2 Functional Genomic Approaches for Mycorrhizal Research

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2.1 Introduction

Mycorrhizal fungi are important and significant biological components of the rhizosphere. These fungi interact with the roots of more than 80% of land plants and form symbiotic associations called mycorrhizas or mycorrhizae (Smith and Read 1997). On the basis of the colonization pattern of host cells, two major types of mycorrhizas can be identified: ectomycorrhizas and arbuscular mycorrhizas. In the ectomycorrhizas the fungus does not penetrate the host cells, but forms a sheath around the roots and only traverses the cortical layers of the roots in the intercellular spaces, forming an interface called the "Hartig Net". However, in endomycorrhizas the fungal hyphae penetrate cells and form intracellular structures like coils or arbuscules (Smith and Read 1997). Mycorrhizal fungi provide improved access to limited soil resources such as minerals and nitrogen to the host plant. In contrast, mycorrhizal fungi receive carbon compounds from host plants to sustain their metabolism and complete the life cycle and also receive protection from other microbes in the rhizosphere.

While the ecology and physiology of mycorrhizal fungi and their uses is well studied, knowledge about cellular and molecular aspects leading to the growth and the development of a mycorrhizal fungi as well as the establishment of a functioning symbiosis is still limited (Harrison 1999; Martin et al. 2001; Podila et al. 2002; Duplessis et al. 2005; Wright et al. 2005). The development of molecular techniques and the recent progress made in the first sequencing of mycorrhizal genomes (Martin et al. 2004) has made it possible to begin to ask important biological questions on the development of symbiotic interactions and the formation of mycorrhizae.

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An appropriate approach to the study of mycorrhizal fungi is to understand the molecular process leading to the host recognition, development and functioning of mycorrhizae through the analysis of expressed genes. With the advent of many high throughput techniques that have been successfully applied to the functional analysis of genes from many organisms, it is now possible to apply similar strategies to study the various aspects of the mycorrhizal symbiosis. In this chapter, we describe protocols applied to study of ectomycorrhizal symbiosis leading to: (1) Yeast two-hybrid methods to determine the interactions of signaling proteins and signaling cascades and (2) transformation system for gene replacement and functional genomic analysis of ectomycorrhizal symbiosis.

2.2 Yeast Two Hybrid: An Approach for Understanding Signaling Pathways

Ectomycorrhiza encompasses a series of complex and overlapping ontogenic process in symbionts, which includes the switching-off of fungal growth mode, initiation of lateral roots, aggregation of hyphae, arrest of cell division in ensheathed roots, and radial elongation of epidermal cells (Feugey et al. 1999). Early events in the interactions are crucial and result in the activation of a cascade of molecular events in each partner. Understanding the process involved during the interaction of plant root with fungal mycelium might provide an insight into the highly complex developmental process of mycorrhiza formation. One of the key points is to study the early induction of signaling genes, which are activated when they perceive signals from each other, leading to their recognition. This process further determines the symbiotic compatibility of fungus towards plant host.

There have been considerable reports of signaling genes and gene response events between the two partners during ectomycorrhizal symbiosis (Barker et al. 1998; Kim et al. 1998; Martin and Tagu 1999; Tagu et al. 2000; Martin et al. 2001; Sundaram et al. 2001). One of the ways to understand the signaling process is to study interactions between the early-induced signaling genes. Novel interacting genes coding for proteins can be screened using signaling genes as bait. For example, cloning and studying the regulation of G protein-coupled receptors (GPCRs) and RAS, which are early induced during the interaction phase, might help in providing some insight into ectomycorrhizal symbiosis. Utilizing yeast two hybrid in many other systems led to the identification of many genes coding for interacting proteins (Fields and Song 1989; Chein et al. 1991; Bartel et al. 1993; Fields 1993; Bendixen et al. 1994; Fields and Strenglanz 1994; Hao et al. 1999) and such a technique has proven to be quite useful. In the yeast two-hybrid assay two fusion proteins are created: the protein of interest "X" which is constructed to have a DNA binding domain attached to its N-terminus, and its potential binding partner "Y" which is fused to an activation domain. If protein

X interacts with protein Y, the binding of these two forms an intact and functional transcriptional activator (Fields and Song 1989). This newly formed transcriptional activator then goes on to transcribe a reporter gene, which is simply a gene whose protein product can be easily detected and measured. In this way, the amount of the reporter produced can be used as a measure of interaction between our protein of interest and its potential partner (Fig. 2.1).

