

Investigation of horizontal gene transfer in poplar/*Amanita muscaria* ectomycorrhizas

Chi ZHANG, Rüdiger HAMPP and Uwe NEHLS*

Universität Tübingen, Botanisches Institut, Physiologische Ökologie der Pflanzen, Auf der Morgenstelle 1, 72076 Tübingen, Germany

Fine roots of forest trees form together with certain soil fungi symbiotic structures (ectomycorrhizas), where fungal hyphae are in intimate contact with plant cells. Due to root cell degeneration, plant DNA is released and could be taken up by the fungus. The possibility that horizontal gene transfer might result in a risk for the environment should be evaluated before a massive release of genetically engineered trees into nature occurs, even though only a few convincing examples of horizontal gene transfer are known. Transgenic poplars containing a construct of the *Streptomyces hygroscopicus bar* gene under the control of the *Cochliobolus heterostrophus* GPD (glyceraldehyde-3-phosphate dehydrogenase) promoter were generated by *Agrobacterium*-mediated transformation. The functionality of this construct in the ectomycorrhizal model fungus *Amanita muscaria* was previously verified by protoplast-based fungal transformation. 35000 ectomycorrhizas, formed between transgenic poplars and non-transgenic *A. muscaria* hyphae, were isolated and transferred to selective agar plates. Putative herbicide-resistant fungal colonies were obtained after the first round of selection. However, none of these colonies survived a transfer onto fresh selection medium, nor did they contain the *bar* gene, indicating that no horizontal gene transfer from poplar to *A. muscaria* occurred during symbiosis under axenic conditions. However, since ectomycorrhizas are associated under natural conditions with viruses, bacteria and other fungi, these additional associations should be evaluated in future.

Keywords: horizontal gene transfer / poplar / ectomycorrhiza / transformation

INTRODUCTION

The introduction of molecular biological methods into plant breeding has offered the possibility to construct genetically modified plants with new qualities. Many different transgenic plants (including trees; Boerjan et al., 1997; Lapierre et al., 1999) have been developed, field tested (Saroja et al., 1998), and planted on at least 15 million ha worldwide since 1998 (Owen, 2000; Warwick et al., 1999). An important necessity with respect to transgenic plants is to ensure that negative effects on the environment must be low or, better, do not occur at all. One potential risk is the spreading of genetic information (e.g. herbicide or antibiotic resistance) from transgenic plants to microorganisms by horizontal gene transfer.

By using total DNA or tissue material from genetically modified plants, transformation of different bacterial species has been demonstrated under optimized laboratory conditions (e.g. Gebhard and Smalla, 1998; Schlüter et al.,

1995) as well as in soil microcosms (e.g. Nielsen et al., 2000). These and other studies showed that the presence of homologous DNA in the bacterial recipient strain is a major prerequisite for stable integration of the DNA taken up. However, De Vries and Wackernagel reported in 2002 that not only homologous recombination but also homology facilitated illegitimate recombination could serve as a mechanism for stable integration.

In contrast to bacterial investigation, approaches to detect horizontal gene transfer to eukaryotic microorganisms (fungi) are sparse. By combining analyses of G/C content and patterns of codon usage, Garcia-Vallvé et al. (2000) suggested that an endoglucanase gene from the rumen bacterium *Fibrobacter succinogenes* had been transferred to the rumen fungus *Orpinomyces joyonii*. Using a dominant selection marker (hygromycin resistance gene) enabled the detection of direct DNA uptake

* Corresponding author: uwe.nehls@uni-tuebingen.de

from dead off-plant material and its integration into the genome by the fungal saprophyte *Aspergillus niger* (Hoffmann et al., 1994).

Since numerous fungi grow in intimate contact with or even within plants (*e.g.* endophytic fungi), uptake of plant DNA by these fungi might be more likely, since the availability of DNA in soil could be a limiting factor for horizontal gene transfer (Gebhard and Smalla, 1999). Some evidence for this comes from the phytopathogenic fungus *Plasmodiophora brassicae*, which takes up host plant DNA during each infection cycle (Bryngelsson et al., 1988).

Tree species usually live in close contact with certain soil fungi, forming together symbiotic organs, the ectomycorrhizas. The function of this symbiosis is the exchange of mineral and organic nutrients for photoassimilates (Smith and Read, 1997). Since root epidermal/cortical cells often degenerate after a certain time of interaction, and fungal hyphae live in intimate contact with plant cells, it is reasonable to assume that plant DNA could be taken up by ectomycorrhizal fungi. DNA uptake by filamentous fungi often leads to its integration into the genome by illegitimate recombination (Magee et al., 2003) and could thus result in a stable horizontal gene transfer.

