726</td>
<td>515</td>
<td>140</td>
<td>27</td>
</tr>
<tr>
<td>Supernodulated TR122 roots</td>
<td>Reverse library (MtSCF)</td>
<td>286</td>
<td>238</td>
<td>43</td>
<td>18</td>
</tr>
<tr>
<td>Root nodules</td>
<td>Reverse library (MtSN4)</td>
<td>436</td>
<td>311</td>
<td>190</td>
<td>61</td>
</tr>
<tr>
<td>Total</td>
<td>…</td>
<td>3,340</td>
<td>2,107</td>
<td>767</td>
<td>36</td>
</tr>
</tbody>
</table>
pected. Approximately a third of the MtSN4 clones (Fig. 1A) gave normalized signal ratios above 1.5 (represented in black) with young nodule material (4 dpi) compared with noninoculated root samples, and almost all of them did so when using functional nodule (10 dpi) probes (Fig. 1B). Similar results were obtained with the MtSTW library, for which probes representing supernodulating roots at 6 dpi revealed a majority of upregulated clones (Fig. 1D), whereas the proportion of induced genes was much lower with 3-dpi samples (Fig. 1C). In the MtSNF library, low levels of induction between NF-treated and control root samples were detected only for a small proportion of genes (Fig. 1E and F).

These results indicate that, in the case of the MtSTW and MtSN4 libraries, the colony-screening procedure allowed a selection of a majority of upregulated genes. It was not really the case for the MtSNF library, for which the SSH approach

Table 4. Selection of upregulated genes coding for potential regulators of the symbiotic *Medicago truncatula-Sinorhizobium meliloti* interaction

<table>
<thead>
<tr>
<th>GenBank accessions</th>
<th>Library origin</th>
<th>MENS Cluster</th>
<th>TIGR8 TC</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AJ847075</td>
<td>MtSTW</td>
<td>MiC40192</td>
<td>TC101434</td>
<td>Dynamin GTPase effector</td>
</tr>
<tr>
<td>AJ847227</td>
<td>MtSTW</td>
<td>MyS00172</td>
<td>...</td>
<td>Microtubule-associated protein</td>
</tr>
<tr>
<td>AJ847037</td>
<td>MtSTW</td>
<td>MyS10591</td>
<td>...</td>
<td>Microtubules GTP-binding protein</td>
</tr>
<tr>
<td>AJ848184</td>
<td>MtSN4</td>
<td>MiC00109.1</td>
<td>TC100208</td>
<td>ADP-ribosylation factor, ARF/SAR superfamily</td>
</tr>
<tr>
<td>AJ847131</td>
<td>MtSTW</td>
<td>MiC00564</td>
<td>TC93927</td>
<td>SKP1-like protein</td>
</tr>
<tr>
<td>AJ847096</td>
<td>MtSTW</td>
<td>MyC0698</td>
<td>TC106531</td>
<td>Ubiquitin carboxyl terminal hydrolase</td>
</tr>
<tr>
<td>AJ8469200</td>
<td>MtSTW</td>
<td>MyS10554</td>
<td>...</td>
<td>Ubiquitin-activating enzyme E1</td>
</tr>
<tr>
<td>AJ843081</td>
<td>MtSN4</td>
<td>MiC02012</td>
<td>TC95554</td>
<td>F-box protein</td>
</tr>
<tr>
<td>AJ843858</td>
<td>MtSN4</td>
<td>MyC02621</td>
<td>TC100437</td>
<td>AgNOD-CP1 and AsNOD32-like cysteine proteinase</td>
</tr>
</tbody>
</table>

Vesicular trafficking, secretion and protein sorting

| AJ848579          | MtSN4         | MyS10215     | ...      | Brassinosteroid biosynthetic protein LKB (DIMINUTO/DWARF-1) |
| AJ848566          | MtSN4         | MiC01086     | TC101963 | Auxin-induced SAUR-like protein |

