Agrobacterium-based gene transfer for post-genomic research in *Laccaria bicolor*

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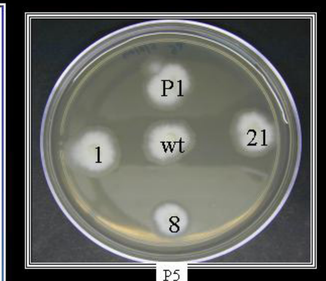

P5

P5 +300μg/ml hygromycin

Introduction

Agrobacterium **is the only known example of inter-kingdom gene transfer in nature. Besides its natural host plants, this Gram-negative bacteria is able to transfer its mobile DNA element (T-strand) to other eukaryots, including fungi. Like already before in plant research, the *Agrobacterium*-mediated gene transfer (AMT) is now proving its usefulness in fungal genetics.**
Sequencing of the genome of the symbiotic basidiomycete *Laccaria bicolor* was recently accomplished by the Joint Genome Institute (http://genome.jgi-psf.org/Lacbi1/Lacbi1.home.html) turning it into the model organism in mycorrhizal research. The fact that a dikaryotic strain of this fungus was also recently shown to be transformed by *Agrobacterium* (Kemppainen *et al.* 2005) makes it truly attractive for functional genomic studies. In order to evaluate the use of AMT as a tool for random insertional mutagenesis in *L. bicolor* we have studied the genomic T-strand integration pattern via a plasmid rescue approach. We have tested the functionality of several heterologous fungal promoters in *Laccaria* and transformed compatible monokaryotic strains. In addition this is the first communication of both cytosolic and localized GFP expression in *L. bicolor* achieved by AMT.

AMT of *Laccaria bicolor*

Agrobacterium –mediated transformation seems to offer an easy and manageable method for genetic transformation of *L. bicolor*. We have optimized a mycelium-based transformation protocol for the dikaryotic strain S238N using hygromycin resistance gene with high efficiency (up to 80% of co-cultivated colonies produced at least one transformed fungal line; Kemppainen *et al.* 2005). In addition compatible monokaryotic strains C11-H82 and C11-H107 have been transformed with the same methodology making possible the construction of double knock-out dikaryotic lines for mycorrhizal studies. In the case of monokaryons the transformation efficiency was slightly lower (40-50%) probably due to their lower hyphae density. We have obtained the best transformation effieciencies using *Agrobacterium* strain AGL1 in combination with pCAMBIA1300 based binary vectors. No spontaneous hygromycin resistance has been detected proving this selection marker reliable for AMT of *Laccaria*.

We have tested various heterologous basidiomycete and ascomycete promoters for their capacity to produce hygromycin resistant lines in *L. bicolor* by AMT (Table 1.) This far the best promoter for *L. bicolor* transformation has been *Agaricus bisporus* Pgpd- promoter but also Pgpd of *Schizophyllum commune* can be used for this fungus.
Interestingly a *Tuber borchii* nitrate transporter promoter can drive *hph* expression but does not seem to be under obvious nitrogen source control in *Laccaria*. *Ustilago maydis* hsp70 promoter does not function in *Laccaria* even though it has been used for transforming ascomycetes.

Promoter	Fungus	Functionality in *Laccaria bicolor*
Pgpd (glyceraldehyde-3-phosphate dehygrogenase)	*Agaricus bisporus*	+
Pgpd	*Schizophyllum commune*	+
Nitrate transporter	*Tuber borchii*	+
TrpC (tryptophane biosynthetic pathway gene)	*Aspergillus nidulans*	-
Pgpd	*Aspergillus nidulans*	-
*hsp*70 (heatshock protein family70)	*Ustilago maydis*	-

