Planta (2002) 214: 653–660
DOI 10.1007/s004250100658

ORIGINAL ARTICLE

Michael Kaldorf · Matthias Fladung · Hans-J. Muhs
François Buscot

Mycorrhizal colonization of transgenic aspen in a field trial

Received: 10 April 2001 / Accepted: 9 June 2001 / Published online: 12 October 2001

Abstract Mycorrhizal colonization of genetically modified hybrid aspen (*Populus tremula* × *P. tremuloides* Michx.) was investigated over 15 months in a field experiment. The aspen carried the *rolC* gene from *Agrobacterium rhizogenes* under control of either the constitutive cauliflower mosaic virus 35S promoter or the light-inducible rbcS promoter. Arbuscular mycorrhizas (AMs) were rare in all root samples, while fully developed ectomycorrhizas (EMs) were found in all samples. No significant differences in the degree of mycorrhizal colonization between aspen lines were seen with either AMs or EMs. The EM community on the release area was dominated by four fungal species that formed more than 90% of all mycorrhizas, while eleven EM types were found occasionally. Mycorrhizal diversity did not differ between transgenic and non-transgenic trees. The structure of mycorrhizal communities was similar for most aspen lines. The sole significant difference was found in the abundance and development of one of the four common EM morphotypes, which was rare and poorly developed on roots from the transgenic aspen line Esch5:35S-*rolC*-#5 compared with non-transgenic controls. This effect is clone specific as the formation of this EM type was not affected by the transgene expression in the other transgenic line, Esch5:35S-*rolC*-#1. This is the first demonstration of a clonal effect influencing the ability of a transgenic plant to form a mycorrhizal symbiosis with a potential fungal partner.

M. Kaldorf · F. Buscot (✉)
Institute of Ecology, Department of
Environmental Sciences, University of Jena,
Dornburger Str. 159, 07743 Jena, Germany
E-mail: francois.buscot@uni-jena.de
Fax: +49-3641-949452

M. Kaldorf · M. Fladung · Hans-J. Muhs
Federal Research Centre for Forestry and
Forest Products, Institute for Forest Genetics and
Forest Tree Breeding, Sieker Landstr.
2, 22927 Grosshansdorf, Germany

Keywords Fungal community · Mycorrhiza · *Populus* (mycorrhiza) · *rolC* · Transgenic tree

Abbreviations AM: arbuscular mycorrhiza · EM: ectomycorrhiza · ITS: internal transcribed spacer · PCR: polymerase chain reaction · RFLP: restriction fragment length polymorphism

Introduction

For most land plants, mycorrhizas represent the crucial link between the root system and soil, enhancing plant nutrition and water acquisition, but also resistance against parasites and other stress factors (Smith and Read 1997). As genetically engineered plants are increasingly being used, it has become essential to assess that their ability to form mycorrhizas is intact, with respect to both the rate of mycorrhiza formation and the diversity of fungal partners. The normal development of arbuscular mycorrhizas (AMs) has been reported for different transgenic tobacco plants cultivated under controlled conditions (Vierheilig et al. 1995; Schellenbaum et al. 1999; Gianinazzi-Pearson et al. 2000), and transgenic aspen were able to form fully developed ectomycorrhizas (EMs) in vitro (Hampp et al. 1996). However, the field development and community structure of the mycorrhizal fungi of transgenic host plants have not yet been investigated. Such investigations are especially important for genetically engineered forest plants, which have to develop over decades without external supports such as fertilization, watering and pesticides to compensate for a putative lack of mycorrhization capability. This accuracy is reinforced because the first transgenic trees are currently being field-tested (Fladung and Muhs 2000; Pullman et al. 1998) and large-scale plantings are likely in the near future.

This communication presents the first study on the mycorrhizal status of transgenic trees in the field. This case study concerns aspens transgenic for the *rolC* gene of *Agrobacterium rhizogenes*, which modifies the

phytohormone balance (Fladung et al. 1997a; Schmülling et al. 1993a). Several workers have demonstrated that phytohormones can play a role in the formation of both EMs (Barker and Tagu 2000) and AMs (Danneberg et al. 1992; Kaldorf and Ludwig-Müller 2000). As aspen forms both kinds of mycorrhiza in nature (Harley and Harley 1987), changes in the formation of EMs or AMs on *rolC*-transgenic aspen seemed likely.

A prerequisite for the monitoring of mycorrhizas is to use tools allowing their definitive characterization at the species level. Methods based on the polymerase chain reaction (PCR) amplification of fungal rDNA regions are suitable for the identification of both EM and AM fungi (Buscot et al. 2000; Redecker 2000). Morphological and anatomical descriptions of EMs according to Agerer (1991) are also often used in large-scale field studies (Visser 1995; Wöllecke et al. 1999). In some cases, however, morpho-anatomically homogeneous EM morphotypes can be formed by different fungi, as revealed by DNA sequence analysis (Pritsch et al. 2000). Therefore, in the present study, morphotype-based sorting of EMs was combined with molecular biological assessment.

