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ORIGINAL ARTICLE

Agrobacterium-mediated insertional mutagenesis in the mycorrhizal fungus *Laccaria bicolor*

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Abstract *Agrobacterium*-mediated gene transfer (AMT) is extensively employed as a tool in fungal functional genomics and accordingly, in previous studies we used AMT on a dikaryotic strain of the ectomycorrhizal basidiomycete *Laccaria bicolor*. The interest in this fungus derives from its capacity to establish a symbiosis with tree roots, thereby playing a major role in nutrient cycling of forest ecosystems. The ectomycorrhizal symbiosis is a highly complex interaction involving many genes from both partners. To advance in the functional characterization of fungal genes, AMT was used on a monokaryotic *L. bicolor*. A collection of over 1200 transgenic strains was produced, of which 200 randomly selected strains were analyzed for their genomic T-DNA insertion patterns. By means of insertional mutagenesis, a number of transgenic strains were obtained displaying differential growth features. Moreover, mating with a compatible strain resulted in dikaryons that retained altered phenotypic features of the transgenic monokaryon. The analysis of the T-DNA integration pattern revealed mostly similar results to those reported in earlier studies, confirming the usefulness of AMT on different genetic backgrounds of *L. bicolor*. Taken together, our studies display the great versatility and potentiality of AMT as a tool for the genetic characterization of *L. bicolor*.

Keywords *Laccaria bicolor* · Monokaryon · *Agrobacterium*-mediated gene transfer · Insertional mutagenesis

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Introduction

Ectomycorrhiza (ECM) has a central role in the nutrient flow in temperate and boreal forest ecosystems. Numerous studies have demonstrated that while engaged in ECM interactions both the fungus and the host plant benefit from the nutrient and water exchange that occur at the symbiotic structures (Smith and Read 2008). The plant receives mainly nitrogen and phosphorus, while the fungus (predominantly basidiomycetes) is provided with carbon in the form of photosynthesis-derived metabolites. Also, it has been proven that the association of the plant with the fungus, whose hyphae spread over much larger extensions of soil than the tree roots alone, gives the former a better access to water as well as to macro- and micro-nutrients (Peterson et al. 2004; Lucic et al. 2008; Nygren et al. 2008; Tatry et al. 2009). Moreover, the interspecific and bidirectional belowground carbon trade between trees through ECM has recently been demonstrated and measured (Klein et al. 2016). This indicates that the forest trees interact with each other via symbiotic fungi in an even more complex manner than previously believed and a comprehensive understanding of the fungal mechanisms involved in the establishment and function of the ECM symbiosis is thus of great importance.

Studies of organisms and their relations with the environment are made through functional analyses, which allow

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the integration of different macro- and micro-scopic, physiological, biochemical, ecological and molecular features.

The development of ECM structures requires a fine space–time coordination in the gene expression of both the fungal and the plant partner (Wright et al. 2005). Also, the formation in the plant's radical apoplast of a Hartig net resembles in certain points the invasion produced by pathogenic fungi. However, a distinctive characteristic of this mutualistic colonization that has long been observed and more recently proven is the absence of a pronounced defensive response from the plant (Martin et al. 2008). This implies that symbiotic fungi have specialized in suppressing and evading the plant's innate defenses, through the production of secreted signal molecules such as effector proteins which can suppress the host immune response, or manipulate host cell physiology (Plett et al. 2011, 2014a, b; Lo Presti et al. 2015). The study of these complex mechanisms demands the existence of precise molecular tools that must be applicable to a wide range of experimental systems.

Gene transfer is a fundamental step in the genetic manipulation of fungi and it is an essential tool for functional genomic approaches. Several methods have been used for the introduction of foreign genetic material into fungal cells. This is a fundamental step in the genetic manipulation of these organisms, essential for functional genomic approaches. The more widely known techniques of electroporation and PEG-mediated transformation have been applied to different mushrooms (Li et al. 2006; Kuo and Huang 2008; Kim et al. 2010; Yin et al. 2012). Their efficiency greatly varies from fungus to fungus but they share a basic obstacle: the need to work with protoplasts. Forming viable protoplast from filamentous fungal material is a laborious process. Protoplasts are very delicate and need carefully monitored osmotic conditions to minimize cell mortality, and the post-transformation regeneration step of mycelia under selective conditions often brings further technical complications. The delivery of the foreign DNA into the fungal protoplasts is usually carried out in the form of non-autoreplicative molecules and the maintenance of this introduced material is dependent on genomic integration of the transgenes. Some autoreplicative vectors are available for gene transfer in fungi, especially for *Aspergillus spp.* (Aleksenko and Clutterbuck 1997; Carvalho et al. 2010), but the scarcity of naturally occurring plasmids strongly suggests that these are not very stable genetic elements in fungi.

The transformation mediated by *Agrobacterium tumefaciens* (AMT) offers an alternative method for genetic manipulation of fungi. AMT takes advantage of the natural capacity of this bacterium to transfer DNA to other organisms, in nature to a plant host, and stably integrate it into its genome. *Agrobacterium* carries a virulence plasmid which encodes both the mobile DNA element (referred as "T-DNA" in its double-stranded form and as "T-strand" in its single-stranded DNA form present during the mobilization), and the protein machinery needed for the transfer process. The T-DNA is flanked by 25 bp long direct, imperfect repeats called the right border (RB) and the left border (LB), and the key for using *Agrobacterium* as a gene delivery vehicle was the finding that the native T-DNA sequence between these borders could be modified without affecting the delivery or the integration process (Bourras et al. 2015).

Ectomycorrhiza is formed by filamentous basidio- and asco-mycetes, and the lack of efficient, protoplast-free, gene transfer method applicable to these fungi has seriously hindered the genetic studies of the ECM interaction.

While widely used in plant genetics since the early 1980s, *Agrobacterium* has been shown, under laboratory conditions, to be able to transfer its T-DNA also to non-plant cells, these including both yeasts and filamentous fungal species (Piers et al. 1996; Shi et al. 2012; Zhang et al. 2014; Aragona and Valente 2015). Furthermore, several ECM basidiomycete species have been demonstrated to be susceptible to AMT, opening totally new possibilities for ECM research (Pardo et al. 2002; Hanif et al. 2002; Müller et al. 2006; Zubieta et al. 2014). One of these fungi is *Laccaria bicolor*, the first ECM basidiomycete with its whole genome sequenced (Kemppainen et al. 2005; Martin et al. 2008).

