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Déterminisme moléculaire des interactions entre le champignon ectomycorhizien *Laccaria bicolor* S238N et des bactéries du sol

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Summary

Ectomycorrhizal fungi have a beneficial impact on tree nutrition and growth by forming symbiotic associations with roots. In their natural environment, they interact physically and metabolically with soil bacterial communities that are beneficial, neutral or antagonistic to the fungus. Since the 80^{ies}, a specific interest has been given to bacterial strains that improve the formation of ectomycorrhizal symbiosis, so-called mycorrhiza helper bacteria. The strain *Pseudomonas fluorescens* BBc6R8 is particularly efficient to enhance the establishment of Douglas fir - *Laccaria bicolor* S238N mycorrhizal symbiosis, by improving the survival and the growth of the pre-symbiotic mycelium in soil. We have used both a global and a targeted approach to investigate the molecular mechanisms of this helper effect. In a first step, we have analysed, using an *in vitro* assay, the morphological and the transcriptomic responses of the ectomycorrhizal fungus to the presence of the helper bacteria at different time of the interaction. Then we have assessed the question of the specificity of the fungal response by studying the effect of non-helper bacterial strains on the fungal behaviour. Finally, we have focused on the role played by several key molecules in the interaction: thiamine, trehalose and the type III secretion system. We suggest that the bacterial strain would exert its helper effect through a combination of mechanisms: an improvement of the nutritional status of the fungus and a preparation of both the plant and the ectomycorrhizal fungus to the root infection.

Résumé

La symbiose ectomycorhizienne a un effet bénéfique sur la nutrition et le développement des arbres. Dans les sols, les champignons ectomycorhiziens interagissent continuellement avec des communautés bactériennes qui peuvent avoir une action bénéfique, neutre ou antagoniste vis-à-vis du champignon. Parmi ces bactéries, une attention particulière a été portée au cours de ces dernières années sur les bactéries auxiliaires de la mycorhization qui favorisent la symbiose ectomycorhizienne. La souche auxiliaire *Pseudomonas fluorescens* BBc6R8 améliore la survie pré-symbiotique et la croissance du champignon ectomycorhizien *Laccaria bicolor* S238N, et favorise son établissement en symbiose avec le Douglas. Mais les mécanismes moléculaires sous-jacents sont inconnus. A l'aide d'un dispositif de confrontation bactérie-champignon *in vitro* et d'outils transcriptomiques, nous avons analysé les réseaux de gènes fongiques impliqués dans l'interaction entre *P. fluorescens* BBc6R8 et *L. bicolor* S238N ainsi que le degré de spécificité de la réponse du champignon. De plus, nous avons examiné le rôle joué par certains métabolites fongiques et bactériens dans l'interaction : thiamine, tréhalose, système de sécrétion de type III. Nos résultats suggèrent que l'effet auxiliaire de la souche BBc6R8 soit dû à une combinaison de mécanismes : d'une part une amélioration du statut nutritionnel du mycélium, d'autre part une préparation des racines et des hyphes à l'infection mycorhienne.

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Au petit rhino…

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General Introduction

Introduction Générale

Préambule

Les études que j'ai réalisées au cours de mon travail de thèse portent sur les mécanismes moléculaires des interactions entre un champignon ectomycorhizien et une bactérie auxiliaire de la mycorhization. Je me suis focalisée sur un champignon parmi des dizaines de milliers d'espèces (100 800 décrites, Lecointre & Le Guyader 2001) et une bactérie parmi des centaines de milliers d'espèces (52 000 par gramme de sol, Roesch *et al.* 2001)

Du fait de leur grande capacité d'adaptation aux modifications de l'environnement et aux conditions de vie extrêmes, champignons et bactéries ont conquis la grande majorité des écosystèmes présents sur Terre, qu'ils soient naturels ou artificiels : aliments issus de l'industrie agroalimentaire, infrastructures (ie. murs, ventilations, tuyauteries…), matériel médical (cathéter, outils chirurgicaux…). Ils jouent donc un rôle majeur dans de nombreux domaines : agriculture, santé humaine et animale, industrie agroalimentaire, environnement, sylviculture, conservation des monuments historiques… et ont un impact économique qui atteint probablement des centaines de millions d'euros. En effet, quel que soit le domaine concerné, certains micro-organismes sont bénéfiques et source de revenus financiers (probiotiques, agents de lutte biocontrôle contre les pathogènes, microbes impliqués dans les processus de fermentation agroalimentaire) tandis que d'autres induisent des nuisances extrêmement coûteuses (infections humaines et animales, contaminations des aliments, santé des cultures, dégradation des bâtiments…). Enfin, ils sont responsables de centaines de milliers de décès dans le monde chaque année. Pour ces raisons, bactéries et champignons ont fait l'objet de multiples recherches. Ces organismes ont longtemps été considérés séparément. Pourtant, ils colonisent souvent les mêmes niches où ils interagissent physiquement et métaboliquement (Wargo & Hogan, 2006). L'absence de prise en compte de ces interactions a mené à l'échec de nombreuses thérapies *in vivo.* L'étude des mécanismes d'interactions entre micro-organismes est aujourd'hui une discipline en plein développement, que ce soit dans le domaine médical, en agronomie ou en agroalimentaire. Toutefois, ces recherches restent, pour le moment, relativement cloisonnées. La grande majorité des micro-organismes étudiés à l'heure actuelle prolifèrent et interagissent naturellement depuis des millions d'années dans le sol. Nous pouvons donc nous attendre à retrouver des similitudes dans les mécanismes d'interaction entre les différents

