Overlaps in the Transcriptional Profiles of *Medicago truncatula* Roots Inoculated with Two Different Glomus Fungi Provide Insights into the Genetic Program Activated during Arbuscular Mycorrhiza[1][w]

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Arbuscular mycorrhiza (AM) is a widespread symbiotic association between plants and fungal microsymbionts that supports plant development under nutrient-limiting and various stress conditions. In this study, we focused on the overlapping genetic program activated by two commonly studied microsymbionts in addition to identifying AM-related genes. We thus applied 16,086 probe microarrays to profile the transcriptome of the model legume *Medicago truncatula* during interactions with *Glomus mosseae* and *Glomus intraradices* and specified a total of 201 plant genes as significantly coinduced at least 2-fold, with more than 160 being reported as AM induced for the first time. Several hundred genes were additionally up-regulated during a sole interaction, indicating that the plant genetic program activated in AM to some extent depends on the colonizing microsymbiont. Genes induced during both interactions specified AM-related nitrate, ion, and sugar transporters, enzymes involved in secondary metabolism, proteases, and Kunitz-type protease inhibitors. Furthermore, coinduced genes encoded receptor kinases and other components of signal transduction pathways as well as AM-induced transcriptional regulators, thus reflecting changes in signaling. By the use of reporter gene expression, we demonstrated that one member of the AM-induced gene family encoding blue copper binding proteins (MtBcp1) was both specifically and strongly up-regulated in arbuscule-containing regions of mycorrhizal roots. A comparison of the AM expression profiles to those of nitrogen-fixing root nodules suggested only a limited overlap between the genetic programs orchestrating root endosymbioses.

Legume plants establish two different endosymbioses with soil microorganisms: the nitrogen-fixing root nodule symbiosis and the arbuscular mycorrhiza (AM). Nodulation is almost exclusively restricted to legumes and requires the organogenesis of a root nodule that houses the rhizobial prokaryotes capable of symbiotic nitrogen fixation (Schultze and Kondorosi, 1998). In contrast, more than 80% of higher plants enter an AM symbiosis with fungi of the phylum Glomeromycota (Schüssler et al., 2001), including *Glomus mosseae* and *Glomus intraradices* as prominent representatives. The AM interaction is characterized by the transfer of minerals, in particular phosphorus, from the soil to the plant in exchange for photosynthates allocated to the fungus.

To initiate the symbiotic interaction, fungal hyphae from an extraradical mycelium penetrate the root epidermis through an appressorium and subsequently proliferate in the inner cortex (Harrison, 1997; Strack et al., 2003). These intraradical hyphae terminate in highly branched, intracellular structures designated arbuscules (Bonfante and Perotto, 1995), which are surrounded by the periarbuscular membrane. With respect to phosphorus and mineral acquisition, arbuscules are regarded as the major site of nutrient uptake, and mycorrhiza-specific phosphate transporters are located at the periarbuscular interface (Harrison et al., 2002). Apart from this novel symbiotic compartment, intraradical hyphae are assumed to be important for the allocation of carbohydrates to the fungus (Shachar-Hill et al., 1995; Bago et al., 2000). Although, in contrast to nodulation, a de novo plant organ is not formed in AM, colonization of roots by endosymbiotic fungi creates an additional carbon sink that alters the physiology of and the metabolite allocation to this symbiotic root system (Wright et al., 1998).

Both rhizobial and fungal microsymbionts colonize plant cells during nodule and AM symbioses, but they remain separated by perisymbiotic membranes controlling nutrient exchange (Provorov et al., 2002). Due to analogies in the infection process, an overlap in the activation of gene expression (vanRhijn et al., 1997;

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Journet et al., 2001; Brechenmacher et al., 2004; Sanchez et al., 2004) and in the recruitment of signal transduction cascades leading to nodulation and mycorrhization (Cullimore and Dénarié, 2003) is not surprising.

A key goal in legume research has been the identification of genes expressed during the development and function of root endosymbioses, an approach that profited from research in the two model legumes *Medicago truncatula* Gaertn and *Lotus japonicus* (Weidner et al., 2003). In contrast to studies dedicated to nodulation, targeted approaches addressing AM have so far revealed a markedly smaller number of genes activated in arbuscules (Franken and Requena, 2001), e.g. the phosphate transporter MtPt4 (Harrison et al., 2002), the plasma-membrane H^+-ATPase Mtha1 (Krajinski et al., 2002), the germin-like protein MtGlp1 (Doll et al., 2003), the glutathione *S*-transferase MtGst1 (Wulf et al., 2003), and the Ser carboxypeptidase MtScp1 (Liu et al., 2003a). In the era of genomics, a more comprehensive view of gene induction during AM should be possible, and experiments making use of suppression subtractive hybridization (SSH) cDNA libraries, cDNA-array hybridizations, and real-time reverse transcription (RT)-PCR experiments identified several dozens of mycorrhiza-related *M. truncatula* genes (Liu et al., 2003a; Wulf et al., 2003; Brechenmacher et al., 2004; Küster et al., 2004; Manthey et al., 2004; Weidmann et al., 2004). On the fungal side, SSH approaches in particular facilitated an identification of Glomus genes expressed during appressorium formation (Requena et al., 2003; Brechenmacher et al., 2004; Breuninger and Requena, 2004), complementing knowledge on AM gene expression during early stages of the symbiosis. Regardless of the progress made, and largely due to the obligate biotrophy of *Glomus* spp. fungi (Franken and Requena, 2001) as well as the presence of different stages of AM formation in mycorrhizal roots (Gianinazzi-Pearson and Brechenmacher, 2004), knowledge on genes activated during AM is still limited in relation to more than a thousand genes identified as up-regulated during different steps of root nodule initiation and function using different cDNA-based macro- and microarrays (Colebatch et al., 2002, 2004; Fedorova et al., 2002; El Yahyaoui et al., 2004; Kouchi et al., 2004; Lee et al., 2004).

To reduce cross-hybridization, cDNA-based arrays are increasingly replaced by 50 to 70-mer oligonucleotide microarrays or in situ synthesized gene chips (Meyers et al., 2004). These arrays are limited in scope only by the number of sequences available for a given organism. Due to their comprehensive character, such microarrays allow us to take up the challenge of specifying the network of genes orchestrating AM formation in legumes to elucidate developmental and physiological processes relevant for this important endosymbiosis.

Expression profiling in mycorrhizal roots so far mainly focused on interactions with a particular AM fungus, leading to the definition of marker genes that subsequently had to be verified in other associations. Since there is evidence that AM fungi are characterized by different degrees of colonization as well as altered carbon allocation and symbiotic efficiency (Klironomos and Hart, 2002; Lerat et al., 2003; Smith et al., 2003b; Munkvold et al., 2004), together leading to an induction of different sets of host genes (Burleigh et al., 2002), the common genetic program for AM interactions still needs to be defined. To identify genuine mycorrhiza-related plant genes as opposed to genes activated by a particular AM fungus, we took advantage of 16,086 probe oligo microarrays, to date the most comprehensive representation of *M. truncatula* genes. We specified 201 *M. truncatula* genes as significantly induced during mycorrhization with the 2 commonly studied AM fungi *G. mosseae* and *G. intraradices*. Our global view of the *M. truncatula* AM transcriptome provides insights into the genetic program orchestrating this root endosymbiosis and facilitates future studies targeted at identifying functions for the encoded gene products during AM.

RESULTS AND DISCUSSION

AM Formation Significantly Alters the Transcriptome of *M. truncatula* Roots

To select mycorrhizal roots infected at a comparable level with the two commonly studied arbuscular mycorrhizal fungi *G. mosseae* and *G. intraradices*, random samples from root systems were stained for fungal structures 28 d post inoculation, and only those roots with similar degrees of mycorrhization were used for RNA isolation. Subsequently, the expression of marker genes for colonization intensity (MtPt4; Harrison et al., 2002) and nodulation (ENOD18; Hohnjec et al., 2003) was checked by real-time RT-PCR. Only those RNA pools with strongest MtPt4 induction and no detectable ENOD18 expression (data not shown) were selected to synthesize targets for hybridization experiments. As controls, nonmycorrhizal roots grown under phosphate limitation were used and here, the absence of MtPt4 and ENOD18 expression was confirmed by RT-PCR (data not shown).

Based on the analysis of 2 biological replicates, we identified several hundred *M. truncatula* genes as at least 2-fold differentially expressed in either interaction with a statistical significance of $P < 0.05$ (Table I), and these genes are included in Supplemental Table I. When comparing these gene expression profiles, we specified 203 genes as coinduced at least 2-fold in *G. mosseae*- as well as in *G. intraradices*-colonized *M. truncatula* roots (Supplemental Table II) and 176 genes as corepressed (Supplemental Table I). For 31 coinduced genes exclusively represented by expressed sequence tags (ESTs) from AM roots, we verified their origin using different approaches (Supplemental Table III). It turned out that two tentative consensus sequences (TCs) were derived from fungal ESTs, and these TCs were not considered further. A similarly low rate of fungal sequences among

Table I. *Overview of the results obtained from AM transcriptome profiling*

To study gene expression in AM, whole root systems of *M. truncatula* colonized with either *G. mosseae* or *G. intraradices* were harvested 4 weeks postinoculation. The table lists the number of probes with a log2 activation or repression ratio *M* larger than 1 or smaller than −1 (2-fold induction or repression), respectively, in relation to noninoculated reference samples grown under conditions of phosphate limitation. For each interaction, two independent biological replicates were studied. The values are based on probes in which at least 5 to 8 replicate spots remained after flagging for empty and poor spots and in which associated *P*-values were $P < 0.05$.

Samples	Induced Probes		Repressed Probes	
	$M > 1$	Maximum *M*	$M < -1$	Maximum *M*
G. mosseae-colonized roots	654	5.85	433	−7.76
G. intraradices-colonized roots	757	7.21	621	−6.86

mycorrhiza-specific TCs was reported by Liu et al. (2003a) for the *M. truncatula-Glomus versiforme* interaction. Our strategy to focus only on those 201 plant genes significantly coregulated at least 2-fold in the 2 interactions studied is supported by the fact that within the list of coinduced genes, we identified well-studied AM-related marker genes. These include the phosphate transporter MtPt4 (Harrison et al., 2002), the germin-like protein MtGlp1 (Doll et al., 2003), the glutathione *S*-transferase MtGst1 (Wulf et al., 2003), the Ser carboxypeptidase MtScp1 (Liu et al., 2003a), the hexose transporter MtSt1 (Harrison, 1996), the 1-deoxy-D-xylulose 5-phosphate synthase MtDXS2 (Walter et al., 2002), and a multifunctional Nodulin 26-like aquaporin (Brechenmacher et al., 2004).

