Isolation and Characterization of a Symbiosis-Regulated *ras* from the Ectomycorrhizal Fungus *Laccaria bicolor*

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Ectomycorrhizae formed by the symbiotic interaction between ectomycorrhizal fungi and plant roots play a key role in maintaining and improving the health of a wide range of plants. Mycorrhizal initiation, development, and functional maintenance involve morphological changes that are mediated by activation and suppression of several fungal and plant genes. We identified a gene, *Lbras*, in the ectomycorrhizal fungus *Laccaria bicolor* that belongs to the *ras* family of genes, which has been shown in other systems to be associated with signaling pathways controlling cell growth and proliferation. The *Lbras* cDNA complemented *ras2* function in *Saccharomyces cerevisiae* and had the ability to transform mammalian cells. Expression of *Lbras*, present as a single copy in the genome, was dependent upon interaction with host roots. Northern analysis showed that expression was detectable in *L. bicolor* 48 h after interaction as well as in the established mycorrhizal tissue. Phylogenetic analysis with other Ras proteins showed that *Lbras* is related most closely to *Aras* of *Aspergillus nidulans*.

Ectomycorrhizae play a key role in maintaining and improving the health of a wide range of plants (Harley and Smith 1983). They are complex symbiotic organs that develop as a result of mutually beneficial interactions between plant roots and ectomycorrhizal fungi. In nature, this type of mutualistic symbiotic association between plant roots and fungi is vital for the health and survival of both partners (Harley and Smith 1983). Ectomycorrhizal fungi penetrate the plant root epidermis and grow intercellularly between cortical cells, forming a network of hyphae that facilitate the exchange of nutrients (Harley and Smith 1983). The plant partner absorbs water and essential nutrients such as phosphorus, nitrogen, and other minerals present in low abundance in the soil through the fun-

gal hyphal network, whereas the fungal partner benefits by obtaining its only carbon energy source in the form of carbohydrates from the plant (Duddridge et al. 1980; Harley and Smith 1983; Kim et al. 1998; Nehls et al. 1998). In addition, mycorrhizal association provides several other benefits to the plant, including increased resistance against disease and pests and the ability to endure unfavorable soil conditions (Duchense et al. 1988; Duchense 1994; Harley and Smith 1983; Marx 1969; Smith and Read 1997; Wilkins and Hodson 1989). By enhancing the utilization of nutrients, ectomycorrhizae improve plant health and vigor, thus contributing to the overall health of the ecosystem.

The formation and maintenance of functional ectomycorrhizae involve a myriad of biochemical events. These include dynamic signaling interactions that take place between the partners, leading to a recognition of each other and the establishment of a functional symbiotic organ (Gianinazzi-Pearson and Gianinazzi 1989; Harley and Smith 1983; Martin et al. 1994). Early events in the interactions are crucial and result in the activation of a cascade of molecular events in each partner. Several genes in each partner are induced and/or suppressed to facilitate the formation of a morphologically distinct symbiotic organ (Hilbert et al. 1991; Kim et al. 1998; Kim et al. 1999a; Kim et al. 1999b; Martin and Hilbert 1991; Martin et al. 1994; Tagu et al. 2000). It is certainly conceivable that genes related to cell growth and proliferation play an important role in this process. There are no reports to date, however, of identification and involvement of such genes in the formation of ectomycorrhizae.

We identified a gene in the ectomycorrhizal fungus *Laccaria bicolor* (an important ectomycorrhizal fungus because of its wide host range and aggressiveness in forming mycorrhizal associations) that appears to belong to the *ras* family of genes found in other systems. The *ras* genes, present in a wide range of eukaryotes, including mammals and yeast, are involved in a variety of cellular signaling pathways that control critical cellular events such as cell cycle progression and cell division (Boguski and McCormick 1993; Bourne et al. 1990; Ellis et al. 1981; Vojtek and Channing 1998). The genes play a central role in the signal transduction pathways that respond to diverse external stimuli, including hormones, growth factors, and other elicitors. Mutations in Ras protein have been observed in more than 30% of human tumors, which are caused by uncontrollable cell growth. In *Saccharomyces cerevisiae* and other fungi, *ras* has been implicated in regulating cell

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GenBank accession nos. used to obtain the sequence for comparisons: *Aspergillus fumigatus ras*, L42229; *Aspergillus nidulans ras*, U03023; *Candida albicans ras1*, AF177670; *Homo sapiens H-ras*, J00277; *Homo sapiens N-ras*, NM_002524; *Lbras* full length cDNA, AF034098; *Neurospora crassa ras*, U33746; *Suillus bovinus ras1*, AF250024; *Lentinus edodes ras*, D00742; *Coprinus cinereus ras*, D13295; *Saccharomyces cerevisiae ras1*, K01970; *S. cerevisiae ras2*, K01971; and *Pisolithus tinctorius ras*, AF329890.

division and proliferation (Katoaka et al. 1984; Powers et al. 1984; Som and Kolaparthi 1994). The establishment of mycorrhizae, which involves the formation of a new symbiotic organ in response to interactions between the fungus and the plant roots, is controlled by molecular events taking place in both partners. Upon recognition, each partner triggers actions that lead to fungal cell division and proliferation to form the mantle and the Hartig net. In this context, it is quite logical that a regulator such as the *ras* gene acts as a mediator in this process.