We have previously examined the frequency and sequence bias of the T-DNA integration in the genome of the dikaryotic *L. bicolor* strain S238 N (Kemppainen et al. 2008). Similarly to plants, the T-DNA integration has been reported to occur preferably as a single copy per genome in fungi (Bundock et al. 1995; Alonso et al. 2003; Schneeberger et al. 2005; Zhang et al. 2007; Choi et al. 2007; Bourras et al. 2012). Our results from *Laccaria* AMT support the simple T-DNA integration pattern and revealed a bias towards coding sequence integrations in the fungal genome. However, no conserved nucleotide motifs were detected between the genomic sites of integration. All these characteristics confirmed AMT as a very appropriate tool for functional genomics in the dikaryotic strain. We have since demonstrated the usefulness of AMT for other objectives, such as gene expression downregulation and overexpression (Kemppainen et al. 2009; Kemppainen and Pardo 2010; Plett et al. 2011; Kemppainen and Pardo 2013; Navarro-Ródenas et al. 2015; Xu et al. 2015, 2016). In all instances, modification of a single nucleus was sufficient to achieve the desired goal. However, there are situations when it would be most useful to be able to obtain dikaryons that have both nuclei modified. Therefore, in the present work we analyzed the T-DNA integration pattern in an insertional mutagenesis library generated by AMT in the monokaryotic *L. bicolor* strain S238 N-H82. We obtained

a collection of over 1200 transgenic strains, of which 200 randomly selected strains were subjected to transgene integration pattern analysis.

Materials and methods

Fungal and bacterial strains

Laccaria bicolor (Maire) Orton (Di Battista et al. 1996) monokaryotic strains S238 N-H82 and S238 N-H107 (Selosse et al. 1996) were used in this study. The monokaryotic strains were kindly provided by Dr François Le Tacon from INRA Nancy, France. The vegetative mycelia were maintained at 22 °C in the dark on Pachlewski (P5)-modified agar medium, according to Kemppainen et al. (2008). The transgenic S238 N-H82 strains, containing the T-DNA of the pHg/pBks binary vector, were cultured in the same medium, with the addition of 300 μg/ml hygromycin B (Invitrogen). *Escherichia coli* TOP10 (Invitrogen) was used for plasmid cloning. *Agrobacterium tumefaciens* AGL-1 was employed in *Laccaria* AMT.

AMT of *L. bicolor*

Laccaria bicolor S238 N-H82 was transformed with *A. tumefaciens* AGL-1 containing the pHg/pBks vector according to a previously established protocol (Kemppainen et al. 2005). The hygromycin resistance (*hph*) carrying transgenic fungal strains were passed three times on GPY medium ($Mg_2SO_4 \cdot 7H_2O$ 0.5 g/l; KH_2PO_4 1 g/l; glucose 20 g/l; maltose 5 g/l; tryptone 5 g/l; peptone 5 g/l; yeast extract 3 g/l; agar–agar 20 g/l; pH: 5.5) with 300 μg/ml hygromycin B before growing mycelia for subsequent use.

Southern blotting

Fungal strains were grown on cellophane membranes on P5 medium with 300 μg/ml hygromycin B. The gDNA was purified with the DNeasy® Plant Mini Kit (Qiagen) and eluted with ddH_2O. Approximately, 6 μg of fungal genomic DNA (gDNA) per sample was digested with SacI (Promega) at 37 °C overnight, separated in 1 % agarose gel and transferred by alkaline capillarity blotting to an Amersham™ Hybond™ N Blotting Membrane (GE Healthcare) according to the manufacturer's protocol. A 1020 bp *hph* probe was generated from pHg/pBks by PCR using primers HpH-For/HpH-Rev (5′ AAGCCTGAACTCACC GCGAC 3′/5′ CTATTCCTTTGCCCTCGGAC 3′) (Invitrogen). The amplification was made according to Kemppainen et al. (2008) and the amplification product was gel purified using the QIAquick® Gel Extraction kit (Qiagen). The labeling of the probe, hybridization and signal detection were performed with the Gene Images AlkPhos Direct Labelling and Detection System (GE Healthcare), according to the manufacturer's instructions, hybridizing at 55 °C overnight and detecting after 6 h of exposure to a film (Agfa New Medical X-ray film).

Vegetative growth screening

The hygromycin-resistant strains obtained by AMT were cultured on solid P5 and GPY media for comparison of growth rates with the wild type strain S238 N-H82. Each screened strain was grown as triplicates on 100 mm diameter Petri dishes with 20 ml of medium at 22 °C in darkness. The vegetative growth was determined by measuring the colony radius every 5 days for 30 days, taking three measures for each colony. Strains with growth rates different from the wild type on one or both media were selected for further studies.

For liquid culture studies, three 1 × 1 mm plugs of mycelium were used to inoculate 125 ml Erlenmeyer flasks containing 20 ml of P5 or GPY media and kept static at 22 °C in darkness. Dry weight was determined every 5 or 7 days for 35 days. For each strain, two independent studies were made, with three replicas for every determination.

Dikaryon formation

The monokaryotic transgenic strains selected based on the growth screening and the wild type strain were mated with the compatible strain S238 N-H107 wt by co-cultivation on solid P5 for 30 days at 22 °C in darkness. The mycelia from the areas of interaction were propagated on the same medium with the addition of 300 μg/ml hygromycin B. The formation of the dikaryon was confirmed by the observation of clamp connections under a Nikon Eclipse 200 microscope.

Plasmid rescue

Fungal strains were grown on cellophane membranes on P5 medium with 300 μg/ml hygromycin B. The gDNA was purified with the DNeasy® Plant Mini Kit (Qiagen) and eluted with ddH_2O. To ascertain the integrity of the hygromycin and ampicillin resistance genes, each gDNA sample was subjected to two PCRs with primers HpH-For/HpH-Rev and Amp-For/Amp-Rev (5′ CCCAAGGTTTGC AAGCAGCAGATTACGCG 3′/5′ CGCGGATCCGCTCA TGAGACAATAACCC 3′) (Invitrogen). The PCRs were carried out according to Kemppainen et al. (2008). One to 3 μg of each gDNA was digested with SacI (Promega) (20–30 U/μg DNA) overnight. The enzymatic reactions were heat-inactivated, precipitated with ammonium acetate/ethanol and dissolved in ddH_2O (Sambrook et al.

