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***Review*:**

Transcriptional snapshots provide insights into the molecular basis of arbuscular mycorrhiza in the model legume *Medicago truncatula*

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Abstract. The arbuscular mycorrhizal (AM) association between terrestrial plants and soil fungi of the phylum *Glomeromycota* is the most widespread beneficial plant–microbe interaction on earth. In the course of the symbiosis, fungal hyphae colonise plant roots and supply limiting nutrients, in particular phosphorus, in exchange for carbon compounds. Owing to the obligate biotrophy of mycorrhizal fungi and the lack of genetic systems to study them, targeted molecular studies on AM symbioses proved to be difficult. With the emergence of plant genomics and the selection of suitable models, an application of untargeted expression profiling experiments became possible. In the model legume *Medicago truncatula*, high-throughput expressed sequence tag (EST)-sequencing in conjunction with *in silico* and experimental transcriptome profiling provided transcriptional snapshots that together defined the global genetic program activated during AM. Owing to an asynchronous development of the symbiosis, several hundred genes found to be activated during the symbiosis cannot be easily correlated with symbiotic structures, but the expression of selected genes has been extended to the cellular level to correlate gene expression with specific stages of AM development. These approaches identified marker genes for the AM symbiosis and provided the first insights into the molecular basis of gene expression regulation during AM.

Keywords: arbuscule-specific genes, EST-sequencing, expression databases, *Glomus* spp., *in silico* transcriptome profiling, microarray-based transcriptome profiling, TIGR *M. truncatula* Gene Index.

Introduction

Some 80% of all terrestrial plants enter an arbuscular mycorrhiza (AM) endosymbiosis with fungi of the phylum *Glomeromycota* (Smith and Read 1997; Schüssler *et al*. 2001). During AM, fungal hyphae from an extraradical mycelium penetrate the root epidermis through an appressorium, pass through the outer cortical cell layers and subsequently proliferate in the inner cortex (Harrison 1997, 2005; Genre *et al*. 2005). In the 'Arum' type of fungal colonisation, these intraradical, intercellular hyphae terminate in highly branched, intracellular structures called arbuscules (Bonfante and Perotto 1995), whereas in the

Abbreviations used: AM, arbuscular mycorrhiza; bcp, blue-copper-binding protein; EST, expressed sequence tag; ET, expressed transcript; GPI, glycosylphosphatidylinositol; SAMS, sequence analysis and management system; SSH, suppressive-subtractive hybridisation; SteN, statistical electronic northern blot; TC, tentative consensus.

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'Paris' type, little or no arbuscule development is observed (Smith and Read 1997).

Arbuscular mycorrhiza interactions are characterised by the bidirectional exchange of nutrients. In return for carbohydrates, fungal microsymbionts transfer minerals, in particular phosphorus, to the plant (Shachar-Hill *et al*. 1995; Smith *et al*. 2001). In the well-studied 'Arum'-type interactions, the uptake of phosphorus and minerals occurs at the peri-arbuscular interface, which encompasses the fungal arbuscular membrane, the periarbuscular matrix, and the plant periarbuscular membrane (Gianinazzi-Pearson 1996; Harrison 1999; Parniske 2000). It has been shown that the uptake of phosphorus is energy-dependent, with plant and fungal plasma membrane H^+-ATPases as well as a plant phosphate transporter being exclusively localised at the arbuscular interface (Gianinazzi-Pearson *et al*. 2000; Ferrol *et al*. 2002; Harrison *et al*. 2002; Requena *et al*. 2003). In contrast to the nutrient uptake by the plant, intraradical hyphae are thought to be responsible for the translocation of carbohydrates to the fungus (Bago *et al*. 2000; Graham and Miller 2005).

Molecular research on AM suffers from two major obstacles: (1) an asynchronous development of the symbioses leading to the concomitant presence of different stages of AM in root systems and (2) the obligate biotrophy of AM fungi. Consequently, molecular information obtained some years ago on genes expressed during AM interactions was limited, and only a few mycorrhiza-induced genes had been reported in different species (Franken and Requena 2001; Gianinazzi-Pearson and Brechenmacher 2004).

With the emergence of plant genomics, the identification of a more comprehensive collection of genes activated during AM became possible. In recent years, expression profiling strategies comprising high-throughput EST-sequencing, *in silico* profiling, and global microarray-based transcriptome profiling were pursued to specify genes activated during mycorrhizal interactions. These transcriptomics approaches benefited from the identification of two model plants from the legume family that proved to be excellent models also for AM, *Medicago truncatula* (Barker *et al*. 1990) and *Lotus japonicus* (Handberg and Stougaard 1992). In addition to well advanced genome sequencing projects and the existence of comprehensive mutant collections (Tadege *et al*. 2005; Udvardi *et al*. 2005; Young *et al*. 2005; Sato and Tabata 2006; Town 2006), more than 330 000 ESTs were generated in the two legumes (Quackenbush *et al*. 2001), and several expression profiling tools have been developed (Colebatch *et al*. 2002, 2004; Kouchi *et al*. 2004; Küster *et al*. 2004; Hohnjec *et al*. 2005; Lohar *et al*. 2006).

