Analysis of *Medicago truncatula* Nodule Expressed Sequence Tags

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Systematic sequencing of expressed sequence tags (ESTs) can give a global picture of the assembly of genes involved in the development and function of organs. Indeterminate nodules representing different stages of the developmental program are especially suited to the study of organogenesis. With the vector λ HybriZAP, a cDNA library was constructed from emerging nodules of *Medicago truncatula* induced by *Sinorhizobium meliloti*. The 5' ends of 389 cDNA clones were sequenced, then these ESTs were analyzed both by sequence homology search and by studying their expression in roots and nodules. Two hundred fifty-six ESTs exhibited significant similarities to characterized data base entries and 40 of them represented 26 nodulin genes, while 133 had no similarity to sequences with known function. Only 60 out of the 389 cDNA clones corresponded to previously submitted *M. truncatula* EST sequences. For 117 cDNAs, reverse Northern (RNA) hybridization with root and nodule RNA probes revealed enhanced expression in the nodule, 48 clones are likely to code for novel nodulins, 33 cDNAs are clones of already known nodulin genes, and 36 clones exhibit similarity to other characterized genes. Thus, systematic analysis of the EST sequences and their expression patterns is a powerful way to identify nodule-specific and nodulation-related genes.

Interaction between rhizobia and their legume host plants results in the development of nitrogen-fixing root nodules. This specialized plant organ hosts its symbiotic bacterium partners, which convert molecular nitrogen to ammonium accessible for the plant. Nodule organogenesis is controlled by molecular signaling between the plant and the bacterium. In the bacterium, host plant flavonoids induce the synthesis and excretion of the lipochitooligosaccharide Nod signals acting as host-specific morphogens capable of reprogramming differentiated root cortical cells for nodule development.

Distinct root cell layers respond differently to Nod factors. In *Medicago* spp., root hairs and outer cortical cells are involved in the infection process, forming infection threads that transport the bacteria toward the inner cortex. These latter cells are induced for division, leading to the formation of the nodule meristem. Then, cells arrested in division differentiate into various cell types, resulting in the development of the characteristic peripheral and central nodule tissues.

Medicago spp. nodules are of the indeterminate type, having a persistent meristem where, due to the continuous conversion of meristematic cells into distinct nodule cell types, all stages of nodule differentiation are represented. The central region of the indeterminate nodules is built up by three major zones: the apical nodule meristem, where cells proliferate (zone I); the invasion zone, where cells stop to divide and undergo differentiation and became infected with rhizobia (zone II); and the nitrogen fixation zone, where bacteria are converted into nitrogen-fixing bacteroids (zone III) (Schultze and Kondorosi 1998; Long 1996).

Understanding of the molecular basis of nodule development is of importance to highlight the mechanisms of symbiotic nitrogen fixation. Moreover, it may give a better insight into general features of plant organogenesis. Until now, only a few genes, characterized by enhanced expression in nodules, have been identified and their functions in nodule development are still largely unknown. So far, differential and subtractive hybridizations and displays (Gamas et al. 1996; Szczygłowski et al. 1997) as well as "cold plaque screening" (Frugier et al. 1998) have been used to isolate symbiosis-related genes. The majority of these approaches resulted in the isolation of cDNA clones of highly expressed genes.

Here we present sequence and expression analysis of 389 expressed sequence tags (ESTs) from a nodule cDNA library of *M. truncatula*, which has become a model plant for studying *Rhizobium*-legume symbiosis (Barker et al. 1990; Blondon et al. 1994; Cook et al. 1997; Hoffmann et al. 1997; Trinh et al. 1998). Systematic sequencing of *M. truncatula* ESTs has been reported so far only for a root hair-enriched cDNA library (Covitz et al. 1998). Our results show that sequencing of ESTs from a nodule cDNA library gives a global view of gene activities in the nodule, and comparison of the expression patterns of cDNAs in the root and nodule provides a powerful way to identify new nodulins or nodule-enhanced genes.

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Nucleotide and/or amino acid sequence data can be found at the EMBL data base under accession numbers AJ388667 to AJ389056.

RESULTS

Construction of an *M. truncatula* cDNA library and sequence analysis of the ESTs.

With polyA⁺ mRNA purified from young, white *M. truncatula* nodules induced by the *S. meliloti* strain 41, a cDNA library was constructed in λ HybriZAP. Primary phages (3.2×10^6) were obtained with an average insert size of about 1,300 bp and an average insert content of $\geq 95\%$. Four hundred and eight clones converted into plasmid DNA were sequenced on their 5' ends. Three hundred eighty-nine out of the 408 clones resulted in readable insert sequences. These sequences have been deposited in the EMBL data base under accession numbers AJ388667 to AJ389056.

