Symbiosis-regulated expression of an acetyl-CoA acetyltransferase gene in the ectomycorrhizal fungus *Laccaria bicolor*

Shiv T. Hiremath, Sujata Balasubramanian, Jun Zheng, and Gopi K. Podila

Abstract: The ectomycorrhiza is a symbiotic organ generated from the intricate association of fungal hyphae and plant root. The establishment of the ectomycorrhiza is a coordinated process of cross-talk between plant and fungus, followed by metabolic, developmental, and structural changes in the fungus, resulting in its growth toward the root. The initial stages of the symbiotic association are significant, since the direction of the association is determined by the gene expression level shifts that occur at this time. We have isolated a *Laccaria bicolor* (Maire) Orton cDNA clone corresponding to acetyl-CoA acetyltransferase (Lb-AAT), which is expressed during interaction with red pine roots and is symbiosis regulated. Acetyl-CoA acetyltransferase (EC 2.3.1.9) is an enzyme of the β-oxidation pathway that degrades long-chain fatty acids to acetyl-CoA. Expression of Lb-AAT is regulated by plant presence, by glucose, and by the presence of acetate or oleate in the medium. It is proposed that the role of Lb-AAT in the symbiosis is generation of two carbon compounds from stored lipids and generation of acetoacetyl-CoA in early interaction facilitating net growth from existing cell material. These results coupled with recent microarray analysis that revealed coordinated expression of malate synthase and other lipid metabolism genes along with Lb-AAT, suggest that this role for Lb-AAT could be an important part of preinfection process in ectomycorrhizal symbiosis and in the transfer and utilization of the carbon in the fungus.

Key words: acetoacetyl-CoA, acetyl-CoA acetyltransferase, β-oxidation, ectomycorrhizae, *Laccaria bicolor*, *Pinus resinosa*, symbiosis.

Résumé : L'ectomycorhize est un organe symbiotique constitué d'une association structurée, entre des hyphes fongiques et une racine. La formation d'une mycorhize est un processus coordonné, basé sur des échanges de signaux entre la plante et le champignon suivis de changements métaboliques, développementaux et structuraux, chez le champignon, entraînant sa croissance vers la racine. Les stades initiaux de l'association symbiotique sont significatifs puisque la direction de l'association est déterminée par les modifications au niveau de l'expression des gènes, qui surviennent à ce moment. Les auteurs ont isolé le cADN du *Laccaria bicolor* (Maire) Orton correspondant à l'acétyltransférase de l'acétylCoA (Lb-AAT), qui s'exprime au cours de l'interaction entre les racines du pin rouge et est régulé en symbiose. L'AAT (EC 2.3.1.9) est une enzyme du sentier d'oxydation β, lequel dégrade les longues chaînes des acides gras en acétylCoA. L'expression du Lb-AAT est régulée par l'essence végétale, le glucose et la présence d'acétate ou d'oléate dans le milieu. On propose que le rôle du Lb-AAT, dans la symbiose, serait la formation de composés à deux carbones à partir des lipides de réserve et la formation d'acétylCoA au début de l'interaction, facilitant une croissance nette, à partir du matériel cellulaire existant. Ces résultats couplés avec des analyses récentes par puces à ADN, qui révèlent une expression coordonnée des gènes de la malate synthase et autres gènes du métabolisme lipidique, incluant le Lb-AAT, suggèrent que ce rôle de la Lb-AAT serait un constituant important du processus de précolonisation de la symbiose ectomycorhizienne, et du transfert et de l'utilisation du carbone dans le champignon.

Mots clés : acétoacétylCoA, acétyltransférase de l'acétylCoA, β-oxydation, ectomycorhizes, *Laccaria bicolor*, *Pinus resinosa*, symbiose.

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Introduction

The ectomycorrhiza is a symbiotic organ formed from coordinated interaction between plant root and fungal hyphae. A vast majority of gymnosperms and angiosperms exhibit this type of association (Smith and Read 1997). While the fungal partner derives fixed carbon from the plant (Wu et al. 2002), the plant partner in turn benefits through improved availability of mineral nutrients like nitrogen and phosphorous (Marschner and Dell 1994; Rousseau et al. 1994; Brun et al. 1995; Perez-Moreno and Read 2000), which confer host plants with higher resistance to biotic stress like pathogens (Duchsene et al. 1989). Tolerance to abiotic stress like drought (Boyle and Hellenbrand 1991) and seedling survivability in reclamation soils is also improved by ectomycorrhizal presence (Richter and Bruhn 1989, 1993).

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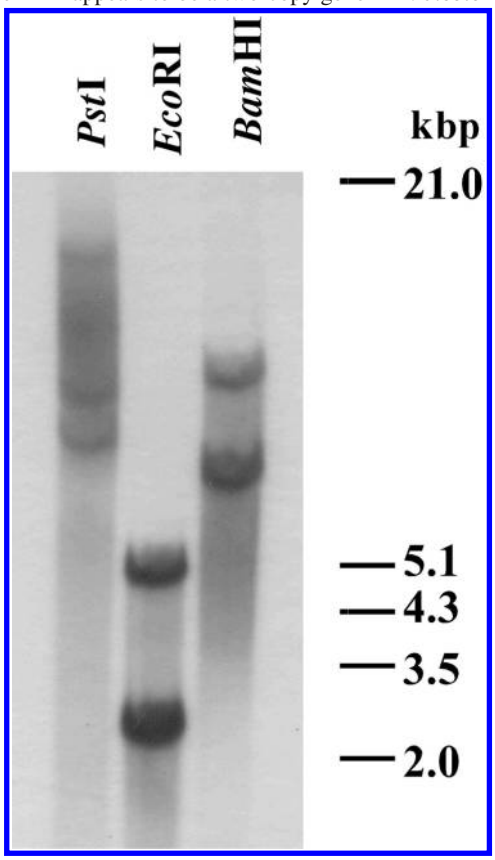
Fig. 1. Southern analysis of *Laccaria bicolor* genomic DNA. Ten micrograms of *L. bicolor* genomic was digested with *Pst*I, *Eco*RI, or *Bam*HI and probed with the 1.1 kb coding sequence of *Lb-AAT* cDNA. *Lb-AAT* appears to be a two-copy gene in *L. bicolor* genome.



The establishment of the ectomycorrhiza at the molecular level is a complex and coordinated process involving both partners. The morphologically observable stages that lead to the formation of the ectomycorrhiza can broadly be divided into preinfection, initiation, differentiation, and mycorrhizal function (Martin and Tagu 1999), which are associated with distinct changes in gene-level regulation of cell biochemistry, physiology, and finally in anatomical structure. The preinfection stage in the fungus involves the perception of the plant host and subsequent response to the presence of the plant host. The responses in both partners determine the direction and progress of the following steps of the symbiosis and the success of the association in the longer term. While the interaction between the partners in the established ectomycorrhiza has received much attention (Martin et al. 1995, 1998; Nehls et al. 1998, 1999, 2001*a*, 2001*b*; Johansson et al. 2004), the crucial preinfection stage is poorly understood at the biochemical and molecular level.