Our observations showed that the *L. bicolor ras* gene (*Lbras*) was induced during early stages of the fungal interaction with its host red pine (*Pinus resinosa*). Further characterization of this gene with the *L. bicolor* × red pine system (Kim et al. 1998; Kim et al. 1999a; Kim et al. 1999b) suggested that the *ras* gene in *L. bicolor* plays a key role in the formation and maintenance of the mycorrhizal condition. Here, we describe the isolation and analysis of *L. bicolor ras*, including its regulation during *L. bicolor* × red pine ectomycorrhizal interactions.

RESULTS

Isolation of *Lbras* cDNA.

Our earlier experiments on gene expression in *L. bicolor* with the mRNA differential display technique indicated that several cDNA fragments are expressed in the fungus in response to signals received from pine roots (Kim et al. 1999b). Sequence analysis of some of these fragments showed that an approximately 580-bp fragment was related to *ras* genes found in eukaryotic systems (data not shown). We cloned this fragment and used it as a probe to screen the *L. bicolor* cDNA library. One of the clones obtained, *Lbras*, contained an insert of approximately 1.5 kbp and appeared to represent a full-length copy of *L. bicolor ras* cDNA. The clone was converted into the phagemid form by autoexcision, and the resultant plasmid pLbras was used for further analyses.

Nucleotide sequencing showed that the insert in pLbras was 1,441 bp in length, without the poly(A) tail (GenBank accession no. AF034098). It contained a single open reading frame,

encoding a protein of 209 amino acids. The 5' and 3' untranslated regions were 403 and 411 bp, respectively.

Lbras has human p21Ras like features.

Characterization of the amino acid sequence encoded by the pLbras (*Lbras*) suggested that it was related closely to the Ras family of proteins (Fig. 1). Sequence identity to the human H-ras (p21Ras) was approximately 60%, with almost 92% identity with the *Aspergillus nidulans ras* cDNA. Conserved functional domains (Fig. 1) such as the phosphate binding loop (GXXXXG motif), the guanine specificity region (NKXD motif), Mg²⁺ binding domain (DXXG motif), and the prenyl binding CAAX box, which are observed in a wide variety of eukaryotic Ras proteins, also were present in *Lbras*. The relative distance between these motifs was identical to that found in the human p21Ras (Bourne et al. 1990; Pai et al. 1989). The sequence of *Lbras*, however, differed from that of human p21Ras in certain areas. The *Lbras* had five extra amino acids at its N terminus and, although all the amino acids shown to be involved in the GTP-binding were present, their relative positions were not the same. Mutations in GTP-binding amino acids have been shown to alter the GTP-binding pocket in the p21Ras, making it insensitive to GTPase-activating proteins, a condition that results in Ras being constitutively active and leads to the transformation of mammalian cells (Pai et al. 1989). The NKCD motif (GTP specific) and the SAK pockets shown to be involved in GTP binding in human p21Ras were separated by 36 amino acids in *Lbras* compared to 35 in the p21Ras (Pai et al. 1989). Additionally, Gly12, Gly13, and Gln61, known to be very critical in the p21Ras gene, were located at positions 17, 18, and 66, respectively, in the *Lbras*. Similarly, the Ser145–Ala146–Lys147 (SAK) pocket was shifted to position 151 to 153 in the *Lbras* (Fig. 1).

Copy number of *Lbras* gene in *L. bicolor* genome.

Southern analysis showed that the probe hybridized to a single fragment in each lane containing genomic DNA digested with restriction enzyme indicated on top of the lanes (Fig. 2). The fragments were 2 to 5 kbp. The pLbras-derived

10	20	30	40
MASKFLREYK	LVVVGGGGEG ¹	KSCLTIQLIQ	SHFVDEYDPT
50	60	70	80
IEESYRKQCV	IDDEVALLDV	LDTAG ² QEEYS	AMREQYMRTG
90	100	110	120
EGFLLVYSIT	SRQSFEEIMT	FQQQILRVKD	KDYFPIIVVG
130	140	150	160
NKCD ³ LDKERV	VSKQEGESLA	RQFGCKFIET	SAK ⁴ SRINVEN
170	180	190	190
AFYDLVREIR	RYNKEMSNPS	GFGARAPDSK	MDVSEPGESA
209			
GCCGKCIVM ⁵			

Fig. 1. Amino acid sequence deduced from the open reading frame in pLbras. The *Lbras* protein had conserved regions present in Ras proteins of other systems. The phosphate binding GXXXXG loop (1: amino acids 15–20), the Mg²⁺-binding motif DXXG (2: amino acids 62–65), and the guanine specificity region NKCD (3: amino acids 121–124) are indicated. Other conserved motifs such as SAK (involved in GTP binding; 4: amino acids 151–153) and the prenyl binding CAAX region (5: amino acids 206–209) are shown.

probe, representing an almost full-length cDNA, did not have any sites for the enzymes that were used to digest the genomic DNA. This indicates that the *Lbras* gene is present as a single copy in the *L. bicolor* genome. When the genomic Southern analysis was performed under very low stringency conditions, we could detect other faint bands (data not shown), suggesting the presence of other genes with some sequence similarity to *Lbras* in the *L. bicolor* genome.

Symbiosis regulation of *Lbras*.

Northern analysis was performed to find out the actual size of the *Lbras* mRNA and to determine whether the expression of *Lbras* mRNA was dependent upon symbiotic interaction. Equal amounts of total RNA extracted from free-living fungus and established mycorrhizal tissue were tested with the p*Lbras* probe. The probe hybridized to an approximately 1.5-kb RNA that was present in samples from the mycorrhizal tissue (Fig. 3, lane Myc). The size of this RNA (size markers not shown) was very similar to the size of the insert in the p*Lbras*. This RNA could not be detected in the RNA extracted from the free-living fungus (Fig. 3, lane Fr), indicating that *Lbras* mRNA in the free-living fungus was absent or below detectable levels.