An important benefit of selecting legumes as AM models derives from the fact that this genus is able to enter a second wide-spread plant–microbe symbiosis leading to the development of nitrogen-fixing root nodules (Brewin 1991; Vessey *et al*. 2005). Based on the existence of common signalling cascades during the early stages of nodulation and mycorrhization (Limpens and Bisseling 2003; Geurts *et al*. 2005; Stacey *et al*. 2006), it is speculated that the comparably young root nodule symbiosis adopted and modified much more ancient signal transduction pathways leading to AM formation (Gianinazzi-Pearson and Dénarié 1997), placing AM research in a general context of molecular research on plant–microbe signalling. Studies on legume mutants defective in early responses to nodulating rhizobia or mycorrhizal fungi (Cullimore and Dénarié 2003; Kistner *et al*. 2005) have recently delivered a detailed molecular understanding of the early infection events leading to nodulation and mycorrhization (Kistner and Parniske 2002). Interestingly, fungal recognition is proposed to require the presence of a Myc-factor that, similar to the action of Nod-factors triggering nodulation, is able to initiate AM formation (Kosuta *et al*. 2003).

Complementing the genomics activities in legume macrosymbionts, a genome project has recently been launched for the AM fungus *Glomus intraradices*. This model fungus is characterised by a haploid and comparably small genome of only ~15 Mb (Hijri and Sanders 2004) containing only few repetitive sequences (Lammers *et al*. 2004; Martin *et al*. 2004). The availability of full genome sequences of two model legumes and a model fungus can be expected to further advance our understanding of AM symbioses in the forthcoming years, although the lack of a transformation system for mycorrhizal fungi remains a major drawback. At the dawn of the whole-genome era of AM research, we summarise here recent advances in the transcriptomics of AM interactions in the model legume *M. truncatula*. These efforts provided a framework of the expressed plant genome during AM and the transcriptional snapshots obtained, together with forward genetics studies on legume mutants, substantially widened our molecular perspective of the AM symbiosis.

Global approach I: high-throughput EST-sequencing provides an overview of the *Medicago truncatula* transcriptome in arbuscular mycorrhiza

Only recently, information on genes expressed during AM interactions was limited (Franken and Requena 2001). In case of the *M. truncatula–Glomus* spp. symbiosis, this situation has improved substantially over the last couple of years owing to the application of untargeted high-throughput EST-sequencing (Gianinazzi-Pearson and Brechenmacher 2004). In the latest release of the TIGR *M. truncatula* Gene Index (http://www.tigr.org/tdb/tgi; verified 16 June 2006; Quackenbush *et al*. 2001), five comprehensive cDNA libraries representing AM roots are listed (Table 1). These libraries stem from studies exclusively covering the AM transcriptome (MHAM and MHAM2 libraries, Liu *et al*. 2003; MtAMP library, Frenzel *et al*. 2005; MtGIM library, Wulf *et al*. 2003; Frenzel *et al*. 2005) as well as from a large-scale project targeted at specifying genes expressed

Table 1. High-throughput EST-sequencing to study the *Medicago truncatula* AM symbiosis
The following five cDNA-libraries were subjected to untargeted EST-sequencing and have been included in the current release 8 of the TIGR *M. truncatula* Gene Index (http://www.tigr.org/tdb/tgi; Quackenbush *et al.* 2001)

Library	Type	ESTs	AM fungus	Library description	Reference
MHAM	Random	7351	*G. versiforme*	Mycorrhizal roots harvested between 10 and 38 d post inoculation	Liu *et al*. (2003)
MHAM2	Random	1679	*G. versiforme*	Mycorrhizal roots harvested between 10 and 38 d post inoculation	Liu *et al*. (2003)
MtBC	Random	8567	*G. intraradices*	Mycorrhizal roots harvested 3 weeks post inoculation	Journet *et al*. (2002)
MtAMP	Random	3448	*G. intraradices*	Mycorrhizal roots harvested 5 weeks post inoculation	Frenzel *et al*. (2005)
MtGIM	SSH	1686	*G. intraradices*	Mycorrhizal roots harvested 3 weeks post inoculation	Wulf *et al*. (2003); Frenzel *et al*. (2005)

both during mycorrhization and nodulation (MtBC library, Journet *et al*. 2002). Whereas the MHAM and MHAM2 libraries contain ESTs from the *M. truncatula–G. versiforme* interaction, all other libraries represent the AM symbiosis between *M. truncatula* and *G. intraradices*.

Together, 22 731 ESTs were generated from the five *M. truncatula* AM libraries mentioned above, ~10% of all ESTs generated in this species so far. This makes the *M. truncatula* / *Glomus* spp. interaction the best-studied model for AM at the level of EST-sequencing. Since AM roots contain polyadenylated transcripts both from the plant and the mycorrhizal fungus, a certain proportion of the transcript sequences obtained can be expected to be of fungal origin. While bioinformatics approaches to *ab initio* predict the origin of AM transcript sequences proved to be more error-prone than for other plant–microbe interactions (Hraber and Weller 2001), analyses based on comparisons to ESTs from non-mycorrhizal tissues or to genomic sequences as well as PCR-amplifications from either *M. truncatula* or *Glomus* spp. genomic DNA indicated that the proportion of fungal sequences in AM-specific TCs or singleton ESTs was between 6.5 and 9% (Liu *et al*. 2003; Hohnjec *et al*. 2005). This is consistent with the observation that only between 4.5 and 8.1% of total RNA from AM roots is of fungal origin (Liu *et al*. 2003). Nevertheless, a definitive proof of the origin of most AM transcript sequences will have to await completion of the *M. truncatula* or the *G. intraradices* genome sequences.