Protein synthesis and processing

| AJ847293          | MtSTW         | MyS10662     | ...      | bZIP transcription factor |
| AJ848040          | MtSN4         | MyS10132     | ...      | C2H2-type zinc finger protein, ID1-like |
| AJ848025          | MtSN4         | MyC10582     | TC95981  | CCAAT-biding transcription factor, subunit B |
| AJ845850          | MtSN4         | MyC00180     | TC106806 | Homeobox-leucine zipper protein |
| AJ847301          | MtSTW         | MyS10664     | ...      | At/Os GRF transcription activators-like |
| AJ848640          | MtSN4         | MyS10222     | ...      | MADS-box transcription factor |
| AJ848228          | MtSN4         | MiD05611     | TC96646  | Myb transcription activator |
| AJ847977          | MtSN4         | MyC0408     | TC109672 | Ethylene response factor (ERF) |
| AJ848045          | MtSN4         | MiD09495     | TC104390 | Response regulator receiver domain protein |
| AJ847047          | MtSTW         | MyC10881     | AW684462 | VSF-1 transcription factor |
| AJ845845          | MtSN4         | MiD01844     | TC102282 | WRKY transcription factor |
| AJ847076          | MtSTW         | MyS10602     | ...      | K Intron Maturase |
| AJ847153          | MtSTW         | MiD04626     | TC104080 | DNA-directed RNA polymerase II |
| AJ845895          | MtSNF         | MyC45563     | TC101140 | RNA-binding protein |
| AJ848359          | MtSN4         | MyS10207     | ...      | Polyphymidine tract binding protein (PTB) |
| AJ847213          | MtSTW         | MyC11003     | TC95298  | Multiple stress-responsive zinc-finger transcription factor |

Gene expression and RNA metabolism

| AJ845532          | MtSNF         | MyC0004      | TC100224 | Extensin LRR receptor kinase |
| AJ845851          | MtSNF         | MyS10364     | ...      | Putative ethylene receptor |
| AJ847918          | MtSTW         | MyC10513     | TC102912 | Serine/threonine protein kinase |
| AJ848389          | MtSN4         | MyC0065.1    | TC95050  | Serine/threonine protein kinase |
| AJ847121          | MtSTW         | MiD02460     | TC108221 | Serine/threonine protein kinase (possibly mitogen activated) |
| AJ844949          | MtSN4         | MyS10201     | ...      | Protein phosphatase 2C (PP2C) |
| AJ846466          | MtSN4         | MiD03675     | TC110302 | WD repeat protein |
| AJ846988          | MtSTW         | MiD15550     | TC103709 | WD-40 repeat protein |
| AJ847074          | MtSTW         | MyS10601     | ...      | Pleckstrin-homology (PH) domain protein |
| AJ848586          | MtSN4         | MiC0355      | TC107257 | Calmodulin-like protein |
| AJ847953          | MtSN4         | MiC0404      | TC107926 | Calmodulin-like protein |
| AJ848497          | MtSN4         | MiC0592      | TC101807 | Calmodulin-like protein 3 |
| AJ848518          | MtSN4         | MiC1428      | TC102961 | Calmodulin-like protein 5 |
| AJ848181          | MtSN4         | MyS00125     | ...      | Calmodulin-like protein |
| AJ848757          | MtSN4         | MyS00135     | ...      | Calmodulin-like protein |
| AJ848450          | MtSN4         | MiC0929      | TC95911  | Calmodulin-like protein 6 |
| AJ848311          | MtSN4         | MyS10178     | ...      | Calmodulin-like protein |
| AJ848358          | MtSN4         | MyS10185     | ...      | Calmodulin-like protein |
| AJ848186          | MtSN4         | MyS10156     | ...      | RAS-related protein |
| AJ847296          | MtSTW         | MiD01053     | TC108046 | RHG GDP-dissociation inhibitor 1 |
| AJ848344          | MtSN4         | MiD04097     | TC102589 | SPX domain (G-protein binding) protein |

