Identification of new potential regulators of the [i]Medicago truncatula[/i]–[i]Sinorhizobium meliloti[/i] symbiosis using a large-scale suppression subtractive hybridization approach

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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 *hcl*. 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

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(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 (Brechenmacher et al. 2004; Weidmann et al. 2004; Wulf et al. 2003), endophytic *Pseudomonas fluorescens* (Sanchez et al. 2005), and a pathogenic oomycete, *Aphanomyces euteiches* (Nyamsuren 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, two libraries, MtSNF and MtSCF, were made by reciprocal subtractions of cDNA populations obtained from either 10^{-8} M NF or mock-treated roots, harvested at 1, 3, 6, 24, and 48 h after treatment. Responses to *S. meliloti* were investigated with the MtSTW and MtSTA libraries, obtained by two-way subtractions between cDNAs representing whole-root systems of a supernodulating mutant, *sunn-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).

SSH library colony screening.

For each forward and reverse library, 4,000 to 5,000 bacterial clones were spotted onto nylon membranes for a first round of screening with different probes (Table 2). In order to assess library quality and to exclude as many known nodulin representatives as possible from our screen, a first series of hybridizations was performed using single or mixed probes made up of 35 known nodulin cDNAs (discussed below). The MtSTW and MtSN4 libraries were found to contain a large proportion of known nodulin cDNAs, (11 and 25% of the clones screened, respectively) (Table 2), substantially higher than in nonsubtracted cDNA libraries (Journet et al. 2002). In contrast, the reciprocal libraries MtSN0 and MtSTA contained only very few nodulin cDNAs (mostly corresponding to genes exhibiting a basal level of expression in roots). We also examined the cDNA representation of *MtPR10.1,* a gene known to be more expressed in roots than in nodules (El Yahyaoui et al. 2004). As expected, we found the corresponding cDNAs to be more represented in reverse MtSTA and MtSN0 than in forward symbiotic MtSTW or MtSN4 libraries.

Only a low percentage of known nodulin cDNAs (0.4%) were found in the MtSNF library. This is not surprising, considering that, in the literature, NF induction has been clearly documented for only a few nodulin genes, such as *RIP1* (Cook et al. 1995) or *MtENOD11* (Charron et al. 2004; Gamas et al. 1996). In all, 2 clones hybridizing with the *MtENOD11* probe

Table 1. Presentation of the seven suppression subtractive hybridization (SSH) libraries used in this study

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

[a] Days postinoculation = dpi.

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

	Forward SSH libraries				Reverse SSH libraries			
Clones	**MtSC4**	**MtSNF**	**MtSTW**	**MtSN4**	**MtSCF**	**MtSTA**	**MtSN0**	**Total**
Screened clones	4,992	5,376	3,680	4,048	5,376	3,680	4,048	26,208
MtENOD11	n.d.	2	10	25	0	0	0	…
MtN6	n.d.	0	1	29	0	0	0	…
MtLb1	n.d.	0	119	305	0	0	0	…
Other nodulin genes	n.d.	19	282	658	4	6	2	…
Total nodulin genes	n.d.	21	412	1,017	4	6	2	1,462
MtPR10-1	n.d.	n.d.	1	1	n.d.	20	71	…
MtNRT2	132	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	…
Spotted clones	843	768	480	690	366	470	324	3,098

[a] n.d. = Not determined.

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 *MtNRT2* 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 semiautomatically 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 *SrENOD2*-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 radiolabeled 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

Roots	SSH library	Number of ESTs	EST clusters	MtS (SSH-specific) clusters	MtS clusters (%)
Nitrogen-starved roots	Forward library (MtSC4)	287	157	43	27
Nod factor-treated roots	Forward library (MtSNF)	726	515	140	27
	Reverse library (MtSCF)	286	238	43	18
Supernodulated TR122 roots	Forward library (MtSTW)	452	317	144	45
	Reverse library (MtSTA)	436	311	190	61
Root nodules	Forward library (MtSN4)	883	350	148	42
	Reverse library (MtSN0)	270	219	59	27
Total	…	3,340	2,107	767	36

