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TECHNICAL NOTE

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T-DNA transfer and integration in the ectomycorrhizal fungus *Suillus bovinus* using hygromycin B as a selectable marker

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Abstract The T-DNA of *Agrobacterium tumefaciens* can be transferred to plants, yeasts, fungi and human cells. Using this system, dikaryotic mycelium of the ectomycorrhizal fungus *Suillus bovinus* was transformed with recombinant hygromycin B phosphotransferase (*hph*) and enhanced green fluorescent protein (*EGFP*) genes fused with a heterologous fungal promoter and CaMV 35S terminator. Transformation resulted in hygromycin B-resistant clones, which were mitotically stable. Putative transformants were analysed for the presence of *hph* and *EGFP* genes by PCR and Southern analysis. The latter analysis proved both multiple- and single-copy integrations of the genes in the *S. bovinus* genome. *A. tumefaciens* transformation should make possible the development of tagged mutagenesis and targeted gene disruption technology for *S. bovinus*.

Keywords Ectomycorrhizal fungus · *Agrobacterium*-mediated transformation · Hygromycin B resistance

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Introduction

Methods of molecular and genetic analysis have progressed more slowly for ectomycorrhizal fungi (ECMF) than for other higher fungi. One of the major constraints in ECMF has been the lack of an efficient transformation system. Although different transformation methods have been tried in ectomycorrhizal fungi, only moderate success has been achieved with protoplast transformation in *Laccaria laccata* (Barret et al. 1990) and *Hebeloma cylindrosporum* (Marmeisse et al. 1992) and with a particle-bombardment method in *Paxillus involutus* and *L. bicolor* (Bills et al. 1995, 1999). T-DNA transfer from the phytopathogen *A. tumefaciens* has succeeded not only in natural plant hosts (Shen et al. 1999), but also in yeast (Bundock et al. 1995), human cells (Kunik et al. 2001) and filamentous fungi (De Groot et al. 1998), including the cultivated mushroom *Agaricus bisporus* (Chen et al. 2000; Mikosch et al. 2001) which, like most of the ECMF, belongs to the basidomycetes. Recently, successful *Agrobacterium*-mediated transformation was performed in the ectomycorrhizal fungi, using the phleomycin resistance gene, but the fate of integration could not be proven (Pardo et al. 2002). The right combination of promoter and selection marker, together with the right choice of *Agrobacterium* strain, is often difficult to make, even when *Agrobacterium*-mediated transformation is used in plants (Hellens et al. 2000).

The current paper is a demonstration of an improvement in the transformation system of the ectomycorrhizal basidiomycete *Suillus bovinus* by changing the selection marker and using a different *Agrobacterium* strain. The transforming plasmid employed in this study, pBGgHg, has been used successfully for the transformation of *A. icus bisporus* (Chen et al. 2000). The vector pBGgHg (9.6 kb) consists of a pCAMBIA1300 backbone containing the *hph* and *EGFP* genes, each of which is joined to the CaMV 35S terminator and placed under the control of the strong and constitutive *gpd* promoter from *A. bisporus*. The hygromycin B resistance gene

(*hph*) coding for a phosphotransferase was originally isolated from *Escherichia coli* (Rao et al. 1983). Resistance to hygromycin B is determined by an aminocyclitol phosphotransferase that modifies hygromycin B and structurally related antibiotics by phosphorylation. Several non-mycorrhizal fungi have been transformed to hygromycin B resistance using *Agrobacterium* and the *hph* gene of *E. coli* as a selective trait (Abuodeh et al. 2000; Chen et al. 2000; Covert et al. 2001; Malonek and Meinhardt 2001; Mikosch et al. 2001; Zwiers and De Waard 2001).

The efficient transformation of the vegetative mycelium of ectomycorrhizal fungi is of extreme importance, since it provides us with a powerful tool for the genetic manipulation of the fungi, which in turn could help us to find the factors controlling mycorrhizal formation (Gorfer et al. 2001; Raudaskoski et al. 2001).