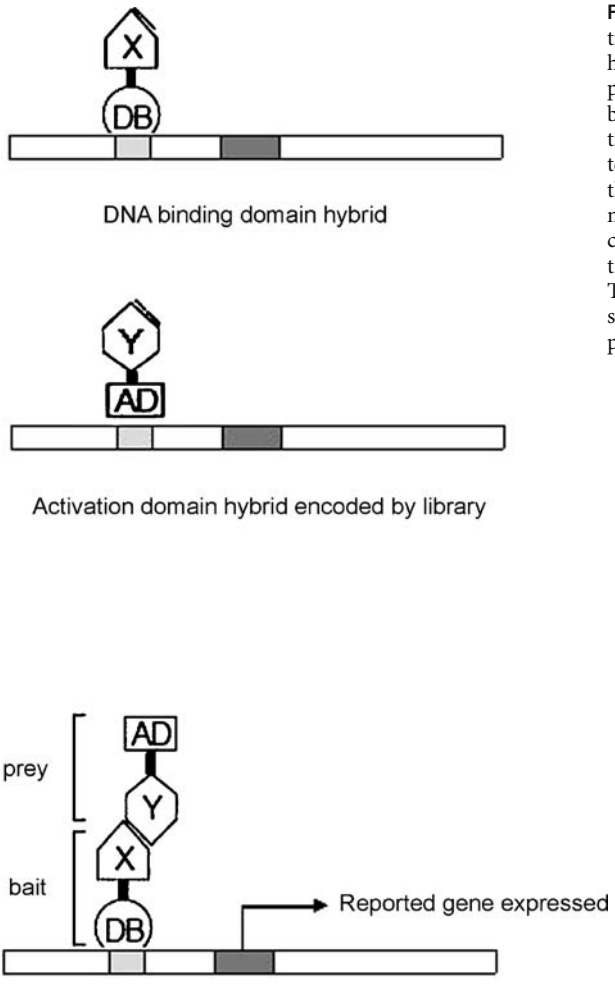

Fig. 2.1 Yeast two-hybrid transcription. The yeast two-hybrid technique measures protein–protein interactions by measuring the transcription of a reporter gene. If protein X and protein Y interact, then their DNA binding domain and activation domain combine to form a functional transcriptional activator (TA). The TA then proceeds to transcribe the reporter gene that is paired with its promoter

The *LbRas* gene cloned from *Laccaria bicolor* has been shown to be regulated during the early stage of the fungal ectomycorrhizal interaction with *Pinus resinosa* (Sundaram et al. 2001). The RAS gene is also expressed in mycorrhizal tissue when compared with free-living fungal mycelium. Such differential expression clearly suggests that *LbRas* plays a key role during ectomycorrhiza formation. Using *LbRas* as a bait and performing yeast two-hybrid interactions with tissue from early stages of *L. bicolor–P. resinosa* led to the isolation of a novel line of Ras-interacting yeast two-hybrid ectomycorrhizal clones (*Rhythm*; Sundaram et al. 2004). One of the important Ras interacting clones showed considerable sequence homology to other eukaryotic clones coding for an AP180-like protein (RhythmA; ~50%). The predicted amino acid sequence of RhythmA shows the presence of an Asn-Pro-Phe (NPF) motif, which is characteristic of all known AP180 proteins (De Camilli et al. 1996; Paoluzi et al. 1998). NPF motifs have been shown to be involved in protein–protein interactions (Paoluzi et al. 1998). AP180 proteins have been shown to play roles in the assembly of clathrin-coated vesicles through protein–protein interactions (Hao et al. 1999). Previously it has been shown the AP180 is also involved in cargo sorting in coated vesicles through its interaction with GTPase (De Camilli et al. 1996). Since establishment of mycorrhizal association is also related to exchange of signals, ligands, and nutrient, one could observe such turnover of vesicles and vesicular trafficking during ectomycorrhizal phenomena. Such regulations were previously reported during the interaction of *L. bicolor–P. resinosa* (Kim et al. 1999).

Similarly performing yeast two-hybrid analysis of pre-infection stage interactions of *L. bicolor* with aspen (*Populus tremuloides*) seedlings led to the isolation of other RAS interacting clones (Table 2.1). Screening the cDNA library prepared from the interaction of *L. bicolor* with *P. tremuloides* led to the isolation of some important RAS interacting clones, including a GPCR, cyclophilin, vacuolar protein sorting (VPR), and biogenesis related protein.