1989). Cut gDNA was self-ligated with 1 U of T4 DNA ligase (Promega) in 50 µl final volume at 4 °C overnight and precipitated with ammonium acetate/ethanol. Samples were dissolved in 10 µl of ddH_2O and 5 µl was used for electroporating *E. coli* by a standard protocol (Sambrook et al. 1989). Electroporated bacteria were plated onto LB agar medium supplemented with 100 µg/ml ampicillin. Six ampicillin-resistant bacterial colonies were picked up for each fungal strain and cultured in LB liquid medium with ampicillin overnight for plasmid purification. The plasmids were linearized with SacI (Promega), which cuts once within the rescued plasmids from the transgenic *L. bicolor* strains. Restriction products were separated in 1 % agarose gels and stained with ethidium bromide. For every fungal transformant, the size of the 6 plasmids was analyzed to identify possible multiple insertional events. If all plasmids had the same size, the analysis was continued with only one bacterial clone. For the fungal strains that failed to produce resistant bacterial colonies at first, the rescue was attempted again by increasing the amount of gDNA.

DNA sequencing and analysis of the T-DNA integration sites

The rescued plasmids were purified for sequencing with the QIAprep® Spin Miniprep Kit (Qiagen). The sequencing was performed with the Post-RB primer (5′-AGCAGCTTGAGCTTGGATC-3′) (Invitrogen), in a 3730XL DNA sequencer (Macrogen Inc, Seoul, Korea). The sequences obtained were compared with the *L. bicolor* genome using the BLASTN algorithm on the JGI genome portal (http://genome.jgi-psf.org/Lacbi2/Lacbi2.home.html). Upstream regions were defined as up to 1500 bp upstream from the start codon and downstream regions up to 500 bp downstream from the stop codon. Sequence alignments were performed using the CLUSTALW2 algorithm (http://www.ebi.ac.uk/Tools/msa/clustalw2/). The alignments were used to create a Sequence Logo with the WebLogo online software available at http://weblogo.berkeley.edu/logo.cgi.

Results

In a previous study, we demonstrated that AMT can be used for gene transfer in dikaryotic *L. bicolor*. The T-DNA integrates primarily as a single copy without sequence bias, making the method suitable for random mutagenesis of the entire fungal genome (Kemppainen et al. 2005, 2008). In the present study, we have used the plasmid rescue binary vector pHg/pBks to investigate the integration pattern in a monokaryotic genetic background of *L. bicolor* and extended the study to a larger transgenic library.

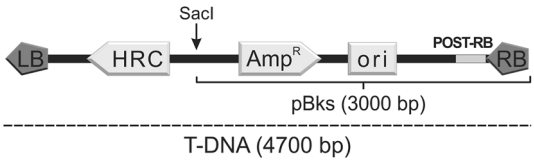

Fig. 1 T-DNA in pHg/pBk. *LB* T-DNA Left Border from pCAMBIA1300. *HRC* Hygromycin resistance cassette, composed by the glyceraldehyde 3-phosphate dehydrogenase promoter from *Agaricus bisporus*, the hygromycin B O-phosphotransferase (hph) coding sequence from *E. coli* (confers resistance to hygromycin B) and the 35S terminator from the cauliflower mosaic virus. *SacI* position of the SacI restriction enzyme recognition site. *AmpR* bla gene form *E. coli*, β-lactamase that confers ampicillin resistance. *ori* replication origin from pBlueScript KS + (pBks). *POST-RB* primer binding site. *RB* T-DNA right border from pCAMBIA1300

Laccaria bicolor monokaryon S238 N-H82 was transformed with *Agrobacterium tumefaciens* AGL-1 carrying the pHg/pBks vector. This vector has no sequence homology with the fungal genome and allows the selection of transgenic strains with hygromycin B. It contains the elements necessary for an easy identification of the integration site by plasmid rescue (Fig. 1). A collection of 1250 transgenic strains was obtained by AMT according to Kemppainen et al. (2005) using GPY medium (glucose-yeast extract-peptone) instead of P5. This change was made to allow the growth of insertional mutants with defective metabolic pathways.

To ascertain if the tendency of single integrations found for the dikaryotic strain existed also in the monokaryon, a Southern blot assay was performed with 9 randomly selected hygromycin-resistant strains. After purification of the gDNAs, the presence of *hph* was tested by PCR, with positive results for every strain (data not shown). The genomes were cut using the restriction enzyme SacI, which has one recognition site inside the T-DNA sequence (Fig. 1), and hybridized with a PCR-generated *hph* probe. At least two-thirds of the strains had a single copy of the T-DNA integrated into the genome (Fig. 2). This percentage appears to be lower than in the case of the dikaryon [90 % single integration events, (Kemppainen et al. 2008)], but it is in keeping with the tendency of single T-DNA integrations.

The next step in the characterization of the integration pattern was the random selection of 200 out of the 1250 transgenic strains. Their gDNA was purified and subjected to PCR with primers specific for the ampicillin resistance cassette, present in the T-DNA. The 190 ampicillin-positive strains were further used for the plasmid rescue protocol according to Kemppainen et al. (2008).

Out of 190 ampicillin-positive fungal strains, 109 produced ampicillin-resistant bacterial colonies. Purified plasmids of the minimum size were then sequenced