Materials and methods

S. bovinus (L.: Fr.) O Kuntze isolate (No 096) was routinely grown on modified Moser 6 medium (Moser 1963) at 20 °C, to obtain mycelium for transformation. For the selection of transformants, the pH of the media was adjusted to 7.5 with 1 M KOH and supplemented with hygromycin at a concentration of 25 μg ml^{-1}. *Agrobacterium* strain AGL-1 carrying the binary plasmid vector pBGgHg used for transformation was kindly provided by Carl Schlagnhaufer, Department of Plant Pathology, Pennsylvania State University. The vector pBGgHg contains both the hygromycin B (*hph*) resistance and enhanced green fluorescence protein (*EGFP*) genes as selection and gene expression markers, respectively (Fig. 1), and a kanamycin resistance gene for selection in *Agrobacterium*.

Bacterial cultivation, media composition, plasmid maintenance and the transformation procedure applied were based on the protocol described by Pardo et al. (2002), with a few modifications. *A. tumefaciens* strain AGL-1 was used instead of LBA1100 and the fungal colonies were grown on dialysis membranes 1×1 cm^2 (CelluSep T3, Membrane Filtration Products, Tex.) placed on the surface of solid Moser 6 media. The membranes were inoculated by small mycelial pieces cut with a Pasteur pipette from the margins of young *S. bovinus* mycelium and transferred to the middle of each membrane overlying the growth media. After the colonies had reached 0.5 cm diameter, which took 7 days, the membranes with colonies were transferred to Moser 6 induction plates with or without 200 μM acetosyringone (AS) and immediately inoculated with 50 μl of *Agrobacterium* culture prepared as follows. *Agrobacterium* was grown overnight in 4 ml of minimal medium containing kanamycin at 50 μg ml^{-1} at 29 °C (OD_{600} ~0.2). Bacteria were collected by centrifugation, resuspended in induction media (200 μM AS plus kanamycin) and grown for 6 h at 29 °C. The co-cultivation plates were incubated at 20 °C for 4 days, after which the membranes with mycelial colonies were transferred to Moser 6 selection plates (pH 7.5) containing 100 μg cefotaxime ml^{-1}, 100 μg ampicillin ml^{-1}, 125 μg tetracycline ml^{-1} and 25 μg hygromycin ml^{-1}, kept at 4 °C overnight and then shifted to 20 °C. Experiments always included controls using non-induced *A. tumefaciens* and non- transformed wild-type *S. bovinus*.

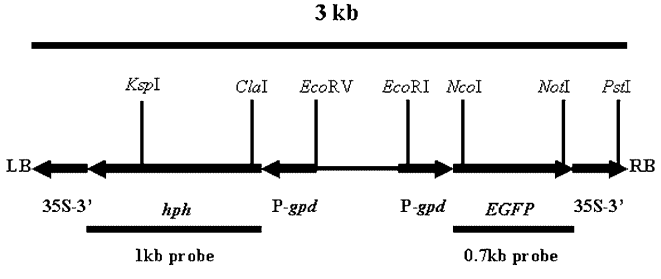

Fig. 1. Schematic drawing of the 3-kb insert containing the hygromycin B (*hph*) resistance and enhanced green fluorescence protein (*EGFP*) cassettes of the binary vector pBGgHg with selected restriction sites (modified from Chen et al. 2000). See text for more details. *LB* Left border, *RB* right border