Global approach II: *in silico* profiles predict several hundred *Medicago truncatula* genes as specifically expressed in arbuscular mycorrhiza

In the latest release of the TIGR *M. truncatula* Gene Index, 226 923 ESTs are assembled. These were generated from 61 cDNA libraries that, apart from representing curated clone collections, covered 53 *M. truncatula* tissues and growth conditions. Automatic EST-clustering was applied to define 36 878 unique sequences, and these were divided into 18 612 TCs ('Tentative Consensus' sequences) and 18 266 singletons (TIGR MtGI, http://www.tigr.org/tdb/tgi/; Quackenbush *et al*. 2001).

The origin of ESTs clustered in a particular TC can be used to mine information on the tissue-specific expression of the corresponding gene (Fig. 1), an approach referred to as eNorthern, *in silico* or digital expression profiling (Alba *et al*. 2004). In addition to simply scoring EST frequencies, mathematical approaches were developed to associate a test statistic with a predicted differential gene expression (Stekel *et al*. 2000). Such *in silico* studies became quite popular in research on root endosymbioses in the last couple of years, and several hundred genes were predicted to be up-regulated during nodulation and mycorrhization (Fedorova *et al*. 2002; Journet *et al*. 2002; Manthey *et al*. 2004; Frenzel *et al*. 2005; Tesfaye *et al*. 2006), based on different subsets of ESTs.

With respect to AM formation, no comparable approach was carried out on the basis of the latest, most comprehensive release of the TIGR *M. truncatula* Gene Index. To specify all *M. truncatula* TCs exclusively composed of ESTs from AM roots, we developed a SteN ('Statistical electronic Northern Blot') tool. SteN builds on a database that stores the TIGR MtGI (release 8) TC composition and incorporates the R-statistic of Stekel *et al*. (2000). Basically, this test statistic allows evaluation of gene expression on the basis of EST frequencies in different cDNA libraries. As a result, R-values are computed that can be used to rank differentially expressed genes proposed by *in silico* predictions, and in particular allows better separation of true- from false-positives (Stekel *et al*. 2000; Journet *et al*. 2002).

When applying the R-statistic (Stekel *et al*. 2000) to identify putative AM-specific genes of *M. truncatula*, we found that 669 TCs were exclusively composed of ESTs generated from the five comprehensive AM libraries listed in Table 1 or of two small collections of suppressive-subtractive hybridisation (SSH) ESTs from AM roots (Brechenmacher *et al*. 2004; Weidmann *et al*. 2004). It should be noted that the TC list contains some sequences that, in addition, rely on ESTs from curated clone collections,