It is known that several signaling mechanisms are triggered, resulting in altered gene expression in the fungus (Martin and Hilbert 1991; Martin et al. 1999; Martin et al. 2001; Sundaram et al. 2001; Podila et al. 2002; Le Quere et al. 2004; Duplessis et al. 2005). *Laccaria bicolor* is a basidiomycete fungus known to associate with several temperate tree species to form ectomycorrhizal associations (Gagnon et al. 1987; Richter and Bruhn 1989; Kropp 1990; Cripps and Miller 1993). We have been using *L. bicolor* as a model ectomycorrhizal fungus to study preinfection stage symbiosis with plant hosts such as *Pinus resinosa* Ait. (red pine) and *Populus tremuloides* Michx. (trembling aspen). Along with genes involved in signaling, the differential expression of genes involved in other processes necessary for symbiosis is also observed in the preinfection stage of ectomycorrhiza formation. *LB-AUT7* related to autophagocytosis (Kim et al. 1999*a*), malate synthase (Balasubramanian et al. 2002), deoxyhypusine synthase, phosphoenolpyruvate carboxykinase, and chitin synthase (Podila et al. 2002) are among those known from the preinfection stage of interaction between *L. bicolor* and *P. resinosa*. From the expression of the above-mentioned genes at the preinfection stage of interaction, we infer a shift in gene expression in response to host perception at a very early stage in the association.

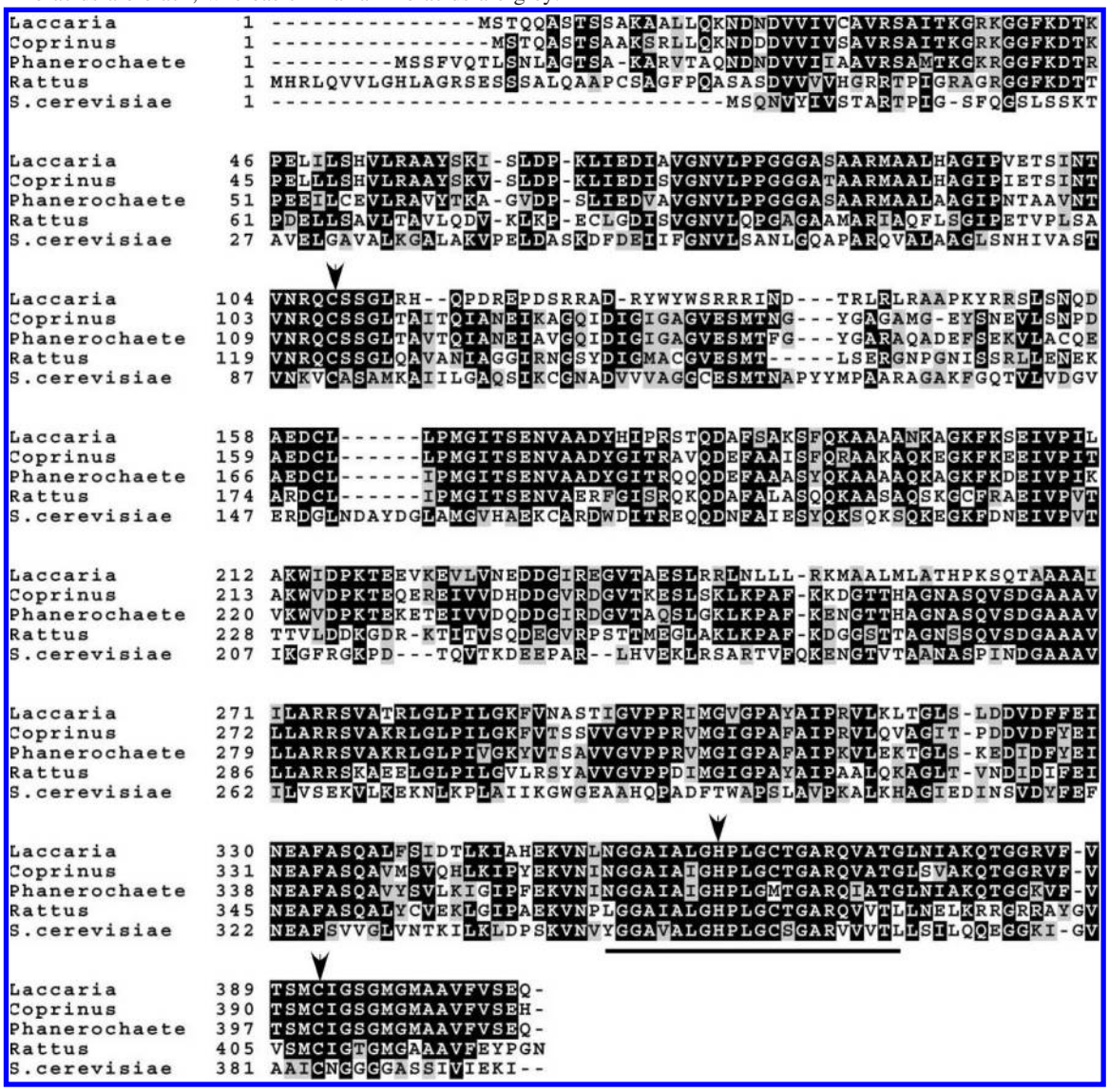
An in vitro system has been established to study preinfection level gene expression in the *L. bicolor – P. resinosa* interaction (Kim et al. 1998, 1999*b*). Using this system, an acetyl-CoA acetyltransferase gene has been identified from *L. bicolor* as differentially regulated during the preinfection stage of ectomycorrhiza formation (Podila et al. 2002). The peroxisomal form of acetyl-CoA acetyltransferase (EC 2.3.1.9) (AAT) is an enzyme of the β-oxidation process by which fatty acids are broken down to two-carbon acetyl-CoA units. Another form of AAT known as thiolase II is involved in sterol biosynthesis (Igual et al. 1992). AAT enzymes are ubiquitous and present in all organisms from bacteria to humans and share high sequence similarity indicating their common origins. These two-carbon units generated by peroxisomal AAT can contribute to energy generation via the tricarboxylic acid pathway or anabolism via the glyoxylate pathway (Nelson and Cox 2000). β-Oxidation is a major catabolic pathway in the vesicular–arbuscular mycorrhiza (Bago et al. 2002), and faulty peroxisome biosynthesis related to defective β-oxidation in the pathogenic fungus *Colletotrichum lageniarum* is implicated in the loss of pathogenic capacity required for invasiveness (Kimura et al. 2001). Zellweger syndrome in humans is also related to β-oxidation defects because of defective thiolase and is lethal (Lazarow and Moser 1989). More recently, the gene expression profiling studies conducted with ectomycorrhizal systems of *Paxillus involutus* × *Betula pendula* (Wright et al. 2005) and *Pisolithus microcarpus* × *Eucalyptus grandis* (Duplessis et al. 2005) revealed the regulated expression of AAT genes and corresponding genes involved in β-oxidation of fatty acids during the development of mycorrhizae. These studies of a variety of systems have proved that the process of β-oxidation and the thiolase function are crucial to proliferating tissue. Thus, in the present study we began to investigate the direction of carbon flux during the early stages of the ectomycorrhizal symbiosis, and explored the utilization of carbon sources by the fungus and their impact on gene expression. We also examined the structure of the *Lb-AAT* gene and its regulation under these conditions and postulated a role for *Lb-AAT* in the mycorrhizal symbiotic interaction.