Based on comparisons to the current releases of the PIR and TrEMBL databases as well as Interpro searches, we reannotated the genes that were differentially expressed in both AM interactions. Subsequently, the proteins encoded by AM-induced *M. truncatula* genes were grouped into functional categories according to Journet et al. (2002). This classification (Fig. 1) illustrates that the genes identified in this study as transcriptionally activated in AM roots specify proteins relevant for different cellular, metabolic, and regulatory processes of AM formation in *M. truncatula*, and these are discussed in the subsequent paragraphs.

AM-Induced Genes Associated with Cell Wall Degradation and Modification

The colonization of a root by AM fungi is accompanied by the reorganization of cell walls and extracellular matrices during (1) the penetration of the epidermis subsequent to appressoria formation, (2) the inter- and intracellular growth of fungal hyphae in the root cortex, and (3) the differentiation of membrane and cell wall structures surrounding arbuscules. It thus makes sense that 15 coinduced genes (Table II) encode a range of enzymes implicated in cell wall degradation and modification, catalytic functions that were reported to be relevant during interactions of plants with AM fungi (Peretto et al., 1995; Liu et al., 2003a).

Concerning cell wall degrading enzymes, 3 different (endo)-glucanases with different preferences for sugar bonds (TC88229, TC81637, and TC86689) as well as 4 different pectolytic or polygalacturonate-degrading enzymes (TC78420, TC88957, TC82059, and TC80800) were coinduced. In addition to an α-D-xylosidase (TC87560) involved in the degradation of complex carbohydrates, these enzymatic functions could modify the extracellular matrix during inter- or intracellular fungal spread as well as during the formation of the periarbuscular matrix, as proposed for the MtCel1 gene in *G. versiforme*-colonized roots (Liu et al., 2003a).

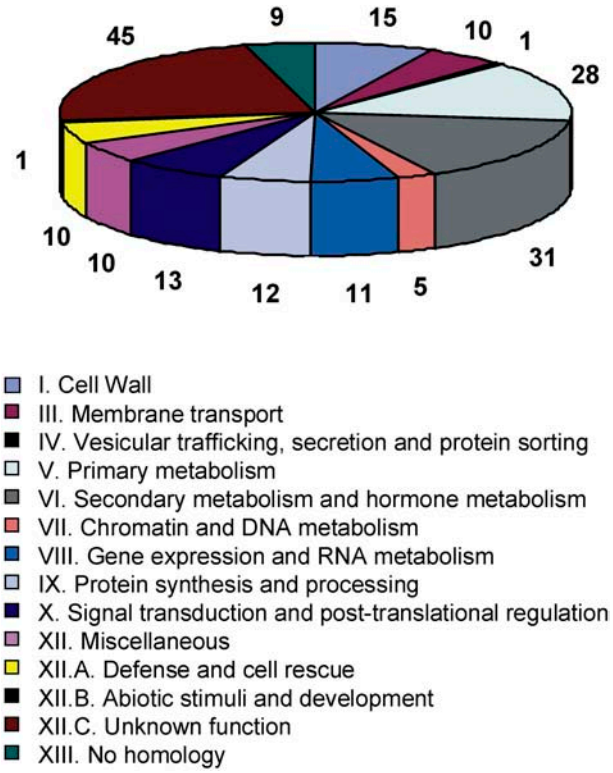

Figure 1. Classification of 201 plant genes found to be at least 2-fold induced in *M. truncatula* roots colonized by the arbuscular mycorrhizal fungi *G. mosseae* and *G. intraradices*. Proteins encoded by AM-induced genes were grouped into the functional categories as defined by Journet et al. (2002). The number of genes allocated to each functional category is indicated, and the functional categories are defined.

Table II. *AM-induced M. truncatula genes related to cell wall degradation and modification*

Genes activated more than 2-fold in both AM interactions are sorted according to the induction level in *G. mosseae*-colonized roots. Oligo ID, Identifier of *M. truncatula* 70-mer oligonucleotides. TIGR ID, Identifier in the TIGR *M. truncatula* Gene Index. Annotation, Updated annotations according to blast 2x hits in PIR and TrEMBL databases as well as Interpro searches. FC, Functional categories as defined by Journet et al. (2002) and in Figure 1. GM and GI, log2 expression ratios in *G. mosseae*- and *G. intraradices*-colonized roots. E-Northern, Expression profiles from the TIGR *M. truncatula* Gene Index. Myc-specific, TIGR TC is exclusively composed of ESTs from AM roots; Myc-induced, TIGR TC is predominantly composed of ESTs from AM roots; Sym-induced, TIGR TC is predominantly composed of ESTs from root nodules, nodulated roots and AM roots; Myc-expressed, TIGR TC is composed of at least one EST from AM roots. Literature, Found to be AM induced in other expression profiling studies; M, Manthey et al. (2004).

Oligo ID	TIGR ID	Annotation	FC	GM	GI	Literature	E-Northern
MT008649	TC87796	Pro-rich protein	I	2.22	1.18		
MT007463	TC77106	Osmotin/thaumatin-like protein	I	2.01	1.18		Myc-expressed
MT003502	TC88229	β-1,3-Glucanase	I	1.97	1.41		
MT001341	TC78420	Pectinesterase	I	1.56	1.41		Myc-expressed
MT003194	TC81637	Endo-1,4-β-glucanase	I	1.51	1.83		
MT000669	TC77589	Arabinogalactan	I	1.42	1.08	M	Myc-expressed
MT002404	TC88957	Polygalacturonase	I	1.34	2.17		
MT000216	TC76827	Pro-rich protein	I	1.29	1.54		Myc-expressed
MT006917	TC82059	Pectinesterase	I	1.22	2.04		
MT007159	TC85575	Arabinogalactan	I	1.22	1.14		
MT000950	TC86689	Endo-1,3–1,4-β-D-glucanase	I	1.16	1.58		Sym-induced
MT007032	TC85309	Extensin	I	1.11	1.64		Sym-induced
MT008602	TC87560	α-D-Xylosidase	I	1.11	1.47		
MT014699	TC89301	Reversibly glycosylated polypeptide (RGP1)	I	1.04	1.20		Myc-expressed
MT002974	TC80800	Pectate lyase	I	1.02	1.58		

The identification of a gene encoding a reversibly glycosylated polypeptide (TC89301) suggests that biosynthesis of xyloglucan and other hemicelluloses is relevant during colonization of roots with AM fungi.

Apart from carbohydrate modification, the identification of 3 genes encoding different Pro-rich proteins and extensins (TC87796, TC76827, and TC85309) as well as 2 genes specifying arabinogalactans (TC77589 and TC88575) point to cell wall alterations by the incorporation of structural or glycosylated proteins, similar to the observation of van Buuren et al. (1999), who localized arabinogalactan gene expression in arbuscule-containing cells of *M. truncatula* roots. Cell wall modification is also evident from the identification of a gene encoding an osmotin/thaumatin-like protein (TC77106). These proteins are known to be resistant to proteases as well as to denaturation, and the corresponding genes are described to be induced in response to osmotic stress or in response to fungi (Ng, 2004).

AM-Induced Genes Associated with Protein Degradation and Plant Defense

Cellular processes related to protein synthesis and processing as well as defense and cell rescue (Table III) were reported to be activated during AM (Dumas-Gaudot et al., 1994; Gianinazzi-Pearson et al., 1996; Salzer et al., 2000).

Concerning protein synthesis, we found evidence for the AM-induced expression of different ribosomal proteins, a finding consistent with an observation of Journet et al. (2001) based on digital expression profiling and, most interestingly, the strong coinduction of a translation initiation factor (TC91847). With respect to protein processing, 3 Ser carboxypeptidases (TC85938, TC85937, and TC79071), a subtilisin proteinase (TC89543), a Cys protease (TC90718), and an oligopeptidase (TC80768) were coinduced. Among these, the arbuscule-induced Ser carboxypeptidase MtScp1 (TC85937; Liu et al., 2003a) was activated most strongly, and 2 signal peptidases (TC77675 and TC88029), one of them also identified by Wulf et al. (2003), could be involved in AM-specific protein translocation to subcellular compartments. In view of the strong induction of proteases, it is tempting to speculate that protein processing is important for arbuscular formation or a reestablishment of cellular structures after arbuscule degradation, processes that occur in parallel during AM.

With respect to defense and cell rescue, we detected 4 different (Kunitz-type) protease inhibitors (TC78105, TC83316, TC86086, and TC84602), a Cys-rich antifungal protein (TC77480), and a xyloglucan-specific fungal endoglucanase inhibitor (TC78600). These proteins could fine-tune protease activity during arbuscule degradation or modulate plant defense responses elicited by the intraradical presence of fungal hyphae. Defense-related gene expression is well documented in AM roots (Salzer et al., 2000) and was also found in other transcriptome profiling studies (Liu et al., 2003a; Brechenmacher et al., 2004).

AM-Induced Genes Encoding Nutrient Transporters

In arbuscular mycorrhizae, the extraradical fungal mycelium acts as an extension of the roots and reaches beyond the root depletion zone, enabling a thorough exploration of the soil for limiting nutrients such as

Table III. *AM-induced M. truncatula genes related to protein degradation and plant defense*

Genes activated more than 2-fold in both AM interactions are sorted according to the induction level in *G. mosseae*-colonized roots. Abbreviations are as defined for Table II. Literature, Found to be AM induced in other expression profiling studies; K, Küster et al. (2004); W, Wulf et al. (2003); L, Liu et al. (2003a).

