Journal of
Experimental
Botany
www.jxb.oxfordjournals.org

RESEARCH PAPER

Composite *Medicago truncatula* plants harbouring *Agrobacterium rhizogenes*-transformed roots reveal normal mycorrhization by *Glomus intraradices*

Cornelia Mrosk[1], Susanne Forner[1], Gerd Hause[2], Helge Küster[3], Joachim Kopka[4] and Bettina Hause[1],*

[1] Leibniz-Institut für Pflanzenbiochemie, Weinberg 3, D-06120 Halle, Germany
[2] Biozentrum, Universität Halle, Weinbergweg 22, D-06120 Halle, Germany
[3] Abteilung IV-Pflanzengenomforschung, Institut für Pflanzengenetik, Leibniz Universität Hannover, D-30419 Hannover, Germany
[4] Max-Planck-Institut für Molekulare Pflanzenphysiologie, Am Mühlenberg 1, D-14476 Potsdam, Germany

Abstract

Composite plants consisting of a wild-type shoot and a transgenic root are frequently used for functional genomics in legume research. Although transformation of roots using *Agrobacterium rhizogenes* leads to morphologically normal roots, the question arises as to whether such roots interact with arbuscular mycorrhizal (AM) fungi in the same way as wild-type roots. To address this question, roots transformed with a vector containing the fluorescence marker DsRed were used to analyse AM in terms of mycorrhization rate, morphology of fungal and plant subcellular structures, as well as transcript and secondary metabolite accumulations. Mycorrhization rate, appearance, and developmental stages of arbuscules were identical in both types of roots. Using Mt16kOLI1Plus microarrays, transcript profiling of mycorrhizal roots showed that 222 and 73 genes exhibited at least a 2-fold induction and less than half of the expression, respectively, most of them described as AM regulated in the same direction in wild-type roots. To verify this, typical AM marker genes were analysed by quantitative reverse transcription-PCR and revealed equal transcript accumulation in transgenic and wild-type roots. Regarding secondary metabolites, several isoflavonoids and apocarotenoids, all known to accumulate in mycorrhizal wild-type roots, have been found to be up-regulated in mycorrhizal in comparison with non-mycorrhizal transgenic roots. This set of data revealed a substantial similarity in mycorrhization of transgenic and wild-type roots of *Medicago truncatula*, validating the use of composite plants for studying AM-related effects.

Key words: *Agrobacterium rhizogenes*, arbuscular mycorrhiza, composite plants, *Glomus intraradices*, isoflavanoids, transcript profiling, transmission electron microscopy.

Introduction

Arbuscular mycorrhiza (AM) represents a widespread mutualistic association between soil-borne fungi of the phylum Glomeromycota and most land plants (Smith and Read, 1997). The fungus assists the plant mainly by improving the supply of water and mineral nutrients, especially phosphate. In return, the obligate biotrophic AM fungus is provided by the plant with assimilates accrued from photosynthesis. In the *Arum*-type interaction, as established, for example, between *Glomus* sp. and *Medicago truncatula* (Brundrett, 2004), the AM fungus colonizes the cortical cells by formation of intra- and intercellular hyphae and very characteristic haustoria-like structures, the highly branched intracellular arbuscules (Smith and Read, 1997). Intracellular fungal structures, however, are separated from the plant cytoplasm by an extension of the plant plasma membrane, forming the periarbuscular membrane surrounding the

arbuscule. These greatly increased membranes of both host and arbuscule offer optimized conditions for effective nutrient exchange via the developed symbiotic interface (for reviews, see Gianinazzi-Pearson *et al.*, 1996; Harrison, 1999). In this respect, the exchange of phosphate via the periarbuscular interface and the regulation of AM by phosphate availability for the plant are well characterized. High levels of available phosphate can suppress the mycorrhization (Mosse, 1973; Jasper *et al.*, 1979). In addition, the inhibition of phosphate exchange by the knockout or down-regulation of AM-inducible phosphate transporters led to an aborted mycorrhization (Maeda *et al.*, 2006; Javot *et al.*, 2007). Due to the importance of phosphate transfer from the fungus to the plant, the level of transcript accumulation of plant mycorrhiza-specific induced phosphate transporter genes can be used to evaluate the amount of functional arbuscules within a mycorrhizal root (Isayenkov *et al.*, 2004; Isayenkov *et al.*, 2005; Floß *et al.*, 2008a).

Among the legumes used for studying symbiotic interactions, *M. truncatula* was proposed as a model system for molecular and genetic studies (Barker *et al.*, 1990). *Medicago truncatula* exhibits a number of characteristics important for these studies, such as autogamy, a small diploid genome, and the well-developed genetic and genomic tools allowing functional genomic approaches (Rose, 2008). Additionally, efficient transformation and regeneration protocols with *Agrobacterium tumefaciens* have been described for at least two ecotypes of *M. truncatula* (Trinh *et al.*, 1998; Chabaud *et al.*, 2002). *In planta* transformation methods were also tested in *M. truncatula*; unfortunately, these protocols could not been reproduced (Somers *et al.*, 2003; Zhou *et al.*, 2004).

In recent years, a transformation method has been developed which is less time consuming in generating transgenic plant tissue. In this case, *Agrobacterium rhizogenes* is used. Unlike *A. tumefaciens*, infection with the soil bacterium *A. rhizogenes* generates adventitious, genetically transformed hairy roots at the site of inoculation in many dicots. Infection at the wounding sites by *A. rhizogenes* results in the transfer and integration of T-DNA from the root-inducing (Ri) plasmid into the plant cells (Grant *et al.*, 1991). Upon expression of the *root locus* (*rol*) genes, roots are formed, of which a certain number are co-transformed with the binary vector that contains the gene of interest (Limpens *et al.*, 2004).

Transformation of roots with *A. rhizogenes* leads to production either of 'composite' plants consisting of a wild-type non-transgenic shoot and transgenic hairy roots (Hansen and Wright, 1999) or of root organ culture (ROC) after clonal propagation of roots as independent organs (Fortin *et al.*, 2002; Bago *et al.*, 2006). Among these systems, ROC systems suffer from the absence of photosynthetic tissues, and the presence of sucrose in the culture medium leading to abnormal subcellular structures as shown for plastids (Lohse *et al.*, 2005). Moreover, the lack of a normal root hormonal balance and incomplete source–sink relationships might alter plant–fungal symbiotic interactions (Fortin *et al.*, 2002).

Protocols for generating transformed hairy roots by *A. rhizogenes*-mediated transformation have been developed (Boisson-Dernier *et al.*, 2001; Rodríguez-Llorente *et al.*, 2003; Limpens *et al.*, 2004) and have been used as rapid tools to test gene function (Limpens *et al.*, 2004; Ivashuta *et al.*, 2005; Javot *et al.*, 2007), promoter activity (Liu *et al.*, 2003; Vieweg *et al.*, 2004; Grønlund *et al.*, 2005; Floß *et al.*, 2008a; Hohnjec *et al.*, 2009), and the role of metabolites, such as phytohormones (Isayenkov *et al.*, 2005; Sun *et al.*, 2006; Ding *et al.*, 2008) and secondary compounds (Wasson *et al.*, 2006; Floß *et al.*, 2008a, b), mainly in the study of root endosymbioses with nitrogen-fixing rhizobacteria and AM fungi in *M. truncatula*. This system provides the advantage of modulating only the plant organ, which is directly involved in the mutualistic interaction. Therefore, this root transformation system has been successfully used to substitute generation of stable transgenics by transformation with *A. tumefaciens*.