In this work, transgenic poplars containing the *Streptomyces hygrosopicus bar* gene (conferring herbicide resistance) expressed under the control of the GPD promoter of *Cochliobolus heterostrophus* were generated. Synthetic mycorrhizas were formed between transgenic poplars and the non-transgenic ectomycorrhizal model fungus *A. muscaria* and isolated mycorrhizas were investigated for horizontal gene transfer events.

RESULTS

Transformation of *Amanita muscaria* with a *bar* gene expression cassette

To investigate the proper functioning of the *bar* selection marker in the ectomycorrhizal model *A. muscaria*, the fungus was transformed with a *HindIII/EcoRI* fragment (containing the *bar* gene behind a fungal GPD promoter) of pBI121/3 by polyethylene glycol-mediated protoplast transformation. Transformed *A. muscaria* protoplasts were embedded in top agarose and plated on agar plates containing 250 $\mu\text{g}\cdot\text{ml}^{-1}$ Basta. The survival rate of protoplasts (under non-selective conditions) was about one per million. After approximately 3 months on the selection medium, herbicide-resistant fungal colonies started to appear. These colonies were transferred to new

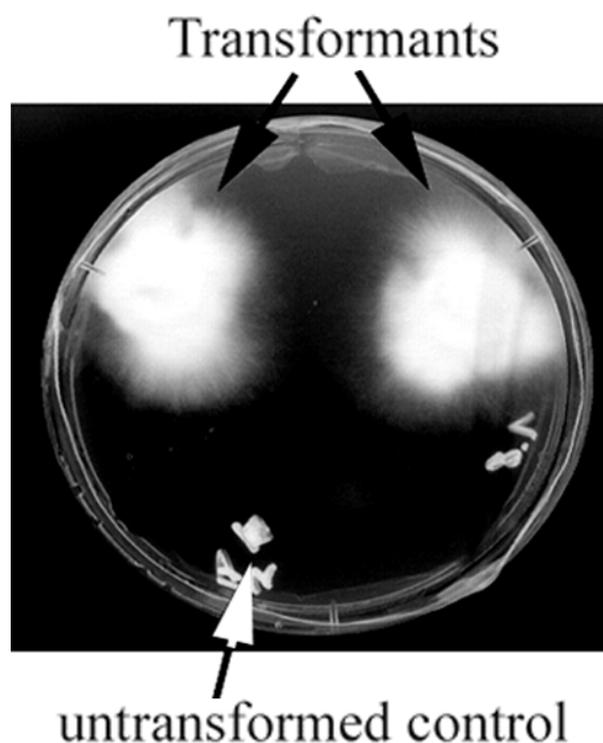


Figure 1. Transformed and wild-type *A. muscaria* colonies on Basta selection medium. Agar pieces with hyphae of two transformants and the parental strain were transferred to a selection plate containing 250 $\mu\text{g}/\text{ml}$ Basta and incubated for 6 weeks.

selective agar plates to ensure a stable transformation. The transformation frequency was about 1 transformant per 10 μg of linearized DNA. Figure 1 shows two transformed Basta-resistant fungal colonies and one non-transformed colony on a selective agar plate.

To investigate whether the *bar* gene was integrated into the genome of *A. muscaria*, PCR amplification from isolated genomic DNA of Basta-resistant fungal colonies was carried out using *bar* gene-specific primers (data not shown) and Southern blot analysis (Fig. 2). As often observed for the transformation of protoplasts of filamentous fungi (Olmedo-Monfil et al., 2004; Ruiz-Diez, 2002), the transformation of *A. muscaria* mainly resulted in the integration of multiple DNA copies into the fungal genome (eight out of nine transformants). Since no extended homology between the *Amanita* genome and the *bar* construct could be expected, the transformants are presumably the results of illegitimate integration events. In accordance with this, the size of restriction fragments containing the *bar* gene differed between different fungal transformants.

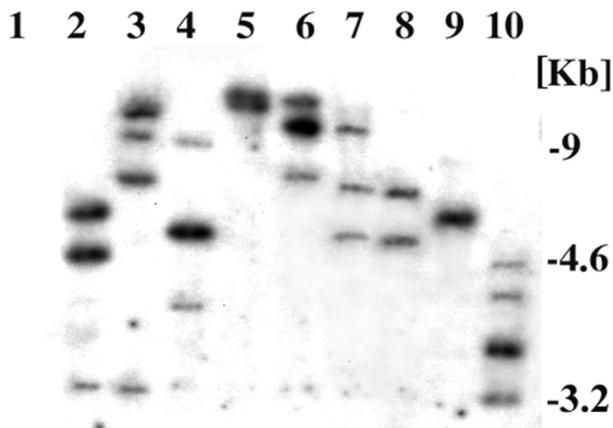


Figure 2. Detection of the *bar* gene in the genome of Basta-resistant *A. muscaria* colonies. Genomic DNA (15 µg) of 9 Basta-resistant fungal colonies was digested with *Hind*III, separated on a 0.8% agarose gel, transferred to a nylon membrane and hybridized with the ³²P-labeled *bar* gene. (Lane 1) DNA isolated from wild-type *A. muscaria* hyphae, (Lanes 2–10) DNA isolated from different transformants.