Oligo ID	TIGR ID	Annotation	FC	GM	GI	Literature	E-Northern
MT009186	TC85938	Ser carboxypeptidase	IX	4.36	2.80	L	Myc-specific
MT009185	TC85937	Ser carboxypeptidase MtScp1	IX	3.99	4.46	L	Myc-specific
MT002165	TC79071	Ser carboxypeptidase	IX	2.39	2.65		
MT012331	TC91847	Translation initiation factor	IX	2.21	2.35		
MT015804	TC89543	Subtilisin-like proteinase	IX	1.76	1.11		
MT007217	AW329656	Ribosomal protein L5	IX	1.75	1.18		
MT011611	TC77675	Signal peptidase	IX	1.67	2.29	W, K	Myc-specific
MT015663	TC88029	Signal peptidase	IX	1.55	1.21		Sym-induced
MT015467	TC86655	40S ribosomal protein S12	IX	1.51	1.82		Myc-expressed
MT002953	TC80768	Prolyl oligopeptidase	IX	1.44	1.15		
MT007237	TC76882	40S ribosomal protein S21	IX	1.33	1.14		Myc-expressed
MT014816	TC90718	Cys protease	IX	1.21	2.08		Sym-induced
MT014645	TC77480	Cys-rich antifungal protein 1	XII.A	4.61	5.34	K	Myc-specific
MT006798	TC78015	Miraculin-trypsin inhibitor Kunitz	XII.A	4.32	4.12	W	Myc-specific
MT013028	TC83316	Protease inhibitor	XII.A	3.62	2.97		Myc-specific
MT015420	TC86086	Kunitz-type proteinase inhibitor	XII.A	3.53	4.39	K	Sym-induced
MT015000	TC84602	Kunitz-type proteinase inhibitor	XII.A	2.62	4.34	K	Myc-specific
MT008080	TC85804	Polygalacturonase-inhibiting protein	XII.A	2.18	2.16		Sym-induced
MT007836	TC86633	LRR protein precursor	XII.A	1.73	1.83		
MT007790	TC86418	SAM:salicylic acid carboxylmethyltransferase	XII.A	1.29	2.05		Myc-expressed
MT015651	TC79023	Resistance protein	XII.A	1.12	1.14		
MT002474	TC78600	Xyloglucan-specific fungal endoglucanase inhibitor	XII.A	1.07	2.07		

phosphorus and minerals (Smith and Read, 1997; Pfeffer et al., 1999). The extensive periarbuscular membrane interface containing plant and fungal H^+-ATPases (Gianinazzi-Pearson et al., 2000; Krajinski et al., 2002; Requena et al., 2003) supports the suggestion that nutrient transfer in particular takes place at this interface, although some studies have shown that nutrient transfer may additionally occur at the intracellular hyphae (Gianinazzi-Pearson, 1996). Nevertheless, the molecular mechanisms underlying the translocation of nutrients in AM are presently not well understood.

In our global profiling approach, we anticipated to obtain information on transport processes relevant for nutrient allocation between fungus and host. We identified 10 genes encoding putative membrane transport proteins (Table IV) as coinduced at least 2-fold in both mycorrhizal associations, and among these, 3 that were previously described to be mycorrhiza induced. Acting as a marker gene, the arbuscule-specific phosphate transporter MtPt4 was strongly induced in both endosymbioses. MtPt4 transcripts and proteins were shown to be most prominent in mature arbuscules, and MtPt4 expression was reported to be positively correlated with the extent of *G. versiforme* colonization (Harrison et al., 2002). Another mycorrhiza-induced gene (TC86110) encodes a previously identified membrane-intrinsic multifunctional aquaporin (Küster et al., 2004; Manthey et al., 2004; Sanchez et al., 2004). One of the two coinduced sugar transporters (TC77798 and TC87421) represents the Mtst1 gene that is known to be highly expressed in arbuscule-containing cells and in cortical cells surrounding infected areas (Harrison, 1996).

Four coinduced genes encoded the first AM-related nitrate transporters (TC78157, TC78158, TC80954, and TC84545) identified in *M. truncatula*. These findings add to a report on an AM-induced nitrate transporter in tomato (Hildebrandt et al., 2002) and suggest mechanisms not only supporting the uptake of ammonium but also the acquisition of nitrate during AM. So far, only an *M. truncatula* nitrate transporter gene (TC79437) down-regulated in roots colonized by *G. mosseae* was identified (Burleigh, 2001) and in our experiments, this repression was confirmed not only in *G. mosseae*- but also in *G. intraradices*-colonized roots. Concerning ion transporters of the MtZIP family (Burleigh et al., 2003), we describe the first mycorrhiza-induced manganese transporter (MtZIP7, TC88701) whose transport properties were recently characterized in yeast expression systems (López-Millán et al., 2004).

It is assumed that a modulation of transporter gene expression may be related to changes in the internal micro- or macronutrient concentrations (Liu et al., 1998). Thus, the coordinated suppression and induction of genes seems to be a common feature reflecting a switch in nutrient supply from direct root uptake to symbiotic uptake. In mycorrhizal roots, the symbiotic pathway accounts for most if not all of the total inorganic phosphate (P_i) acquisition (Pearson and Jakobsen, 1993) and recent studies support a

Table IV. *AM-induced M. truncatula genes related to membrane transport*

Gene activated more than 2-fold in both AM interactions are sorted according to the induction level in *G. mosseae*-colonized roots. Abbreviations are as defined for Table II. Literature, found to be AM induced in other expression profiling studies: M, Manthey et al. (2004); K, Küster et al. (2004); H96, Harrison (1996); S, Sanchez et al. (2004); H02, Harrison et al. (2002); W, Wulf et al. (2003).

Oligo ID	TIGR ID	Annotation	FC	GM	GI	Literature	E-Northern
MT009707	TC85743	Inorganic phosphate transporter MtPt4	III	4.98	5.06	H02, W, K	Myc-specific
MT002501	TC78158	High-affinity nitrate transporter	III	4.76	1.03		
MT009589	TC78157	High-affinity nitrate transporter	III	2.51	1.14		Myc-expressed
MT007526	TC86110	Multifunctional Nodulin 26-like aquaporin	III	2.06	1.97	K, M, S	Myc-expressed
MT008596	TC87421	Hexose transporter MtSt1	III	1.42	2.05	H96	Myc-expressed
MT002866	TC88701	Manganese transporter MtZIP7	III	1.32	1.76		
MT003256	TC80954	High-affinity nitrate transporter	III	1.21	1.91		
MT001072	TC77763	Proton pump interactor	III	1.14	3.07		Myc-expressed
MT007819	TC77798	Hexose transporter	III	1.12	1.22		Myc-expressed
MT006556	TC84545	Nitrate transporter	III	1.01	1.18		

hypothesis that in the *M. truncatula-G. intraradices* symbiosis, the entire P_i was delivered via the mycorrhizal rather than the direct pathway (Smith et al., 2003a). In accordance with this, the 2 high-affinity phosphate transporters, MtPT1 and MtPT2, representing the direct pathway of P_i acquisition and shown to be down-regulated during both *G. versiforme* and *G. intraradices* AM (Versaw et al., 2002), were both repressed in *G. intraradices* AM. On the other hand, these genes were still slightly induced or not regulated during *G. mosseae* AM, suggesting possible differences in the regulation of phosphate transporters in different AM interactions. Apart from these genes, our study revealed an additional strongly cosuppressed P_i transporter gene (TC84790), which could be a representative of the direct uptake system in addition to MtPT1 and MtPT2.

Even on the basis of approximately 16,000 *M. truncatula* probes, MtPt4 remains the only phosphate transporter (PT) gene strongly induced in both AM analyzed, while several other members of the PT gene family were not. This exclusive focus on one single symbiotic PT gene agrees with Paszkowski et al. (2002), who identified an entire set of 13 high-affinity P_i transporter genes in *Oryza sativa*, in which only one (OsPT11) responded to mycorrhizal colonization. At present, this is also the case for *Lycopersicon esculentum* (LePT1) and *Solanum tuberosum* (StPT3; Liu et al., 1998; Rosewarne et al., 1999; Rausch et al., 2001). Thus, the evolution of a single AM-induced phosphate transporter gene might reflect a common strategy.

In contrast, this principle seems not to be favored for the regulation of nitrate transporter genes. Here, stringent conditions uncovered at least 4 genes as being coinduced in AM, while other nitrate transporter genes were down-regulated to different extents (TC88300 and TC82201). A similar situation of up- and down-regulation is evident for the hexose transporter gene family. While MtSt1 (Harrison, 1996) and the hexose transporter specified by TC77798 are coinduced, we identified counterparts in TC77077 and TC83509, both down-regulated by AM fungi. Surprisingly, the hexose transporter gene represented by TC77077 was previously described to be up-regulated during AM formation between *M. truncatula* and *G. versiforme* using macroarray hybridizations (Liu et al., 2003a). This raises the possibility that different AM fungi might activate different members of the hexose transporter gene family during symbiotic uptake mechanisms. Alternatively, different sugar transporters might be recruited during specific stages of the symbiosis. Whereas it seems likely that AM-induced mineral transporters function as nutrient importers to supply minerals to the plant, the situation is more complex with respect to up-regulated sugar transporters. Here, either an export function to supply hexoses to the symbiotic fungus or an import function to fine-tune the amount of carbohydrates allocated to the mycorrhizal fungus has to be considered.

AM-Induced Genes Associated with Primary Metabolism

The establishment of an arbuscular mycorrhiza significantly alters the metabolism of a plant root. In accordance with this, 28 genes up-regulated in both AM interactions encoded gene products related to primary metabolism (Table V). In plants, Suc serves as the major transport molecule for source-to-sink carbon allocation (Hawker, 1985). Since Suc is a stable disaccharide, it has to be cleaved into hexoses prior to further metabolic degradation by either Suc synthases or different invertases (Copeland, 1990), both regarded as key enzymes responsible for carbon partitioning. In roots colonized by AM fungi, there is evidence for an up-regulation of Suc synthase, soluble acid invertase,

Table V. *AM-induced M. truncatula genes related to primary metabolism*

Genes activated more than 2-fold in both AM interactions are sorted according to the induction level in *G. mosseae*-colonized roots. Abbreviations are as defined for Table II. Literature, Found to be AM induced in other expression profiling studies; K, Küster et al. (2004).

Oligo ID	TIGR ID	Annotation	FC	GM	GI	Literature	E-Northern
MT015669	TC88539	MtBcp1	V	4.28	4.89	K	Myc-specific
MT000134	TC76657	PSII oxygen-evolving complex protein 2	V	3.95	3.62		Myc-expressed
MT003225	TC87415	Bcp	V	2.94	2.35		Myc-specific
MT004678	TC82671	Cys synthase	V	2.81	4.54		
MT010383	TC90212	Leucoanthocyanidin dioxygenase	V	2.36	1.28		Sym-induced
MT004097	TC87185	Allyl alcohol dehydrogenase	V	2.36	2.12		Myc-induced
MT015668	TC88539	MtBcp1	V	1.94	2.68	K	Myc-specific
MT015793	TC81478	UDP-glucoronosyl/UDP-glucosyl transferase	V	1.91	1.62		
MT008396	TC78334	L-Ascorbate oxidase	V	1.72	1.67		
MT000147	TC76664	Patatin	V	1.70	1.27		
MT002011	TC88442	β-Hydroxyacyl-ACP dehydratase	V	1.60	1.86		
MT004744	TC90058	Putative dehydrogenase	V	1.42	1.31		Myc-expressed
MT000961	TC86557	Branched-chain amino acid aminotransferase	V	1.40	2.56		Myc-expressed
MT008259	TC87200	Acetylornithin aminotransferase	V	1.38	1.40		
MT000454	TC86072	Cys synthase	V	1.37	1.71		
MT007664	TC86426	L-Ascorbate oxidase	V	1.37	1.48		Myc-expressed
MT007807	TC86231	Protein disulfide isomerase	V	1.34	1.34		
MT004004	TC77871	Malonyl-CoA: acyl carrier protein transacylase	V	1.33	1.71		Myc-expressed
MT002830	TC80501	Chlorophyll *b* synthase	V	1.28	2.43		
MT004707	TC81108	Glucosyltransferase	V	1.22	1.72		
MT015293	TC85778	NFU1 iron-sulfur cluster assembly factor	V	1.21	1.66		
MT010141	TC80652	α-Fucosidase	V	1.20	1.66		
MT015305	TC85814	Ω-6 desaturase	V	1.20	1.31		Myc-expressed
MT000873	TC77795	Pyruvate dehydrogenase E1 α-subunit	V	1.19	1.59		Myc-expressed
MT000207	TC76829	Fructokinase	V	1.14	1.49		Sym-induced
MT007901	TC77591	Putative phosphatase	V	1.10	1.16		Sym-induced
MT000450	TC77284	Bcp	V	1.05	1.51		Sym-induced
MT007472	TC86035	Triacylglycerol lipase	V	1.02	1.15		Sym-induced

and alkaline invertase in regions surrounding arbuscules and even within arbuscule-containing cells (Blee and Anderson, 2002; Hohnjec et al., 2003; Ravnskov et al., 2003). Cleavage of Suc by either enzyme yields hexoses that after phosphorylation enter glycolysis and the tricarboxylic acid cycle. Opposite to the induction of genes corresponding to this pathway in root nodules (El Yahyaoui et al., 2004), the expression levels of most genes related to glycolysis and the tricarboxylic acid cycle are not significantly altered in AM on the basis of pooled tissue samples.