For PCR and Southern analysis, hygromycin-resistant and wild-type *S. bovinus* mycelia were harvested from Moser 6 plates after 1 week of growth with and without hygromycin (25 μg ml^{-1}) and frozen in liquid nitrogen. The mycelia were then ground with pestle and mortar; and genomic DNA was isolated by the CTAB method (Russo et al. 1992). PCR amplification for detection of the *hph* gene in the putative transformants was carried out using primers hph-F (5′-AAGCCTGAACTCACCGCGAC-3′) and hph-R (5′-CTATTCCTTTGCCCTCGGAC-3′). Amplification included an initial denaturation at 94 °C for 2 min, followed by 30 cycles of 30 s denaturation at 94 °C, 30 s annealing at 60 °C and 1 min polymerisation at72 °C, followed by a final elongation of 5 min at 72 °C. The *EGFP* gene was detected using the primers 123-1 (5′-ATGGTGAGCAAGGGC-3′) and 123-2 (5′-TTACTTGTACAGCTCGTCC-3′). PCR was also carried out to check the presence of vector sequences outside the T-DNA borders in the transformants, using primers kan-F (5′-GGTCATGCATTCTAGGTACT-3′) and kan-R (5′-AATGGCTAAAATGAGAATAT-3′). Amplification conditions for the above two pairs of primers (kanamycin and *EGFP*) were as used for the *hph* primers, except that the annealing temperature was 54 °C.

The PCR products obtained with the primers for *hph* and *EGFP* from the putative transformant SbI were cloned into pCR 2.1-TOPO (Invitrogen) for sequencing. The sequencing was done automatically with the ABI Prism 377 DNA sequencer (Perkin-Elmer, Foster City, Calif.) and samples were prepared with the BigDyes terminator cycle sequencing ready reaction kit (Perkin-Elmer). Southern blot analysis was carried out with a ~1-kb fragment of the *hph* gene and a ~ 0.7-kb fragment of the *EGFP* gene (both α-^{32}P-labelled) as probes and with *Eco*RV- and *Bam*HI-digested genomic DNA from transformed and non-transformed *S. bovinus* strains.

For the formation of mycorrhiza, methods described by Niini et al. (1996) and Tarkka et al. (1998) were followed with some modifications. *Pinus sylvestris* L. seeds were incubated overnight in distilled water at 4 °C, surface-sterilised in 30% H_2O_2 for 30 min and rinsed at least five times with sterile water. Seeds were germinated on 1% water-agar plates for 2 weeks at room temperature in darkness and then seedlings were transferred to 60-ml test tubes with 10 ml of slanted growth medium for trees (Brown and Wilkins 1985). Once the radicles penetrated into the medium, they were covered with washed and sterilised burnt clay gravel (Leca; Lohja Rudus Oy, Helsinki, Finland). The tubes were plugged with cotton wool and placed in a growth cabinet (Fiotron 600H, Gallenkamp Industrial, Loughborough, UK) with an 18-h photoperiod. The temperature was 21 °C and the photosynthetic photon flux of 100 μmol m^{-2} s^{-1} was supplied by fluorescent lamps (36 W, warm-white; Airam Electric Oy, Helsinki, Finland). The seedlings were watered with sterile water every week. When lateral roots developed, they were inoculated with a 5 mm plug of wild-type or transformed *S. bovinus* mycelium. The mycorrhizal seedlings were allowed to grow for 2 months until the test tube was fully colonised by mycelium and mycorrhizae had developed in the entire root system. Short mycorrhizal roots from the seedlings inoculated with wild-type or transformed *S. bovinus* mycelium were cut out and transferred to Moser 6 medium with or without 25 μg hygromycin ml^{-1}. The growth of mycelium from the mycorrhizal short roots was followed for 1 month.

Results and discussion

The sensitivity of *S. bovinus* to hygromycin was tested by bringing small inocula of the dikaryotic mycelium onto Moser 6 plates with different concentrations of hygromycin (0, 10, 15, 30, 40 μg ml^{-1}). All colonies grew on 0 μg ml^{-1}, only two out of six grew on 10 μg ml^{-1} and growth was totally inhibited on 15 μg ml^{-1}. On the basis of these tests, 25 μg hygromycin B ml^{-1} was chosen for the selection of resistant colonies in the following transformation experiments.