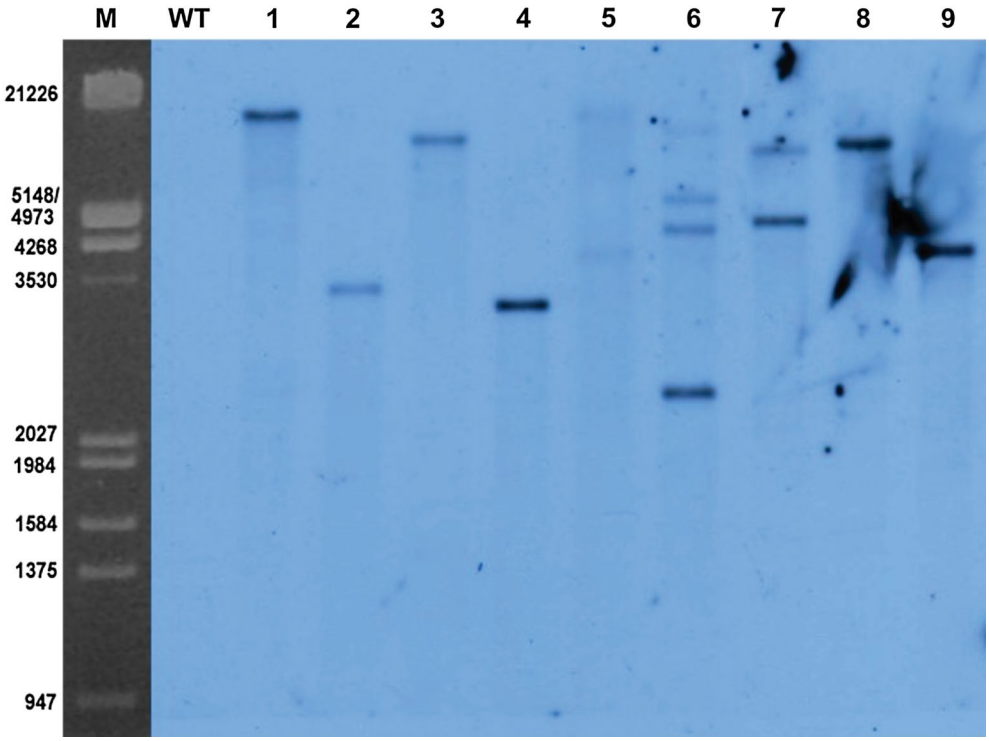

Fig. 2 Southern blot analysis of *L. bicolor* S238 N-H82 transgenic and wild type strains. From *left* to *right*, *M* molecular size marker (λ-EcoRI/HindIII); *WT* wild type strain; *1–9* Randomly selected transgenic strains from the library

Fig. 3 Frequency *plot* of the right border of the T-DNA right border (RB)—gDNA junction in the *L. bicolor* S238 N-H82 transgenic strains. All the nucleotides that appear in a given position are shown. The *height* of each *letter* represents the frequency of occurrence of the nucleotide in that position. The analysis was performed with sequences from 80 rescued plasmids, each one obtained from independent fungal transgenic strains. The RB sequence is over-lined. Positions 1–5 correspond to recovered gDNA sequences

using the Post-RB primer (Fig. 1) yielding 99 successful reactions. The majority of these (87/99) contained identifiable sequences from the *L. bicolor* genome and had a significant degree of conservation of the RB (Fig. 3). The remaining 12 sequences corresponded to empty plasmidic vectors or did not carry enough genomic sequence to allow the identification of a unique integration site. Few sequences belonged to transposable elements, which are found throughout the fungal genome and could, therefore, not be mapped to a single locus. A BLASTN search was performed by means of the JGI *Laccaria* genome portal (http://genome.jgi.doe.gov/Lacbi2/Lacbi2.home.html) to identify the precise T-DNA–gDNA junctions. The integrations were mapped in intergenic or coding regions of the gene models annotated in the genome by automated predictions or manually curated.

In the monokaryon, similarly to the *Laccaria* dikaryon, the T-DNA integrations took place predominantly in the sequences annotated as putative genes (75.9 %, 66/87), while the minority of the integrations happened within intergenic regions (24.1 %, 21/87). When the sequences of the 66 interrupted genes were examined in more detail, we found that 22.7 % had integration sites in the upstream region (15/66), 39.4 % in an exon (26/66), 13.6 % in an intronic sequence (9/66) and 24.2 % (16/66) in the downstream region. Only nine of these genes had no known homologs in other organisms and half of them had a predicted associated function or encoded a conserved functional protein domain (Table 1).

The entire collection of 1250 strains was used in growth assays on solid P5 and GPY media, out of which 13 are shown in Fig. 4. The diameters of the resulting colonies, as well as the shape of the growth borders, were compared with those obtained from the wt strain S238 N-H82. A total of 34 transgenic strains were selected based on a slightly differential growth pattern on one or both of the solid media.

Table 1 Genomic T-DNA integration sites in the monokaryotic *Laccaria* strains

Strain ID/integration site	BLASTp best hit	Predicted function
Transgenic strains with ORF integrations		
46–exon integration Protein ID: 582699	Hypothetical protein CC1G_05762 [*Coprinopsis cinerea okayama*7#130] Score 266 E-value 1e-69	Peptidase
151–exon integration Protein ID: 589926	Hypothetical protein K443DRAFT_112936 [*Laccaria amethystina* LaAM-08-1] Score 1290 E-value 0.0	Ectomycorrhiza-induced ankyrin-domain/NACHT-domain containing protein
159–exon integration Protein ID: 605640	No match	Predicted protein
162–exon integration Protein ID: 438367	Hypothetical protein STEHIDRAFT_73988 [*Stereum hirsutum* FP-91666 SS1] Score 55.5 E-value 3e-06	Predicted protein
165–exon integration Protein ID: 596861	Hypothetical protein AURDEDRAFT_22663 [*Auricularia delicata* TFB-10046 SS5] Score 212 E-value 2e-59	Predicted protein
174–intron integration Protein ID: 438847	Predicted protein [*Postia placenta* Mad-698-R] Score 120 E-value 3e-08	Predicted protein HAT dimerization
289–intron integration Protein ID: 703227	Hypothetical protein CC1G_00439 [*Coprinopsis cinerea okayama*7#130] Score 354 E-value 2e-95	Ubiquitin
305–exon integration Protein ID: 497384	No match	Predicted protein
332–intron integration Protein ID: 657839	Hypothetical protein K443DRAFT_273853 [*Laccaria amethystina* LaAM-08-1] Score 213 E-value 7e-67	Predicted protein
346–exon integration Protein ID: 640010	Hypothetical protein SCHCODRAFT_114815 [*Schizophyllum commune* H4-8] Score 202 E-value 5e-50	Protein kinase
350–exon integration Protein ID: 477109	Glycoside hydrolase family 30 protein [*Schizophyllum commune* H4-8] Score 372 E-value 8e-101	Glycoside hydrolase
453–exon integration Protein ID: 462922	2OG-Fe(II) oxygenase [*Coprinopsis cinerea okayama*7#130] Score 672 E-value 0.0	Oxygenase
461–intron integration Protein ID: 675910	Hypothetical protein GALMADRAFT_228557 [*Galerina marginata* CBS 339.88] Score 271 E-value 9e-78	Oligopeptide transporter
498–intron integration Protein ID: 464093	Hypothetical protein K443DRAFT_683457 [*Laccaria amethystina* LaAM-08-1] Score 139 E-value 9e-36	Predicted protein
566–exon integration Protein ID: 435729	Hypothetical protein SERLADRAFT_374879 [*Serpula lacrymans var. lacrymans* S7.9] Score 607 E-value 6e-15	Predicted protein
592–exon integration Protein ID: 571322	Hypothetical protein CC1G_09502 [*Coprinopsis cinerea okayama* 7#130] Score 366 E-value 2e-12	Major facilitator superfamily MFS-1; MFS general substrate transporter
677–exon integration Protein ID: 442667/328128	Hypothetical protein K443DRAFT_682668 [*Laccaria amethystina* LaAM-08-1] Score 73.6 E-value 4e-12	Predicted protein
743–exon integration Protein ID: 470940	WD40 repeat-like protein [*Stereum hirsutum* FP-91666 SS1] Score 850 E-value 5e-32	Predicted protein WD40 repeat
788–exon integration Protein ID: 448252	Hypothetical protein FG03324.1 [*Gibberella zeae* PH-1] Score 97.8 E-value 3e-18	P-loop GTPase
850 A–intron integration Protein ID: 294445	Hypothetical protein K443DRAFT_431644 [*Laccaria amethystina* LaAM-08-1] Score 1286 E-value 0.0	Hypothetical protein Protein kinase
853–exon integration Protein ID: 480565	Glutaminyl-peptide cyclotransferase-like protein [*Coprinopsis cinerea okayama*7#130] Score 138 E-value 4e-23	Peptidase
855–intron integration Protein ID: 680545	Hypothetical protein K443DRAFT_91227 [*Laccaria amethystina* LaAM-08-1] Score 885 E-value 0.0	Synaptic vesicle protein EHS-1 and related EH domain proteins
871–intron integration Protein ID: 576765	No match	Hypothetical protein

