Expressed Sequence Tags from Roots and Nodule Primordia of *Lotus japonicus* Infected with *Mesorhizobium loti*

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Messenger RNA from young *Lotus japonicus* roots carrying root nodule primordia appearing after inoculation with *Mesorhizobium loti* bacteria were used to construct a cDNA expression library. Single-pass sequencing employing colony-polymerase chain reaction (PCR) and analysis of PCR products established a total of 2,397 new expressed sequence tags (ESTs). We have putatively identified 1,236 known and 484 hypothetical proteins coded by the corresponding mRNAs. The remaining cDNAs are unknown (316) or redundant overlapping cDNAs (361). We hope that this batch of ESTs will assist in the recognition of plant genes involved during development of nitrogen-fixing root nodules.

Additional keywords: legumes, root nodule development.

Legume research employs several model species, one of which is *Lotus japonicus* (Handberg and Stougaard 1992; Szczygłowski et al. 1997). *L. japonicus* is diploid, has a small genome and a short generation time, and represents the determinate root nodule type. In addition, *L. japonicus* has advantageous properties in tissue culture and is a capable recipient of *Agrobacterium tumefaciens*-mediated DNA transfer and transposon tagging (Quaedvlieg et al. 1998; Schauser et al. 1998; Wegel et al. 1998). These procedures have yielded mutations that led to the identification of a key regulatory gene in root nodule inception, *Nin* (Schauser et al. 1999). Efforts in genome mapping using molecular markers and sequence polymorphism in different populations (N. N. Sandal, J. Stougaard, *personal communication*), and chemical mutagenesis programs for analyses of root nodule development or arbuscular mycorrhiza (Imazumi-Anraku et al. 2000; Senoo et al., 2000) are underway. With these approaches, map-based cloning may identify new genes. Differential display cDNAs (Szczygłowski et al. 1997) and analysis of expressed sequence tag (EST)–cDNA sequences from libraries of different organs and development stages, employing micro array or DNA-chip technology, will suggest other genes of importance. In order to contribute to such a project, we have begun sequencing cDNAs from *L. japonicus* *Mesorhizobium loti*-infected roots and root nodule primordia (Ljirnpst cDNA library).

RESULTS AND DISCUSSION

We report the single-pass sequencing of 3,684 cDNAs from *L. japonicus*, of which 2,397 sequences were inserted into six National Center for Biotechnology Information (NCBI) GenBank batch submission forms. These sequences went through a NCBI GenBank BlastN or BlastX procedure for identification of the gene or the protein. Whenever possible, the sequences were annotated before submission. GenBank accession numbers for six batches of ESTs from the Ljirnpst cDNA library are: AI967297 to AI967914 plus AI967920 to AI967923, AW163869 to AW164183, AW428653 to AW428932, BE122430 to BE122644, BF177456 to BF177921, and BG661984 to BG662483. The first three batches (1,221 ESTs) were used for blast searches in the non-redundant (NR) nucleotide sequence database (BlastN: nucleotide sequence homology at the level of genes and mRNAs, and BlastX: homology at the level of nucleotide sequences translated into protein primary structure). After the release of large numbers of ESTs from soybean, *Medicago truncatula*, and *L. japonicus* in early 2000, systematic BlastN searches in the NCBI/dbest database were performed on all sequences in the last three batches to pinpoint cDNAs/mRNAs specific for our library and confirm legume sequences not present in nonlegume databases. We observe that, of the identified sequences among these 1,176 ESTs, 95% hit other plant EST primary targets, 81% hit legume EST primary targets, and approximately 25% of the tags correspond to other *L. japonicus* ESTs (Asamizu et al. 2000; Endo et al., 2000; (M. K. Udvardi, *unpublished data*).

Summaries of search results available online focus on putative protein identifications in alphabetical order. The primary blast hits of BlastN NR, BlastX NR, and BlastN dbest searches that seem to give specific identification are entered in columns. When this was not possible, the “unknown” tag was given. Also, we show the approximate cDNA size and putative cDNA variant number, possibly reflecting gene polymorphism. Direct links to NCBI from this site will aid further identification in the future.

