Combined Transcriptome Profiling Reveals a Novel Family of Arbuscular Mycorrhizal-Specific *Medicago truncatula* Lectin Genes

André Frenzel,¹ Katja Manthey,² Andreas M. Perlick,² Folker Meyer,³ Alfred Pühler,² Helge Küster,² and Franziska Krajinski¹

¹Lehrgebiet Molekulargenetik, Universität Hannover, Herrenhäuser Str. 2, D-30419 Hannover, Germany; ²Lehrstuhl für Genetik, Universität Bielefeld, Postfach 100131, D-33501 Bielefeld, Germany; ³BRF, Center for Biotechnology (CeBiTec), Universität Bielefeld, D-33594 Bielefeld, Germany

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The large majority of plants are capable of undergoing a tight symbiosis with arbuscular mycorrhizal (AM) fungi. During this symbiosis, highly specialized new structures called arbuscules are formed within the host cells, indicating that, during interaction with AM fungi, plants express AM-specific genetic programs. Despite increasing efforts, the number of genes known to be induced in the AM symbiosis is still low. In order to identify novel AM-induced genes which have not been listed before, 5,646 expressed sequence tags (ESTs) were generated from two *Medicago truncatula* cDNA libraries: a random cDNA library (MtAmp) and a suppression subtractive hybridization (SSH) library (MtGim), the latter being designed to enhance the cloning of mycorrhiza-upregulated genes. In silico expression analysis was applied to identify those tentative consensus sequences (TCs) of The Institute for Genomic Research *M. truncatula* gene index (MtGI) that are composed exclusively of ESTs deriving from the MtGim or MtAmp library, but not from any other cDNA library of the MtGI. This search revealed 115 MtAmp- or MtGim-specific TCs. For the majority of these TCs with sequence similarities to plant genes, the AM-specific expression was verified by quantitative reverse-transcription polymerase chain reaction. Annotation of the novel genes induced in mycorrhizal roots suggested their involvement in different transport as well as signaling processes and revealed a novel family of AM-specific lectin genes. The expression of reporter gene fusions in transgenic roots revealed an arbuscule-related expression of two members of the lectin gene family, indicating a role for AM-specific lectins during arbuscule formation or functioning.

Additional keywords: eNorthern, expression profiling.

More than 80% of all terrestrial plants, including legumes, are capable of forming a tight symbiosis with fungi of the phylum *Glomeromycota* (Schüssler et al. 2001). This symbiosis with arbuscular mycorrhizal (AM) fungi is of great economic importance, because an established AM symbiosis leads to better mineral nutrition of the host plants (Harrison 1999). Furthermore, plants harboring an AM symbiosis often show

higher levels of resistance against abiotic stress (Augé 2001) and bioprotection against pathogen attacks (Cordier et al. 1996; Newsham et al. 1995; Slezacek et al. 2000). In recent years, notable research efforts were carried out in order to advance our insights into the molecular genetics of AM symbiosis. One main aspect in this field of research focused on the analysis of differential gene expression in host plants after colonization by different *Glomus* spp. microsymbionts (Franken and Requena 2001). In legumes, recent studies on differential gene expression during AM symbiosis centered on the two genetic models *Medicago truncatula* (Barker et al. 1990) and *Lotus japonicus* (Handberg and Stougaard 1992), where genome sequences currently are being established (VandenBosch and Stacey 2003). Legumes have the unique capacity to form a second important root endosymbiosis, the legume–*Rhizobium* spp. symbiosis leading to symbiotic nitrogen fixation; therefore, comparative profiling of gene expression during AM formation and nodulation was initiated (Küster et al. 2004, Manthey et al. 2004) in addition to the profiling of gene expression exclusively under AM conditions (Liu et al. 2003). These high-throughput expression profiling experiments significantly advanced our knowledge of genes upregulated during AM formation. Prior to the identification of mycorrhiza-induced genes by transcriptome profiling, only a limited number of genes were identified as being upregulated or specifically expressed in mycorrhizal roots (e.g., encoding a plasma membrane ATPase [Krajinski et al. 2002], a glutathione-S-transferase [Wulf et al. 2003], a chitinase [Salzer et al. 2000], and mycorrhiza-specific phosphate transporters (Harrison et al. 2002; Paszkowski et al. 2002; Rausch et al. 2001)).

In recent years, high-throughput DNA sequencing technologies significantly transformed molecular genetics approaches in legumes and, once whole genome sequences are available, the complete gene inventory of these plants can be studied concerning gene regulation under different symbiotic conditions. A genome sequencing project for *M. truncatula* is underway (VandenBosch and Stacey 2003); however, thus far, the complete genome sequence is not available. Nevertheless, high-throughput expressed sequence tag (EST) sequencing projects identified approximately 190,000 *M. truncatula* ESTs from more than 40 different cDNA libraries which can serve as a source of information for functional genomics approaches. One of the applications of such EST collections is the identification of genes whose expression differs between the tissue sources from which the libraries were derived (Bortoluzzi and Danielli 1999; Stekel et al. 2000). Using such in silico strate-

H. Küster and F. Krajinski contributed equally to this work.

Corresponding author: F. Krajinski; Telephone: +49-511-762 5548; Fax: +49-511-762 4088; E-mail: krajinski@lgm.uni-hannover.de

gies, several genes predicted to be up- or downregulated during AM symbiosis were identified in EST populations of two different cDNA libraries originating from AM tissues (Journet et al. 2002). A similar strategy was adopted to identify a notable number of nodule-specific tentative consensus sequences (TCs), which seemed to represent genes solely expressed in root nodules during the symbiosis of legumes with rhizobia (Fedorova et al. 2002). Based on a subset of the *M. truncatula* and *L. japonicus* sequences available in EST databases, microarrays were constructed which now enable experimental expression studies of several thousand genes in parallel (Colebatch et al. 2002; El Yahyaoui et al. in press; Küster et al. 2004).

Because a significant number of plant genes show a very tight regulation with respect to the timing and the localization of expression, they tend to be underrepresented in random cDNA libraries and, hence, are difficult to detect on the basis of cDNA-based microarray hybridizations. Thus, it can be assumed that more sophisticated strategies are useful to detect as many differentially expressed genes as possible in a particular tissue. Suppressive subtraction hybridization (SSH) enables the construction of cDNA libraries enriched for sequences upregulated under certain conditions (Diatchenko et al. 1996); therefore, we have, in addition to constructing a random cDNA library, focused our analysis on an SSH-cDNA library from AM roots. That way, we aimed to identify novel, so-far uncharacterized *M. truncatula* genes that are expressed specifically during AM. We report here on the identification of novel genes that previously were not known to be upregulated in *M. truncatula* arbuscular mycorrhiza. Apart from genes connected to different transport as well as signaling processes, a novel family of AM-specific lectin genes was identified. We were able to show that AM-specific lectin sequences did not match previously described *M. truncatula* lectins involved in root-nodule symbiosis, and reporter gene fusions revealed an arbuscule-specific expression of two members of this gene family, indicating a role for AM-specific lectins during arbuscule formation or functioning.