Table 1 continued

Strain ID/integration site	BLASTp best hit	Predicted function
878–intron integration Protein ID: 568491	Hypothetical protein CC1G_11649 [*Coprinopsis cinerea Okayama* 7#130] Score 67 E-value 1e-08	Hypothetical protein
883–exon integration Protein ID: 473557	No match	Hypothetical protein
954 A–exon integration Protein ID: 605975	Hypothetical protein K443DRAFT_115054 [*Laccaria amethystina* LaAM-08-1] Score 1058 E-value 0	Hypothetical protein
954 B–intron integration Protein ID: 621992	Hypothetical protein K443DRAFT_115054 [*Laccaria amethystina* LaAM-08-1] Score 1241 E-value 0.0	Hypothetical protein
959–exon integration Protein ID: 463764	Hypothetical protein SCHCODRAFT_67901 [*Schizophyllum commune* H4-8] Score 927 E-value 0.0	Multifunctional beta-oxidation protein
970–exon integration Protein ID: 442480	Hypothetical protein K443DRAFT_90406 [*Laccaria amethystina* LaAM-08-1] Score 172 E-value 3e-42	Predicted protein
1007–exon integration Protein ID: 444532	Small oligopeptide transporter [*Coprinopsis cinerea okayama*7#130] Score1211 E-value 0.0	Oligopeptide transporter
1015–exon integration Protein ID: 293613	Hypothetical protein K443DRAFT_673722 [*Laccaria amethystina* LaAM-08-1] Score 196 E-value 1e-57	Predicted protein
1078–exon integration Protein ID: 446870	Hypothetical protein K443DRAFT_675267 [*Laccaria amethystina* LaAM-08-1] Score 677 E-value 0.0	WD40 repeat-containing predicted protein
1097–exon integration Protein ID: 524483	Hypothetical protein K443DRAFT_86395 [*Laccaria amethystina* LaAM-08-1] Score 385 E-value 6e-126	Nuclear pore complex protein
1194–exon integration Protein ID: 659637	Hypothetical protein K443DRAFT_676336 [*Laccaria amethystina* LaAM-08-1] Score 155 E-value 6e-41	Predicted protein
1205–exon integration Protein ID: 442667/328128	Hypothetical protein K443DRAFT_682668 [*Laccaria amethystina* LaAM-08-1] Score 73.6 E-value 4e-12	Predicted protein
Transgenic strains with upstream integrations		
115–5′ UTR integration Protein ID: 687231	Hypothetical protein K443DRAFT_683457 [*Laccaria amethystina* LaAM-08-1] Score 122 E-value 5e-31	Predicted protein
185–5′ UTR integration Protein ID: 700799	Hypothetical protein K443DRAFT_681172 [*Laccaria amethystina* LaAM-08-1] Score 330 E-value 3e-108	Drug/metabolite transporter
338–5′ UTR integration 5′ UTR Protein ID: 301981	Peptide transporter PTR2B [*Hebeloma cylindrosporum*] Score 954 E-value 0.0	Peptide transporter
5′ UTR Protein ID: 583775	Hypothetical protein K443DRAFT_112055 [*Laccaria amethystina* LaAM-08-1] Score 567 E-value 0.0	Predicted protein
347–5′ UTR integration Protein ID: 571638	Hypothetical protein M378DRAFT_89621, partial [*Amanita muscaria* Koide BX008] Score 137 E-value 3e-37	Predicted protein HAT dimerization
355–5′ UTR integration Protein ID: 593948	Hypothetical protein K443DRAFT_604541 [*Laccaria amethystina* LaAM-08-1] Score 323 E-value 4e-109	Predicted protein
473–5′ UTR integration Protein ID: 465514	Helicase [*Coprinopsis cinerea okayama*7#130] Score: 1536 E-value: 0.0	DNA helicase
498–5′ UTR integration Protein ID: 687231	Hypothetical protein K443DRAFT_683457 [*Laccaria amethystina* LaAM-08-1] Score 122 E-value 5e-31	Predicted protein
517–5′ UTR integration Protein ID: 708933	Nucleus protein [*Coprinopsis cinerea okayama*7#130] Score 776 E-value 0.0	Fungal specific transcription factor
521–5′ UTR integration Protein ID: 459899	No match	Predicted protein
630–5′ UTR integration Protein ID: 450921	Hypothetical protein K443DRAFT_614189 [*Laccaria amethystina* LaAM-08-1] Score 50.1 E-value 2e-05	Predicted protein