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

GenBank accessions	Library origin[a]	MENS Cluster[b]	TIGR8 TC	Description
Cytoskeleton				
AJ847075	MtSTW	MtC40192	TC101434	Dynamin GTPase effector
AJ847227	MtSTW	**MtS00172**	…	Microtubule-associated protein
AJ847037	MtSTW	**MtS10591**	…	Microtubules GTP-binding protein
Vesicular trafficking, secretion and protein sorting				
AJ848184	MtSN4	MtC00109.1	TC100208	ADP-ribosylation factor, ARF/SAR superfamily
Protein synthesis and processing				
AJ847131	MtSTW	MtC00564	TC93927	SKP1-like protein
AJ847096	MtSTW	MtC90698	TC106531	Ubiquitin carboxyl terminal hydrolase
AJ846920	MtSTW	**MtS10554**	…	Ubiquitin-activating enzyme E1
AJ848231	MtSN4	MtC20212	TC95554	F-box protein
AJ848385	MtSN4	MtC60261	TC100437	AgNOD-CP1 and AsNODf32 -like cysteine proteinase
Secondary metabolism and hormone metabolism				
AJ848579	MtSN4	**MtS10215**	…	Brassinosteroid biosynthetic protein LKB (DIMINUTO/DWARF-1)
AJ848566	**MtSN4**	MtC61086	TC101963	Auxin-induced SAUR-like protein
Cell cycle				
AJ847046	MtSTW	**MtS10593**	…	Prohibitin
AJ845403	MtSNF	MtD24972	TC97683	Cell division control protein 48-like protein
Gene expression and RNA metabolism				
AJ847279	**MtSTW**	MtD19113	TC108540	Basic-helix-loop-helix protein
AJ845785	**MtSNF**	MtD19688	TC112026	bHLH transcription factor
AJ847293	MtSTW	**MtS10662**	…	bZIP transcription factor
AJ848040	**MtSN4**	**MtS10132**	…	C2H2-type zinc finger protein, ID1-like
AJ848025	**MtSN4***	MtC10582	TC95981	CCAAT-binding transcription factor, subunit B
AJ845850	MtSNF	MtC60180	TC106806	Homeobox-leucine zipper protein
AJ847301	MtSTW	**MtS10664**	…	At/Os GRF transcription activators-like
AJ848640	MtSN4	**MtS10222**	…	MADS-box transcription factor
AJ848228	MtSN4	MtD05611	TC96646	Myb transcription activator
AJ847977	**MtSN4***	MtC50408	TC109672	Ethylene response factor (ERF)
AJ848045	MtSN4	MtD04945	TC104390	Response regulator receiver domain protein
AJ847047	MtSTW	MtC10881	AW684462	VSF-1 transcription factor
AJ848145	**MtSN4**	MtD01844	TC102282	WRKY transcription factor
AJ847076	MtSTW	**MtS10602**	…	K Intron Maturase
AJ847153	MtSTW	MtD04626	TC104080	DNA-directed RNA polymerase II
AJ845895	MtSNF	MtC45563	TC101140	RNA-binding protein
AJ848529	MtSN4	**MtS10207**	…	Polypyrimidine tract binding protein (PTB)
AJ847213	MtSTW	MtC11003	TC95298	Multiple stress-responsive zinc-finger transcription factor
Signal transduction and posttranslational regulation				
AJ845532	MtSNF	MtC93004	TC100224	Extensin LRR receptor kinase
AJ845851	MtSNF	**MtS10364**	…	Putative ethylene receptor
AJ847198	**MtSTW**	MtC10513	TC102912	Serine/threonine protein kinase
AJ848389	MtSN4	MtC20065.1	TC95050	Serine/threonine protein kinase
AJ847121	MtSTW	MtD02460	TC108221	Serine/threonine protein kinase (possibly mitogen activated)
AJ848494	MtSN4	**MtS10201**	…	Protein phosphatase 2C (PP2C)
AJ848646	MtSN4	MtD03675	TC110302	WD repeat protein
AJ846988	MtSTW	MtD15550	TC103709	WD-40 repeat protein
AJ847074	MtSTW	**MtS10601**	…	Pleckstrin-homology (PH) domain protein
AJ848586	MtSN4	MtC60355	TC107257	Calmodulin-like protein
AJ847953	MtSN4	MtC60404	TC107926	Calmodulin-like protein
AJ848497	MtSN4	MtC60592	TC101807	Calmodulin-like protein 3
AJ848518	MtSN4	MtC61428	TC102961	Calmodulin-like protein 5
AJ848181	MtSN4	**MtS00125**	…	Calmodulin-like protein
AJ848757	MtSN4	**MtS00135**	…	Calmodulin-like protein
AJ848450	MtSN4	MtC60929	TC95911	Calmodulin-like protein 6
AJ848311	MtSN4	**MtS10178**	…	Calmodulin-like protein
AJ848358	MtSN4	**MtS10185**	…	Calmodulin-like protein
AJ848186	MtSN4	**MtS10156**	…	RAS-related protein
AJ847296	MtSTW	MtD01053	TC108046	RHO GDP-dissociation inhibitor 1
AJ848344	MtSN4	MtD04097	TC102589	SPX domain (G-protein binding) protein