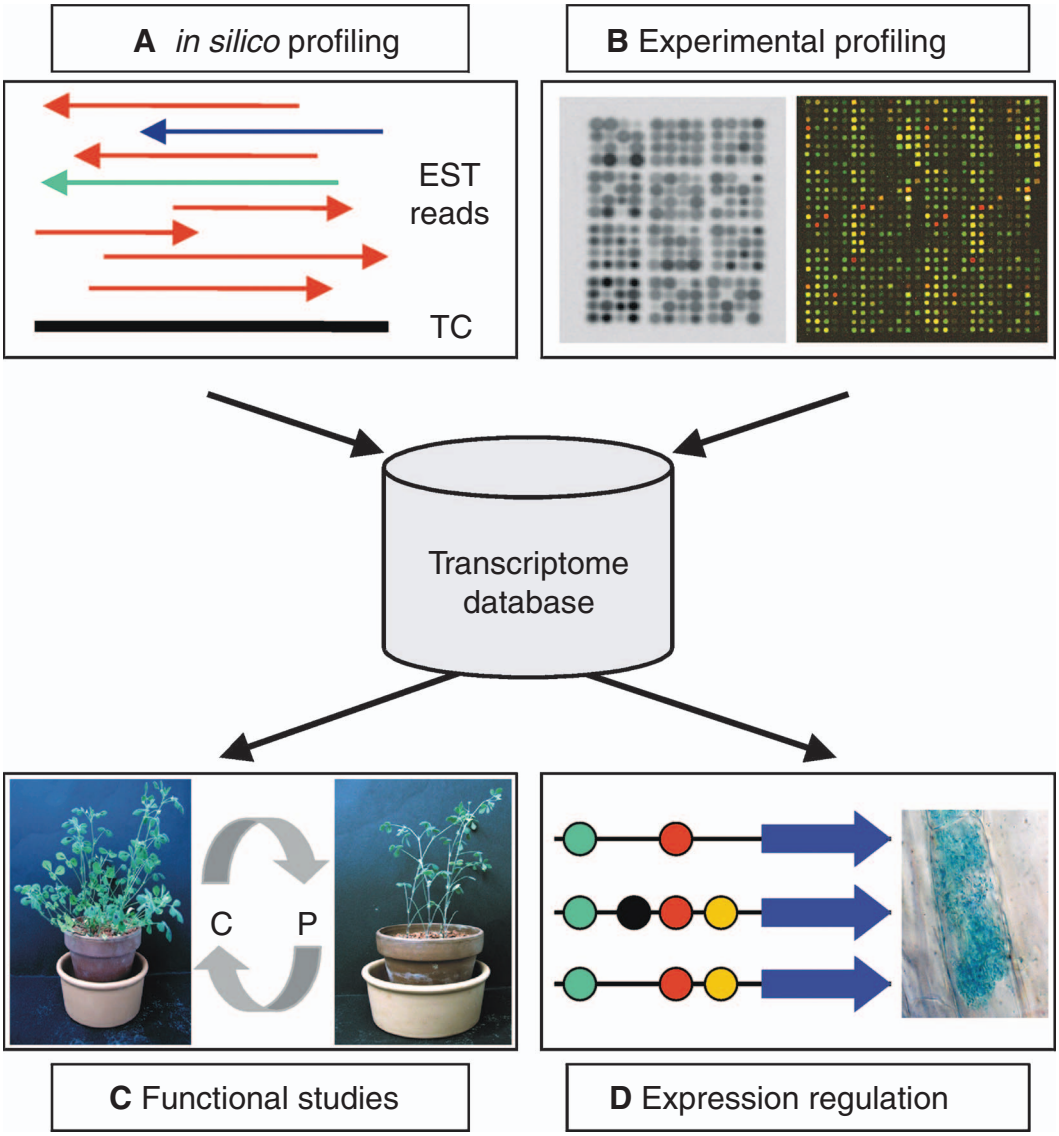

Fig. 1. Untargeted transcriptome profiling facilitates functional analyses of arbuscular mycorrhiza formation in *Medicago truncatula*. (*A*) An *in silico* expression profiling experiment, where the EST-composition of a particular TC is traced to derive information on the expression of the corresponding gene. In the example shown, red arrows denote ESTs from AM roots, whereas arrows with different colours represent ESTs from non-AM tissues. For the TC sequence assembled from these ESTs, an AM-induced expression can be predicted. (*B*) Experimental expression profiling approaches based on macro- and microarray hybridisations. Together, *in silico* and experimental expression profiling strategies lead to the identification of genes differentially regulated during AM, and these data can be queried based on the use of appropriate transcriptome databases storing EST compositions as well as hybridisation data. Relying on the mining of transcriptome profiles from such databases, more targeted studies on the function (*C*) and transcriptional regulation (*D*) of AM-activated genes can be performed. Here, (*C*) illustrates a phenotypic study that compares a wild type plant with a line mutated in an AM-relevant gene, with the underlying effect of the mutation on AM efficiency being symbolised by the exchange of phosphorus for carbon compounds. Finally, (*D*) summarises approaches to study the expression regulation of genes activated in arbuscule-containing cells. Typically, these experiments rely on the expression analysis of reporter genes (symbolised by blue arrows) controlled by different promoter regions of genes expressed in arbuscules, as indicated by the reporter gene activity on the right. These regions can be compared to identify common motifs that mediate expression regulation. Motifs are symbolised by circles with different colours. In the example shown, the green and red motifs can be identified in all promoters tested and hence constitute putative binding sites for AM-specific transcriptional regulators. Abbreviations: C, carbon compound; EST, expressed sequence tag; P, phosphorus; TC, tentative consensus sequence.

such as the 6 k unigene library, and the leguminosin library (http://www.tigr.org/tdb/tgi/). Since those ESTs also derive from AM libraries, this does not interfere with the prediction of an AM-specific expression. In addition, a few TCs contain expressed transcripts (ETs) e.g. deriving from targeted sequencing to characterise full-length cDNAs of AM-expressed genes.

In supplementary Table 1 (accessory publication, see website of Functional Plant Biology), all 669 TCs encoding putative AM-specific genes of *M. truncatula* are listed, together with their EST-composition and with R-values calculated according to Stekel *et al*. (2000). Looking at the distribution of EST-frequencies in the AM-specific TCs, it is evident that 128 TCs are composed of at least four ESTs (24 TCs with more than 10 ESTs), whereas the other TCs (541) are represented by, at most, three transcript sequences. The high proportion of TCs consisting of only a few ESTs is probably related to a dilution of AM structures in whole roots used for library construction and reflects the fact that most AM-activated genes are up-regulated only locally, e.g. in developing arbuscules.

To provide updated annotations for the predicted AM-specific genes, we used the 'Sequence Analysis and Management System' (SAMS) tool that is based on the GenDB software for automatic annotation of prokaryotic genomes (Meyer *et al*. 2003). In order to improve the automatic annotation of TCs, frameshift-corrected open reading frames were calculated from TIGR MtGI TC sequences using the frameD software (Schiex *et al*. 2003). Together with the TIGR MtGI 8 annotation, updated automatic annotations are included in supplementary Table 1.

When inspecting the EST contribution from the different AM cDNA libraries, 68 TCs were exclusively composed of MHAM or MHAM2 ESTs, whereas 86 TCs were composed of MtGIM and 14 TCs of MtAMP ESTs. A surprisingly high number of 343 TCs were only built from ESTs of the MtBC library. This number can be explained by the fact that MtBC is the only library where 5′ and 3′ ESTs were generated, yielding 287 TCs exclusively composed of two MtBC ESTs. In most cases, those TCs are composed of ESTs defining the ends of only one cDNA. The existence of only 125 TCs where ESTs were obtained from at least two cDNA libraries most likely reflects differences in colonising fungi or in growth conditions of the AM roots used for library construction. Being confirmed by different experimental setups, these TCs are obviously prime candidates for AM-induced *M. truncatula* genes.

Global approach III: suppressive subtractive hybridisations provide shortcuts to *Medicago truncatula* genes activated in arbuscular mycorrhiza

Since random libraries contain a high proportion of strongly expressed genes and a limited proportion of genes expressed at a low level (Diatchenko *et al*. 1996), the analysis of SSH cDNA libraries can be expected to ease the identification of candidate genes with an AM-induced expression. One such library is MtGIM, generated from *M. truncatula* / *G. intraradices* AM roots (Table 1). During MtGIM construction, transcript sequences from uninfected roots, phosphate-supplemented roots, *Sinorhizobium meliloti*-infected roots and *Aphanomyces euteiches*-infected roots were subtracted to enrich for AM-induced genes. This strategy proved to be successful, given the comparably high proportion of 86 MtGIM-specific TCs generated from a moderate collection of 1686 ESTs (supplemental Table 1, Frenzel *et al*. 2005). In addition, the specific expression of most novel genes — involvement in transport as well as signalling processes — was verified by real-time RT–PCR, thus providing an independent confirmation of the *in silico* data (Frenzel *et al*. 2005).