Because the Northern analysis suggested that expression of *Lbras* mRNA in the fungus was dependent upon interaction with the roots, we investigated the temporal regulation of the *Lbras* gene during *L. bicolor* × *P. resinosa* interactions. Total RNA was extracted from fungal tissues that had interacted with roots for different periods of time and the presence of *Lbras* in them was determined by Northern analysis, as described above. RNA from the mycelium incubated in the absence of roots was used as a control. The *Lbras* mRNA was detectable only in samples from the fungus that had interacted for 48 h (Fig. 4A). This mRNA was either absent or below detectable levels in early interaction tissues and the free-living

fungus. The level of expression from interaction samples beyond 48 h, however, could not be detected easily with Northern blot. To increase sensitivity, the RNA samples were reverse transcribed and the first strands synthesized were used as template for polymerase chain reaction (PCR) with *Lbras* gene-specific primers. The resultant PCR products were subjected to a Southern blot analysis (Fig. 4B) with a *Lbras* cDNA probe. The results confirmed the Northern blot analysis data. No expression was observed when the fungus was grown under starvation conditions or with other carbon sources (data not shown), and the expression continued in 72-h interaction samples.

We performed Western blot analysis to further confirm that expression of the *Lbras* in *L. bicolor* was a response to the signals or elicitors from pine roots (Fig. 5A and B). Proteins were extracted from mycorrhizae (Myc), nonmycorrhizal roots (Root), and *L. bicolor* that was incubated in the interaction medium in the presence (24, 36, 48, and 72 h) and absence of pine roots (Fr). Purified recombinant *Lbras* was used as control (R). The *Lbras* antibody was able to detect in mycorrhizal- and red pine-interacted mycelial extracts (72 h) an approximately 26-kDa protein, which presumably was the *Lbras* (Fig. 5A, Myc lane). The size of the *Lbras* appeared to be very similar to the recombinant protein expressed in *Escherichia coli*, suggesting that the *Lbras* does not undergo significant posttranslational modifications in the fungus. The *Lbras* could not be detected in the fungus that had not interacted with the pine roots (Fig. 5A, Fr lane). Also, the pine roots themselves did not contain any protein that cross reacted with the *Lbras* antibodies (Fig. 5A, Root lane).

Immunolocalization analyses with transmission electron microscopy (TEM) showed that the *Lbras* protein is located

Fig. 2. Southern analysis of *Laccaria bicolor* genomic DNA showing the presence of a single copy of *Lbras*. Ten micrograms of genomic DNA was digested with indicated restriction enzymes, and fragments were separated by electrophoresis on 1% agarose gels. After transfer to Hybond-N nylon membranes, the fragments were hybridized to the ³²P-labeled cDNA probe (insert from p*Lbras*).

Fig. 3. Northern analysis showing the expression of *Lbras* in mycorrhizae. Twenty micrograms (each) of RNA from the free-living *Laccaria bicolor* (Fr) and *L. bicolor* × *Pinus resinosa* mycorrhizae (4 months old) (Myc) were subjected to denaturing electrophoresis on 1% agarose gels. They were transferred to Hybond-N membranes and hybridized to the ³²P-labeled cDNA probe (insert from p*Lbras*). Arrow points to the approximately 1.5-kb *Lbras* mRNA that is present in the mycorrhizal tissue but absent in the free-living fungus. Equal loading of RNA can be seen on the agarose gel picture (lower panel).

primarily near the inner surface of the fungal cell wall (Fig. 6). The immunogold particles coupled to the Lbras antibody could easily be visualized (shown by arrowhead) in fungal hyphae (H) (Fig. 6A). It appeared that the protein was bound to components inside the cell wall area (Fig. 6C). Some signal also could be seen in the cytoplasmic area (Cy) of the fungi and the intercellular region in the Hartig net area, although it was not very significant. The fungal cells that had not been treated with the primary antibody did not show significant presence of gold particles, indicating that the Lbras antibody was specific (compare Fig. 6A and C). No significant presence of gold particles was observed near the root cell wall (W) or within the root cells (data not shown), which was expected because the Western analysis had indicated the absence in these samples of proteins cross reacting with the antibody. Fungal cells in the mantle area (Fig. 6D) as well as in the Hartig net (Fig. 6A and B) were expressing Lbras because immunogold signal was noticeable in both of these regions.

Lbras is functionally homologous to yeast and mammalian Ras proteins.

In order to test whether Lbras functions as a Ras protein, we performed complementation studies with the *S. cerevisiae* mutant SR843. This mutant strain requires for its survival a functional *S. cerevisiae ras2* gene that is provided through plasmid pRS316RAS2. The latter also contains a Ura3 marker and, therefore, the mutant strain cannot grow in a medium containing 5-fluoro orotic acid (5-FOA; see below) (Chen and Powers 1995). Our transformations were designed to replace the pRS316RAS2 with the input plasmids by selecting trans-

formants on 5-FOA containing media. Under these conditions, only transformants devoid of pRS316RAS2 and containing a plasmid encoding a functional *S. cerevisiae ras2* gene can grow. We transformed SR843 with YEp13RAS2 (a construct containing yeast *ras2* without the Ura3 marker) and a construct containing *Lbras*-coding sequences. As expected, the untransformed SR843 did not grow in the medium containing 5-FOA (Fig. 7). It regained its ability to grow in the presence of 5-FOA after transformation with YEP13RAS2. The SR843 strain, after transformation with the *Lbras* sequence, also was able to grow under these conditions, suggesting that the *Lbras* and *S. cerevisiae ras2* were functionally similar. Western blot analysis with anti-Lbras antiserum on proteins extracted from these transformants showed the presence of the Ras-related protein (data not shown).