PCR analysis of transgenic poplar plants

Poplar was transformed with pBI121/3 using an *A. tumefaciens*-based protocol (Tuominen et al., 1995). After regeneration, twenty plants were isolated, which originated from different calli. Single leaves were excised and used for the preparation of genomic DNA. To ensure that the transgenic plants contained the entire *bar* gene expression cassette, PCR amplifications were carried out using GPD promoter- (data not shown) and *bar* gene-specific primers (Fig. 3). Amplification products of the expected size were obtained from 19 out of a total of 20 putative transgenic poplar plants. A negative control using genomic DNA from wild-type poplar as a template did not yield any PCR fragment. Isolated PCR fragments of three

clones were subjected to sequencing, revealing the sequences of the introduced GPD promoter and the *bar* gene (data not shown).

Screening of ectomycorrhizas for horizontal gene transfer events

To detect horizontal gene transfer events, ectomycorrhizas were formed under aseptic conditions between transgenic poplars and *A. muscaria* (Fig. 4). A total of 1000 transgenic poplar plants, representing 15 independent clones, were used for *in vitro* synthesis of ectomycorrhizas. About 35000 ectomycorrhizas were isolated under axenic conditions and investigated on selective agar plates. 102 ectomycorrhizal fungal colonies were formed under selective conditions (Fig. 5). However, when parts of these fungal colonies were transferred to fresh selection plates, the hyphal growth stopped, indicating that these colonies were not truly herbicide-resistant. Genomic DNA isolated from fungal hyphae that were initially growing on selection medium was investigated for the presence of the *bar* gene using PCR. No *bar*-specific fragment could be obtained from any of the clones investigated (Fig. 6a). However, the utilization of primers of a single-copy gene of *A. muscaria* (SCIV038; Nehls et al., 1999) revealed PCR-fragments in all samples (Fig. 6b), indicating that no inhibitors of the PCR reaction were present in the genomic DNA preparations.

DISCUSSION

Tree species have biological properties that differ from that of most crop plants, e.g. a long life time and the tight association of their fine roots with certain ectomycorrhizal fungi. In this symbiosis, fungi form a single-layer thick hyphal network within the plant root cortex (Hartig net), revealing a strongly increased plasma membrane surface (Kottke and Oberwinkler, 1987). During the interaction,

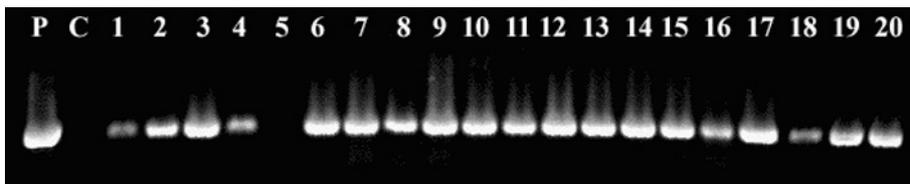


Figure 3. Analysis of genomic DNA isolated from kanamycin-resistant poplar transformants. PCR amplification was performed on genomic DNA using primers specific for the *bar* gene, generating a fragment of 550 bp in length. (Lanes 1–20) DNA isolated from putative transformants, (P) positive control with diluted plasmid DNA of pBI121/3, (C) DNA isolated from a non-transformed poplar plant.