Table 1. Functionality of heterologous fungal promoters in *Laccaria bicolor*

T-strand integration pattern in *Laccaria bicolor*

As the whole genome sequence of *L. bicolor* is now available, studies of T-DNA integration patterns can be conducted in order to understand the integration mechanisms involved and to evaluate the AMT as an insertional mutagenesis tool for *Laccaria*.
We have studied the integration pattern and conservation of the right border regions of the T-DNAs in *Laccaria* genome via a plasmid rescue approach.
The plasmid-rescue binary vector was constructed using pBGgHg (Chen *et al.* 2000). The orginal sGFP-box was removed from the T-DNA with *Sac*I and *Xba*I and ligated with pBluescript KS+ (Stratagene) complete plasmid creating the ampicillin rescue-plasmid pHg/pBks where the right border (RB) can be rescued with *Sac*I (Fig.1). 47 out of 51 analyzed fungal lines (92%) were PCR-positive for both hygromycin and ampicillin resistance genes, indicating that the 4.7Kb T-DNA integrated mostly complete. In addition the Southern blot analysis of a subset of samples proved a dominantly single integration pattern in different genomic sites (data not shown). Of the 47 transformed fungal lines 40 (85%) produced ampicillin resistant bacterial colonies in plasmid rescue. Consistently *Sac*I linearizable plasmids from 35 fungal lines were subjected to sequencing with the Post-RB primer and 29 sequences were obtained.
Aligned RB sequences showed that it conserves completely or with minor 1-5 bp deletions during T-strand integration (Fig. 2). No clear micro-homologies were indentified between different genomic integration sites or between the integration sites and LB or RB sequences.
19 T-strand integration sites were located in *Laccaria* genome in predicted genes (in exons or introns), in upstream elements (up to 1500 bp upstream from ATG) and downstream elements (up to 500 bp downstream from TAG) or in intergenic regions. Four sequences, due to their repetitive nature present in various genomic scaffolds, could not be located with absolute certainty. These were considered as intergenic integrations.
Twelve gene interruptions were found, 10 of which were in exons and 2 in predicted intronic sequences. Three proposed promoter and two downstream ORF integration were found. One integration was intergenic (Table 2).

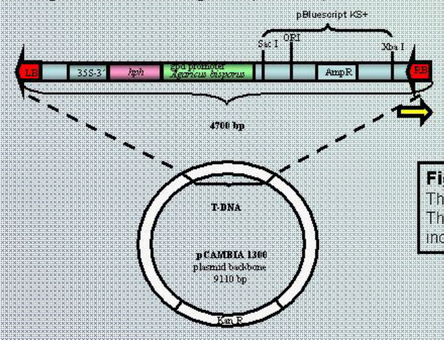

Fig. 1
The pHg/pBks –rescue plasmid.
The binding site of the Post-RB primer is indicated by a yellow arrow.