Table 1 continued

Strain ID/integration site	BLASTp best hit	Predicted function
661–5′ UTR integration Protein ID: 703675	Hypothetical protein K443DRAFT_81327 [*Laccaria amethystina* LaAM-08-1] Score 252 E-value 6e-82	Zinc finger
670–5′ UTR integration Protein ID: 306423	Hypothetical protein K443DRAFT_677108 [*Laccaria amethystina* LaAM-08-1] Score 418 E-value 2e-144	Predicted protein
857–5′ UTR integration Protein ID: 697935	Hypothetical protein K443DRAFT_112644 [*Laccaria amethystina* LaAM-08-1] Score 625 E-value 0.0	Hypothetical protein
903–5′ UTR integration Protein ID: 552445	No match	Predicted protein
1014–5′ UTR integration Protein ID: 592119	Hypothetical protein M413DRAFT_144266 [*Hebeloma cylindrosporum* h7] Score 149 E-value 2e-37	Oxygenase
Transgenic strains with downstream integrations		
279–3′ UTR integration Protein ID: 384388	Hypothetical protein K443DRAFT_683665 [*Laccaria amethystina* LaAM-08-1] Score 1060 E-value 0.0	WD40 repeat
280–3′ UTR integration Protein ID: 438341	Hypothetical protein K443DRAFT_13482 [*Laccaria amethystina* LaAM-08-1] Score 310 E-value 1e-100	Hypothetical protein
353–3′ UTR integration Protein ID: 616185	No match	Hypothetical protein
366–3′ UTR integration Protein ID: 469892	Hypothetical protein K443DRAFT_673875 [*Laccaria amethystina* LaAM-08-1] Score 288, E-value 5e-91	HEAT repeat
641–3′ UTR integration Protein ID: 573843	Hypothetical protein M413DRAFT_19123 [*Hebeloma cylindrosporum* h7] Score 154 E-value 1e-39	P-loop NTPase
775–3′ UTR integration Protein ID: 604761	Hypothetical protein JAAARDRAFT_32950 [*Jaapia argillacea* MUCL 33604] Score 67.4 E-Value 2e-11	HrpA-like RNA helicase
797–3′ UTR integration Protein ID: 309955	Hypothetical protein K443DRAFT_8187 [*Laccaria amethystina* LaAM-08-1] Score 106 E-value 2e-44	Predicted protein
801–3′ UTR integration Protein ID: 591928	Hypothetical protein K443DRAFT_74767 [*Laccaria amethystina* LaAM-08-1] Score 164 E-value 6e-43	Predicted protein
850 B–3′ UTR integration Protein ID: 465540	Hypothetical protein M413DRAFT_29658 [*Hebeloma cylindrosporum* h7] Score 184 E-value 8e-48	Protein kinase
864–3′ UTR integration Protein ID: 684810	Peptide transporter PTR2B [*Hebeloma cylindrosporum*] Score 954 E-value 0.0	Peptide/h + symporter protein
873–3′ UTR integration Protein ID: 686252	Hypothetical protein K443DRAFT_670871 [*Laccaria amethystina* LaAM-08-1] Score 807 E-value 0.0	Predicted protein
956–3′ UTR integration Protein ID: 439025	Hypothetical protein K443DRAFT_645534 [*Laccaria amethystina* LaAM-08-1] Score 642 E-value 0.0	P-loop NTPase
989–3′ UTR integration Protein ID: 482081	Hypothetical protein K443DRAFT_670871 [*Laccaria amethystina* LaAM-08-1] Score 807 E-value 0.0	Predicted protein
992–3′ UTR integration Protein ID: 611915	Eukaryotic translation initiation factor SUI1 family protein [*Coprinopsis cinerea okayama*7#130] Score 597 E-value 0.0	Translation initiation factor
1189–3′ UTR integration Protein ID: 441064	No match	Predicted protein
250–scaffold_11:395347 3′ UTR protein ID: 708196	Hypothetical protein K443DRAFT_685172 [*Laccaria amethystina* LaAM-08-1] Score 339 E-value 1e-115	Predicted protein
5′ UTR protein ID: 611935	Glycoside hydrolase family 31 protein [*Laccaria amethystina* LaAM-08-1] Score 1687 E-value 0.0	Glycoside hydrolase

In the case of gene integrations, the JGI protein ID is indicated according to the *Laccaria* genome database. When no sequence homology or functional domain was found, the function is listed as "predicted protein"

Letters A and B are used for plasmids of different sizes obtained from the same fungal transformant

To further explore the potential of AMT as a functional genetics tool for *L. bicolor*, these selected monokaryons were mated with the compatible strain S238 N-H107 wt. The resulting dikaryons were then used in growth assays in liquid as well as on solid P5 or GPY media. Two dikaryons were found to have similar growth characteristics as the parental transgenic H82 monokaryon (strains 151 and 903; Fig. 5). In the case of the monokaryon 151, the difference in growth was originally observed as an alteration in mycelial morphology on solid medium as well as a slightly diminished growth. However, dry weight had no significant variation from the values attained for the wild type monokaryon. On the contrary, the dikaryon obtained with strain 151 had a markedly diminished dry weight when compared to the wild type dikaryon and still a slight difference when growing on solid media. For the monokaryon 903, a difference could be detected in the final dry weight in liquid media grown mycelia but not for solid media cultures. Surprisingly, the dikaryon showed a decreased growth capacity in both liquid and solid media.

Discussion

In this study, we employed the previously constructed binary vector pHg/pBks for the rescue of RB–gDNA junctions in the monokaryotic strain S238 N-H82 of the basidiomycete *L. bicolor* after transformation by *A. tumefaciens*. We had already successfully employed this vector system for a T-DNA integration pattern analysis in a dikaryotic strain of the same fungus (Kemppainen et al. 2008). Those analyses demonstrated a high conservation of the RB during the integration of the T-DNA in the *L. bicolor* genome (Fig. 3), confirming that the RB is a good target for the T-DNA–gDNA plasmid rescue. However, if this approach should fail, the plasmid pHg/pBks has the elements required for the rescue of the LB with several restriction enzymes as well as for the simultaneous rescue of both borders by means of restriction enzymes without recognition sites within the T-DNA.

With the RB plasmid recue, we obtained positive results for the rescue of 63 % of the transgenic monokaryotic strains analyzed. Out of these, 79 % could be sequenced with the POST-RB primer, which represents 50.5 % of the total of rescues attempted. The absolute majority of these had a highly conserved RB, although several showed truncations of up to five bases. This phenomenon has been reported for plants and other fungi transformed by AMT (Oosumi et al. 2010; Maruthachalam et al. 2011; Kemski et al. 2013; Yu et al. 2015). The genomic integration sites of the rescued transgenic strains had no evident homology with the RB or LB sequences. Also, no nucleotide conservation could be found among the different integration sites, not even at single positions, microdomains or AT/CG biases.