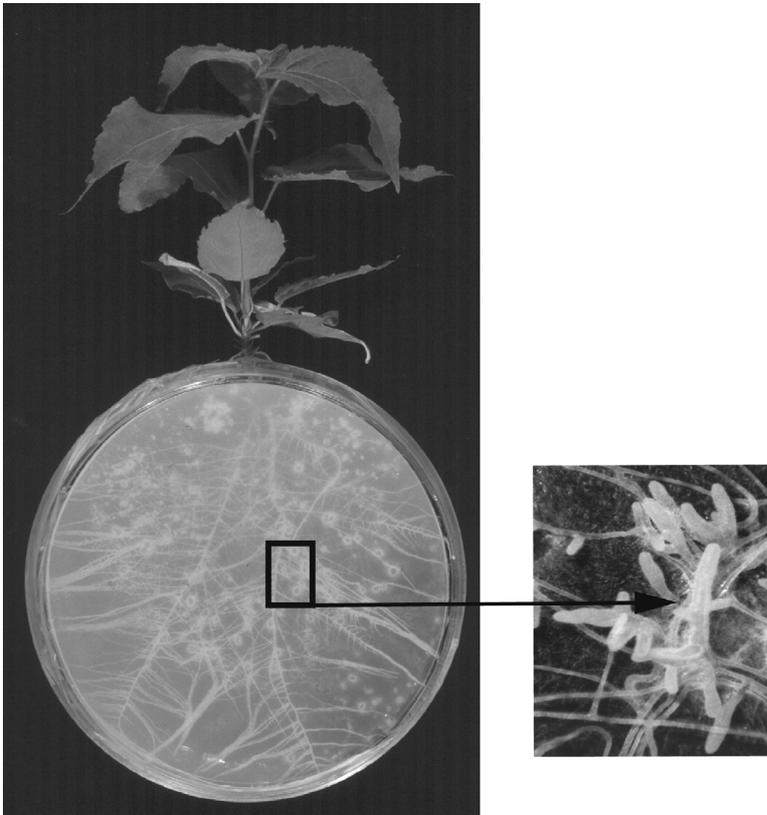


Figure 4. Axenically synthesized mycorrhizas. Rooted poplar cuttings were transferred to Petri dishes and 5 ml homogenized fungal mycelium were added to the roots system. Mycorrhiza formation (see close-up on the right) occurred after 6 to 8 weeks.

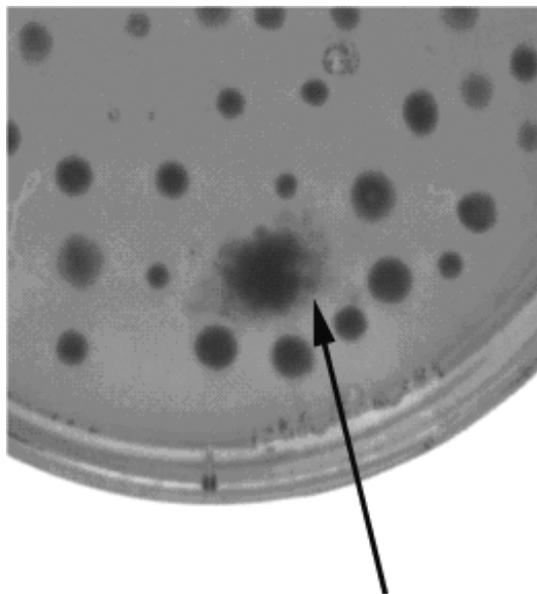


Figure 5. Formation of fungal colonies from mycorrhizas transferred to selection agar plates. Mycorrhizas were axenically excised from the root system, transferred to selection agar plates and incubated for two months. A colony revealing strong background growth is labeled with an arrow.

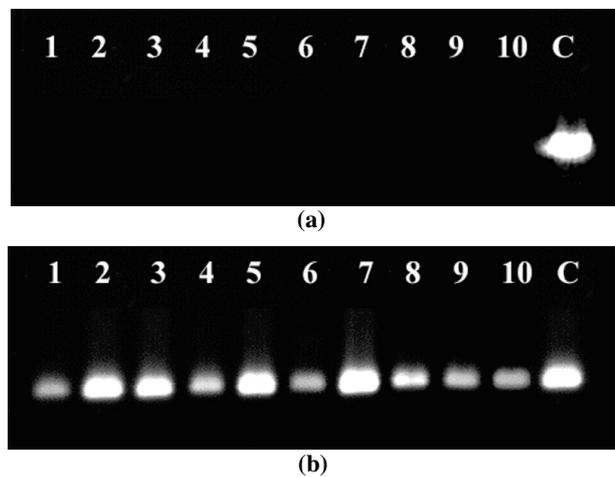


Figure 6. Investigation of the presence of the *bar* gene in genomic DNA of 10 initially Basta-resistant fungal clones. Genomic DNA was isolated from fungal colonies that revealed an enhanced growth after the first round of selection as well as of control hyphae. PCR-amplification of (A) the *bar* gene or (B) the *SCIV038* gene (Nehls et al., 1999) using gene-specific primers. A positive control (C, diluted plasmid DNA of the *bar* gene [Fig. 6a] or diluted plasmid DNA of the *SCIV038* gene [Fig. 6b]) was included to determine the proper size of the expected PCR fragments.

root cells are frequently degenerating and plant DNA might be taken up more easily by Hartig net hyphae, due to their modified anatomy and the tight contact with root cells. Since DNA uptake by filamentous fungi often leads to its integration into the genome (Fincham, 1989), a horizontal gene transfer from the plant to the fungal partner could result.

Horizontal gene transfer events have been reported from plant materials to bacteria (Nielsen et al., 2000) as well as to saprophytic fungi (Hoffmann et al., 1994), using a dominant selectable marker strategy similar to that used in our study. The utilization of a dominant marker has the advantage that a large sample number can be simultaneously screened while only a small number has to be re-investigated in detail (those that were able to grow under selective conditions).