RESULTS

Generation of EST clusters from two *M. truncatula*–*Glomus intraradices* AM cDNA libraries.

Two different cDNA libraries were constructed and used for EST sequencing. The first one, designated MtAmp, was generated from *M. truncatula* roots 5 weeks after inoculation with *Glomus intraradices*. At this time point, all developmental stages of an AM fungus (arbuscules, inter- and intracellular hyphae, appressoria, and vesicles) were present. Mycorrhiza parameters (Trouvelot et al. 1986) were as follows: frequency of infection (F) = 30%, mycorrhization intensity of the whole root system (M) = 16%, mycorrhization intensity within infected root areas (m) = 58%, arbuscule frequency within infected root areas (a) = 63%, and arbuscule frequency of the whole root

system (A) = 10%. From this library, a total of 3,805 ESTs was generated, with an average length of 479 bp. All ESTs with a length of at least 100 bp were submitted to the EMBL database under accession numbers AJ500944 to AJ504393. The second library, MtGim, was generated by SSH and 1,841 ESTs with an average length of 416 bp were obtained. An initial characterization of 34 cDNAs of the MtGim library revealed the presence of different mycorrhiza-induced genes in this library (Wulf et al. 2003); however, so far, the remaining cDNAs have not been characterized in detail. All MtGim ESTs that were at least 50 bp in length were submitted to the EMBL database and can be accessed under AJ499169 to AJ500943.

Clustering of the MtAmp ESTs revealed 534 TCs and 1,857 singletons, whereas clustering of MtGim ESTs resulted in the identification of 297 TCs and 611 singletons. Of the 534 MtAmp TCs, only 4.9% were novel compared with all other libraries in The Institute for Genomic Research (TIGR) *M. truncatula* gene index (MtGI). In contrast, a notably higher number of the 297 MtGim TCs encoded novel genes, because 34.7% of the MtGim TCs did not match any other TIGR MtGI TC. To analyze all TCs derived from both libraries in a single step, all 5,646 MtGim and MtAmp ESTs were clustered to one population of 833 TCs and 2,349 singletons. Our clustering protocol was identical to that used by TIGR (Quackenbush et al. 2000); therefore, in order to ease cross-referencing of our data, we used TIGR MtGI TC numbers as references for our MtAmp and MtGim TCs.

In silico screening for novel AM-specific transcripts.

In silico expression analysis was applied to identify those TCs that were composed of ESTs exclusively deriving from the MtGim or MtAmp libraries, but not from any other cDNA library of the TIGR MtGI. Therefore, we compared MtGim and MtAmp EST clusters via a BLAST (Altschul et al. 1997) homology search against the TIGR (Liang et al. 2000) sequences. Those of our clusters that hit only sequences of the MtAmp or MtGim library but not of any other library of this database were assumed to represent novel, not previously listed, mycorrhiza-specific genes of *M. truncatula*. This search revealed 115 MtAmp- or MtGim-specific TCs. These TCs, likely to represent novel mycorrhiza-specific or at least mycorrhiza-related genes, were annotated and grouped into functional categories according to Journet and associates (2002). After BLASTX searches, 10 TCs showed the highest homologies to fungal genes, indicating that these might represent *G. intraradices* genes (Table 1). A large group of 52 TC sequences did not reveal homologies to any other sequence after blastx and also did not contain significant matches to the PROSITE database (Table 2). To obtain first information about the genomic origin of these sequences, the available *M. truncatula* bacterial artificial chromosome (BAC) sequences were searched using blastn. This confirmed an *M. truncatula* origin for seven of these sequences.

Table 1. MtGim- or MtAmp-specific tentative consensus sequences (TCs) with highest homologies to sequences of fungal origin^a

MtGI-TC	bp	Annotation	GenBank entry	E value	N _{MtAmp}	N _{MtGim}
TC64085	559	mRNA for histone H4 (<i>Agaricus bisporus</i>)	X94189	7e-32	...	4/4
TC68248	362	Isolate RWM_243 28S ribosomal RNA (<i>Glomus mosseae</i>)	AF396793	e-173	...	5/5
TC68246	561	Isolate RWC_658 28S ribosomal RNA (<i>G. caledonium</i>)	AF396794	e-115	...	45/45
TC75488	641	Strain DAOM212595 small subunit ribosomal RNA gene (<i>G. mosseae</i>)	U96143	9e-41	...	2/2
TC67625	669	Cytochrome P-450 cyp509A1 (<i>Cunninghamella elegans</i>)	Q9P493	5e-41	...	2/2
TC59129	487	Glo8 isolate M3 small subunit ribosomal RNA gene (<i>Glomus</i> sp.)	AY129636	0.0	...	2/2
TC75848	458	mRNA for hypothetical protein (<i>G. mosseae</i>)	AJ242917	0.0	...	2/2
TC74547	717	Histone H3 (<i>Mortierella alpina</i>)	Q9HDN1	6e-67	1/2	1/2
TC68247	390	Isolate SI_141 28S ribosomal RNA (<i>G. intraradices</i>)	AF396797	e-177	...	11/11
TC75784	596	Vesicular transport protein sec18 homolog (<i>Schizosaccharomyces pombe</i>)	T50122	1e-12	...	2/2

^a MtGI = *Medicago truncatula* gene index; N = number of expressed sequence tags in a library.

The remaining group of 53 TCs displayed significant homologies to plant genes (Table 3). Of these, 20 were homologous to proteins of unknown function of other plants. Thus, these proteins probably encode *M. truncatula* proteins; however, predictions of their putative functions are not possible. Finally, 33 TCs revealed the highest homologies to plant genes with assigned functions. Of those, five encoded putative transporter proteins, four encoded lectin-like proteins, and six gene products involved in signal transduction.

RNA accumulation studies.

All 33 TCs with significant similarities to plant genes were selected for experimental expression profiling using quantitative real-time reverse-transcription polymerase chain reaction (RT-PCR). For this approach, *M. truncatula* plants were inoculated with *G. intraradices*. Two independent sets of plants were grown and used as biological replicates in RNA accumu-

lation analyses. Mycorrhiza parameters of this plant sets were as follows: for set 1, $F = 100\%$, $M = 34.73\%$, $m = 34.73\%$, $a = 64.42\%$, and $A = 23.42\%$; and for set 2, $F = 77.78\%$; $M = 40.56\%$, $m = 52.14\%$, $a = 61.92\%$, and $A = 25.11\%$.

The *MtEf-1 α* gene encoding the constitutively expressed translation elongation factor 1 α was chosen for normalization. Specific primer pairs were designed for each gene in a way that selected primer sequences were compared with the TIGR MtGI to extensively verify their specificity. All primers first were used to amplify genomic DNA of *M. truncatula*. Amplification products were obtained for all genes except TC 60540 and TC 67079 (data not shown), proving that at least the remaining 31 genes originated from *M. truncatula* and not from the microsymbiont. Prior to RNA-accumulation analyses, all RNA preparations were checked for DNA contaminations by PCR using primer combinations that span intron sequences of known gene sequences (data not shown).