Next, we classified the putatively identified proteins according to their presumed function in a variety of cellular processes (Table 1). From the 2,397 ESTs submitted, we have identified 1,236 different nonredundant cDNAs, which identify a similar number of apparently different proteins. These hit all imaginable classes of protein function, ranging from DNA and RNA metabolism to transcription factors, mRNA translation, and cellular protein compartmentalization. Also, various enzymes appearing in metabolic processes and signal transduc-

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tion processes were identified, as well as proteins that may play roles in stress or infection. We note a high number of different Cab (light-harvesting chlorophyll a/b binding protein) ESTs, together with other photomorphogenesis-related mRNAs, in the library from nonphotosynthetic tissues. Interestingly, we find only two different ESTs, derived from ribulose 1,5-bisphosphate carboxylase-oxygenase (RUBISCO) *rbcS* mRNAs ubiquitous in aerial parts of light-grown plants. Also, several ESTs for thioredoxin, glutaredoxin, and cytochrome P450 monooxygenases were isolated, reflecting a high demand for electron flow and oxidoreductive processes. We observe a number of diverse Myb-like proteins, ubiquitous factors in the cellular transcription machinery. Finally, a group of ESTs represent "other" proteins, to which we cannot assign clear roles in a plant context (e.g., calcineurin, presenilin, separation anxiety protein, synaptobrevin-like, and others). In general, this distribution pattern is also reflected in other *L. japonicus* EST data collections (Asamizu et al. 2000; Endo et al. 2000).

We have isolated 10 cDNAs established as specific for early root nodule development (e.g., those encoding the nod factor-binding lectin-nucleotide phosphohydrolase) (Roberts et al.

1999), eNOD40 (Flemetakis et al. 2000; Yang et al. 1993), eNOD8, eNOD18 (Hohnjec et al., 2000), and the previously mentioned NIN regulatory protein. Interestingly, leghemoglobin cDNAs were absent, although homologs of a few other "late" nodulins were sequenced: NOD21, NOD26 [aquaporin, membrane intrinsic protein (MIP)], SAT1 (Kaiser et al. 1998), annexin (De Carvalho Niebel et al. 1998), and the unknown protein MTD1 (Curioni et al. 2000).

The fact that we dealt with very early root nodule differentiation events somewhat diluted by mRNA populations of overall root tissues may explain why we have isolated only a few ESTs corresponding to mRNAs that already have been implicated in root-nodule events. However, a large proportion of gene products probably will be found to be root-nodule enhanced or root-nodule specific when analyzed in greater detail. Examples of these could be calmodulins (Webb et al. 2000) and other signal transduction proteins such as G-proteins (Pingret et al. 1998), protein phosphatases (Kapranov et al.

Table 1. Protein classes identified from expressed sequence tag collection^a

Classes	Number found
80S ribosomal proteins	90
70S ribosomal proteins	4
Translation factors	38
Chaperones, assembly	34
Protein sorting, secretion, endocytosis	23
Membrane assembly and function	19
Plasma membrane and cell wall	26
Transporters and channels	58
Nuclear matrix	28
Filament and cytoskeleton structure and function	31
RNA-binding	24
DNA-binding	15
RNA- and DNA-metabolism	19
Hormonal regulation, infection and stress	117
Growth regulators and signal transduction	16
Transcription complex proteins and factors	53
Cell cycle and cell division	9
GTP binding and GTPases	25
GTPase regulators	8
Protein kinases and receptors	58
Kinase regulators	16
Unspecified regulators	11
Protein phosphatase related	10
N-metabolism	4
Amino acid -metabolism	39
S-metabolism	3
Nucleotide-metabolism	8
Lipo-compound metabolism	33
Carbon- and carbohydrate-metabolism	51
Energy-conversion and coupling, ATPases	28
Electron transport, Redox proteins	42
Vitamin metabolism	13
Synthesis of secondary metabolites	49
Embryogenesis and Seeds	15
Photomorphogenesis and Photosynthesis	22
Nodule Development and Function	10
Flower Development	3
Metal binding	11
Apoptosis*	3
Ubiquitin-complex and protein-metabolism	71
Others	100
Putatively identified proteins	1,236
Hypothetical proteins	484
Unknown	316
Redundant ESTs, multiply covering mRNAs	361
Total	2,397

^a Table information is presented in greater detail online.