Half a dozen dominant selection markers are frequently used for fungal transformation. Most frequently, the *hph* gene (conferring hygromycin resistance) is used as dominant selection marker. However, since *A. muscaria* is resistant to up to 1 mg.ml⁻¹ hygromycin B (data not shown), this marker could not be used for selection. In contrast to hygromycin, *A. muscaria* mycelia are highly sensitive to the herbicide Basta (at concentrations above 200 µg.ml⁻¹). Thus, a PEG-based protoplast transformation protocol was developed to introduce the *bar* gene selection system (Straubinger et al., 1992) into the ectomycorrhizal model fungus *A. muscaria*.

Compared to other protoplast-based protocols for ectomycorrhizal fungi (e.g. *Hebeloma cylindrosporum*, five transformants/µg DNA; Marmeisse et al., 1992), the efficiency of the *A. muscaria* protoplast-based transformation was rather low (one transformant per 10 µg DNA). One explanation for this could be the rather low regeneration frequency of *A. muscaria* protoplasts, even under non-selective conditions.

Integration of foreign DNA occurred at different locations within the *A. muscaria* genome. Most filamentous fungi do not contain extra-chromosomal plasmids, and incoming DNA is unstable unless it integrates into the host genome (for reviews see: Fincham, 1989; Magee et al., 2003). Fungi differ in their ability to integrate foreign DNA by either homologous or illegitimate recombination. While DNA integration preferentially occurs via homologous recombination in *Saccharomyces cerevisiae* or *Candida glabrata*, illegitimate recombination clearly dominates in *Histoplasma capsulatum* or *Blastomyces dermatitidis* (for a review see Magee et al., 2003). In the case of ectomycorrhizal fungi, no systematic evaluation of DNA integration mechanisms was performed. However, in none of the transformation experiments with ectomyc-

orrhizal fungi performed so far was evidence for the integration of foreign DNA by homologous recombination found (Barret et al., 1990; Bills et al., 1999; Combier et al., 2004; Hanif et al., 2002; Marmeisse et al., 1992; Pardo et al., 2002).

After confirming that the *bar* selection system worked well in *A. muscaria*, transgenic poplars containing the marker construct were used for mycorrhiza formation with non-transgenic *A. muscaria* hyphae under axenic conditions. From a total of 35000 investigated poplar/*A. muscaria* ectomycorrhizas, 102 fungal colonies were obtained that were able to grow on the selection medium. Nevertheless, none of these fungal clones contained the *bar* gene, indicating that these fungal hyphae were not truly Basta-resistant. The reason for these false positives was most probably the too low herbicide concentration in the growth medium. A concentration of 200 µg.ml⁻¹ Basta (as used in this study) could result in a fungal background growth, especially when large mycorrhizas were transferred to selection plates. This relatively low Basta-concentration was chosen to enable recognition of horizontal gene transfer events in cases where the *bar* gene without its heterologous promoter was transferred. Under these conditions, the *bar* gene might integrate behind a weak *A. muscaria* promoter, resulting in only weak herbicide resistance.

What are the limitations of this study?

(1) A successful *A. muscaria* protoplast transformation system is not proof that the fungus is able to take up plant DNA in degenerating mycorrhizas, nor does it tell anything about the transformation frequency under these conditions. However, (non-optimized) protoplast transformation of filamentous fungi, as in the case of *A. muscaria*, is often rather inefficient compared to other techniques (Olmedo-Monfil et al., 2004).

(2) The 35000 mycorrhizas investigated in this study represent only a limited sample number. Nevertheless, each mycorrhiza contains a large number (about 35000) of fungal cells in direct contact with plant root cells (about 700 hundred per mycorrhiza) that degenerated under selection conditions. Considering the transformation efficiency and protoplast regeneration rate observed by protoplast transformation of *A. muscaria*, about 7.3×10^6 DNA molecules were necessary to generate one transformant. 35000 mycorrhizas contained about 2.45×10^7 plant cells that were in direct contact (and additionally about 1×10^8 plant cells that were not in direct contact) with fungal hyphae. Thus, 35000 mycorrhizas represent about 3.4 (only cells in direct contact with fungal hyphae) to 17 times (all root cells) the number of plant genomes necessary to obtain one fungal transformant (based on

protoplast transformation frequency). Furthermore, microscopically observation of ectomycorrhizal sections indicate that each plant cell of the interface is surrounded by about 50 coenocytic fungal cells (Kottke and Oberwinkler, 1987; Nehls, unpublished) that could take up the liberated plant DNA.

(3) The poplar/*Amanita* system used in this contribution represents only one out of about a few million possible plant/fungus combinations. Thus, it cannot be ruled out that in other mycorrhizas horizontal gene transfer might occur at observable frequencies.

