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LB-AUT7, a Novel Symbiosis-Regulated Gene from an Ectomycorrhizal Fungus, *Laccaria bicolor*, Is Functionally Related to Vesicular Transport and Autophagocytosis

SUNG-JAE KIM,[1] DANIELA BERNREUTHER,[2] MICHAEL THUMM,[2] AND GOPI K. PODILA[1]*

Department of Biological Sciences, Michigan Technological University, Houghton, Michigan 49931,[1] *and Institut fuer Biochemie, Universitaet Stuttgart, D-70569 Stuttgart, Germany*[2]

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We have identified *LB-AUT7*, a gene differentially expressed 6 h after ectomycorrhizal interaction between *Laccaria bicolor* and *Pinus resinosa*. LB-Aut7p can functionally complement its *Saccharomyces cerevisiae* homolog, which is involved in the attachment of autophagosomes to microtubules. Our findings suggest the induction of an autophagocytosis-like vesicular transport process during ectomycorrhizal interaction.

Ectomycorrhizal fungi such as *Laccaria bicolor* are ubiquitous symbiotic fungi found all over the world (16, 29, 30). The host range of these fungi includes most gymnosperm and angiosperm trees as well as economically important timber-producing species (27). Mycorrhizal fungi play an important role in the health and survival of many tree species. Mycorrhizal symbiosis is especially important for transplanted trees and seedlings and when the soil conditions are unfavorable (6, 34). The fungi form a network of hyphae called the Hartig network, penetrating between the cortical cells of the root system. The ectomycorrhizal fungi provide the plant several benefits, which include the enhanced ability to absorb water and important ions such as phosphorus and nitrogen (8, 13, 33), and protection from soilborne root pathogens such as *Fusarium oxysporum* (10, 11) and heavy metals (9, 14). Mycorrhizal fungi form diverse interactions, and their beneficial effects also differ from species to species and under various soil and environmental conditions (20, 21, 39). In order to better utilize ectomycorrhizal fungi for forest tree health and increased yield of timber, it is important to understand the mechanisms by which the fungi and plants recognize and respond to each other for the formation of symbiotic ectomycorrhizas.

The molecular mechanisms that control the formation of symbiotic ectomycorrhizas are not well understood. The interaction between the fungus and roots must involve a series of events resulting in an integrated and functioning structure, namely, the mycorrhiza. These interaction events are likely mediated by the switching on and off of several genes in both the fungus as well as the host plant (7, 15). The root cells, presumably, produce certain elicitors that regulate the expression of fungal genes involved in the establishment of symbiosis (2, 18). This includes turning on genes responsible for attachment to the root surface and developmental processes, such as the formation of the Hartig network and hyphal mantle (7, 31, 32). It may also include turning off fungal genes encoding factors that elicit host plant defense reactions during early stages of interaction. Salzer et al. (31) have shown that specific elicitors present in ectomycorrhizal fungi were inactivated by chitinases secreted by roots during interaction, without affecting the fungus, thus enabling formation of ectomycorrhizas without eliciting a defense response from the plant.

Our objective in this study was to identify and characterize symbiosis-regulated genes from the ectomycorrhizal fungus *L. bicolor* that are differentially expressed in interaction with red pine seedling roots. Primarily we are interested in genes whose expression is triggered during very early stages of the interaction. Towards this goal, we have developed an in vitro *L. bicolor* × *Pinus resinosa* interaction model system (17) that allows us to identify symbiosis-regulated genes by the mRNA differential display technique (24). We describe here cDNA cloning and characterization of *LB-AUT7*, an *L. bicolor* gene that is turned on as early as 6 h after interaction with pine. *LB-AUT7* has significant homologies with *AUT7* from *Saccharomyces cerevisiae*. *AUT7* in yeast has been identified as a gene essential for autophagocytosis (23). Autophagocytosis is a degradative pathway, transporting proteins from the cytoplasm to the lysosome (vacuole) (12, 37). During autophagy cytosol-containing, double-membrane layered vesicles (autophagosomes) are formed and delivered to the vacuole, where they are degraded together with their cytosolic content. Most recently it has been shown that Aut7p forms a protein complex with the microtubule-associated Aut2p. Aut7p and Aut2p are necessary for the attachment of autophagosomes (vesicles) to microtubules, to allow their directed movement to the vacuole (23).