Table I.1. Examples of studies performed on mechanisms of the interactions between bacteria and fungi in various fields of research.

Mechanisms	Plant nutrition	Animal & Human nutrition	Plant health	Animal & Human health	Food Processing	Biochemical cycles	Environmental protection	Archaeological patrimony protection
pH	Romano & Kolter 2004	Chaucheyaras-Durand & Fonty 2001	Sarniguet et al. 2006	Aruscavage et al. 2006, O'May et al. 2005	Corsetti *et al.* 2001, Versari *et al.* 1999			
Nutrient competititon		Chaucheyras-Durand *et al.*, 2005	Kamilova *et al.* 2001, van Dijk *et al.*2000, Whipps *et al.* 2001, Kaufmann *et al.* 2005		Alexandre *et al.* 2004	Moller *et al.* 1999		
Detoxification process	Duponnois and Garbaye, 1990, Rainey, 1991	Newbold *et al.*1996	Tsukamoto *et al.* 2002		Guilloux-Benatier *et al.* 1998			Allemand, 2003
Biofilm	Nurmiaho-Lassila *et al.* 1997			O'May et al. 2005, Adam et al. 2002, Thurnheer et al. 2003, Al-fattani et al. 2004, Lamfon et al. 2005			Johnsen *et al.* 2005, Seneviratne *et al.* 2006	
Adhesion processes	Cook *et al.* 2000		Cook *et al.* 2000	Ofek et al. 1996, van der Mei et al. 2000	Verchtert *et al.* 1990, Lavermicocca *et al.* 2005			
Nutritional effect	Duponnois & Garbaye 1990, Olsson & Wallander 1998	Chaucheyras-Durand & Fonty 2001, Marvin-Sikkema *et al.* 1990., Chaucheyras-Durand *et al.* 2005		Frases et al. 2006	Guilloux-Benatier *et al.* 1998	Rikhvanov *et al.* 1999		
Antibiosis	Sbrana *et al.* 2000, Selim *et al.* 2005, Lehr *et al.* 2007	Elahi et al. 2005	Nielsen *et al.*1998, Tambon & Höfte 2001, Tran *et al.* 2007, de Boer *et al.* 2007	Cruz *et al.* 2001, Minkwitz & Berg 2001, Cardoso Furtado *et al.* 2002, Noverr *et al.* 2004	Corsetti *et al.* 1998, Ström *et al.* 2005, Comitini *et al.* 2005			
Response of pathogens to antagonism			Levy *et al.* 1992, Duffy *et al.* 2003, Schouten *et al.* 2004	Korczynska *et al.* 2007, Zguraskay *et al.* 2003, Hogan *et al.* 2004, Rasmussen *et al.* 2005				
Type III secretion sytems			Rezzonico *et al.* 2004, Rezzonico *et al.* 2005					
Cross regulation	Schrey *et al.* 2005, Hildebrandt *et al.* 2006, Deveau *et al.* 2007		Notz *et al.* 2002, Schoonbeek *et al.* 2002, van Rij 2005	Hogan *et al.* 2002, Rasmussen *et al.* 2002	Melin *et al.* 2002			

modèles d'étude (Table I.1). Afin de promouvoir la transversalité disciplinaire dans le domaine des interactions bactéries-champignons, P. Frey-Klett *et al.* ont entrepris la rédaction d'un article de revue sur les avancées de la recherche sur les interactions bactéries-champignons, toute discipline confondue. La partie qui suit et qui m'a été confiée concerne les mécanismes des interactions entre bactéries et champignon.

Publication n°1. Fungal-bacterial interactions : a hyphen between environment, plant, animal and food microbiologists.

I. Publication n°1. Fungal-bacterial interactions: a hyphen between environment, plant, animal and food microbiologists.

Frey-Klett P.[1*], Deveau A.[1], Barret M.[2], Garbaye J.[1] and Sarniguet A.[2]

Manuscript in preparation.