(4) Under natural conditions, ectomycorrhizas are associated with viruses, bacteria and pathogenic as well as saprophytic fungi, which might significantly influence horizontal gene transfer. Agrobacteria are, for example, able to transfer DNA not only into plants but also into ectomycorrhizal fungi (e.g. Combier et al., 2004; Hanif et al., 2002; Pardo et al., 2002). However, even when genetic information could be passed into ectomycorrhizal fungi via *Agrobacterium*, an import of plant DNA into associated Agrobacteria has never been shown, and a direct horizontal gene transfer from plants towards ectomycorrhizal fungi via Agrobacteria is thus rather unlikely.

In summary, the investigation of 35000 ectomycorrhizas formed under axenic conditions between transgenic poplar plants and *Amanita muscaria* revealed no hint of horizontal gene transfer in symbiosis. In contrast to the situation with some phytopathogenic fungi, these data thus indicate that plant DNA is not routinely taken up by ectomycorrhizal fungal hyphae. However, a field trial is currently under progress to investigate whether horizontal gene transfer from poplar to ectomycorrhizal fungi could be observed under natural conditions.

MATERIALS AND METHODS

Biological materials

Amanita muscaria ([L.] ex Fr.) Hooker strain CS83 was isolated from fruiting bodies (Schaeffer et al., 1996). Mycelia were grown in liquid culture for 4 weeks according to Chen and Hampp (1993) with glucose as sole carbon source.

The *Populus tremula* × *Populus tremuloides* clone T89 (Tuominen et al., 1995) was used.

The axenic formation of mycorrhizas was performed in a Petri dish system according to Hampp et al. (1996). Mycorrhiza formation took about four to six weeks. For

each batch, proper Hartig net formation was verified by microscopy of cross sections of mycorrhizal samples taken at random.

Construction of the shuttle vector: pBI121/3

To generate the plant transformation vector pBI121/3, pBI121 (Clontech, Palo Alto, CA, USA) was linearized with *EcoRI* and *XbaI*, and a 1250 bp *EcoRI/XbaI* fragment of pBG (Straubinger et al., 1992) containing the *Streptomyces hygroscopicus bar* gene under the control of the *Cochliobolus heterostrophus GPD1* promoter was inserted.

Transformation of *A. muscaria*

Isolation of *A. muscaria* protoplasts was performed according to Hampp et al. (1998) with modifications. Fungal mycelia of *A. muscaria* were grown in liquid MMN medium (Kottke et al., 1987) under agitation at 20 °C in the dark. Three days before use, the mycelial suspension was homogenized with an Ultra-Turrax T25 (Janke & Kunkel, Staufen, Germany) for 5 seconds at 8000 rpm. One gram of mycelia was collected by filtration on a 100 µm nylon mesh in a funnel rinsed with protoplast medium (0.5% (w/v) BSA, 0.2% (w/v) ascorbic acid, 2 mM MgSO₄, 1 mM CaCl₂, 0.55 M sorbitol, 5 mM MES-KOH pH 5.6) and suspended in 10 ml protoplast medium in a Petri dish containing 5 mg.ml⁻¹ Novozyme 234 (InterSpex, Foster City, CA, USA). After incubation for 3 hours at 20 °C under gentle agitation, protoplasts were separated from the remaining mycelia by filtration over a 100 µm nylon mesh. The protoplasts were collected by centrifugation (10 minutes at 3000 × g) and washed 3 times with 1 ml protoplast medium. Finally, the protoplasts were resuspended in protoplast medium to a density of 1 × 10⁹ protoplasts.ml⁻¹.

Protoplast transformation was carried out according to Nehls et al. (1992) in 2.2 ml Eppendorf tubes. 200 µl protoplasts suspension (2 × 10⁸ protoplasts) were subsequently mixed with 10 µl 1M CaCl₂, 1.25 µl DMSO, 10 µl (5 µg) DNA and 100 µl 40% polyethylene glycol 4000. The mixture was incubated for 1 hour on ice, and then for 20 minutes at 22 °C. The protoplasts were transferred into 10 ml tubes, carefully mixed with 4 ml melted (36 °C) top agarose (MMN, 0.55 M sorbitol, 0.5% (w/v) BSA, 0.7% (w/v) low melting agarose) and plated onto MMN agar plates containing 250 µg.ml⁻¹ Basta (BASF, Mannheim, Germany). The plates were incubated for up to six months at 18 °C in the dark until fungal colonies had regenerated from protoplasts.

Poplar transformation

The *Agrobacterium tumefaciens* strain C58/pMP90 (Koncz et al., 1992) was transformed by electroporation with the binary vector pBI121/3. This transgenic *Agrobacterium* strain then was used to transform the hybrid poplar *Populus tremula* × *tremuloides* strain T89 (Tuominen et al., 1995) according to Leplé et al. (1992) using leaves from 6 week old plants.