Although transformed roots are morphologically indistinguishable from untransformed roots and suitable to be colonized by AM fungi, the question arises as to whether transgenic roots of 'composite' plants respond similarly to wild-type roots in respect to morphological and molecular features of mycorrhization. It should be noted that *Agrobacterium rol* genes co-transformed with the transgene of interest may disturb the hormonal balance of the plants (Schmülling *et al.*, 1988). This leads to an often observed altered phenotype of transgenic plants regenerated from hairy roots (Christey and Braun, 2005). Therefore, the effect of *A. rhizogenes*-mediated root transformation on mycorrhization was examined. Using different microscopical techniques, the degree of mycorrhization and the morphology of mycorrhizal structures were investigated. Furthermore, transcript and metabolite accumulation in mycorrhizal transgenic roots in comparison with mycorrhizal wild-type roots was analysed. The results show clearly that mycorrhization of transgenic roots corresponds to mycorrhization of wild-type roots. Thus, *A. rhizogenes*-transformed *M. truncatula* roots represent a valid and highly versatile model to enhance our understanding of AM interaction by targeted genetic manipulation.

Materials and methods

Plant material and fungal inoculation

Medicago truncatula (L.) Gaertn. var. Jemalong A17 was grown in a phytochamber with a 16 h/8 h cycle (22 °C/18 °C) at 375 μM light intensity and in pots filled with expanded clay of 2–5 mm particle size (Original Lamstedt Ton; Fibo ExClay, Lamsted, Germany). Plants were watered with deionized water three times per week. Fertilizer (10 ml of Long Ashton medium containing 20% phosphate) was applied once per week. Five-week-old plants were inoculated with the AM fungus *Glomus intraradices* Schenk & Smith isolate 49 (Maier *et al.*, 1995) by transfer to clay substrate containing 15% (v/v) inoculum.

The inoculum was enriched in propagules by cultivation with mycorrhizal leek (*Allium porrum* cv. Elefant). As non-mycorrhizal controls, plants were mock-inoculated with the same amount of inoculum, which was sterilized by auto-claving.

Hairy root transformation and selection of composite plants

Transgenic hairy roots in *M. truncatula* have been generated using a modified protocol from Vieweg *et al.* (2004) as described by Floß *et al.* (2008*a*) using *A. rhizogenes* harbouring the vector pRedRoot encoding the fluorescent marker protein DsRED 1 (Limpens *et al.*, 2004). Five weeks after *A. rhizogenes* injection the first screen for DsRED1 was performed using a fluorescence stereomicroscope (Leica MZ FLIII with DsRED1-Filter; Leica Camera AG, Solms, Germany). All non-fluorescent roots were removed and plants bearing transgenic roots were inoculated with *G. intraradices* and cultivated under the conditions described above.

Microscopic visualization of fungal structures in roots

Roots were stained with 5% (v/v) ink (Shaeffer Skrip jet black, Sheaffer Manufacturing, Madison, WI, USA) in 2% acetic acid according to Vierheilig *et al.* (1998). Fungal structures were assessed using a stereomicroscope and the colonization rate was evaluated with the computer program 'Mycocalc' according to Trouvelot *et al.* (1986).

For the investigation of arbuscule developmental stages, root fractions were stained with acid fuchsin according to Dickson *et al.* (2003). Subsequently, stained roots were rinsed in water and transferred to slides with 100% glycerol. The fungal structures within the roots were visualized by confocal laser scanning microscopy (LSM 510 Meta; Zeiss, Jena, Germany) using the 543 nm laser line for excitation.

For bright field and electron microscopy, small pieces of mycorrhizal roots were dissected and fixed in 3% glutardial-dehyde in 0.1 M sodium cacodylate buffer (SCB, pH 7.0) for 3 h at room temperature. After rinsing with buffer, samples were post-fixed with 1% (w/v) osmium tetroxide in SCB for 30 min, rinsed again, and dehydrated in a graded series of ethanol. Ethanol was then substituted by epoxy resin (Spurr, 1969). Semi-thin sections (1 μm) were stained with 0.5% toluidine blue and observed with a bright field AxioImager microscope equipped with an AxioCam digital camera (Zeiss, Jena, Germany). Ultrathin sections (90 nm) were stained with uranyl acetate/lead citrate and observed with an EM 900 transmission electron microscope (Zeiss, Oberkochen, Germany). Electron micrographs were taken with a slow scan camera (Variospeed SSCCD camera SM-1k-120, TRS, Moorenweis, Germany). All micrographs were processed through the Photoshop 8.0.1 program (Adobe).

Isolation of RNA and synthesis of cDNA

Total RNA was prepared from roots with the Qiagen RNeasy® Plant Mini Kit (Qiagen, Hilden, Germany) followed by DNase digestion (RNase-free DNase Set; Qiagen). For real-time RT-PCR analyses, 1 μg of total RNA was converted into cDNA with M-MLV reverse transcriptase, RNase H Minus, Point Mutant (Promega, Madison, WI, USA) according to the manufacturer's protocol using oligo(dT)$_{19}$ primer. Prior to real-time PCR the cDNA was diluted to fixed quantities (15 ng per reaction of reverse-transcribed total RNA). For microarray analyses, RNA was concentrated using Microcon-30-colums (Milli-pore, Billerica, MA, USA) according to the supplier's instructions. RNA integrity was analysed by gel electrophoresis. A 20 μg aliquot of RNA was then used to synthesize Cy3- and Cy5-labelled cDNA as described (Hohnjec *et al.*, 2005).

Microarray analysis

Microarray analysis was performed using four independent mycorrhizal transgenic plants. RNA from two non-mycorrhizal transgenic plants were combined and served as control. The cDNAs were hybridized to four Mt16kO-LI1Plus microarrays. These microarrays were composed of 16 470 70mer oligonucleotide probes representing tentative consensuses (TCs) of the DFCI *M. truncatula* Gene Index 5 (Küster *et al.*, 2007). Hybridization conditions, image acquisition, and data processing were performed according to Hohnjec *et al.* (2005). Lowess normalization and *t*-test statistical analyses were performed using the EMMA array analysis software (Dondrup *et al.*, 2003).

Realtime RT-PCR

For semi-quantitative detection of transcripts a SYBR Green-based PCR assay (Applied Biosystems, Warrington, UK) was performed. All primers used are listed in Supplementary Table S1 available at *JXB* online. The assay mix contained 15 ng of reverse-transcribed total RNA and 100 nM primers. The reactions were performed in a Mx 3005P QPCR system (Stratagene, La Jolla, CA, USA) with the following protocol: 95 °C for 10 min followed by 40 cycles of 95 °C for 30 s, 60 °C for 1 min, and 72 °C for 30 s, and a subsequent standard dissociation protocol. As control for genomic DNA contamination, 15 ng of total non-transcribed RNA was used under the same conditions as described above.