Materials and methods

Cultures and media

Laccaria bicolor (Maire) Orton DR170 is a basidiocarp

Fig. 2. Multiple sequence alignment of Lb-AAT with peroxisomal thiolases. AAK48841.1, *Laccaria bicolor*; Phchr1/scaffold_3:19324–20933, *Phanerochaete chrysosporium*; CC1G_12895.1, *Coprinus cinereus*; BAA14107.1, *Rattus norvegicus*; YPL028W, *Saccharomyces cerevisiae*. Arrows indicate catalytic triad amino acids, and the peroxisomal acetyl-CoA acetyltransferase signature sequence is underlined. Identical amino acids are black, whereas similar amino acids are grey.



isolate obtained from mixed forest red pine – aspen plantations in the Upper Peninsula of Michigan. The fungal mycelium was cultured on modified Melin Norkans (MMN) growth medium (Marx 1969). For interaction experiments and alternative carbon source experiments, *L. bicolor* cultures were grown on MMN growth medium over a cellophane membrane for convenience in transferring. Interaction experiments were set up according to Kim et al. (1999*b*) in magenta boxes using the seedling-over-mesh technique. Seedlings were first acclimatized in interaction medium (Wong and Fortin 1989), which is a minimal mineral medium for 24 h before transfer of fungal mycelium into the set-up. Interactions were also conducted in the presence of supplemental glucose at 2.5 mmol/L. Starvation controls for interaction studies included incubation of transferred mycelia in interaction medium alone, interaction medium supplemented with 0.5 mmol/L glucose, and interaction medium supplemented with 2.5 mmol/L glucose. The levels of glucose used were selected after several time course experiments were conducted to determine the amount of glucose required to cause catabolite suppression and to last through the experiment, as indicated by glucose oxidase assays. To establish an alternative carbon source, cellophane-grown cultures were transferred to interaction medium in Petri dishes containing the relevant carbon supplement. Sodium salts of acetate, glyoxylate, isocitrate, and oleate (SigmaAldrich, St. Louis, Missouri) at 10 mmol/L were used as sole carbon sources and in combination with 2.5 mmol/L glucose in the interaction medium.

Cloning and sequence of acetyl-CoA acetyltransferase

The acetyl-CoA acetyltransferase from *L. bicolor* was identified from a cDNA library made in lambda screen phagemid vector (Novagen, Madison, Wisconsin), which

represented fungal mRNA from the first 72 h of interaction (Sundaram et al. 2001). The library was screened using a mRNA probe generated from subtractive hybridization of control RNA and subsequent plaque screening using polymerase chain reaction (Podila et al. 2002). Sequencing of the cDNA clone was done using the ABI-310 automated sequencing system from PE Applied Biosystems (Foster City, California). DNA sequencing analysis was done using Sequencing Analysis software version 3.3 from PE Applied Biosystems, MacDNAsis software version 3.7 (Hitachi Instruments, San Jose, California), and BLAST from the National Center for Biotechnology Information (NCBI) (Altschul et al. 1997). Full-length clone was obtained using the 5′ and 3′ rapid amplification of cDNA Ends kit (Roche Diagnostics, Indianapolis, Indiana). Two 5′ antisense nested primers were designed from the known sequence AAT416A 5′TGTGTCATTGATTCGACGCCGGCT3′ and AAT309A 5′GACGGTATTGATGGAGGTTTCGAC3′. Phylogenetic analysis for homology comparison was carried out using PAUP software version 4.0b8 with bootstrap values of 100 (Farris 1983; Swofford 2001). Multiple sequence alignment was done using the Multialin tool (Corpet 1988) against human, representative fungal, bacterial, and plant peroxisomal thiolases. The alignment output was inserted into the boxshade tool of the embnet server for visualization. Sequence analysis to study protein properties was performed using the Scanprosite tool (Bucher and Bairoch 1994; Hoffman et al. 1999) of the ExPASY tool listing from the Swiss Institute of Bioinformatics (http://www.expasy.ch).

Southern and Northern hybridization analysis

Genomic DNA was extracted as described previously (Kim et al. 1998) from 2- to 3-week-old cultures of *L. bicolor* grown on MMN medium, then harvested and ground in liquid nitrogen. Ten micrograms each of genomic DNA, digested with *Bam*HI, *Eco*RI, and *Pst*I, was used for the Southern hybridization. Hybridization was carried out at 42 °C as described in Sambrook et al. (1989) and Kim et al. (1998). Total RNA for Northern analysis from tissue harvested at 4, 8, 12, and 24 h for interaction, and 12 and 24 h for alternative carbon treatment, was extracted as described in Balasubramanian et al. (2002). Radioactive probes for both Northern and Southern analyses were generated from a 400-base pair fragment of *Lb-AAT* cDNA 3′-end, including the untranslated region and a small portion of coding sequence. This piece is isolated using restriction digestion and purification from the full-length cDNA clone. ^{32}P-dCTP-labeled probe was made using the RediprimeII Random primer labeling kit (Amersham Pharmacia Biotech, Piscataway, New Jersey). RNA blots were scanned using the Bio Rad Phosphorimager FX (Bio Rad, Foster City, California).

Quantification of signal intensity from the northern hybridization analyses was done using Quantity One software (Bio Rad). Each experiment was replicated three times. RNA extracted from each of three sets of treated tissue was used for Northern analysis. The signal detected at the control depicting background levels of expression was used as standard and assigned a value of 1. This value is obtained using all the pixels spanning the RNA signal. The ribosomal RNA signal from all lanes, in comparison with the control, was quantified to obtain a normalization factor. Quantifications from the RNA blots probed with *Lb-AAT* were then divided by this factor to obtain accurate fold-regulation values. Three densitometric scans per blot were averaged for final values.