Materials and methods

Plant material and experimental site

The first German release experiment with transgenic trees was started in 1996 on an experimental field of approx. 1,500 m^2 at the Federal Research Center for Forestry and Forest Products (Grosshansdorf, Germany; Fladung and Muhs 2000). Eleven aspen lines, eight of them transgenic, were planted out in a loamy, sandy soil homogenized by ploughing and harrowing. Each aspen line was represented by four randomized blocks with eight plants in each. One clone of hybrid aspen (*Populus tremula* × *P. tremuloides* Michx., clone Esch5) and three independent transgenic lines (Esch5:35S-*rolC*-#1, Esch5:35S-*rolC*-#5, and Esch5:rbcS-*rolC*-#4) were chosen for detailed analyses of their mycorrhizal status. The aspen lines Esch5:35S-*rolC*-#1 and Esch5:35S-*rolC*-#5 had been obtained by transformation of Esch5 (Fladung et al. 1997b) with the plasmid pPCV002-35S-*rolC* (Spena et al. 1987). The construct carried was the *rolC* gene from *Agrobacterium rhizogenes* under control of the cauliflower mosaic virus 35S promoter. For the generation of aspen line Esch5:rbcS-*rolC*-#4, the plasmid pPCV002-rbcS-*rolC* (Schmülling et al. 1993b) was used, in which *rolC* is under control of the light-inducible *rbcS* promoter from potato.

Sampling of mycorrhizal aspen roots

For the investigation of mycorrhizal colonization of the different aspen lines, it was essential to ensure to which individual tree the fine roots taken out belonged. As the aspen root systems overlapped, investigation of soil cores would not have allowed the fine roots to be assigned unambiguously to individual trees. Thus, each sample consisted of one root of approximately 5–8 mm diameter and 100–120 cm length that was traced from the trunk of an individual tree and carefully removed together with its derived fine rootlets. The root samples were stored in plastic bags filled with water for up to 4 weeks at 4 °C until analysis.

Samples were taken in September and November of 1998 and in March, May, September, and November of 1999. Each time, nine plants from different blocks of the experimental field were sampled, representing the lines Esch5, Esch5:rbcS-*rolC*-#4, and Esch5:35S-*rolC*-#5. Samples from aspen line Esch5:35S-*rolC*-#1 were additionally collected on the last two dates in 1999 and in March 2000. Further 35S-*rolC* transgenic lines were occasionally investigated.

Determination of AM colonization

Aspen roots were cleared in 10% (w/v) KOH for 1 h at 95 °C and bleached with 30% H_2O_2 for 5 min, followed by staining with lactophenol blue as described by Phillips and Hayman (1970). AM colonization was quantified microscopically by counting, for each sample, the presence or absence of AM structures in 200 microscope fields of 2 mm diameter at 100× magnification.

Morphological description of EM types

EM types were described on the basis of fresh ectomycorrhizal root tips as recommended by Agerer (1991). The first step was to examine morphological characters such as color, shape, branching type and surface structure using a dissecting microscope. In addition, the anatomy of the hyphal mantle and Hartig net was described from tangential and longitudinal hand sections observed with a light microscope.

Characterization of EM types by PCR-restriction fragment length polymorphism (PCR-RFLP)

Genomic DNA for PCR was extracted as described by Doyle and Doyle (1990) from single ectomycorrhizal roots tips (fresh weight 0.1–1 mg) homogenized with micro-pestles in 100 µl of CTAB DNA extraction buffer. Purified DNA was dissolved in 100 µl of sterile water and stored at 4 °C. The PCR assays contained 5 µl of 10× *Taq* DNA polymerase buffer (Promega, Heidelberg, Germany), 4 µl of 25 mM $MgCl_2$, 10 nmol of each deoxynucleotide, 50 pmol of each of the oligonucleotide primers ITS1 and ITS4 (White et al. 1990) and 1 µl of template DNA in a total volume of 50 µl. After 10 min. of denaturation at 95 °C, PCR was started by adding 2 U *Taq* DNA polymerase (Promega). The reaction was performed in 32 cycles with 40 s at 92 °C, 40 s at 52 °C, and 40 s at 72 °C. PCR products were cut with *Alu*I, *Eco*RI, *Bsu*RI, *Hinf*I, or *Msp*I (all from MBI Fermentas, St. Leon-Rot, Germany). Lengths of amplificates and restriction fragments were determined by electrophoresis on 2% agarose gels at 10 V/cm.

Quantification of EM types in root samples

Root clusters with vital fine roots were transferred to Petri dishes with water and divided into EM units, in which all mycorrhizal root tips were colonized by the same EM fungus. Most root samples contained 100–200 EM units, each consisting of 1–10 individual root tips, and there were only a few bigger EM clusters. Out of each root sample, 100 EM units representing all bigger EM units and over 50% of the randomly taken smaller EM units were sorted into different EM types under the stereomicroscope. Verification of the differentiation by detailed examination of hand sections was only necessary in a few cases. For each EM unit, the number of mycorrhizal and non-mycorrhizal root tips and the EM type were recorded. Differences in total mycorrhization and the abundance of different EM morphotypes were assessed by Student's *t*-test.

In the second period of analysis between May 1999 and March 2000, one morphotype (EM5) was investigated more thoroughly. Four EM classes involving the EM5 mycobiont were differentiated: fully developed pure EMs (= EM5), pure EMs with degenerated or missing external hyphae (= EM5*) and EMs of morphotypes EM6.1 and EM6.2 secondarily colonized by the mycobiont of EM5 (= EM6.3* and EM6.3**). A weighted mycorrhization intensity

was calculated for all EMs involving the EM5 mycobiont, by giving the coefficients 1 to EM5, 0.5 to EM5* and 0.25 to EM6.3* and EM6.3**.