We found that the insertion sites were located primarily in putative genes (~76 %), with nearly half of the integration events occurring within the ORFs. These results are in concordance with the findings of our previous work, where we applied the same RB rescue protocol to a dikaryotic strain of *L. bicolor* (Table 2). There are several models proposed for the molecular mechanisms of the T-DNA integration into the cell's genome (for a review, see Bourras et al. 2015). A common feature of the models is the participation of multiple proteins in the process directing the insertion of the T-DNA. These proteins mostly perform the recognition of the target site (alone or in association with other cellular factors) and generally belong to the transcription regulation machinery of the transformed cell. Therefore, it is possible that the absence of a conserved sequence pattern is a consequence of the involvement of different proteins, each with its particular sequence recognition. This could also explain the preference of integration in transcriptionally active genes rather than in intergenic regions, since the T-DNA interaction partners would rarely associate with the latter. Similar studies for other fungi found that integration of the T-DNA preferentially occurred in gene-rich regions and transcriptionally active loci (Bourras et al. 2012; Kemski et al. 2013). This could be interpreted as a further support for the specific role of T-DNA integration proteins in directing the transgenes into transcriptionally active sites. Alternatively, the detected integration site bias can also be a simple artifact, a result from the selection conditions used in the isolation of the transgenic strains. As the T-DNA carries the genetic element, the transcriptional activity of which is a pre-requirement for survival and further growth of the transformed cells under selection conditions, the T-DNA integrations in euchromatic regions of the chromosomes with high transcriptional activity could be expected to allow a higher fungal survival than the heterochromatic integrations events. The results of the genome-wide T-DNA integration analysis of *Arabidopsis thaliana* under non-selective conditions strongly support this T-DNA integration bias (Kim and Veena 2007).

Another interesting observation is the number of integrations per genome. If the sequence data alone is regarded, there is a very high level of single integration events (more than 90 %). However, the number decreases significantly when using the Southern blot analysis (about 75 %). This difference could be due to the low number of strains employed for the latter assay when compared to the number of strains used in the rescue protocol. Also, it is possible that the transgenic strains, which produced multiple transgene signals in the Southern blot analysis, presented mixed colonies with nuclei of different T-DNA integration events. Be that as it may, the conditions employed for the AMT protocol clearly favored a single integration event per genome.

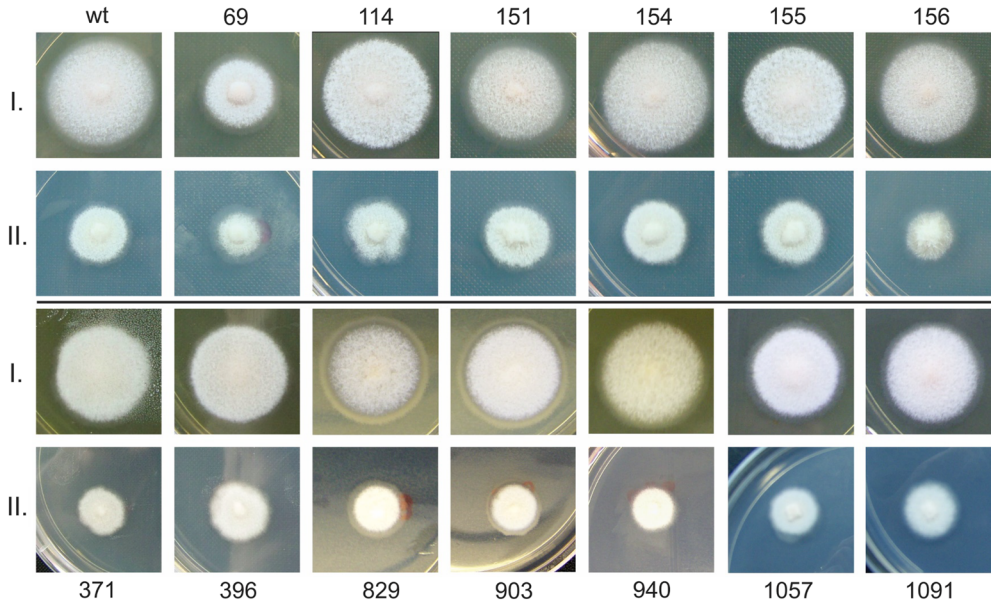

Fig. 4 Phenotypes of some *L. bicolor* S238 N-H82 monokaryotic T-DNA tagged strains on solid media. *I* GPY medium. *II* P5 medium. *wt* wild type strain. The *numbers above* and *below* the *photos* refer to the ID number of the transgenic strain

When compared to our previous results from the dikaryotic *L. bicolor*, we found that the percentage of successful plasmid rescue with the monokaryon was lower, even though a much larger strain set was analyzed (Table 2). On the contrary, the fraction of sequences obtained from the rescued strains was somewhat higher in the present study. The relative number of unresolved sequences remained practically identical. These correspond to strains where the integration site of the T-DNA is located very closely to a SacI recognition site or within a transposable element. In both cases, the recovered sequence is insufficient to allow the identification of a unique insertion site in the genome. Nevertheless, it is noteworthy that three out of the five insertions in transposable elements occurred within the putative coding region of the reverse transcriptase. This is in keeping with the general tendency of the T-DNA integration in gene-rich regions.

The percentage of the intergenic integrations did not vary between the AMT of monokaryotic or dikaryotic strains. Our results point to a highly reproducible outcome when using AMT on different *L. bicolor* genetic backgrounds and demonstrate the great potentiality of this genome manipulation technique. The starting material for a particular AMT-mediated functional genomics assay will, therefore, be determined not by the limitations of the gene transfer technique but by the desired information. As it does not require the preparation of special forms of fungal cells and employs easily cultivable mycelium, there are almost no restrictions for the use of AMT in *L. bicolor* functional genomics.