[a] 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).

[b] MtS suppression subtractive hybridization–specific clusters are shown in bold.

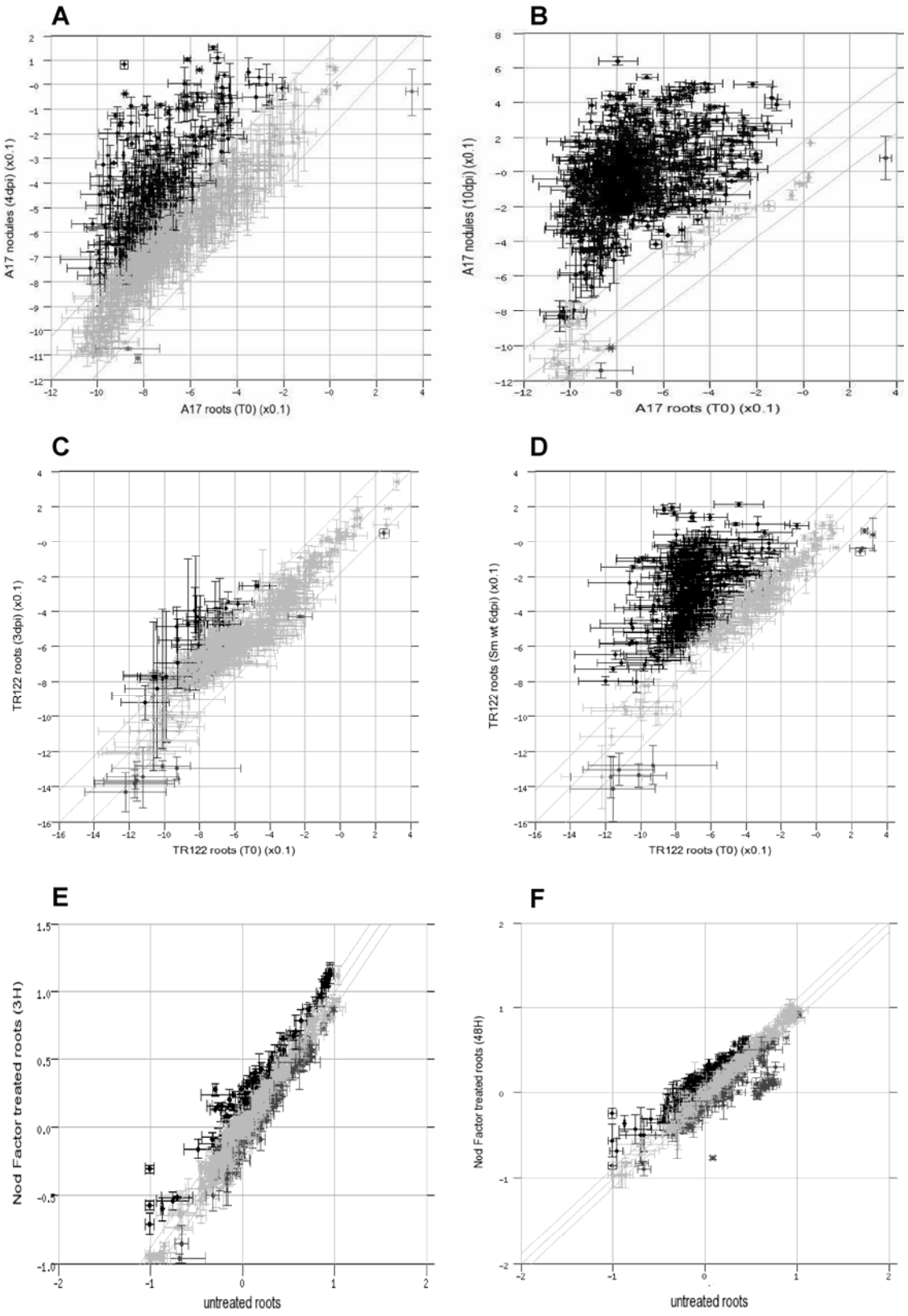


Fig. 1. Scatter plots of gene expression levels in three *Medicago truncatula* suppression subtractive hybridization (SSH) libraries: **A** and **B,** MtSN4; **C** and **D,** MtSTW; and **E** and **F,** MtSNF. In all, 768, 480, and 690 cDNA inserts from the MtSN4, MtSTW, and MtSNF library, respectively, 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 transduction. 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 clusters correspond to genes not previously characterized as upregulated during nodulation. In addition, this list includes 19 MtS clusters (36%), thus representing potential novel symbiotic 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 corresponded to four potential transcription factor genes belonging 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 exhibited a moderate but reproducible upregulation at 48 h after NF treatment is shown in Figure 2A. The induction ratios obtained for the other five selected genes derived from the MtSN4 and MtSTW libraries were examined in isolated developing 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 distinguished, revealing the diversity of symbiotic stages covered by the SSH libraries. The MtD19113 bHLH TF gene