* Bold indicates genes tested by quantitative reverse-transcription-polymerase chain reaction in this study. Bold and an asterisk (*) indicates genes tested in the study by El Yahyaoui and associates (2004).
* MtS suppression subtractive hybridization-specific clusters are shown in bold.
Fig. 1. Scatter plots of gene expression levels in three *Medicago truncatula* suppression subtractive hybridization (SSH) libraries: A and B, MtSN4; C and D, MtSTW; and E and F, MtSNF. In all, 768, 480, and 690 cDNA inserts from the MtSN4, MtSTW, and MtSNF library, respectively, were selected after colony hybridization, polymerase chain reaction amplified, spotted on nylon macroarrays, and hybridized. Figures on the vertical axis represent normalized gene expression levels obtained for induced samples (logarithmic scale) whereas the horizontal axis represent normalized expression levels obtained for control samples, as follows: A and B, 4- and 10-day-old isolated nodules versus uninoculated roots; *Sinorhizobium meliloti*-inoculated roots from supernodulating mutant sunn-2 (originally TR122) at C and D, 3 and 6 days postinoculation versus sunn-2 uninoculated roots; and 10^{-8} M Nod factor-treated roots after E and F, 3 and 48 h versus untreated roots. The data presented are the averages of independent duplicate membrane hybridizations. The outer diagonals indicate the limits for two-fold (A, B, C, and D) or 1.5-fold (E and F) expression ratios.
or the macroarray analysis did not allow us to overcome the
dilution of NF-responding cells among nonresponding root
tissues.

A selection of 52 upregulated genes coding for potential
regulators of the symbiotic S. meliloti–M. truncatula interaction
is shown in Table 4. This includes 14 clones representing
putative transcription factors belonging to various classes,
and 21 coding for proteins potentially involved in signal trans-
duction. Two of these transcription factor genes, MtC10582
and MtC50408, have been identified previously as induced in
nodule tissue by macro- or microarray analyses and by
quantitative reverse-transcription (qRT-PCR) experiments (El
Yahyaoui et al. 2004). The 31 remaining MtC or MtD clus-
ters correspond to genes not previously characterized as up-
regulated during nodulation. In addition, this list includes 19
MtS clusters (36%), thus representing potential novel sym-
biotic genes.

**Six novel putative regulatory genes are induced
in symbiotic tissues.**

Six genes not reported before to be induced following NF
treatment or during nodulation were studied further. They cor-
responded to four potential transcription factor genes belong-
ing to the WRKY (MtD01884), C2H2-type zinc finger protein
(MtS10132), and the bHLH (MtC19688 and MtD19113) families.
We also selected a gene coding for a Ser/Thr protein
kinase (MtC10513) potentially involved in signal transduction
and a last one coding for an auxin-induced SAUR-like protein
(MtC61086). All of them had a low EST representation in the
standard cDNA libraries (from 0 to 6 ESTs in MENS release
2003).

Average induction levels in NF-treated root (Fig. 2A) or in
nodule samples (Fig. 2B through F) compared with control
roots were determined by qRT-PCR analysis. The response to
purified Nod factors of a MtSNF clone, MtD19688, that ex-
hibited a moderate but reproducible upregulation at 48 h after
NF treatment is shown in Figure 2A. The induction ratios ob-
tained for the other five selected genes derived from the
MtSN4 and MtSTW libraries were examined in isolated de-
veloping young nodules 4 dpi and in mature nitrogen-fixing
nodules at 10 and 14 dpi compared with noninoculated roots
(Fig. 2B through F). Three expression patterns could be dis-
tinguished, revealing the diversity of symbiotic stages cov-
ered by the SSH libraries. The MtD19113 bHLH TF gene

![Fig. 2](image-url)

**Fig. 2.** Expression analysis by quantitative reverse-transcription polymerase chain reaction (qRT-PCR) of six putative regulatory genes after Nod factor
treatment or during nodule development. The expression of six genes, isolated from the forward suppression subtractive hybridization libraries and predicted
by macroarray analysis to be upregulated were tested by qRT-PCR for expression A, after NF treatment or B through F, in excised nodules compared with
control roots. The relative cDNA concentrations normalized by EF1-α. are expressed in arbitrary units on the Y axis whereas A, 0, 6, 24, and 48 on the X axis
represent hours after Nod factor treatment and B through F, 0, 4, 10, 14 represent days postinoculation. R is the ratio between the expression values
obtained in treated samples compared with the initial values at T0.
(Fig. 2B) and the MtC10513 kinase gene (Fig. 2C) showed a maximum expression in young nodules (induction levels of 3.6 and 2, respectively). A second group was composed of the MtS10132 zinc finger protein gene (Fig. 2D) and the bHLH TF gene MtD01844 (Fig. 2E), for which an induction of comparable intensity could be seen in young and older nodules. Both genes, but especially MtS10132, were strongly activated in nodules compared with roots (65- and 5-fold, on average, for MtS10132 and MtD01844, respectively). Finally, MtC61086 (Fig. 2F), an auxin-induced SAUR-like protein gene, exhibited differential expression only at later symbiotic stages, with the highest induction levels of all tested clones (135- and 124 fold at 10 and 14 dpi, respectively). Except for MtC61086, the absolute expression values were quite weak for these genes (from $10^{-4}$ to $10^{-1}$ compared with EF1-α expression levels used to normalize the data), showing that the SSH approach can, indeed, lead to the identification of weakly expressed genes.