All assays using four independent transgenic or non-transgenic root systems were performed in three technical replicates each. ΔCt-values of the respective gene and the *Translation Elongation Factor 1α* (*MtTEF1α*) gene, respectively, were calculated by subtracting Ct-values of mycorrhizal samples from the Ct-value of the non-mycorrhizal samples. ΔΔCt-values were calculated by subtracting ΔCt-values obtained for the respective gene from the ΔCt-value of *MtTEF1α*.

Profiling analyses of metabolite fractions enriched with secondary metabolites

To determine levels of isoflavonoids and apocarotenoids, homogenized and freeze-dried material of transgenic mycorrhizal and non-mycorrhizal roots was extracted with 80% aqueous methanol. Aliquots of the extracts were subjected to analytical HPLC as described by Schliemann *et al.* (2008) using a modified gradient system consisting of 1.5% aqueous H_3PO_4 (A) and MeCN (B): linear gradient from 5% to 25% of B in (A+B) within 40 min and linear gradient from 25% to 80% of B in (A+B) within 20 min.

Results

Colonization of transgenic roots with *G. intraradices*

After *A. rhizogenes*-mediated transformation using the vector pRedRoot, plants harbouring transgenic roots were selected by their red fluorescence and inoculated with *G. intraradices*. Determination of mycorrhization rates using the method of Trouvelot *et al.* (1986) demonstrated nearly complete colonization of the roots 5 weeks after inoculation (Fig. 1A). There was no detectable difference between transgenic and wild-type roots in all parameters determined, such as, for example, colonization frequency and arbuscule abundance. Evaluation of fungal structures in toluidine blue-stained cross-sections of roots resulted in a similar pattern for both types of roots (Fig. 1B, C). In both, cells of the inner cortex surrounding the vascular cylinder contained arbuscules. Additionally, a dense hyphal network was visible in the intercellular space of the outer cortex. To uncover potential changes in arbuscule morphology, confocal images of arbuscules were evaluated following acid fuchsin staining (Fig. 1D, E). Three stages of arbuscule development were defined according to Floß *et al.* (2008*a*) and their proportions determined by counting. Again, neither the appearance of these developmental stages nor the proportion between them changed in transgenic roots compared with wild-type roots.

For detailed ultrastructural analyses, the focus was on inner cortex cells harbouring arbuscules. Mycorrhizal regions of wild-type and transgenic roots were processed for transmission electron microscopy (TEM). Analysis of electron micrographs revealed that typical developmental stages of arbuscules were visible in both types of roots (Fig. 2). Mature arbuscules are characterized by a high number of hyphal branches filled with cytoplasm. These arbuscular branches are surrounded by the plant periarbuscular membrane. They are embedded in a dense plant cytoplasm containing the nucleus (Fig. 2A, B) and organelles such as mitochondria, plastids, and endoplasmic reticulum (Fig. 2A–D). Collapsed arbuscules were visible as an accumulation of fungal cell walls without fungal cytoplasm, but 'enclosed' in plant cytoplasm (Fig. 2E, F). Analysing preparations of two and four different mycorrhizal wild-
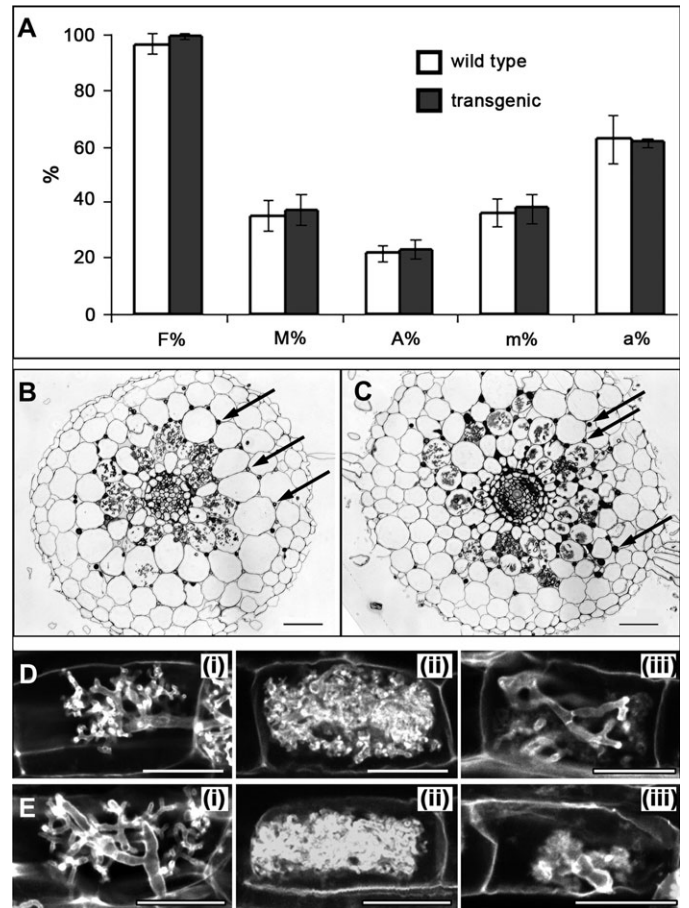

Fig. 1. Mycorrhization 5 weeks after inoculation of wild-type and transgenic roots of *M. truncatula*. To generate transgenic roots, root transformation was performed using *A. rhizogenes* carrying pRedRoot. For analysis of transgenic roots, only roots with strong red fluorescence have been selected. (A) Degree of colonization as determined from ink-stained roots according to Trouvelot *et al.* (1986). F%, frequency of colonization in the root system; M%, intensity of the mycorrhizal colonization in the root system; A%, arbuscule abundance in the root system; m%, density of colonization in mycorrhizal root fragments; and a%, arbuscule abundance in the mycorrhizal root part. For each parameter, the mean ±SD is shown (*n*=6). (B and C) Representative semi-thin cross-sections of mycorrhizal wild-type (B) and transgenic (C) roots showing fungal structures in the inner cortex. The intercellular space contains numerous hyphae (arrows). Bars represent 20 μm. (D and E) Representative confocal images of the arbuscule developmental stages in wild-type (D) and transgenic (E) roots visualized by staining with acid fuchsin. For each, (i) a young, developing arbuscule (characterized by small size and restricted dichotomous branching); (ii) a fully developed arbuscule (exhibiting extensive branching and almost completely filling the host cortical cell); and (iii) a degenerating arbuscule (characterized by septa formed on arbuscule branches) is shown. Bars represent 20 μm.