Because the Lbras differed in its structure from the human p21Ras in regions associated with the normal functioning of the p21Ras protein, we tested the ability of Lbras to elicit oncogenic response to transform mammalian cells (Fig. 8). The mouse embryonic fibroblast (MEF) cells were transfected with Lbras-encoding constructs that also contained sequences coding for a green fluorescent protein (EGFP), which was included to facilitate cell sorting and analysis of recombinants. The MEF cells transfected with the plasmid construct that had only EGFP sequences were used as controls. The control cells were grown on cover slips for live imaging and visualized with a phase contrast microscope (Fig. 8A) and an inverted confocal microscope (Fig. 8B). The growth of control cells was normal. The cells maintained the typical elongated shape and grew in monolayers. The Lbras-containing mouse cells,

Fig. 4. Temporal regulation of Lbras expression during early stages of *Laccaria bicolor*–*Pinus resinosa* interactions. **A**, Northern analysis with RNA samples from *L. bicolor* subjected to interaction with red pine roots for 24, 36, and 48 h was carried out as described (Fig. 3). RNA from *L. bicolor* incubated for 48 h in the absence of pine roots was used as control (Fr). Presence of the approximately 1.5-kb Lbras mRNA was detectable only after interaction with the roots for 48 h. **B**, 50 ng of RNA from each of the samples was used for Northern analysis as template for reverse transcription. A polymerase chain reaction (PCR) with *Lbras* gene-specific primers was performed on the first strands synthesized, and the resultant products were subjected to Southern blot analysis (Fig. 2). Presence of the expected size fragment (approximately 500 bp) was detected only in the lane corresponding to 48 and 72 h *L. bicolor* × *P. resinosa* interactions. PLbras plasmid DNA was used as control for the PCR (lane R).

however, differed significantly in their rate of growth and shape. They were able to proliferate faster, differentiate, and gradually lost their elongated shape (Fig. 8C and D). Fluorescence imaging through an inverted confocal microscope clearly showed that the cells underwent morphological changes and became spherical, similar to cells observed in carcinogenic transformants (Fig. 8E). Gradually they lost cell-cell contact inhibition and started piling up into a mass, a phenotype typically observed in transformed tumorigenic cells (visualized with a 4',6-diamidino-2-phenylindole [DAPI] filter) (Fig. 8F).

DISCUSSION

The *ras* family genes have been shown to be key regulators of cell growth in a variety of eukaryotic organisms (Bourne et al. 1990; Fernandez-Sarbia and Bischoff 1993; Katoaka et al. 1985; Vojtek and Channing 1998). Our data presented here suggests that the *Lbras* gene is indeed a homolog of *ras* genes found in other systems and plays an important role in the formation and maintenance of mycorrhizae.

Our main interest has been to identify and characterize fungal genes that are involved in the initiation and maintenance of the symbiotic relationship between the plant roots and the fungus. We employed the differential display of mRNA technique (DDRT-PCR) for this purpose because this technique has been utilized successfully in many other systems to identify interaction-specific genes (Liang and Pardee 1998). We

identified and characterized several symbiosis-specific genes from *L. bicolor* with this system (Kim et al. 1998; Kim et al. 1999a; Kim et al. 1999b). Because mycorrhizal development requires cell growth and proliferation, we were expecting that genes associated with such functions would be included in the pool of genes identified by DDRT-PCR. The sequence of one of the DDRT-PCR clones obtained by this technique significantly matched *ras* family genes found in other systems. Therefore, we pursued the isolation and characterization of the full-length cDNA corresponding to this fragment.

Southern analysis showed that there is a single type of *Lbras* gene in *L. bicolor*. This was surprising because most organisms, including *S. cerevisiae*, contain at least two types of *ras* genes, which are characterized by the presence of a significant portion of common sequences. Because the *L. bicolor* mycelium used was dikaryotic, the single-copy nature of the *Lbras* gene indicates that the gene might be present only in one of the nuclei. It is possible that *L. bicolor* has other genes (such as trimeric G-proteins involved in signaling) (Posas et al. 1998) with a similar function as *ras* but do not have significant sequence similarity to *Lbras*. We believe that *L. bicolor* also might have other *ras* or *ras*-like genes, which show some sequence similarity to *Lbras* but not enough similarity to be detected in a high-stringency Southern analysis. Based on the Southern data, it appears that there is a single copy of the *Lbras* gene in the *L. bicolor* genome. Only *A. nidulans* appears to contain a single gene, as demonstrated here for *L. bicolor* (Som and Kolaparthi 1994). It is interesting to note

Fig. 5. A, Detection of *Lbras* protein in mycorrhizal tissue and **B**, *Pinus resinosa*-interacted *Laccaria bicolor* mycelial tissue with Western analysis. Protein extracts (20 µg) from *L. bicolor* × *P. resinosa* mycorrhizal roots (4 months old) (Myc), nonmycorrhizal *P. resinosa* roots (Root), and *L. bicolor* grown in the interaction medium in the presence of pine roots for 24, 36, 48, and 72 h as well as in the absence of pine roots (Fr) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis in 12% polyacrylamide gels and transferred to nitrocellulose membranes. Antibody generated with the recombinant *Lbras* was employed for immunological analysis. Purified recombinant *Lbras* protein (100 ng) was included in the analysis as a control (R). Positions where the 25- and 32-kDa size markers migrated in the gel are indicated by arrows. The approximately 26-kDa *Lbras* was detectable only in the mycorrhizal sample (**A**) and in the *L. bicolor* mycelial sample that interacted with *P. resinosa* for 72 h (**B**).

that the Lbras has the highest degree of sequence similarity to the *A. nidulans* gene (*Aras*) compared to *ras* genes from other systems, suggesting that these two systems are evolutionarily closer to each other (see below).