Expression of novel putative regulatory genes in symbiotic Nod⁻ plant mutants.

To better characterize the stage of the symbiotic interaction in which these putative regulatory genes could be involved, we analyzed their expression profile in three Nod⁻ M. truncatula mutants blocked at early steps of nodulation. We studied the expression of the five genes most likely to be involved in early symbiotic steps according to their expression profile (MtD19688, MtD19113, MtC10513, MtS10132, and MtD01844) and compared them with the expression of the early nodulin gene MtENOD11. We first analyzed gene expression in an nsp1 line, mutated in a gene coding for a GRAS transcriptional regulator (Smit et al. 2005) and blocked for certain NF-induced responses but not for root hair branching or calcium spiking (Wais et al. 2000). Additionally, we used an hcl mutant that shows symbiotic root hair deformation and normal expression of several nodulin genes in response to NF treatment, but no hair curling (Catoira et al. 2001). Finally, we examined gene expression in the D8 mutant, which is allelic to the lin mutant (Kuppusamy et al. 2004; C. Gough and J. F. Arrighi, unpublished results). In D8, S. meliloti-induced root hair curling was observed but infection threads formed only rarely. We used entire root systems inoculated with wild-type S. meliloti or, as a control, with a non-nodulating (nodA⁻) S. meliloti mutant (unable to produce NFs), at 3 or 7 dpi, or both.

Induction of the MtD19688 gene (MtSNF library) was undetectable in S. meliloti-inoculated roots, as predicted from its lack of expression in nodules (data not shown). In contrast, we...
could detect transcriptional activation of the four nodule-induced genes from MtSN4 and MtSTW, similarly to MtENOD11, in wild-type S. meliloti-infected roots at 3 and 7 dpi, but not in nodA- S. meliloti-inoculated samples (Fig. 3).

None of the genes analyzed was found to be induced in nsp1 at 3 dpi with S. meliloti. In the hcl background, MtENOD11 was clearly induced at 3 dpi, although less than in wild-type plants, probably as a result of the lack of infection in this mutant (Fig. 3A) (Catoira et al. 2001). Similarly, MtD19113 was weakly but consistently upregulated in wild-type and hcl roots at 3 dpi (Fig. 3B). In the D8/tin background, an upregulation was observed for MtD19113, MtD01844, and MtS10132 (Fig. 3B through D). No induction in any of the tested mutants was observed for the Ser/Thr protein kinase gene MtC10513 (Fig. 3E), suggesting that its activation requires a subsequent step of infection or nodule development.

**DISCUSSION**

We carried out a large cDNA subtractive library sequencing project with the aim to discover new regulatory genes involved in the M. truncatula-S. meliloti symbiotic interaction. A series of SSH libraries representing early to late symbiotic stages was constructed and differentially screened, resulting in the analysis of over 31,000 SSH clones and the sequencing of more than 3,300 of them. There are several reports about the use of SSH to study mycorrhizal symbiotic interactions (Brechenmcher et al. 2004; Ouzi et al. 2005; Voilb et al. 2001; Wulf et al. 2003); however, to our knowledge, there is only one other report of an SSH analysis of the rhizobial symbiosis so far, but on a much smaller scale (Chou et al. 2006).