Similarities to already characterized data base entries were found for 256 clones, while the rest (133 clones) represented novel genes (Fig. 1A). Ninety-two cDNAs from the latter, unknown category showed no homology to data base entries while 41 did so to ESTs or hypothetical genes without assigned functions from different genome projects. Comparison of the sequences of unknown clones with each other revealed that six genes were represented by two independent cDNA clones and one by a set of three, while the others are probably distinct genes.

The 256 clones with putative functions refer to 221 different genes. These clones were sorted into 12 categories as indicated in Figure 1A and listed alphabetically in Table 1. Clones

encoding the same function but representing distinct members of a gene family are presented in separate rows. Beside genes involved in primary metabolism (54 clones/51 genes), protein biosynthesis and processing (57 clones/45 genes), nodulins represented the third, most numerous category, with 40 clones of 26 genes.

In the categories of genes with putative functions, 20 genes were represented with more than one cDNA clone as indicated in Table 1. The most abundant ones were the leghemoglobin *MtLB1* (Gallusci et al. 1991) and one type of the 40S ribosomal protein S8 gene (five clones for each), while *MtLB2* was represented by four clones. The overlap of the 389 nodule ESTs with the previously published ESTs of an *M. truncatula* root hair-enriched library (Covitz et al. 1998) was restricted to about 15% of the clones (60 out of 389). Fifty-six of them possessed significant similarity to genes, particularly in the category of protein synthesis (ribosomal proteins) and primary metabolism (Fig. 1B), while four ESTs with unknown function (MtNo55, 245, 352, 423) were present in both libraries (Covitz et al. 1998). In the category of nodulins, 17 ESTs corresponded to already described nodulins from *M. truncatula* such as MtN1, 4, 5, 16, 20, 21, 22, and 27 isolated by Gamas et al. (1996) via subtractive hybridization as well as the leghemoglobins *MtLB1* and *MtLB2* (Gallusci et al. 1991).

Fig. 1. Distribution of *Medicago truncatula* EST (expressed sequence tag) clones. **A**, Functional categories based on data base similarity. Black bar: number of clones in category. White bar: number of genes represented by the clones. **B**, Overlap of the nodule EST set with the root hair-enriched EST set of *M. truncatula* cv. Jemalong. "Others" represent those functional groups represented only by 1 to 2 clones. **C**, Distribution of the nodule-enhanced ESTs according to their expression level in the nodule. Black bar: clones with putative function, other than nodulin. White bar: clones similar to known nodulins. Hatched bar: putative new nodulins.

Table 1. Clones showing similarity to already characterized data base entries

Putative gene products by category	Clone no.	Relative transcript level ^a	Relative induction ^b
Cell division cycle			
Ankyrin-like protein	174	Low	1.1
cdc 48	56	Very high	22
Hem C / Dim1p homolog	153	Low	0.8
Cell wall structure or metabolism			
Beta-xylosidase	136	ND	NA
Endo-1,4-beta-glucanase	117	ND	NA
Expansin	280	High	1.9
Extensin like protein	425	Medium	1.2
Hydroxyproline-rich glycoprotein HRGP1	22	Medium	1.0
Pectinacetylsterase	106	Very high	110
Pectinacetylsterase (putative)	252	Very high	21
Polygalacturonase precursor (pectinase)	204	Low	1.0
Proline-rich protein PRP4	401	Medium	0.3
Proline-rich protein (PRP1)	403	Very high	0.7
Proline-rich protein	96	Medium	0.2
Cytoskeleton			
Actin-like protein 2	397	Low	3.3
Adhesin protein	77	Low	1.2
Annexin	152	Low	1.2
Putative myosin heavy chain	151	Low	1.0
Tubulin beta-1-chain	367	Very high	1.8
Defense and cell rescue			
Acidic chitinase	103	Medium	7.1
Acidic chitinase precursor	159	Medium	>14
Acidic endochitinase class III	216	Low	1.6
Ascorbate peroxidase	156	Very high	7.1
Cadmium induced protein	139	Medium	>5.6
Cationic peroxidase 2	73	Medium	0.6
Cyanase	71	Low	0.8
Ferritin	358	Medium	7.4
Isoflavonoid reductase	44	Very high	2.7
Metallothionein	295	High	1.6
Peroxidase ATP 20a	246	Low	0.8
Phenylalanine ammonia-lyase	371	Low	0.7
RAD6	188	NM	NA
Superoxide dismutase	213	Low	<0.4
Wound-induced protein	250	Low	1.4
Zeta-crystallin oxidoreductase	314	Low	>1.6
Gene expression, chromatin organisation			
ALY transcriptional activator	141	Low	<1.2
ATP dependent RNA helicase	281 / 292	Medium / Low	0.7 / <1.2
bZIP-like protein	125	ND	NA
DNA topoisomerase III	181	Low	>7.5
Glycine-rich RNA binding protein	51	High	0.6
Histone H1	327	NM	NA
Histone H4	391	Low	1.2
PcMYB1 protein	329	Low	2.1
PcMYB1 protein	394	Medium	7.0
Poly (A) binding protein	165	Low	1.0
Poly(ADP-ribose) polymerase	268	Low	>1.9
Polyadenylate binding protein 2	186	Low	1.6
Putative poly(A)-binding protein	43	Very high	6.8
RING zinc finger protein	76	Low	1.3
RNA polymerase I common subunit	296	High	9.0
RSZp22 / 21 splicing factor	349	Medium	0.8
Scarecrow-like protein	307	Low	>0.9
SnRNP core protein	270	ND	NA
SPF1-like DNA-binding protein	161	Low	<1.0
WRKY3 DNA binding protein	332	Very high	97
Zinc finger protein like	426	Low	2.2
Membrane transport, intracellular trafficking			
ATPase	283	Low	1.4
Chloroplast outer envelope protein	178	Very high	1.1

