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Identification of a Small Heat-Shock Protein Associated with a *Ras*-Mediated Signaling Pathway in Ectomycorrhizal Symbiosis

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Abstract

Initiation, development, and establishment of a functional ectomycorrhiza involve a series of biochemical events mediated by a number of genes from the fungus as well as the host plant. We have identified a heat-shock protein gene from *Laccaria bicolor* (*Lbhsp*) that appears to play a role in these events. The size and characteristics of *Lbhsp* suggest that it belongs to the family of small heat-shock proteins described in the literature. Nucleotide sequencing of an almost full length cDNA indicated that the *Lbhsp* mRNA is about 611 nucleotides long and codes for a single protein of ~ 17 kDa. Isolation and characterization of the *Lbhsp* gene showed that it was made up of three exons separated by two small introns. Southern analysis suggested that the *L. bicolor* genome contains at least two copies of the *Lbhsp* gene. Temporal expression analyses revealed that the gene is expressed within 4 to12 hours after interaction with red pine roots. The yeast two-hybrid studies showed that the *Lbhsp* was closely associated with the ras gene (*Lbras*) described earlier. The data suggest that Lbhsp may play a supporting role in *ras*-mediated mycorrhizal signaling pathways during various stages of ectomycorrhizal development.

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INTRODUCTION

Initiation, development, and establishment of a functional ectomycorrhiza involve a myriad of biochemical and genetic events in each partner that eventually culminate in the formation of a new functioning symbiotic organ (Duplessis et al. 2005, Hilbert et al. 1991, Johansson et al. 2004, Martin and Tagu 1999, Martin et al. 2007, Nehls et al. 1999, Polidori et al. 2001, Voiblet et al. 2001). A number of dynamic signaling interactions take place between the host plant roots and the fungus leading to recognition of each other (Podila et al. 2002). If compatible, further changes in gene expression are initiated to bring about morphological changes needed for the development of the functioning ectomycorrhiza. Thus, early events in the interaction are crucial and determine the final outcome. They have been shown to involve activation of a cascade of molecular events in each partner. Because ectomycorrhiza represents a completely distinct new organ formed after the interaction, it is highly conceivable that genes related to cell growth and proliferation play an important role.

We identified a gene *Lbras* from the ectomycorrhizal fungus *Laccaria bicolor* that belongs to the *ras* family of genes found in other systems (Kim et al. 1998, Sundaram et al. 2001). The *ras* genes, present in a wide range of eukaryotes, including mammals and yeast, are involved in a variety of cellular signaling pathways controlling critical cellular events such as cell cycle progression and cell division (Broek et al. 1985, Ha et al. 2003, Rocha et al. 2001). The *Lbras* cDNA complemented *ras2* function in *Saccharomyces cerevisiae* and had the ability to transform mammalian cells (Sundaram et al. 2001). Expression of *Lbras*, present as a single copy in the *L. bicolor* genome, was dependent upon interaction with host roots. Northern analysis showed that expression was detectable in the fungus as early as 12 to 24 hours after interaction with plant roots and continued to be expressed even in the established mycorrhizal tissue. Phylogenetic analysis with other *ras* proteins showed that *Lbras* is most closely related to *Aras* gene of *Aspergillus nidulans*.

Our examinations of early changes in gene expression in *L. bicolor* during its interaction with red pine roots indicated the presence of a heat-shock protein related gene whose expression followed a pattern similar to that of *Lbras* and other signal pathway related genes we have identified. The similar pattern of expression as well as known relationships between heat-shock proteins and *ras* in other systems prompted us to undertake further studies to characterize the *L. bicolor* heat-shock protein (*Lbhsp*) gene. This protein was also identified as an interacting protein with *Lbras* during yeast two-hybrid screening of a cDNA library from *L. bicolor* prepared after interaction with red pine seedling roots. We describe here the isolation and characterization of the *Lbhsp* cDNA, its gene, and determination of its expression pattern under different physiological conditions. The data indicate that it is indeed a heat-shock protein gene and may have a role in *ras*-mediated signaling pathwaysfor effecting development and formation of the ectomycorrhiza.