I. Mechanisms of interaction between bacteria and fungi

I.1 Impact of the interaction on the microbial environment.

I.1.1 Effect of pH modifications

Fungi and bacteria can affect each other indirectly through modifications of the physico-chemical properties of their environment. One of the most common effect is pH alteration. Although some microorganisms (Streptococci, lactobacilli, candidas, O'May *et al.* 2005) can live in broad range of pH, most of them are susceptible to low pH (< 4). As a consequence, pH alteration can affect microbial community structure by either promoting or inhibiting the growth of non acidic tolerant organisms, as demonstrated in gastric, plant, wine, or cheese environments (O'May *et al.* 2005, Aruscavage *et al.* 2006, Versari *et al.* 1999, Corsetti *et al.* 2001). For example, on the cheese surface, the lactate metabolism and the production of alkaline metabolites such as ammonia by yeasts lead to a deacidification which favours the growth of less acidic tolerant bacterial strains that are essential for cheese ripening (Corsetti *et al.* 2001). The same phenomenon is involved in the increased proliferation of *Salmonella enterica* on tomatoes in the presence of *Alternaria* or *Cladosporium* fungi (Aruscavage *et al.* 2006). In opposition, the strain *Pseudomonas fluorescens* Pf29A inhibits the growth of the pathogenic fungus of the wheat *Gaeumannomyces tritici* through the production of organic acids (Sarniguet *et al.* unpublished). Environmental pH also affects key microbial functions among which pathogenicity (Calvo *et al.* 2002, Peñalva *et al.* 2002): for example, the production of aflotoxin by *Aspergillus parasiticus* is higher under acidic growth conditions while alkaline medium increases the production of penicillin by *Aspergillus nidulans.*

I.1.2 Nutrient competition

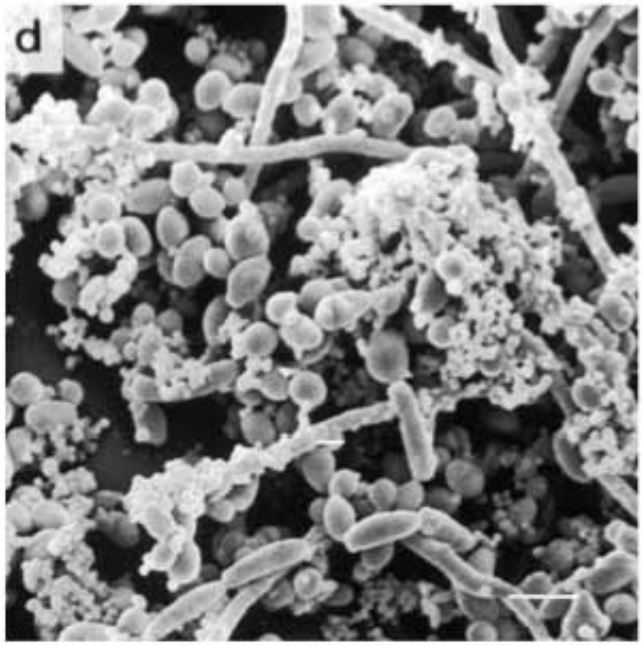
A second major alteration of the microbial environment comes from the nutrient uptake by microorganisms. Up to now, trophic competition between fungi and bacteria has been mainly described in plant rhizosphere where competition for carbon (van Dijk *et al.* 2000) or iron (Whipps *et al.* 2001, Kaufmann *et al.* 2005) is a well-known biocontrol mechanism against the development of fungal plant pathogens. Competition for carbon substrate between fungi and bacteria also impairs the decomposition of leaves by these microorganisms (Moller *et al.* 1999). However, trophic competition between bacteria and fungi occurs in a wide range of environments. In wine-making process, the uptake and release of nutrients by yeast, during alcoholic fermentation, greatly influence the further development of malolactic bacteria and therefore the quality of wine (Alexandre *et al.* 2004). Competition for nutrients between the feed additive *S. cerevisiae* CNCM I-1077 and rumen bacteria is also partly involved in the diminution of protein degradation activities of rumen microbes (Chaucheyras-Durand *et al.,* 2005).

I.1.3 Detoxification process

In complex microbial communities, some microorganisms can favour the survival and the growth of other microorganisms by metabolizing toxic molecules from the environment. These compounds can be autotoxic as shown for the polyphenols secreted by the ectomycorrhizal fungus *Paxillus involutus* (Duponnois and Garbaye, 1990) or the gluconate produced by *Pseudomonas putida* ZK3093 growing on grapes (Romano & Kolter, 2005). The degradation of the metabolite by another microorganism reduced the autotoxicity. Similarly, bacterial degradation of fungal self-inhibitory compounds was suspected to be involved in the induction of fruiting-body formation of the edible mushroom *Agaricus bisporus* (Rainey, 1991). The phenomenon could also be partly responsible for the probiotic activity of yeast cultures on anaerobic bacteria of the rumen (Newbold *et al.*1996). Many rumen microorganisms are highly sensitive to the presence of O_2. Peaks of O_2 concentration appear when animals are feeding. The respiratory activity of *S. cerevisiae* allows it to scavenge O_2, thus protecting the strictly anaerobic bacteria.