Northern and Western data clearly indicate that expression of *Lbras* is dependent upon interaction with the plant roots. The approximately 26 kDa protein detected in the mycorrhizal tissues was Lbras on the basis of the size and cross reactivity with the antibody. It is possible that red pine roots contained a *ras* homolog that is expressed only after interaction with the

fungus, similar to that of the fungal gene. If so, the Lbras detected in the mycorrhizal tissue would be of plant and fungal origin. This was not the case, however, because the TEM analyses clearly showed that all Lbras-related protein present in the mycorrhizal tissue was associated with the fungus. No significant gold label was detected in root cells or near their cell wall. Therefore, the Lbras mRNA and protein detected in the mycorrhizal tissue was of fungal origin. If the plant roots did contain a Ras-like protein, it differed significantly from the Lbras in its sequence and did not cross react with Lbras antibodies.

Fig. 6. Immunolocalization of Lbras protein in the mycorrhizal tissue (4 months old) through transmission electron microscopy analysis. Antibody generated against the recombinant Lbras was used in the analyses. **A**, Hartig net region. Immunogold label (indicated by arrowheads) could be detected along the wall area of the fungal hyphae (H). The root wall (W) also is shown in the picture. **B**, Hartig net region similar to the one seen in **A**, but treated with preimmune serum instead of the antibody. **C**, Higher magnification of Hartig net region shows gold particles along the fungal cell wall. **D**, Mantle region of the mycorrhizal roots after treatment with the antibody followed by the immunogold label. Cy and W refer to the fungal cytoplasmic region and root wall, respectively. Bars = 400 nm (**A**, **B**, and **D**) and 200 nm (**C**).

The data suggest that certain soluble elicitors synthesized by the roots are responsible for starting up the *Lbras* gene. The interaction system has the advantage of separating the symbiotic partners after incubation in the medium for a short period of time. Because mycorrhizal tissue does not form in a short time, it is presumable that soluble elicitors secreted by the roots into the medium mediate stimulation of the fungal gene. Appearance of *Lbras* mRNA in the fungus was not detectable until 48 h after interaction, suggesting that it is probably not one of the very early genes affected by the plant elicitors. For example, the PF6.2 gene is initiated as early as 6 h after interaction (Kim et al. 1999b). It is possible that the *Lbras* stimulation is actually a secondary event, which is dependent upon the activation and expression of other early genes in the fungus, the plant, or both. Furthermore, it has been shown in other systems that *ras* expression levels fluctuate during fungal development. It is quite reasonable to assume that *Lbras* expression in *L. bicolor* also rises and falls during the process of initiation and formation of ectomycorrhizae. A detailed study with a synchronous interaction culture for more interaction time points (2 h to a few days) is necessary to determine changes in *Lbras* levels and their relationship to physiological conditions. Expression within 48 h as well as in the established mycorrhizal tissue strongly suggests that *Lbras* plays a critical role in the development and maintenance of the mycorrhizal association.

The *ras* gene from *L. bicolor* probably has a function very similar to the *ras* genes found in *S. cerevisiae* and other systems. The gene is able to complement the *ras2* in *S. cerevisiae*, although it has only a 66% homology to its yeast counterpart. Involvement of *ras* in several cellular functions during growth and development has been well demonstrated in *S. cerevisiae* and several other fungal systems (Bourne et al. 1990; Daniel et al. 1994; Robbins et al. 1991; Roze et al. 1999; Van Hasstert et al. 1991; Vojtek and Channing 1998). In *A. nidulans*, a well-coordinated expression of *Aras* is essential for the organism to go through different stages of development. Levels of *ras* expression appear to determine the developmental path the fungus follows (Som and Kolaparthi 1994).

Similarly, levels of *Ras* have been shown to control cellular function and morphogenesis in *Mucor racemosus*, *Dictyosporium discoideum*, and yeast (Daniel et al. 1994; Robbins et al. 1991; Roze et al. 1999). Based on complementation studies and structural similarities, the *Lbras* appears to have a similar function in *L. bicolor*, although the mycorrhizal system is more complex compared to other fungi and yeast. In mycorrhizae, there is a cross flow of nutrients between partners, and the fungi are dependent upon the host roots for their carbon energy sources. This type of symbiotic interaction involves extensive morphological changes in the fungus as well as the roots. In this context, it is presumable that the *Lbras* has wide-ranging functions in regulating events in the initiation, formation, and maintenance of the symbiotic organ. Determination of its precise roles in symbiosis will need further investigations with knockout mutants and/or mutants that express varying levels of *Lbras*.

The *Lbras* protein appears to be associated with the cell membrane components in *L. bicolor*. The TEM analyses clearly showed that the bulk of *Lbras* was localized near the cell membrane. This was the case whether the fungus was in the mantle area or in the Hartig net area, suggesting that its mode of action could be similar in both these tissues. It is not clear whether some of the protein is secreted out of the fungal cell because gold label was detectable in the intercellular region in the Hartig net area. The amount of label in this region, however, was not significant. The *Ras* protein in mammalian systems has been shown to reside at the inner surface of the plasma membrane, interacting with tyrosine kinase receptors (Vojtek and Channing 1994). Through this interaction, the *Ras* influences various cellular signaling pathways controlling critical cellular events. A similar situation might exist in *L. bicolor*, where *Lbras* controls cellular functions by interacting with components present in the fungal cell wall and membrane. More work is needed, however, to determine the cell wall's components and their interaction with *Lbras*.