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MATERIALS AND METHODS

Media, Cultures, and *Laccaria bicolor-Pinus resinosa* Interactions

The *Laccaria bicolor* (Maire) Orton DR170 strain used for our studies was a basidiocarp isolate associated with the roots of red pine obtained from Michigan's Upper Peninsula (provided by D. Richter, School of Forestry, Michigan Technological University, Houghton). The fungus was grown and maintained on MMN medium as previously described (Bills et al. 1995, Marx 1969). *In vitro* interactions and harvesting of interacted fungal tissue were carried out as described previously (Kim et al. 1998, 1999). Synthesis of mycorrhizal tissue in soil was performed according to Bills et al. 1999.

Isolation of cDNA Clones Coding for *Lbras* Interacting Proteins

Isolation of total RNA from *L. bicolor-P.resinosa* ectomycorrhizal tissue was carried out as described previously (Kim et al. 1998). The Smart cDNA Synthesis Kit (Clontech, CA) was used to synthesize cDNA ligated with *Sfi* arms as per manufacturer's instructions. LexA Match Maker Library kit (Clontech, CA) was used to perform the yeast two-hybrid screens as described (Fields and Song 1989, Sundaram et al. 2004).

For protein interaction studies, the mycorrhizal cDNA synthesized with the Smart cDNA kit was cloned into the Match Maker Library AD vector, full length *Lbras* cDNA was cloned into the Match Maker BD vector, and two-hybrid interactions were performed as per manufacturer's instructions. A previously well characterized *ras* clone (*Lbras*) from the ectomycorrhizal fungus *L. bicolor* (Kim et al. 1998) was used as bait in a yeast two-hybrid interaction system. About 10,000 colonies were screened for positive interactions by selection for Leu+ followed by a standard blue/white selection for expression of the β-galactosidase. Plasmids containing interacting clones were rescued from the yeast as per manufacturer's instructions (Clontech, CA). *E. coli* DH5 alpha cells were transformed with the rescued plasmids, and resultant plasmid DNAs were isolated and subjected to further analysis. Selected clones were also tested for authenticity by one-on-one interaction to confirm their interaction with *Lbras*. The strength of the interaction was determined using the liquid β-galactosidase assays (Pandey et al. 2006).

Sequence and Phylogenetic Analyses

Nucleotide sequencing was carried out on an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, CA) and Dynamic ET sequencing kit (GE Health Systems, NJ). For sequence analyses, Sequencing Software version 3.0 (PE Applied Bio-systems, CA) and MacDNAsis software version 5.0 (Hitachi Instruments, San Jose, CA) were used. Multiple alignments of the sequences were done with the Mutalin (www.toulouse.inra.fr). A parsimony-based phylogenetic analysis was done on *hsp* class proteins using the software PAUP version 4.0b.8 (Sinauer Associates, MA) as previously described (Berlocher and Swofford 1997, Farris 1983, Sundaram et al. 2001).

Northern and Southern Analyses

Northern analyses were carried out essentially as described earlier (Kim et al. 1998). Total RNA from *L. bicolo*r subjected to interaction with pine seedling roots for 12, 24, 36, 48, 72 and 96 hours was electrophoresed on agarose gels and transferred to Hybond-N membranes (GE Health Systems, NJ). Total RNA from free-living *L. bicolor* was used as control. Twenty μg RNA was loaded in each lane; gels were stained with SYBR Green, and the intensity of color was observed to confirm equal amounts of RNA being present in each lane for comparisons.

The *L. bicolor* genomic DNA isolated from ground mycelia (Kim et al. 1998, Sundaram et al. 2004) was digested with restriction enzymes. About 10 μg genomic DNA was used for each restriction digestion. After the fragments were resolved by agarose gel electrophoresis, they were transferred to a Hybond-N^+ membrane. The cDNA fragment of *Lbhsp* labeled with ^{32}P-dCTP using the Rediprime DNA Labeling Kit (GE Health Systems, NJ) served as a probe in Southern hybridization analysis (Kim et al. 1999).