mRNA differential display and identification of symbiosis-regulated genes from *L. bicolor*. The in vitro ectomycorrhizal model system (17) and mRNA differential display (24) were used to study the plant-fungus interaction versus that with free-living fungus. In this system, fungal genes that are differentially expressed during the early stages of fungus-pine interaction were identified. Figure 1 shows part of a differential-display reverse transcriptase PCR (DDRT-PCR) gel showing *L. bicolor* cDNA clones which are differentially expressed after 6 h of interaction with red pine seedlings. Based on preliminary results from Northern hybridizations using DDRT-PCR clones and DNA sequence analysis, three clones were selected, PF6.1, PF6.2, and PF6.3 (Fig. 1). Of these three DDRT-PCR clones, PF6.3 (Fig. 1) was selected for further characterization because of its sequence characteristics and significant homology to yeast *AUT7*. Thus, this system provided us with a powerful tool to study the molecular basis of ectomycorrhizal symbiosis during the very early stages of interaction. Since the establishment of mycorrhizal roots is a long-term process, soil-based or other

* Corresponding author. Mailing address: Department of Biological Sciences, Michigan Technological University, Houghton, MI 49931. Phone: (906) 487-3068. Fax: (906) 487-3167. E-mail: gkpodila@mtu.edu.

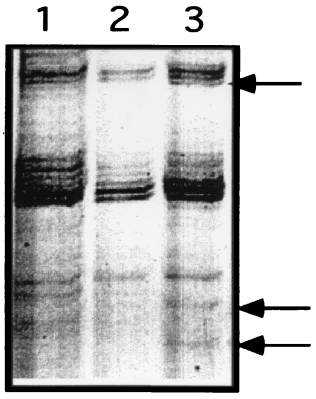


FIG. 1. Autoradiogram of part of an mRNA differential display gel showing the differences between fungus induced in response to pine signals and free-living mycelium as a control at 6 h. Shown are RNA from *L. bicolor* at the beginning of experiment, (lane 1), RNA of free-living fungus at 6 h (lane 2), and RNA of fungus after 6 h of interaction with red pine seedlings (lane 3). Arrows indicate selected differentially expressed cDNAs after 6 h of interaction. The arrow at the bottom corresponds to the PF6.3 clone.

solid-medium-based systems can be used to establish mycorrhizal roots as we have done and can be used to study gene expression in synthesized ectomycorrhizal roots. A solid-agar-based system was used by Tagu and colleagues (35, 36) to study gene expression from mycorrhizal roots formed by interaction of the ectomycorrhizal fungus *Pisolithus tinctorius* and its plant host, eucalyptus.

Isolation and characterization of *LB-AUT7* cDNA. RNA extracted from *L. bicolor* cocultivated with pine seedlings for 6 to 48 h was pooled and used to isolate poly(A)$^+$ RNA by employing the Oligo-dT mRNA purification system (New England Biolabs, Beverly, Mass.). This poly(A)$^+$ RNA was used to construct a cDNA library in the phagemid vector lambda-ZAP (Stratagene, La Jolla, Calif.). The cDNA library was screened with a cDNA fragment, corresponding to *LB-AUT7*, isolated by DDRT-PCR. A total of 10^5 recombinant phages were screened, and seven positive plaques were isolated; the largest cDNA clone was selected and named *LB-AUT7*. DNA sequence analysis of *LB-AUT7* cDNA and a subsequent open reading frame search using MacDNAsis software (Hitachi Corp.) have indicated that *LB-AUT7* cDNA contained an open reading frame encoding a 197-amino-acid protein with a predicted molecular mass of 22.8 kDa and a pI of 8.2. The *LB-AUT7* cDNA clone also contained 57 nucleotides of the 5′ untranscribed region and 84 nucleotides of the 3′ untranscribed region, for a total length of 732 bases (GenBank accession no. U93506).