Table 2. MtGim- or MtAmp-specific tentative consensus sequences (TCs) without significant homologies after blastx search^a

MTGI-TC	bp	N_{MtAmp}	N_{MtGim}	Result of blastn search of <i>Medicago truncatula</i> BAC sequences	E value
TC63455	921	1/5	4/5	No hit	...
TC71585	649	1/5	4/5	No hit	...
TC71978	460	...	5/5	No hit	...
TC72388	610	1/4	3/4	No hit	...
TC72546	296	...	4/4	No hit	...
TC71536	575	...	5/5	No hit	...
TC72266	343	...	4/4	No hit	...
TC74813	280	...	2/2	Clone mth2-36h10	0.15
TC64951	675	3/3	...	No hit	...
TC73661	617	1/3	2/3	No hit	...
TC73656	615	...	3/3	No hit	...
TC64827	350	...	3/3	No hit	...
TC65527	202	...	3/3	No hit	...
TC73785	147	...	3/3	No hit	...
TC73242	387	...	3/3	No hit	...
TC64807	632	...	3/3	No hit	...
TC64793	611	1/3	2/3	No hit	...
TC74270	300	...	2/2	No hit	...
TC65745	463	...	2/2	No hit	...
TC67236	385	...	2/2	No hit	...
TC74867	221	...	2/2	No hit	...
TC66251	251	...	2/2	No hit	...
TC67552	434	...	2/2	No hit	...
TC67547	611	...	2/2	No hit	...
TC67067	371	...	2/2	No hit	...
TC67791	408	...	2/2	No hit	...
TC65735	411	...	2/2	No hit	...
TC75233	517	2/2	...	Clone mth2-12h1	0.79
TC67349	377	...	2/2	No hit	...
TC75302	497	...	2/2	Clone mth2-19c18	0.048
TC66690	570	...	2/2	Clone mth2-1413	0.00
TC65891	633	...	2/2	No hit	...
TC75970	428	...	2/2	No hit	...
TC74472	655	...	2/2	No hit	...
TC74247	460	...	2/2	No hit	...
TC67260	586	...	2/2	No hit	...
TC75969	802	...	2/2	No hit	...
TC74463	296	...	2/2	No hit	...
TC74186	487	1/2	1/2	No hit	...
TC65823	493	...	2/2	Clone mth2-11n13	0.19
TC73954	304	...	2/3	Clone mth2-27m3	0.00
TC67344	582	...	2/2	No hit	...
TC65995	541	...	2/2	No hit	...
TC74126	418	...	2/2	No hit	...
TC75555	594	...	2/2	No hit	...
TC67674	631	...	2/2	Clonemth2-175h23	0.00
TC65854	374	...	2/2	No hit	...
TC70769	667	...	7/7	No hit	...
TC75097	612	1/2	1/2	No hit	...
TC75784	428	2/2	...	No hit	...
TC66878	601	...	2/2	No hit	...
TC75914	368	...	2/2	No hit	...

^a MtGI = *Medicago truncatula* gene index; N = number of expressed sequence tags in a library.

MtEf-1α was found to be the most abundant gene showing a constant expression in control and mycorrhizal roots. After RT-PCR, melting curves were analyzed, and in all reactions, single amplification products were present. Gene induction levels calculated on the basis of real-time RT-PCR are shown in Table 4. Of the 33 TCs analyzed for AM-induced expression, 18 were

verified to be at least threefold induced in both samples of mycorrhizal roots. As expected, the AM-specific phosphate transporter gene *MtPt4* was more than 10⁴-fold induced in both sets of mycorrhizal roots. This corresponds well to the detection of 53 *MtPt4* ESTs in the MtGim library, in contrast to only 6 *MtPt4* ESTs identified in the random cDNA libraries MtBC

Table 3. MtGim- or MtAmp-specific tentative consensus sequences (TCs) with homologies to plant sequences^a