On the other hand, 4 genes encoding enzymes related to fatty acid metabolism were induced: malonyl-CoA: Acyl carrier protein transacylase (TC77871), β-hydroxyacyl-ACP dehydratase (TC88442), Ω-6 desaturase (TC85814), and triacylglycerol lipase (TC86035). These enzymes might be implicated in the biosynthesis and modification as well as the metabolic degradation of lipids from the membranes surrounding arbuscules. Since arbuscules are transient structures with a life span of only a few days, both establishment and degradation of periarbuscular membranes occur in parallel in mycorrhizal roots and require both an active fatty acid biosynthesis and the degradation of fatty acids.

Finally, 3 different members (TC88539, TC87415, and TC77284) of a gene family encoding different blue copper proteins (Bcp) were coinduced, 2 of which were already identified on the basis of cDNA microarrays (Küster et al., 2004). Bcps contain a single copper atom and are implicated in electron transfer reactions. The most characterized members of this class of proteins are chloroplastic plastocyanins that exchange electrons with cytochromes. So far, the role of Bcps in AM roots is elusive, but it is tempting to speculate that they are involved in electron transfer

processes taking place in the network of plastids surrounding arbuscules in mycorrhizal roots. Here, plastids are known to be essential compartments for fatty acid biosynthesis, nitrogen assimilation, and starch deposition (Fester et al., 2001). The cell-specific movement of plastids to a close vicinity of arbuscules is associated with extensive cytoskeleton reorganizations, supported by an arbuscule-related expression of the *M. truncatula* β-tubulin gene MtTubb1 (Manthey et al., 2004). These correlations prompted us to inspect the genomic organization of the Bcp gene family and we found that 7 genes encoding Bcps are located in tandem in a region of the *M. truncatula* genome represented by bacterial artificial chromosome (BAC) mth2-15c20 (GenBank accession no. AC126009). For one of these genes, designated MtBcp1 (TC88539), 1,180 bp of promoter sequence were PCR-amplified and used to evaluate the cellular localization of *gus*Aint expression in transgenic roots mycorrhized with *G. intraradices*. As shown in Figure 2, β-glucuronidase (GUS) staining is most prominent in regions where arbuscule formation takes place and correlates with the degree of mycorrhization. Intensely mycorrhized areas characterized by a high density of arbuscule-containing cells exhibited strongest reporter gene expression in the whole root tissue including outer cortical cells. Counterstaining with ink demonstrated that, depending on the mycorrhization status, pMtBcp1-driven *gus*Aint expression is strongest in the arbuscule-containing cells but is, although to a lesser extent, additionally present in adjacent cortical cells. Thus, the observed promoter activity indicates a correlation of MtBcp1 expression with root colonization and supports the assumption that Bcps serve as mediators of electron transfer processes in arbuscule-containing cells and their close vicinity.

AM-Induced Genes Associated with Secondary Metabolism and Hormone Action

Secondary metabolism and phytohormone biosynthesis are processes with major relevance for AM roots

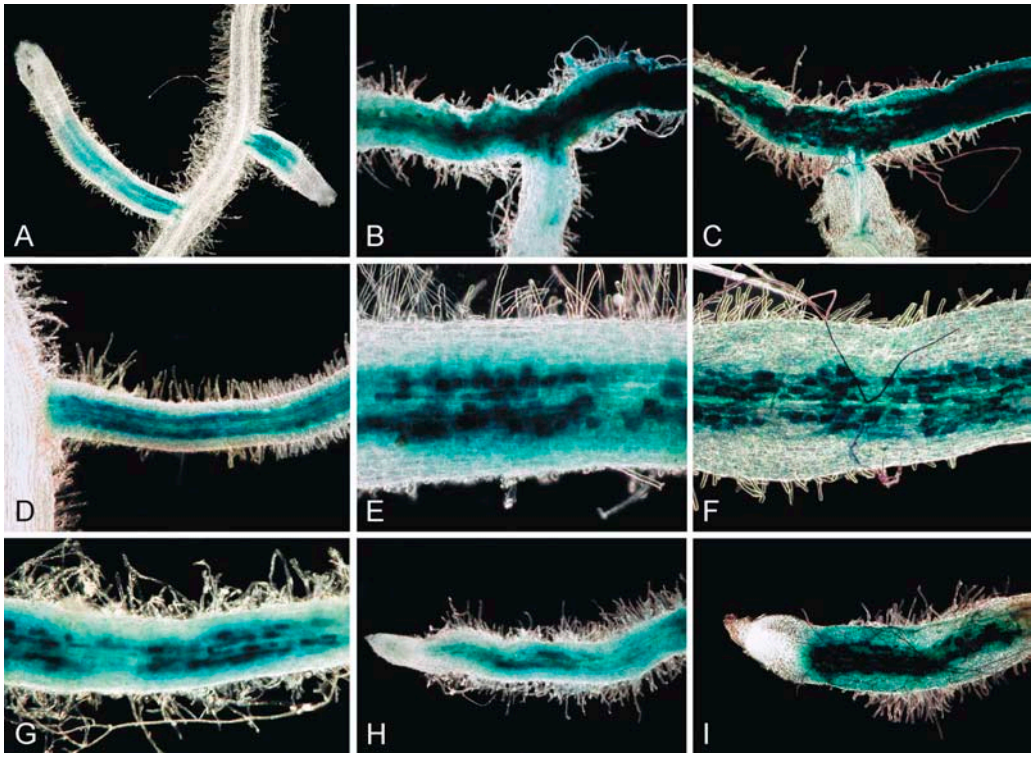

Figure 2. Histochemical localization of GUS activity in transgenic roots of *M. truncatula* colonized with *G. intraradices* and expressing the −1,181/-2 pMtBcp1-*gus*Aint fusion. Prior to dark-field microscopy, roots shown in A, B, D, E, G, and H were stained for GUS activity exclusively, while those in C, F, and I were additionally stained with ink to visualize fungal structures. A, Distinct GUS activity in cortical cells surrounding the vasculature of young, lateral roots. B, Root exhibiting strongest GUS staining on the right, extending to staining of lesser extent on the left. Here, intensive coloration is confined to inner cortical cells. C, Double-staining of the same mycorrhizal root fragment as shown in B. Fungal structures (arbuscules and hyphae) are most prominent in the intensely infected area on the right and decrease toward the left. Parts of the root that do not exhibit GUS coloration are free of fungal infection. D, E, G, and H, Strong local GUS expression represented by single cells of the innermost cortical layers and surrounded by cells that are stained to a lower extent and do not exhibit distinct dark spots. F, Double-stained root fragment. The ink staining reveals arbuscule-containing cells that overlay the single blue spots of intensively GUS coloration. I, Lateral root tip with infected parts corresponding to pMtBcp1-activated cells, surrounded by outer cortical cells exhibiting GUS staining without harboring fungal structures.

(Fester et al., 1999; Strack et al., 2003). In our study, apart from the AM-induced glutathione *S*-transferase gene MtGst1 (Bestel-Corre et al., 2002; Wulf et al., 2003) that might be involved in detoxification processes, 30 AM-activated genes (Table VI) encoded enzymes involved in the synthesis of secondary metabolites such as terpenes (TC76892 and TC80795), flavonoids (TC81553, TC85753, TC87789, and TC88553), ascorbate (TC78334), and amygdalin (TC76723), compounds known to be present in AM (Liu et al., 2003b; Strack et al., 2003).

Important members of the terpene family are carotenoid tetraterpenes, and their biosynthesis was reported to be partially controlled on the transcriptional level in mycorrhizal roots (Fester et al., 2002). It is well documented that cleavage products deriving from carotenoids are known to form the yellow pigment that is characteristic of AM roots in many plants (Strack et al., 2003). Two genes related to the biosynthesis of terpenes were identified here: TC77051 encoding mevalonate disphosphate decarboxylase, an important component of the mevalonate pathway of carotenoid production, and TC78589 encoding 1-deoxy-D-xylulose 5-phosphate synthase 2 (DXS2; Walter et al., 2002), a key enzyme of the mevalonate-independent pathway of carotenoid biosynthesis (Strack et al., 2003). Although either pathway results in the formation of isopentenyl diphosphate, the key intermediate of terpene biosynthesis, only the DXS2-related pathway

Table VI. *AM-induced M. truncatula genes related to secondary metabolism and hormone action*

Genes activated more than 2-fold in both AM interactions are sorted according to the induction level in *G. mosseae*-colonized roots. Abbreviations are as defined for Table II. Literature, Found to be AM induced in other expression profiling studies; K, Küster et al. (2004); S, Sanchez et al. (2004); W, Wulf et al. (2003).