A. tumefaciens strain AGL-1 with the binary vector pBGgHg was used to transform *S. bovinus*. The map of the *hph* resistance and *EGFP* cassettes of the vector pBGgHg is shown in Fig. 1. Co-cultivation of *S. bovinus* with *Agrobacterium* resulted in hygromycin-resistant colonies after 10–14 days (Fig. 2). The transformation was performed twice (independent experiments 1, 2) and the efficiency of transformation was exactly the same in both experiments. The percentage of regenerating mycelial colonies on selective medium was in both cases 66.7% (8 out of 12 in experiment 1, 14 out of 21 in experiment 2). No transformants were detected when AS, which is necessary for the induction of *vir* genes (Sheng and Citovsky 1996; Tzfira et al. 2000; Zupan et al. 2000), was omitted from the treatments.

Putative *S. bovinus* transformants showing resistance to hygromycin, obtained from both experiments, were first screened by PCR analysis. Using the hph-F and hph-R oligonucleotide primers, which include the start and stop codons of the *hph* gene, it was possible to amplify a PCR product of the expected size (~1 kb) from all transformants (Fig. 3A). Similarly, all transformants also showed the expected DNA amplification pattern (~ 0.7 kb) when primers 123-1 and 123-2, specific for the *EGFP* gene, were used (Fig. 3B). In the controls, no such fragments were obtained, neither by omitting the fungal DNA from the reaction mixture (Fig. 3A, B, lane 10) nor by using DNA from wild-type, non-transformed *S. bovinus* as the PCR template (Fig. 3A, B, lane 11). Cloning, sequencing and comparisons with public databases indicated that the ~1-kb and ~0.7-kb PCR products from the transformants were identical with the *hph* and *EGFP* genes, respectively. The *hph* gene could be detected in all hygromycin-resistant clones tested by PCR. However, using a phleomycin marker, only 73% of the phleomycin-resistant clones could be verified by PCR (Pardo et al. 2002). This could be attributed to: (1) better growth of mycelium on Moser 6, (2) higher sensitivity of *S. bovinus* to the new selectable marker, (3) higher stability of hygromycin B, compared with phleomycin during the long incubation periods necessary for selection of transformants from ECMF, or (4) the *A. bisporus gpd* promoter which seems to function efficiently in *S. bovinus*.

In order to confirm the PCR results and to determine the copy number and fate of the transferred T-DNA, Southern blot analysis was performed for eight transformants, four from each experiment, giving the PCR products shown in Fig. 3A and B. Genomic DNA from the transformants was digested with *Eco*RV and individually probed with 1-kb *hph* and 0.7-kb *EGFP* gene fragments. *Eco*RV cuts once within the T-DNA but does not cut inside the *hph* or *EGFP* genes and therefore multiple hybridising bands would be indicative of multiple T-DNA copies. According to this criterion, hybridisation patterns of the four transformants from the

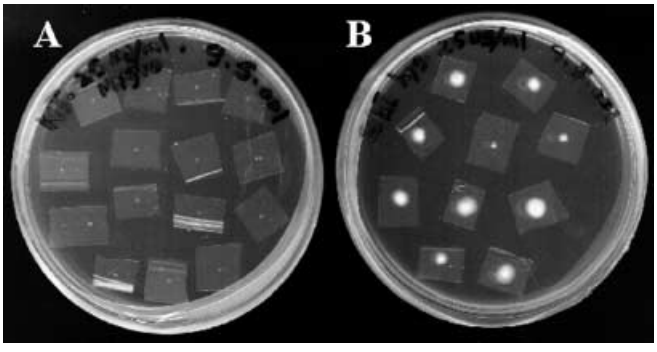

Fig. 2. Mycelial clones from *S. bovinus* wild type (**A**) and the transformant SbI (**B**) grown for 2 weeks on dialysis membranes overlying Moser 6 medium with hygromycin (25 μg ml^{-1}). SbI clones (**B**) were obtained after transformation with *A. tumefaciens* strain AGL-1 carrying the vector pBGgHg containing *hph* and *EGFP* genes. No growth of the untransformed mycelium (**A**) is seen on hygromycin-containing medium