Homologies of *LB-AUT7* to known sequences. Sequences similar to those of *LB-AUT7* have not been previously isolated from ectomycorrhizal fungi. However, LB-Aut7p has significant homologies to a number of proteins (Fig. 2), including Aut7p from *Saccharomyces cerevisiae* (23), which is 77% identical (Fig. 2) based on analysis performed with CLUSTAL software. The hydrophobicity plot of LB-Aut7 protein also shows a potential membrane insertion domain starting at amino acid 175.

Differential expression and genomic organization of *LB-AUT7*. In order to confirm the patterns of gene expression obtained from differential display for *LB-AUT7*, a full-length cDNA clone of *LB-AUT7* was used as a probe to perform Northern analysis. RNA from free-living mycelium and/or mycelium after interaction with red pine seedlings was isolated as described previously (17). To obtain RNA from mycorrhizal roots, red pine seeds were surface sterilized and allowed to germinate under conditions described by Richter and Bruhn (30). After seedlings had formed fine roots (4 to 6 weeks), synthesis experiments between *L. bicolor* and red pine seedlings were conducted as described previously (5). After 3 to 4 months, ectomycorrhiza formation was observed under a microscope prior to extraction of RNA for analysis. For northern analysis, 10 μg each of total RNA was electrophoresed on 1% agarose gels with formaldehyde and transferred to Hybond N$^+$ membranes (Amersham Corp., Arlington Heights, Ill.). Prehybridization, hybridization, and washings were done as described before (38). RNA gels were also stained with SYBR Green II (Molecular Probes, Eugene, Oreg.) to confirm the quality of RNA and also equality of loadings in each lane. As seen in Fig. 3A, the expression of *LB-AUT7* was dependent upon interaction with red pine seedlings, and its expression was not detectable in free-living mycelium. We also confirmed the in vivo relevance of the *L. bicolor LB-AUT7* cDNA clone as symbiosis regulated. RNA from red pine-*L. bicolor* ectomycorrhizal roots showed differential expression of *LB-AUT7* compared to RNA isolated from free-living *L. bicolor*. As shown in Fig. 3B, *LB-AUT7* transcript was detected only from mycorrhizal roots and not from free-living mycelium or from nonmycorrhizal pine roots.

The fact that *LB-AUT7* is differentially expressed even in mycorrhizal roots indicates that this gene is regulated by interaction with a plant host and that a continual expression of this gene is needed for the maintenance of ectomycorrhizae in red pine by *L. bicolor*. As per the eucalyptus × *P. tinctorius* model system proposed by Tagu and colleagues (35, 36), the preinfection stage (0 to 12 h) may have an important role in signal exchange and continuation to the next stage of symbiotic interaction. We have identified differentially expressed cDNA clones from *L. bicolor* after 24 h of interaction also and are currently characterizing them. These clones may represent determinants of establishment of symbiotic association between *L. bicolor* and red pine. Clones of 6-h interaction may serve as essential genes in the establishment of recognition events and developmental processes for ectomycorrhizae formation as well as maintenance of the symbiotic interaction.

We have also extracted RNA from mycorrhizal roots of aspen (*Populus tremuloides*) colonized by *L. bicolor* and larch (*Larix decidua*) colonized by *Sullius* spp. and subjected the RNA to Northern analysis using *LB-AUT7* cDNA as a probe. A weak signal was detected with aspen and larch (data not shown). Thus, it seems that the *LB-AUT7* expression may be specific to or regulated by symbiotic interaction with various plant hosts. Further screening of ectomycorrhizal roots formed by various ectomycorrhizal fungi on their respective plant hosts needs to be performed to determine the expression of *LB-AUT7* homologues.