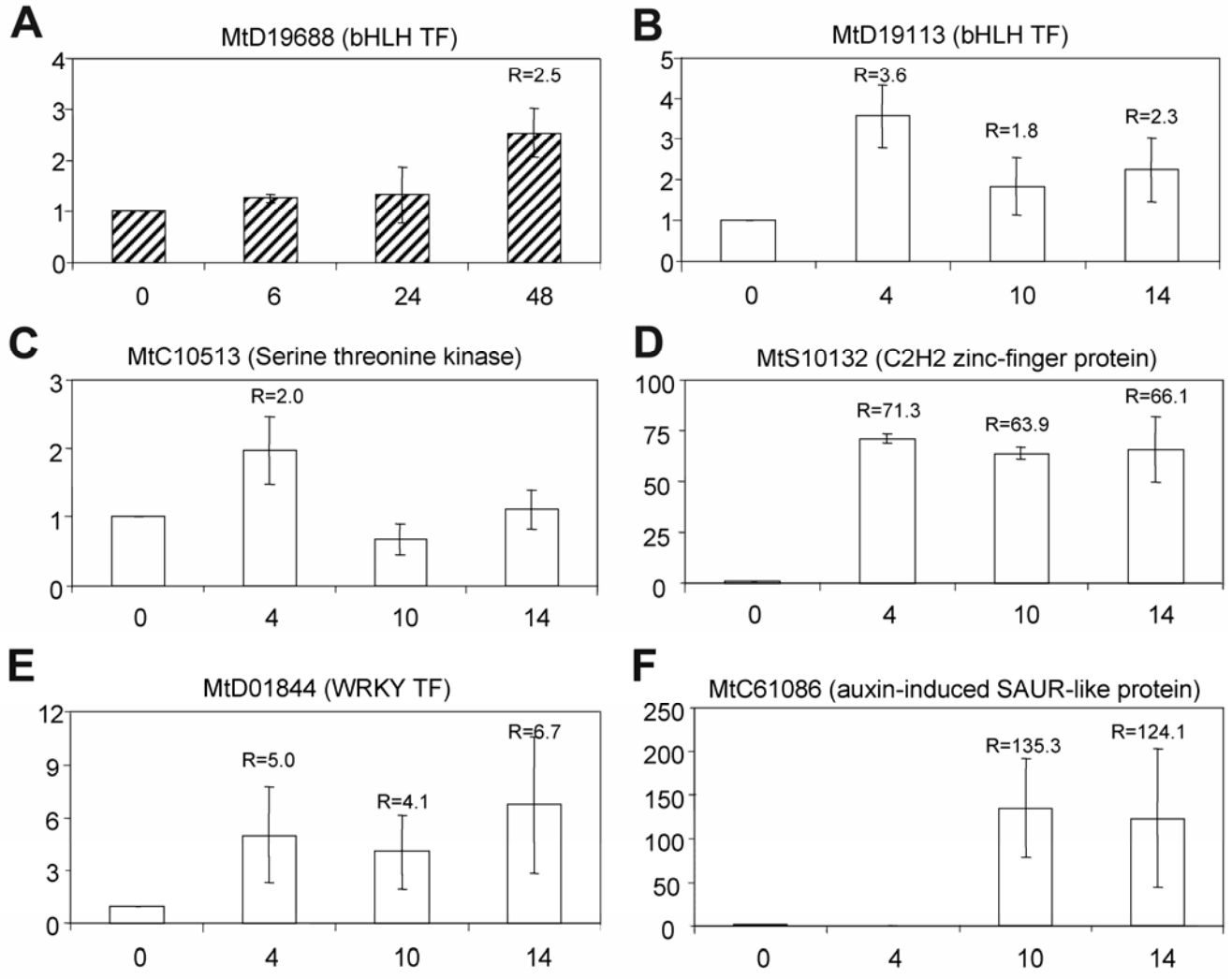