The long-standing investment of many laboratories in nodulin gene identification and EST sequencing make it possible to evaluate to what extent this SSH approach was efficient to find both known and novel nodulation gene markers complementary to existing EST or cDNA data. For example, it is interesting to recall that differential screening of 30,000 clones from a standard M. truncatula nodule 4-dpi cDNA library led to the selection of 473 nodule-induced clones (1.6% of the total library), including MtLb and MtENOD genes, and 29 new nodulin genes called MtEN-29 (Gamas et al. 1996). For comparison, from the 4,048 nodule SSH clones that we screened (MtSN4 library), 1,017 clones (25%) corresponded to a similar set of known nodulin genes. Thus, we can roughly evaluate the enrichment factor in known nodulin ESTs of the nodule SSH library to 15-fold compared with previous non-SSH nodule cDNA libraries. This illustrates the power of the normalization-subtraction approach that allows the screening of a lower number of clones without substantially altering gene diversity in the library. Indeed, from 31,200 total clones initially screened, 2,107 clusters were identified from 3,340 ESTs, indicating a low redundancy and an effective gene coverage in the seven libraries. Moreover, the SSH strategy allowed the identification of 767 potentially novel MtS clusters among only 3,340 sequences performed (23% of putative new genes). These genes are good candidates to be upregulated during symbiotic interactions because they were selected twice on macroarrays with a P value threshold of 0.05. However, not all the genes recently identified as being activated during nodulation by microarray analyses (Barnett et al. 2004; El Yahyaoui et al. 2004; Mitra and Long 2004; Mitra et al. 2004) were found in the sequenced SSH clones, indicating that this resource is complementary to others but cannot substitute for them. Of course, the number of predicted MtS clusters should be considered with caution, notably because, in SSH libraries, several nonoverlapping inserts can be generated from a single transcript (by restriction enzyme hydrolysis before the cloning step).

Whereas identifying upregulated genes was successful for two of three forward SSH libraries (MtSN4 and MtSTW), this turned out to be a difficult task for the third one, MtSNF. Indeed, many of the clones selected as potentially upregulated following the first colony screening could not be confirmed by the second screening on macroarrays. This might be due to the fact that the NF-responding cells were actually too poorly represented in the material used to screen these macroarrays. The SSH procedure could be reaching its limits in this case. One way to solve this problem could be to enrich the biological samples in NF-responding cells; for example, by using NF-treated isolated root hairs (Sauvage et al. 2005).

A potentially important aspect of gene regulation, so far poorly explored in the context of the symbiotic program, deals with root genes that need to be downregulated for the nodulation and infection to occur. Using reverse subtracted SSH libraries (enriched for downregulated gene cDNAs), 768 clusters potentially corresponding to genes expressed at a lower level in nodules or NF-treated roots compared with control roots were identified by colony screening, including 292 novel MtS clusters. While lower gene expression in nodules than in roots might simply reflect the fact that two different organs are being compared, TF genes from these reverse libraries could correspond to negative regulators of one or more nodule-specific pathways that need to be turned down to allow nodulation. The genes identified here represent useful candidates with which to explore such hypotheses.

Bearing in mind our original goal to find new regulatory genes, 52 candidate genes belonging to very diverse classes were identified from the forward libraries. They correspond either to putative new genes (MiS) or to previously identified genes (MiC or MiD) not known to be induced during symbiotic interactions. They add to the 34 putative regulatory symbiotic genes that we previously identified (El Yahyaoui et al. 2004). Clearly, SSH libraries cannot be comprehensive and, therefore, should be considered as complementary to other large-scale transcriptomic approaches such as gene expression analyses on microarrays (Lohar et al. 2006) or on Affymetrix chips (Mitra et al. 2004).

The differential expression of six putative regulatory genes, encoding four different TFs, a kinase, and an early auxin-induced gene, was tested in Nod mutant plants affected in early steps of nodulation and infection (nsp1, hcl, and lin, in the predicted order of blockage). Although all the genes that we tested showed an absolute need for NSP1, they behaved differently in hcl and lin background, thus representing useful markers for molecular phenotyping.