To determine the gene copy number, we performed Southern analysis. Genomic DNA from *L. bicolor* was isolated as described by Reymond (28), and 10 μg each of genomic DNA was digested with restriction enzymes *Bam*HI, *Eco*RI, and *Pst*I. These digested DNA fragments were electrophoresed, transferred to a Hybond N$^+$ membrane (Amersham Corp.), and cross-linked by using a UV cross-linker (Fisher Biotech, Itasca, Ill.). Hybridization to the probe and washing of the filter were carried out under relatively high stringency conditions as described previously (17). Genomic Southern blotting of *LB-AUT7* has indicated that there is possibly only one copy of this gene (Fig. 3C). We also detected a second, faint band in the *Eco*RI digest at the 6-kb range, which may be due to hybridization to another related gene with limited homology in *L.*

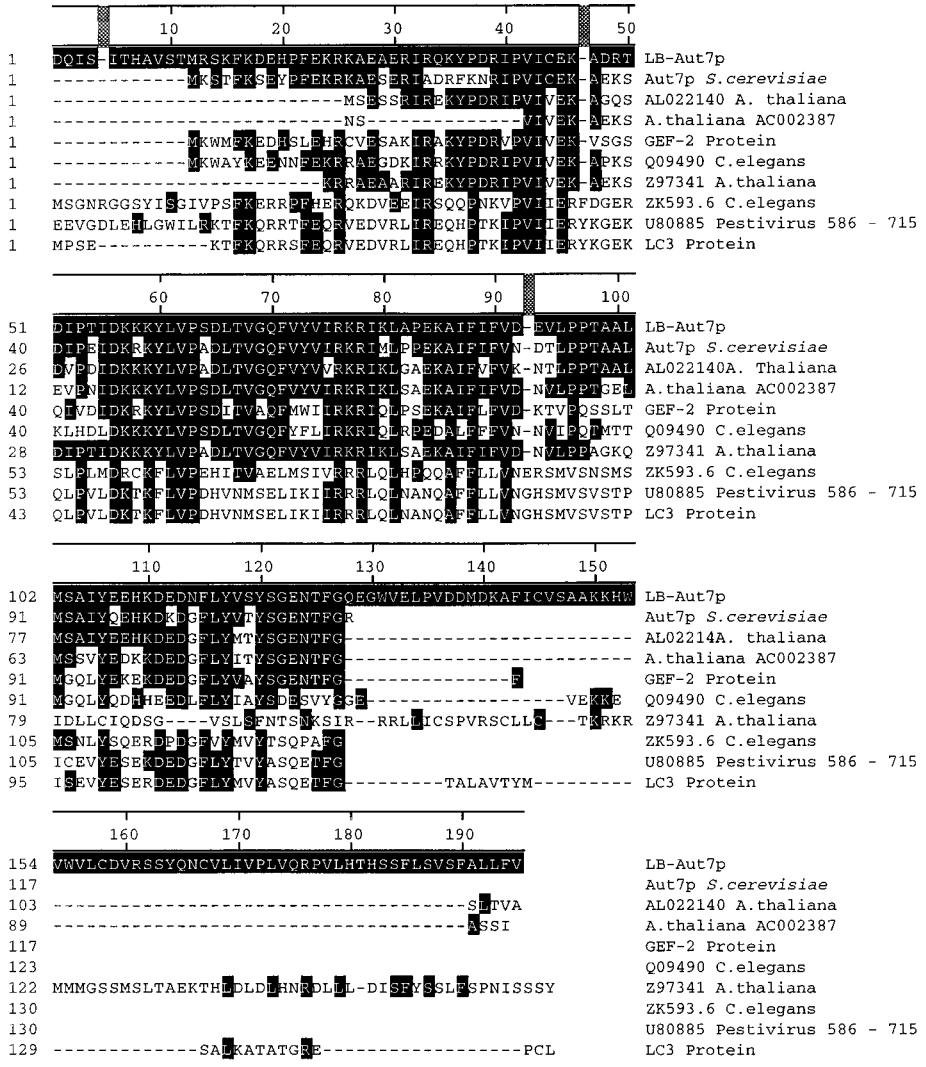
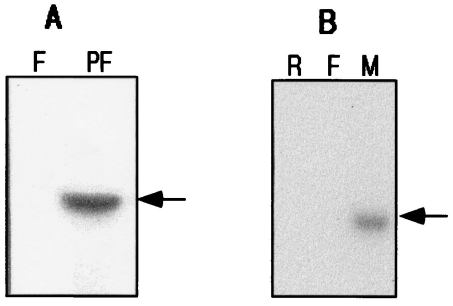