Oligo ID	TIGR ID	Annotation	FC	GM	GI	Literature	E-Northern
MT009013	TC85868	Glutathione *S*-transferase MtGst1	VI	5.02	5.86	W, S, K	Myc-specific
MT004625	TC81595	Ent-kaurene synthase A	VI	3.25	2.05		Myc-expressed
MT006682	TC84637	Cytochrome P450	VI	3.12	1.12		
MT000228	TC85641	Cytochrome P450	VI	2.45	2.61		Myc-expressed
MT000634	TC77465	Auxin-regulated protein GH3	VI	2.44	3.13		
MT002829	TC89260	Cytochrome P450	VI	2.43	3.00	K	Myc-specific
MT003018	TC88553	Flavanone 3-hydroxylase	VI	2.43	1.64		
MT015424	TC76892	Terpene synthase	VI	2.38	2.45		Sym-induced
MT007595	TC77355	SRG1-like oxidoreductase (ethylene-forming enzyme)	VI	2.23	2.68		
MT000541	TC86263	Δ-Aminolevulinic acid dehydratase	VI	2.16	2.01		Myc-expressed
MT007257	TC76942	Narbonin	VI	1.84	1.45		Sym-induced
MT000424	TC77154	Narbonin	VI	1.81	1.30		Sym-induced
MT003200	TC80866	Narbonin	VI	1.69	1.37		
MT008518	TC78110	SRG1-like oxidoreductase (ethylene-forming enzyme)	VI	1.50	1.12		Sym-induced
MT006856	TC78989	Cytochrome P450	VI	1.49	1.38		Myc-expressed
MT003010	TC89135	Cytochrome P450	VI	1.42	1.52		Myc-expressed
MT003219	TC80795	5-α-Taxadienol-10-β-hydroxylase	VI	1.35	1.05		Sym-induced
MT002731	TC78764	1-Aminocyclopropane-1-carboxylic acid oxidase	VI	1.33	1.79		Sym-induced
MT015692	TC88126	Cytochrome P450	VI	1.27	1.73		
MT000598	TC85270	Benzoyl-CoA:benzyl alcohol benzoyl transferase	VI	1.25	1.38		Myc-expressed
MT015525	TC78048	GA-regulated protein GASA4	VI	1.24	1.54		
MT003683	TC81553	Flavonoid 3′-hydroxylase	VI	1.23	1.04		
MT015597	TC87789	UDP-glycose:flavonoid glycosyltransferase	VI	1.21	1.43		
MT005972	TC78620	Cytochrome P450	VI	1.19	1.38		Myc-expressed
MT003693	TC89459	Oxidase-like protein	VI	1.18	1.29		
MT007295	TC85753	Flavon momnoamine oxidase	VI	1.11	1.02		Sym-induced
MT013911	TC90420	Chalcone *O*-methyltransferase	VI	1.11	1.43		Sym-induced
MT002771	TC78589	DXS2	VI	1.08	1.27		Myc-expressed
MT000153	TC76723	Amygdalin hydrolase isoform AH I	VI	1.05	2.51		Sym-induced
MT000313	TC77051	Mevalonate disphosphate decarboxylase	VI	1.03	1.72		Sym-induced
MT002711	TC78989	Cytochrome P450	VI	1.03	1.44		Myc-expressed

was previously reported to be AM induced and related to mycorrhadicin production (Walter et al., 2002).

Strikingly, and in line with the induction of a range of genes associated with secondary metabolism, 8 different cytochrome P450 genes were identified as AM induced. P450-type cytochromes comprise a range of different families, and are, for example, involved in the oxidation of different isoflavonoids, phenylpropanoid metabolites characteristic of legumes (Dixon and Sumner, 2003). Recently, Liu et al. (2003b) reported on P450 cytochromes that function as isoflavone 2′- and 3′-hydroxylases, and similar to our observations, one of them showed elevated expression during AM. Interestingly, the action of hydroxylated isoflavonoids, e.g. the phytoalexin medicarpin, is related to pathogen- as well as insect-induced responses, respectively (Dixon, 1999), and the synthesis of the phytoalexin medicarpin was shown to be transiently induced also during AM (Harrison and Dixon, 1993).

Concerning phytohormone biosynthesis and action, different coinduced genes can be related to GA_3, auxin, and ethylene. In case of GA_3, genes were up-regulated that specify the GA biosynthesis enzyme ent-kaurene synthase A (TC81595) and the GA-regulated protein GASA 4 (TC78048), indicating the synthesis of GA_3 in AM roots. This is opposite to the situation in root nodules, where a down-regulation of these genes was reported (El Yahyaoui et al., 2004). With respect to auxin hormones, the auxin-regulated protein GH3 (TC77465) was induced. Finally, AM-induced genes involved in ethylene biosynthesis were identified: 2 ethylene-forming enzymes (TC77355 and TC78110) and an aminocyclopropane carboxylic acid oxidase (TC78764). Whereas auxin biosynthesis was reported to occur in AM roots (Kaldorf and Ludwig-Müller, 2000), data on the effect of ethylene and GA_3 on AM formation are conflicting and rather limited (El Ghachtouli et al., 1996; Geil and Guinel, 2002). Nevertheless, the expression data reported here support the involvement of these phytohormones at least during particular stages of AM, as shown for cytokinins, abscisic acid, or jasmonic acid (Strack et al., 2003).

AM-Induced Genes Encoding Components of Signal Transduction Pathways

With *dmi1*, *dmi2*, and *dmi3*, 3 plant genes relevant for the common early stages of signal transduction during nodulation and AM formation were identified by positional cloning (Cullimore and Dénarié, 2003). Apart from the well-studied *dmi* genes, several mutations with specific defects in AM development were identified (Marsh and Schultze, 2001), but in most cases, the genes responsible for the mycorrhiza-deficient phenotypes are not known. Thus, there is a need to increase the knowledge on genetic determinants of plant signal perception prior to the action of *dmi* genes as well as on genes involved in signaling during later stages of AM development.

In this study, we identified 13 such genes as induced in both *G. mosseae*- and *G. intraradices*-colonized roots, and these can be related to different aspects of plant signal perception and signal transduction (Table VII). First, 2 genes (TC78350 and TC87043) encoding different lectins were strongly up-regulated in AM, and this expression pattern is supported by data from cDNA-based microarrays (Küster et al., 2004), by the identification of corresponding ESTs in a *G. intraradices* SSH library (Wulf et al., 2003), and by digital expression profiling (Supplemental Table II). Legume lectins were described as determinants of specificity during nodule symbioses by gain-of-function studies (van Rhijn et al., 1998), and their strong activation in AM roots points to a role during signal perception also in AM.

Concerning AM-related receptors, genes encoding a Ser/Thr receptor kinase (TC86597) and a Leu-rich

Table VII. *AM-induced M. truncatula genes related to signal transduction*

Genes activated more than 2-fold in both AM interactions are sorted according to the induction level in *G. mosseae*-colonized roots. Abbreviations are as defined for Table II. Literature, Found to be AM induced in other expression profiling studies; K, Küster et al. (2004).

Oligo ID	TIGR ID	Annotation	FC	GM	GI	Literature	E-Northern
MT003520	TC78350	Man/Glc-binding lectin	X	4.35	5.20		Myc-induced
MT013816	TC87043	Man/Glc-binding lectin	X	3.87	2.92	K	Myc-specific
MT006551	TC82283	Putative phytosulfokine LRR-type receptor kinase	X	2.55	4.02		
MT004715	TC80630	Ser/Thr protein kinase	X	2.05	1.71		Myc-specific
MT014872	TC89285	NBS/LRR resistance protein	X	1.99	3.68		Myc-specific
MT005914	TC93498	33-kD secretory protein	X	1.78	1.37		Myc-expressed
MT014665	TC86597	Ser/Thr receptor kinase	X	1.77	2.52		Sym-induced
MT003662	TC80490	Ser/Thr protein kinase	X	1.28	1.04		Myc-expressed
MT005857	TC92439	Phosphoinositol-specific phospholipase C	X	1.24	4.89		
MT002595	TC80104	LRR receptor-like protein kinase	X	1.15	3.03		Sym-induced
MT006436	TC93373	ERG GTPase	X	1.14	1.05		Myc-induced
MT007943	TC86792	Putative two-component response regulator	X	1.08	2.33		Myc-expressed
MT003494	TC88105	Ser/Thr protein kinase	X	1.02	1.59		

repeat (LRR) receptor-like protein kinase (TC80104) were identified. LRR-type receptor-like kinases are characterized by extracellular LRR domains mediating protein-protein interactions. These receptors act by binding extracellular ligands and transducing this signal to intracellular protein kinase domains (Chen, 2001). In addition to classical receptor-like kinases, a gene (TC93498) encoding a homolog of the Arabidopsis (*Arabidopsis thaliana*) 33-kD Cys-rich secretory proteins deserves attention. These proteins resemble extracellular domains of receptor-like protein kinases, but in contrast to these, they do not contain a transmembrane and an intracellular protein kinase domain. Thus, it was proposed that they may interact with membrane-bound receptor-like protein kinases during signal perception (Chen, 2001). The identification of a putative phytosulfokine LRR-type receptor kinase (TC82283) seems also to be noteworthy, since phytosulfokine constitutes a sulfated intercellular peptide signal during cellular differentiation and proliferation in plants (Matsubayashi et al., 2002).

With respect to signal transduction cascades initiated after signal perception, we identified an AM-induced gene (TC92439) encoding a phosphoinositol-specific phospholipase C, an enzyme that generates the second messengers inositol triphosphate and diacylglycerol through hydrolysis of membrane-bound PIP_2. The second messengers initiate further signal transduction events, e.g. the release of Ca^{2+} from intracellular stores, a process relevant during initial stages of Nod-factor perception during nodulation. Since plant phosphoinositol-specific phospholipase Cs mediate different stress and pathogen responses (Repp et al., 2004), an involvement in signaling during AM is also possible. Downstream components of signal transduction cascades are protein kinases that, for example, facilitate signal amplification, and here we identified 3 different AM-induced genes (TC80630, TC80490, and TC88105) coding for Ser/Thr protein kinases with possible relevance for this process.

During initial stages of AM formation, Myc-factors are postulated to be perceived by the plant (Cullimore and Dénarié, 2003), but the molecular processes mediating this perception are unknown. Subsequent to the very early stages of signaling, diffusible low-M_r compounds activate expression of the early nodulin gene ENOD11 (Kosuta et al., 2003), and the AM-induced genes reported here are candidates for legume genes required for the recognition and transduction of such signals.

AM-Induced Genes Encoding Transcriptional Regulators

As detailed above, the colonization of plant roots by AM fungi results in an extensive reorganization of cellular structures and in specific alterations of metabolism. These changes prerequisite differential gene expression, a process primarily mediated by transcriptional regulators. In accordance with this, 11 coinduced genes encoding transcription factors were identified (Table VIII). Highest transcript accumulation was detected for a Myb-family transcription factor (TC78253) already identified as induced by different AM fungi using macroarrays (Liu et al., 2003a; Küster et al., 2004; Sanchez et al., 2004). Similarly, a TINY AP2 domain transcription factor gene (TC78355) induced during *M. truncatula-G. versiforme* associations (Liu et al., 2003a) exhibited elevated expression in *G. mosseae* and *G. intraradices* AM.