One gene, MtC10513, whose induction by S. meliloti was HCL and LIN dependent, encodes a putative protein of the PERK family of receptor kinases (Silva and Goring 2002). This family consists of 15 members in A. thaliana, exhibiting diverse expression patterns (Nakhamchik et al. 2004) and showing an intriguing structure with a proline-rich, extensin-like extracellular domain, probably anchored in the cell wall, a transmembrane domain, and an intracellular kinase domain. This structure suggests an implication in sensing and transducing external signals that could be linked to development, cell-to-cell communication, mechanical stress, or microbial infection. In *Brassica napus* plants, this gene is indeed induced transiently in leaf, stem, and root tissues after wounding and in leaves infected by a fungal pathogen (Silva and Goring 2002). Thus, our results suggest that MtC10513 expression could act as a sensor of *S. meliloti* infection or nodule development during later stages of the symbiotic interaction.
Two genes, MtS10132 and MtD01844, were induced in lin (in which infection threads initiate but fail to develop and grow properly) but not in hcl (affected in root hair curling, cortical cell activation, and infection initiation) (Catoira et al. 2001). MtS10132 encodes a new potential M. truncatula TF highly homologous to the indeterminate 1 (ID1) protein, a plant-specific C2H2 zinc finger TF (Colasanti et al. 1998, 2006). Genetic analysis and expression studies demonstrated that ID1 plays a key role in regulating the transition from vegetative to reproductive growth in maize by controlling the production or transmission of leaf-derived floral inductive signals (Colasanti et al. 1998). More generally, C2H2 zinc finger TFs represent a very large family of TFs involved in various aspects of plant development such as flower or seed development (Ito et al. 2003; Luo et al. 1999) or response to abiotic stress (Milla et al. 2006). Of all upregulated TF clones, MtS10132 showed the highest induction level in nodules (>70-fold) and in nodulated roots (58-fold) compared with control roots. This suggests that MtS10132 could encode a key regulator controlling nodule initiation and functioning once rhizobial infection has been initiated. MtC61086 encodes a protein with homology to the SAUR family of genes rapidly induced (within minutes) by auxin treatment (Milla et al. 2006). Although this contrast with the late expression pattern of this gene (Fig. 2F), MtC61086 could play a role in auxin-mediated processes specific to mature nodules.

MtD01844 encodes a potential TF of the WRKY family, with closest homology to WRKY28 from A. thaliana, of unknown function. WRKY-type TFs have been shown to be important in developmental processes such as leaf senescence, trichome development, or gibberellin signaling; however, this family is particularly well known for its involvement in defense responses (Dong et al. 2003; Ulker and Somssich 2004). It will be interesting to test whether its expression is associated to rhizobial infection, and whether it also could be induced by plant pathogens as other M. truncatula symbiotic TFs (T. Vernié, P. Gamas, and A. Niebel, unpublished data).

Finally, MdD19113 encodes a predicted protein belonging to the large bHLH superfamily of TFs that bind DNA as dimers, giving them the potential to participate in a variety of combinatorial interactions. In plants, they are involved in a large range of developmental to metabolic pathways, such as phytochrome-mediated light signaling or anthocyanin biosynthesis (Toledo-Ortiz et al. 2003). MdD19113 was the only tested TF gene to be still expressed in the hcl mutant, which is blocked much earlier in the NF signal transduction, before infection initiation. Together with three putative TFs identified by Mitra and associates (2004), MdD19113 is thus one of the few TFs known to be upregulated by S. meliloti in hcl. Consequently, MdD19113 could play a role during the preinfection stage, associated with either cortical cell divisions or preparation of infection.

In conclusion, using a large-scale SSH approach, we were able to significantly enrich the current M. truncatula EST data collection and to identify and initially characterize various new potential symbiotic regulators. This set of clones clearly represents a wealth of new information that should be helpful not only for the exploration of the complex symbiotic regulatory networks but also for the entire scientific community interested in legume biology.

**MATERIALS AND METHODS**

**Biological material.**

S. meliloti RCR2011 pXLGD4 (GMI 6526) referred as wild-type strain) and S. meliloti RCR2011 nodA::Tn5 pXLGD4 (GMI 6702) (Ardourel et al. 1994; Debellé et al. 1986) referred to as nodA strain, were grown in tryptone-yeast medium supplemented with 6 mM calcium chloride at 30°C using tetracycline at 10 μg ml⁻¹ for GMI 6526 and tetracycline (10 μg ml⁻¹) and neomycine (100 μg ml⁻¹) for GMI 6702.