In other cases, the interaction involves three organisms: a toxin produced by the pathogen *Pseudomonas tolaasii* induces necrosis on its host *Pleurotus ostreatus* but its activity is suppressed by a third organism, *Mycotecola tolaasinivorans* that degrades the toxin (Tsukamoto *et al.* 2002). The same mechanism is also involved in resistance against antibiotic treatments as it was already demonstrated in mixed bacterial biofilms (Burnmølle *et*

Figure 1. Scanning electron micrograph of mixed species biofilm of *C. albicans* and *S. epidermidis* on PVC catheter disks (from Adam *et al.* 2002). Bar: 10 μm.



al. 2006). It was also responsible of the inefficiency of the antibiotic treatment against a fungal contamination in the prehistoric painted cave of Lascaux (Dordogne, France, Allemand 2003).

I.1.4 Mycelium as a bacterial carrier

Filamentous fungi, thanks to the slim film of water that currently surrounds mycelium and the continuous surface provided by hyphae, can facilitate the displacement of bacteria in various environments, notably in soil and serve, by the way, as carriers. This could play a role in cleaning up process by giving access the bacteria to substrates (Johnsen *et al.* 2005).

I.1.5 Biofilms as a protected niche

Finally, interactions between fungi and bacteria can lead to the creation of new microcosms when forming mixed biofilms (Figure 1). Biofilms are matrix-enclosed microbial organized accretions in which cell phenotype and physiology profoundly differ from that of planktonic cells. Biofilms create a protective and homeostatic environment that can be completely different from the surrounding milieu (Stoodley *et al*. 2002). This explains why non-acidic tolerant bacteria like the opportunistic pathogens *Escherichia coli* and *Klebsiella pneumoniae* are able to multiply at the very low gastric pH (O'May *et al.,* 2005). But biofilms do not only generate new environments, they also create a complex barrier that protects the microorganisms against toxic compounds diffusion. As an exemple, the extracellular polymer produced by *Staphylococcus epidermis* inhibits the penetration of the antifungal fluconazole inside the *S. epidermis*-*Candida albicans* mixed biofilms while, at the same time, the presence of *C. albicans* reduces the diffusion of the antibiotic vancomycin used against *S. epidermis* (Adam *et al.* 2002, Thurnheer *et al.* 2003, Al-fattani *et al.* 2004). One should ask if mixed fungal-bacterial biofilms also exist in the rhizosphere of crop plants and if fungal phytopathogens could be protected from pesticides because of their localization in these mixed biofilms. Besides their protective effect, mixed biofilms can also favour the colonization of surfaces hardly accessible to some microorganisms. For instance, bacterial cell walls are hydrophilic and they poorly adhere to hydrophobic surfaces like polyethylens, contrary to filamentous fungi that are generally able to attach to hydrophobic surfaces through the production of hydrophobic proteins. As a consequence, the formation of mixed biofilms allows the colonization of new environments by bacteria (Seneviratne *et al.* 2006). This process has been described in a polyethylen biodegradation context but may also occur during catheter colonization.

I.2 Metabolite-mediated relationships between fungi and bacteria

I.2.1 Chemotaxis

The successful colonization of environments by microorganisms largely depends on their timely response to environmental stimuli and the competition or cooperation with other microbes. In this context, motility and chemotaxis may provide a significant advantage. Chemotaxis is thought to be the first step of bacterial colonization of roots of several plant species (Turnbull *et al.* 2001, Sood *et al.* 2003). It plays also an important role in the symbiotic interactions between rhizobacteria and legumes (Currier & Strobel, 1977). So far, only few examples of chemotaxis of bacteria towards fungal exudates have been reported However, bacteria are able to colonize all the fungal tissues: hyphae (Grewal & Rainey 1991, Artursson & Jansson 2003, Seneviratne & Jayasinghearachchi 2003, Toljander *et al.*, 2006), fruiting bodies (Garbaye & Bowen, 1989, Danell *et al.* 1993, Sbrana *et al.* 2000, Russo *et al.* 2003), spores (Roesti *et al.* 2005), mycorrhizae (Frey *et al.* 1997) and sclerotia (Arora *et al.* 1983). This colonization is probably quite often mediated by chemotaxis. Fungal exudates can be antibiotic such as the fusaric acid (de Weert *et al.* 2003) or fungal nutrients. Trehalose, a disaccharide accumulated in the mycelium of the ectomycorrhizal fungus *L.bicolor* is responsible for the chemoattraction of the mycorrhiza helper bacterial strain *P. fluorescens* BBc6R8 (unpublished results). Morevover, trehalose is also suspected to mediate attraction and selection of trehalose degrading *P. fluorescens* populations in the mycorrhizosphere of the Douglas fir (Frey *et al.* 1997).