The sequence analyses and complementation studies clearly indicate that *Lbras* belongs to the *ras* gene family reported from other systems. On the basis of sequence, *Lbras* is related

Fig. 7. Complementation studies in *Saccharomyces cerevisiae*. The *S. cerevisiae* SR843 strain that requires a functional *ras* to be supplied through a plasmid for its growth was transformed with the plasmid containing the entire coding region of *Lbras* and the transformants were able to grow on Leu⁻ medium containing **B**, 5-fluoro orotic acid (5-FOA), which counterselects against the Ura3 marker and SR843 cells harboring plasmid pRS316RAS2 (which contains a *S. cerevisiae* *ras2* insert). Thus, the ability of the strain to grow on 5-FOA containing medium indicates the presence of a functional *ras* other than *S. cerevisiae*'s own and demonstrates the ability of *Lbras* to functionally complement the *S. cerevisiae* *ras2* gene. **A**, Plasmid containing a *S. cerevisiae* *ras2* insert, but a URA3 marker was not used as a positive control. **C**, Wild-type SR843 strain was used as a negative control.

more closely to the fungal *ras* genes than to its counterparts in mammalian systems. We performed a phylogenetic analysis employing PAUP software, version 4.0 (Sinauer Associates, Sunderland, MA, U.S.A.) (Farris 1983), with *Ras* sequences from various species, including yeast and mammals. The analysis indicated that, among the fungi for which *ras* genes have been characterized, the *Lbras* was related closely to the *Aras* of *A. nidulans* (Table 1 and Fig. 9). Even with only a 66% homology, however, *Lbras* was able to complement the *ras2* function in *S. cerevisiae*, indicating that critical sequences and structures were conserved through evolution. The data presented here suggest that *A. nidulans* and *L. bicolor* genes probably represent the ancestral forms from which *ras* genes of higher systems are derived. It is tempting to speculate that initially there might have been a single gene system, as found in these fungi, which later evolved into a system of multiple, related families of genes in higher mammalian forms. The regulatory function might have evolved, keeping in line with the more complex cellular functions of the higher systems. A more exhaustive phylogenetic analysis of all known *Ras* protein sequences is required before one can conclude about the evolution of this important set of proteins. The *L. bicolor* *Ras* protein is bigger than its mammalian counterparts and contains an extra five amino acids at the N-terminal end. This, coupled with critical differences in the spacing of GTP-binding amino acids within the *Lbras* molecule, causes the protein to resemble mutated forms of human p21^{Ras}. Such mutated forms cause disruption in the normal regulation of *Ras* function in mammalian cells, causing transformation and tumor formation, which is why we tested the possibility that *Lbras* functions as a transformation agent in the mammalian systems.

Our experiments showed that *Lbras* was capable of transforming mammalian cells. The *Lbras* containing cells showed all of the morphological characteristics exhibited by transformed cells: they lost their usual elongated morphology; attained spherical shape; and grew into a mass of cells, as occurs in tumor-forming cells. We believe this is the first report demonstrating the ability of a fungal *ras* gene to transform mammalian cells.

The data presented here clearly suggests that *Lbras* has a mammalian *ras*-like function in *L. bicolor*. To understand its precise role(s) in cellular signaling pathways in the initiation and establishment of mycorrhizal symbiosis, however, it is necessary to further characterize the gene and its expression under different physiological conditions. Such characterization will help provide an understanding to molecular events underlying host recognition as well as compatibility, and development of symbiosis under different environmental conditions. This could be achieved through generation and characterization of knockout mutants and mutant strains of *L. bicolor* expressing varying amounts of *Lbras*.

MATERIALS AND METHODS

Media, cultures,

and *Laccaria bicolor* × *Pinus resinosa* interactions.

The basidiospore isolate *Laccaria bicolor* (dikaryotic) (Maire Orton) DR170 (obtained from the roots of red pine trees from Michigan's Upper Peninsula and provided by D. Richter, School of Forestry, Michigan Technological University, Houghton, U.S.A.) was grown and maintained on MMN

medium (Bills et al. 1995). In vitro interactions and harvesting of interacted fungal tissue have been described previously (Kim et al. 1998; Kim et al. 1999b; Wong et al. 1990).

Isolation of cDNA clone.

Isolation of total RNA from *L. bicolor* mycelial tissue that interacted with red pine seedlings for 6 to 72 h, isolation of poly(A) RNA with the Oligo-dT mRNA Purification Kit (New England Biolabs, Beverly, MA, U.S.A.), and mRNA differential display techniques were carried out as described (Kim et al. 1998; Liang and Pardee 1998). The poly(A) RNA was converted to cDNA with the cDNA Synthesis Kit (Stratagene, La Jolla, CA, U.S.A.) and cloned into the Lambda Screen phagemid vector (Novagen, Madison, WI, U.S.A.) to construct the cDNA library. Screening the library to obtain a full-length *ras* clone was performed with a probe derived from the differential display experiments (Kim et al. 1998; Kim et al. 1999b).

Sequence analyses.