The power of suppressive subtractive approaches to specify differentially expressed genes is also evident from two further SSH libraries covering early and late stages of the *M. truncatula* / *G. mosseae* interaction (Brechenmacher *et al*. 2004; Weidmann *et al*. 2004). In both cases, deep EST sequencing was not performed, but all cDNAs identified by reverse northerns or RT–PCR as mycorrhiza-induced were analysed in detail, revealing 29 *M. truncatula* genes activated during the appressorium stage and 12 genes induced during later stages of the symbiosis. Interestingly, 11 genes related to signalling, transcription and translation were not activated in a *M. truncatula* mutant where fungal infection of the root is impossible, although appressoria are formed. This indicated that these genes are related to AM establishment (Weidmann *et al*. 2004).

Owing to the fact that a high proportion of AM-activated genes was detected by SSH, these approaches bridge untargeted EST sequencing and global transcriptome profiling. It must be stressed that, in particular, owing to the potential of identifying rare transcripts or genes expressed locally, SSH is usually not redundant but quite complementary to microarray-based transcriptome profiling.

Global approach IV: array-based transcriptome profiling identifies *Medicago truncatula* genes activated in arbuscular mycorrhiza

Three global transcriptome studies applying cDNA- and 70mer oligonucleotide-based macro- and microarrays (Fig. 1) have so far been reported in *M. truncatula*.

First, Liu *et al*. (2003) applied a macroarray based on 2268 unique cDNAs from the *M. truncatula* / *G. versiforme* MHAM library mentioned above (Table 1) to identify genes up-regulated in response to *Glomus versiforme* at different stages of AM development. Based on hierarchical clustering, the authors identified two distinct temporal gene expression patterns in the course of the symbiotic interaction. Whereas the first group showed a peak in gene induction during

early stages, including defence- and stress-response genes, genes from the second group displayed a constant increase in transcription that correlated with fungal colonisation. Since components of signal transduction pathways were transcriptionally induced in the latter group, novel AM-related signalling pathways have been proposed. These signalling processes can be related to the maintenance and sustainability of fungal structures in root tissues and the specific activation of gene expression in arbuscule-containing cells.

Second, Manthey *et al*. (2004) performed a global transcriptome approach to identify genes activated in the *M. truncatula–G. intraradices* AM. This study relied on the Mt6kRIT (*M. truncatula* 6 k Root Interaction Transcriptome) macro- and microarrays (Küster *et al*. 2004) that represent ~6000 EST clusters from an EST-sequencing project covering mycorrhization and nodulation ('MENS–*Medicago* EST Navigation System', http://medicago.toulouse.inra.fr/Mt/EST/; verified 16 June 2006; Journet *et al*. 2002). A major focus of this approach was to identify symbiotically induced genes up-regulated both during mycorrhization and nodulation. Here, earlier targeted studies indicated that, in particular, genes expressed early during nodulation, e.g. the nodulin genes ENOD2, ENOD40, ENOD5, ENOD11, and ENOD12, are also transcriptionally activated during AM (van Rhijn *et al*. 1997; Albrecht *et al*. 1998; Chabaud *et al*. 2002; Journet *et al*. 2001). Based on Mt6kRIT array hybridisations, several hundred genes were detected as being up-regulated during different stages of nodule development (El Yahyaoui *et al*. 2004; Küster *et al*. 2004; Manthey *et al*. 2004), now allowing a more global comparison of the expression profiles during nodulation and mycorrhization. Although the development of the two endosymbioses shares common mechanisms such as the formation of intracellular infection structures, the intracellular colonisation of root tissues by symbiotic microorganisms, and the generation of symbiotic carbon sinks (Provorov *et al*. 2002; Genre *et al*. 2005), only a limited overlap of the genetic program activated during nodulation and mycorrhization was found, with 75 genes being co-induced in the two interactions (El Yahyaoui *et al*. 2004; Manthey *et al*. 2004). To a certain extent, this low overlap is due to a lack of cellular resolution and a dilution in particular of genes activated early during mycorrhization by non-mycorrhizal areas of the roots.

In a recent study, the more comprehensive Mt16kOLI1 70-mer oligonucleotide microarrays were applied to specify the overlapping genetic program activated by two commonly studied microsymbionts, *Glomus mosseae* and *Glomus intraradices*. In total, 201 plant genes were significantly co-induced at least 2-fold in either interaction (Hohnjec *et al*. 2005), using normalisation functions and statistical analysis pipelines implemented in EMMA, a database designed to store and evaluate expression profiles from spotted microarrays (Dondrup *et al*. 2003). A range of well known AM marker genes were found to be co-activated, thus validating the transcriptomics data. Among those were 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 serine carboxypeptidase MtScp1 (Liu *et al*. 2003), the hexose transporter MtSt1 (Harrison 1996), the 1-deoxy-D-xylulose 5-phosphate synthase MtDXS2 (Walter *et al*. 2002), and a multifunctional aquaporin (Brechenmacher *et al*. 2004).