Genomic library screening

A genomic library of *L. bicolor* genomic DNA was constructed using *Bam*HI arms in the lambda bluestar vector (Novagen, Madison, Wisconsin). This library was screened with the 1.1-kilobase coding region of *Lb-AAT* cDNA using the fragment amplified by AAT3S modified to include an *Nde*I site 5′ CACTCCATCACACATATGTCAATCCAACAAGCTTCCACC3′ and AAT1252A 5′ with a *Sal*I site. 5′CCTGGAGGCGTCGACCTGCTCACTAACGAAAACAG CAGC3′ to identify putative genomic clones. Inserts were subcloned into bluescript vector (Stratagene, LaJolla, California) and sequenced using the ABI-310 automated sequencing system from PE Applied Biosystems. DNA sequencing analysis was done using Sequencing Analysis software version 3.3 from PE Applied Biosystems and BLAST from the NCBI (Altschul et al. 1997). Exons were identified based on the GENESCAN and TwinScan tools (Burge and Karlin 1997; Korf et al. 2001) and comparison with the cDNA sequence. Potential transcription start, TATAA box, and several elements characteristic of promoters of lipid metabolism genes were identified using the Neural Network promoter prediction tool (Adams et al. 2000) and Mat Inspector software (Cartharius et al. 2005).

Results

Gene structure of Lb-AATg

Based on Southern analysis, *Lb-AAT* is a two-copy gene (Fig. 1). Hybridization of radiolabeled probe was observed in two large-sized fragments in each of the three digests. In addition, there were no *Bam*HI or *Pst*I sites in the genomic sequence of *LbAATg* (GenBank accession No. AF340027). The genomic sequence of *Lb-AATg* from *L. bicolor* was 3.3 kb in length. It had five exons 104, 288, 383, 326, and 124 bp in length and four introns ranging in length from 55 to 68 bp. The TATAA box and transcription start was predicted to be located between bp 801 and 851 in the 0.86 kb upstream region. The region downstream of the stop site was about 1 kb in length. The five exons were identified between 862 and 2330 bp of the 3.3 kb sequence. Promoter sequence analysis also indicated the presence of conserved sequence elements for cAMP response element binding protein (CREB) (between 138 and 158 bp) and carbohydrate response element binding (CHREBP) (between 613 and 629 bp) in the 0.86 kb upstream region. A survey by the US Department of Energy Joint Genome Institute (DOE-JGI) of the recently released sequence from the haploid genome of *L. bicolor* (haploid strain H82 derived from diploid strain SN238) (Lammers et al. 2004) indicated that there is only one sequence highly similar to the AAT gene sequences cloned from *L. bicolor* strain DR170. While some other sequences exhibit some similarity, they do not have the features characteristic of LbAAT. Thus, it is likely that the diploid *L. bicolor* genome contains two copies of acetyl-CoA acetyltransferase gene.

Fig. 3. Phylogenetic analysis comparing Lb-AAT to other known and putative thiolases. National Center for Biotechnology Information (NCBI) accession numbers to sequences used in phylogenetic analysis: AAK48841.1, *Laccaria bicolor*; Phchr1/scaffold_3:19324–20933, *Phanerochaete chrysosporium*; CC1G_12895.1, *Coprinus cinereus*; AAW41040.1, *Cryptococcus neoformans* var. *neoformans*; XP_324153.1, *Neurospora crassa*; EAK83613.1, *Ustilago maydis* 521; AA62739.1, *Aspergillus nidulans*; EAL89695.1, *Aspergillus fumigatus*; CAA49605.1, *Yarrowia lipolytica*; AAH72706.1, *Danio rerio*; BAA04143.1, *Candida tropicalis*; EAK99762.1, *Candida albicans*; BAA14107.1, *Rattus norvegicus*; CAA47926.1, *Cucumis sativus*; NP_666342.1, *Mus musculus*; XP_468412.1, *Oryza sativa* (japonica cultivar-group); CAG88359.1, *Debaryomyces hansenii*; AAQ77242.1, *Helianthus annuus*; AAM20592.1, *Arabidopsis thaliana*; CAA46270.1, *Homo sapiens*; AAQ93070.1, *Glycine max*; CAA63598.1, *Brassica napus*; S72532, cucurbit.