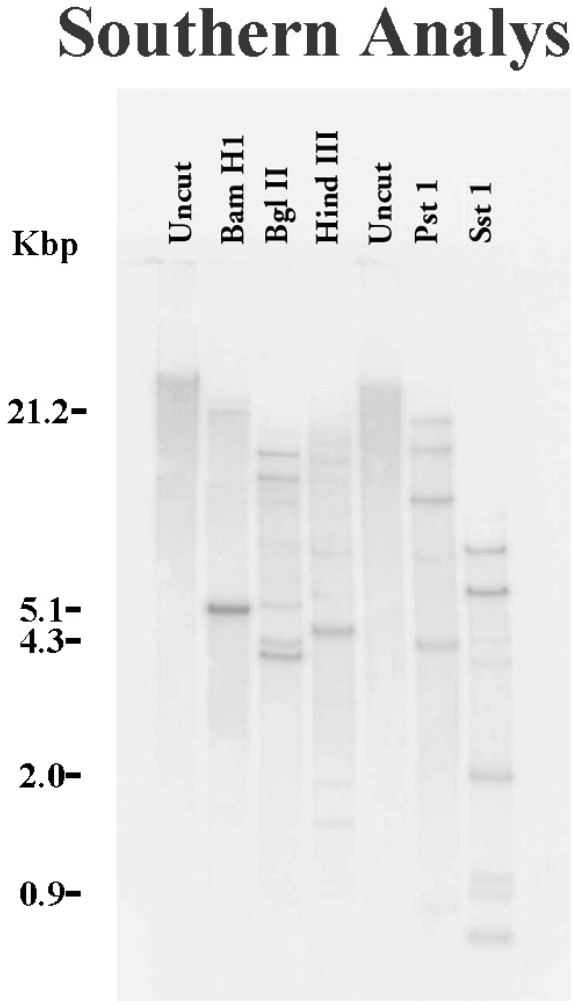

Figure 1.—Southern hybridization of *L. bicolor* genomic DNA for *Lbhsp* sequences. Hybridization of the genomic blot was done with *Lbhsp*-specific probe. Restriction enzymes used for digesting *L. bicolor* genomic DNA and position of molecular weight markers are indicated.

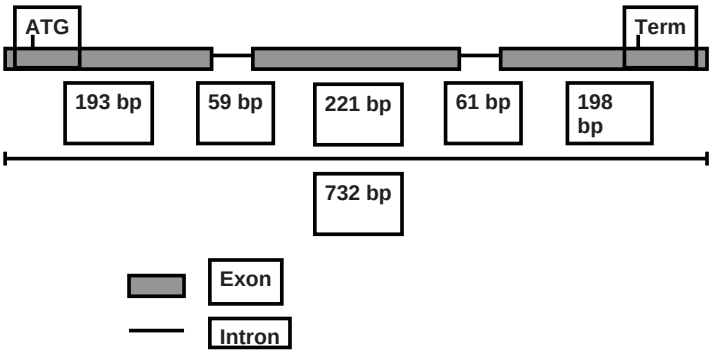

Figure 2.—The *Lbhsp* gene isolated from the *L. bicolor* genomic library. Complete nucleotide sequencing was done to identify the structure of the gene. Locations and sizes of introns and exons are indicated. The location of the initiation codon (ATG) and termination codon (Term) are also shown.