Fungal lines with interrupted ORF (genomic site)	Best homology hit	Predicted or known functions	Cellular functional group
Line 2: exon integration in eu2.Lbscf0011g0140 (11:306616-309168)	gi|70981392|ref|XP_731478.1|reverse transcriptase RNase H (*Aspergillus fumigatus* Af293)	Reverse transcriptase	Transposon activty
Line 4: exon integration in eu2.Lbscf009g01110 (9:362315-364169)	gi|61860534|ref|ZP_585369.1|PREDICTED: similar to zinc finger, MYND domain containing 17 (*Bos taurus*)	Predicted histone tail methylase containing SET domain	Chromatin stucture and dynamics
Line 23: exon integration in eu2.Lbscf0081g00300 (81:71160-116835)	Not known homologies	Not known	Not known
Line 24: exon integration in eu2.Lbscf0001g06630 (1:1402862-1406907)	jgi|Phchr|7351|fgenesh1_pg.C_scaffold_13000139 (*Phanerochaete chrysosporium*)	ARK protein kinase family	Signal transduction mechanisms
Line 28: exon integration in estExt_fgenesh2_pg.C_80221 (8:912830-914182)	Not known homologies	Not known	Not known
Line 33: exon integration in gwh1.41.127.1 (41:16451-318018)	jgi|Phchr1|90269|gwh2337.1 (*Phanerochaete chrysosporium*)	von Willebrand factor and related coagulation proteins	Defence mechanisms
Line 34: exon integration in eu2.Lbscf0078g00200 (78:60246-63024)	gi|6684748|gb|EAL87807.1| reversetranscriptase, RnaseH (*Aspergillus fumigatus* Af293)	Reverse transcriptase	Transposon activty
Line 35: exon integration in eu2.Lbscf0003g5410 (3:1759882-1763683)	jgi|Phchr|8500|fgenesh1_pg.C_scaffold_17000065 (*Phanerochaete chrysosporium*)	Uncharacterized conserved protein	Unknown
Line 36: intron integration in eu2.Lbscf0095g00140 (95:28724-30106)	jgi|Phchr1|4314|fgenesh1_pg.C_scaffold_7000127 (*Phanerochaete chrysosporium*)	Na+/dicarboxylate, Na+/tricarboxylate and phosphate transporter	Inorganic ion transport and metabolism
Line 39: intron integration in estExt_fgenesh2_pg.C_90079 (9:331027-334257)	gi|2493240|sp|O10341|Y091_NPVOP Hypothetical 29.3 kDa protein(ORF92) (*Orgyia pseudotsugata* MNPV	von Willebrand factor and related coagulation proteins	Defence mechanism
Line 43*: exon integration in Eugenev2.0000270017 (27:107609-108702) or eu2.Lbscf0330g00010 (330:1-1345)	Not known homologies	Not known	Not known
Line 51: exon integration in fgensh3_pg.C_scaffold_7000288 (7:977692-980017)	jgi|Phchr1|8345|fgenesh_pg.C_scaffold16000127 (*Phanerochaete chrysosporium*)	Not known	Not known
Fungal lines with upstream integrations (genomic site)	Best homology hit	Predicted or known functions	Cellular functional group
Line 11 fgensh3_pg.scaffold_54000052 (54:228552-229443)	No know homologies	Not known	Not known
Line 12 eu2.Lbscf0014g03590 (14:1013226-1013872)	No known homologies	Not known	Not known
Line 49 eu2.Lbscf0068g00660 (68:176274-178292)	jgi|Phchr1|4876|fgenesh1_pg.C_scaffold_8000051 (*Phanerochaete chrysosporium*)	Hypothetical protein	Not known
Fungal lines with downstream integrations (genomic site)	Best homology hit	Predicted of known functions	Cellular functional group
Line 16 estExt_fgenesh2_pg.C_120134 (12:426088-427106)	gi|27805755|sp|O74196|UBC1_COLGL Ubiquitin-conjugating enzyme E2-16kDa (Ubiquitin-protein ligase)(Ubiquitin carrier protein) (*Colletotrichum* hard-surface-induced protein1) (*Gloerenella cingulata*)	Ubiquitin protein-ligase	Post-transcriptional modification, protein turnover, chaperone
Line 18 Eugenv2.0000100099 (10:228001-231240	spo:SPAC27D7.03c mei2; mei2 protein (*Schizosaccharomyces pombe*)	RNA-binding	Control of meiosis

Table 1. 18 T-strand integration sites in *Laccaria* genome located in ORFs and their upstream or downstream elements.
* Line 43 sequence is presented twice in *Laccaria* genome in two different scaffolds

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pCAMBIA1300 TATCAGTGTTTGA ..............
        16B TATCAGTGTTTGA GTTTCG
        11A TATCAGTGTTTGA TGCATT
        17A TATCAGTGTTTGA GGGAG
        24A TATCAGTGTTTGA TTATGA
        27B TATCAGTGTTTGA ATTATT
        33A TATCAGTGTTTGA GCTGAT
        43A TATCAGTGTTTGA TGCGAT
        47A TATCAGTGTTTGA GCCCCG
        49B TATCAGTGTTTGA TAATAC
         4E TATCAGTGTT t g a AAGTCT
        23A TATCAGTGT t t g a CTTGGG
        12A TATGACTGTTTG a GTCGAA
        28A TATCAGTGTTTG a CTCGTA
        29A TATCAGTGTTTG a GACAGG
        34A TATCAGTGTTTG a GTGTAT
         2A TATCAGTGTTTG G CATTCT
        44A TATCAGTGTTTG G ATGGC
         3A TATCAGTGTTTC CCTGACT
        38A TATCAGTGTT t A CACCAGA
        35A TATCAGTGTT AGCATCCCC
        36A TATCAGTGTT AGATCCTTG
        50A TATCAGTGTT AACCAGACA
        39A TATCAGTGTt GGCCATGCC
        51C TATCAGTG AAGGGTGCATT
        18A TATCAGTCTTCCCGCGCCTT
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Fig. 2. Right border conservation of 25 *Agrobacterium* transformed *Laccaria* lines. The pCAMBIA 1300 sequence is presented till the RB nick site.
Letters in italics represent bases that could orginate from both RB or genomic DNA.