FIG. 2. Multiple sequence alignment of LB-Aut7p by the CLUSTAL method with PAM250 residue weight table. Residues identical with LB-Aut7p are shown on a black background. From U80885 only amino acids 586 to 715 are included in the alignment. A. thaliana, *Arabidopsis thaliana*; C. elegans, *Caenorhabditis elegans*.

bicolor. In *S. cerevisiae* no additional Aut7p homologues encoded by the genome are found, but since in rat two Aut7p homologues (LC3 and GEF-2) have been detected (also in *Arabidopsis* spp. there are several homologous proteins), this suggests the existence of several related proteins in the same organism. All these proteins are most likely involved in vesicle transport or the attachment of vesicles to microtubules. We could detect only one type of transcript for *LB-AUT7* under the experimental conditions used or from the mycorrhizal roots. Further characterization of genomic clones of *LB-AUT7* is currently in progress.

***LB-AUT7* can functionally substitute for its yeast homologue during autophagy.** In yeast, Aut7p is implicated in autophagy. It functions by binding to microtubules via Aut2p and thus mediates attachment of autophagosomes for their transport to the vacuole (23). We looked for the ability of *LB-AUT7* to complement the autophagic defects seen in *aut7*Δ yeast cells (23). *LB-AUT7* was expressed from centromeric plasmids under the control of the *CYC1*, *ADH*, and *TEF* promoters (26). *LB-AUT7* cDNA was inserted as an *Eco*RI-*Spe*I fragment into the respective sites of pRS416-TEF (26). *Eco*RI-*Xba*I fragments from *LB-AUT7* were similarly inserted in the respective sites of pRS416-CYC1 and pRS416-ADH (26). Cells were grown to the stationary phase and after Western blotting were analyzed with antibodies directed against aminopeptidase I as described previously (23). Under control of the *TEF* promoter an almost complete complementation of the maturation defect of proaminopeptidase I was detected (Fig. 4, lane 4). Expression using the weaker *CYC1* and *ADH* promoters resulted in only partially processed aminopeptidase I (Fig. 4, lanes 3 and 5). Expression of *LB-AUT7* in *aut7*Δ yeast cells with the *TEF* promoter further resulted in complementation of the reduced survival rate during starvation for nitrogen and the defect in accumulation of autophagic vesicles in the vacuole during starvation in the presence of phenylmethylsulfonyl fluoride (data not shown).

Since symbiotic initiation is a process of developmental regulation and differential gene expression in response to plants (35, 36), *LB-AUT7* may play an important role in the differentiation and development of *L. bicolor* in response to plant signals. In addition, since there will be changes in the nutritional status of the fungus during the establishment of symbi-



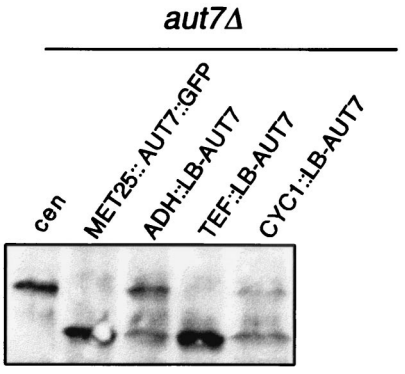


FIG. 4. Complementation of the maturation defect of proaminopeptidase I in *aut7*Δ yeast cells by *LB-AUT7* expressed under control of the *TEF* promoter. *LB-AUT7* cDNA was inserted in pRS416-TEF, pRS416-CYC1, or pRS416-ADH. Cells were grown to the stationary phase and after Western blotting were analyzed with antibodies directed against aminopeptidase I.