GPCR proteins are a very important group of genes and are one of the largest protein families in human and other animal genomes (Pin et al. 2005). However, in most fungal genomes, the number of GPCRs identified is very low (Kulkarni

Table 2.1 Interacting partners of Lbras. Yeast two-hybrid interactions were performed with tissue from early stages of *L. bicolor–P. tremuloides* using *LbRas* as bait. The table describes some of the interacting clones and their potential function

Description	Putative role
Cyclophilin (peptidylprolyl isomerase-like 2 isoform)	Phosphorylation and dephosphorylation process
Vacuolar protein sorting and organization and biogenesis related protein	Transport, signaling
Cullin-1	Protein targeting, processing, and degradation
GPCR – STE3 like	Receptor and signaling

et al. 2005). GPCRs are involved in a variety of signaling mechanisms of various organisms and are known to be associated with critical biological functions. In fungi, GPCRs have been shown to be involved in signaling (Riquelme et al. 2005), including pheromone signaling, receptors involved in host recognition (Marsh and Herskowitz 1988), and pathogenicity (Kulkarni et al. 2005).

Cyclophilin have peptidyl-prolyl *cis-trans* isomerase (PPIase) and are chaperones and folding catalysts with the ability to catalyze the *cis-trans* isomerization of prolyl bonds, a rate-limiting step in protein folding. Cyclophilin were shown to be involved in different phosphorylation processes of RAF/RAS, thus regulating the signaling events (Dougherty et al. 2005). Northern analysis of cyclophilin showed they are induced at the very early stages of interaction and are present until the time of interaction (Fig. 2.2).

Another Lbras interacting clone showed a high match with VPS. Vacuolar protein sorting genes encode proteins that are involved in protein sorting to the vacuole. A region of vacuolar protein sorting Vps9p from yeast is related to human proteins (Rin1, JC265) that were shown to negatively regulate Ras-mediating signaling in *S. cerevisiae* (Han and Colicelli 1995). In fact Rin1 has been shown to bind directly to RAS in a manner that competes with the binding of RAF (a downstream effector of RAS). This suggests that an effector domain of RAS is a principal binding site for Rin1 and that Rin1 may act as a downstream effector of RAS. There have been previous reports of vesicular traffic protein regulation during ectomycorrhizal interaction and vesicular turnover in ectomycorrhiza (Cole et al. 1998; Kim et al. 1999). Though such functional evidence is lacking to show their role during ectomycorrhiza formation, the involvement of vacuolar sorting proteins in signaling and transport of signals/information cannot be ruled out.

Cloning of the signaling genes like GPCRs, Raf, and Rho and testing their intensity of interaction with each other to build a protein–protein interaction network model could provide us evidence during the upstream and downstream signaling process in ectomycorrhiza formation.

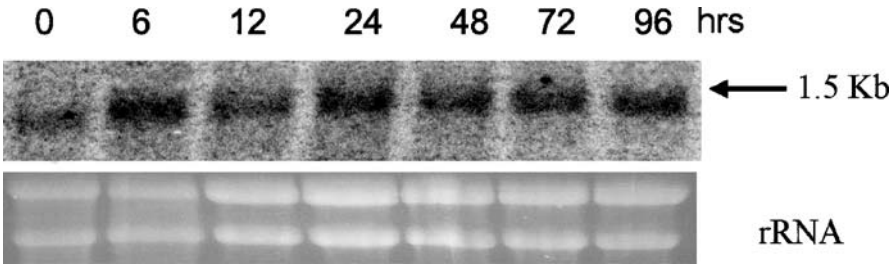

Fig. 2.2 Temporal regulation of mRNA expression for cyclophilin-like protein during the early stages of interaction between *L. bicolor* and *P. tremuloides*. Northern analysis with 10 μg of total RNA samples from *L. bicolor* subjected to interaction with *P. tremuloides* for 6, 12, 24, 48, 72, 96 h was carried out as described in the Materials and Methods. Ethidium bromide staining of rRNA was used to verify the loaded amount of total RNA