type and transgenic roots, respectively, no differences in ultrastructure of the plant cells or of the fungal structures (hyphae and arbuscules) could be detected comparing both types of roots.
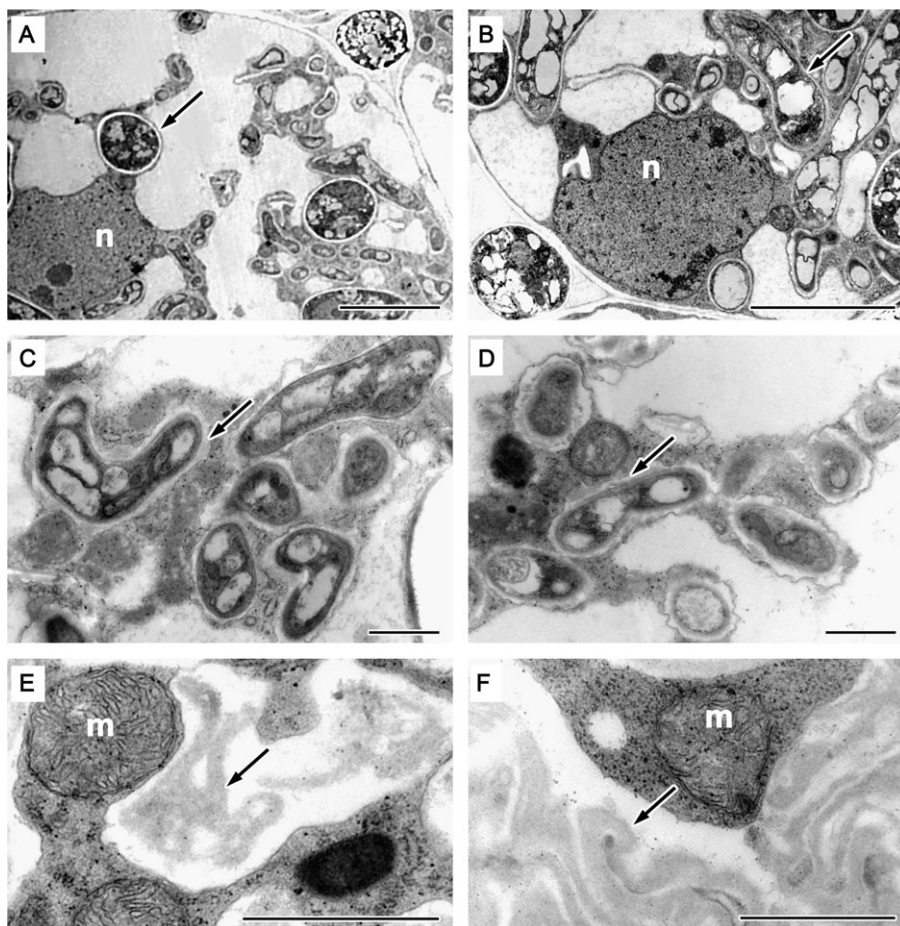
Fig. 2. Representative transmission electron micrographs of mycorrhizal wild-type (A, C, and E) and transgenic (B, D, and F) roots. To generate transgenic roots, root transformation was performed using *A. rhizogenes* carrying pRedRoot. For analysis of transgenic roots, only roots with strong red fluorescence have been selected. (A and B) Cortex cells harbouring a functional arbuscule with well-separated hyphal structures (arrow) in near proximity to the plant cell nucleus (n). (C and D) Details of arbuscular branches. Note the organelle-rich plant cytoplasm surrounding each arbuscule branch, which is enclosed by the periarbuscular membrane (arrow). (E and F) Degenerating arbuscules. Note the collapsed fungal cell walls (arrow) clearly separated from the plant cytoplasm containing organelles such as mitochondria (m). Bars represent 5 μm in A, B, and 1 μm in C–F.

Transcript accumulation in non-mycorrhizal and mycorrhizal transgenic roots

To obtain a first impression about the extent of altered transcript accumulation in mycorrhizal transgenic roots, transcriptome analyses were performed from four mycorrhizal transgenic roots compared with a pooled sample from two non-mycorrhizal transgenic roots using Mt16kOLI1-Plus microarrays (Thompson *et al.*, 2005). A total of 222 genes could be identified showing at least a 2-fold induction due to mycorrhization, whereas 73 genes exhibited less than half the expression level in mycorrhizal roots in comparison with non-mycorrhizal roots (Supplementary Table S2 at *JXB* online). Comparing data presented here for transgenic roots with those for mycorrhizal wild-type roots previously published (Hohnjec *et al.*, 2005), 107 and 29 genes are commonly up- and down-regulated, respectively. This corresponds to ~50% and 40% of genes, which are identified as regulated in transgenic roots and are regulated in the same way in wild-type roots.

This result prompted us to pay particular attention to genes previously reported as specifically up-regulated in *M. truncatula*/*G. intraradices*-colonized roots (Hohnjec *et al.*, 2005; Liu *et al.*, 2007). Out of the *M. truncatula* genes co-induced at least 2-fold by mycorrhization, nine genes were selected and directly compared between transgenic and wild-type roots (Table 1). This includes well-known marker genes for cells containing functional arbuscules, such as *MtPT4* (Harrison *et al.*, 2002), *MtSCP1* (Liu *et al.*, 2003), *MtGST1* (Wulf *et al.*, 2003), *MtGLP1* (Doll *et al.* 2003), *MtLEC7* (Frenzel *et al.*, 2005), and *MtBCP1* (Hohnjec *et al.*, 2005). Moreover, TC101060 annotated as defensin and TC112474 annotated as a proteinase inhibitor were taken into account. All these genes showed a mycorrhiza-specific induction in transgenic as well as in wild-type roots. The levels of transcript accumulation in transgenic and wild-type roots deduced from array analyses were in some cases different. This might be due to different inoculation periods and degree of fungal colonization of the plants used

Table 1. Expression of selected mycorrhiza-specific and mycorrhiza-induced marker genes deduced from microarrays and validated by real-time RT-PCR

M-values representing the fold induction deduced from array hybridization [$\log_2$ expression ratios ($P \leq 0.05$) of four individual mycorrhizal transgenic roots in comparison with two non-mycorrhizal transgenic roots (pooled)] are from transgenic roots 3 weeks post-inoculation. Data for wild-type roots are from Hohnjec *et al.* (2005) and were obtained 4 weeks post-inoculation. $\Delta\Delta$Ct values were obtained by comparing the mean value of mycorrhizal roots with non-mycorrhizal roots for transgenic and wild-type roots, both 5 weeks post-inoculation. Statistical analyses were performed with Student's *t*-test, resulting in $P \leq 0.05$ ($n=4$).

Oligo-ID	DFCI MtGI8 Annotation	Annotation	M-value, transgenic roots	M-value, wild-type roots	$\Delta\Delta$Ct, transgenic roots	$\Delta\Delta$Ct, wild-type roots
MT009707	TC94453 UP|Q8GSG4 (Q8GSG4) Phosphate transporter PT4, complete	*MtPT4*[a]	1.37	5.06	14.49	13.65
MT015668	TC96500 weakly similar to UP|Q9LUM8 (Q9LUM8) Blue copper-binding protein-like, partial (38%)	*MtBcp1*[b]	1.42	2.68	8.20	8.13
MT009704	TC95018 UP|Q84XR7 (Q84XR7) Germin-like protein 1, complete	*MtGlp1*[a]	1.92	4.29	9.92	7.65
MT009185	TC106954 similar to UP|PRTP_HUMAN (P10619) Lysosomal protective protein precursor (Cathepsin A) (Carboxypeptidase C) (Protective protein for beta-galactosidase), partial (16%)	*MtScp1*[a]	1.97	4.46	6.61	6.73
MT007813	TC94919 homologue to UP|Q76FS3 (Q76FS3) Beta-tubulin, partial (98%)	*MtTubb1*[b]	2.07	2.20	2.77	2.52
MT003520	TC95567 similar to UP|P93536 (P93536) Bark lectin II precursor (LECSJABMII) (B-SJA-II) (Fragment), partial (40%)	*MtLec7*[b]	2.27	5.20	11.69	8.16
MT009013	TC100720 UP|Q8H1Y6 (Q8H1Y6) Glutathione-*S*-transferse-like protein, complete	*MtGst1*[a]	3.54	5.86	11.48	7.66
MT014645	TC101060 weakly similar to PIR|S66221|S66221 defensin AMP1–*Dahlia merckii (Dahlia merckii)*, partial (88%)	*Defensin*[a]	4.10	5.34	15.44	9.23
MT015000	TC112474	*Proteinase inhibitor*[a]	4.44	4.34	11.57	10.70
–	–	*GiβTub1*	–	–	17.30	15.83

Oligo ID, identifier of *M. truncatula* 70mer oligonucleotides.
[a] Mycorrhiza specific.
[b] Mycorrhiza induced.

for microarray experiments rather than to differences in plant nutrition and the cultivation conditions.