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^a Relative transcript levels were classified on the basis of median value of hybridization signals obtained for all clones. NM = not measured due to masking effect of neighboring signals. ND = not detected.

^b Nodules per root. Calculated as the normalized ratio of transcript levels observed in nodule and in root. NA = the ratio cannot be calculated (lack of detection either in the root or both in the root and nodule).

Table 1. (continued from preceding page)

Putative gene products by category	Clone no.	Relative transcript level ^a	Relative induction ^b
Copper transporter protein	240	NM	NA
Deep orange / PEP3 protein	63	ND	NA
Kinesin heavy chain isolog	324	Very high	16
Low affinity sulphate transporter	129	NM	NA
MEM3 / vacuolar transport protein	66	Low	>1.5
Membrane channel protein	143	Medium	0.8
MRP-like ABC transporter	259	Low	>2.1
Putative ammonium transporter	415	Low	0.9
SDF2 secretory protein	57	Low	>2.9
Sec12p like protein	128	Very high	32
SEC61 transport protein	111 / 145 / 396	Medium	1.4 / 1.5 / 1.5
Small GTP-binding protein (RAB1)	404	Low	>2.1
Small GTP-binding protein SAR1	378	Low	1.1
Small GTP Binding protein SAR1A	134	Low	1.8
Vacuolar protein sorting homolog, vps45	218	ND	NA
Nodulins			
Early nodulin (N-75)	229	High	>43
Early nodulin protein ENOD2	288	Very high	>110
ENOD40	309	High	1.9
ENOD93	120	Low	>1.8
Leghemoglobin 1	113 / 130 / 225 / 232 / 269	Very high	>510 / >2100 / 170 / 190 / 260
Leghemoglobin 2	20 / 107 / 140 / 354	Very high	68 / >670 / >360 / 230
Leghemoglobin 3	215 / 228	NM / Very high	NA / 12
Leghemoglobin 4	108 / 264 / 298	Very high	>380 / >150 / >130
Leghemoglobin 5	146 / 168	Very high	>930 / >300
Leghemoglobin 6	224	High	>27
Leginsulin, MtN16	248	Medium	0.7
MtN1 gene	237	ND	NA
MtN4 gene	410	Medium	3.6
MtN5 gene	210	Low	>1.7
MtN20	74	Low	>9.0
MtN21	62	ND	NA
MtN22	24	Very high	>950
MtN27	279	Medium	>9.2
nms22	304	High	>350
nms22 / GRP5 related	388 / 422	High	69 / 62
Nodulin 25 like	94	Medium	>12
Nodulin / GRP5 related	376	Medium	8.8
Nodulin 25 precursor	154 / 272	Very high	>150 / 110
Nodulin 26	418	Medium	12
Nodulin 8	81 / 127	Very high	120 / >160
Soybean mRNA for late nodulin	408	Low	>4.8
Primary metabolism			
3-Deoxy-D-arabino-heptulosonate-7-phosphate synthase	320	Low	1.7
3-Hydroxy-3-methylglutaryl-coenzyme A	116	Low	1.7
Acetoacetyl-CoA-thiolase	375	Medium	1.2
Aconitate hydratase	88	ND	NA
Acyl carrier protein	330	Low	>1.0
Adenine nucleotide translocator	301	Medium	0.6
Adenosine kinase	346	Low	0.8
Adenylate kinase	417	Medium	1.8
Alpha amylase precursor	231	Low	<1.0
Aminolevulinic acid dehydratase	183	Low	1.0
Arabinosidase	39	ND	NA
Asparagine synthetase	37	High	3.8
ATP synthase beta chain	299	Medium	0.8
ATP synthase delta chain	389	Medium	1.5
Carbonic anhydrase	131 / 336	Very high / Medium	45 / 6.8
Chloroplast localized GSII	29	Low	1.6
Choline kinase	61	Low	2.1
Cysteine synthase	267 / 395	Low	1.3 / 1.5
Cysteine synthase	421	Low	1.0
CytC reductase hinge prot. Subunit	35	Low	0.9
Cytochrome b5	201	Low	0.4
Cytosolic fructose-1,6-bis-phosphate aldolase	65	Medium	0.7
Dihydrofolate reductase	180	Low	0.5
Enolase	60	Medium	0.8
Enolase	414	ND	NA

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Leghemoglobins.