GFP expression in *Laccaria bicolor*

Laccaria hyphae show autofluorescence when aged or grown on solid media. In liquid culture this autofluorescence is remarkable lower and expression of GFP protein can be evaluated. We have been able to produce cytosolic GFP expressing monokaryotic (C11-H82) *Laccaria* lines (Photo panel 1) with the binary vector pBGgHg (Chen *et al.* 2000). In addition, the lines showing fluorescence in liquid cultures grew on solid media as more compact, greenish coloured colonies (data not shown). Not all the hyphae seem to express the transgene and frequently the expressing hyphae show an altered morphology (thick and branchy) proposing a toxic effect of the GFP protein in *Laccaria*. Moreover, the GFP expression tends to shut down in fungal lines when subcultured, limiting though the long term use of this marker. If this lost of expression is due to inactivation or elimination of the GFP-transgene is not known yet.

We have also studied localized GFP expression in the monokaryotic strain C11-H82 by using the mitochondrial localization signal of the citrate synthase gene of *A. niger* (plasmid pRS54, Suelmann & Fischer 1999). A localized fluerescence signal was effectively detected within the hyphae of transformed *Laccaria* lines (Photo panel 2). As seen with cytosolic GFP expression not all the hyphae showed this signal and the number of expressing hyphae could not be increased by subculturing the lines with elevated concentrations of selective antibiotic. More remarkably, lines with localized GFP signal showed clear signs of the toxic effect of this protein in *Laccaria*. The growth rate of GFP expressing lines was strongly reduced compared to non-expressing transformants and this was gradually reversed when GFP expression was lost (like already seen in the case of cytosolic GFP expression). This lost of expression was permanent and could not be regained with a cold treatment indicating that it is probably not methylation related.

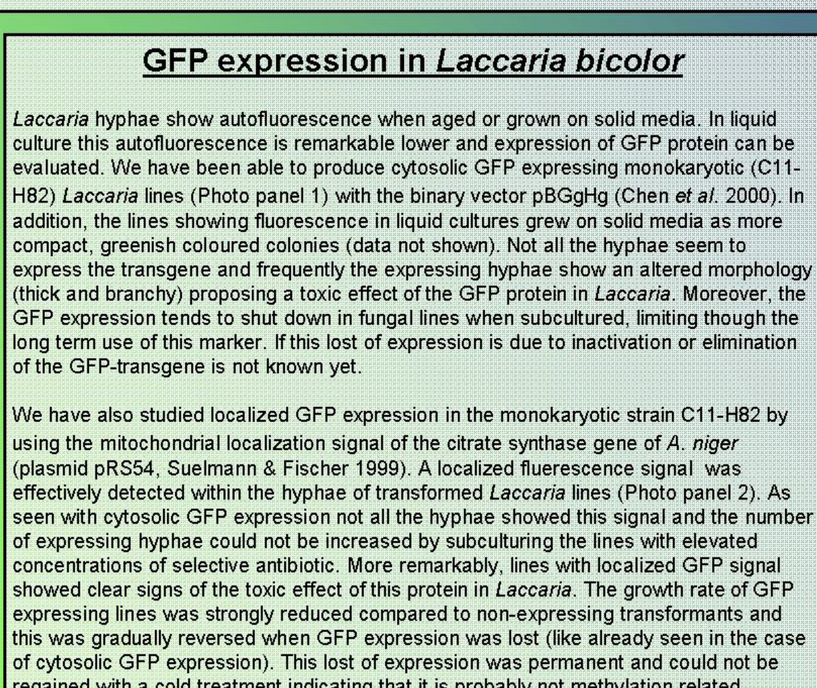

Photo panel 1. Cytosolic expression of GFP in *Laccaria bicolor* C11-H82 grown in liquid medium. Wild type: photos A-B, pBGgHg transformed lines showing green fluorescence: photos C-I. Magnification 1000x.