To validate the microarray results, real-time RT-PCR analyses were carried out on the selected genes (Table 1). For this purpose, plants containing transgenic roots and wild-type plants were inoculated simultaneously. All genes selected showed a strong increase in transcript levels in mycorrhizal roots relative to non-mycorrhizal roots. For example, TC101060 and TC112474 exhibited the strongest increase in transcript accumulation in both the array and real-time RT-PCR data. The high induction values revealed by real-time RT-PCR for *MtPT4* are due to the fact that this gene exhibits a strictly mycorrhiza-specific expression and transcripts are close to the detection limit in non-mycorrhizal roots (Harrison *et al.*, 2002). Similar results were obtained for *GiβTub1* ($\Delta\Delta$Ct >15), which is a fungal gene and is by definition not detectable in non-mycorrhizal roots. Differences between M-values obtained by array analyses and $\Delta\Delta$Ct obtained by real-time RT-PCR can be ascribed to the higher dynamic range of real-time RT-PCR analyses (Huggett *et al.*, 2005) and the typical variability of mycorrhizal colonization experiments. Nevertheless, the detected increase in the transcript accumulation of selected marker genes is similar in transgenic and wild-type roots, pointing to similar transcriptional responses of both types of roots during colonization.

Accumulation of secondary metabolites in non-mycorrhizal and mycorrhizal transgenic roots

The relative content of secondary metabolites, known to occur in increased amounts in mycorrhizal roots, was determined by HPLC (Table 2). The levels of isoflavonoids, such as ononin and malonylononin, increased in mycorrhizal transgenic roots in comparison with non-mycorrhizal transgenic roots. The increase in the levels of both compounds reached nearly the same degree as reported for wild-type roots (Schliemann *et al.*, 2008). Moreover, apocarotenoids accumulated in mycorrhizal roots, but do not occur in non-mycorrhizal roots (Table 2). Among them, the 'yellow pigment complex', mycorradicin, and three cyclohexenone derivatives have been identified. All of them occurred exclusively in mycorrhizal roots, both in transgenic and in wild-type roots.

Discussion

Generation of 'composite' plants consisting of wild-type shoots and transgenic roots is a fast and easy method for functional genomics in legume research concerning biotic interactions of the root. Although roots transformed using *A. rhizogenes* have been described as morphologically similar to wild-type roots, it cannot be excluded that a disturbed hormone balance in the hairy root may have an influence on biotic interactions and gene expression, especially when phytohormone-regulated transgenes are analysed in such roots. Therefore, the mycorrhization of transgenic 'hairy' roots in comparison with wild-type roots in respect to mycorrhization rate, morphology of arbuscules and cortex cells, gene expression, and metabolite accumulation was analysed. Roots of *M. truncatula* cv. Jemalong A17 were transformed using *A. rhizogenes* strain *Arqua*1 (Quandt *et al.*, 1993). This strain bears a pA4 type plasmid and induces only 1–3 roots per wound, leading to plants

similar to wild-type plants as far as the shoot/root proportion was concerned (Quandt *et al.*, 1993). Using a plasmid encoding the fluorescent protein DsRED1 as a non-destructive selectable marker for transformed roots (pRedRoot, Limpens *et al.*, 2004), non-transgenic, chimeric, and homogeneously transformed roots could be discriminated. Only completely transformed roots have been selected and used for the analyses.

Medicago truncatula has proven to be a useful model for investigating the AM symbiosis with the obligate biotroph *Glomus* spp. (Liu *et al.*, 2003; Hohnjec *et al.*, 2006). Using *G. intraradices*, both *M. truncatula* wild-type and transgenic roots exhibited a nearly complete colonization at 5 weeks after colonization. All fungal structures typical for the symbiosis were apparent, including intercellular hyphae, arbuscules, vesicles, and extraradical hyphae. The typical mycorrhization parameters did not differ in both types of roots, and the abundance of arbuscules did not show significant differences in all plants analysed. Since arbuscules are transient structures developing and subsequently degenerating within 6–10 d (Alexander *et al.*, 1989; Dickson and Smith, 2001), arbuscule formation and decay are ongoing during the time course of mycorrhization. Therefore, AM roots always contain a population of developing and degenerating arbuscules and regularly all developmental stages are visible within one and the same mycorrhizal root. This is also the case for transgenic roots (Fig. 1), where all developmental stages were detected in a proportion similar to that within wild-type roots. Additionally, TEM analysis of arbuscular structures and root cortex cells has provided evidence supporting the similar appearance of living as well as collapsing arbuscules in transgenic and wild-type roots. The ultrastructure of neither the symbiotic interface nor the plant cytoplasm including organelles differed between both types of roots (Fig. 2). The cytoplasm of arbuscule-containing cells is usually characterized by a high number of organelles, such as dictyosomes (Balestrini *et al.*, 1996), endoplasmatic reticulum (Hause and Fester,

Table 2. Analysis of mycorrhiza-specific and mycorrhiza-induced secondary metabolites from mycorrhizal and non-mycorrhizal roots of *M. truncatula*

Metabolites were identified by their retention time (R_t) and absorption maxima (λ_{max}) according to Schliemann *et al.* (2008). Isoflavonoids exhibited significantly increased levels in mycorrhizal roots compared with non-mycorrhizal roots, whereas apocarotenoids not occurring in non-mycorrhizal roots are described as mycorrhiza-specific (myc-spec). All plants were analysed 5 weeks after inoculation with *G. intraradices* ($n=7$).

Metabolite	R_t (min)	λ_{max} (nm)	*P*	x-fold (myc/non-myc), transgenic	x-fold (myc/non-myc), wild-type
Isoflavonoids					
Ononin	37.96	251, 300sh	0.00004	2.83	3.52
Malonylononin	45.23	257, 300sh	0.00127	1.79	2.45
Apocarotenoids					
13-Hydroxyblumenol C 9-*O*-glycoside	21.28	246	–	myc-spec	myc-spec
Blumenol C 9-*O*-glycoside	27.96	243	–	myc-spec	myc-spec
Blumenol C 9-*O*-malonylglycoside	37.49	245	–	myc-spec	myc-spec
Mycorradicin derivative 3	44.31	281, 360sh, 378, 397sh	–	myc-spec	myc-spec
Yellow pigment	45.52–53.51	315, 360sh, 382, 404	–	myc-spec	myc-spec

2005), and elongated plastids and mitochondria (Lohse *et al.*, 2005), which were also present in colonized transgenic roots. It can therefore be concluded that generation of transgenic roots influences neither the amount of fungal structures, the life span and the morphology of arbuscules, nor the structure of the arbuscule-containing plant cortex cells.