RESULTS

Heat-shock Protein (*Lbhsp*) mRNA and Gene

An interaction-specific cDNA library, constructed using *L. bicolor* that had interacted with red pine roots for 4 to 72 hours (Kim et al. 1998), was subjected to differential screening to identify cDNAs that show significant changes in expression during early interaction (data not shown). One of the clones with an insert of ~ 600 bp was designated as the heat-shock protein cDNA (*Lbhsp*) after further characterization. The cDNA insert from this clone was subcloned and its complete nucleotide sequence was determined. It had 611 nucleotides without the poly(A) tail and encoded a single protein of 155 amino acids (Genbank Database Accession Number AY117412). The predicted size of the protein was ~ 17 kDa.

Southern analysis was performed using the *Lbhsp* cDNA insert as a probe on restriction enzyme digested *L. bicolor* genomic DNA (Fig.1). The restriction enzymes used in the analysis did not have any sites in the cDNA used as the probe. As seen in the figure, the pattern suggested that at least two copies of the *Lbhsp* genes were present in the *L. bicolor* genome.

The cDNA insert was used as a probe to screen an *L. bicolor* genomic library to identify and isolate the *Lbhsp* gene. The isolated gene was characterized by mapping with restriction enzymes, and its complete nucleotide sequence was determined (Fig. 2). Analysis of the nucleotide sequence showed that the *Lbhsp* gene is made up of three exons of 193, 221, and 198 bp separated by two small introns of 59 and 61 bp.

Comparison to known sequences deposited in the Genbank indicated that the nucleotide sequence and the derived amino acid sequence had significant similarity to the small heat-shock proteins previously identified in yeast and other organisms. Alignments showed significant identity in conserved portions of the protein (Fig. 3). Phylogenetic analysis of closely related *hsp16* sequences showed that the *Lbhsp*, while related to other

Similarity to yeast heat-shock protein

Score = 76.3 bits (186), Expect = 9e-14
Identities = 44/110 (40%), Positives = 62/110 (56%), Gaps = 4/110 (3%)

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Sp hsp 16   LKPRMDLHEDKEKNLVTATFEFPGSKKEDVHLEIHNGRLVVSVE--NKISEEHDE 99
            L P +D+HE K+   V+   E PG KKEDV +   +G+L +S E  N+   E  E
Lbhsp       LSPSIDVHEGKDT--VSVDVELPGVKKEDVQVHYDSGKLTISGEVVNERKNESTE 87

Sp hsp 16   SGYAVRERRYGKFSRTLQLPQGVKDDEIKAGMEDGVLTVTFPKSGAELAPKKISI 154
                  ERR+G FSRT+ +P  +  D I+A   +G+LTVT PK       K+I+I
Lbhsp       GNQRWSERRFGSFSRTITIPAKIDADRIEANFSNGLLTVTLPKVEKSQTKKQIAI 142
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Figure 3.—Comparison of *Lbhsp* amino acid sequence to those in the Genbank. Comparison analysis indicated that the amino acid sequence had significant similarity to heat-shock proteins previously identified in the yeast and other organisms. Alignments showed significant identity in conserved portions of the protein. The alignment with the amino acid sequence of the *hsp 16* from *Schizosaccharomyces pombe* (*Sp hsp16*) is shown. The identities are shown with the amino acid marker and + indicates a similar amino acid. Other details such as total identities, similarities, and gaps are indicated.

hsp16 sequences, falls on a different branch (Fig. 4). This suggests that *Lbhsp* may be functionally different from other proteins in the *hsp* family.

Expression of *Lbhsp*

Expression analysis of *Lbhsp* gene was carried out for the free-living fungus (on agar plates) as well as for fungus interacting with the red pine roots (In vitro interaction system described earlier: Kim et al. 1999). For assays involving free-living fungal tissues, the fungus was first grown on a cellophane membrane placed on MMN agar medium and later transferred to agar plates containing the interaction medium.