Nucleotide sequencing was carried out with the Big Dye Terminator Cycle Sequencing Ready Reaction kit (PE Applied Biosystems, Foster City, CA, U.S.A.) and the ABI PRISM 310 Genetic Analyzer (PE Applied Biosystems). For sequence analyses, Sequencing software, version 3.0 (PE Applied Biosystems, CA), and MacDNAsis software, version 5.0 (Hitachi Instruments, San Jose, CA, U.S.A.), were used. Phylogenetic analysis was carried out with PAUP software, version 4.0 (Farris 1983).

Complementation studies in yeast.

Complementation studies with a *S. cerevisiae* mutant lacking a functional *ras2* gene was carried out as described (Chen and Powers 1995). The entire coding region of the *L. bicolor* *ras* cDNA was cloned into the yeast expression vector pyx243 (Novagen) and used in complementation studies. The mutant yeast strain *S. cerevisiae* SR843 (MATa/aras1::HIS3/ras1::HIS3 ras2Δ/ras2Δura3-52/ura3-52 leu2::hisG/leu2::hisG his3::hisG/his3::hisG trp1::hisG/trp1::hisG) containing plasmid pRS316RAS2 (CEN, Ura3) was provided by S. Rupp, Whitehead Institute for Biomedical Research, Cambridge, MA, U.S.A. After transformation of the SR843 (Ausubel et al. 1996), clones were selected by growing on leucine-minimal plates containing 5-FOA, an uracil analog. Under these conditions, the yeast mutant strain does not reproduce unless a functional *ras* is provided through a plasmid vector. The plasmid construct YEp13RAS2 (2μ LEU2) carrying the *S. cerevisiae* *ras2* gene (Reymond 1987) was used as a control in these experiments.

Oncogenic *ras* response in mammalian cells.

pLbras cDNA was used as a template in a PCR with sense primer 5'-CGA CGA ATT CCA TGG CTT CAA AGT TTC T-3' and antisense primer 5'-CGA CGAATT CTT TTT TTT TTT TTT-3' to obtain a fragment containing the entire coding region and the 3' untranslated region. This fragment was cloned into the TA vector pCR2.1 (Invitrogen, San Diego, CA, U.S.A.). The *EcoRI* fragment from this plasmid containing *Lbras*-related sequences was then subcloned into the mammalian expression vector pEGFP-C1 (Clontech, Palo Alto, CA, U.S.A.), and constructs having the inserts in proper orien-

Fig. 8. *Lbras* from *Laccaria bicolor* has the ability to transform mammalian cells. The mouse embryonic fibroblast (MEF) cells transfected with the control plasmid pCMV-EGFP (containing only the sequences encoding a green fluorescent protein) was grown in Delbecco medium and visualized through **A**, a phase contrast microscope or **B**, an inverted confocal microscope. **C and D**, MEF cells transfected with *Lbras* sequences observed through a phase contrast microscope. These cells lost their characteristic elongated shape and began to become spherical. This was clearer in the fluorescence imaging of these cells with inverted confocal microscopy, which showed **E**, the spherical transformed cells and **F**, their continued growth into a mass of cells rather than maintaining a monolayer phenotype (live cells were viewed with a 4',6'-diamidino-2-phenylindole [DAPI] filter). Magnification: 400× (**A and C–E**); 1000× (**B and F**).

tation for expression were selected through restriction enzyme mapping. The resultant construct, pCMV-EGFP-*Lbras*, which would yield an EGFP-*Lbras* translational fusion protein, was used to transfect MEF cells (mouse embryonic fibroblast cell line, p53 null), employing the Effectene Transformation Reagent (Qiagen, Valencia, CA, U.S.A.) in accordance with the manufacturer's instructions. The MEF cells were grown in DMEM-F12 medium (GIBCO-BRL, Rockville, MD, U.S.A.) supplemented with 10% fetal bovine serum as control. Cells were transfected with the pCMV-EGFP vector. The transfected cells were subjected to sorting through fluorescence-activated cell sorting, and stable clones expressing GFP were grown on cover slips in Delbecco's medium for live-cell imaging. Observations were made with an inverted confocal fluorescence microscope (Fang et al. 1999; Galbraith et al. 1999; Lybarger and Chervenak 1999a; Lybarger and Chervenak 1999b). The GFP-specific filter was used to visualize

Table 1. Amino acid sequence comparison of *Lbras* with Ras proteins from other systems

Other Ras sequences analyzed	% homology to <i>Lbras</i>
<i>Aspergillus nidulans</i> Ras	92
<i>Aspergillus fumigatus</i> Ras	87
<i>Pisolithus tinctorius</i> Ras	70
<i>Candida albicans</i> Ras	69
<i>Neurospora crassa</i> Ras	67
<i>Suillus bovinus</i> Ras1	67
<i>Lentinus edodes</i> Ras	67
<i>Coprinus cinereus</i> Ras	66
<i>Saccharomyces cerevisiae</i> Ras1	66
<i>S. cerevisiae</i> Ras2	66
<i>Saccharomyces pombe</i> Ras	62
Human N-RAS	60
Human H-RAS	60

EGFP expression, whereas a DAPI filter was used to visualize the cancerous cell mass formed.

Northern and Southern analyses.