Results

Density of mycorrhiza on aspen roots

Rootlets from the three regularly sampled aspen lines Esch5, Esch5:rbcS-*rolC*-#4 and Esch5:35S-*rolC*-#5 were screened in May 1999 and September 1999 for AM colonization. In May, 1,255 root segments were investigated, of which 94 (7.5%) contained AM structures. In two samples (line Esch5:rbcS-*rolC*-#4 plant #7 and Esch5:35S-*rolC*-#5 plant #7), AM colonization reached 20%, while in the remaining seven samples it was below 3%. Vesicles and intra-radical hyphae were found in roots from all 3 aspen lines, but only 13 root segments from Esch5 plant #2 and Esch5:35S-*rolC*-#5 plant #7 contained arbuscules. In September, AM colonization was between 0–4% and thus lower than in May. Altogether, less than 5% of all root segments were AM colonized. Differences between the three aspen lines were not statistically significant.

The colonization of roots by ectomycorrhizal (EM) fungi was similar in the three aspen lines. EMs were found on $67.9 \pm 6.2\%$ (mean $\pm$ SD) of the root tips in Esch5 ($n = 12$ samples from different trees; 5,686 root tips counted), $64.3 \pm 4.8\%$ in Esch5:rbcS-*rolC*-#4 ($n = 11$; 5,430 root tips) and $72.7 \pm 11.3\%$ in Esch5:35S-*rolC*-#5 ($n = 9$; 4,332 root tips). The differences between the aspen lines were not statistically significant ($P = 0.05$).

Classification of EM types using morphological characters and PCR-RFLP

Fifteen EM morphotypes were distinguished by combination of morphological and anatomical characters (Table 1). Eleven of them could be differentiated solely by their morphological features, while the unequivocal differentiation of two highly similar morphotype pairs (EM1-EM6.1 and EM2-EM10) necessitated anatomical investigations.

Roughly 30% of all examined EMs appeared to be colonized by more than one mycobiont. With few exceptions, these mixed EMs (= EM6.3) corresponded to secondary colonization of EM6.1 and EM6.2 by a loose hyphal network formed by the mycobiont of EM5. In some cases the secondary mycobiont came to form a regular hyphal mantle. To verify the differentiation based on anatomical and morphological features, the major morphotypes (EMs 2, 3, 5, 6.1, 6.2, 10, 14, and 15) that formed over 98% of the observed EMs, were characterized by PCR-RFLP. From 112 analyzed individual mycorrhizal roots, 71 gave amplificates, of which 37 displayed interpretable

Table 1 Description of hybrid aspen (*Populus tremula* × *P. tremuloides* Michx.) EM types occurring at the release area. The nomenclature corresponds to Agerer (1991)

Colour	Shape of mycorrhiza	Surface of mantle	Emanating hyphae	Thickness of mantle	Structure of mantle	Type
Beige	Unbranched, straight	Smooth	Rare	3–5 layers	outer layers Pseudoparenchymatic type L, inner layers plectenchymatic type H-E	EM1
Dark brown	Branched, straight	Rough	Missing	1–3 layers	Plectenchymatic type H	EM2
Dark brown	Branched, straight	Hairy	Branched, with clamps	3–5 layers	Pseudoparenchymatic type M	EM3
Dark brown	Branched, straight	Smooth	Branched, septate, no clamps	3–5 layers	Pseudoparenchymatic type L	EM4
Black	Unbranched, straight	Hairy	Unbranched, septate, no clamps	4–6 layers	Plectenchymatic type B-E	EM5
White–beige	Unbranched, straight	Smooth	Missing	5–7 layers	Plectenchymatic type H	EM6.1
White–beige	Branched, slightly bent	Smooth	Rare, with clamps	5–7 layers	Plectenchymatic type H	EM6.2
Brown	Branched, bent	Woolly	Rare, branched, with clamps	2–4 layers	Plectenchymatic type B	EM7
Redish-brown	Unbranched, bent	Hairy	Straight, not septate	1–2 layers	Pseudoparenchymatic type M	EM9
Redish-brown	Branched, straight	Rough	Rare	1–3 layers	Pseudoparenchymatic type O-P	EM10
Beige	Unbranched, straight	Hairy	Unbranched, not septate	2–3 layers	Plectenchymatic type A	EM11
Brown	Unbranched, straight	Woolly	Branched, septate, no clamps	4–6 layers	Pseudoparenchymatic type Q	EM12
Brown	Branched, slightly bent	Rough	Branched, with clamps	2–3 layers	Plectenchymatic type B	EM13
Brown	Unbranched, straight	Rough	Branched, with clamps	3–5 layers	Plectenchymatic type E-H	EM14
Grey	Branched, straight	Smooth	Rare, with clamps	5–7 layers	Plectenchymatic type B-E	EM15

banding patterns after restriction analysis (Table 2). Six of the eight analyzed morphotypes showed typical and distinguishable restriction patterns (EMs 3, 5, 6.1, 6.2, 14 and 15; Fig. 1). EM3 and EM14 were only separated by *Alu*I digestion and EM6.1/EM15 only by *Hinf*I. For each of the highly similar morphotypes

Table 2 Results of PCR/RFLP analysis of eight EM morphotypes. The ITS region was amplified by PCR using the oligonucleotide primer pair ITS1/ITS4 and genomic DNA isolated from single ectomycorrhizal root tips. PCR products were checked by agarose gel electrophoresis, and all good PCR products were used for restriction analysis with *Alu*I, *Eco*RI, *Bsu*RI, *Hinf*I and *Msp*I. The respective number of each restriction pattern corresponds to the EM morphotype in which it was first detected