Among nodulins, leghemoglobins were represented by 17 cDNA clones. Multiple alignment of these clones formed six distinct groups, indicating that at least six different leghemoglobin genes (LB1–LB6) were expressed in young nodules of *M. truncatula*. All leghemoglobin cDNA clones contained a 280-bp-long sequence coding for amino acid residues from positions 47 to 139 (LKDSAG . . . AYDALA) in the MtLB1 protein. The phylogram of the leghemoglobins shown in Figure 2 was based on this region. Group 1 corresponds to the MtLB1 and group 2 to the MtLB2 (Gallusci et al. 1991), while the other four groups have not been reported yet in *M. truncatula*. Group 3 was the one most similar to the *M. sativa* le-

ghemoglobin 1 (Barker et al. 1988) and groups 4 and 5 to the *M. sativa* leghemoglobin 4 (Löbner and Hirsch 1992), while group 6 (MtNo224) showed the highest similarity to the *Vicia faba* VLB29 at protein level (Frühling et al. 1997).

Expression of ESTs in roots and nodules.

cDNA inserts of the 389 clones were excised from plasmid DNAs with *EcoRI* and *XhoI* digestion and separated by agarose gel electrophoresis. For reverse Northern (RNA) hybridizations two sets of filters were prepared: one was hybridized with nodule-derived and the other with root-derived ³²P-labeled, first-strand cDNAs synthesized from nodule and root total RNA, respectively. As an example for signals on reverse

Table 1. (continued from preceding page)

Putative gene products by category	Clone no.	Relative transcript level ^a	Relative induction ^b
Glyoxalase II isozyme	409	Low	2.0
Inorganic pyrophosphatase	177	Low	0.8
Inosine-5'-monophosphate dehydrogenase	70	Low	>4.4
Kinetoplast methionine synthase	58	Medium	0.4
Lipoxygenase	115	Medium	2.0
Lysophosphatidic acid acyltransferase	95	Very high	64
Malate dehydrogenase	97	Medium	1.8
Malate dehydrogenase precursor	72	Low	1.0
N-carbamoyl-D-amino acid amidohydrolase	172	Low	0.7
NADH dehydrogenase	234	Low	1.0
NADH-ubiquinone oxidoreductase	101	Low	1.6
Nucleoside diphosphate kinase	353	Medium	1.2
Nucleoside triphosphatase	313	Medium	2.0
Phospholipid glutathione peroxidase	289	Very high	10
Pyrophospho-fructose-6-phosphate 1-phosphotransferase	278	Low	1.2
Prolyl-4-hydroxylase alpha subunit	197	Medium	1.8
Pyridoxine kinase	258	Low	1.4
S-adenosyl-methionine decarboxylase	90	Low	1.2
S-adenosyl-methionine synthetase	135	High	2.0
S-adenosyl-methionine synthetase	363	High	3.0
Sucrose synthase	257 / 369	Medium	1.3 / 0.8
Thiazole biosynthetic enzyme	203	Low	1.0
Trehalose-6-phosphate phosphatase	79	Low	>3.3
Triosephosphate isomerase	233	NM	NA
UDP-glucuronyltransferase	321	High	1.6
Uridyl transferase	392	Low	2.1
Protein synthesis and processing			
26S Protease regulatory subunit 4	100	Medium	1.7
Cysteine proteinase	99 / 393	High	>130 / 33
Cysteine proteinase inhib. (cystatin)	387	Low	0.9
Elongation factor 1-alpha	41, 222	Medium	1.5 / 0.6
Initiation factor 2-alpha	137	Low	1.6
Initiation factor 3-p40	42	ND	NA
Initiation factor 5A-4	337	Low	0.4
Initiation factor 4A-11	331	Low	1.0
Serine carboxypeptidase isolog	207	Low	>1.0
Ubiquitin/CEP52	338	Low	0.4
Ubiquitin	416	Medium	0.5
Ubiquitin carrier protein E2	149	Low	1.1
Ubiquitin conjugating enzyme E2	413	Very high	0.8
40S ribosomal protein S2	40	Low	1.1
40S ribosomal protein S2, putative	377	High	3.7
40S ribosomal protein S3B	290	NM	NA
40S ribosomal protein S8	105 / 132 / 293 / 328 / 339	Low	0.8 / <0.8 / NA / >1.9 / 0.5
40S ribosomal protein S8	236	Low	0.5
40S ribosomal protein S13	98	Low	0.9
40S ribosomal protein S14	32	Medium	0.6
40S ribosomal protein S15	196	Low	1.1
40S ribosomal protein S19	23 / 374	Medium / Low	0.8 / 0.7
40S ribosomal protein S20	274	Low	<1.0
40S ribosomal protein S26, putative	53 / 370	Very high	NA / 0.7