Table 2. Examples of probable multigene families^{a, b}

Protein	Number of tags
Actin	5
Amino acid permease/carrier	6
Ankyrin	4
Annexin	4
Aquaporin	18
Ascorbic acid peroxidase	5
Arf1 small GTP-binding	7
Calmodulin	6
Calreticulin	4
Cytochrome P450/monooxyg.	16
Dormancy associated protein	4
DnaJ-type chaperone	11
Elongation factor 1a	6
Endo 1,4-beta-D-glucanase	6
Epoxide Hydrolase	4
Expansin	4
14-3-3 protein	10
Glutaredoxin	4
Glutathione S-transferase	8
Glycine rich RNA-binding	4
Histone deacetylase	4
Heat Shock Protein 70/71	7
Heat Shock Protein 80	3
Isoflavone Reductase	4
Major Latex Protein	6
Late Embryogenesis Related	5
Light Harvesting Chl a/b (Cab)-PS2	11
Lipoxygenase	9
Lysophospholipase	3
Methionine synthase	6
Metallothionein	3
Myb factor	10
NAD dehydrogenase	4
Peroxidase	16
Protein phosphatase 2c	4
Profilin	2
Proline rich (HRP, extensin)	16
Serine amino transferase	2
Serpins	2
Subtilisin-like	4
Sugar transporter	5
Thiol Protease	10
Thioredoxin	4
Alpha-tubulin	3
Beta-tubulin	3
Ubiquitin	10
Ubiquitin conjugating enzyme	12

^a Number of nonoverlapping expressed sequence tags (ESTs) coding for the specified protein are shown. Some of the protein species of these families are represented by overlapping ESTs.

^b Table information is presented in greater detail online.

1999), and phospholipase C and phosphatidyl inositol-4-phosphate-5-kinase (PIP) (den Hartog et al. 2001). We also sequenced cDNAs for a large number of protein kinase-like proteins. Protein kinases participate in the regulation of metabolism and in both signal perception and the funneling of signals into developmental pathways; therefore, they should be closely investigated. ESTs for the SAR1, Arf1 SMG-proteins and an LjRab5A GTPase (Borg et al. 1997), together with many other ESTs possibly involved in vesicle trafficking, may also be of importance during rhizobial invasion and symbiosome formation. Many of the ESTs in the classes of hormone regulation, infection, stress, and environmental response may show up during root nodule development.

A total of 484 unique ESTs are homologous to hypothetical proteins, encoded primarily in the *A. thaliana* genome sequence (Arabidopsis Genome Initiative 2000). For another 316 unique ESTs, we did not identify homologs in the NR or dbest databases. It appears that this group of cDNAs contain very short poly(A+)-proximal sequences or longer sequences that cover 5' noncoding information. Much larger sets of ESTs, or extensive genome sequence information from *L. japonicus*, are necessary for identification or verification of these ESTs. The classes of hypothetical and, in particular, the unknown mRNAs should be investigated with respect to root nodule development.

The last group of cDNAs consists of 361 redundant ESTs, that overlap and have partially identical sequences when compared to the remaining 2,036 ESTs. It is clear that the sequences in this group are derived from independent cDNA synthesis and cloning events. We have not established, per se, how many different mRNAs this group encompasses. It appears that the ESTs here stem from mRNAs that are abundant in the cells of the tissue used for RNA isolation (e.g., mRNAs for the aquaporin-like proteins, extensins [proline/hydroxyproline-rich proteins], the RD22-like dehydration responsive protein, major latex-like [ripening-related] proteins, late embryogenesis abundant [LEA-like] proteins, ubiquitin, the ubiquitin conjugating enzyme, and finally, some ribosomal protein mRNAs are represented by multiple ESTs).

Interestingly, the redundant cDNAs overlap the list of mRNAs and gene products expressed from putative multigene families. Table 2 presents the number of EST classes for some putatively polymorphic proteins, which may reflect gene multiplication. Complete sequencing of all these cDNAs and a full sequence alignment study will resolve this. We have not compared the putative multigene family members at the level of protein primary structures, although the overlapping of protein sequences derived from nonaligning mRNAs would sustain the suggested gene diversity. To aid such procedures, it is also likely that the Kazusa DNA Research Institute *L. japonicus* database, encompassing approximately 30,000 ESTs, can be used for assembly of tentative consensus (TC) sequences (Quackenbush et al. 2001), clusters of ESTs derived from specific gene transcripts.