Among the other regulatory genes, we identified another putative Myb family transcription factor (TC77052). So far, only the Myb gene Mt-phan was described in *M. truncatula*, and this gene was expressed in lateral root initials, in nematode-induced giant cells, and in root nodules (Koltai et al., 2001). Apart from genes encoding Myb transcription factors, a homeobox-Leu zipper (TC91273) and a basic-Leu zipper (bZIP) transcription factor (TC81463) were identified. Both families of proteins are involved in pathogen defense (Zhou et al., 2000) and hormone action (Fukazawa et al., 2000). Interestingly, members of the TGA class of bZIP factors were discussed to regulate auxin-induced glutathione *S*-transferases (Johnson et al., 2001), and with MtGst1,

Table VIII. *AM-induced M. truncatula genes encoding transcriptional regulators*

Genes activated more than 2-fold in both AM interactions are sorted according to the induction level in *G. mosseae*-colonized roots. Abbreviations are as defined for Table II. Literature, Found to be AM induced in other expression profiling studies; K, Küster et al. (2004); S, Sanchez et al. (2004); L, Liu et al. (2003a).

Oligo ID	TIGR ID	Annotation	FC	GM	GI	Literature	E-Northern
MT001930	TC78235	Myb-like transcription factor	VIII	5.15	3.09	L, S, K	Myc-specific
MT007392	TC77052	Myb-family transcription factor	VIII	2.15	1.26		Myc-expressed
MT011784	TC91273	Homeodomain-zip protein	VIII	1.96	1.27		Myc-expressed
MT006389	TC92797	Zinc-finger, CCHC type	VIII	1.86	1.47		
MT005076	TC92211	AT-rich interaction domain protein	VIII	1.82	1.72		Sym-induced
MT005698	TC92089	YABBY protein transcription factor	VIII	1.74	3.36		Myc-specific
MT001444	TC78204	Zinc-finger RNA binding protein	VIII	1.70	1.16		Myc-expressed
MT002458	TC79248	Zinc-finger RNA binding protein	VIII	1.22	1.14		Myc-expressed
MT005542	TC92282	Zinc-finger, C2H2 type	VIII	1.20	1.94		Myc-specific
MT001680	TC78355	TINY-like protein	VIII	1.16	2.91	L	Sym-induced
MT003698	TC81463	bZIP transcription factor	VIII	1.04	1.50		Sym-induced

an AM-induced glutathione *S*-transferase (TC85868) was recently characterized by Wulf et al. (2003).

TC92089 corresponds to a strongly induced gene encoding a YABBY transcription factor. These regulators form a small protein family known to be responsible for the specification of abaxial cell fate in Arabidopsis lateral organs as well as axis formation (Bowman et al., 2002). Additional genes (e.g. TC92282) encoded DNA- or RNA-binding proteins containing zinc fingers, suggesting a role for these proteins in the regulation of gene expression in AM.

From 5 genes that were activated more than 2-fold in the opposite direction in the 2 AM interactions studied (Supplemental Table I), 3 genes specified a Myb transcription factor (TC86301), a RING zinc finger protein (TC89100), and an AP2-domain DNA-binding protein (TC88292). The identification of differentially regulated genes encoding putative transcription factors supports the observation that in addition to common genetic mechanisms, specific sets of host genes are induced by different AM fungi.

In Arabidopsis, different families of transcription factors, each containing distinct DNA binding domains, were implicated in plant stress responses since their expression is modulated under particular stress conditions (Shinozaki and Yamaguchi-Shinozaki, 2000). This supports the notion that in addition to mediating early signal transduction during fungal colonization, the AM-induced transcription factors reported here could regulate cellular responses evoked by the intraradical presence of these two microsymbionts, processes certainly placed downstream of the initial recognition of mycorrhizal symbionts. With respect to this, there is increasing evidence for the existence of regulatory mechanisms that govern arbuscule-specific gene expression (Harrison et al., 2002; Doll et al., 2003; Liu et al., 2003a; Hohnjec et al., 2003; Wulf et al., 2003; Vieweg et al., 2004), and together with the components of signal transduction mentioned above, the AM-induced transcriptional regulators could e.g. mediate gene expression in arbuscule-containing cells.

AM-Induced Genes Encoding Proteins of Unknown Function or No Homology

A total of 54 genes up-regulated in AM roots either encode gene products matching hypothetical gene products, proteins only characterized by Interpro domains, or proteins with no homology (Supplemental Table II). Among these are four putative F-box containing proteins and a putative ubiquitin C-terminal hydrolase, possibly related to protein degradation and processing via the ubiquitin ligase complex. Protein degradation apart from general protein turnover processes is known to play important regulatory roles (Hellmann and Estelle, 2002) by controlling protein degradation during the differentiation of symbiotic structures. Heavy metal detoxification is another important feature of mycorrhizal roots, also with respect to phytoremediation (Vassilev et al., 2004). A coinduced gene (TC78576) encoding a putative heavy metal transport/detoxification protein is thus of interest, and the encoded protein could be involved in the uptake of heavy metals from the soil.

Digital Expression Profiling Generally Validates AM-Related Gene Expression

Digital expression profiling approaches have become increasingly popular not only for identification of differentially expressed genes, but also for the validation of high-throughput expression profiling data (Alba et al., 2004). In the case of *M. truncatula*, almost 190,000 ESTs derived from more than 40 different conditions are deposited in The Institute for Genomic Research (TIGR) *M. truncatula* Gene Index, and among these, 21,049 ESTs are derived from arbuscular mycorrhiza roots (http://www.tigr.org/tdb/tgi/mtgi). As shown in Supplemental Table II, apart from typical mycorrhiza-specific marker genes (e.g. MtPt4, Harrison et al., 2002; MtDxs2, Walter et al., 2002; MtScp1, Liu et al., 2003a; MtGst1, Wulf et al., 2003; MtGlp1, Doll et al., 2003), most genes reported here to be up-regulated in AM roots are either mycorrhiza specific, mycorrhiza induced, or are at least expressed in cDNA libraries constructed from mycorrhizal roots. In addition, digital expression profiling indicated that several genes are up-regulated in endosymbioses including AM. Due to the different biological material and the different growth conditions of plants used for cDNA library construction and considering that with *G. versiforme* a different fungus was used to obtain 7,351 of the 21,049 AM ESTs (Liu et al., 2003a), these in silico data generally support our expression profiles.

Real-Time RT-PCR Experiments Support the AM-Induced Expression of Selected Genes

Using 4 additional biological samples of *G. mosseae* and *G. intraradices* colonized roots in comparison to nonmycorrhized control roots, we performed real-time RT-PCR experiments to verify the expression of 20 coinduced genes corresponding to a range of functional categories and expression ratios (Table IX). Based on these samples, we confirmed the AM-induced expression for 19 of 20 genes tested. Whereas 15 genes were coinduced more than 2-fold in either interaction, 4 genes were identified as coinduced more than 1.7-fold in *G. mosseae* and more than 2-fold in *G. intraradices* colonized roots (Table IX). In some cases, expression ratios based on real-time RT-PCR were significantly higher than those ratios obtained from microarray hybridizations, which is a common phenomenon for specifically expressed genes. In other cases, expression ratios obtained by real-time RT-PCR were comparably low. Similar to results reported by Manthey et al. (2004), this was evident in particular for those genes only weakly induced on the basis of microarray hybridizations. A low induction ratio had

Table IX. *Verification of AM-induced M. truncatula genes by real-time RT-PCR*

The expression of 20 AM-induced genes belonging to a range of functional categories was verified by real-time RT-PCR. Expression ratios are given as log2 values to allow a comparison to the expression ratios derived from microarray hybridizations. All expression ratios except one are significant with $P < 0.05$. Abbreviations are as defined in Table II.

Oligo ID	TIGR ID	Annotation	FC	Microarray		Real-Time RT-PCR	
				GM	GI	GM	GI
MT003502	TC88229	β-1,3-Glucanase	I	1.97	1.41	1.30	1.32
MT001341	TC78420	Pectinesterase	I	1.56	1.41	1.93	1.41
MT002501	TC78158	High-affinity nitrate transporter	III	4.76	1.03	0.93	1.12
MT009589	TC78157	High-affinity nitrate transporter	III	2.51	1.14	1.35	1.30
MT002866	TC88701	Manganese transporter MtZIP7	III	1.32	1.76	2.74	2.46
MT006556	TC84545	Nitrate transporter	III	1.01	1.18	0.74	1.37
MT015669	TC88539	MtBcp1	V	4.28	4.89	12.03	9.95
MT003225	TC87415	Bcp	V	2.94	2.35	2.36	1.53
MT004004	TC77871	Malonyl-CoA:acyl carrier protein transacylase	V	1.33	1.71	1.40	1.00
MT000207	TC76829	Fructokinase	V	1.14	1.49	0.20[a]	0.85
MT004625	TC81595	Ent-kaurene synthase A	VI	3.25	2.05	0.87	1.90
MT000634	TC77465	Auxin-regulated protein GH3	VI	2.44	3.13	4.36	3.33
MT002731	TC78764	1-Aminocyclopropane-1-carboxylic acid oxidase	VI	1.33	1.79	0.78	1.51
MT002771	TC78589	DXS2	VI	1.08	1.27	1.59	1.90
MT001930	TC78235	Myb-like transcription factor	VIII	5.15	3.09	13.75	11.18
MT005542	TC92282	Zinc-finger, C2H2 type	VIII	1.20	1.94	3.76	9.95
MT014816	TC90718	Cys protease	IX	1.21	2.08	10.20	7.48
MT003520	TC78350	Man/Glc-binding lectin	X	4.35	5.20	4.42	3.93
MT004715	TC80630	Ser/Thr protein kinase	X	2.05	1.71	1.52	1.03
MT013028	TC83316	Protease inhibitor	XII.A	3.62	2.97	6.79	5.39

[a]Expression ratio not significant.

to be expected for several genes since in contrast, for example, to nodulation where many nodule-induced genes do not exhibit a basal transcription in roots, a significant expression level can be detected in nonmycorrhized tissues for many AM-related genes. In summary, our real-time RT-PCR data, obtained from samples, support our microarray-based expression profiles. The differences observed are likely to result from the fact that the extent and efficiency of mycorrhizal colonization is difficult to control and that pooled tissue samples have been studied, making it inevitable to average over different cell types.

M. truncatula Genes Down-Regulated in AM Are Largely Related to Stress Responses

In total, 176 genes were identified as down-regulated more than 2-fold in response to both *G. intraradices* and *G. mosseae* colonization (Supplemental Table I), and the 50 most strongly down-regulated genes are listed in Table X. Since nonmycorrhizal roots used as controls were grown under conditions of phosphate limitation (20 μM), it is not surprising that several of the AM down-regulated genes coded for proteins involved in stress responses.