To elucidate whether more subtle distinctions do exist between transgenic and wild-type roots, accumulation of transcripts was analysed using non-mycorrhizal and mycorrhizal transgenic roots. Information on changes in the plant root transcriptome due to the generation of 'composite' plants followed by mycorrhization is still limited. In most cases, wild-type roots have been analysed to identify genes activated specifically in the *M. truncatula–G. intraradices* AM (Manthey *et al.*, 2004; Liu *et al.*, 2007) or genes commonly induced by different AM fungi (Hohnjec *et al.*, 2005; Grunwald *et al.*, 2009). To date, transcript profiling of transgenic roots has been carried out primarily to identify genes differentially regulated due to a knock-down of a gene under functional analysis (Floß *et al.*, 2008*a*). In this case, changes in transcript accumulation have been analysed between different types of transgenic roots, e.g. RNA interference (RNAi) versus empty vector.

To obtain first insights, Mt16kOLI1Plus 70mer oligonucleotide microarrays (Hohnjec *et al.*, 2005) were applied and data were compared with data available for mycorrhizal *M. truncatula* wild-type roots after hybridization to the Mt16kOLI1 microarray (Hohnjec *et al.*, 2005) containing the same core set of 16 086 70mer oligos (Küster *et al.*, 2007). It turned out, however, that in mycorrhizal transgenic roots the genes showing enhanced and decreased transcript accumulation represent only about the half of genes which were described as regulated by mycorrhization in wild-type roots. This relatively low match could be due to the mycorrhization period, which was different between both approaches (3 and 4 weeks for transgenic and wild-type roots, respectively). Moreover, it has to be stressed that a relatively high number of genes could not be evaluated due to missing signals or to insufficient statistical significance of the data obtained from the four biological replicates used in the transgenic approach. This is, however, different in both types of roots inoculated for the same time period. Here, at least the subset of the nine best characterized mycorrhiza-specific and mycorrhiza-induced marker genes exhibited very similar levels of increased transcript accumulation in mycorrhizal roots as revealed by real-time RT-PCR (Table 1). This also points to the similarity of transgenic and wild-type roots in respect to mycorrhization.

Regarding secondary metabolites, two isoflavonoids (ononin and malonylononin) exhibited higher levels in mycorrhizal than in non-mycorrhizal transgenic roots (Table 2). This is in agreement with data from *M. truncatula* wild-type roots, which accumulate isoflavonoids starting from 35 d after inoculation (Harrison and Dixon, 1994; Schliemann *et al.*, 2008), corresponding to the increased expression of an isoflavonoid glucosyltransferase gene (Liu *et al.*, 2007). Isoflavonoids are known to be synthesized *de novo* in response to biotic or abiotic stresses (Dixon and Steele, 1999). Their function in AM, however, still remains unclear. As during early stages of AM, plant defences are induced, but later repressed, it has been speculated that the enhanced levels of flavonoid compounds stimulate the growth of AM fungi rather than function as antimicrobial compounds (Hause and Fester, 2005).

Unlike the constitutively occurring isoflavonoids, the apocarotenoids (cyclohexenone and mycorradicin derivatives) accumulate specifically during AM. No other biotic interaction such as infections of roots with pathogens and endophytes and no treatment with abiotic stressors such as heat, cold, high light intensities, and heavy metals led to the accumulation of these compounds (for a review, see Akiyama and Hayashi, 2008). Thus, apocarotenoids may represent the most specific metabolic marker of mycorrhization known today. In mycorrhizal transgenic roots of *M. truncatula* several derivatives of apocarotenoids accumulated, all known from mycorrhizal *M. truncatula* wild-type roots (Schliemann *et al.*, 2008). Usually, apocarotenoids accumulate to high levels particularly in the late stages of the symbiosis, and their occurrence might be related to the degradation of arbuscules (Strack and Fester, 2006; Walter *et al.*, 2007). The main compounds, three blumenol glycosides, one mycorradicin derivative, and the 'yellow pigment', have been detected in mycorrhizal transgenic roots, pointing to a normal mycorrhization including synthesis of secondary compounds in such roots.

Summarizing the presented results, it can be stated that composite plants harbouring transgenic roots after transformation with *A. rhizogenes* interact with AM fungi in the same way as wild-type plants. There are no general differences detectable in the mycorrhization rate, in the morphology of fungal structures, or in the molecular response of the plant root to the interaction with the fungus. Therefore, we conclude that the hairy root transformation system represents a valid and easy to handle model to modify genetically and study the molecular physiology of the mycorrhizal interaction between *M. truncatula* and *G. intraradices*.

Supplementary data

Supplementary data are available at *JXB* online.

Table S1. Summary of PCR primer sequences.

Table S2. Compilation of $\log_2$ expression ratios of mycorrhizal versus non-mycorrhizal transgenic roots from an analysis on Mt16kOLI1Plus microarrays. Data for wild-type roots mycorrhized with *G. intraradices* are from Hohnjec *et al.* (2005).

Acknowledgements

References

Akiyama K, Hayashi H. 2008. Plastid-derived strigolactones show the way to roots for symbionts and parasites. *New Phytologist* **178,** 695–698.

Alexander T, Toth R, Meier R, Weber H. 1989. Dynamics of arbuscule development and degeneration in onion, bean, and tomato with reference to vesicular-arbuscular mycorrhizae in grasses. *Canadian Journal of Botany* **67,** 2505–2513.

Bago A, Cano C, Toussaint J-P, Smith S, Dickson S. 2006. Interactions between the arbuscular mycorrhizal (AM) fungus *Glomus intraradices* and nontransformed tomato roots of either wild-type or AM-defective phenotypes in monoxenic cultures. *Mycorrhiza* **16,** 429–436.

Balestrini R, Hahn MG, Faccio A, Mendgen K, Bonfante P. 1996. Differential localization of carbohydrate epitopes in plant cell walls in the presence and absence of arbuscular mycorrhizal fungi. *Plant Physiology* **111,** 203–213.

Barker D, Bianchi S, Blondon F, et al. 1990. *Medicago truncatula*, a model plant for studying the molecular genetics of the *Rhizobium–legume* symbiosis. *Plant Molecular Biology Reporter* **8,** 40–49.

Boisson-Dernier A, Chabaud M, Garcia F, Bécard G, Rosenberg C, Barker D. 2001. *Agrobacterium rhizogenes*-transformed roots of *Medicago truncatula* for the study of nitrogen-fixing and endomycorrhizal symbiotic associations. *Molecular Plant-Microbe Interactions* **14,** 695–700.

Brundrett M. 2004. Diversity and classification of mycorrhizal associations. *Biological Reviews* **79,** 473–495.