We observe that mRNAs for aquaporins, extensins, peroxidases, lipoxygenases, thiol proteases, ubiquitins, and ubiquitin-conjugating enzymes contribute the largest number of variant cDNAs. This probably reflects the fact that cells in the organs and tissues used for library construction demand both transcript variation and the presence of particular mRNAs in high amounts, a consequence of local requirements for a given protein species. This can be further studied by creating TCs, described above, to be used for blast analyses of tissue- and organ-specific EST libraries, resulting in electronic Northern blots (Quackenbush et al. 2001). These may give hints about gene expression patterns based on relative EST abundance. However, we believe that the number of *L. japonicus* ESTs

and libraries is not yet sufficient for such an exercise and, further, may rapidly be overtaken by results of microarray and DNA-chip analyses.

In this research, we have only scratched the surface of gene complexity in *L. japonicus*. However, in a study combining and comparing *L. japonicus* data with data from other legumes, from *Arabidopsis thaliana*, and from other nonleguminous plant resources found on NCBI or The Institute for Genomic Research websites, the genetic ore that contributes to formation of the important legume symbiotic organ will be more fully revealed.

MATERIALS AND METHODS

Messenger RNA was prepared from seedling roots infected with *Mesorhizobium loti* culture during seed germination. The roots were harvested between 5 and 12 days after inoculation (Borg et al. 1999; Jorgensen et al. 1999). Synthesis of cDNA was primed with *Xho*I site-tagged oligodT, and cDNAs were 5' tagged with 5'-GAATTCGGCACGAG-3' (*Eco*RI). Tagged cDNAs were inserted in the Stratagene λ HybriZAP vector. The library was manipulated in *Escherichia coli* XL1-Blue MRF' and in *E. coli* XL0LR cells, as described in the HybriZAP manual (Stratagene, La Jolla, CA, U.S.A.). Ampicillin-resistant colonies from 0.7-ng library plasmid DNA, pAD-GAL4 recombinant plasmids obtained after HybriZap mass-excision and transformed into *E. coli* XL1-Blue MRF', were transferred onto 45 Luria-Bertani-Amp plates in a microtiter plate (MTP) replica format. Colony-polymerase chain reaction (PCR) employed the 21-mer T7 sequencing primer 5'-GTAATACGACTCACTATAGGG-3' and the 26-mer GAL4AD primer 5'-CCA-CTACAATGGATGATGTATATAAC-3'. Thermocycling was initiated at 94°C for 4 min, followed by 35 cycles at 94, 50, and 72°C for 1, 1, and 2 min, respectively, and terminated at 72°C for 7 min. PCR fragments were purified by passage through Sephadryl S-400. Amplicons obtained using the GAL4AD primer and the ThermoSequenase sequencing reagents (Amersham Biosciences UK, Little Chalfont, Buckinghamshire, U.K.) were sequenced using ABI310 and ABI377 Genetic Analyzers (PerkinElmer Inc., Boston). MTP-format liquid cultures were transferred to 45 sealed 96-well microtiter plates, and pools of 10- μ l aliquots of single colony cultures mixed in cryotubes were stored at -80°C. Corresponding digital sequence files were edited on Power Macintosh computers employing Sequencher 2.0.1 and MacDNAsis 3.5 software to eliminate reading errors, bad sequences, untrustworthy sequence segments, and duplicated cDNAs.

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AUTHOR RECOMMENDED INTERNET RESOURCES

The Institute for Genomic Research TIGR database: <http://www.tigr.org/tdb/index.shtml>

Kazusa DNA Research Institute Department of Plant Gene Research *L. japonicus* database: <http://www.kazusa.or.jp/en/plant/lotus/EST/>

National Center for Biotechnology Information GenBank: <http://www.ncbi.nih.gov>

Carsten Poulsen search result summaries and Table 2 data found at <http://www.mbio.aau.dk/~chp/ABZlist.html>

Carsten Poulsen Table 1 presented in greater detail at <http://www.mbio.aau.dk/~chp/Table1.html>.