(continued on next page)

Northern, hybridization of 16 clones (from MtNo295 to MtNo310) with root and nodule cDNA probes is shown in Figure 3. The expression level of the nodulin genes leghemoglobin 4 and *nms22* in the nodules was high as well as that of the MtNo302 and, at a lesser extent, MtNo297, which might be putative new nodulins. The RNA polymerase I common subunit transcript also exhibited a high accumulation level in the nodules. The other clones did not show significant change in their transcript levels; most of them appeared to be constitutive while expression of the AMP-activated protein kinase was at the background level. The vector DNA also gave a nonspecific background signal with the probes that were found even in those samples in which no hybridization was detected with the cDNA insert. This result indicates that separation of the vector and the insert DNA might be essential in the expression stud-

ies, since nonspecific signals by the vector DNA may mask the weak specific signals or distort their quantification.

The hybridization signal intensities were quantified by STORM with the ImageQuant software. They were regarded as "detected" if the average value of the hybridization signal of a cDNA fragment was at least 10% higher than the local background. ESTs were sorted in four categories based on their "relative transcript levels" in the nodule. This was calculated by comparing the expression of each EST with the median value of all detected signals: 50% of the ESTs possessing weaker signals than the median value were classified in the "low" expression category and the others in the "medium," "high," and "very high" categories when the signal intensities were one to fivefold, five- to 25-fold, or >25-fold higher, respectively, than the median value.

Table 1. (continued from preceding page)

Putative gene products by category	Clone no.	Relative transcript level ^a	Relative induction ^b
40S ribosomal protein S28	126	Medium	1.4
40S ribosomal protein S29	28	Medium	1.2
40S ribosomal protein S30	300	Medium	1.5
60S ribosomal protein L5	82	Low	0.9
60S ribosomal protein L6	54 / 158 / 261	Not det. / NA / Low	NA / NA / 0.9
60S ribosomal protein L6 / L41	266	Medium	1.0
60S ribosomal protein L9	310	Medium	0.9
60S ribosomal protein L14	198	Low	<0.3
60S ribosomal protein L10	85	Medium	1.6
60S ribosomal protein L10A	241	Very high	9.8
60S ribosomal protein L10A	382	Low	0.9
60S ribosomal protein L12	192	Low	0.6
60S ribosomal protein L13a	38	Medium	0.7
60S ribosomal protein L15	253 / 306	Low / Medium	0.6 / 0.5
60S ribosomal protein L18	308	Medium	0.3
60S ribosomal protein L18A, putative	326	Very high	24
60S ribosomal protein L29	348	Medium	1.0
60S ribosomal protein L32	112	Medium	0.8
60S ribosomal protein L35	114	Low	0.8
60S ribosomal protein L37B	119	Low	<0.4
Ribosome-associated protein p40	138 / 171	Low	>1.0 / 0.8
Secondary metabolism			
Cytochrome P450	184	Low	1.3
Cytochrome P450 monooxygenase	160 / 194	ND / Low	NA / 1.0
Tropinone reductase	187	Low	1.0
Signal transduction			
14-3-3 brain protein homolog	179 / 219	Medium	0.7 / 0.7
AMP activated protein kinase beta	303	ND	NA
Calmodulin	344	Medium	8.0
Calmodulin-binding protein	243	Very high	7.8
Calreticulin	199	Low	0.6
GTPase-activating protein	176	ND	NA
MAP kinase	157	Very high	26
NPK1-related protein kinase	316	Low	>3.0
protein kinase homolog	214	Low	>0.9
Putative receptor protein kinase	399	Low	2.3
Putative serine/ threonine kinase	400	Low	>5.1
Ras-GTPase-activating protein SH3	368	Low	1.6
Serine/threonine kinase	249	Low	>1.8
Small GTP-binding protein	282	Low	1.2
Miscellaneous			
A3 protein	277	Low	0.9
Axi1 homolog	155	ND	NA
Cucumber basic protein	287	High	0.5
GA and darkness activated protein	383	Low	1.3
Leginsulin	142 / 420	High	3.2 / 0.4
Membrane component B-box protein	80	Low	1.1
Oxysterol-binding protein	170	Low	0.7
Patatin	317	Low	0.8
USF protein homolog	238	Low	<0.5

For estimation of nodule/root signal ratios hybridization of the cDNA fragments was evaluated by quantification of both the hybridizing bands (object volumes) and the peak values of the signals. The latter was used to avoid errors in the first measurement due to distorted running of DNA fragments. Ratios of both the nodule/root “object volumes” and “object peak” values were calculated and their mean served for the quantification of the relative induction levels. In the case of 53 cDNAs, no hybridization signal was detected with the root-derived probe, indicating that either the corresponding genes were not expressed in roots or their transcript level was below detection. In these cases, the counts detected on the blot at the expected band position were used to calculate the relative enhancement of the transcript level. Differences in the strengths of the probes were filtered out by selecting 37 cDNA clones of constitutively expressed genes including genes of various ribosomal proteins, translation elongation factors, ubiquitins, as well as *Msc27* (Crespi et al. 1994) used frequently as constitutive controls for *Medicago* spp. root and nodule RNA, which served as internal controls for normalizing the signal intensities on filters.