MtGI-TC	bp	Annotation ^b	GenBank entry	E value	N _{MtAmp}	N _{MtGim}
Signal transduction						
TC64428	494	NBS-LRR type disease resistance protein (<i>Phaseolus vulgaris</i>)	Q93W58	3e-17	...	3/3
TC66505	491	Lectin-like protein kinase (<i>Arabidopsis thaliana</i>)	Q9FG33	9e-42	...	2/2
TC74096	615	Receptor like protein (<i>A. thaliana</i>)	T49121	6e-56	2/2	...
TC74325	1,046	Seven transmembrane protein MLO2 (<i>Oryza sativa</i>)	Q93XC8	e-123	1/2	1/2
TC76060	625	Serine protein kinase-like (<i>A. thaliana</i>)	T47560	6e-20	...	2/2
TC71483	1,236	Protein kinase (<i>A. thaliana</i>)	G96593	2e-74	...	5/5
Metabolism						
TC65799	909	Dihydrodipicolinate synthase (<i>Glycine max</i>)	S50750	2e-94	1/2	1/2
TC67209	591	Phosphoenolpyruvate carboxylase (<i>A. thaliana</i>)	Q9CA39	4e-76	...	2/2
TC74500	206	β-Galactosidase (Lactase) (<i>Fragaria ananassa</i>)	Q93X57	3e-19	...	2/2
TC67079	555	Epoxide hydrolase homolog (<i>G. max</i>)	Q39856	2e-72	...	2/2
TC76132	438	Blue-copper-binding protein (<i>O. sativa</i>)	AAO37971	1e-21	...	2/2
TC60541	960	Germin-like protein (<i>Medicago truncatula</i>)	AAO32795	1e-72	...	4/4
TC63954	473	Germin-like protein (<i>A. thaliana</i>)	Q9FMA8	1e-32	...	4/4
Post-transcriptional^c						
TC68426	519	Serin carboxypeptidase-like protein (<i>A. thaliana</i>)	T49188	7e-43	...	2/2
TC66580	596	Cysteine proteinase precursor (<i>Astragalus sinicus</i>)	Q9FS02	7e-71	...	2/2
TC75569	526	Prolyl endopeptidase (<i>Arabidopsis thaliana</i>)	Q8RXQ7	1e-55	...	2/2
TC71486	643	Phosphoprotein phosphatase (<i>M. sativa</i>)	T09544	1e-86	...	4/5
TC60540	665	Ribonuclease T2 (<i>Cicer arietinum</i>)	Q9ZQX1	2e-13	...	2/2
Cell structure, extracellular^d						
TC68166	1,373	Agglutinin II precursor (<i>C. lutea</i>)	Q39529	4e-54	...	29/29
TC69333	1,033	Bark lectin II precursor (<i>Sophora japonica</i>)	P93536	5e-49	...	6/6
TC75254	606	Agglutinin II precursor (<i>C. lutea</i>)	Q39529	3e-23	...	2/2
TC75360	599	Lectin (<i>S. japonica</i>)	P93536	8e-37	...	2/2
Transport						
TC70909	672	MDR-like ABC transporter (<i>O. sativa</i>)	Q8GU68	7e-46	...	7/7
TC63901	950	Copper transporter (<i>A. thaliana</i>)	AAM67571	1e-17	...	4/4
TC66155	242	Zn transporter ZNT4 (<i>Thlaspi caerulescens</i>)	Q9FPW7	4e-15	...	2/2
TC73332	405	Ammonium transporter (<i>O. sativa</i>)	BAC65232	1e-12	...	3/3
TC65476	552	Sugar transporter (<i>A. thaliana</i>)	AAM78192	1e-65	...	3/3
Unclassified, function unkn^e						
TC72502	439	Nodulin-like protein (<i>A. thaliana</i>)	Q9FHJ9	1e-18	...	4/4
TC67410	536	RING zinc finger protein (<i>A. thaliana</i>)	Q9M8K4	3e-21	2/2	...
TC76092	337	Myotubularin (<i>A. thaliana</i>)	Q9CAF1	3e-20	2/2	...
TC59827	722	Cytokinin-specific binding protein (<i>Vigna radiata</i>)	Q9ZWP8	1e-31	1/6	5/6
TC64306	963	Vesicle-associated membrane protein (<i>A. thaliana</i>)	O23429	e-109	1/3	2/3
TC67060	708	Mitochondrial inner membrane protein (<i>A. thaliana</i>)	Q9S724	1e-52	2/2	...
Hypothetical, unkn proteins^e						
TC71232	497	Hypothetical protein (<i>O. sativa</i>)	AAP03413	2e-43	...	6/6
TC59539	958	Hypothetical protein (<i>O. sativa</i>)	AAO39879	6e-36	...	7/7
TC63326	715	Hypothetical protein OSJNB0016H12.7 (<i>O. sativa</i>)	AAP03413	7e-66	...	5/5
TC63653	614	Hypothetical protein (<i>O. sativa</i>)	Q8H7L0	3e-32	...	4/4
TC73773	539	Hypothetical protein (<i>Plasmodium falciparum</i>)	Q8IEM0	4e-15	...	3/3
TC64871	646	Hypothetical protein (<i>A. thaliana</i>)	Q8LCS0	9e-35	3/3	...
TC73566	658	Hypothetical protein F12B17.70 (<i>A. thaliana</i>)	F12B17.70	3e-75	3/3	...
TC64610	298	Hypothetical protein (<i>A. thaliana</i>)	T20K12.120	6e-11	...	3/3
TC73173	672	Hypothetical protein [imported] (<i>A. thaliana</i>)	At2g34680	8e-56	...	3/3
TC64788	515	OSJNB0014K08.26 protein (<i>O. sativa</i>)	Q8LIY8	2e-40	...	2/2
TC67169	629	Hypothetical protein (<i>A. thaliana</i>)	At1g10490	6e-26	...	2/2
TC66818	655	Hypothetical protein F9N12.8 (<i>A. thaliana</i>)	F9N12.8	2e-36	...	2/2
TC65882	504	Hypothetical protein (<i>A. thaliana</i>)	T16L1.40	5e-19	...	2/2
TC63150	497	Hypothetical protein (<i>A. thaliana</i>)	F14L2.70	3e-19	2/2	...
TC66584	516	Hypothetical P0501G01.24 protein (<i>O. sativa</i>)	Q9AX93	2e-59	...	2/2
TC67556	725	Gb AAC80617.1 (<i>A. thaliana</i>)	Q9FFD5	7e-16	2/2	...
TC75304	645	Unknown protein (<i>A. thaliana</i>)	AY088569.1	2e-90	2/2	...
TC66240	450	Hypothetical protein (<i>A. thaliana</i>)	At2g38010	3e-39	...	2/2
TC66672	627	Hypothetical 71.0-kDa protein	Q8W4D0	1e-53	...	2/2
TC75639	723	Hypothetical protein (<i>A. thaliana</i>)	At2g46080	1e-71	2/2	...

^a MtGI = *Medicago truncatula* gene index; N = number of expressed sequence tags in a library.

^b NBS-LRR = nucleotide binding site-leucine-rich repeat.

^c Post-transcriptional modification, protein processing.

^d Cell structure and extracellular space.

^e Unknown (unkn).

Table 4. Induction levels of genes represented by MtGim- or MtAmp-specific tentative consensus sequences (TCs)^a

MtGI-TC	Induction level ^b		Annotation ^c	Similarities ^d
	BR 1	BR 2		
Signal transduction				
TC64428	10.7	59.2	NBS-LRR type disease resistance protein	...
TC66505	3.1	15.8	Lectin like protein kinase	...
TC74096	1.1	3.1	Receptor like protein	...
TC74325	3.6	6.1	Seven transmembrane protein MLO2	61% identity to MtC50782.1 ^e
TC76060	n.d.	0.4	Serine protein kinase like	...
TC71483	n.d.	267.9	Probable protein kinase	...
Metabolism				
TC65799	12.4	21.0	Dihydrodipicolinate synthase	...
TC67209	0.6	2.0	Phosphoenolpyruvatcarboxylase	...
TC74500	3.9	18.8	β-Galactosidase	...
TC67079	5.3	18.7	Epoxide hydrolase homologue	...
TC76132	24,505.2	69,515.6	Blue copper binding protein	79% identity to MtGIM13 (AW584704) ^f
Post transcriptional modification, protein processing				
TC68426	42.5	485.0	Serin carboxypeptidase-like protein	79% identity to MtSCP1 ^g
TC66580	9.1	1,681.3	Cystein proteinase precursor	...
TC75569	None	n.d.	Prolyl endopeptidase	...
TC71486	0.6	1.8	Phosphoprotein phosphatase	...
Cell wall and extracellular space				
TC75254	None	None	Agglutinin II precursor	64% identity to MtGIM14 (TC78055)/MtLEC6 ^f 46% identity to MtGIM3 (TC87043)/MtLEC5 ^f 31% identity to MtGIM11 (TC78315)/MtLEC7 ^f
TC68166	None	1,586.2	Agglutinin II precursor	75% identity to MtGIM3 (TC87043)/MtLEC5 ^f 51% identity to MtGIM14 (TC78055)/MtLEC6 ^f 42% identity to MtGIM11 (TC78315)/MtLEC7 ^f
TC75360	139.8	114.6	Lectin	50% identity to MtGIM3 (TC87043)/MtLEC5 ^f 47% identity to MtGIM14 (TC78055)/MtLEC6 ^f 47% identity to MtGIM11 (TC78315)/MtLEC7 ^f
TC69333	7482.2	7477.6	Bark lectin II precursor	75% identity to MtGIM14 (TC78055)/MtLEC6 ^f 43% identity to MtGIM3 (TC87043)/MtLEC5 ^f 49% identity to MtGIM11 (TC78315)/MtLEC ^f
TC60541	5971.8	8699.7	Germin-like protein	68% identity to MtGIM31 (TC77375)/MtGLP1 ^{t/h}
TC63954	80.6	115.2	Germin-like protein	36% identity to MtGIM31 (TC77375)/MtGLP1 ^{t/h}
Transport				
TC70909	n.d.	46.1	MDR-like ABC-transporter	...
TC63901	56.3	164.4	Copper transporter	...
TC66155	0.4	n.d.	Zinc transporter	...
TC73332	130.3	450.0	Ammonium transporter	...
TC65476	5.4	16.3	Sugar transporter	...
Unclassified, unknown function				
TC72502	1.1	7.0	Nodulin-like protein	...
TC67410	0.6	2.4	RING zinc finger protein	...
TC76092	1.5	2.4	Myotubularin	...
TC59827	1.0	2.9	Cytokinin-specific binding protein	...
TC64306	0.8	4.0	Vesicle-associated membrane protein	...
TC67060	1.3	1.7	Mitochondrial inner membrane protein	...