It was interesting that several gene families were identified as activated in phosphate-starved roots, e.g. encoding two caffeic acid *O*-methyltransferases involved in the biosynthesis of lignin cell wall precursors (Zubieta et al., 2002), 4 Δ-1-pyrroline-5-carboxylate synthetases (the first enzymes of the Pro biosynthetic pathway) known to be induced under salt stress (Ginzberg et al., 1998), and 5 different late embryogenesis abundant proteins relevant for desiccation tolerance (Wise and Tunnacliffe, 2004). Further examples for genes related to stress responses are a desiccation-responsive protein (TC77899), a dehydrin-like protein (TC77327), and a desiccation-responsive low temperature-induced protein (TC81698). The observation that the expression level of different stress-induced genes is obviously much lower in AM-colonized roots adds to reports that efficient mycorrhization increases the capability of plants to alleviate abiotic stress conditions (Smith and Read, 1997). With respect to metabolism, the identification of 2 phosphate starvation-induced genes encoding the Suc synthases MtSucS2 and MtSucS4 is in accordance with real-time RT-PCR experiments showing elevated expression of these genes only in phosphate-starved roots (Hohnjec et al., 2003).

In addition to transcription factors, the identification of a down-regulated peptidoglycan-binding LysM receptor kinase (TC83559) is intriguing, since LysM receptor kinases mediate Nod-factor perception during nodulation. Although there are common steps in early signaling during nodulation and mycorrhization, the initial signal perception of the host is specific for either symbiosis and requires a Nod-factor or a hypothetical Myc-factor (Cullimore and Dénarié, 2003). The strong down-regulation of this LysM-type

Table X. *M. truncatula genes repressed in AM roots*

The 50 genes most strongly repressed in both AM interactions are listed according to the functional categories as defined by Journet et al. (2002) and are sorted within these classes according to the induction level in *G. mosseae*-colonized roots. Abbreviations are as defined in Table II.

Oligo ID	TIGR ID	Annotation	FC	GM	GI
MT007074	TC76646	Caffeic acid *O*-methyltransferase	I	−4.73	−2.96
MT005666	TC83381	Caffeic acid *O*-methyltransferase	I	−3.80	−2.75
MT000246	TC85734	Pro-rich protein	I	−3.15	−1.11
MT000967	TC77984	Expansin-related protein	I	−2.58	−1.22
MT003596	TC88633	Isoprenylated heavy metal transport/detoxification protein	III	−3.66	−2.73
MT003439	TC90076	RAB1 GTP-binding protein	IV	−2.92	−1.71
MT015170	TC76593	Δ-1-Pyrroline-5-carboxylate synthetase	V	−4.58	−3.17
MT014053	TC76589	Δ-1-Pyrroline-5-carboxylate synthetase	V	−4.52	−3.48
MT015169	TC76589	Δ-1-Pyrroline-5-carboxylate synthetase	V	−4.37	−4.58
MT000507	TC77268	Cinnamoyl-CoA reductase	V	−3.54	−2.30
MT000869	TC77949	Putative copper-binding protein	V	−2.96	−3.78
MT005126	TC78918	Suc synthase MtSucS2	V	−2.70	−2.38
MT011868	TC82744	Suc synthase MtSucS4	V	−2.68	−2.35
MT007444	TC86064	Glutamate decarboxylase	V	−2.59	−2.07
MT014842	TC90894	Anthocyanin 5-aromatic acyltransferase-like protein	V	−2.52	−2.56
MT007047	TC76593	Δ-1-Pyrroline-5-carboxylate synthase	V	−2.49	−2.50
MT012479	TC83483	Malate dehydrogenase	V	−2.38	−3.89
MT004582	TC82770	Chalcone reductase	VI	−5.46	−3.65
MT003716	TC80105	Ferritin	VI	−4.05	−2.93
MT008389	TC78616	Probable stress and abscisic acid induced protein	VI	−3.31	−2.31
MT009487	TC88609	NAC domain protein NAC2	VIII	−2.98	−2.52
MT008226	TC78257	Homeobox-Leu zipper protein	VIII	−2.97	−1.78
MT000799	TC86417	NAM-like regulatory protein	VIII	−2.59	−2.28
MT014768	TC90326	bZIP transcription factor	VIII	−2.45	−2.52
MT004368	TC91563	γ-Glutamyltranspeptidase	IX	−3.89	−3.41
MT000029	TC76371	Protein kinase C, phorbol ester/diacylglycerol binding	X	−4.13	−1.66
MT012651	TC83559	Peptidoglycan-binding LysM receptor kinase	X	−3.60	−3.13
MT007340	TC85793	Annexin	X	−3.52	−3.27
MT014127	TC85429	Nonspecific lipid-transfer protein	XII	−4.07	−3.07
MT015187	TC85327	Proteinase inhibitor	XII.A	−2.82	−2.88
MT015189	TC85327	Proteinase inhibitor	XII.A	−2.54	−3.88
MT014313	TC76866	Late embryogenesis abundant protein	XII.B	−7.76	−3.61
MT014312	TC76867	Late embryogenesis abundant protein	XII.B	−6.77	−4.20
MT005274	TC84691	Late embryogenesis abundant protein	XII.B	−6.43	−3.56
MT014784	TC81698	Desiccation-responsive low-temperature-induced protein	XII.B	−5.53	−3.50
MT007558	TC85950	Late embryogenesis abundant protein	XII.B	−5.47	−3.12
MT000542	TC77327	Dehydrin-like protein	XII.B	−5.38	−3.08
MT007538	TC85220	Late embryogenesis abundant protein	XII.B	−5.03	−3.17
MT008432	TC78666	Alkaline α-galactosidase II	XII.B	−3.70	−2.51
MT015129	TC76537	Cold acclimation responsive protein	XII.B	−3.41	−2.42
MT007556	TC85949	Seed maturation protein LEA 4	XII.B	−3.28	−3.89
MT007269	TC76315	Cold acclimation response protein	XII.B	−2.65	−3.26
MT014258	TC76698	Dehydrin-like protein	XII.B	−2.62	−2.32
MT000966	TC86847	Thaumatin-like protein	XII.B	−2.46	−1.29
MT004870	TC89522	Hypothetical protein	XII.C	−6.26	−4.15
MT007837	TC77899	Desiccation-responsive protein	XII.C	−3.26	−3.15
MT001853	TC88006	Hypothetical protein	XII.C	−2.77	−2.79
MT012402	TC92349	Hypothetical protein	XII.C	−2.63	−3.24
MT002839	TC80357	Hypothetical protein	XII.C	−2.47	−2.07
MT005919	TC86466	No significant homology	XIII	−2.64	−4.98

receptor kinase in AM argues against a role during mycorrhization, but might indicate a function related to nodule initiation.

AM-Induced Gene Expression Is Not Mediated by Phosphate

To assess if the AM-induced genes identified in this study are also activated by exogenously supplied phosphate, we studied gene expression in *M. truncatula* roots in response to high phosphate concentrations (2 mM). Although phosphate acquisition is one of the major benefits of the plant in mycorrhiza symbioses, these experiments revealed that from 201 genes found to be coinduced in 2 AM interactions, only 8 genes were up-regulated more than 2-fold in roots grown under conditions of high phosphate supply (Supplemental Table II). Among these were 1 of the 2 high-affinity nitrate transporter (TC78158) and 1 of the 4 Kunitz-type protease genes (TC78015), indicating a differential response of members of gene families to external phosphate concentrations. The low overlap between AM- and phosphate-induced gene expression shows that the transcriptional changes observed are largely due to the colonization of roots by AM fungi and cannot be regarded as a mere consequence of a mycorrhiza-improved phosphorus nutrition, a finding in accordance with the observations by Liu et al. (2003a) for the *M. truncatula-G. versiforme* symbiosis.

AM- and Nodulation-Induced Gene Expression Overlap Only to a Limited Extent

Since there is evidence for common gene expression during AM and nodulation in the early stages of these interactions, we compared the AM-induced transcription profiles with those derived from nitrogen-fixing root nodules. Although large-scale transcriptomics studies during *M. truncatula* nodulation were published (El Yahyaoui et al., 2004; Manthey et al., 2004), expression data derived from different probe sets are difficult to relate, since oligonucleotide probes can match multiple EST clusters, depending on the clustering strategies applied.

To assess the overlap between nodulation- and mycorrhization-related gene expression, we performed reference hybridizations using mature, nitrogen-fixing nodules and uninfected roots of comparable age. A relation of the expression profiles revealed that from 201 plant genes significantly induced in AM roots, 27 were also up-regulated at least 2-fold in mature root nodules (Supplemental Table II). Among these were 3 nodulin genes encoding a multifunctional Nodulin 26-like aquaporin known to be up-regulated in both root nodules and AM (TC86110; Brechenmacher et al., 2004), an MtN21-like putative membrane protein (TC78291; Gamas et al., 1996), and a symbiosome membrane nodulin (TC90920). From the up-regulation of the symbiosome membrane nodulin gene originally identified in soybean in response to *Bradyrhizobium japonicum* inoculation (Winzer et al., 1999), it can be inferred that the peribacteroid and periarbuscular membranes share common structural properties.

An induction of TC88957 encoding a polygalacturonase and TC86689 encoding an endo-1,3 to 1,4-β-D-glucanase indicates the recruitment of similar cell wall modifying enzymes in root nodules and AM, whereas an activation of the auxin-regulated protein GH3 (TC77465) is consistent with the synthesis of auxin in root nodules. Interestingly, different TCs encoding enzymes involved in protein processing were identified: TC85937 representing the Ser carboxypeptidase MtScp1, TC90718 specifying a Cys protease, and TC88029 that codes for a signal peptidase. The identification of a symbiosis-induced signal peptidase is intriguing, since this either indicates an involvement of similar genes in the translocation of proteins across perisymbiotic membranes or extends the hypothesis of Mergaert et al. (2003) that cleaved signal peptides of conserved structure might act as signals in root nodules. Finally, one gene (TC79248) encoding a zinc-finger RNA binding protein was identified that represents a candidate for a symbiotic regulator of gene expression.

The comparably low overlap in gene induction during mature stages of two different root endosymbioses is reminiscent of the observations reported by Manthey et al. (2004) and El Yahyaoui et al. (2004) on the basis of less comprehensive *M. truncatula* cDNA microarrays. In conclusion, only a limited overlap in transcriptional activation exists between mature stages of the two root endosymbioses arbuscular mycorrhiza and root nodule.

CONCLUSIONS AND PERSPECTIVES

Following on different cDNA-based arrays (Liu et al., 2003a; VandenBosch and Stacey, 2003; Küster et al., 2004), 70-mer oligonucleotide microarrays designated Mt16kOLI1 now constitute the most comprehensive expression profiling tool available for this model plant. Using these microarrays, we here present a global overview on common gene expression in AM by studying the interaction of *M. truncatula* with two different mycorrhizal fungi. By focusing on those genes that were reproducibly induced in the two interactions studied, gene expression related to the inoculum or the fungal species should have been minimized, allowing us to specify genuine mycorrhiza-related genes. On the other hand, AM formation is an asynchronous process suffering from dilution effects and a mixture of different developmental stages. Although it is likely that more AM-related genes can be identified by applying cell-specific expression profiling techniques, our collection of AM-related genes already forms a comprehensive resource for functional characterizations in the T-DNA or TILLING mutant collections currently established for different legume species.