By inspecting the phosphorimager data and the traditional X-ray exposures, we estimated that a two- to 2.5-fold difference in signal intensity between root and nodule hybridizations revealed significant differences in transcript levels. To be on the safe side and to avoid overestimation of stimulated expression, clones were regarded as “nodule enhanced” only if the ratio of nodule/root signals was ≥ 3 . Based on this criterion, 117 out of the 389 clones showed increased transcript levels in nodules, compared with roots. The relative changes

in transcript levels for all known clones are shown in Table 1, while putative novel nodulins are listed in Table 2. As shown in Figure 1C, in all four transcript level categories nodule-enhanced cDNA clones were found.

Thirty-three out of the 40 known nodulin or nodulin-homolog cDNA clones showed the expected, nodule-enhanced expression pattern. However, the *MtN1* and *MtN21* transcripts (Gamas et al. 1996) were not detectable by reverse Northern and the expression of the *MtN16*/leginsulin homologue clone even showed a slight decrease. The increases in transcript levels for the *enod40* (Crespi et al. 1994), *enod93* (Kouchi and Hata 1993), and *MtN5* early nodulin genes were below the threefold arbitrary limit. Several-hundred-fold relative increases were observed for transcript levels of most leghemoglobin genes, *MtN22* (Gamas et al. 1996), and an *nms22*-like clone (Ganter et al. 1998).

Thirty-six clones showing similarity to genes encoding known function also showed nodule-enhanced expression. In agreement with a previous report (Coba de la Pena et al. 1997), carbonic anhydrase exhibited strong activation in the nodule. A similar increase in transcript level was observed for pectinacetylsterases, cysteine proteinases, the WRKY3 pathogenesis-induced DNA-binding protein, and the lysophosphatidic acid acyltransferase. Several-fold increase was observed for transcripts of defense-related proteins like chitinases, ascorbate peroxidase, ferritin, and a cadmium-induced protein. The transcript levels were also elevated for DNA topoisomerase III, a poly-A binding protein and RNA polymerase. Eightfold higher mRNA levels were detected for calmodulin and calmodulin-binding protein genes. Remarkable increases were also observed for certain protein kinases, indicating that signaling pathway components might be transcriptionally activated in the nodule.

In the category of unknown genes, nodule-enhanced expression was observed for 48 clones (Table 2). However, based on their sequence comparisons these clones may represent at most 42 different transcripts since one gene was represented by three clones while four genes were represented by two clones. Three clones exhibited more than hundred-fold in-

Fig. 2. A phylogram of *Medicago truncatula* nodule expressed sequence tags (ESTs) coding for leghemoglobin.

Fig. 3. Comparison of transcript levels of EST (expressed sequence tag) clones MtNo295–MtNo310 in roots and nodules by autoradiography of reverse Northern (RNA) blots.

creases in the transcript level and another five showed a more than 50-fold increase.

DISCUSSION

Here we show that systematic sequencing of cDNA clones prepared from young indeterminate nodules, in combination with reverse Northern experiments, is an efficient way to identify new nodulin genes. Compared with previous methods based on various screening approaches, random sequencing of ESTs better reflects the global gene activities involved in nodule development and function. This study demonstrated that 30% of the ESTs exhibited nodule-enhanced expression, and resulted in the identification of many potential nodulin genes.

The remarkably high ratio of the nodule-enhanced ESTs versus all clones is not surprising in light of the altered cellular structure and physiology of the nodule, compared with root cells (Brewin 1998). For instance, the infection process necessitates the formation of the infection threads, thereby synthesis of new cell wall and matrix components, as well as restructuring

of the plant cell cytoskeleton. Proline-rich proteins, which are the major components of the infection thread matrix, were also identified as early nodulins (*enod12*, *enod10*, *enod11*). Moreover, nodule cells produce large amounts of membrane materials for the formation of peribacteroid membrane required to envelop the rhizobia when they are released into the cytoplasm of the host cells. The surface of the peribacteroid/symbiosome membrane can exceed that of the plasma membrane by 20- to 50-fold and it also determines the type and flux of metabolites exchanged between the nitrogen-fixing bacteroids and the plant cell, integrating thereby the bacteroid and plant carbon and nitrogen metabolism. The low oxygen tension is essential for symbiotic nitrogen fixation and the nodule structure provides the microaerobic environment where the oxygen-carrier leghemoglobin represents the most abundant nodulin. Nodule organogenesis also necessitates the activation and differential regulation of the cell cycle at different stages of nodule development.