^a MtGI = *Medicago truncatula* gene index.^b BR = biological replicate; n.d. = no products detected using cDNA of nonmycorrhizal or mycorrhizal roots; None = no polymerase chain reaction (PCR) products detectable after amplification of cDNA deriving from non-mycorrhiza roots; PCR products were obtained after amplification of cDNA of mycorrhizal roots^c NBS-LRR = nucleotide binding site–leucine-rich repeat.^d Similarities to previously described arbuscular mycorrhizal-induced genes.^e Manthey et al. 2004.^f Wulf et al. 2001.^g Liu et al. 2003.^h Doll et al. 2001.

and MHAM (TIGR MtGI), and indicates that the MtGim cDNA collection represents a resource for novel mycorrhiza-induced genes that are summarized in Table 4.

A family of *M. truncatula* lectin genes is induced in mycorrhizal roots.

Notably, four novel lectin-like sequences were strongly induced in mycorrhizal roots (Table 4). Previous reports on *M. truncatula* lectin genes (*MtLec1-4*) were focused on genes induced during root nodule development and nitrogen fixation (Bauchrowitz et al. 1996; Mitra and Long 2004). During the initial characterization of the MtGim library (Wulf et al. 2003), three AM-induced lectins represented by TIGR TC 69503 (*MtLec5*), 69334 (*MtLec6*), and 69628 (*MtLec7*) were identified. In the approach presented here, four novel AM-specific lectin-like sequences were found, represented by TIGR TC 68166 (*MtLec8*), 69333 (*MtLec9*), 75254 (*MtLec10*), and 75360 (*MtLec11*). Hence, seven different AM-induced lectin-sequences have been identified in *M. truncatula* up to now. Amino acid sequences of the AM-induced lectins *MtLec5*- to -*11* were aligned and compared with the previously described nodule-induced lectin sequences. A further nodule-specific lectin-like sequence (TC78397) identified from the TIGR MtGI by in silico expression profiling also was included in this analysis. The multiple alignment revealed that AM-induced lectin sequences group in one branch when compared with the lectin sequences induced during the root nodule symbiosis (Fig. 1), indicating a putative subfamily of AM-specific members within the group of *M. truncatula* lectin-like sequences.

To obtain information on the localization of AM-specific lectins, the promoter sequences of two genes (*MtLec5* and *MtLec7*) were used in reporter gene studies. The genomic sequence of *MtLec5* was amplified from BAC clone mth2-20e4 (GenBank accession no. AC126010), whereas the *MtLec7* promoter sequence was cloned by inverse PCR. For reporter gene

studies, 1,059 bp of the *MtLec5* promoter (position -1060 to -1 relative to the ATG) and 1,503 bp (position -1504 to -1 relative to the ATG) of the *MtLec7* promoter were fused to the *gus* reporter gene. With both constructs, a comparable pattern of promoter activity was observed. No reporter gene activities were found in roots of nonmycorrhizal plants (data not shown); however, strong reporter gene activities occurred in transgenic roots colonized by *G. intraradices* (Fig. 2). Both promoters showed high activity in arbuscule-containing cells, but no activity in the vicinity of appressoria, intercellular or intracellular fungal hyphae, and vesicles. Thus, *MtLec5* and *MtLec7* seem to be specifically activated in arbuscule-containing cells.

A comparison of the MtLEC5 and MtLEC7 amino acid sequences showed that both sequences share 37.8% identity (Fig. 3). For these two lectins, a cleavable N-terminal signal sequence and a putative apoplastic localization was predicted with a certainty of 0.8 using the PSORT software (Nakai and Kanehisa 1992), suggesting an extracellular localization of these proteins.

DISCUSSION

Data presented in this report contribute to the theory that plants, which are susceptible to AM fungi, possess a set of genes specifically induced after colonization with AM fungi. A notable number of ESTs were generated from two cDNA libraries (MtAmp and MtGim) of *M. truncatula* roots colonized by the AM fungus *G. intraradices*. The resulting population of tentative consensus sequences was used in an in silico screen for novel AM-specific sequences by searching for TCs within the MtGI exclusively composed of ESTs derived from the MtAmp or MtGim library. In silico strategies also have been used by Journet and associates (2002) to identify genes regulated during root nodule and AM endosymbiosis using likelihood ratio (*R*-value) statistics by comparing EST counts in

Fig. 1. Unrooted tree based on ClustalW alignment. The following sequences were included: *MtLec1* (X60386), *MtLec2* (X60387), *MtLec3* (X82216), *MtLec4* (TC77066), *MtLec5* (TC69503), *MtLec6* (TC69334), *MtLec7* (TC6928), *MtLec8* (TC68166), *MtLec9* (TC69333), *MtLec10* (TC75254), *MtLec11* (TC75360), and the nodule-specific lectin-like sequence encoded by TC78397.

cDNA populations of different tissues. This is in contrast to the here-applied strategy of screening for a novel set of AM-specific genes which are exclusively composed of MtGim or MtAmp ESTs.

This stringent search strategy revealed 115 novel AM-specific TCs. Annotation of these sequences indicated that 10 of them

showed the highest similarities to fungal sequences and most likely are derived from the microsymbiont *G. intraradices*. Most of these putative fungal genes encoded ribosomal RNAs but one sequence was highly homologous to a previously characterized non-rRNA gene of an AM fungus, *G. mosseae* (Franken et al. 2000).

Fig. 2. Promoter activities in *Glomus intraradices*-colonized hairy roots. Histochemical analysis of transgenic *Medicago truncatula* roots carrying **A**, **B**, **C**, and **D**, the *MtLec5* GUS or **E**, **F**, **G**, and **H**, *MtLec7*-GUS fusions. *M. truncatula* plants with *Agrobacterium rhizogenes*-transformed hairy roots were colonized by *G. intraradices* and stained for GUS activity 3 weeks after colonization. **B** through **H**, Double stainings for GUS activity and subsequent acid fuchsin staining to detect *G. intraradices*. **A**, Intense *MtLec5* promoter activity is detectable in root parts colonized by the arbuscular mycorrhizal fungus (magnification bar: 2 mm). **B** and **D**, Arbuscules showing intense *MtLec5* promoter activity (magnification bars: 10 and 25 µm, respectively). **C**, *MtLec5* activity is localized in inner cortical cell layers where arbuscules have been formed; no GUS activity can be detected at the root surface after contact with *G. intraradices* hyphae (magnification bar: 20 µm). No *MtLec7* promoter activity was observed in the vicinity of **E**, vesicles (magnification bar: 25 µm) or **F**, appressoria (magnification bar: 50 µm); **G** and **H**, arbuscules showing intense *MtLec7* promoter activity (magnification bars: 25 µm).