Plants of wild-type Medicago truncatula Gaertn cv. Jemalong genotype A17 of the supermodulating M. truncatula sunn-2 mutant (originally TR122) (Sagan et al. 1995; Schnabel et al. 2005), and nspl (B85 allele), hcl (B56 allele), and lin (D8 allele) A17 mutants (Catoira et al. 2001; Kuppusamy et al. 2004; C. Gough and J. F. Arrighi, unpublished results) were germinated on 2% glose for 24 h. At 20°C, and seedlings with 1 cm of root length were grown aeroponically, as described previously (El Yahyaoui et al. 2004). For NF experiments, A17 plants were also treated with 10⁻⁸ M Nod factor after 4 days of nitrogen starvation. Whole-root systems were harvested before and at 1, 3, 6, 24, and 48 h after treatment, and were frozen in liquid nitrogen. In the case of nitrogen experiments, unstarved roots were harvested after 10 days in the medium containing 10 mM NH₄NO₃, just before nitrogen starvation was started by replacing this medium with the nitrogen-free medium. Whole-root systems were then harvested 8, 24, 48, and 96 h after nitrogen starvation and were frozen in liquid nitrogen. Total RNA was extracted with Trizol (Invitrogen, Cergy Pontoise Cedex, France) for SSH library construction, or by using the SV total RNA isolation system (Promega, Madison, WI, U.S.A.) for qRT-PCR experiments. Poly A+ RNA was purified using Dynabeads oligoDT (Dynal AS, Oslo, Norway).

**SSH library construction.**

cDNA was prepared from 2 μg of poly A+ RNA using the Smart PCR cDNA synthesis kit (Clontech). The SSH procedure was carried out using the PCR-Select Subtractive Hybridization Kit (Clontech) according to the manufacturer’s recommendations. The resulting subtracted cDNA was cloned into the pGemT vector (Promega pGem-T cloning kit) and transformed into competent Escherichia coli XL1blue cells (Stratagene, La Jolla, CA, U.S.A.). For each library, 4,000 to 5,000 bacterial clones were picked using a Biopick Robot (BioRobotics, BioRobotics Limited, Comberton, Cambridge) in 384-well microtiter plates and grown in Luria-Bertani medium supplemented with ampicillin (100 μg ml⁻¹) and glycerol (8%). These core libraries were duplicated and stored at –80°C. The presence and size of inserts of 100 random clones was checked by PCR amplification.

**SSH library colony screening.**

The bacterial clones were spotted on Immobilon-N⁺ membranes (Millipore, Bedford, MA, U.S.A.) using a Biogrid robot (BioRobotics, BioRobotics Limited, Comberton, Cambridge) placed on 22-by-22-cm petri dishes (NalgeNunc international, Rochester, NY, U.S.A.) containing Luria-Bertani medium with ampicillin (100 μg ml⁻¹). DNA from bacterial colonies was released onto the filters by alkaline lysis (Nizetic et al. 1991). Membranes were hybridized in Church buffer at 65°C and washed under stringent conditions (final wash: 0.1 SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate] and 0.5% sodium dodecyl sulfate, 15 min at 65°C), with 32P-labeled probes generated by random labeling (Ready To Go; Amersham Pharmacia Biotech, Uppsala, Sweden) from known nodulin genes (early nodulin genes: MEnOD11, MEnOD12, MEnOD16, MEnOD20, MEnOD40, RIP1, Vol. 20, No. 3, 2007 / 329
MtANN1, and MtN1 to MtN20; and later-induced genes: MtLb1 and MtN21 to MtN26), or from the driver and tester cDNAs corresponding to each library. Hybridization signals were then revealed on a Phosphorimager (Storm 840; Molecular Dynamics, Sunnyvale, CA, U.S.A.) and quantified by XDotsReader (COSE, Dugny, France), and analyzed by Bioplot program developed at platform “Biopuces” of the Toulouse Genopole. The mean intensity signal of each spot was corrected by subtracting the local background and negative values were floored to a value of 10. Net signal values were normalized by the mean of all spot intensities. Normalized log intensities were averaged first for spot duplicates, then for biological replicates (two to three biological replicates). The log ratios between experiment and control mean values were calculated. Only signals with a fold change of at least 1.5 were considered. Statistical significance of differential expression was evaluated by a bi-tail two-sample t test, choosing a threshold P value of 0.05.