The cDNA library used in this study was made of young, emerging nodules collected before visible accumulation of leghemoglobin. In the category of known genes, 11 cDNA encoded functions are likely implicated in cell wall or cell wall matrix synthesis, five in cytoskeleton structure, 17 in membrane transport and intracellular trafficking, and 21 in gene expression and chromatin structure. Another 45 cDNAs correspond to genes playing a role in protein synthesis and processing while 51 cDNAs may be involved in primary metabolism and 16 in plant defense and cell rescue. Twenty-six cDNAs showed homology to known nodulin genes, most of them belonging to the group of early nodulins, indicating that in the library the late nodulin genes were not overrepresented. So far only a few cDNAs represent cell cycle genes while a relatively high number of ESTs code for functions that might be implicated in signal transduction pathways. Thus, this relatively small-scale analysis of ESTs appears to be consistent with the expected nodule functions.

The overlap of these nodule ESTs with the ESTs derived from a root hair-enriched cDNA library of *M. truncatula* (Covitz et al. 1998) is restricted to 15% (60 clones). The majority of these clones code for proteins involved in protein biosynthesis (mainly ribosomal proteins) or in the primary metabolism, which are of universal importance regardless of the cell type. The function of the remaining 4 common clones is unknown. The proportion of the overlapping clones with unknown function (4/133) is about sevenfold lower than that of the overlapping clones with putative functions (56/256). This indicates that sequencing of EST libraries representing distinct organs, tissues, or cell types may reflect drastic and characteristic differences in gene activities.

In reverse Northern hybridizations, the expression profiles of ESTs with the root and nodule probes revealed elevated transcript levels in the nodules for several clones in each category of the known genes, including primary metabolism and protein synthesis. Due to basal levels of expression, the housekeeping genes exhibiting stimulated activity in the nodules could have been lost via subtractive approaches.

The majority of clones classified as nodulins on the basis of sequence similarities showed elevated expression in nodules, indicating that the reverse Northern hybridization is reliable for the identification of new nodulin genes. However, demonstration of the involvement of these putative nodulins in dif-

Table 2. List of putative nodulin expressed sequence tag (EST) clones

Clone name	Expression	Relative induction	Insert length ^a
MtNo 68	Very high	>242.7	0.63
MtNo 123	Very high	>187.0	0.5
MtNo 356	Very high	183.7	1.77
MtNo 302 / 335 / 263	High	>78 / 30 / >28	0.48 / 0.49 / 1.22
MtNo 312	High	>71.1	0.64
MtNo 366	Medium	>69.7	0.86
MtNo 164	High	>61.9	0.56
MtNo 102 / 297	High, Medium	>56 / >16	0.44 / 0.48
MtNo 52	Very high	34.3	0.88
MtNo 166	Very high	33.5	0.69
MtNo 59	Medium	>33.5	0.54
MtNo 21	Very high	32.3	0.2
MtNo 318 / 133	High	>31 / >29	0.34 / 0.45
MtNo 93	High	>26.3	0.44
MtNo 239	Medium	>26.3	0.41
MtNo 333	Very high	23.4	1.0
MtNo 217	Medium	>23.2	NM
MtNo 212	High	21.4	0.41
MtNo 87	Very high	19.9	0.4
MtNo 276	Very high	19.6	0.32
MtNo 92	High	16.9	NM
MtNo 262	Medium	>16.3	0.7
MtNo 347	Medium	>14.3	0.5
MtNo 193	Medium	>13.4	NM
MtNo 286	Very high	13.4	0.54
MtNo 169 / 372	Very high	13 / 3.6	NM / NM
MtNo 230	Medium	11.3	0.49
MtNo 49 / 208	Medium	>8.8 / 5.5	0.75, 0.74
MtNo 390	Medium	8.2	0.4
MtNo 407	High	7.3	0.49
MtNo 150	Medium	7.0	0.56
MtNo 385	Low	>6.6	4.31
MtNo 273	Very high	6.1	0.65
MtNo 91	High	5.5	0.62
MtNo 89	Medium	5.2	NM
MtNo 360	Low	5.1	1.05
MtNo 118	High	4.7	1
MtNo 144	Low	>4.7	1.25
MtNo 361	Low	4.0	1.32
MtNo 294	Medium	3.4	NM
MtNo 124	Low	>3.3	NM
MtNo 381	Low	>3.0	0.37

^a NM = not measured.

ferent phases of nodule development needs more detailed expression studies by focusing on individual genes of interest. Leghemoglobins, identified previously as late nodulins, have been described recently as one of the earliest Nod factor-induced nodulins during nodule organogenesis (Heidstra et al. 1997); therefore, their presence in the young nodule library is not surprising. It was, however, unexpected that they represented six types of leghemoglobin genes and four of them were identified for the first time from *M. truncatula*. The majority of nodulin genes isolated by conventional screening techniques belong to the highly transcribed category. In this study, the putative new nodulin genes were expressed at different levels (from very low to very high), which is another strong argument for the usefulness of the EST transcript profile analysis providing a more comprehensive and reliable way for the isolation of novel nodulins. Our study on the nodule ESTs resulted in the identification of only a fraction of the changes that may occur in gene activities during nodule organogenesis; however, it provided an important glimpse into the likely complexity of gene expression during nodule development.