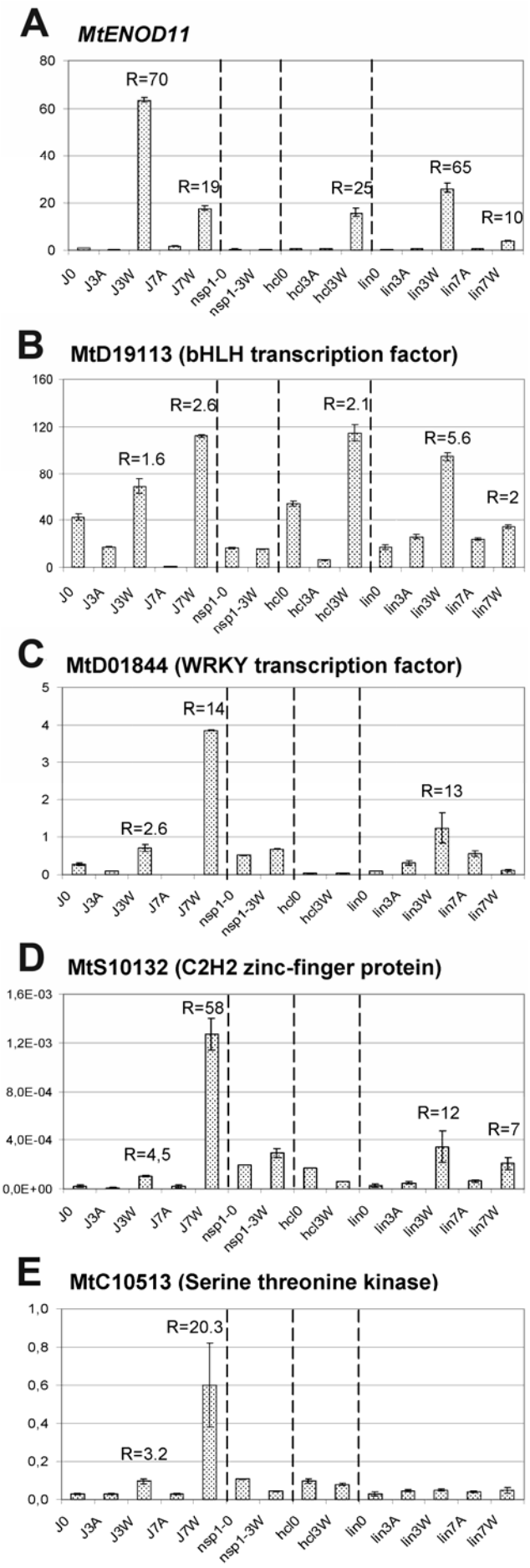