Northern analyses showed the presence of a ~ 600 b mRNA, which was consistent with the size predicted by the nucleotide sequence of the cDNA (Fig. 5). The *Lbhsp* mRNA was not detectable in samples obtained from free-living (agar plates) fungus (Lane Fr). However, samples from fungus that had interacted with the roots for 6 to 72 hours showed the presence of this mRNA. Only traces could be detected in samples from the 6-hour interaction; however, after 12 hours it was clearly seen and its levels kept increasing until 72 hours. In other experiments we have detected mRNA as early as 4 hours after interaction by using more RNA in Northern analysis (data not shown). We also investigated the effect of 2.5 mM glucose added to the interaction medium and found that it did not have any significant effect on free-living and/or interacted tissues.

Protein-protein Interaction of *Lbhsp* and *Lbras*

We used the yeast two-hybrid screening to determine the relationship between *Lbhsp* and *Lbras*. Screening the *L. bicolor* library using *Lbras* as bait, we were often able to isolate *Lbhsp*, suggesting that *Lbhsp* is closely linked and presumably has a role in the functioning of *Lbras*. Out of more than 50 positive yeast two-hybrid clones isolated as interacting partners of *Lbras, Lbhsp* was identified more than six times. We repeated the experiment with individual clones (one-on-one) to confirm that this association was indeed true. Liquid beta-galactosidase assays performed to determine the strength of the interaction showed that it is a very strong interaction.

DISCUSSION

Significant changes in gene expression are initiated in *L. bicolor* during its interaction with red pine roots (Kim et al. 1998, Podila et al. 2002). We have identified a number of genes that are turned on as early as 4 hours after interaction. Many of these were related to genes that have been shown to be involved in signal transduction events in other systems. Formation of a functioning

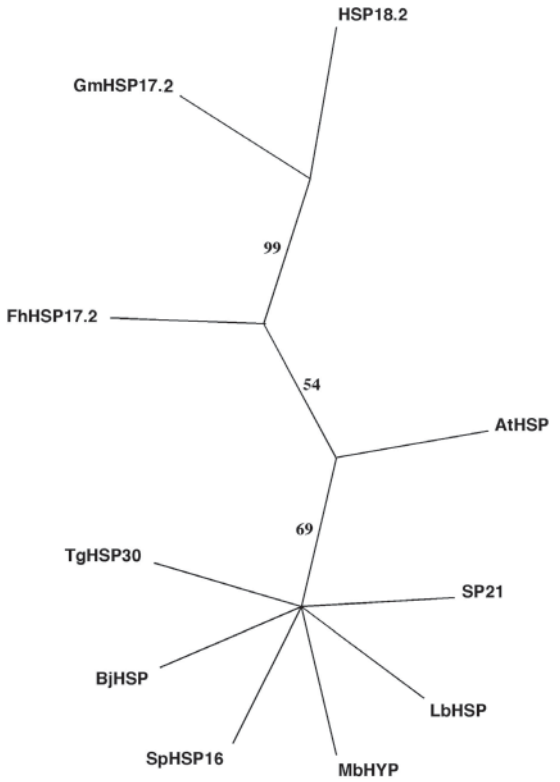

Figure 4.—Phylogenetic analysis of *Lbhsp* with PAUP 4.0 software shows its relationship to other *hsp* proteins. The following protein sequences were used for generating the unrooted tree. The Genbank accession number for sequences of the corresponding protein are indicated in parentheses. HSP18.2: *Medicago sativa* (P27880); GmHSP17.2: Glycine max (P04794); FhHSP17.2: *Funaria hygrometrica* (CAC81966); AtHSP: *Arabidopsis thaliana* (NP_190209); SP21: *Gemmata obscuriglobus* (ZP_02734271); LbHSP: *Laccaria bicolor* hsp16.2 (AAM78595); MbHYP: (NP_853922); SpHSP16: *Schizosaccharomyces pombe* (SPBC3E7.02c); BjHSP: *Bradyrhizobium japonicum* (NC_00945.1); TgHSP30: *Toxoplasma gondii* (AAM62131).