One of the most surprising outcomes of this study was the identification of 52 TCs without homologies on an amino acid level to previously identified sequences. In addition to comprehensive EST collections, several plant genome sequences that either sequenced completely or sequenced to a large extent are available to date; therefore, it might be anticipated that *M. truncatula* ESTs at least weakly match sequences from other plant EST or genome sequence data. A blastn search within the presently available *M. truncatula* BAC sequence collection, confirmed a plant origin for seven of these sequences. For these seven genes, no putative homologues have been identified so far in other plant genomes; therefore, they are extremely interesting for further studies in AM research and could reveal novel mechanisms. A notable number of the 52 TCs without significant homologies also probably correspond to hitherto unknown fungal genes. To gain information on the genetic program of *M. truncatula* activated in mycorrhizal roots, only plant-derived genes which could be assigned to putative functions have been further studied concerning their RNA accumulation pattern in mycorrhizal and control roots, and an induced expression in mycorrhizal roots was confirmed for the majority of the analyzed genes.

The exchange of nutrients is supposed to be the key element of an AM fungus. In the past, numerous efforts have been made to identify plant genes involved in these transport processes (Harrison 1996; Hildebrandt et al. 2002; Liu et al. 1998). Very recently, mycorrhiza specifically expressed phosphate transporters have been identified in several plant species (Harrison et al. 2002; Paszkowski et al. 2002; Rausch et al. 2001). In this study, sequence annotation raised evidence for an involvement of copper and ammonium transporters during symbiotic nutrient transfer. Studies using ^{15}N showed that AM fungi are able to take up ammonium as well as nitrate from the soil environment (George et al. 1992; Johansen et al. 1992) and the proteins presented here could be responsible for the allocation of copper or N-compounds in mycorrhizal roots. For copper transporters, an involvement during the root nodule symbiosis could be demonstrated already: a nodule-specific copper transporter was identified by an in-silico approach (Fedorova et al. 2002), indicating that *M. truncatula* possesses both a mycorrhiza- and nodule-specific copper-transporter gene, which probably are localized in specific tissues.

Within the group of genes involved in signalling processes, a gene with similarities to nucleotide binding site (NBS) leucine-rich repeat (LRR) resistance-like protein was found to be more than 10-fold induced in mycorrhizal roots. NBS-LRR resistance genes represent the largest class of disease resistance genes (Hammond-Kosack and Jones 1996) and have been shown to be represented by at least 58 members within the NBS-LRR resistance gene family in *M. truncatula* (Zhu et

al. 2002). NBS-LRR resistance-like genes encode receptors with high specificities and can be involved in different signal transduction pathways (Ellis et al. 2000). Thus, the AM-induced NBS-LRR resistance-like gene identified here could represent a receptor involved in AM-specific signaling processes which is transcriptionally activated during AM symbiosis. A second upregulated gene which also is involved in signaling processes shows similarities to members of the plant-specific seven-transmembrane MLO family. Members of this protein family are suggested to have a function similar to that of G protein-coupled receptors of animal and fungi (Devoto et al. 2003).

Annotation of several AM-induced sequences showed that they belong to sequence families for which AM-induced members already have been described. The phenomenon that several members of the same gene family show increased RNA accumulation in mycorrhiza compared with control roots was observed for the family of germin-like sequences (Doll et al. 2003; Wulf et al. 2003), serine-carboxypeptidases (Liu et al. 2003), blue-copper binding proteins, and lectins (Wulf et al. 2003).

In this study, two germin-like proteins (GLPs) have been demonstrated to be upregulated in mycorrhizal roots. A further GLP gene of *M. truncatula*, MtGLP1, recently was described to be upregulated specifically in AM fungi (Doll et al. 2003; Wulf et al. 2003). Together with the two GLP-like TCs presented here, three different AM-induced GLPs seem to be involved in AM symbiosis. A serine carboxypeptidase-like sequence was found to be more than 40-fold induced in AM roots compared with control roots; however, this gene is not identical to the AM-induced serine carboxypeptidase which was identified by array hybridizations (Liu et al. 2003). The gene represented by TC 76132, which more than 10^4 -fold upregulated in mycorrhizal roots compared with control roots, also belongs to a family of AM-specific sequences. These sequences show similarities to blue-copper binding proteins, or plantacyanins. Although nothing is known about functions of these copper-binding domain-containing proteins, some blue-copper binding proteins have been identified as nodulins (Fedorova et al. 2002; Greene et al. 1998; Jiménez-Zurdo et al. 2000) and a blue-copper binding protein-like sequence has been described to be AM-specifically expressed (Wulf et al. 2003) or induced in AM roots (Journet et al. 2002). Hence, it might be anticipated that members of this gene family play specific roles during both root nodule and AM endosymbiosis.

The most important result of this study is probably the identification of a group of seven AM-specific lectin-like sequences in *M. truncatula*. In a previous report, three different *M. truncatula* lectin-like sequences were described to show an increased expression in mycorrhizal roots (Wulf et al. 2003). Four different new, lectin-like sequences have been identified

MtLEC5	MANSIPKLLATQNPFSVLSIFFLLLLINNVKSDLSLNFNPKFDTDALNIVIDGDAKTT	60
MtLEC7	MAINTSR---TQILF---ITIISFLILAQN-VNS--AAFTVSNFDPYKTNIELEGNAFIS	51
MtLEC5	GGVLQLTKKDQFGNPSPHSVGFSAFFGAIQLSDKQSGKVADFTTEFSFVNPKGSQHLGD	120
MtLEC7	DGSIHLT-----NVIPNSAGRASWGGPVRLWDADTGNLAGFTSVFSFEVAPAGPLIGD	105
MtLEC5	GFIFYIASLDYDFPEKSSDGGFLGLFDKETAFNTSKNSIVAVEFDSFA-----NEWDPNS	175
MtLEC7	GITFFIAPFNSHIPKNSS-GGFLGLFNAETALNTYQNRIVAVEFDSFGGNSGGNPWDPAY	164
MtLEC5	PHIGIDINTIESSISVPWPIDRQPQGTIGKARISYNTASKDLSVFVTYP--NSPAKVDVI	233
MtLEC7	PHVGIDVNSIASVTTAPWKTSILTGFAIAFVNYEPVEKNLSVVVRYPPGNFVNGTSNS	224
MtLEC5	VSYPIDFASVLSSEWVYVGFSGATGQVAETHDILSWSFVSNL-	274
MtLEC7	VSFIIDLRTVLPWVRIGFSGATGQLVELHKILSWTFKSSFQ	266