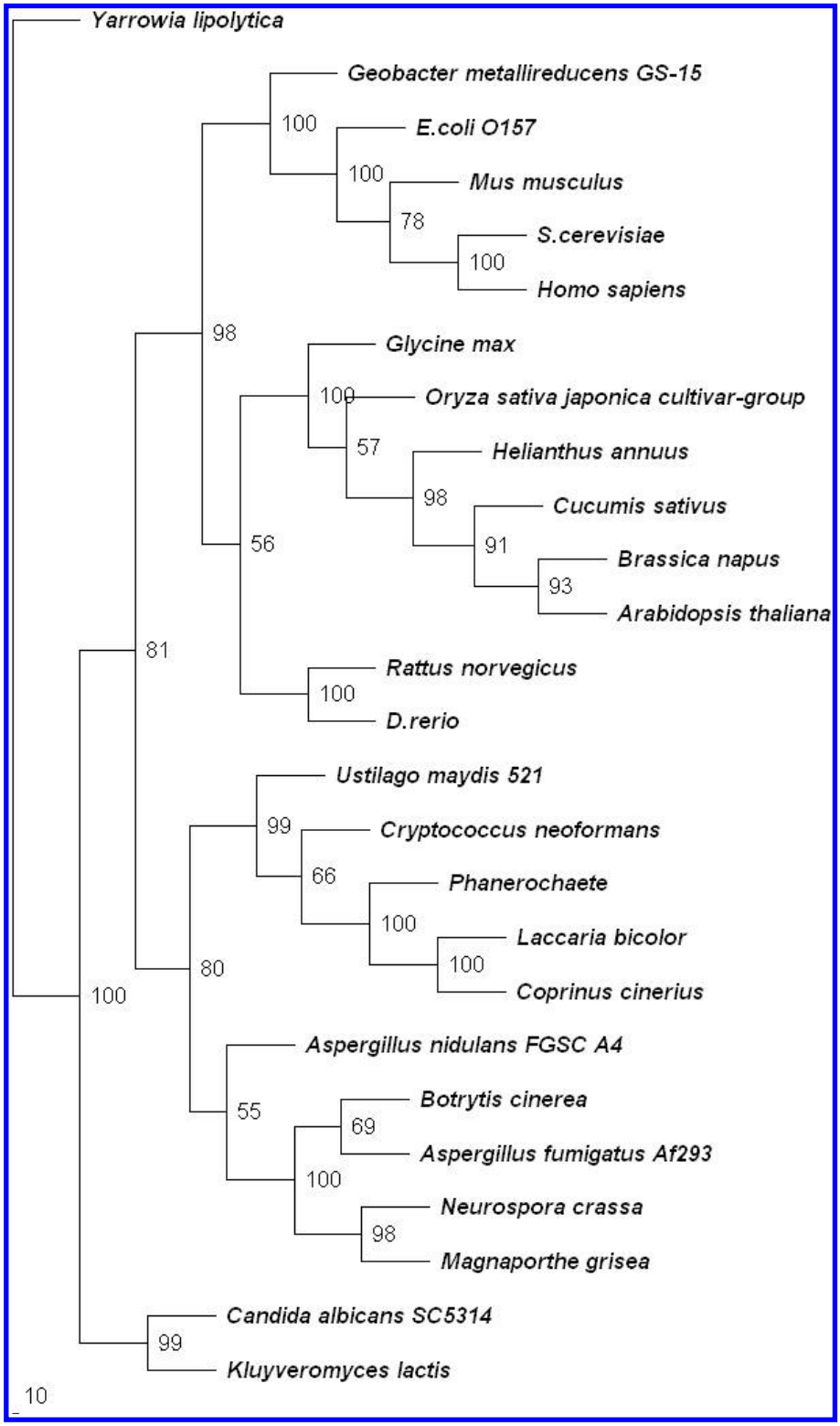
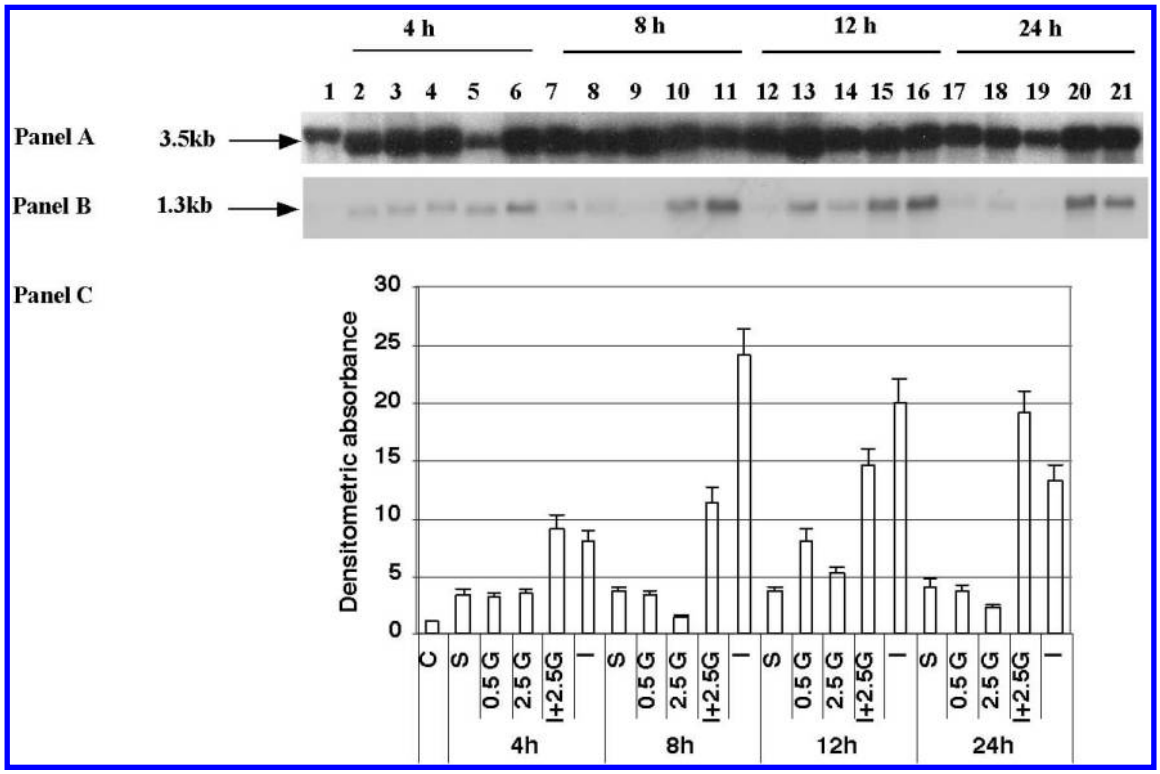


Fig. 4. Northern hybridization analysis of total RNA from interaction treated cultures of *Laccaria bicolor* with red pine. Where supplemented, glucose is at 2.5 mmol/L or 0.5 mmol/L at zero time point. In interaction, glucose at start of interaction is 2.5 mmol/L. (A) RNA analysis using *L. bicolor* ribosomal cDNA fragment as probe (signal at approx. 3.5 kb). (B) RNA analysis using acetyl-CoA acetyltransferase cDNA fragment as probe (signal at approx. 1.4 kb). Lanes: 1, glucose-grown control; 2, 4 h starvation, no glucose; 3, 4 h at 0.5 mmol/L glucose; 4, 8 h at 2.5 mmol/L glucose; 5, 4 h interaction with glucose; 6, 4 h interaction; 7, 8 h starvation, no glucose; 8, 8 h at 0.5 mmol/L glucose; 9, 8 h at 2.5 mmol/L glucose; 10, 8 h interaction with glucose; 11, 8 h interaction; 12, 12 h starvation, no glucose; 13, 12 h at 0.5 mmol/L glucose; 14, 12 h 2.5 mmol/L glucose; 15, 12 h interaction with glucose; 16, 12 h interaction; 17, 24 h starvation, no glucose; 18, 24 h at 0.5 mm glucose; 19, 24 h at 2.5 mmol/L glucose; 20, 24 h interaction with glucose; 21, 24 h interaction. (C). Quantifications from Northern analysis was done using Phosphorimager and Quantity One software. Data analyzed from triplicate experiments. Representative RNA blots are shown.



cDNA and protein sequence analysis of acetyl-CoA acetyltransferase from *Laccaria bicolor*