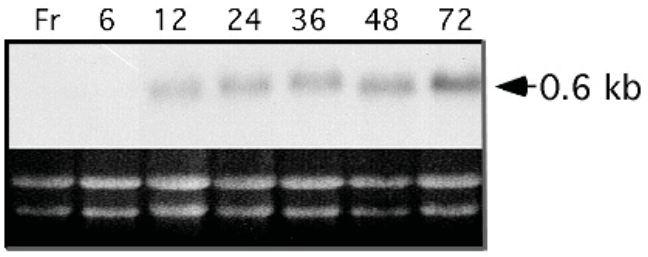

Figure 5.—Temporal regulation of *Lbhsp* expression during early stages of *L. bicolor x P. resinosa* interactions. Northern analysis using RNA samples from *L. bicolor* subjected to interaction with red pine roots for 6, 12, 24, 36, 48, and 72 hours. The bottom panel shows the picture of the gel after staining with SYBR stain to visualize the ribosomal RNA in samples that were transferred to the Hybond membrane. The relative intensity of these bands indicates that roughly the same amount of total RNA was present in each lane. The top panel shows the autoradiogram after hybridization to the *Lbhsp* cDNA probe. A single mRNA of ~ 600 b hybridized to the cDNA probe and was seen in samples that had interacted with plant roots for 12 to 72 hours. The sample in lane Fr refers to total RNA isolated from free-living fungus grown on MMN media on agar plates.

ectomycorrhiza requires turning on of key genes that control events such as cell growth and proliferation. These actions eventually lead to formation of a novel symbiotic organ. The *Lbhsp* gene reported here appears to belong to the same key group of genes involved in mediating cell growth and proliferation events.

To identify important genes involved in early interaction between *L. bicolor* and red pine roots, we used a differential screening procedure to generate a subset of cDNA clones that would represent an interaction-specific cDNA library. The genes in this library would be the ones whose expression is significantly induced due to interaction with host roots. To achieve this, we made two cDNA probes using poly(A) RNA from *L. bicolor* that had interacted with plant roots either for 0 hours or for 72 hours. We screened the cDNA library described above with both these probes separately. Comparison of the results from these screenings allowed us to select the cDNA clones that represented *L. bicolor* mRNA that was expressed only after interaction with the roots. This subset of clones was designated as the interaction-specific cDNA library, and some of these were subjected to further analyses.

The *Lbhsp* cDNA clone isolated represented an almost full length copy of the *Lbhsp* mRNA. The deduced protein from the nucleotide sequence showed significant homology to the small heat-shock proteins described in the genome database. Particularly, *Lbhsp* protein was very similar to the yeast *hsp16* protein. Southern analysis showed the presence of at least two copies of the *Lbhsp* gene in the fungal genome. This is consistent with the recent finding of a single copy found in the haploid strain H82 of *L. bicolor* (Martin et al. 2004, http://genome.jgi-psf.org/Lacbi1/Lacbi1.home.html). Because the strain DR 170 we used for analyses is a diploid, our results showed two copies.

Northern analysis confirmed that *Lbhsp* is expressed only in response to interaction with the host roots. A single mRNA of ~ 600 b was detected in the samples. The size of the mRNA was consistent with the nucleotide sequences of *Lbhsp* cDNA and the gene. The mRNA was not detectable in the free-living fungus grown on agar plates. But, within hours after interaction, it started appearing. We have been able to detect its presence as early as 4 hours after interaction as well as in the established ectomycorrhiza (data not shown). The data suggest that *Lbhsp* is essential not only for initiation and development of the symbiotic organ, but also for its maintenance.