MATERIALS AND METHODS

Construction of the cDNA library.

The cDNA library was constructed in the phage λ HybriZAP with the synthesis and cloning kit of Stratagene (La Jolla, CA). Seedlings of *M. truncatula* line R108 were grown in aeroponic tanks and inoculated with the wild-type *S. meliloti* strain Rm41 as described by Hoffmann et al. (1997). Poly-A⁺ mRNA was purified from young root nodules 4 to 8 days after *Rhizobium* infection. The cDNA synthesis of 1.6 μ g of poly-A⁺ mRNA was primed by oligo-(dT)-XhoI adapter primer with MMLV-reverse transcriptase while the second strand was synthesized via polymerase I–ribonuclease H coincubation. EcoRI adapter was added to the blunted, double-stranded cDNA, followed by XhoI digestion. cDNAs longer than 400 bp were directionally cloned in EcoRI-XhoI digested λ HybriZAP phage vector. The library was excised in vivo according to the manufacturer's instructions and pADGal4 phagemid carrying individual bacterial colonies was obtained.

Sequencing and analysis of the EST clones.

Plasmid DNA minipreps of 408 randomly picked bacterial colonies were made by a modified alkaline lysis method (Feliciello and Chinali 1993). Single run sequencing on the 5' ends of each cDNA clone was performed on an Applied Biosystems 373A sequencer (Applied Biosystems, Foster City, CA) with the BigDyeTerminator kit and AcGal3SE primer (5' ATT CGA TGA TGA AGA TAC CCC 3') located in the Gal4 activator domain sequence of the phagemid upstream of the EcoRI cloning site. Fifty-five clones were also sequenced on the 3' ends with standard T7 primer (5' GTA ATA CGA CTC ACT ATA GGG C 3').

Classification of the clones was based on their similarity to data base entries. In general, 450 to 500 nucleotides following the EcoRI adapter sequence were taken for data base comparisons with the BlastN program for nucleic acids and the BlastX program for proteins at the NCBI web server (Altschul et al. 1997). Default parameters of the programs were used in both cases. Usually, similarities having random probabilities higher

than 10^{-7} were considered to be significant. Comparisons and alignments of the EST sequences were performed with the GCG program package (Genetics Computer Group, Madison, WI).

Reverse Northern hybridization.

Plasmid DNA (0.5 to 1 μ g) of each clone was digested with EcoRI and XhoI restriction enzymes. For preparation of two sets of filters, equal volumes from each digestion were subjected to electrophoresis on two identical 1% TBE (Tris-borate-EDTA) agarose gels. Gels were transferred via alkaline downward blotting to BioTrans (ICN, Costa Mesa, CA) nylon membranes (Chomczynski 1992) and immobilized via UV crosslinking according to the manufacturer's instructions.

Radiolabeled cDNA probes were synthesized by reverse transcription of 15 μ g of total RNA for 1 h in the presence of 50 μ Ci α [³²P]-dCTP with the Superscript reverse transcriptase kit (Gibco-BRL/Life Technologies, Gaithersburg, MD) as described by Charon et al. (1997). The total RNA samples from roots and nodules were isolated 21 days after the inoculation of the plants with the Nod⁻ mutant *S. meliloti* strain ZB138 (Kondorosi et al. 1984) and the wild-type strain Rm41, respectively. To complete cDNA synthesis, reactions were prolonged for 45 min with 1 mM cold dCTP. Unincorporated dNTPs were removed by purification with a Sephadex G-50 column and labeling efficiency was measured by scintillation counting. The filters were hybridized, one set with each probe, in 0.5 M Na-phosphate pH7.2, 7% SDS (sodium dodecyl sulfate) buffer (Church and Gilbert 1984) for 36 h at 65°C, then washed for 30 min each with 2 $\times$ SSC (1 $\times$ SSC: 0.15 M NaCl, 0.015 M sodium citrate) / 0.1% SDS and 1 $\times$ SSC / 0.1% SDS. Hybridization signals were observed first on Kodak X-ray films and then quantified on a STORM phosphorimager (Molecular Dynamics, Sunnyvale, CA) after 2 to 20 h of exposure.

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