Morphotypes	PCRs	PCR products				Restriction patterns								Unclear patterns
		None	Weak	Double	Good	2	3	5	6.1	6.2	10	14	15	
EM2	25	10	5	2	8	3					4			1
EM3	6	4			2		2							
EM5	16	2	7	2	5			5						
EM6.1	20	8	2	3	7				6					1
EM6.2	12	6	2		4					4				
EM10	25	9	6		10	6					3			1
EM14	4			1	3							3		
EM15	4	2	1		1								1	
All	112	41	23	8	40									

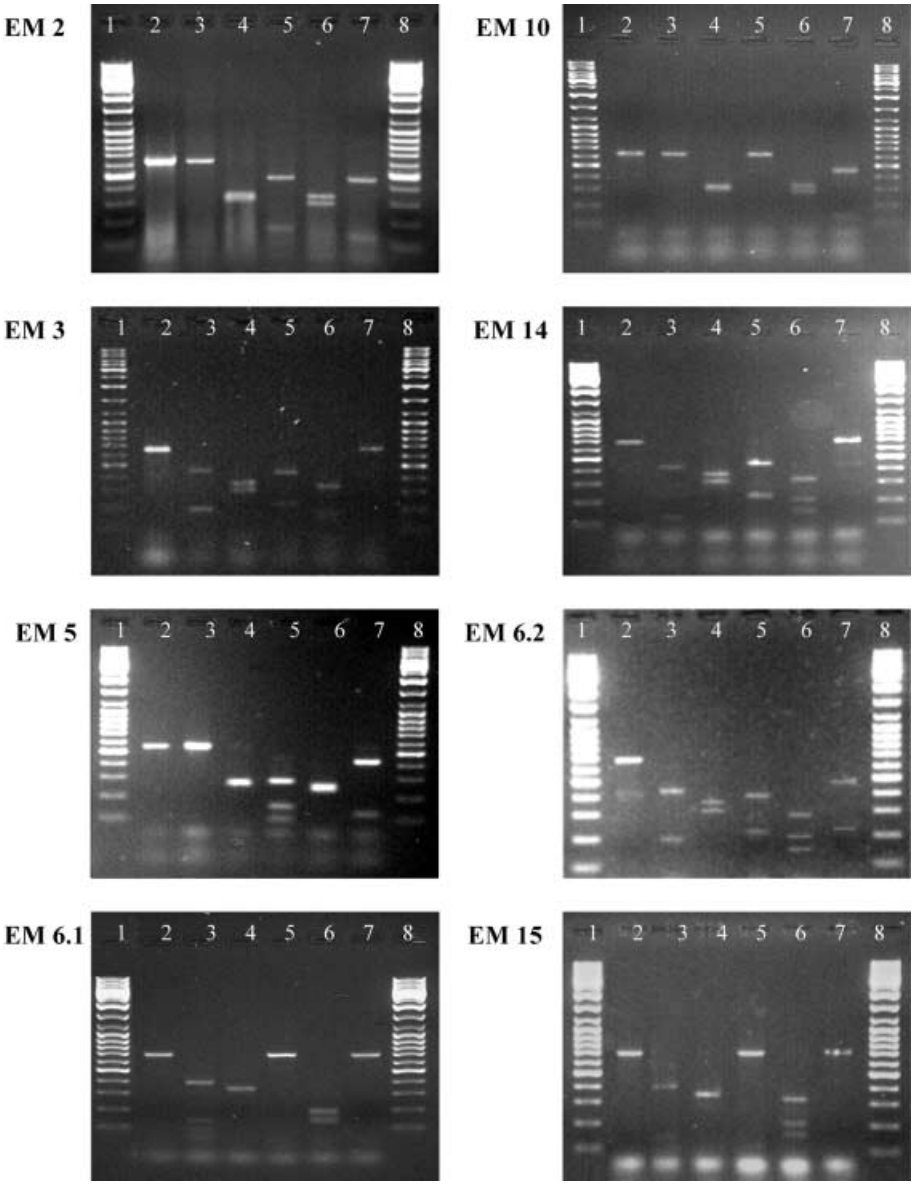

Fig. 1 RFLP analysis of the most common ectomycorrhizal (*EM*) morphotypes from the hybrid aspen (*Populus tremula* × *P. tremuloides* Michx.) release area. PCR-amplified ITS regions of single mycorrhizal root tips representing eight different morphotypes (*lane 2* on each agarose gel) were restricted by *Alu*I (*lanes 3*), *Eco*RI (*lanes 4*). *Bsu*RI (*lanes 5*), *Hae*III (*lanes 6*) and *Msp*I (*lanes 7*). Restriction fragments were separated on 2% agarose gels. *Lanes 1, 8* GeneRuler DNA Ladder Mix (MBI Fermentas)

EM2 and EM10, the same two restriction patterns were found, only differing by the presence or absence of one *Bsu*RI restriction site.

EM colonization patterns of different aspen lines

The four morphotypes EM2, EM5, EM6.1, EM6.2 and the mixed morphotype EM6.3 represented 94% of all EMs in the release area (Table 3). With the exception of EM2, these dominant morphotypes were found at all sampling dates on each of the lines investigated. All other morphotypes were rare and only occurred occasionally. Additionally, they were not found on all probed trees of the samples concerned, since eight (EMs 1, 3, 4, 9, 11, 12, 13, and 14) were found on fewer than five trees, while EM7 (found on nine trees), EM10 (on seven), and EM15 (on nine) had a wider distribution.