I.2.2 Adhesion processes

In many fungi-bacteria interactions, attachment of bacteria to living fungal hyphae is an important prerequisite. It plays a fundamental role in multiple fields of application notably plant and human health (Cook *et al.* 2000, Nikawa *et al.* 2001). The artificial reduction of the attachment ability of *Pseudomonas* sp. decreased its biocontrol activity against the plant pathogen *Phytophtora parasitica* (Yang *et al.* 1994). In human, the coaggregation of *Candida* to bacteria of the oral cavity increases fungal infections as the colonization of oral surfaces serves as a reservoir of the pathogen. In many cases, adhesion process involves cell-wall glycoproteins (mannose, galactose, raffinose) that bind to lectin-type sugar receptors on the neighbouring cells (Nikawa *et al.* 2001). Conversely, interaction between probiotic bacteria and fungi can be used to reduce adherence of yeast biofilms. If bacteria like *Streptococcus sanguis* are prerequisite for the adherence of *C. albicans* on acrylic, other bacterial strains compete effectively with yeasts in their surface colonisation by releasing biosurfactants that

interfere with the adhesion of yeasts. These properties have been used to limit the establishment of yeast biofilm on silicone rubber voice prostheses (van der Mei *et al.* 2000).

I.2.3 Nutritional effect

Both fungi and bacteria are famous for their production of a wide variety of compounds. Many are deleterious but some enhance the survival of the surrounding organisms by providing biosynthesis precursors or cofactors to cells that are unable to synthesize them. It is the case of the thiamine (vitamine B1) that was found to mediate either the bacterial or fungal growth-promoting effect in several interaction models: between the thermophilic yeast *Debaryomyces vanriji* and the *Bacillus* sp. TB-1 strain (Rikhvanov *et al.* 1999), between *S. cerevisae* and the lamb rumen cellulolytic bacteria (Chaucheyras-Durand & Fonty, 2001) and between the ectomycorrhizal fungus *Laccaria bicolor* S238N and the helper bacteria *Pseudomonas fluorescens* BBc6R8 (Deveau *et al.* unpublished data). In the case of the human pathogen *Cryptococcus neoformans*, nutritional cooperation with *K. aerogenes* permits the synthesis of melanin and thus leads to a reduced fungal susceptibility to insults from the environment and to an enhanced virulence against mammalian hosts. The fungus uses p-hydroxyphenylpyruvate (HGA) and dopamine produced by *K. aerogenes* as precursors for melanin synthesis (Frases *et al.* 2006, Frases *et al.* 2007). In other cases, one partner provides to the other carbon skeletons for energy metabolism. Several mycorrhiza helper bacteria of the ectomycorrhizal fungus *L. bicolor* secrete citric acid that is metabolized by the fungus and promotes its growth (Duponnois & Garbaye, 1990). Conversely, ectomycorrhizal fungi produce organic acids or sugars that can affect associated bacterial communities composition and growth (Frey *et al.*1997, Olsson & Wallander, 1998).

I.2.4 Antibiosis

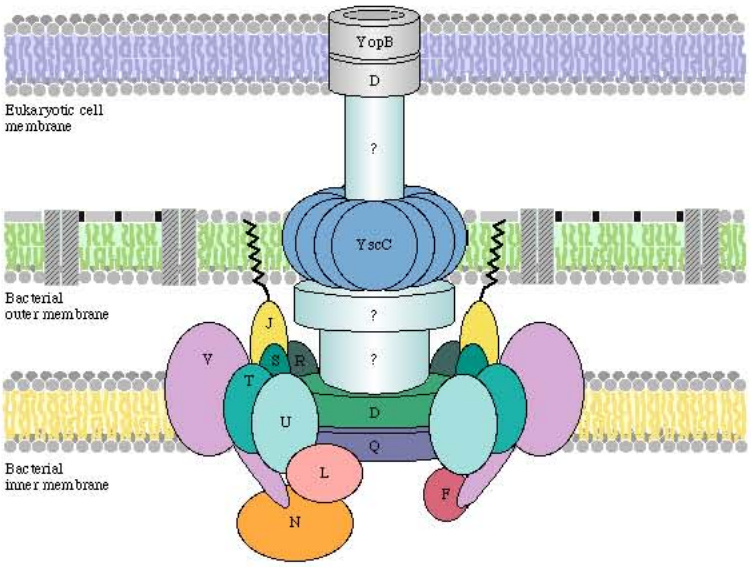
Antibiosis is a well known ubiquitous process used by both bacteria and fungi to inhibit the growth or the metabolic activity of other organisms. Antibiotics and antifungal compounds encompass a heterogeneous group of organic, low molecular weight compounds: penicillins, phenazines, diacetylfluoroglucinols, fusaric acid, cyclic lipopeptides, polymyxin, peptides, butyric acid, cell wall-degradation enzymes. If many are diffusible, it should not be forgotten that a lot of volatile compounds have also antibiotic activities (hydrogen cyanide, β-phenylethanol, benzothiazol, trimethyl disulfide). Because of their diversity, antibiotics have a broad range spectrum of activities. Some of them are inhibitors of key cellular functions such as cellular respiration (hydrogen cyanide, fusaric acid), cell wall synthesis (penicillin, butyric

