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

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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*, isoflavonoids, 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 arbusculae 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 be 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; Rodriguez-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 autoclaving.

**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.* (2008a) using *A. rhizogenes* harbouring the vector pRedRoot encoding the fluorescent marker protein DsRED1 (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 (Sheaffer 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% glutaraldehyde 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 AxiosImager 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 SM1k-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)15 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-columns (Millipore, Billerica, MA, USA) according to the supplier’s instructions. RNA integrity was analysed by gel electrophoresis. A 20 μl 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-L11Plus 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. ΔCt-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$_3$PO$_4$ (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. (2008a) 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 peri-arbuscular 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-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. 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.
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; 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.

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). 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.
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* (ΔΔCt >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 ΔΔCt 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.

<table>
<thead>
<tr>
<th>Oligo-ID</th>
<th>DFCI MtGi8 Annotation</th>
<th>Annotation</th>
<th>M-value, transgenic roots</th>
<th>M-value, wild-type roots</th>
<th>ΔΔCt, transgenic roots</th>
<th>ΔΔCt, wild-type roots</th>
</tr>
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<tr>
<td>MT009707</td>
<td>TC94453 UPIQ8GSG4 (Q8GSG4) Phosphate transporter PT4, complete</td>
<td><em>MtPT4</em>&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.37</td>
<td>5.06</td>
<td>14.49</td>
<td>13.65</td>
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<tr>
<td>MT015668</td>
<td>TC96500 weakly similar to UPIQ9LUM8 (Q9LUM8) Blue copper-binding protein-like, partial (38%)</td>
<td><em>MtBcp1</em>&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.42</td>
<td>2.68</td>
<td>8.20</td>
<td>8.13</td>
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<tr>
<td>MT009704</td>
<td>TC96118 UPIQ84XR7 (Q84XR7) Germin-like protein 1, complete</td>
<td><em>MtGlp1</em>&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.92</td>
<td>4.29</td>
<td>9.92</td>
<td>7.65</td>
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<tr>
<td>MT092185</td>
<td>TC106954 similar to UPIPRTP_HUMAN (P10619) Lyssosomal protective protein precursor (Cathepsin A) (Carboxypeptidase C) (Protective protein for beta-galactosidase), partial (16%)</td>
<td><em>MtScp1</em>&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.97</td>
<td>4.46</td>
<td>6.61</td>
<td>6.73</td>
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<td>MT007813</td>
<td>TC94919 homologue to UPIQ76FS3 (Q76FS3) Beta-tubulin, partial (98%)</td>
<td><em>MtTubb1</em>&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.07</td>
<td>2.20</td>
<td>2.77</td>
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<td>MT003520</td>
<td>TC96567 similar to UPIQ93536 (P93536) Bark lectin II precursor (LECSJABMII) (B-SJA-II) (Fragment), partial (40%)</td>
<td><em>MtLec7</em>&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.27</td>
<td>5.20</td>
<td>11.69</td>
<td>8.16</td>
</tr>
<tr>
<td>MT009013</td>
<td>TC100720 UPIQ8H1Y6 (Q8H1Y6) Glutathione-S-transferase-like protein, complete</td>
<td><em>MtGst1</em>&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.54</td>
<td>5.86</td>
<td>11.48</td>
<td>7.66</td>
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<td>MT014645</td>
<td>TC101060 weakly similar to PIR6S6221S66221 defensin AMP1—Dahlia merckii (Dahlia merckii), partial (88%)</td>
<td><em>Defensin</em>&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.10</td>
<td>5.34</td>
<td>15.44</td>
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<tr>
<td>MT015000</td>
<td>TC112474</td>
<td>Proteinase inhibitor&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>4.34</td>
<td>11.57</td>
<td>10.70</td>
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<tr>
<td>–</td>
<td>–</td>
<td><em>GiβTub1</em>&lt;sup&gt;a&lt;/sup&gt;</td>
<td>–</td>
<td>–</td>
<td>17.30</td>
<td>15.83</td>
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Oligo ID, identifier of *M. truncatula* 70mer oligonucleotides.

<sup>a</sup> Mycorrhiza specific.

<sup>b</sup> Mycorrhiza induced.

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<sub>2</sub> expression ratios (P ≤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. ΔΔCt 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 ≤0.05 (n=4).
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 Arqual (Quandt et al., 1993). This strain bears a pA4 type plasmid (pRedRoot, Limpens et al., 2004) 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, 2008), and endomembrane systems.

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

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>R&lt;sub&gt;t&lt;/sub&gt; (min)</th>
<th>λ&lt;sub&gt;max&lt;/sub&gt; (nm)</th>
<th>P</th>
<th>x-fold (myc/non-myc), transgenic</th>
<th>x-fold (myc/non-myc), wild-type</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Isoflavonoids</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ononin</td>
<td>37.96</td>
<td>251, 300sh</td>
<td>0.00004</td>
<td>2.83</td>
<td>3.52</td>
</tr>
<tr>
<td>Malonylononin</td>
<td>45.23</td>
<td>257, 300sh</td>
<td>0.00127</td>
<td>1.79</td>
<td>2.45</td>
</tr>
<tr>
<td><strong>Apocarotenoids</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13-Hydroxyblumenol C 9-O-glycoside</td>
<td>21.28</td>
<td>246</td>
<td>–</td>
<td>myc-spec</td>
<td>myc-spec</td>
</tr>
<tr>
<td>Blumenol C 9-O-glycoside</td>
<td>27.96</td>
<td>243</td>
<td>–</td>
<td>myc-spec</td>
<td>myc-spec</td>
</tr>
<tr>
<td>Blumenol C 9-O-malonylonoglycoside</td>
<td>37.49</td>
<td>245</td>
<td>–</td>
<td>myc-spec</td>
<td>myc-spec</td>
</tr>
<tr>
<td>Mycorradicin derivative 3</td>
<td>44.31</td>
<td>281, 360sh, 378, 397sh</td>
<td>–</td>
<td>myc-spec</td>
<td>myc-spec</td>
</tr>
<tr>
<td>Yellow pigment</td>
<td>45.52–53.51</td>
<td>315, 360sh, 382, 404</td>
<td>–</td>
<td>myc-spec</td>
<td>myc-spec</td>
</tr>
</tbody>
</table>
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., 2008a). 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 malonylmalononin) 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 mycorradin 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 mycorradin 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 log2 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).

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References