Figure 2 gives an example of the percent occurrence of five morphotypes among different trees of the non-transgenic aspen line Esch5 on three sampling dates in 1999. While EM5, EM6.1, and EM6.2 were present in all samples with a more or less equal occurrence on individual trees, the distributions of EM2 and especially of the rare morphotype EM3 were patchy. These types were missing in the majority of root samples, but were found to be frequent in single samples (Fig. 2). This example illustrates the fact that the distribution pattern of rare morphotypes did not allow statistical assessment of differences in their abundance between the different aspen lines.

Between September 1998 and March 1999, the sole significant difference found between the mycorrhization of aspen lines was a lower occurrence of EM5 on Esch5:35S-*rolC*-#5 (Table 3). In order to elucidate this phenomenon, mycorrhization by the EM5 mycobiont was investigated more thoroughly between May 1999 and March 2000 on four aspen lines (Table 4). Compared to the wild-type line Esch5, a significant reduction of EM5 and EM5* was found for the transgenic line Esch5:35S-*rolC*-#5, constitutively expressing the *rolC* gene in the whole plant. In contrast, the number of EM6.3* morphotypes was significantly higher in Esch5:35S-*rolC*-#5 than in Esch5. Thus, the total number of mycorrhizas involving the EM5 mycobiont was similar in Esch5 and Esch5:35S-*rolC*-#5, but the partition between the four classes differed (Table 4). The weighted mycorrhization intensity calculated for all EMs involving the EM5 mycobiont, appeared to be significantly lower for the transgenic line Esch5:35S-*rolC*-#5 than for all other lines (Table 4).

Discussion

After the first observations had shown EMs to constitute the major mycorrhizal type on all aspen lines, the study of the population structure was restricted to EMs. Of the 15 encountered morphotypes, 13 could be differentiated unambiguously by morphological and anatomical characters. The morphotype pair EM2-EM10 could only be separated by minor morphological and anatomical features, and two closely related RFLP patterns were found in both morphotypes. As the mycorrhizal root tips used for the PCR could only be sorted morphologically, mixing of both morphotypes in the PCR study

Table 3 Abundance of different EM morphotypes as a percentage of total mycorrhizal root tips removed from the release area between September 1998 and November 1999. Abbreviations for aspen lines: *rbcS* #4 = Esch5:rbcS-*rolC*-#4, 35S #5 = Esch5:35S-*rolC*-#5, and 35S #1 = Esch5:35S-*rolC*-#1. *n.d.* Not determined, *n.id.* not identified

Aspen line	Date	Total number	EM morphotype																
			1	2	3	4	5	6.1	6.2	6.3	Σ 6	7	9	10	11	12	13	14	15	n.id.
Esch5	9/98	719	0	4.0	0	1.7	18.2	n.d.	n.d.	n.d.	69.0	1.1	5.1	0	0	0	0	0	0	1.3
Esch5	11/98	1,081	0	34.7	0	0	19.8	4.1	6.7	31.8	42.6	1.9	0	0	0	1.1	0	0	0	0
Esch5	3/99	1,040	0	14.5	0	0	15.1	23.4	22.3	21.7	67.4	0	0	1.2	0	0	0.6	0	1.1	0
Esch5	5/99	916	0	1.0	0	0	14.0	7.5	28.4	49.1	85.0	0	0	0	0	0	0	0	0	0
Esch5	9/99	1,172	0	1.0	0	0	9.1	11.4	50.7	23.8	85.9	0	0	0	0	0	0	0	2.5	0
Esch5	11/99	978	0	17.3	17.5	0	15.6	7.1	34.0	8.5	49.6	0	0	0	0	0	0	0	0	0
rbcS #4	9/98	680	0	6.2	0	0.4	26.8	n.d.	n.d.	n.d.	65.4	1.0	0	0.1	0	0	0	0	0	0
rbcS #4	11/98	812	0	2.5	0	0	37.1	3.3	34.5	21.8	59.6	0.5	0	0	0	0	0	0	0	0.4
rbcS #4	3/99	975	0	25.0	0	0.1	30.8	4.5	4.8	27.8	37.1	0	0	3.7	0	0	0.3	0	3.0	0
rbcS #4	5/99	587	0	0.2	0	0	11.2	14.1	18.2	54.2	86.5	0	0	0	0	0	0	0	1.5	0.5
rbcS #4	9/99	921	0	2.7	0	0	29.2	7.5	11.4	35.2	54.1	0	0	0	0	0	0	0	14.0	0
rbcS #4	11/99	990	0	3.1	0	0	24.8	10.2	48.3	13.5	72.0	0	0	0	0	0	0	0	0	0
35S #5	9/98	1,233	0	22.5	0	0	0.9	n.d.	n.d.	n.d.	71.5	1.9	0	2.2	1.1	0	0	0	0	0
35S #5	11/98	1,450	0	0.3	0	0	19.2	10.0	27.7	36.2	73.9	1.2	0	0	0	2.7	2.6	0	0	0
35S #5	3/99	1,103	0	11.4	0	0	9.4	11.9	12.3	47.8	72.0	0	0	3.4	0	0	0	3.8	0	0
35S #5	5/99	662	0	0	0	0	1.8	39.4	16.6	42.1	98.1	0	0	0	0	0	0	0	0	0
35S #5	9/99	347	0	0	0	0	3.5	13.0	57.3	26.3	96.6	0	0	0	0	0	0	0	0	0
35S #5	11/99	985	0	6,6	27.2	0	3.2	10.6	26.6	25.8	63.0	0	0	0	0	0	0	0	0	0
35S #1	9/99	376	7.7	0	0	0	25.0	3.5	31.1	31.6	66.2	0	0	0	0	0	0	0	1.1	0
35S #1	11/99	1,232	0	0.2	0	0	11.5	15.9	45.3	27.1	88.3	0	0	0	0	0	0	0	0	0
Σ		18,171	0.2	8.7	2.4	0.1	16.1	11.2	27.1	31.3		0.4	0.4	0.4	0.1	0.3	0.2	0.2	1.2	0.1