acid), transport sytems (β-phenylethanol), while others impair the integrity of cell membranes (hydrolysis enzymes, cyclic lipopeptides, polymyxin, β-phenylethanol, tolaasin). But for many of them, especially those described in soil environments, their targets remain unknown.

There are so many cases of antibiosis that have been described in all the different fields of studies that we will not report all. We have decided to focus on some examples that illustrate the importance of comparative biology for the understanding of molecular interactions between fungi and bacteria. The existence of common patterns governing molecular interactions between fungi and bacteria relies on their origin from a common environment, the soil where they have co-evoluted into interactions for millions of years. Indeed, soil is considered as a major reservoir for human pathogens (Staib *et al.* 1980, Summerbell *et al*. 1989). The opportunistic human pathogen *P. aeruginosa* is also in some cases a plant pathogen (Rahme *et al* 1995) and in other cases a potential plant biocontrol (Tambong & Hofte, 2004). Similarly, *Aspergillus fumigatus* is both a major human opportunistic pathogen and a wheat root pathogen (Tekaia & Latgé, 2005). Lactic acid bacteria are also found in a variety of environment from food (yoghurt, cheese, fermented sausage, bread, wine) to mammalian microflora. Interestingly, studies which have been performed on the antagonism of lactic acid bacteria against various fungi such as the human pathogen *C. albicans* (Noverr & Huffnagle, 2004) or the three fungi responsible of bread spoilage *A. niger, C. herbarum* and *P. verrucosum* have provided the same result: whatever the environment, lactic acid bacteria produce short chain fatty acids (mainly butyric, acetic and caproic acids) which are responsible for the reported antifungal activities. Another interesting comparison is the biological control of plant root pathogens (Thomashow 1996, Tambong & Höfte 2004, Chaurasia *et al.* 2005, Selim *et al*. 2005, Haas & Défago 2005) and the use of probiotics to prevent human and animal infectious diseases (Elahi *et al.* 2005, Chaucheras-Durand *et al.* 2006, Madsen 2006). The two strategies, which have been developed in parallel, rely in the same principle: the introduction of selected strains to prevent infectious diseases. Strikingly, mechanisms of action are quite similar: it currently involves antibacterial activity and a stimulation of immune response. The two fields of study could take advantage from the advances made in each one.

Many studies dedicated to the understanding of mechanisms of interactions between fungi and bacteria are performed in controlled microcosms with a limited number of interacting microorganisms. However, natural interactions generally involve a complex community of microorganisms and antibiotic production can be strongly modified by the community. de Boer *et al*. (2007) observed that mixed soil bacteria reduce the expansion of pathogenic fungi

Figure 2. Schematic overview of the *P. aeruginosa* type III secretion system (© KEGG database).



while, taken separetly, the different bacterial species have no effect on the growth of the fungi. The authors suggested that the cohabitation of the bacterial strains triggered antibiotic production via interspecific interactions. The method was used by Furtado *et al.* (2002) to discover new antibiotics for therapeutics against various human pathogens. They used a pool of bacteria to enhance the antimicrobial activity of the fungus *A. fumigatus.*

I.2.5 Pathogen response to antibiosis.

Because it is one of the main mechanisms of control of animal and plant diseases, and because of its practical relevance for food preservation, antibiosis has received a lot of attention in the last decade. But most of studies have focused on the mechanisms of the antibiotic synthesis and less studies have addressed the question of the impact of the antibiotics on the biology and the physiology of the targeted pathogen. Pathogens have developed a wide variety of mechanisms to counteract antibiosis. Many studies have been performed *in vitro* to understand fungal or bacterial resistance to therapeutics antibiotics and thus have not been realized in a context of interaction between fungi and bacteria. But evidences indicate that the same mechanisms occur in natural interactions (Duffy *et al.* 2003). It includes detoxification of antibiotics by enzymatic degradation (Korczynska *et al.* 2007), repression of genes involved in antibiotic synthesis in the antagonistic organism (Lutz *et al.* 2003, van Rij *et al.*, 2005), active efflux of antibiotics (Schoobeek *et al.* 2002, Zguraskay *et al.* 2003), mutation (Cruz *et al.* 2001) and interference (Hogan *et al.* 2004, Rasmussen *et al.* 2005).