Apart from AM marker genes, more than 150 novel AM-induced genes were reported, among them genes specifying novel AM-related transporters, enzymes involved in secondary metabolism, and different proteases as well as protease inhibitors. With respect to signalling, several co-induced genes encoded putative components of signal transduction pathways as well as ten novel AM-induced transcriptional regulators (Hohnjec *et al*. 2005), including a Myb-like transcription factor initially reported by Liu *et al*. (2003). Several hundred genes were additionally up-regulated during the *Glomus mosseae* or the *Glomus intraradices* AM, adding to the global profiling data from Liu *et al*. (2003) as well as Manthey *et al*. (2004), and implying that the plant genetic program activated during AM to some extent depends on the microsymbiont or the batch of inoculum used.

Subsets of genes identified by the global transcriptome profiling studies were subjected to real-time RT–PCR for verification of their symbiosis-induced expression (Liu *et al*. 2003; Manthey *et al*. 2004; Hohnjec *et al*. 2005). These experiments allowed the conclusion that microarray-based expression data in general appear to be more reliable than *in silico* predictions (Manthey *et al*. 2004), an observation corroborating a theoretical consideration by Allison *et al*. (2006). Also, this conclusion resembles findings of Fedorova *et al*. (2002) and Tesfaye *et al*. (2006), who demonstrated that a TC has to be composed of a minimal number of ESTs to allow a robust expression prediction of the corresponding gene in root nodules.

Mt16kOLI1 oligonucleotide microarrays were designed on the basis of all TIGR MtGI release 5 TCs. Thus, expression data obtained with these arrays could be related to the *in silico* prediction of AM-induced genes from the TIGR MtGI. In supplemental Table 1, expression ratios of *M. truncatula* genes during the AM with *G. mosseae* and *G. intraradices* (Hohnjec *et al*. 2005) have been included. Since additional ESTs (including those from the MHAM2, the MtAMP and the MtGIM library; Table 1) were incorporated into the TIGR MtGI release 8, not all current TCs match oligonucleotide probes on Mt16kOLI1 microarrays. Nevertheless, for those TCs represented by many ESTs, there is a good overlap between *in silico* and experimental transcriptome profiles (supplemental Table 1). Not surprisingly, TCs represented by only a few ESTs were not reproducibly detected across the different biological replicates used for microarray hybridisation. In these cases, either the *in silico* profiles suffer from a lack of biological

repetition or the microarray hybridisations fail to detect locally expressed genes that were detected by deep EST-sequencing.

Cellular expression of *Medicago truncatula* genes in arbuscular mycorrhizal roots

Adding to the identification of AM-induced genes by more targeted approaches (e.g. the proline-rich protein MtEnod11, Journet *et al*. 2001; the 1-deoxy-D-xylulose 5-phosphate synthase MtDXS2, Walter *et al*. 2002; the aquaporin Mtaqp1, Krajinski *et al*. 2000), untargeted *in silico* and experimental transcriptome profiling identified several hundred *M. truncatula* genes with a mycorrhiza-specific or a mycorrhiza-induced expression. While post-transcriptional mechanisms of expression regulation should not be neglected, this collection of genes forms a comprehensive basis for functional analyses of the encoded gene products.

Although a method allowing the rapid, synchronous root colonisation by AM fungi was reported by Rosewarne *et al*. (1997), it has not been widely applied in molecular AM research. Consequently, mycorrhizal roots containing different symbiotic structures were used for the transcriptomics experiments described above, and the expression profiles obtained cannot easily be connected to specific stages of the AM symbiosis. It is thus evident that global expression profiling approaches that rely on the analysis of pooled tissue samples suffer from a significant loss of biological information. Ultimately, the combination of high-throughput transcriptomics, e.g. by constructing cell-specific SSH cDNA libraries or by studying cell-specific gene expression via microarray hybridisations (Kehr 2003; Kerk *et al*. 2003), will be required.

Although so far, cellular high-throughput expression profiles are not available for the AM symbiosis, *in situ* localisation techniques have allowed specification of a cellular localisation of selected genes found to be transcriptionally activated during AM (Fig. 1; Table 2). These approaches relied on *in situ* transcript and protein localisations as well as on the expression of promoter–reporter gene fusions in transgenic roots.

On the basis of EST analyses and macroarray hybridisations, Harrison *et al*. (2002) and Liu *et al*. (2003) studied the spatial expression pattern of three AM-induced genes by expressing reporter gene fusions in transgenic *M. truncatula* roots. Whereas the phosphate transporter MtPt4 and the endoglucanase MtCel1 were found to be expressed exclusively in arbuscule-containing cells, the serine carboxypeptidase MtScp1 was up-regulated in discrete cell files of the roots, even if only some cells contained arbuscules. Based on these distinct expression patterns, Harrison (2005) proposed that gene expression in mycorrhizal roots is signalled both by cell-autonomous (expression in arbuscule-containing cells) and by non-cell-autonomous signals (expression in arbusculated cell files or additionally in cells surrounding arbuscules), a finding also supported by the promoter studies mentioned below.