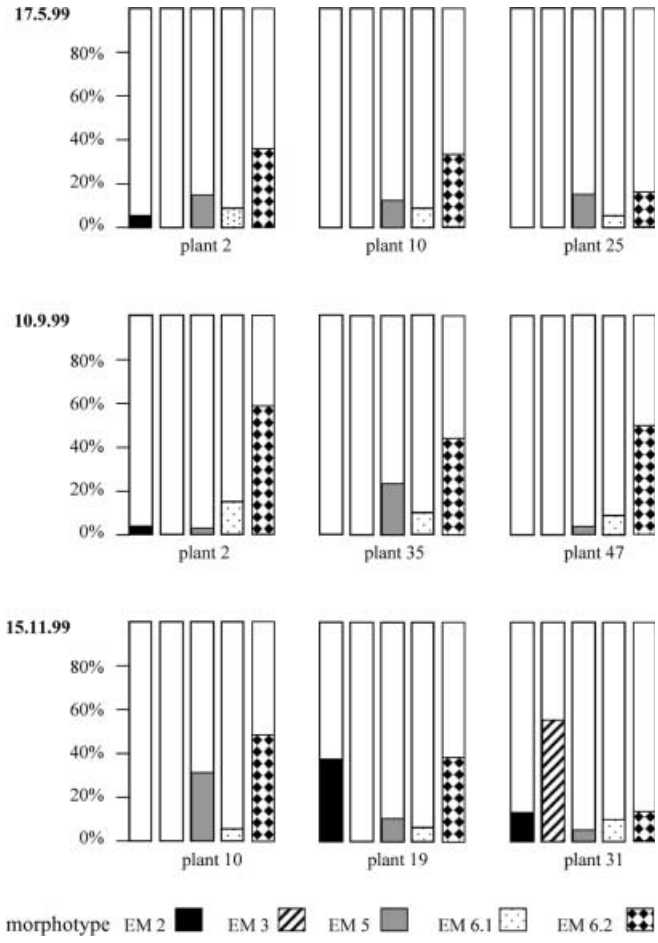

Fig. 2 Distribution of five different ectomycorrhizal (*EM*) morphotypes in samples from single trees of hybrid aspen line Esch5, collected between May 1999 and November 1999. The abundance of the different EM types is given as a percentage of about 300 mycorrhizal root tips counted for each sample

cannot be excluded. However, their great similarities in both morpho-anatomy and PCR-RFLP signature indicate a close systematic relationship of the mycobionts, which lowers the impact of this confusion. Another rational but less probable explanation is that EM2 and EM10 corresponded to different developmental stages of a single mycorrhizal type. In this case, the two related RFLP patterns have to be interpreted as an ITS (internal transcribed spacer) polymorphism within one fungal species, which has been reported for several EM fungal species (Farmer and Sylvia 1998; Henrion et al. 1992).

Except for this critical morphotype pair, the combined methodical approach suggested by Pritsch and Buscot (1996) allowed an unequivocal separation of the encountered EM types. For example, the respective RFLP pattern differences within the morphotype pairs EM3-EM14 and EM6.1-EM15 could have been overlooked by indirect comparison of different electrophoresis gels, while the morpho-anatomical separation in each pair was obvious. Thus, comparison of the mycorrhization patterns of the genetically modified aspen lines and even statistical assessments of the major morphotypes were possible.

The fact that despite the changed phytohormone balance in *rolC*-transgenic aspen (Fladung et al. 1997a) only minor and non-significant alteration of AM and EM formation was observed is a major result of this work. The maintained mycorrhization level of the *rolC* transgenic aspen indicates that the mycorrhization capability of transgenic plants is not basically altered, even when the transformation concerns genes known to influence the phytohormonal balance. However, this provisional conclusion needs to be assessed. First, the role of hormones in mycorrhizal symbioses is not clear. Second, hormone production by the fungus seems to be more important for the symbiosis than that by the host plant. Auxin-overproducer mutants of *Hebeloma* have an increased mycorrhizal activity (Gay et al. 1994), while transgenic aspen expressing auxin-biosynthetic genes did not display a modified mycorrhization (Hampp et al. 1996).