Screening of ectomycorrhizas for horizontal gene transfer events

Ectomycorrhizas were formed under aseptic conditions using 15 independent transgenic poplar clones and wild-type *A. muscaria* hyphae as fungal partner. Vital ectomycorrhizas were excised from the root systems under axenic conditions using a stereo microscope, and transferred to MMN agar plates containing 200 µg.ml⁻¹ Basta.

Extraction of genomic DNA

The extraction of genomic DNA from poplar leaves was performed according to Murray and Thompson (1980). Isolation of fungal genomic DNA was performed according to Nehls et al. (1992).

PCR-amplification of the *bar* gene

PCR-reactions (32 cycles, 1 min 91 °C 45 sec 48 °C, and 1.5 min 72 °C) were performed in a Progene thermocycler (Techne, Cambridge, UK) in a total volume of 50 µl, using 40 pmol of gene-specific primers and 1 U *Taq* polymerase (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

bar gene primers: 5'-GAAACCCACGTCATGCC-3';
5'-AGCCCAGAACGACGCC-3'.

GPD promoter primers: 5'-ATGGCCTGACATCATGTTG-3';
5'-AAGCTCCGAGGGCTGTA-3'.

ACKNOWLEDGEMENTS

We thank Nina Grunze for critical reading of the manuscript. This work was supported by the Bundesministerium für Bildung und Forschung (bmb+f), Schwerpunktprogramm "Biologische Sicherheitsforschung".

Received March 21, 2005; accepted February 7, 2006.

REFERENCES

- Barret V, Dixon RK, Lemke PA (1990) Genetic transformation of a mycorrhizal fungus. *Appl. Microbiol. Biot.* **33**: 313–316
- Bills S, Podila GK, Hiremath S (1999) Genetic engineering of an ectomycorrhizal fungus *Laccaria bicolor* for use as a biological control agent. *Mycologia* **91**: 237–242
- Boerjan W, Baucher M, Chabbert B, PetitConil M, Leple JC, Pilate G, Cornu D, Monties B, Inze D, VanDoorsselaere J, Jouanin L, VanMontagu M, Tsai CJ, Podila GK, Joshi CP, Chiang VL (1997) Micropropagation, genetic engineering, and molecular biology of *Populus*. US Department of Agriculture, Forest Service, Rocky Mountain Forest and Range Experiment Station, Fort Collins, CO
- Bryngelsson T, Gustafsson M, Green B, Lind C (1988) Uptake of host DNA by the parasitic fungus *Plasmodiophora brassicae*. *Physiol. Mol. Plant P.* **33**: 163–171
- Chen XY, Hampp R (1993) Sugar uptake by protoplasts of the ectomycorrhizal fungus, *Amanita muscaria* (L. ex fr.) Hooker. *New Phytol.* **125**: 601–608
- Combiér JP, Melayah D, Raffier C, Pepin R, Marmeisse R, Gay G (2004) Nonmycorrhizal (myc-) mutants of *Hebeloma cylindrosporum* obtained through insertional mutagenesis. *Mol. Plant Microbe Int.* **17**: 1029–1038
- De Vries J, Wackernagel W (2002) Integration of foreign DNA during natural transformation of *Acinetobacter* sp. by homology-facilitated illegitimate recombination. *Proc. Natl. Acad. Sci. USA* **99**: 2094–2099
- Fincham JR (1989) Transformation in fungi. *Microbiol. Rev.* **53**: 148–170
- Garcia-Vallvé S, Romeu A, Palau J (2000) Horizontal gene transfer of glycosyl hydrolases of the rumen fungi. *Mol. Biol. Evol.* **17**: 352–361
- Gebhard F, Smalla K (1998) Transformation of *Acinetobacter* sp. strain BD413 by transgenic sugar beet DNA. *Appl. Environ. Microbiol.* **64**: 1550–1554
- Gebhard F, Smalla K (1999) Monitoring field releases of genetically modified sugar beets for persistence of transgenic plant DNA and horizontal gene transfer. *FEMS Microbiol. Ecol.* **28**: 261–272
- 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. *Trees* **11**: 59–64
- Hampp R, Stülten C, Nehls U (1998) Isolation and regeneration of protoplasts from ectomycorrhizal fungi. In Varma A, ed, *Modern manual of mycorrhiza*, Springer-Verlag, Berlin Heidelberg, Germany, pp 115–126
- Hanif M, Pardo AG, Gorfer M, Raudaskoski M (2002) T-DNA transfer and integration in the ectomycorrhizal fungus *Suillus bovinus* using hygromycin B as a selectable marker. *Curr. Genet.* **41**: 183–188