PCR amplification of selected SSH clones and spotting on macroarrays.

cDNAs were amplified using YieldAce DNA Polymerase (Stratagene, La Jolla, CA, U.S.A.) isopropanol precipitated and resuspended in water to a concentration of at least 300 ng/μl. An aliquot was used for sequencing. Another aliquot was spotted after addition of dimethyl sulfoxide (50% final) in duplicate on nylon membranes using a Biogridd robot. DNA was fixed to the membrane using UV irradiation using a Stratalinker (Stratagene).

Macroarray hybridizations.

Reverse transcription and hybridizations were performed as described by El Yahyaoui and associates (2004). Macroarray data then were analyzed as described above for bacterial clones screening, except for the analyses of MtSTW and MtSN4 SSH clones, where hybridization signals were normalized by the mean of eight housekeeping gene signals.

EST sequencing and clustering.

ESTs were sequenced from one end using SSH nested PCR primers 1 (TCGAGCGGCGGCGGGCCAGGT) or 2 (AGCGTGGTCGCGGCCGAGGT) for forward SSH libraries, and SP6 for reverse libraries. These raw sequences were edited to mask vector and cloning adaptor sequences, and submitted to the EMBL with the following accession numbers (MtSC4: AJ845978-AJ846265; MtSNF: AJ845245-AJ845970; MtSTW: AJ846898-AJ847350; MtSN4: AJ847919-AJ848801; MtSCF: AJ846292-AJ846578; MtSTA: AJ847417-AJ847852; and MtSN0: AJ846579-AJ846848). The 3,340 sequences were clustered with Mt. truncatula ESTs available in public databases using the CAP3 assembling program, as previously described (Journet et al. 2002) and added to the MENS database.

qRT-PCR.

For nodule expression data, qRT-PCR reactions were performed in optical 384-well plates using an ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems, Foster City, CA, U.S.A.) and SYBR Green to monitor template amplification. Reactions contained 3 μl of 2x SYBR Green Master Mix reagent (Applied Biosystems), approximately 0.5 ng of cDNA, and 1 pmol of each gene-specific primer in a final volume of 6 μl. RNAs were tested by qRT-PCR for genomic DNA contamination prior to cDNA synthesis. PCR conditions were used as described by Czechowski and associates (2004). Data were normalized to EF1α expression. For Nod- mutant expression data, qRT-PCR experiments were performed as described by El Yahyaoui and associates (2004), except that the values were normalized with an external control consisting of purified in-vitro-transcribed desmin RNA (80 pg) added to 4 μg of total RNA before cDNA synthesis. qRT-PCR analysis was performed on different biological samples than the ones used for the library construction.

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LITERATURE CITED


Weidmann, S., Sanchez, L., Descombin, J., Chatagnier, O., Gianinazzi, S.,
and Gianinazzi-Pearson, V. 2004. Fungal elicitation of signal transduc-
tion-related plant genes precedes mycorrhiza establishment and requires
17:1385-1393.
Wulf, A., Manthey, K., Doll, J., Perlick, A. M., Linke, B., Bekel, T.,
tional changes in response to arbuscular mycorrhiza development in the
model plant Medicago truncatula. Mol. Plant-Microbe Interact. 16:306-
314.
J., Bai, Y., and Wang, G. 2004. Isolation and analysis of water stress in-
duced genes in maize seedlings by subtractive PCR and cDNA macro-

AUTHOR-RECOMMENDED INTERNET RESOURCES

BioPlot User’s guide:
biopuce.insa-toulouse.fr/ExperimentExplorer/doc/BioPlot
MENS (Medicago EST Navigation System) database:
medicago.toulouse.inra.fr/Mt/EST/
TIGR database:
www.tigr.org/tigr-scripts/tgi/T_index.cgi?species=medicago