2.3 *Agrobacterium*-Mediated Transformation in *Laccaria bicolor*

Agrobacterium tumefaciens is a well known bacterium that causes crown galls on plants by transferring part of a tumor-inducing plasmid into their genomes. Such *Agrobacterium*-mediated transformation has been well developed for gene transfer in plant systems (Tinland 1996) and also in variety of other organisms like yeast, filamentous fungi, and human cells (Bundock et al. 1995; de Groot et al. 1998; Kunik et al. 2001). To date *Agrobacterium*-mediated transformation has been reported in many fungal species, like *Botrytis cinerea*, *Aspergillus awamori*, *Magnaporthe grisea*, *Fusarium oxysporum* (Gouka et al. 1999; Rolland et al. 2003; Khang et al. 2005). Methods of molecular and genetic analysis have progressed more slowly for ectomycorrhizal fungi than for higher fungi. Still, successful transformation has been reported in *Suillus bovinus*, *Agaricus bisporus*, *Paxillus involutus*, *Hebeloma cylindrosporum*, and *Laccaria bicolor* (Pardo et al. 2002, 2005; Hanif et al. 2002; Combier et al. 2003). But the functional aspects and utilization of such a technique for insertional mutagenesis or genetic transformation are largely missing.

Recently, *Agrobacterium*-mediated transformation was used for insertional mutagenesis in the symbiotic ectomycorrhizal fungus *Hebeloma cylindrosporum* (Combier et al. 2003). Though there can be single or multiple insertions, the frequency of single insertions can be controlled by treating bacteria with acetosyringone (AS) prior to co-cultivation, an experimental condition which slightly reduces transformation efficiency. In fact, a higher percentage (60%) of single insertion was obtained in *H. cylindrosporum* using AS-treated bacteria when compared with protoplast-based transformation, which generally led to an unpredictable number of plasmid integration per genome (Marmeisse et al. 1992; Amey et al. 2002). Kemppainen et al. (2005) obtained successful transformation of *L. bicolor* S238N with an efficiency of 55% using the AGL-1 strain.

Using this transformation procedure, a non-mycorrhizal mutant of *H. cylindrosporum* was obtained (Combier et al. 2003). Further, it will be interesting to study the key regulatory steps of mycorrhizal functioning by directing precise silencing of symbiosis-regulated genes using siRNA/RNA interference technology. Though such techniques are well developed in phytopathogens (Fitzgerald et al. 2004), they are still lacking in mycorrhizal fungi.

We have used *Agrobacterium*-mediated transformation of *L. bicolor* strain S238N with a vector for the selection and expression of green fluorescent protein (GFP) reporter gene (Fig. 2.3). In addition, we have also obtained gene replacement for the PF6.2 gene earlier found to be induced very early in interaction between *L. bicolor* and red pine (Kim et al. 1998). This is the first instance of gene replacement in mycorrhizal fungi. Figure 2.4 shows the replacement of the PF6.2 gene in *L. bicolor* transformants. One of these transformants has been tested in its ability to form mycorrhizae on *P. tremuloides* seedlings. The trans-