The EM community on the release area was dominated by four EM fungi, as would be expected for a monoculture of young trees of similar age. Community structures with five or less morphotypes forming over 90% of all EMs have been described for young seedlings of Sitka spruce (Flynn et al. 1998) and for six-year-old jack pines (Visser 1995). One of the four dominating morphotypes, EM5, showed a significantly decreased ability to form EM on the transgenic aspen line Esch5:35S-*rolC*-#5. Two other transgenic lines, Esch5:rbcS-*rolC*-#4 and Esch5:35S-*rolC*-#1, were not affected, indicating that neither the presence, nor the expression of the *rolC* gene interfered with the formation

Table 4 Occurrence and developmental state of the ectomycorrhizal fungus EM5 in root samples from different hybrid aspen lines. The respective proportions of fully developed EMs of type EM5 (EM5, column 4), type EM5 with degenerated or missing external hyphae (EM5*, column 5), type EM6.1 secondarily colonized by hyphae of EM5 (6.3*, column 6), type EM6.2 secondarily colonized by hyphae of EM5 (6.3**, column 7) and of the sum of all EMs formed by EM5 (Σ EM5, column 8) are given as a percentage (± SD) of the total classified EMs. EM intensity was calculated as follows: EM intensity = (EM5) + 0.5(EM5*) + 0.25 (6.3* + 6.3**). Values with different letters within columns are significantly different at $P < 0.05$ (student's *t*-test)

Aspen line	Samples	Total no. of EMs	EM5	EM5*	EM6.3*	EM6.3**	Σ EM5	EM intensity
Esch5	($n = 12$)	4,006	10.9 ± 8.3^{a}	2.6 ± 1.5^{a}	3.9 ± 2.8^{a}	1.8 ± 2.7^{a}	19.0 ± 13.6^{a}	0.68 ± 0.14^{a}
E 2/1	($n = 7$)	2,133	11.8 ± 5.9^{a}	1.7 ± 1.2^{ab}	20.1 ± 21.2^{ab}	2.6 ± 5.0^{a}	36.7 ± 25.0^{a}	0.57 ± 0.17^{a}
E 2/5	($n = 10$)	3,016	1.6 ± 1.5^{b}	1.0 ± 0.9^{b}	11.5 ± 10.7^{b}	3.3 ± 4.7^{a}	17.6 ± 16.4^{a}	0.37 ± 0.08^{b}
E 14/4	($n = 11$)	3,484	18.7 ± 18.2^{a}	3.3 ± 2.5^{a}	7.8 ± 5.7^{ab}	1.2 ± 1.5^{a}	29.3 ± 16.7^{a}	0.63 ± 0.21^{a}

of EM5. The effect seems to be clone specific, which is reinforced by single observations on further lines of *rolC*-transgenic aspen (W52:35S-*rolC*-#2, Brauna11:35S-*rolC*-#2, Esch5:35S-*rolC*-#3, and Esch5:35S-*rolC*-#16) released at the experimental field, which also displayed high levels of EM5 formation (data not shown). The changes in mycorrhization by EM5 in Esch5:35S-*rolC*-#5 could be explained by the occurrence of an unrelated genomic change known as somaclonal variation, or by a position effect of the insert. However, no features of coding regions have been identified in flanking sequences of the insert in Esch5:35S-*rolC*-#5 (Fladung 1999), making a position effect less likely.

This first example of a clonal effect on the ability of transgenic plants to form mycorrhiza with one fungal partner is the second major result of this work. The ecological importance of the finding should not be overestimated, as forest trees form EMs with a high diversity of fungal species with probable redundant ecological functions. A depressed mycorrhization by one species should have no consequences, as we found that the released transgenic trees displayed a normal mycorrhization without real modification in the spectrum of fungal partners. However, it should be pointed out that reduction of mycorrhization by one mycobiont can be of importance in nurseries or just after transplantation of young trees in forests, two conditions under which mycorrhization only involves few mycobionts, all of which may be crucial for survival.

Two further lessons for the handling of transgenic plants can be drawn from our findings. First, it appears that minute physiological modifications not directly related to the function of the cloned gene can occur. In our case, this results in a lowered frequency of one EM type. Second, this result questions the biological assessment procedures for genetically engineered plants before their release in nature. Testing a limited number of transgenic lines cannot rule out unexpected and unpredictable physiological modifications in other lines.

Acknowledgements This work was kindly supported by a grant from the Bundesministerium für Bildung, Wissenschaft, Forschung und Technologie (Förderkennzeichen: 0311387). Many thanks to D. Boedecker, K. Dahlügge, M. Wellern and J. Kuhn for fieldwork and to Dr. A. Davis and two anonymous reviewers for critical reading and improvement of the manuscript.

References

Agerer R (1991) Characterization of ectomycorrhiza. In: Norris JR, Read DJ, Varma AK (eds) Methods in microbiology, vol 23. Academic Press, London San Diego New York, pp 25–73

Barker SJ, Tagu D (2000) The roles of auxins and cytokinins in mycorrhizal symbioses. J Plant Growth Regul 19:144–154

Buscot F, Munch J-C, Charcosset JY, Gardes M, Nehls U, Hampp R (2000) Recent advances in exploring physiology and biodiversity of ectomycorrhizas highlight the function of these symbioses in ecosystems. FEMS Microbiol Rev 699:1–14

Danneberg G, Latus C, Zimmer W, Hundeshagen B, Schneider-Poetsch H, Bothe H (1992) Influence of vesicular-arbuscular mycorrhiza on phytohormone balances in maize (*Zea mays* L.). J Plant Physiol 141:33–39

Doyle JJ, Doyle JL (1990) Isolation of plant DNA from fresh tissue. Focus 12:13–15