- Hoffmann T, Golz C, Schieder O** (1994) Foreign DNA sequences are received by a wild type strain of *Aspergillus niger* after co culture with transgenic higher plants. *Curr. Genet.* **27**: 70–76
- Koncz CJ, Schell J, Redei GP** (1992) Methods in *Arabidopsis* research, World scientific, London
- Kottke I, Guttenberger M, Hampp R, Oberwinkler F** (1987) An *in vivo* method for establishing mycorrhizae on coniferous tree seedlings. *Trees* **1**: 191–194
- Kottke I, Oberwinkler F** (1987) The cellular structure of the Hartig net: coenocytic and transfer-cell like organization. *Nord. J. Bot.* **7**: 85–95
- Lapierre C, Pollet B, Petit-Conil M, Toval G, Romero J, Pilate G, Leplé JC, Boerjan W, Ferret VV, De Nadai V, Jouanin L** (1999) Structural alterations of lignins in transgenic poplars with depressed cinnamyl alcohol dehydrogenase or caffeic acid O-methyltransferase activity have an opposite impact on the efficiency of industrial kraft pulping. *Plant Physiol.* **119**: 153–164
- Leplé JC, Brasileiro ACM, Michel MF, Delmotte F, Jouanin L** (1992) Transgenic poplars: Expression of chimeric genes using four different constructs. *Plant Cell Rep.* **11**: 137–141
- Magee PT, Gale C, Berman J, Davis D** (2003) Molecular genetic and genomic approaches to the study of medically important fungi. *Infect. Immun.* **71**: 2299–2309
- Marmeisse R, Gay G, Debaud JC, Casselton LA** (1992) Genetic transformation of the symbiotic basidiomycete fungus *Hebeloma cylindrosporum*. *Curr. Genetics* **22**: 41–45
- Murray MG, Thompson WF** (1980) Rapid isolation of high molecular weight plant DNA. *Nucleic Acids Res.* **8**: 4321–4325
- Nehls U, Friedrich T, Schmiede A, Ohnishi T, Weiss H** (1992) Characterization of assembly intermediates of NADH: ubiquinone oxidoreductase (complex I) accumulated in *Neurospora* mitochondria by gene disruption. *J. Mol. Biol.* **227**: 1032–1042
- Nehls U, Mikolajewski S, Ecke M, Hampp R** (1999) Identification and expression analysis of two fungal cDNAs regulated by ectomycorrhiza and fruit body formation. *New Phytol.* **144**: 195–202
- Nielsen KM, Elsas JD, Smalla K** (2000) Transformation of *Acinetobacter* sp. strain BD413 (pFG4DnptII) with transgenic plant DNA in soil microcosms and effects of kanamycin on selection of transformants. *Appl. Environ. Microb.* **66**: 1237–1242
- Olmedo-Monfil V, Cortes-Penagos C, Herrera-Estrella A** (2004) Three decades of fungal transformation: key concepts and applications. *Meth. Molec. Biol.* **267**: 297–313
- Owen MDK** (2000) Current use of transgenic herbicide-resistant soybean and corn in the USA. *Crop Prot.* **19**: 765–771
- Pardo AG, Hanif M, Raudaskoski M, Gorfer M** (2002) Genetic transformation of ectomycorrhizal fungi mediated by *Agrobacterium tumefaciens*. *Brit. Mycol. Soc. Trans.* **106**: 132–137
- Ruiz-Diez B** (2002) Strategies for the transformation of filamentous fungi. *J. Appl. Microbiol.* **92**: 189–195
- Saroha MK, Sridhar P, Malik VS** (1998) Glyphosate-tolerant crops: Genes and enzymes. *J. Plant Biochem. Biot.* **7**: 65–72
- Schaeffer C, Johann P, Nehls U, Hampp R** (1996) Evidence for an up-regulation of the host and a down-regulation of the fungal phosphofructokinase activity in ectomycorrhizas of Norway spruce and fly agaric. *New Phytol.* **134**: 697–702
- Schlüter K, Fütterer J, Potrykus I** (1995) “Horizontal” gene transfer from a transgenic potato line to a bacterial pathogen (*Erwinia chrysanthemi*) occurs – if at all – at an extremely low frequency. *Biotechniques* **13**: 1094–1098
- Smith SE, Read DJ** (1997) Mycorrhizal symbiosis: Second edition, Academic Press, London
- Straubinger B, Straubinger E, Wirsal S, Turgeon G, Yoder O** (1992) Versatile fungal transformation vectors carrying the selectable bar gene of *Streptomyces hygroscopicus*. *Fungal Genet. Newsl.* **39**: 82–83
- Tuominen H, Sitbon F, Jacobsson C, Sandberg G, Olsson O, Sundberg B** (1995) Altered growth and wood characteristics in transgenic hybrid aspen expressing *Agrobacterium tumefaciens* T-DNA indoleacetic acid-biosynthetic genes. *Plant Physiol.* **109**: 1179–1189
- Warwick SI, Beckie HJ, Small E** (1999) Transgenic crops: new weed problems for Canada? *Phytoprotection* **80**: 71–84