We wanted to determine the possible role of *Lbhsp* in the ectomycorrhiza formation. Therefore, we undertook further studies to determine its relationship to other genes expressed during early events of interaction. Since it has been shown in other systems that heat-shock proteins may interact with *ras* proteins, we investigated the possible relationship between *Lbhsp* and *Lbras*. The *Lbras* gene from *L. bicolor* is also turned on after interaction with plant roots as in the case of *Lbhsp* (Sundaram et al. 2001). In addition, expression of both followed a very similar pattern. The data also showed that *Lbhsp* was linked to *Lbras* not only at the gene expression level, but also at the protein level. In fission yeast, *Schizosaccharomyces pombe*, Danjoh and Fujiyama showed that expression of a low molecular weight heat-shock protein, *hsp16*, is regulated by a *ras*-mediated signaling pathway (1999). It is possible that in the present case the interaction between *Lbras* and *Lbhsp* is also needed for the activation of the *ras* protein. In *S. pombe,* it appeared that *hsp16* expression is regulated by the *ras*1-mediated pathway and not by the heat shock response. Similarities of *Lbhsp* to the *hsp16* suggest that a similar phenomenon might exist in *L. bicolor* in its interaction with pine roots.

Analysis using the yeast two-hybrid system indicates that the *Lbhsp* protein indeed interacts with *Lbras*. However, it is not clear what precise functional role *Lbhsp* plays during signaling or interaction of *L. bicolor* with red pine. Because many *hsp* proteins are known to have a role as a chaperone, it is likely that the *Lbhsp* is involved in the protein folding leading to the activation of *Lbras*. In such a situation, the functioning of *Lbras* will depend upon simultaneous expression of *Lbhsp* and its interaction with *Lbras*. The coordinated expression of *Lbhsp* with *Lbras* in *L. bicolor* during its interaction with host roots supports this notion. Furthermore, in our previous studies with *L. bicolor,* we showed that *Lbras* may be transported to different locations within the cell and may also be transported across the dolipore septum from one cell to the other (Sundaram et al. 2004). It has been shown in other systems that *ras* proteins are made initially in an inactive form and subsequently transported to different membrane organelles or locations in the cell where they are transformed into active forms through proper refolding of the protein (Hirotsu et al. 2000). Thus, it is conceivable that *Lbhsp* may bind *Lbras* soon after its synthesis and keep it inactive until it is transported to a target location. The *Lbhsp* shares all the features of yeast *hsp16* motifs, but at the sequence level is not highly homologous to the other *hsp16* proteins. Thus, it is possible that *Lbhsp* is specifically involved in signaling events in association with *Lbras*. In our macroarray gene expression studies, we have seen coordinated expression of *Lbhsp* with *Lbras* (Podila et al. 2002). Taken together, all these results suggest that *Lbras* may have different signaling circuits depending upon the developmental stage of the ectomycorrhiza. Further studies using GFP tagged *Lbhsp* and pull down experiments with *Lbras* are required to determine the precise dynamics of *Lbhsp* and *Lbras* interactions during symbiosis. Similarly, extension of future functional genomic studies of *L. bicolor* (Pandey et al. 2006) using *Lbras* and *Lbhsp* genes will provide important details of the functional role of *Lbhsp* and its importance for symbiosis.

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Initiation, development, and establishment of a functional ectomycorrhiza involve a series of biochemical events mediated by a number of genes from the fungus as well as the host plant. We have identified a heat shock protein gene from *Laccaria bicolor* (*Lbhsp*) that appears to play a role in these events. The size and characteristics of *Lbhsp* suggest that it belongs to the family of small heat-shock proteins described in the literature. Nucleotide sequencing of an almost full length cDNA indicated that the *Lbhsp* mRNA is about 611 nucleotides long and codes for a single protein of ~ 17 kDa. Isolation and characterization of the *Lbhsp* gene showed that it was made up of three exons separated by two small introns. Southern analysis suggested that the *L. bicolor* genome contains at least two copies of the *Lbhsp* gene. Temporal expression analyses revealed that the gene is expressed within 4 to 12 hours after interaction with red pine roots. The yeast two-hybrid studies showed that the *Lbhsp* was closely associated with the ras gene (*Lbras*) described earlier. The data suggest that *Lbhsp* may play a supporting role in *ras*-mediated mycorrhizal signaling pathways during various stages of ectomycorrhizal development.

KEY WORDS: mycorrhizal fungi, heat-shock protein, symbiosis, *ras*-mediated

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