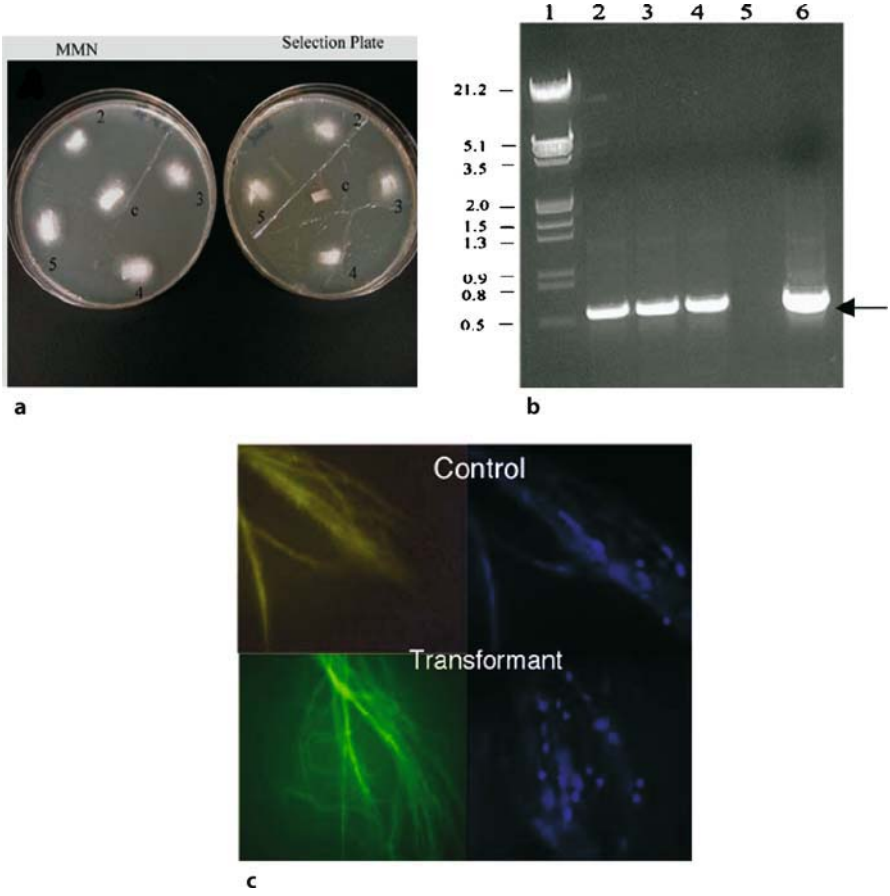

Fig. 2.3 *Agrobacterium*-mediated transformation of *L. bicolor* and expression of selection marker and reporter gene GFP. Panel **a** shows *L. bicolor* wild type (*C*) and transformants grown on non-selective MMN medium (*left dish*). The *right dish* shows the selection of transformants on 300 μg/ml hygromycin. Panel **b** shows colony PCR of transformants selected on hygromycin. The *arrow* points to the GFP PCR product. *Lane 1* DNA molecular weight marker, *lanes 2, 3, 4* transformant DNA samples, *lane 5* blank, *lane 6* positive control from pBGgHg plasmid. Panel **c** shows the expression of GFP protein in the transformants and the DAPI staining of nuclei, compared with a non-transformed control *L. bicolor*

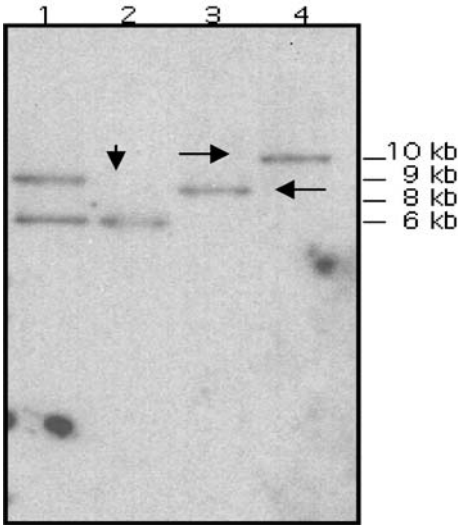

Fig. 2.4 Southern analysis of PF6.2 gene displacement in *L. bicolor*. Genomic DNA samples (10 μg each) from *L. bicolor* were digested with *Bam*H1. Hybridization was done with a ^{32}P-labeled PF6.2 cDNA fragment as described by Kim et al. (1998). Molecular weight markers are indicated in kilobasepairs. *Lane 1* Wild-type *L. bicolor*, *lanes 2–4 L. bicolor* transformants 1, 2, 3, respectively. *Arrows* point to the deletion of one copy of PF6.2 in transformant 1 and displacement in transformants 3 and 4

formant was able to form mycorrhizal roots, but was defective in stopping the formation of root hairs on mycorrhizal roots (Fig. 2.5), which is a common feature under normal conditions. This suggests that the displacement in PF6.2 in the *L. bicolor* genome and reduction in its copy number impacted the symbiosis process. These results also corroborate the earlier hypothesis that PF6.2 from *L. bicolor* may be involved in a signaling process (Kim et al. 1998). Thus, the *Agrobacterium*-mediated gene transformation methods open up the possibility of using gene silencing or ectopic expression techniques in mycorrhizal fungi to study the process of symbiosis.