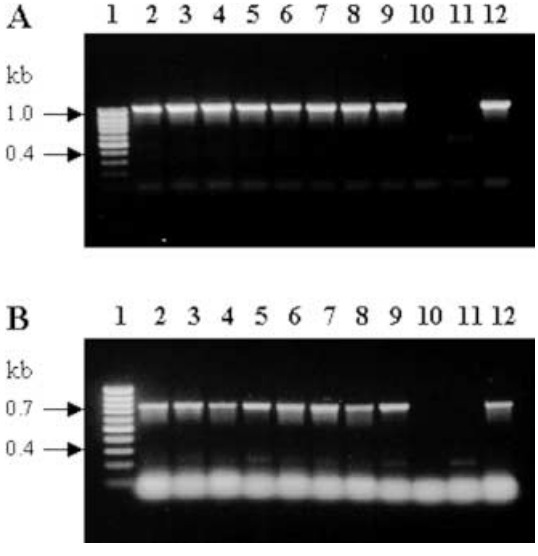

Fig. 3A, B. Polymerase chain reaction (PCR) of *S. bovinus* transformants (*lanes 2–9*), negative control without DNA (*lane 10*) and with wild-type DNA (*lane 11*) and positive control with vector pBGgHg (*lane 12*). PCR analysis was carried out using genomic DNA and primers hph-F and hph-R defining the 1-kb *hph* gene fragment (**A**) and primers 123-1 and 123-2 amplifying a 0.7-kb fragment corresponding to the *EGFP* gene (**B**). *Lane 1* DNA molecular size markers, *lanes 2–5* DNA isolated from the putative transformants SbI, SbII, SbIII and SbVI (experiment 1) and *lanes 6–9* DNA isolated from transformants SbAI, SbAII, SbAIII and SbAIV (experiment 2)

first experiment present a multiple integration event (3–8 copies of T-DNA) using *hph* as a probe (Fig. 4A). A corresponding pattern of multi-copy integration was also observed using *EGFP* as a probe (data not shown). The multiple hybridising patterns could be due to the problem we had when killing *A. tumefaciens* in the first experiment after transformation. During the selection of transformants, *A. tumefaciens* started to grow again, which could increase the frequency of T-DNA transfer. The growth of *A. tumefaciens* ceased only after the transformed mycelial colonies were transferred to new selection plates with a fresh cocktail mixture of antibiotics (Pardo et al. 2002). The high copy number of inserts could also be due to the insertion of T-DNA at different locations in each of the two haploid nuclei present in each hyphal cell of the dikaryotic mycelium of *S. bovinus*. Dedikaryotisation (Raper 1966) of the transformants to monokaryotic mycelium is still needed to determine the precise copy number per nucleus.

In the second experiment, the same protocol was used as in the first, but without any trouble in eliminating *A. tumefaciens* cells from the fungal transformants. Southern analysis of the transformants from the second experiment showed single-copy insertions both for *hph* and *EGFP* genes (Fig. 4B, C), indicating a single transfer of the whole T-DNA. The variability in the size of the *hph* and *EGFP* hybridisation fragments is due to differences in the size of the flanking genomic DNA. When *Bam*HI, which is not assumed to cut within the T-DNA, was used instead of *Eco*RV, the hybridisation pattern was exactly the same with the *hph* and *EGFP* probes in three of the transformants, confirming the single transfer of the whole T-DNA (Fig. 4D, E, lanes 2, 3, 5). Only in one of the transformants, SbAIII, did poor hybridisation with the *EGFP* probe not allow us to draw any conclusion (Fig. 4E, lane 4). The fragments varied in size among the different transformants, indicating that the T-DNA integrated into diverse sites within the *S. bovinus* genome (Fig. 4A–E). No hybridisation was found with the wild-type DNA (Figs. 4A–E, lane 1).