Fig. 3. MtLEC5 and MtLEC7 amino acid sequence alignment. Identical amino acids are marked by gray background. The predicted N-terminal signal sequences are indicated by bold letters.

in this study; all of them were proven to be strongly induced in AM. Thus, seven different AM-specific lectin-like sequences of *M. truncatula* have been identified so far. Lectins are proteins that reversibly bind to specific mono- or oligosaccharides (Peumans and van Damme 1995). Genes encoding lectins have been found in plants, animals, microbes, and viruses. The sequences differ remarkably with respect to their molecular structure and are divided in different subfamilies. For some members of the legume lectins, a function as determinants of specificity in the root nodule symbiosis could be demonstrated by gain-of-function analyses (Diaz et al. 1989; Hirsch et al. 1995; van Rhijn et al. 1998). Nevertheless, sequences of this subfamily also must have other functions, because sequences with high similarity to legume lectins also have been identified in nonleguminous plants and animals (Wang et al. 2003). The identification of numerous different lectin-like sequences presented here which all show increased RNA accumulation in mycorrhizal roots indicates an important role for these proteins during AM symbiosis. Reporter gene studies showed an arbuscule-specific transcription for two members of the AM-specific lectin family, suggesting a role during arbuscule formation and functioning during late stages of the AM symbiosis. No promoter activities were detected in the vicinity of appressoria which represent early steps of AM development. Appressoria are formed only at epidermal cells of host plants (Giovanetti et al. 1993; Nagahashi and Douds 1997), indicating a host plant–fungus recognition at this step. Thus, the two lectins *MtLec5* and *MtLec7* do not play a role during recognition at early steps of symbiosis development. One possible function of AM-specific lectins could be a direct interaction with the fungal microsymiont during arbuscule formation by binding of fungal surface carbohydrates. Because AM fungi possess a thick and rigid cell wall, it is unlikely that plant lectins can directly bind to glucoconjugates on the fungal membranes and, thus, directly influence intracellular processes in the fungus; however, plant lectins could bind to carbohydrates exposed to fungal cell wall surfaces and, therefore, indirectly regulate molecular processes during symbiosis. Biochemical studies targeted at identifying the carbohydrates which are recognized by the AM-specific lectins remain to be done to strengthen this hypothesis. Another possible function of lectins during AM symbiosis is a role as storage for organic nitrogen. Immunolocalizations have shown that lectins accumulate in vacuoles, parenchyma cells, and the extracellular matrix of peanut nodules, indicating a role as storage proteins (VandenBosch et al. 1994). An extracellular localization was predicted for the two AM-specific lectins *MtLec5* and *MtLec7* pointing to a similar function of AM-specific lectins. Recent studies by Bauchrowitz and associates (1996) showed that three *M. truncatula* lectin genes (*MtLec1* to -3) are expressed throughout nodule development. Except for *MtLec2*, which is presumably a pseudogene (Bauchrowitz et al. 1992), these lectins also were transcribed in nonsymbiotic tissues. In contrast, in silico expression profiling using the MtGI revealed the identification of one nodule-specific gene, *MtLec4*, which shows an expression correlated to bacterial penetration of the cortex cells and sustained cell divisions (Mitra and Long 2004). Amino acid comparison of AM-specific, nodule-specific, and nodule-correlated lectins revealed that seven AM-specific sequences form a unique branch in the corresponding tree, suggesting that AM-specific and nodule-enhanced lectins might have different functions during root endosymbioses.

The strategy of coupling EST sequencing with in silico and experimental expression analysis has proven to be a powerful tool for identifying novel *M. truncatula* genes specifically regulated in endomycorrhizae. Our results suggest an involve-

ment of several lectins in the AM symbiosis and, although their individual role remains to be elucidated, a specific role during arbuscule development or functioning can be proposed.

MATERIALS AND METHODS

Plant growth and inoculations.

For RT-PCR, plants were grown as described by Wulf and associates (2003). Briefly, seed of *M. truncatula* cv. Jemalong A17 were sterilized by a 10-min treatment with concentrated sulfuric acid, washings with distilled water, and a 5-min incubation in 6% NaClO. Germination of the seed occurred at room temperature in the dark for 2 days. For AM inoculation, seedlings were planted into a 1:2 mixture of expanded clay and vermiculite. Inoculations with *G. intraradices* were carried out with a commercially available inoculum (Biorize Sarl, Dijon, France). After 3 weeks, all plants were harvested. Mycorrhization was quantified as described by Trouvelot and associates (1986).

cDNA libraries and sequencing.

The MtGim SSH library was generated as described earlier (Wulf et al. 2003). The MtAmp library was generated by MediGenomix (Martinsried, Germany) from mycorrhizal roots of *M. truncatula* using a directional cloning strategy with a cutoff of 500 bp. cDNA was prepared by reverse transcription of poly A+ enriched RNA. The cDNA was directionally ligated into the pGEM-T vector (Promega, Madison, WI, U.S.A.) using GCATGCGGCCGAGGCGGCCGACATG and CTGCAGGCCATTATGGCCGGG adaptors. Plasmids containing cDNA inserts were propagated in *Escherichia coli* DH10B cells. cDNA sequences were obtained using standard protocols on the MegaBace 1000 sequencer (Amersham Biosciences, Freiburg, Germany).

Sequence processing, annotation, and clustering.

To cluster and annotate the ESTs obtained, the BioMake software package was used. BioMake processes sequence data as follows: beginning with the raw EST trace files, a normalization step using phred (Ewing et al. 1998) was performed. Afterward, low-quality regions were removed according to the phred 13 quality definition (Ewing and Greene 1998). Finally, the vector content needed to be clipped, leading to EST reads in fasta file format. Clustering of the EST reads also was performed by the BioMake software package according to the TIGR clustering algorithm (Liang et al. 2000). The sequence data obtained consist of TCs and single (unclustered) EST reads, called singletons. Comparisons to the TIGR MtGI were done in order to identify identical TCs. BLAST comparisons against the GenBank nonredundant database and annotations also were done using the BioMake software. BLASTX searches were carried out in order to identify homologous genes in other organisms (Altschul et al. 1997).

In silico analysis of gene expression.

To identify TCs representing putative mycorrhiza-specific genes, an electronic Northern (eNorthern) algorithm was developed. We compared MtGim and MtAmp EST clusters via a BLAST (Altschul et al. 1997) homology search against TIGR (Liang et al. 2000) using an e-value cut-off of $1e^{-10}$ and sequence identity of at least 95%. Those of our clusters that hit only the MtAmp or MtGim library were assumed to represent novel mycorrhiza-specific genes of *M. truncatula*.

Quantitative real-time RT-PCR.

Primer design and quantitative RT-PCR was carried out according to Wulf and associates (2003). All quantitative real-

time RT-PCR experiments were repeated (technical control) in order to verify RNA-accumulation values. Primer sequences used for RT-PCR are shown in Table 5.

Isolation of lectin promoters from BAC sequences and by inverse PCR.