Farmer DJ, Sylvia DM (1998) Variation in the ribosomal DNA internal transcribed spacer of a diverse collection of ectomycorrhizal fungi. Mycol Res 102:859–865

Fladung M (1999) Gene stability in transgenic aspen (*Populus*). I. Flanking DNA sequences and T-DNA structure. Mol Gen Genet 260:574–581

Fladung M, Muhs H-J (2000) Field release with *Populus tremula* (*rolC*-gene) in Großhansdorf. In: Umweltbundesamt (ed) Release of transgenic trees – present achievements, problems, future prospects. Humboldt-Universität Berlin, pp 40–45

Fladung M, Grossmann K, Ahuja MR (1997a) Alterations in hormonal and developmental characteristics in transgenic *Populus* conditioned by the *rolC* gene from *Agrobacterium rhizogenes*. J Plant Physiol 150:420–427

Fladung M, Kumar S, Ahuja MR (1997b) Genetic transformation of *Populus* genotypes with different chimaeric gene constructs: transformation efficiency and molecular analysis. Transgen Res 6:111–121

Flynn D, Newton AC, Ingleby K (1998) Ectomycorrhizal colonization of Sitka spruce [*Picea sitchensis* (Bong.) Carr] seedlings in a Scottish plantation forest. Mycorrhiza 7:313–317

Gay G, Normand L, Marmeisse R, Sotta B, Debaud JC (1994) Auxin overproducer mutants of *Hebeloma cylindrosporum* Romagnesi have increased mycorrhizal activity. New Phytol 128:645–657

Gianinazzi-Pearson V, Arnould C, Oufattole M, Arango M, Gianinazzi S (2000) Differential activation of H^+-ATPase genes by an arbuscular mycorrhizal fungus in root cells of transgenic tobacco. Planta 211:609–613

Hampp R, Ecke M, Schaeffer C, Wallenda T, Wingler A, Kottke I, Sundberg B (1996) Axenic mycorrhization of wild type and transgenic hybrid aspen expressing T-DNA indoleacetic acid-biosynthetic genes. Tree 11:59–64

Harley JL, Harley EL (1987) A check-list of mycorrhiza in the British flora. New Phytol [Suppl] 105:1–102

Henrion B, LeTacon F, Martin F (1992) Rapid identification of genetic variation of ectomycorrhizal fungi by amplification of ribosomal RNA genes. New Phytol 122:289–298

Kaldorf M, Ludwig-Müller J (2000) AM fungi might affect the root morphology of maize by increasing indole-3-butyric acid biosynthesis. Physiol Plant 109:58–67

Phillips JM, Hayman DS (1970) Improved procedures for clearing roots and staining parasitic and vesicular-arbuscular mycorrhizal fungi for rapid assessment of infection. Trans Br Mycol Soc 55:158–161

Pritsch K, Buscot F (1996) Biodiversity of ectomycorrhizas – from morphotypes to species. In: Azcon-Aguilar C, Barea JM (eds) Proceedings of the 4th European symposium on mycorrhizae: mycorrhizas in integrated systems from genes to plant development. Office for official publications of the European Communities, Luxembourg, pp 9–14

Pritsch K, Munch J-C, Buscot F (2000) Identification and differentiation of mycorrhizal isolates of black alder by sequence analysis of the ITS region. Mycorrhiza 10:87–93

Pullman GS, Cairney J, Peter G (1998) Clonal forestry and genetic engineering: where we stand, future prospects, and potential impacts on mill operations. Tappi J 81:57–63

Redecker D (2000) Specific PCR primers to identify arbuscular mycorrhizal fungi within colonized roots. Mycorrhiza 10:73–80

Schellenbaum L, Sprenger N, Schüepp H, Wiemken A, Boller T (1999) Effects of drought, transgenic expression of a fructan synthesizing enzyme and of mycorrhizal symbiosis on growth and soluble carbohydrate pools in tobacco plants. New Phytol 142:67–77

Schmülling T, Fladung M, Grossmann K, Schell J (1993a) Hormonal content and sensitivity of transgenic tobacco and potato

plants expressing single *rol* genes of *Agrobacterium rhizogenes* T-DNA. Plant J 3:371–382

Schmülling T, Röhrig H, Pilz S, Walden R, Schell J (1993b) Restoration of fertility by antisense RNA in genetically engineered male sterile tobacco plants. Mol Gen Genet 237:385–394

Smith SE, Read DJ (1997) Mycorrhizal symbiosis, 2nd edn. Academic Press, San Diego London New York

Spena A, Schmülling T, Koncz C, Schell J (1987) Independent and synergistic activity of *rol A*, *B* and *C* loci in stimulating abnormal growth in plants. EMBO J 6:3891–3899

Vierheilig H, Alt M, Lange J, Gut-Rella M, Wiemken A, Boller T (1995) Colonization of transgenic tobacco constitutively expressing pathogenesis-related proteins by the vesicular arbuscular mycorrhizal fungus *Glomus mosseae*. Appl Environ Microbiol 61:3031–3034

Visser S (1995) Ectomycorrhizal fungal succession in jack pine stands following wildfire. New Phytol 129:389–401

White TJ, Bruns T, Lee S, Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds) PCR protocols: a guide to methods and applications. Academic Press, San Diego, pp 315–322

Wöllecke J, Münzenberger B, Hüttl RF (1999) Some effects of Non ectomycorrhizal diversity of Scots pine (*Pinus sylvestris* L.) in northeastern Germany. Water Air Soil Pollut 116:135–140