The characteristic peroxisomal thiolase signature NlnGGaIAlGHPlGcTG is found in Lb-AAT between the amino acids 352 and 368 (Fig. 2) (Bucher and Bairoch 1994; Hoffman et al. 1999). Crystal structure and mutagenesis studies of peroxisomal 3-ketoacyl-CoA thiolase (acetyl-CoA acetyltransferase) from pig heart revealed that two cysteines (Cys) and one histidine (His) residues are catalytically crucial (Mathieu et al. 1997). These are residues of the active site pocket of the thiolase. The conserved catalytic residues of *Saccharomyces cereviseae* AAT protein, Cys 125, His 375, and Cys 403 (Mathieu et al. 1997), correspond with Cys 108, His 362, and Cys 392 in Lb-AAT seen through multiple sequence alignments with yeast and rat thiolases (Fig. 2). Structure and motif analyses of the acetyl-CoA acetyltransferase from *L. bicolor* by the ScanProsite tool (Bucher and Bairoch 1994; Hoffman et al. 1999) enabled identification of six putative protein kinase C phosphorylation sites (AA10–12, 122–124, 132–134, 187–189, 241–243, 343–345), four putative casein kinase II phosphorylation sites (44–47, 154–157, 181–184, 320–323), and one tyrosine kinase phosphorylation site (123–130). Strong protein level homology with other closely related peroxisomal acetyl-CoA acetyltransferases (β-ketothiolases) from basidiomycete fungi *Coprinus cinereus* and *Phanerochaete chrysosporium* is evident (Fig. 2). Phylogenetic analysis also indicated that Lb-AAT is very similar to AATs from other basidiomycetes such as *Coprinus cinereus, Phanerochaete chrysosporium, Cryptococcus neoformans*, and *Ustilago maydis* (Fig. 3). It is also clear from phylogenetic analysis that the basidiomycete AATs were divergent from ascomycete AATs. Lb-AAT has 75% identity with basidiomycete fungal AAT from *C. cinereus,* 72% identity with *Phanerochaete chrysosporium*, 65% identity with *U. maydis* and *Cryptococcus neoformans*, 49% identity with *Candida tropicalis* acetyl-CoA acetyltransferases, 49% identity with *Aspergillus nidulans* and *A. fumigatus*, respectively (Fig. 3). The amino acid level identity was about 47% with rat and human and plants such as Arabidopsis, rice, and cucumber.

Based on Northern analysis, the transcript length of *Lb-AAT* was about 1.4 kb (Fig. 4). The transcript had a 1.1 kb open reading frame, coding for a 407-amino acid protein (GenBank accession No. AF346762.1).

Regulation of expression of *Lb-AAT* under interaction conditions

Based on time course experiments, the peak expression of

Fig. 5. Northern hybridization analysis of total RNA isolated from *Laccaria bicolor* cultures treated with alternative carbon sources. Glucose at zero time is at zero for an alternative sole carbon source or 2.5 mmol/L in conjunction with an alternative carbon source. Alternative carbon source is at 10 mmol/L. (A) RNA analysis using *L. bicolor* ribosomal cDNA fragment as probe (signal at approx. 3.5 kb). (B) RNA analysis using acetyl-CoA acetyltransferase cDNA fragment as probe (signal at approx. 1.4 kb). Lanes: 1, glucose-grown control; 2, acetate with glucose 12 h; 3, acetate 12 h; 4, acetate with glucose 24 h; 5, acetate 24 h; 6, isocitrate with glucose 12 h; 7, isocitrate 12 h; 8, isocitrate with glucose 24 h; 9, isocitrate 24 h; 10, glyoxylate with glucose 12 h; 11, glyoxylate 12 h; 12, glyoxylate with glucose 24 h; 13, glyoxylate 24 h; 14, oleate with glucose 12 h; 15, oleate 12 h; 16, oleate with glucose 24 h; 17, oleate 24 h; 18, glucose 12 h; 19, starvation without glucose 12 h; 20, glucose 24 h; 21, starvation without glucose 24 h. (C) Quantifications from Northern analysis was done using Phosphorimager and Quantity One software. Data analyzed from triplicate experiments. Representative RNA blots are shown.

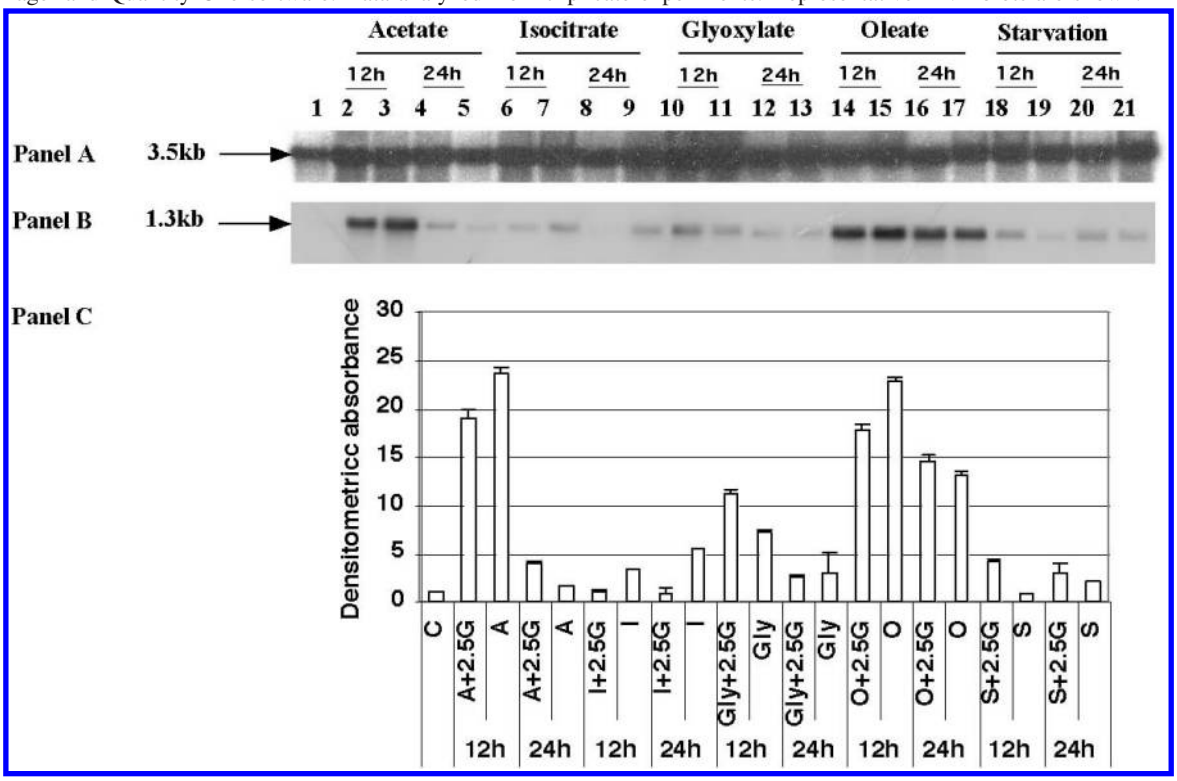
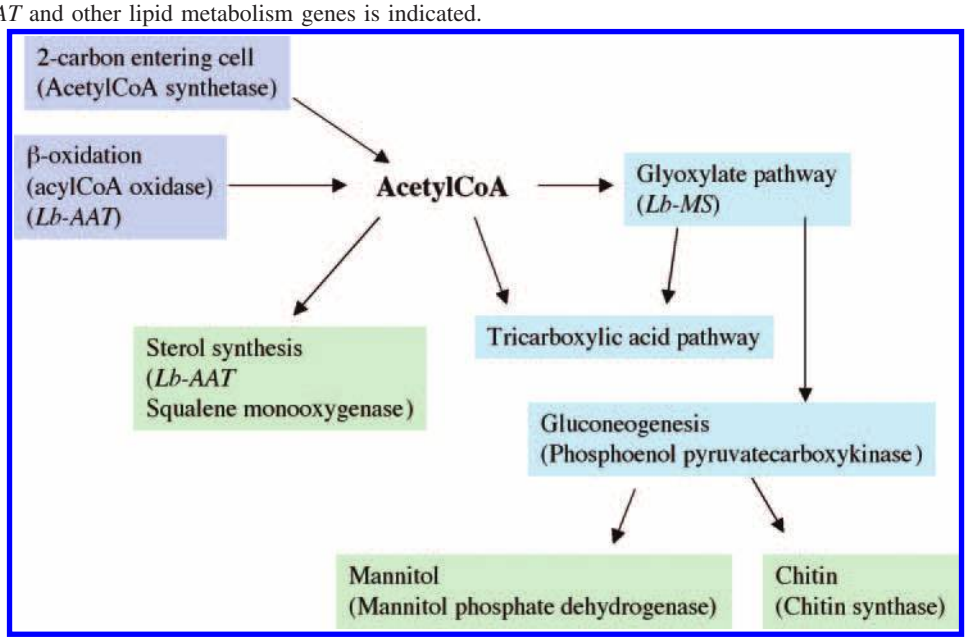