PCR was carried out with all transformants, using primers specific for the kanamycin resistance cassette outside the T-DNA. No sequence could be amplified in all the tested transformants, showing that T-DNA is the only DNA transferred (data not shown), as was shown previously (Pardo et al. 2002). This result contradicts Covert et al. (2001), who reported the transfer of vector sequences external to the T-DNA in *Agrobacterium*-mediated transformation of the fungus *Fusarium circinatum*. On the basis of the present PCR and Southern hybridisation results, it is suggested that the T-DNA integrates into the *S. bovinus* genome by non-homologous recombination and that non-homologous end-joining proteins are probably required for T-DNA integration into the genome, as in the yeast *Saccharomyces cerevisiae* (Van Attikum et al. 2001). Whether the addition of homologous sequences into T-DNA has an impact on transformation in *S. bovinus* needs to be

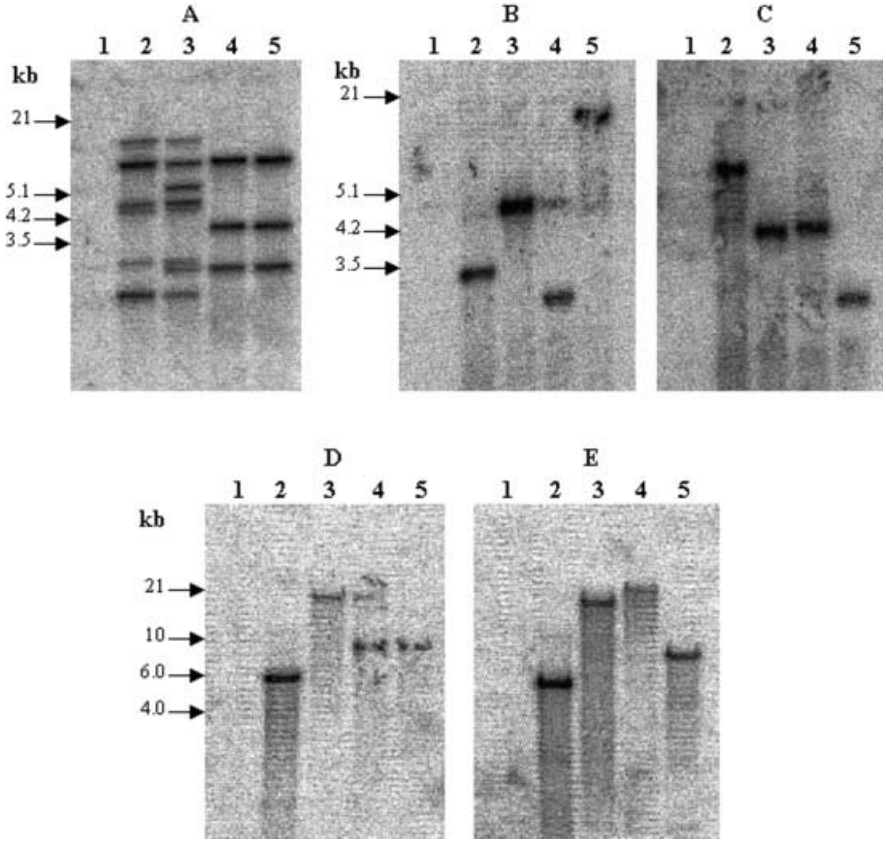

Fig. 4A–E. Southern blot analysis of the *S. bovinus* wild type and eight transformants. **A**, **B** and **D** were probed with the *hph* (1 kb) probe and **C** and **E** were probed with the *EGFP* (0.7 kb) probe. DNA in **A**, **B** and **C** was digested with *Eco*RV and in blots **D** and **E** with *Bam*HI. In **A–E** no signal was obtained from untransformed *S. bovinus* (*lane 1*). **A** Hybridisation of transformants from experiment 1: SbI (*lane 2*), SbII (*lane 3*), SbIII (*lane 4*) and SbIV (*lane 5*) showed multiple integration of the *hph* gene. **B–E** Hybridisation of transformants from experiment 2: SbAI (*lane 2*), SbAII (*lane 3*), SbAIII (*lane 4*) and SbAIV (*lane 5*) suggested single-copy integration of *hph* (**B**, **D**) and *EGFP* (**C**, **E**) genes, except in *lane 4* in **E**, where no clear signal was visible when DNA from the transformant SbAIII was probed with *EGFP*. Each lane contained 4 μg of restricted genomic DNA. *Numbers on the left* in **A**, **B** and **D** indicate the position and size of molecular size markers

tested in the future. T-DNA-based homologous recombination has been reported for *Aspergillus awamori*, *Kluyveromyces lactis*, *Mycosphaerella graminicola* and *S. cerevisae* (Bundock et al. 1995, 1999; Gouka et al. 1999; Zwiers and De Waard 2001).