A range of other AM marker genes of *M. truncatula* can be grouped into these two categories as well. Whereas arbuscule-specific gene expression was reported for the MtGlp1 gene encoding a germin-like protein (Doll *et al*. 2003), the annexin gene MtAnn2 (Manthey *et al*. 2004), and the lectin genes MtLec5 and MtLec7 (Frenzel *et al*. 2005), expression in the arbuscule-containing as well as the surrounding root cells were reported for the glutathione-S-transferase gene MtGst1 (Wulf *et al*. 2003), the trypsin inhibitor gene MtTi1 (Grunwald *et al*. 2004), the β-tubulin gene MtTubb1 (Manthey *et al*. 2004), the truncated hemoglobin gene MtTrHb2 (Vieweg *et al*. 2005), and the MtBcp1 gene specifying a blue-copper-binding protein (bcp) (Hohnjec *et al*. 2005). Whereas MtTrHb2, MtTubb1 and MtAnn2 were also activated during nodulation, the other genes appear to be specifically up-regulated during mycorrhization.

The first AM-activated gene with relevance to the physiology of arbuscule-containing cells were two plasma membrane H^+-ATPases found to be expressed in arbuscule-containing cells of tobacco roots and the corresponding protein and enzyme activities were localised to the periarbuscular membrane (Gianinazzi-Pearson *et al*. 2000). In *M. truncatula*, the H^+-ATPase MtHa1 protein has recently been localised to the periarbuscular membrane by proteomics approaches (Valot *et al*. 2006). In addition to being expressed in AM roots (Krajinski *et al*. 2002) and similar to other AM-induced genes, MtHa1 was also found to be induced during nodulation, illustrating the common requirement for an acidification of the perisymbiotic space in either root endosymbiosis (Manthey *et al*. 2004), probably to support energy-dependent transport processes.

A particularly interesting arbuscule-specific gene is MtBcp1. This gene is part of a family of tandemly arranged genes, all of them specifically and strongly induced in AM (Wulf *et al*. 2003; Frenzel *et al*. 2005; Hohnjec *et al*. 2005). MtBcp1 encodes a bcp with a predicted glycosylphosphatidylinositol (GPI) anchor (Valot *et al*. 2006). Bcp proteins usually contain a single copper atom and are implicated in electron transfer reactions, such as the chloroplastic plastocyanins that exchange electrons with cytochromes (Hohnjec *et al*. 2005). Together with the plasma membrane ATPase MtHa1 (Krajinski *et al*. 2002), MtBcp1 was recently localised in the periarbuscular membrane, probably being inserted via its GPI-anchor (Valot *et al*. 2006).

Whereas the biological function of some AM-induced genes is obvious, e.g. the modification of cytoskeletal structure for MtTubb1, the acidification of the periarbuscular space for MtHa1 and phosphate transfer to the plant for MtPT4, the function of MtBcp1 remains elusive. This also holds true for other arbuscule-specific or arbuscule-induced genes that were connected to detoxification processes (MtGst1) and the suppression of plant defence (MtTi1, MtTrHb2), or that were related to

Table 2. Arbuscule-related AM marker genes in the model legume *Medicago truncatula*

Genes are listed where an induced expression in AM was identified by global transcriptome profiling and where the cellular localisation of gene expression was subsequently determined by *in situ* RNA and protein localisations or the analysis of promoter-reporter gene fusions. The original mode of identification and the proposed function of these genes during AM are indicated

Gene	Identifier	Annotation	Identification	Localisation	Proposed functions	Reference
MtAnn2	TC95776	Annexin	Microarray hybridisation	Arbuscule-containing cells	Membrane reorganisation	Manthey *et al*. (2004)
MtBcp1	TC96500	Blue-copper-binding protein	Microarray hybridisation	Arbuscule-containing cells and surrounding cortical cells	Electron transfer	Hohnjec *et al*. (2005)
			Proteomics	Periarbuscular membrane	Electron transfer	Valot *et al*. (2006)
MtCel1	TC102782	Cellulase	Macroarray hybridisation	Arbuscule-containing cells	Cell wall modifications	Liu *et al*. (2003)
MtGlp1	TC95018	Germin-like protein	EST-sequencing, reverse northern	Arbuscule-containing cells	Oxalate oxidase, extracellular signal perception	Doll *et al*. (2003)
MtGst1	TC100720	Glutathione-S-transferse	EST-sequencing, reverse northern	Arbuscule-containing cells and surrounding cortex	Stress responses	Wulf *et al*. (2003)
MtHa1	TC95400	Plasma membrane H^+-ATPase	Proteomics	Periarbuscular membrane	Acidification of the periarbuscular space to create a proton gradient	Valot *et al*. (2006)
MtLec5	TC101749	Lectin	*in silico* profiling	Arbuscule-containing cells	Recognising fungal structures; nitrogen storage protein	Frenzel *et al*. (2005)
MtLec7	TC95567	Lectin	*in silico* profiling	Arbuscule-containing cells	Recognising fungal structures; nitrogen storage protein	Frenzel *et al*. (2005)
MtPt4	TC94453	Phosphate transporter	EST databases	Arbuscule-containing cells	Phosphate transfer	Harrison *et al*. (2002)
			Immunolocalisations	Periarbuscular membrane	Phosphate transfer	Harrison *et al*. (2002)
MtScp1	TC106954	Serine carboxypeptidase	Macroarray hybridisation	Arbuscule-containing cells and surrounding cortex	Signalling during fungal infection	Liu *et al*. (2003)
MtTi1	TC106351	Trypsin inhibitor	Macroarray hybridisation	Arbuscule-containing cells and surrounding cortex	Stress responses	Grunwald *et al*. (2004)
MtTubb1	TC94919	β-tubulin	Microarray hybridisation	Arbuscule-containing cells and surrounding cortex	Cytoskeleton reorganisation	Manthey *et al*. (2004)

signalling (MtGlp1, MtLec5 and MtLec6). Detailed reverse genetics studies using mutants or RNAi approaches (Tadege *et al*. 2005; Isayenkov *et al*. 2005; Ivashuta *et al*. 2005) will be required to establish biological functions for these genes during AM (Fig. 1). It should be kept in mind that several AM-induced genes belong to large gene families, a finding e.g. reported by Salzer *et al*. (2000) for eight *M. truncatula* chitinase genes that were differentially expressed not only in AM, but also during other symbiotic and pathogenic plant–microbe interactions. Studying gene families opens the challenging opportunity to identify more fine-tuned functional properties of those members specifically activated during AM.