MATERIALS AND METHODS

Plant Production

Medicago truncatula Gaertn cv Jemalong genotype A17 seeds were surface-sterilized and scarified as described previously (Hohnjec et al., 2003). Subsequently, they were spread on 0.8% (w/v) water agar (Sigma agar-agar, Sigma-Aldrich, Steinheim, Germany) in petri dishes wrapped with parafilm and aluminum foil. Seeds were vernalized at 4°C for 4 d, transferred to RT for 2 d, and finally incubated for one more day at RT in the light. The seedlings were grown in 27-cm^3 pots in clay granules (Seramis, Masterfoods, Verden, Germany) at 22°C with 60% relative humidity and an 18-h/6-h-light/-dark alteration until the plantlets developed at least one trifoliate. Afterward, they were transferred to 600-cm^3 pots half-filled with clay granules that were covered with approximately 1 cm of sand. An inoculum containing *Glomus mosseae* (Granular AMF Inoculum *G. mosseae* BEG 12, Biorize R&D, Dijon, France) or *Glomus intraradices* (Premier Tech Biotechnologies, Rivière-de-Loup, Québec, Canada), respectively, was directly applied to the root system before pots were filled to a maximum volume with clay granules. Control plants were treated comparably except for inoculum application. Plants were watered twice a week with one-half-strength Hoagland solution containing 20 μM phosphate (Arnon and Hoagland, 1940). At 28 d post inoculation, mycorrhizal roots as well as nonmycorrhizal control roots were harvested and immediately frozen in liquid nitrogen. At this time, randomly selected mycorrhizal roots were stained for colonization measurements using the gridline intersection method according to McGonigle et al. (1990). The percentage of root length colonization (AMF hyphae, spores, vesicles, or arbuscules) ranged between 50% and 80%, whereas the relative arbuscule frequency within colonized fragments varied between 55% and 75%, respectively.

To obtain phosphate supplied roots, plants were grown under the conditions described above and fertilized twice a week using one-half-strength Hoagland solution (Arnon and Hoagland, 1940) supplemented with 2 mM phosphate. Twenty-eight days after being transferred into 600-m^3 pots, roots grown under 2 mM phosphate, and corresponding control roots grown under conditions of phosphate limitation (one-half-strength Hoagland solution, 20 μM phosphate) were harvested and immediately frozen in liquid nitrogen.

To collect nitrogen-fixing root nodules, vernalized seedlings were grown aeroponically using a nitrogen-rich medium (Journet et al., 2001) with 40 plants/250 L caisson. After 2 weeks, this medium was exchanged for a medium lacking combined nitrogen. Three days later, plants were inoculated with a *Sinorhizobium meliloti* RCR2011 pXLGD4 (Journet et al., 2002) culture diluted to a final concentration of approximately 5×10^5 bacteria/milliliter medium. After 20 d, nitrogen-fixing root nodules were collected. As a control, noninoculated roots grown to the same age in nitrogen-rich medium (Gallusci et al., 1991) were harvested from approximately 4 cm below the crown. All collected tissues were immediately frozen in liquid nitrogen.

Isolation of Total RNA, Real-Time RT-PCR Experiments, and Genomic PCR-Amplifications

Total RNA was prepared using the RNeasy Plant Mini kit and DNase I on-column digestion according to the manufacturer's instructions (Qiagen, Hilden, Germany) from different pools of six to eight roots (or different root nodule pools) to provide biological replicates for expression profiling experiments. The resulting RNA preparations were concentrated to 1.25 μg/microliter using Microcon-30 columns (Millipore, Schwalbach, Germany) and stored at −80°C until use. The integrity of total RNA was checked on agarose gels and its quantity as well as purity was determined spectrophotometrically.

Real-time RT-PCR experiments were performed according to a protocol reported by Hohnjec et al. (2003) using gene-specific primers (Supplemental Table IV). The results for Glomus-inoculated versus nonmycorrhizal roots were averaged over four biological replicates and the expression ratios were tested for statistical significance using a Student's *t* test.

To prove the plant origin of AM-induced genes, gene-specific primers (Supplemental Table IV) were used to PCR-amplify the corresponding genomic regions from total *M. truncatula* DNA (isolated from leaves) using *Taq* DNA polymerase (Qiagen) as recommended by the manufacturer.

Scope, Layout, and Printing of Mt16kOLI1 Microarrays

Mt16kOLI1 microarrays contain 16,086 70-mer oligonucleotide probes (Qiagen) representing all TCs of the TIGR *M. truncatula* Gene Index 5 (http://www.tigr.org/tdb/mtgi) as well as different GAPDH controls (Küster et al., 2004). An assessment of background levels and unspecific hybridizations was performed on the basis of 226 probes containing spotting buffer and 12 70-mer probes that serve as negative controls. The layout of Mt16kOLI1 microarrays comprises 48 grids arranged in 12 metarows and 4 metacolumns. Each grid contains 702 spots in 27 rows and 26 columns with 24 columns carrying 27 probes and 2 columns carrying 20 probes. For each *M. truncatula* probe, duplicate spots are present in the same grids throughout Mt16kOLI1 microarrays.

Oligonucleotides were dissolved in 1.5 M betaine, 3× SSC to a concentration of 40 μM and were printed onto QMT epoxy slides (Quantifoil, Jena, Germany) using a MicroGrid II 600 spotter (BioRobotics, Cambridge, UK) with 48 SMP3 stealth pins (TeleChem International, Sunnyvale, CA). We estimate that each spot contains approximately 300 fmol of oligonucleotides. DNA was cross-linked to the slide by incubation for 105 min at 80°C. Slides were kept in sealed plastic bags containing desiccation packs at 18°C to 22°C for up to 6 months with no loss of quality. For validation of microarray printing and efficient coupling to the slide, one microarray from each series was hybridized with Alexa 555-labeled random nonamers (Molecular Probes, Leiden, The Netherlands) as described (Küster et al., 2004). Mt16kOLI1 array definition files were deposited in ArrayExpress under accession A-MEXP-85.

Cy-Labeling of Hybridization Targets, Hybridization, and Image Acquisition

Twenty micrograms of total RNA was used to synthesize Cy3- or Cy5-labeled cDNA targets according to Küster et al. (2004) using a mixture of 2.5 μg of double-anchored oligo$(dT)_{15}$VN primers and 5 μg of random hexamers. Labeled cDNA was purified using CyScribe GFX columns (Amersham Biosciences, Freiburg, Germany), and labeling efficiency was checked as described in Küster et al. (2004). Immediately before use, microarrays were rinsed once for 5 min in 0.1% (v/v) Triton X-100, twice for 2 min each in 250 mL MilliQ water containing 29 μL 32% (v/v) HCl, once for 10 min in 0.1 M KCl, and once for 1 min in MilliQ water (at 20°C each). Slides were blocked for 15 min at 50°C in 200 mL 1 × QMT (Quantifoil) solution containing 46 μL 32% (v/v) HCl. Subsequently, slides were rinsed in MilliQ water (20°C, 1 min) and dried by centrifugation (185*g*, 5 min, 20°C). Hybridization was performed in an ASP hybridization station (Amersham Biosciences) in a sample volume of 250 μL DIG EasyHyb solution (Roche, Mannheim, Germany) supplemented with 15 μg sonicated salmon sperm DNA (Amersham Biosciences). Immediately before injection, samples were denatured for 5 min at 65°C. After 16 h of hybridization at 42°C, microarrays were washed once in 2× SSC, 0.2% (w/v) SDS (1 min, 42°C), twice in 0.2× SSC, 0.1% (w/v) SDS (1 min, 20°C), twice in 0.2 × SSC (1 min, 20°C), and once in 0.1 × SSC (1 min, 18°C). Slides were dried by centrifugation (185*g*, 5 min, 20 C) and scanned with a resolution of 10 μm using the ScanArray 4000 (PerkinElmer, Boston). Original expression profiling data were deposited in the ArrayExpress database under accession numbers E-MEXP-218, E-MEXP-237, and E-MEXP-238.

Analysis of Image Data from Microarray Hybridizations

Image processing was performed using the ImaGene 5.5 software (BioDiscovery, Los Angeles). The mean intensities of signal pixels and the mean intensities of local background pixels were calculated for each spot in both channels, and spots were flagged "empty" in the case of $R \leq 0.7$ for both channels. The $R \leq 0.7$ threshold resulted in the removal of signals corresponding to negative controls and empty wells. In addition, manual flags were set for spots that were within hybridization artefacts. Data files were imported into the *EMMA1.1* array analysis software (Dondrup et al., 2003), and flagged spots were discarded during import. Subsequent to Lowess normalization with a floor value of 20, regulated genes were identified using a *t*-statistics. Genes were regarded as significantly differentially expressed if $P \leq 0.05$ and $M \geq 1$ or if $P \leq 0.05$ and $M \leq -1$ with M specifying the expression ratio.

Construction and Histological Analysis of Transgenic Hairy Roots

The promoter of the MtBcp1 gene (TC88539) was PCR-amplified with *Pwo* DNA polymerase (Roche) from BAC mth2-15c20 (GenBank accession no. AC126009) using gene-specific primers containing appropriate restriction

sites. The amplified fragment covered the −1,181/−2 region (position 29,680 to 28,501 of BAC mth2-15c20) relative to the start codon and was cloned as *Sph*I/*Eco*RI fragment in front of the *gus*Aint gene of pGUSINT (Hohnjec et al., 2003). Subsequently, the promoter-*gus*Aint fusion was excised using *Spe*I, filled in with Klenow polymerase, and cloned into the *Sma*I site of the binary plasmid pRedRoot (Limpens et al., 2004). The resulting plasmid was electroporated into *Agrobacterium rhizogenes* ARqua1, and this strain was used to generate hairy roots on *M. truncatula* cv Jemalong A17 according to Vieweg et al. (2004). Four weeks after mycorrhization with *G. intraradices*, GUS assays were performed as described by Hohnjec et al. (2003) and subsequent stainings for fungal structures as described by Vierheilig et al. (1998), except that transgenic roots were preselected using dsRed fluorescence (Leica MZ FL III, Wetzlar, Germany). Examination of tissues was performed by light microscopy and documented with a digital camera (Olympus C-4040Z, Hamburg, Germany).

Distribution of Materials

Upon request, all novel materials described in this publication will be made available in a timely manner for noncommercial research purposes, subject to the requisite permission from any third-party owners of all or parts of the material. Obtaining any permissions will be the responsibility of the requestor.

Sequence data from this article have been deposited with the EMBL/GenBank data libraries under accession number AC126009.

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