2.4 Materials and Methods

2.4.1 Interaction Studies of *Laccaria bicolor* with Aspen (*Populus tremuloides*) Seedlings

To construct the cDNA library of *L. bicolor* undergoing interaction with *P. tremuloides*, aspen seeds were incubated overnight at 4 °C and were surface-sterilized using 10% hydrogen peroxide. The sterilized seeds were transferred to Petri dishes (diam. 75 mm) containing woody plant medium (WPM) agar (Sigma, Mo., USA) and was incubated for 1 week at 25 °C. Five seedlings of aspen were

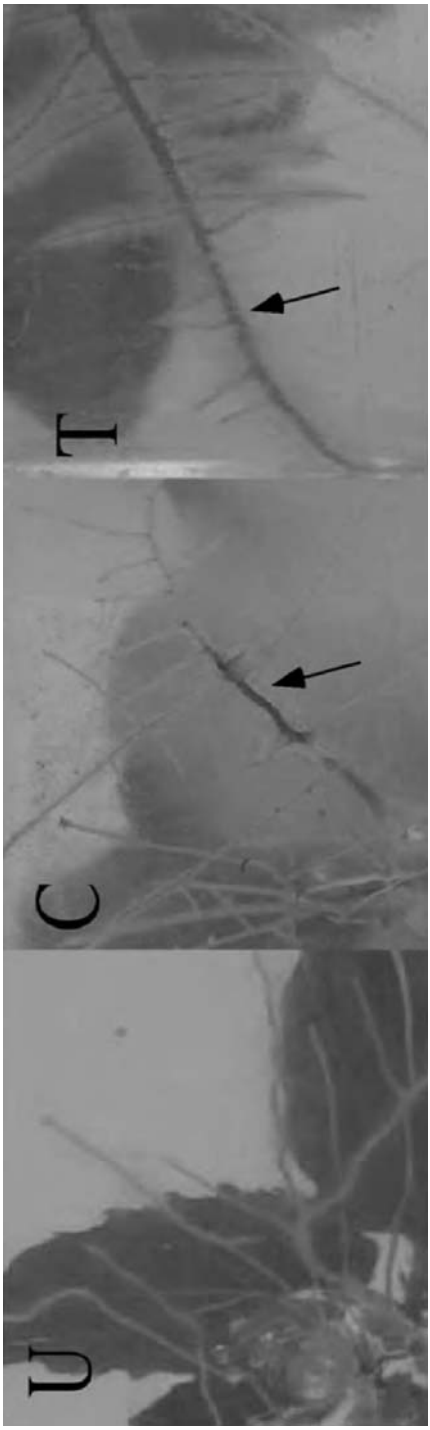

Fig. 2.5 Phenotypic changes in the mycorrhizae formed by *L. bicolor* PF6.2 transformant. **U** Un-inoculated roots. **C** Roots inoculated with control *L. bicolor*. **T** Roots inoculated with transformant 3 showing mantle formation but no loss of root hairs. The loss of root hairs is a hallmark of ectomycorrhizal development

transferred to each magenta box containing WMP media overlaid with a cellophane sheet. The seedlings were incubated in a growth chamber with a cycle of 16 h light and 8 h dark at 25 °C for 4–5 weeks. The *L. bicolor* culture was maintained on MMN medium (Podila et al. 2002) in MMN-filled Petri dishes (diam. 150 mm) at 22 °C. The fungal culture for inoculation of aspen roots was grown from agar plugs of mycelium placed on cellophane-covered MMN medium. Mycelial strips of approximately 15×5 mm were excised from the edge of the culture. Strips were then placed on the cellophane just above the root and were grown for different time intervals (viz. 0, 6, 12, 24, 48, 72, 96 h). This allowed for diffusion of root signals, but prevented physical contact between the roots and the mycelium to study the gene expression before physical contact was made between the fungus and the roots.

2.4.2 Yeast Two-Hybrid Protocol

The yeast two-hybrid experiment was performed using BD Matchmaker library construction and screening kits and protocols (BD Biosciences, Clontech, Calif., USA).

2.4.2.1 cDNA Synthesis and Bait Construction

All RNA samples were treated with RNase-free DNase at 37 °C for 30 min using the DNA-free kit (Ambion, Austin, Tex.) prior to cDNA synthesis, to ensure that the amplicon template originated from RNA and not DNA. Two micrograms of DNA-free RNA was used for first-strand cDNA synthesis for all samples belonging to interaction time points, carried out simultaneously using the BD Smart cDNA synthesis kit. *Lbras* was used to construct a DNA-BD fusion vector using a BD-cloning vector (pGBKT7). The GAL4AD fusion library was constructed using vector pGADT7-Rec and the constructed interaction library. The GAL4AD fusion library samples along with the bait (BD vector) were co-transformed in yeast strain AH109 (as described in the BD Biosciences protocol).