Total RNA from *L. bicolor*, subjected to interaction with pine seedling roots for 24, 36, and 48 h, was electrophoresed on agarose gels and transferred to Hybond-N membranes (Amersham Pharmacia Biotech, Piscataway, NJ). Total RNAs from free-living *L. bicolor* and established mycorrhizal tissue (4 months old) were used as controls. Gels were stained with SYBR Green to determine equal loadings and intensity of RNA. RNA (50 ng) was used as template for reverse transcription reaction with oligo(dT) primers (Clontech). *Lbras* gene-specific primers 5'-GCT TTT TAC GAC CTT GTG CGT G-3' and 5'-CTA TTG GCG CGC CTA TCC TC-3' were used for the PCR, and products were resolved electrophoretically on 1% agarose gel and transferred to Hybond-N membranes. *L. bicolor* genomic DNA isolated from ground mycelia (Reymond 1987) was digested with restriction enzymes *Hind*III, *Pst*I, and *Xba*I. After resolving the fragments by agarose gel electrophoresis, they were transferred to Hybond-N membranes.

The cDNA fragment of p*Lbras* was labeled with ³²P with the DECAprime II DNA Labeling Kit (Ambion, Austin, TX, U.S.A.) and used as a probe in the hybridization analyses of the membrane-bound nucleic acids (Kim et al. 1999a; Kim et al. 1999b; Sambrook et al. 1989). For low-stringency Southern analysis, hybridization and washings were performed at 37°C.

Protein expression and antibody production.

The *Lbras* cDNA fragment was cloned into expression vector pet22b⁺ (Novagen) through a PCR fragment of p*Lbras* generated with primers 5'-CCC CCA AGG TTC TCA GTC TCA C-3' and 5'-GCC GAG CTC ATA ACA ATA CAT TTC CCG C-3' (Rosenberg et al. 1987; Studier and Moffatt 1986; Van Hasstert et al. 1991). This construct facilitated expression of *Lbras* with 3'-histidine tags. The construct was used to transform *E. coli*, and expression of the protein encoded by the plasmid was carried out per the manufacturer's instructions. The recombinant protein was purified from *E. coli* extracts by affinity chromatography with the His-Bind metal chelation resin (Novagen).

Purified recombinant protein was used to generate polyclonal antibodies in rabbits. The specificity of antibodies was confirmed with purified *Lbras* recombinant protein. When

Fig. 9. Phylogenetic analysis of LbRas with PAUP 4.0 software shows its relatedness to other Ras proteins. A bootstrap value of 1,000 was used, and human H and N-Ras were defined as out groups. LbRas was closest to the *Aspergillus nidulans* Ras (AnRas). NcRas1, *Neurospora crassa*; SbRas, *Suillus bovinus* Ras1; LERas, *Lentinus edodes* Ras; CcRas, *Coprinus cireneus* Ras; ScRas1, *Saccharomyces cerevisiae* Ras1; ScRas2, *S. cerevisiae* Ras2; CaRas, *Candida albicans*; SpRas, *Saccharomyces pombe*; PtrRas, *Pisolithus tinctorius*; AfRas, *Aspergillus fumigatus*; AnRas, *A. nidulans*; LbRas, *Laccaria bicolor*; HHRas, Human H-Ras; HNRas, Human N-Ras.

Lbras antibodies were preincubated with an excess of recombinant Lbras protein and used for Western blot analysis, it effectively eliminated any signal. After confirming the specificity of the antibodies, they were used for Western blot analysis of protein samples extracted from free-living *L. bicolor* and *L. bicolor* interacted with red pine roots and mycorrhizal and nonmycorrhizal red pine roots (Dudareva et al. 1996).

Protein was extracted from the tissues with 50 mM Tris-HCl, pH 6.9, containing 10% glycerol, 2% sodium dodecyl sulfate, 1% polyvinylpyrrolidone, and 1 mM phenyl methyl sulfonyl fluoride (Dudareva et al. 1996). Western blot analyses were carried out after transferring proteins resolved on 12% polyacrylamide gels to Hybond-C pure nitrocellulose membranes (Amersham Pharmacia Biotech). The BCA Protein Assay Kit (Pierce, IL, U.S.A.) was used to determine the concentration of protein samples (Lowry et al. 1951), and an equal amount of total protein from each sample was used for the analyses.

Immunolocalization with TEM.

Preparation of samples for TEM analyses was performed essentially as described (Moore et al. 1991). Briefly, small (< 4 mm) mycorrhizal and nonmycorrhizal root pieces were cut and fixed in 2.5% glutaraldehyde (10 mM sodium phosphate, pH 7.2) by incubating for 16 to 20 h at 40°C and then placed under vacuum at room temperature for 2 h. After washing (3 × 15 min) with 10 mM sodium phosphate, pH 7.2, samples were treated with 1% osmium tetroxide for 1 h and washed with water. This was followed by the gradual removal of water from the samples through treatments with 30% etha-

nol, gradually increasing the ethanol concentration. After final treatment with 100% ethanol, LR white resin infiltration was performed (Moore et al. 1991).

Cross sections of roots (100 to 120 nm) generated with an ultramicrotome equipped with a diamond knife were collected on nickel grids and subjected to immunological analysis. After an initial blocking step in TBS (50 mM Tris-HCl, pH 7.6, containing 0.5 M sodium chloride) containing 1% bovine serum albumin (BSA) and 1:30 dilution of rabbit preimmune serum, the grids were incubated for 16 to 20 h with 1:1000 dilution of anti-Lbras antiserum in TBS at room temperature. After washing with TBS, they were treated with TBS containing 1% BSA and 1:20 dilution of gold-labeled second antibody (goat antirabbit immunoglobulin Polygold 10 nM; Polysciences, Warrington, PA, U.S.A.). After incubation for 1 h, root sections were washed several times with TBS and finally with water and dried. Poststaining with uranyl acetate was performed with some of the samples as described (Moore et al. 1991). The grids were examined with a JEOL JEM-1010 TEM operated at 80 kV.

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