Towards a molecular basis of gene expression regulation in arbuscular mycorrhiza

So far, hundreds of AM-induced *M. truncatula* genes have been identified by *in silico* and experimental expression profiling experiments, and the full-size promoters of several genes have been tested for gene expression in AM (see Table 2). Although these studies led to a collection of *M. truncatula* promoters that are exclusively or predominantly expressed in arbuscule-containing cells of mycorrhizal roots (e.g. Journet *et al*. 2001; Harrison *et al*. 2002; Krajinski *et al*. 2002; Wulf *et al*. 2003; Grunwald *et al*. 2004; Manthey *et al*. 2004; Vieweg *et al*. 2004; Frenzel *et al*. 2005; Hohnjec *et al*. 2005), little is known about the promoter elements that mediate gene expression in arbuscule-containing cells.

In the promoter of the MtPt4 gene encoding the AM-specific phosphate transporter of *M. truncatula*, Harrison *et al*. (2002) identified a sequence sharing some similarity with a mycorrhiza- and resistance-related element (MRR1) reported for the promoter of the AM-specific phosphate transporter StPT3 from potato (Rausch *et al*. 2001), but this element was not reported to be present or functional in other genes activated in arbuscule-containing cells.

Recently, Boisson-Dernier *et al*. (2005) presented a detailed loss-of-function study of the MtEnod11 promoter. The MtEnod11 gene encoding a proline-rich protein is particularly interesting, since it is activated by Nod-factors, *S. meliloti* infection, diffusible factors from AM fungi and during AM formation (Journet *et al*. 2001; Kosuta *et al*. 2003). The loss-of-function study specified the −257 promoter region as sufficient to drive gene expression in AM roots. In this region, an AT-rich regulatory sequence known from other early nodulin gene promoters was identified. Interestingly, site-directed mutagenesis of this element led to a significant reduction of gene expression both during *S. meliloti* infection and in AM. Since the −257 promoter region is apparently not sufficient for Nod-factor-induced gene expression, it appears that different modules of the promoter are responsible for different symbiotic gene activations (Boisson-Dernier *et al*. 2005).

A similar observation was made for the promoter of the *Vicia faba* leghemoglobin gene VfLb29. This gene was initially identified by reverse northern hybridisations (Frühling *et al*. 1997) and was recently found to be exclusively expressed in the infected cells of root nodules and in the arbuscule-containing cells of arbuscular mycorrhizal roots of different legume and non-legume plants (Vieweg *et al*. 2004). A loss-of-function approach led to the identification of an 85-bp region (position −410 to −326 relative to the start codon) that is required for expression in arbuscule-containing cells, although being dispensable for gene activation in nodules. To investigate an autonomous function of the 85-bp region in arbuscule-containing cells, gain-of-function approaches were performed with chimeric promoter constructs. Since a *trans*-activation by the 85-bp region was not achieved, it appears that the activation of the VfLb29 promoter in arbuscule-containing cells is governed by a complex system requiring at least two regulatory modules (Fig. 1; Fehlberg *et al*. 2005). Thus, although detailed promoter studies in AM roots are obviously far from comprehensive, it emerges that the situation is more complex than in the root nodule symbiosis where organ-specific-elements (OSEs) were reported to be sufficient to direct gene expression to the infected cells of root nodules (Stougaard *et al*. 1987).

Clearly, the detailed molecular analysis of more AM-specific promoters will be required to specify core elements responsible for gene activation in arbuscule-containing cells. The global transcriptome profiling experiments presented above delivered a collection of AM-induced genes that, together with upcoming information on promoters from the *M. truncatula* genome sequence, can be the basis for more genome-wide analyses of promoter motifs regulating gene expression during AM.

Conclusions

The application of untargeted transcriptome profiling has provided novel insights into the molecular basis of AM development, but the lack of cellular resolution in these high-throughput expression approaches makes it difficult to determine the symbiotic stage or the cell types where AM-activated genes are important. A major future challenge will thus be to develop and apply single-cell profiling techniques to specify the transcriptome of characteristic symbiotic cells, e.g. epidermal cells in contact with appressoria, cells surrounding the infecting hyphae, and cells harbouring arbuscules of different developmental stages. The second challenge will be to overcome the lack of knowledge on transcriptional regulators controlling the formation and sustainability of the AM symbiosis. The identification of such regulators will be facilitated by the development of real-time RT–PCR platforms targeted at the robust detection of transcription factor gene expression (Czechowski *et al*. 2004). Finally, it should be kept in mind that an understanding

of the 'system' AM will only be possible by analysing the transcriptional reprogramming in the two genomes that mastermind the development of AM roots. The ongoing genome project in the model AM fungus *G. intraradices* is a first step towards this direction and the future availability of whole-genome microarrays covering the genomes of the macro- as well as the microsymbiont can be expected to jump-start another era in AM research.

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