2.4.2.2 Preparation of Competent Yeast Cells – LiAc Method

1. Inoculate fresh yeast strain AH109 (<4 weeks old, 2–3 mm diam.) into 3 ml of YPDA medium and incubate at 30 °C with shaking for 8 h.

2. Transfer 5 µl of the culture to a 250-ml flask containing 50 ml of YPDA. Incubate at 30 °C with shaking at 230–250 rpm for 16–20 h. The OD_{600} should reach 0.15–0.2.
3. Centrifuge the cells at 700 *g* for 5 min at room temperature. Discard the supernatant and resuspend the cell pellet in 100 ml of YPDA.
4. Incubate at 30 °C for 3–5 h (OD_{600} = 0.4–0.5). Centrifuge the cells at 700 *g* for 5 min at room temperature.
5. Discard the supernatant and resuspend the cell pellet in 60 ml of sterile, deionized H_2O. Centrifuge the cells at 700 *g* for 5 min at room temperature.
6. Discard the supernatant and resuspend the cells in 3 ml of 1.1× TE/LiAc solution.
7. Divide the resuspension between two 1.5-ml microcentrifuge tubes (1.5 ml per tube).
8. Centrifuge each tube at high speed for 15 s. Discard the supernatant and resuspend each pellet in 600 µl of 1.1× TE/LiAc solution.

Competent cells should be used for transformation immediately following preparation; however, if necessary they can be stored at room temperature for a few hours without significantly affecting the competency.

2.4.2.3
Transformation of Yeast Strain AH109 with dscDNA and pGADT7-Rec

1. In a sterile, prechilled, 15-ml tube combine the following: 20 µl dscDNA (from protocol Section IX.I, step 16), 6 µl pGADT7-Rec (0.5 µg/µl), 5 µg GBKT7/bait plasmid DNA, 20 µl herring testes carrier DNA, denatured*. (* Transfer ~50 µl of herring DNA to a microcentrifuge tube and heat at 100 °C for 5 min. Then, immediately chill the DNA by placing the tube in an ice bath. Repeat once more before adding the DNA to the 15-ml reaction tube.)
2. Add 600 µl of competent cells to the DNA. Gently mix by vortexing. Add 2.5 ml PEG/LiAc Solution. Gently mix by vortexing. Incubate at 30 °C for 45 min. Mix cells every 15 min.
3. Add 160 µl DMSO, mix, and then place the tube in a 42 °C water bath for 20 min. Mix cells every 10 min. Centrifuge at 700×*g* for 5 min.
4. Discard the supernatant and resuspend in 3 ml of YPD plus liquid medium.
5. Incubate at 30 °C with shaking for 90 min. Centrifuge at 700×*g* for 5 min. Discard the supernatant and resuspend in 6 ml of NaCl solution (0.9%).
6. Spread the co-transformation mixture on selection media. Transformants expressing interacting proteins were selected on triple dropout medium: SD/–His/–Leu/–Trp and quadruple dropout medium: SD/–Ade/–His/–Leu/–Trp.

Colonies become visible after 2–3 days, but plates should be incubated 5 days to allow slower growing colonies to appear.

To identify the gene responsible for a positive two-hybrid interaction, rescue the gene by plasmid isolation or by PCR colony-screening.

Further, AD/library cDNA insert can be sequenced using the AD LD-insert screening amplimer set, a T7 sequencing primer, or the 3' AD sequencing primer provided with the BD Matchmaker two-hybrid kit (Clontech, Calif., USA).

2.4.2.4
Northern Analysis

Total RNA from *L. bicolor*, subjected to interaction with *P. tremuloides* seedling roots for 6, 12, 24, 48, 72, 96 h, respectively, was electrophoresed on formaldehyde–agarose gels and transferred to Hybond-N membranes (Amersham Pharmacia Biotech, Piscataway, N.J., USA), as described by Kim et al. (1998). Total RNA from free-living *L. bicolor* was used as control. A 10-µg sample of RNA was loaded in each lane; and gels were stained with ethidium bromide (Sigma, USA) to determine equal loadings and intensity of RNA. The cDNA fragment coding for cyclophilin-like protein was labeled with ^{32}P-dCTP with the Rediprime DNA labeling kit (Amersham Pharmacia Biotech) and used as a probe in the hybridization analyses of the membrane-bound nucleic acids, as described previously (Sambrook et al. 1989; Kim et al. 1999).