Although integration of the *EGFP* gene into the *S. bovinus* genome was clearly indicated by Southern hybridisation, we could not detect its expression by GFP fluorescence. Also, Chen et al. (2000) could not detect the expression of the EGFP protein in *A. bisporus* transformed with pBGgHg. The expression of EGFP has succeeded in the basidiomycetes, *Phanerochaete chrysosporium* (Ma et al. 2001) and *Schizophyllum commune* (Lugones et al. 1999), by the addition of an intron either to the coding region (Ma et al. 2001) or to the 3′ end of the transcriptional unit of the *EGFP* gene (Lugones et al. 1999). In filamentous fungi, the addition of introns appears not to be the only means to induce the expression of a gene, since in *S. commune* the addition of one or more introns to the *hph* gene improved its expression only after an AT-rich stretch in the *hph* gene was modified to a higher GC content (Scholtmeijer et al. 2001). In the present work in *S. bovinus* and previously in *A. bisporus* (De Groot et al. 1998; Chen et al. 2000; Mikosch et al. 2001), hygromycin B-resistant clones were isolated after using an unmodified *hph* gene in *Agrobacterium*-mediated transformation. These results indicate that the unmodified *hph* gene is expressed in *A. bisporus* and *S. bovinus*, which could result from a higher A and T nucleotide content in the genome of *S. bovinus* than in *S. commune*. This seems to be the case, at least when the nucleotide content of the actin-encoding genes of *S. commune* and *S. bovinus* are compared (Tarkka et al. 2000). If *S. bovinus*, unlike other basidiomycetes (Raeder and Broda 1984; Schuren and Wessels 1990; Russo et al. 1992), has a higher A and T content in the genome, this could be a reason for the lack of expression of the *EGFP* gene, which has a G and C content of 59%. In the yeast *Candida albicans*, GFP expression required the construction of a synthetic gene (*yEGFP*) in which all 238 codons in the open reading frame were optimal for translation (Cormack et al. 1997).

Pure culture synthesis with seedlings determined that there was no difference in ectomycorrhizal development between the transformants and wild-type *S. bovinus*. The mitotic stability of the transferred T-DNA was tested by bringing pieces of mycorrhizal roots with the transformed mycelium to Moser 6 plates containing 25 μg hygromycin ml^{-1}. The growth of mycelium from the roots indicated that resistance to hygromycin B was retained in the symbiotic mycelium, as previously shown by using a phleomycin marker (Pardo et al. 2002).

In conclusion, the present experiments show that the unmodified *hph* gene can be used as a selection marker in *Agrobacterium*-mediated transformation of the ectomycorrhizal fungus *S. bovinus*. In future, the single-copy integration of T-DNA, achieved in the second set of *S. bovinus* transformants, can be utilised for functional studies of genes thought to be necessary for the establishment of symbiosis. We isolated and characterised actin genes (Tarkka et al. 2000) and several genes encoding small GTPases from *S. bovinus*, including *SbCdc42* and *SbRac1* (Gorfer et al. 2001), and *SbRas1* and *SbRas2* (Raudaskoski et al. 2001). The introduction of in vitro mutagenised dominant forms of these genes into the *S. bovinus* genome by the *Agrobacterium*-mediated transformation described here will greatly advance our understanding of the function of the actin cytoskeleton and small GTPases in vegetative and symbiotic hyphae of *S. bovinus*. The current studies corroborate our previous work (Pardo et al. 2002) regarding *Agrobacterium*-mediated transformation and open a way to a better understanding of the molecular genetics of *Pinus sylvestris–S. bovinus* ectomycorrhiza, which could also be applied to other ectomycorrhizal fungi.

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