Fig. 6. Model for preinfection stage shift in carbon metabolism in *L. bicolor* – red pine symbiotic interaction. A flowchart of potential involvement of *LbAAT* and other lipid metabolism genes is indicated.



Lb-AAT was determined to be at 24 h in response to the plant interaction or in response to oleate as a carbon source. (Fig. 4). Controls, including glucose in the presence or absence of the plant, were used to eliminate the possibility of a starvation response. Time-points within the first 24 h of interaction with plant exudates showed an increase in expression over the glucose-grown control from 4 h with a peak at 8 h (Fig. 4, lanes 6, 11, 16, and 21), decreasing at 12 h, and further declining at 24 h. This upregulation of *Lb-AAT* was not a starvation response (Fig. 4, lanes 2, 7, 12, and 17), because the transcript levels from culture grown in MMN medium (lane 1) were clearly diminished in comparison with culture grown in the presence of plant. The addition of glucose, at either 0.5 or 2.5 mmol/L, caused a peak in upregulation at 12 h. While this is the peak for the glucose treatment, the levels of *Lb-AAT* were clearly two- to several-fold lower than the plant interaction response. The presence of glucose during the interaction attenuated *Lb-AAT* upregulation, compared with the interaction without glucose. The peak in upregulation of *Lb-AAT* expression occurred 12 h after interaction, and decreased by 24 h.

Regulation of expression of *Lb-AAT* under alternative carbon source treatments

Carbon sources other than glucose were supplied to the mycelium when transferred to interaction medium, and examined for expression of *Lb-AAT* at 12 h and 24 h. Acetate, isocitrate, glyoxylate, and oleate as sole carbon sources and in conjunction with glucose were supplied in the medium (Fig. 5). Controls included starvation with no glucose or with 2.5 mmol/L glucose as a supplement. The starvation treatment did not show significant upregulation with almost no detectable levels of *Lb-AAT* transcript at 12 h under glucose starvation (Fig. 5). The presence of acetate in the medium as the sole carbon source resulted in a transient upregulation of *Lb-AAT* at 12 h, followed by a decline at 24 h in the presence or absence of glucose. The presence of isocitrate caused limited upregulation of *Lb-AAT* at 12 h, whereas glucose caused comparatively greater upregulation than in its absence. At 24 h, transcript levels, in the absence of glucose, resembled initial levels prior to start of treatment (glucose-grown control, lane1). The signal from the isocitrate treatments, however, did not exceed those of the starvation treatment. The presence of glyoxylate caused approximately 11-fold upregulation of *Lb-AAT* at 12 h, followed by a decline at 24 h; its effects were comparable both in the presence and absence of glucose. Oleate in the medium generated a profound upregulation of transcript both in the presence (approx. 17-fold) and absence of glucose (approx. 23-fold), which remained upregulated through 24 h.

Discussion

Fatty acid metabolism is a major component of the physiology of the mycorrhizal fungi (Martin et al. 1984; Pfeffer et al. 1999; Bago et al. 2002). Fatty acids in fat droplets are a major carbon storage compound in free-living, glucose-grown ectomycorrhizal fungi (Martin et al. 1984). Lipid bodies are generated and used in spatially separated regions of the arbuscular mycorrhiza (Pfeffer et al. 1999; Bago et al. 2002). Laczko et al. (2003) showed strong changes in lipid profile from the first 21 d postinoculation in the *Pinus sylvestris – Pisolithus tinctorius* interaction. Our observation of the expression of acetyl-CoA acetyltransferase in the *L. bicolor – P. resinosa* interaction suggests fatty acid metabolism to be an active metabolic component of the early stages of interaction in the establishment of the ectomycorrhiza. The establishment of the symbiotic ectomycorrhiza is a complex and intricate process that is subject to a very stringent temporal and signal-response control, proceeding through the preinfection, initiation, differentiation, and functioning stages. In each of these stages, there is a significant gene expression modulation. Especially in the case of preinfection, there is a major shift in gene expression from the saprobic to the symbiotic stage. Several saprobic genes are downregulated, and others are upregulated through the early interaction. Several compounds (Smith and Read 1997) have been implicated indirectly in the regulation of the symbiosis, but the actual signaling molecules are not specifically identified. This is further complicated by the lack of host specificity in ectomycorrhizal interactions (Smith and Read 1997). Since there is a definite host preference (Richter and Bruhn 1993; Smith and Read 1997), it is certain that the given signaling compounds for a particular interaction can be identified.