For amplification of the *MtLec5* promoter, 1 ng of genomic DNA was amplified with specific primers (5' to 3': GAA TTC GGG AAA ATT GAC TAA CGT TGT GAA A and GGA TTC GGT ATT ATG TTG TGT ATG ATG C) deduced from the corresponding BAC sequence. The PCR product was cut with *EcoRI* and *BamHI* and cloned into the corresponding sites into the pLP 100 vector. For the inverse PCR to amplify the *MtLec7* promoter region, 2 µg of genomic *M. truncatula* DNA was cut with the restriction enzymes *EcoRI*, *XbaI*, *XhoI*, *PvuI*, *PaeI*, or *HindIII*. Circularization of 50 ng of digested DNA was carried out in 30 µl using 0.6 U of T4-DNA ligase at 16°C. The flanking regions were amplified using 10 ng of recircularized DNA and *MtLec7*-specific outward orientated primers (5' to 3': gagatgaattacattcttca and gatggaatgaaaaggatttgatcc). Of the resulting PCR fragment, a 1,503-bp *MtLec7* promoter region was amplified using specific primers (5' to 3': GGG GAC AAG TTT GTA CAA AAA AGC AGG CTC ACC GCG CAT AAT GAT TGA GG and GGG ACC ACT TTG TAC AAG AAA G CT GGG TGA ATC CGC GAT GGT TCT AAA TTA GTG) and 1 ng of genomic DNA as template. The resulting PCR product was cloned into the pMDC163 vector (Curtis and Grossniklaus 2003).

Agrobacterium rhizogenes-mediated transformation of *M. truncatula*.

Using sequence-specific primers with restriction enzyme recognition sites, 1,059 bp of the *MtLec5* promoter region

were cloned in front of the GUS gene in the binary vector pLP100 (Szabados et al. 1995). A 1,503-bp *MtLec6* promoter fragment was cloned in front of the GUS gene of the pMDC163 binary vector (Curtis and Grossniklaus 2003) using the Gateway cloning system (Invitrogen, Karlsruhe Germany). *Agrobacterium rhizogenes* strain ARqua 1 (Quandt et al. 1993) was transformed with the binary plasmids via electroporation. *M. truncatula* roots were transformed according to a modified protocol of Vieweg and associates (2004).

Histochemical analysis of transgenic roots.

Histochemical assays for GUS activity were performed as described by Jefferson and associates (1997). Roots were incubated in the staining solutions in the dark at 37°C for 1 to 2 h and subsequently cleared in 70% ethanol for 1 h.

Online resources.

Sequences have been analyzed concerning conserved short protein patterns using the PROSITE (Gattiker et al. 2002; Sigrist et al. 2002) database at ExPASy. Searches for sequence similarities were done using Blast2X (Altschul et al. 1997). *M. truncatula* genomic sequence data were obtained from the *M. truncatula* Genome Sequencing Project at the Advanced Center for Genome Technology of the University of Oklahoma, (Norman, OK, U.S.A.) and the Noble Foundation (Ardmore, OK, U.S.A.). The N-terminal sequences were identified using the PSORT protein sorting predication tool (Nakai and Kanehisa 1992).

Multiple amino acid sequence alignments.

Open reading frames of TIGR TCs were translated into amino acid sequences and aligned using ClustalW (Jeanmougin et al. 1998) at the EMBnet ClustalW server. Of the aligned

Table 5. Primer sequences used for quantitative real-time reverse-transcription polymerase chain reaction

MtGI-TC ^a	Left primer (5'-3')	Right primer (5'-3')
TC64428	tacgaaatcaaacatttcac	gcagagatgatgacaataaga
TC66505	atctctttattgtttccaagg	aggatagaggatcaaatca
TC74096	aattcaacctttatccatt	attatcttgctcgaattctc
TC74325	aataagatggatagtgccatt	aattgtaaacgttcataagca
TC76060	ttcacaagttctctcattcag	agaacaccttcaattttagg
TC71483	tcctatcttcccaagtaagag	aattgctgtaagcaacataa
TC65799	tgggataacctctaaagaag	tttgggtgatacaagagtg
TC67209	accagaatcagaatatccaac	ttgcagtagagcttttacaga
TC74500	gtatgcacttctatcagccgt	catctatgatttcaagtttcg
TC67079	aacttccttttagcaatttcag	gtttctgtgagctcatgattg
TC68426	aaaaatgggtcactggttat	gtcagatgagatacttgatgt
TC66580	atgaattcttaaccaaccaat	tttccgtaactattgatgcta
TC75569	gaaccagaagacataaggag	aatgcttattgcatcaagata
TC71486	gctttaattttggaagagc	gtatacgtttggtgctgataa
TC60540	agaagtgtccacacacaagta	caacactttatggaaaatgaa
TC68166	agtgaagaccatgaaagaat	ataagttataatcctgcctcg
TC69333	caatgtgagatattgagatg	tcttttatcgtgaacacaaac
TC75254	gatactgacatgccaattta	aactgggtgaacacatgata
TC75360	ctatcaaatcaacaaggaca	aactcaaacggtagacaagat
TC73332	accatttgaagttcacat	ctacaacgttttaagacagag
TC65476	gaacacaacagtcacaaac	atagtcagccattattcag
TC63901	aaataaaaattttggtggtgt	gacataccaataagcacttga
TC66155	tgatagcagctgatttctta	atctaccaaaattgaattgga
TC70909	caaaattgtagttggagaa	ataaatgcattgtgttga
TC72502	attctattgcatcatcatca	ggatcttctgcttcaaac
TC76132	tcttcccataggttgaat	ttttagtggagatgaaa
TC67410	tccctctcatcaataact	cagagtgtgtttatgcaagt
TC76092	gattaacgacaaaagatct	gcttgaataactgtcatcaa
TC59827	atgtcttggtaggttgact	gtaaattatcaaggaggtg
TC60541	ttgctagcacattgttaact	agttacatcaaccatttca
TC63954	agctctttcatttttagctgt	gcaagactggagttactct
TC64306	cataaccatctcaacaagaa	actcgttacagagcacaatg
TC67060	tacttctactgtctctgatcg	ctggttatgacgattaacaaa

^a MtGI = *Medicago truncatula* gene index; TC = tentative consensus sequence.

amino acid sequences, 3' and 5' extremities as well as gap columns were removed and the remaining sequence block was used to calculate a tree by the neighbor-joining method.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

- European Bioinformatics Institute EMBnet ClustalW server:
www.ebi.ac.uk/clustalw
- ExPASy PROSITE database: www.expasy.ch
- The Institute for Genomic Research (TIGR) *M. truncatula* gene index:
www.tigr.org/tdb/mtgi
- The *Medicago truncatula* Genome Sequencing Project, Advanced Center for Genome Technology of the University of Oklahoma and the Noble Foundation: www.genome.ou.edu/medicago.html
- National Center for Biotechnology Information Blast2X server:
www.ncbi.nlm.nih.gov/BLAST
- PSORT protein sorting predication tool: psort.nibb.ac.jp
- The University of California-Davis *Medicago truncatula* physical and genetic map BAC sequences: mtgenome.ucdavis.edu/index.html