Chabaud M, Venard C, Defaux-Petras A, Becard G, Barker DG. 2002. Targeted inoculation of *Medicago truncatula in vitro* root cultures reveals *MtENOD11* expression during early stages of infection by arbuscular mycorrhizal fungi. *New Phytologist* **156,** 265–273.

Christey M, Braun R. 2005. Production of hairy root cultures and transgenic plants by *Agrobacterium rhizogenes*-mediated transformation. *Methods in Molecular Biology* **286,** 47–60.

Dickson S, Schweiger P, Smith F, Soderstrom B, Smith S. 2003. Paired arbuscules in the Arum-type arbuscular mycorrhizal symbiosis with *Linum usitatissimum*. *Canadian Journal of Botany* **81,** 457–463.

Dickson S, Smith SE. 2001. Cross walls in arbuscular trunk hyphae form after loss of metabolic activity. *New Phytologist* **151,** 735–742.

Ding Y, Kalo P, Yendrek C, Sun J, Liang Y, Marsh JF, Harris JM, Oldroyd GED. 2008. Abscisic acid coordinates Nod factor and cytokinin signaling during the regulation of nodulation in *Medicago truncatula*. *The Plant Cell* **20,** 2681–2695.

Dixon R, Steele C. 1999. Flavonoids and isoflavonoids—a gold mine for metabolic engineering. *Trends in Plant Science* **4,** 394–400.

Dondrup M, Goesmann A, Bartels D, Kalinowski J, Krause L, Linke B, Rupp O, Sczyrba A, Pühler A, Meyer F. 2003. EMMA: a platform for consistent storage and efficient analysis of microarray data. *Journal of Biotechnology* **106,** 135–146.

Floß DS, Hause B, Lange PR, Küster H, Strack D, Walter MH. 2008a. Knock-down of the MEP pathway isogene *1-deoxy-D-xylulose 5-phosphate synthase-2* inhibits formation of arbuscular mycorrhiza-induced apocarotenoids, and abolishes normal expression of mycorrhiza-specific plant marker genes. *The Plant Journal* **56,** 86–100.

Floß DS, Schliemann W, Schmidt J, Strack D, Walter MH. 2008b. RNA interference-mediated repression of *MtCCD1* in mycorrhizal roots of *Medicago truncatula* causes accumulation of C27 apocarotenoids, shedding light on the functional role of CCD1. *Plant Physiology* **148,** 1267–1282.

Fortin L, Bécard G, Declerck S, Dalpé Y, St-Arnaud M, Coughlan A, Piché Y. 2002. Arbuscular mycorrhiza on root-organ cultures. *Canadian Journal of Botany* **80,** 1–20.

Frenzel A, Manthey K, Perlick A, Meyer F, Pühler A, Küster H, Krajinski F. 2005. Combined transcriptome profiling reveals a novel family of arbuscular mycorrhizal-specific *Medicago truncatula* lectin genes. *Molecular Plant-Microbe Interactions* **18,** 771–782.

Gianinazzi-Pearson V, Dumas-Gaudot E, Gollotte A, Tahiri-Alaoui A, Gianinazzi S. 1996. Cellular and molecular defence-related root responses to invasion by arbuscular mycorrhizal fungi. *New Phytologist* **133,** 45–57.

Grant JE, Dommisse EM, Christey MC, Conner AJ. 1991. Gene transfer to plants using Agrobacterium. In: Murray D, ed. *Advanced methods in plant breeding and biotechnology*. Wallingford, UK: CAB International, 50–73.

Grønlund M, Roussis A, Flemetakis E, Quaedvlieg NEM, Schlaman HRM, Umehara Y, Katinakis P, Stougaard J, Spaink HP. 2005. Analysis of promoter activity of the early nodulin Enod40 in *Lotus japonicus*. *Molecular Plant-Microbe Interactions* **18,** 414–427.

Grunwald U, Guo W, Fischer K, Isayenkov S, Ludwig-Müller J, Hause B, Yan X, Küster H, Franken P. 2009. Overlapping expression patterns and differential transcript levels of phosphate transporter genes in arbuscular mycorrhizal, Pi-fertilised and phytohormone-treated *Medicago truncatula* roots. *Planta* **229,** 1023–1034.

Hansen G, Wright M. 1999. Recent advances in the transformation of plants. *Trends in Plant Science* **4,** 226–231.

Harrison M. 1999. Molecular and cellular aspects of the arbuscular mycorrhizal symbiosis. *Annual Review in Plant Physiology and Plant Molecular Biology* **50,** 361–389.

Harrison M, Dewbre G, Liu J. 2002. A phosphate transporter from *Medicago truncatula* involved in the acquisition of phosphate released by arbuscular mycorrhizal fungi. *The Plant Cell* **14,** 2413–2429.

Harrison M, Dixon R. 1994. Spatial patterns of expression of flavonoid/isoflavonoid pathway genes during interactions between roots of *Medicago truncatula* and the mycorrhizal fungus *Glomus versiforme*. *The Plant Journal* **6,** 9–20.

Hause B, Fester T. 2005. Molecular and cell biology of arbuscular mycorrhizal symbiosis. *Planta* **221,** 184–196.

Hohnjec N, Henckel K, Bekel T, Gouzy J, Dondrup M, Goesmann A, Küster H. 2006. Transcriptional snapshots provide insights into the molecular basis of arbuscular mycorrhiza in the model legume *Medicago truncatula*. *Functional Plant Biology* **33,** 737–748.

Hohnjec N, Lenz F, Fehlberg V, Vieweg MF, Baier MC, Hause B, Küster H. 2009. The signal peptide of the *Medicago truncatula* modular nodulin MtNOD25 operates as an address label for the specific targeting of proteins to nitrogen-fixing symbiosomes. *Molecular Plant-Microbe Interactions* **22,** 63–72.

Hohnjec N, Vieweg MF, Pühler A, Becker A, Küster H. 2005. 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. *Plant Physiology* **137,** 1283–1301.

Huggett J, Dheda K, Bustin S, Zumla A. 2005. Real-time RT-PCR normalisation; strategies and considerations. *Genes and Immunity* **6,** 279–284.

Isayenkov S, Fester T, Hause B. 2004. Rapid determination of fungal colonization and arbuscule formation in roots of *Medicago truncatula* using real-time (RT) PCR. *Journal of Plant Physiology* **161,** 1379–1383.

Isayenkov S, Mrosk C, Stenzel I, Strack D, Hause B. 2005. Suppression of allene oxide cyclase in hairy roots of *Medicago truncatula* reduces jasmonate levels and the degree of mycorrhization with *Glomus intraradices*. *Plant Physiology* **139,** 1401–1410.

Ivashuta S, Liu J, Liu J, Lohar DP, Haridas S, Bucciarelli B, VandenBosch KA, Vance CP, Harrison MJ, Gantt JS. 2005. RNA interference identifies a calcium-dependent protein kinase involved in *Medicago truncatula* root development. *The Plant Cell* **17,** 2911–2921.

Jasper D, Robson A, Abbott L. 1979. Phosphorus and the formation of vesicular-arbuscular mycorrhizas. *Soil Biology and Biochemistry* **11,** 501–505.