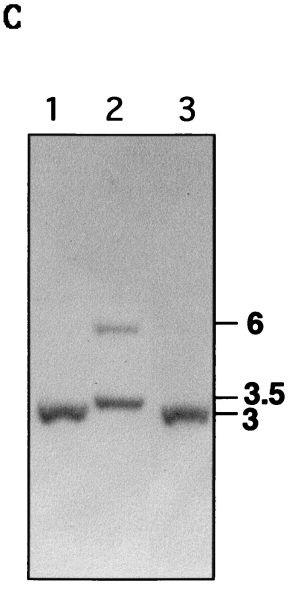


FIG. 3. (A) Northern analysis of *LB-AUT7* expression. Shown is an RNA blot of *L. bicolor* RNA isolated from free-living mycelium (lane F) and after 6 h of interaction with red pine seedlings (lane PF). Ten micrograms of total RNA was used per lane. Full-length ^{32}P-labeled *LB-AUT7* cDNA was used as a probe for hybridization. (B) Northern analysis using total RNAs from nonmycorrhizal red pine roots (lane R), free-living *L. bicolor* (lane F), and mycorrhizal roots (lane M) was performed as described for panel A. (C) Southern analysis of *LB-AUT7* from *L. bicolor*. Ten micrograms each of *L. bicolor* genomic DNA digested with *Bam*HI (lane 1), *Eco*RI (lane 2), and *Pst*I (lane 3) was used per lane. Hybridization was done with ^{32}P-labeled *LB-AUT7* cDNA. Molecular sizes are shown in kilobases.

osis, expression of proteins such as LB-Aut7p may serve as a means to recycle the cellular components to synthesize new macromolecules for growth of the fungus. It has been shown with several ectomycorrhizal fungi that there is an increase in the production and turnover of vesicles during symbiotic interaction with their plant hosts (19), and proteins such as LB-Aut7p are probably involved in this vesicular transport. Also in yeast an essential function of Aut7p was found for the cell differentiation process of sporulation (23). Motif searches using PRODOM and PSORTII indicated that the LB-Aut7p also has several tyrosine kinase phosphorylation sites. Thus, it is possible that LB-Aut7p activity is regulated through phosphorylation state and that in turn regulates other proteins involved in vesicle transport and differentiation. This may also involve cyclic AMP-mediated pathways which are regulated by the nutritional status of the fungus during interaction. Further analysis through in situ localization will allow the unraveling of the definitive role LB-Aut7p plays during and after the establishment of symbiotic interaction of *L. bicolor* with red pine.

Isolation of *LB-AUT7* out of a cDNA library prepared from the RNA of symbiotic fungal cells confirms expression of this gene during symbiosis. Since the expression of the *LB-AUT7* gene in free-living fungus cannot be detected (Fig. 3B), its expression seems to be dependent on a symbiotic interaction with its plant host. Due to the 77% identity between LB-Aut7p and yeast Aut7p (Fig. 2), together with the functionality of *LB-AUT7* in *aut7* deletion mutant yeast cells during autophagy (Fig. 4), we also expect a function of *LB-AUT7* in the transport of vesicular intermediates or the attachment of vesicles to microtubules. Taken together this might lead to the hypothesis that autophagy itself or a related vesicle transport process is induced during symbiosis.

Initiation of ectomycorrhizal development might involve activation of primary or universal regulator genes to transcribe specific target genes for mycorrhiza formation. This may include expression of genes for signal transduction as well as genes involved in metabolism and biosynthesis. The ectomycorrhizal signal transduction pathway at the very early stages of symbiosis in response to plant signals may include signaling and transcription cascades which can be modulated by feedback modulation as seen in yeast and other fungal systems (3, 25). Once the initial stages of interaction have occurred through the primary cascade of gene expression between both partners, further development of ectomycorrhizae may proceed through expression of secondary gene expression cascades, resulting in the formation of ectomycorrhizae (4). This may also result in limited host defense response to limit further infection by other mycorrhizal fungi (1, 22). Thus, involvement of genes such as *LB-AUT7* may play a key role in preparing the fungus for entry into the plant host root by providing necessary components for hyphal biogenesis and differentiation. Currently we are in the process of characterization of genomic clones of *LB-AUT7* and its regulatory elements to establish its functional significance for ectomycorrhiza formation as well as to determine the regulation of this gene by signals from red pine seedlings.

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