I.2.6 Type III secretion system

The type III secretion system (TTSS) is widely distributed among proteobacterial pathogens of plants, animals and humans. It functions as a molecular syringe for the introduction of virulence factors directly into eukaryotic host cells (Figure 2). The virulence factors are then able to subvert host cell functions in a way that is beneficial to the invading bacteria (Hueck *et al.* 1998). The TTSS system is essential for the induction of disease in the susceptible host cell (Alfano & Collmer, 1997). The role of TTSS system in bacteria-fungi interactions remains poorly known yet. But two recent studies have evidenced its involvement in the antagonism of some bacteria against fungi. First, the inactivation of the TTSS gene *hrcV* in *P. fluorescens* KD strongly reduced its biocontrol activity against the oomycete *Pythium ultimum* (Rezzonico *et al.*, 2005). Secondly, Preston *et al.* (unpublished results) showed that, when expressed as a transgene, a TTSS-secreted effector from a biocontrol *P. fluorescens*

Table 1. Transcriptomic and proteomic regulations of the interactions between fungi and bacteria.

Environment	Fungal species	Bacterial species	Type of interaction	Technique	Organism analyzed	Nature of the inducer	Number of regulated genes/proteins	Regulated functions	Reference
Food	*Aspergillus nidulans*	*Streptomyces* sp.	-	Proteome analysis	F	Concanamycin	20	Control of global amino acid synthesis and sexual development Carbon metabolism Lovostatin synthesis Hypothetical proteins (CipA, CipC, CipD)	Melin *et al.* 2002
Food	*Aspergillus nidulans*	*Streptomyces* sp.	-	SSH	F	Bafilomycin	5	Zinc binding protein Cell wall mannoprotein Hypothetical proteins	Melin *et al.* 1999
Human	*Candida albicans*	*Pseudomonas aeruginosa*	-	Mutants	B	Unknown	Multiple	Type IV pili Hemolytic phospholipase C synthesis Phenazine synthesis Virulence factor regulator GacA, LasR, RhIR, RpoN	Hogan *et al.* 2002
Human	*Penicillium* sp.	*Pseudomonas aeruginosa*	-	DNA microarrays	B	Patulin, penicilic acid	Multiple	QS regulated virulent factors	Rasmussen *et al.*2005
Plant	*Botritis cinerea*	*Pseudomonas spp.*	-	Northern blot	F	DAPG, phenazines	2	ABC tranporters (BcatrB, BcatrD)	Schoonbeek *et al.* 2002
Rhizosphere	*Amanita muscaria*	*Streptomyces* AcH505	+	SSH	F	Auxofuran	58	Signaling pathways Metabolism Cell structure Cell growth reponse	Schrey *et al.* 2005
Rhizosphere	*Glomus intraradices*	*Paenibacillus validus*	+	SSH	F	Unknown	19	Cellular signalling pathway	Hildebrandt *et al.* 2006
Rhizosphere	*Glomus mosseae* BEG12	*Bacillus subtilis* NR1	+	Differential RNA display	F	Unknown	1	Catabolism of long-chain fatty acids	Requena *et al.* 1999

Rhizosphere	*Fusarium oxysporum*	*Pseudomonas fluorescens* CHA0	-	Tn5 ::lacZ mutant	B	Fusaric acid	1	DAPG synthesis	Notz *et al.* 2002
Rhizosphere	*Fusarium oxysporum*	*Pseudomonas chlororaphis* PCL1391	-	cDNA microarray	B	Fusaric acid	46	Phenazine-1-carboxamide synthesis QS sensing regulatory genes	van Rij *et al.* 2005
Rhizosphere	*Laccaria bicolor*	*Streptomyces* sp. strains	+ -	Proteome analysis	F	Unknown	several	Unknown	Becker *et al.* 1999
Rhizosphere	*Laccaria bicolor* S238N	*Pseudomonas fluorescens* BBc6R8	+	cDNA microarray	F	Unknown	104	Protein synthesis Stress response Post translation modifications Chromatine structure & modifications Energy metabolism Lipid metabolism Transport Cytoskeleton	Deveau *et al.* 2007
Rhizosphere	*Laccaria bicolor*	N-HSL QS producing bacteria	Unknown	cDNA microarray	F	N-HSL	230	Energy and carbon metabolism Growth process Signal transduction Transcription regulation Stress response	Deveau *et al.* unpublished
Rhizosphere	*Phytophtora parasitica*	*Pseudomonas putida* 06909	-	IVET	B	Unknown	5	Cell surface carbohydrate biosynthesis ABC transporter Outer membrane porins	Lee & Cooksey 2000
Rhizosphere	*Pythium ultimum*	*Pseudomonas fluorescens* F113	-	Tn5 ::lacZ mutant library	B	Unknown	7	RNA operons Unknown	Smith *et al.* 1999 Fedi *et al.* 1997