Another important point is the possibility of mating transgenic strains with a compatible monokaryon. In general, the dikaryotization reverted the altered growth phenotypes of the transgenic monokaryons, which indicates that the gene function driven from the second nucleus is sufficient for compensating the genetic defect of the T-DNA tagged nucleus. However, we identified two monokaryotic strains that conserved a differential growth rate even after dikaryotization. This phenomenon will have to be studied in more detail, since there are several possible explanations. One option is that the T-DNA integration results in a truncation of the gene and not its complete elimination. This could yield a partially active protein resulting in a dominant phenotype. Another option could be through gene silencing, if the truncated messenger molecule is recognized as an RNA-silencing trigger. In this case, the messengers from the second gene copy could also be affected. The realm of possibilities is wide and the only way to narrow it is through further studies.

The use of monokaryons in ectomycorrhiza studies is very limited. Normally, the dikaryotic form is the one establishing symbiosis with the plant. However, there are some reports of successful mycorrhizations with monokaryons for *Hebeloma cylindrosporum* (Gay et al. 1994) and *Pisolithus spp*. (Lamhamedi et al. 1990; Costa et al. 2010). The dikaryotization of two compatible transgenic monokaryons would allow the creation of strains defective in more than one gene of choice or in both copies of the same gene.

We have now used AMT on monokaryotic and dikaryotic strains for insertional mutagenesis, protein overexpression and gene downregulation. These studies have demonstrated

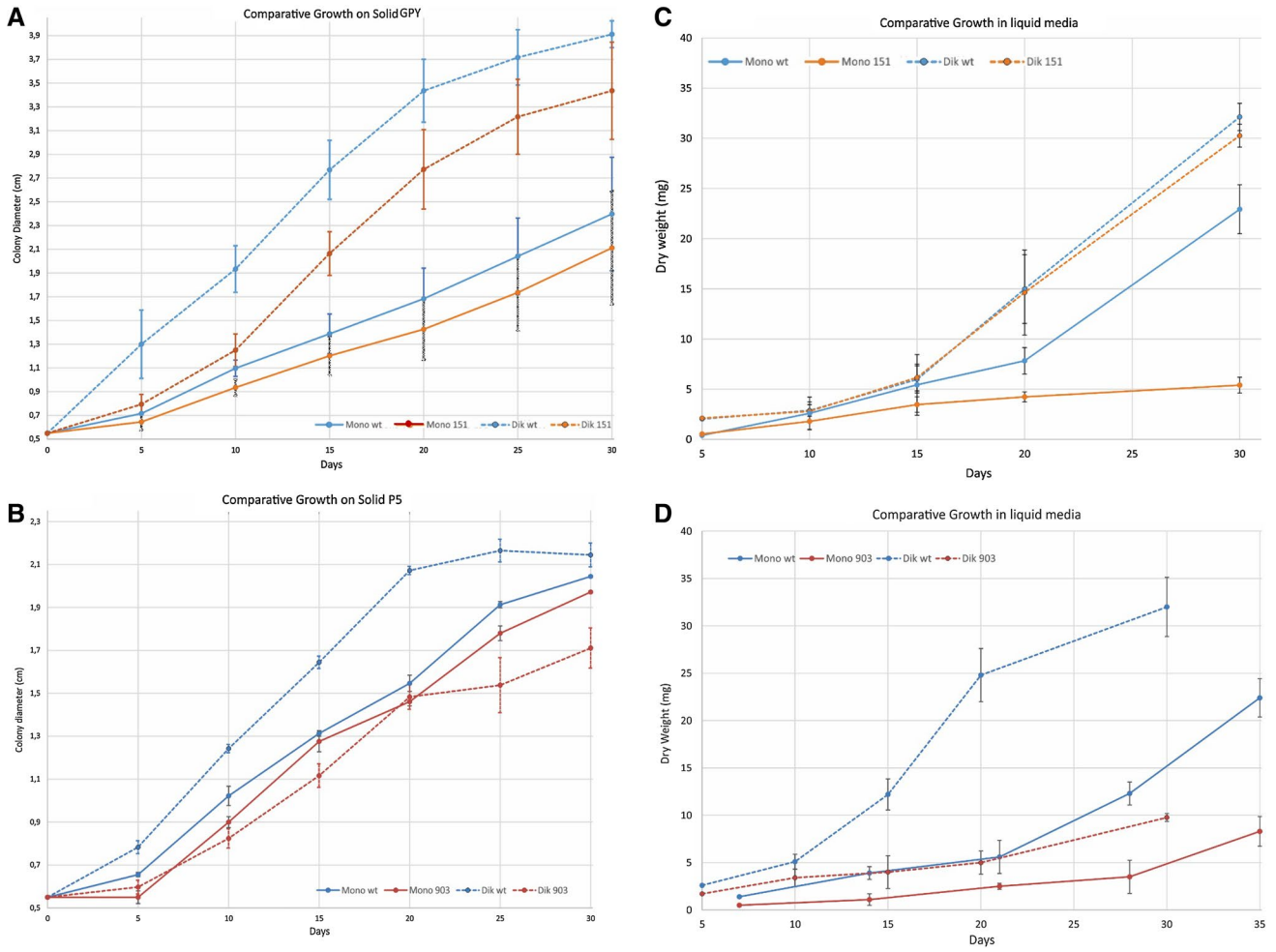

Fig. 5 Growth assays of the transgenic monokaryotic strains 151 and 903 of *L. bicolor* S238 N-H82 and the dikaryons resulting from the mating with strain S238 N-H107 wt. **a** Growth of wt and 151 strains on solid GPY medium. **b** Growth of wt and 903 strains on solid P5 medium. **c** Growth of wt and 151 strains in liquid GPY. **d** Growth of wt and 903 strains in liquid GPY

Table 2 Comparison of plasmid rescue results in different genetic backgrounds of *L. bicolor*

	Monokaryon (H82)	Dikaryon (S238 N)[a]
Library size	1250	500
Analyzed strains	200	51
Transgene positive	190	47
Attempted rescues	190	47
Successful rescues	57 % (109/190)	74 % (35/47)
Sequences obtained	91 % (99/109)	83 % (29/35)
Unresolved sequences	12 % (12/99)	14 % (4/29)
Identified sequences	88 % (87/99)	86 % (25/29)
Sequences within genes	76 % (66/87)	75 % (19/25)
Sequences in intergenic regions	24 % (21/87)	25 % (6/25)

[a] Taken from Kemppainen et al. (2008)

some of the possible uses of this gene transfer tool in functional genomics of *L. bicolor*. Such great dynamic potential is necessary when attempting to unravel the highly complex genetic mechanisms that control the establishment and function of the ectomycorrhizal symbiosis.

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