Fig. 2. Expression analysis by quantitative reverse-transcription polymerase chain reaction (qRT-PCR) of six putative regulatory genes after Nod factor treatment or during nodule development. The expression of six genes, isolated from the forward suppression subtractive hybridization libraries and predicted 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

Fig. 3. Expression analysis by quantitative reverse-transcription polymerase chain reaction of four putative regulatory genes in wild-type *Medicago truncatula* Jemalong and nodulation mutant backgrounds upon *Sinorhizobium meliloti* inoculation. The relative cDNA concentrations normalized by desmin (purified in-vitro-transcribed desmin RNA [80 pg] added to 4 µg of total RNA before cDNA synthesis) are expressed in arbitrary units on the Y axis. Expression was examined in the following root samples: wild-type Jemalong roots before inoculation (J0), after inoculation with wild-type *S. meliloti* at 3 or 7 days postinoculation (dpi) (J3W and J7W), or control non-nodulating *nodA*⁻ *S. meliloti* at 3 or 7 dpi (J3A and J7A); nsp1 roots before (nsp1-0) or 3 days after inoculation with wild-type *S. meliloti* (nsp1-3W); hcl roots before (hcl0) or 3 days after inoculation with wild-type *S. meliloti* (hcl3W) or control non-nodulating *nodA*⁻ *S. meliloti* (hcl3A); lin roots before (lin0) or 3 (lin3W) or 7 days (lin7W) after inoculation with wild-type *S. meliloti* or control *nodA*⁻ *S. meliloti* at 3 (lin3A) or 7 dpi (lin7A). R is the ratio between the expression values obtained in treated samples compared to the initial values at T0.

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 *nsp*1 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/*lin* 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 (Brechenmarcher et al. 2004; Ouziad et al. 2005; Voiblet 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 *MtN1-29* (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 (Sauviac 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 (MtS) or to previously identified genes (MtC or MtD) 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 contrasts 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, MtD19113 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). MtD19113 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 indentified by Mitra and associates (2004), MtD19113 is thus one of the few TFs known to be upregulated by *S. Meliloti* in *hcl*. Consequently, MtD19113 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 30ºC 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 supernodulating *M. truncatula sunn-2* mutant (originally TR122) (Sagan et al. 1995; Schnabel et al. 2005), and *nsp1* (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% gloze for 24 h. at 20°C, and seedlings with 1 cam of root length were grown aeroponically, as described previously (El Yahyaoui et al. 2004), for 10 days in a medium containing 10 mM NH_4NO_3. The culture medium was then replaced by a nitrogen-free medium for 4 days and the nitrogen-starved plants were subsequently inoculated by *S. meliloti* strains, also as described previously (El Yahyaoui et al. 2004). Whole root or nodule materials were then harvested. In the case of 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 NH_4NO_3, 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 –80ºC. 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 65ºC 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 65ºC), with ^{33}P-labeled probes generated by random labeling (Ready To Go; Amersham Pharmacia Biotech, Uppsala, Sweden) from known nodulin genes (early nodulin genes: *MtENOD11*, *MtENOD12, MtENOD16, MtENOD20, MtENOD40*, *RIP1,*

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 then were 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 higher than 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.

cDNA inserts 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 Biogrid robot. DNA was fixed to the membrane using UV radiation 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 (TCGAGCGGCCGCCCGGGCAGGT) or 2R (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 *M. 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 2× 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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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