Acetyl-CoA acetyltransferase or β-ketothiolase is an enzyme of β-oxidation of fatty acids (Nelson and Cox 2000). It catalyzes the final thiolytic cleavage of acetoacetyl-CoA to two molecules of acetyl-CoA. Regulation of metabolism-related gene expression by 4 h during interaction with the plant suggests that the fungus is able to respond to plant signals in a very short time. The lack of significant upregulation in the starvation controls, with or without supplemental glucose, indicates that the expression of *Lb-AAT* is interaction dependent and is upregulated as early as 4 h into the interaction (Fig. 4). Addition of glucose to the interaction treatment caused an upregulation pattern similar to the absence of glucose. However, the peak in upregulation is delayed, suggesting that glucose-based repression is overcome by interaction. Differential regulation of gene expression in the saprobic glucose-grown state, and the mycorrhizal state, is also seen in the *Amanita–Populus* interaction (Nehls et al. 2001*b*). The hexose transporter from *Amanita muscaria* (*AmMst*) (Nehls et al. 1998, 2001*b*) is upregulated in the presence of high glucose levels in the medium. The phenylalanine ammonium lyase gene (*AmPAL*) (Nehls et al. 1999, 2001*b*) is upregulated under low ambient glucose, but both genes become upregulated in the mycorrhiza. The perception of the host seems to cause the downregulation of several saprophytic mode genes during the mycorrhizal symbiosis (Hilbert and Martin 1988; Duplessis et al. 2005) and is also likely to override some or many of the responses to glucose-based repression.

Alternative carbon sources are able to upregulate *Lb-AAT* expression significantly (Fig. 5). Since fungi are very versatile in their metabolism, it is difficult to define what form these compounds are in when they enter the cell and should be a consideration for future study. Whether the compounds enter the cells, are metabolized by the cells, or simply act as signaling mediators, will be known once the preinfection is

completely dissected. The presence of oleate is seen to upregulate the enzymes of β-oxidation in *Neurospora crassa* (Kionka and Kunau 1985) and yeast (McCammon et al. 1990; Smith et al. 2000). Upregulation of *Lb-AAT* in the presence of oleate thus follows the similar regulation pattern. Oleate is a strong inducer of β-oxidation, since the presence of glucose along with oleate did not result in lower levels of *Lb-AAT* transcript. The upregulation of *Lb-AAT* in the presence of acetate at 12 h and subsequent downregulation at 24 h may be due to the depletion of the alternative substrate. Acetate is a two-carbon compound that is likely to contribute to the acetyl-CoA pool and subsequently, to the synthesis of acetoacetyl-CoA. Acetoacetyl-CoA can generate acetoacetate, which is a four-carbon compound that can be used as carbon currency or for the synthesis of sterols. Low levels of upregulation of *Lb-AAT* in the presence of glyoxylate and isocitrate may be due to the specificity of the former to an enzyme like malate synthase (Balasubramanian et al. 2002) and the involvement of the latter in other pathways and does not specifically upregulate *Lb-AAT* alone.

The observed expression of *Lb-AAT* along with acylCoA oxidase in preinfection (Podila et al. 2002, 2004) suggests that β-oxidation of reserve lipids is an active process at this time. This breakdown is essential for growth. The upregulation of *Lb-AAT* in the presence of acetate suggests that it may be also involved in the synthesis of acetoacetyl-CoA from acetyl-CoA. Acetoacetate is an early precursor in the acetate–mevalonate pathway of sterol synthesis in vesicular–arbuscular mycorrhiza (Fontaine et al. 2001). The synthesis of sterols is suggested by the expression of squalene monooxygenase in preinfection (Podila et al. 2002) in the *L. bicolor – P. resinosa* interaction. These sterols may be signal molecules or may be incorporated into the cell membrane that is synthesized in the fungus and grows toward the plant host (Nelson and Cox 2000; Fontaine et al. 2001). Expression of *Lb-AAT* and squalene monoxygenase in preinfection may be the beginning of the lipid profile change seen in later stages reported by Laczko et al. (2003).

The expression of *Lb-AAT* at the onset of the symbiosis when the fungus grows toward the plant host suggests that the breakdown of reserve food sources may be used to generate viable substrates for biosynthesis. This indicates an overall shift in cellular carbon metabolic processes. The products of *Lb-AAT* are then channeled into the glyoxylate pathway (Lammers et al. 2001; Balasubramanian et al. 2002), which replenishes the substrates of the tricarboxylic pathway while also providing intermediates for pathways like amino acid biosynthesis and gluconeogenesis. Recent gene expression studies from different soil compartments of ectomycorrhizal fungus *P. involutus* mycorrhizal with *B. pendula* showed that β-oxidation-related genes from *P. involutus* such as acetyl-CoA dehydrogenase and AAT are upregulated in the rhizomorph part of the fungus (Wright et al. 2005). This is analogous to the fungus receiving signals from the plant in our experiments, as the fungus is connected to the plant, but not inside the plant roots. Sterol biosynthesis through acetoacetate either for membrane biogenesis or signaling is another possible role of *Lb-AAT* at preinfection. It has been well established that many ectomycorrhizal fungi are capable of using fatty acids and lipids as carbon sources (Hattori et al. 2003). It can be inferred that fatty acids like oleate and two-carbon compounds like acetate, which can be reduced to acetyl-CoA, and also glucose can regulate the expression of *Lb-AAT*. Alternatively, these compounds or their analogs may be the plant root signals that are presented to the fungus. Rhizobacteria and streptomyces species are also known to produce compounds that induce beta oxidation enzyme genes in mycorrhizal fungi (Requena et al. 1999; Schrey et al. 2005). In the *Laccaria* – red pine system, interaction overrides the effects of glucose in *Lb-AAT* upregulation. Coordinated regulation of expression of metabolic genes like *Lb-AAT,* together with malate synthase, acetyl CoA oxidase, and isocitrate lyase (Blaudez et al. 1998; Balasubramanian et al. 2002; Podila et al. 2002; 2004) support the idea that metabolic changes are manifested well ahead of structural changes during the preinfection stage of symbiosis, and that β-oxidation of lipids followed by gluconeogenesis may be used for net growth of the fungus towards the plant host. Cell component recycling is indicated by the expression of *LB-AUT7* (Kim et al. 1999*a*), a protein involved in autophagocytosis. Based on our observations, we propose a putative model of how some of these genes are involved in the preinfection and establishment of symbiosis process (Fig. 6). It is clear that carbon economy is important in the fungus entering into the symbiosis, and that there is a marked shift in metabolism in the fungus from saprobic to symbiotic mode. It is clear from this and other studies (Kim et al. 1999*a*; Balasubramanian et al. 2002; Podila et al. 2002, 2004) that the fungus redirects its resources towards growth and establishment of the ectomycorrhiza within hours of perception of the host. Further analyses on the various genes upregulated at preinfection and closer observation of temporal expression through the symbiosis would provide a more detailed global overview of the symbiosis.

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