Javot H, Penmetsa RV, Terzaghi N, Cook DR, Harrison MJ. 2007. A *Medicago truncatula* phosphate transporter indispensable for the arbuscular mycorrhizal symbiosis. *Proceedings of the National Academy of Sciences, USA* **104,** 1720–1725.

Küster H, Becker A, Firnhaber C, *et al*. 2007. Development of bioinformatic tools to support EST-sequencing, *in silico-* and microarray-based transcriptome profiling in mycorrhizal symbioses. *Phytochemistry* **68,** 19–32.

Limpens E, Ramos J, Franken C, Raz V, Compaan B, Franssen H, Bisseling T, Geurts R. 2004. RNA interference in *Agrobacterium rhizogenes*-transformed roots of *Arabidopsis* and *Medicago truncatula*. *Journal of Experimental Botany* **55,** 983–992.

Liu J, Blaylock L, Endre G, Cho J, Town C, VandenBosch K, Harrison M. 2003. Transcript profiling coupled with spatial expression analyses reveals genes involved in distinct developmental stages of an arbuscular mycorrhizal symbiosis. *The Plant Cell* **15,** 2106–2123.

Liu J, Maldonado-Mendoza I, Lopez-Meyer M, Cheung F, Town CD, Harrison MJ. 2007. Arbuscular mycorrhizal symbiosis is accompanied by local and systemic alterations in gene expression and an increase in disease resistance in the shoots. *The Plant Journal* **50,** 529–544.

Lohse S, Schliemann W, Ammer C, Kopka J, Strack D, Fester T. 2005. Organization and metabolism of plastids and mitochondria in arbuscular mycorrhizal roots of *Medicago truncatula*. *Plant Physiology* **139,** 329–340.

Maeda D, Ashida K, Iguchi K, Chechetka SA, Hijikata A, Okusako Y, Deguchi Y, Izui K, Hata S. 2006. Knockdown of an arbuscular mycorrhiza-inducible phosphate transporter gene of *Lotus japonicus* suppresses mutualistic symbiosis. *Plant and Cell Physiology* **47,** 807–817.

Maier W, Peipp H, Schmidt J, Wray V, Strack D. 1995. Levels of a terpenoid glycoside (blumenin) and cell wall-bound phenolics in cereal mycorrhizas. *Plant Physiology* **109,** 465–470.

Manthey K, Krajinski F, Hohnjec N, Firnhaber C, Pühler A, Perlick A, Küster H. 2004. Transcriptome profiling in root nodules and arbuscular mycorrhiza identifies a collection of novel genes induced during *Medicago truncatula* root endosymbioses. *Molecular Plant-Microbe Interactions* **17,** 1063–1077.

Mosse B. 1973. Plant growth responses to vesicular-arbuscular mycorrhiza. *New Phytologist* **72,** 127–136.

Quandt HJ, Pühler A, Broer I. 1993. Transgenic root nodules of *Vicia hirsuta*: a fast and efficient system for the study of gene expression in indeterminate-type nodules. *Molecular Plant-Microbe Interactions* **6,** 699–706.

Rodríguez-Llorente I, Pérez-Hormaeche J, Dary M, Caviedes M, Kondorosi A, Ratet P, Palomares A. 2003. Expression of *MsPG3–GFP* fusions in *Medicago truncatula* 'hairy roots' reveals preferential tip localization of the protein in root hairs. *European Journal of Biochemistry* **270,** 261–269.

Rose R. 2008. *Medicago truncatula* as a model for understanding plant interactions with other organisms, plant development and stress biology: past, present and future. *Functional Plant Biology* **35,** 253–264.

Schliemann W, Ammer C, Strack D. 2008. Metabolite profiling of mycorrhizal roots of *Medicago truncatula*. *Phytochemistry* **69,** 112–146.

Schmülling T, Schell J, Spena A. 1988. Single genes from *Agrobacterium rhizogenes* influence plant development. *EMBO Journal* **7,** 2621–2629.

Smith SE, Read DJ. 1997. *Mycorrhizal symbiosis*. San Diego: Academic Press.

Somers D, Samac D, Olhoft P. 2003. Recent advances in legume transformation. *Plant Physiology* **131,** 892–899.

Spurr A. 1969. A low-viscosity epoxy resin embedding medium for electron microscopy. *Journal of Ultrastructure Research* **26,** 31–43.

Strack D, Fester T. 2006. Isoprenoid metabolism and plastid reorganization in arbuscular mycorrhizal roots. *New Phytologist* **172,** 22–34.

Sun J, Cardoza V, Mitchell DM, Bright L, Oldroyd G, Harris JM. 2006. Crosstalk between jasmonic acid, ethylene and Nod factor signaling allows integration of diverse inputs for regulation of nodulation. *The Plant Journal* **46,** 961–970.

Thompson R, Ratet P, Küster H. 2005. Identification of gene functions by applying TILLING and insertional mutagenesis strategies on microarray-based expression data. *Grain Legumes* **41,** 20–22.

Trinh T, Ratet P, Kondorosi E, Durand P, Kamaté K, Bauer P, Kondorosi A. 1998. Rapid and efficient transformation of diploid *Medicago truncatula* and *Medicago sativa* ssp. *falcata* lines improved in somatic embryogenesis. *Plant Cell Reports* **17,** 345–355.

Trouvelot A, Kough J, Gianinazzi-Pearson V. 1986. Mesure du taux de mycorhization VA d'un système radiculaire. Recherche des méthodes d'estimation ayant une signification fonctionnelle. In: Gianinazzi-Pearson V, Gianinazzi S, eds. *The mycorrhizae: physiology and genetic.* Paris: INRA Presse, 217–221.

Vierheilig H, Coughlan AP, Wyss U, Piché Y. 1998. Ink and vinegar, a simple staining technique for arbuscular-mycorrhizal fungi. *Applied Environmental Microbiology* **64,** 5004–5007.

Vieweg M, Frühling M, Quandt H-J, Heim U, Bäumlein H, Pühler A, Küster H, Perlick A. 2004. The promoter of the *Vicia faba* L. leghemoglobin gene *VfLb29* is specifically activated in the infected cells of root nodules and in the arbuscule-containing cells of mycorrhizal roots from different legume and nonlegume plants. *Molecular Plant-Microbe Interactions* **17,** 62–69.

Walter MH, Floß DS, Hans J, Fester T, Strack D. 2007. Apocarotenoid biosynthesis in arbuscular mycorrhizal roots: contributions from methylerythritol phosphate pathway isogenes and tools for its manipulation. *Phytochemistry* **68,** 130–138.

Wasson AP, Pellerone FI, Mathesius U. 2006. Silencing the flavonoid pathway in *Medicago truncatula* inhibits root nodule formation and prevents auxin transport regulation by Rhizobia. *The Plant Cell* **18,** 1617–1629.

Wulf A, Manthey K, Doll J, Perlick AM, Linke B, Bekel T, Meyer F, Franken P, Küster H, Krajinski F. 2003. Transcriptional changes in response to arbuscular mycorrhiza development in the model plant *Medicago truncatula*. *Molecular Plant-Microbe Interactions* **16,** 306–314.

Zhou X, Chandrasekharan MB, Hall TC. 2004. High rooting frequency and functional analysis of GUS and GFP expression in transgenic *Medicago truncatula* A17. *New Phytologist* **162,** 813–822.