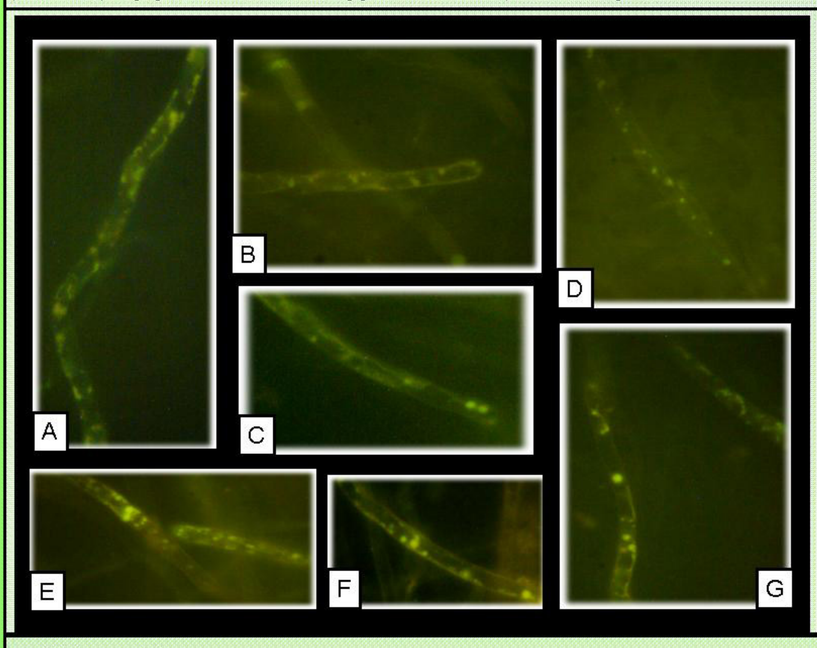

Photo panel 2. Localized GFP expression in *Laccaria bicolor* C11-H82 grown in liquid medium (A-G). Mitochondrial localization signal: *A.niger* citrate synthetase 5'- region. Magnification 1000x.

Conclusions

We have studied the use of different heterologous fungal promoters in *Laccaria* transformation. Among them the Pgpd from *A. bisporus* and *S. commune*, and nitrate transporter promoter from *T. borchii* were shown to be functional. The promoters from *Aspergillus*, even though used with other tranformation methods in *Laccaria*, are probably not strong enough for AMT of this fungus which produced mostly a single copy integration of the transgene. We have demonstrated that the plasmid rescue method can be used for resolving T-DNA integration sites in *Laccaria.* Our integration site analysis showed no clear sequence homology between different sites or the sites and the T-strand border sequences indicating non-homologous and random integration of the transgenes in this organism. Majority (74%) of integrations were located in potentially transcribed sites (interrupted ORFs or their upstream and downstream elements). Our results demonstrate that *Agrobacterium*- mediated transformation can be a powerful tool for gene validation in *Laccaria*. The appearance of two interrupted transposomal protein coding genes and integrations in repetitive sequences in our data set may suggest an elevated tendency of integration in sites rich in transposons or zones under active DNA repair in *Laccaria* genome as proposed for *Saccharomyces* (Bundock *et al* . 2002). We have also demostrated that cytosolic and organelle localized GFP expression can be established in *Laccaria*. However our findings indicate a toxic effect of this protein when constitutively expressed limiting its use to short term studies or with an inducible promoter. Currently we are evaluating the requirements for directed gene disruption and RNA interference by *Agrobacterium* –mediated tranformation in *L. bicolor.*

References: *Bundock *et al.* (2002) Yeast 19:529-536 *Chen X *et al.* (2000) Appl Environ Microbiol 66:4510-4513 *Kemppainen M *et al.* (2005) Mycorrhiza 16:19-22 *Suelmann R & Fisher R (2000) Cell Motil Cytoskeleton 45:42-50