2.4.3
Agrobacterium-Mediated Transformation in *Laccaria bicolor*

2.4.3.1
Preparation of Fungal Material

Laccaria bicolor mycelium was freshly cultured on a cellophane sheet overlaid on low glucose-MMN (2% glucose) agar medium at 22 °C for 1 week, as described by Balasubramanian et al. (2002).

2.4.3.2
Induction of *Agrobacterium*

Agrobacterium tumefaciens strain AGL1 containing plasmid pBGgHg or pBG-6.2 was grown overnight in 4 ml of minimal medium containing kanamycin at 50 µg/ml at 29 °C (until the cell density reached OD = 0.2).

Bacterial cells were collected by centrifugation (3000 *g* at 4 °C for 5 min) and resuspended in induction medium (200 μM AS plus kanamycin at 50 μg/ml) and grown for 6 h at 29 °C.

2.4.3.3
Transformation, Co-Cultivation, and Selection

After the fungal colonies reached 0.5 cm diameter, which took 7 days, the membranes with colonies were transferred to induction media plates with or without 200 μM AS.

Laccaria mycelium was then inoculated with 50 μl of induced *Agrobacterium*. The co-cultivation plates were incubated at 22 °C for 5 days in the dark.

The membrane with mycelia colonies were then transferred to MMN or Moser 6 selection plates (pH 7.5; containing antibiotics mix and hygromycin 300 μg/ml). The plates were incubated at 4 °C overnight and then shifted to 22 °C for 10 days in the dark.

After 2 weeks, the growing colonies were repeatedly subcultured on the selection plates containing antibiotics and tested to make sure no residual agrobacteria were present.

DNA isolation was performed from the putative transformant colonies growing on cellophane membrane using the methods described by Kim et al. (1998). The positive transformants were selected using primers which specifically amplify hygromycin (*hph*) or modified EGFP gene.

Further analyses of T-DNA integration were done by Southern analysis, as described by Kim et al. (1998).

1. Minimal medium: K_2HPO_4 10.5 g, KH_2PO_4 4.0 g, $(NH_4)_2SO_4$ 1.0 g, Na_3-citrate $2H_2O$ 0.5 g, $MgSO_4.7H_2O$ 0.2 g, thiamine-HCl 1.0 mg, glucose 2.0 g, plus 50 μg/ml kanamycin.
2. Induction medium: minimal medium, plus 40 mM MES and 0.5% glycerol, pH 5.3.
3. Induction agar: induction medium, plus 2% agar.
4. Antibiotics mix: cefotaxime (Sigma, USA) 100 μg/ml, ampicillin (Amresco, USA) 100 μg/ml, tetracyclin (Amresco, USA) 125 μg/ml, hygromycin (Roche, USA) 300 μg/ml.

2.4.3.4
Fungal DAPI Staining and Visualization of GFP Expression

Actively growing fungal hyphae from the edges of the colony were collected and transferred on the slides using a sterile needle or the forceps. Using the blunt end of forceps the cover slip was tapped gently to spread the mycelia uniformly.

Fungal mycelia were mounted in Vectashield plus 4,6-diamidino-2-phenylindole (DAPI) as per the manufacturer's instructions (Molecular Probes, USA). Hyphae were observed under a Nikon E600 microscope equipped with a Qi-Cam digital camera (Q Imaging, USA).

The GFP fluorescence was observed under the Nikon E600 microscope. The filters used were B2A (excitation filter wavelength: 450–490 nm) for green fluorescence and UV-2A (excitation filter wavelength: 330–380 nm) for DAPI stain at 100× magnification.

2.4.3.5
Fungal DNA PCR

DNA was extracted from the mycelia actively growing on selection medium containing hygromycin.

DNA was diluted to 10% and hot start PCR was performed using EGFP primers (Clontech, Calif., USA).

PCR consisted of 30 cycles of amplification on an Eppendorf Mastercycler gradient PCR machine. Each cycle consisted of 1 min of melting at 94 °C, 30 s of annealing at 55 °C, and 1 min of extension at 72 °C. Prior to the first cycle, the samples were heated to 94 °C for 3 min. The last cycle was followed by a final extension at 72 °C for 5 min.

Amplification products were detected by electrophoresis on 1.2 % agarose gels that were stained with ethidium bromide and were visualized with a UV trans-illuminator. The identity of PCR products was further confirmed by DNA sequence analysis.

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