SSH: Substractive Hybridization, IVET: In Vitro Expression Technology

strain alters the morphology of *Neurospora crassa*. If it is well known that *P. aeruginosa* has a functional TTSS system playing a major role in its pathogenesis and that the bacteria is frequently associated with fungi. We could wonder whether it is necessary for its antagonism towards fungi like *C. albicans.*

I.3 Transcriptomic and proteomic regulation during fungal-bacterial interactions

I.3.1 Cross regulation of gene expression and protein synthesis

The cell functioning is tightly regulated at several levels, notably at the transcriptional and post-translational levels. Fungal and bacterial responses to modifications of many environmental parameters are known to be under the control of transcriptional regulation (pH, nutrients availability, oxidative stress, heat shock, drug treatment, anaerobic growth…, Gasch *et al.* 2002, Babu *et al.* 2006). Evidences indicate that gene expression and protein synthesis are also modulated during interactions between bacteria and fungi. Around fifteen examples of such cross regulations have been reported so far in the literature (Table 1). Most of them concern the effect of bacteria on the fungal transcriptome of rhizosphere organisms. These studies have been realized using various transcriptomic (northern blot, microarrays, substractive hybridization) or proteomic tools (2D-SDS page-electrophoresis) and mutagenesis technologies. Interactions with bacteria frequently lead to a modification of lipid metabolism involved in either synthesis or turnover of membrane lipids (Requena *et al.* 1999, Melin *et al.* 2002, Lee *et al.* 2000, Schrey *et al.* 2005, Deveau *et al.* 2007). This regulation is generally correlated with modifications of hyphal growth and morphology. Alteration of the expression of transporter proteins is also retrieved in several organisms (Lee *et al.* 2000, Schoonbek *et al.* 2002, Deveau *et al.* 2007). This should be related, for a part, to drug efflux strategies used by some antagonistic microorganisms to resist against antibiotics. Finally, intermediate molecules of signal transduction pathways are often found transcriptionaly regulated. The number of studies is yet too low to identify general patterns of response but we can expect that this number will increase in the next years, because of the generalization of the usage of these molecular tools.

I.3.2 Inducers of gene expression during fungal-bacterial interactions.

The word « signal » is commonly used to refer to a variety of substances produced by a *cell A* that elicits a response in *a cell B*. However we should distinguish true signals involved in a real dialog between two organisms, such as quorum sensing between bacteria, from non-communication molecules produced by an organism which induces directly or indirectly a

Table 2. Functional groups of genes affected by sub inhibitory antibiotics (from Davies *et al.* 2006)

Antibiotic	Organism	Effect
Tetracyclines	*Bacteroides sp*	Enhanced gene transfer (conjugation of antibiotic resistance genes)
	S. epidermidis	Stimulation of bacterial adhesion
	Streptococcus sp	Changes in exoprotein secretion
β-lactams	*Staphylococcus sp*	Decreased biofilm formation
Cerulenin	*S. aureus*	Inhibition of protein secretion
Aminoglycoside	*P. aeruginosa*	Increased biofilm formation
	S. pneumoniae	Increased mutation frequency
Fluoroquinolones	*E. coli*	Reduced hemolytic activity
		Induction of colicin synthesis
	S. aureus	Increased adhesion
	S. pneumoniae	Increased mutation frequency
	Mycobacterium fortuitum	Increased mutation frequency
Macrolides	*Mycobacterium avium*	Decreased biofilm formation
	P. aeruginosa	Inhibition of quorum sensing (virulence suppression)
Lincosamides	*Bacillus fragilis*	Altered cell morphology and increased DNA fragmentation
	S. aureus	Changes in exoprotein expression
Oxazolidinone	*S. aureus*	Decreased secretion of virulence factors
Mupirocin	*P. aeruginosa*	Reduced biofilm formation
		Reduced flagellin expression
Rifampicin	*E. coli*	Reduced toxin secretion

behaviour change in another organism (Diggle *et al.* 2007). In microbiology, this distinction is difficult to take into account because, generally, when a molecule that elicites a regulation in another organism is discovered, the existence of a molecular "answer" to this first molecule is unknown. Three categories of fungal-bacterial inducers of transcriptomic cross regulations have been described until today: antibiotics, the auxofuran and quorum sensing molecules (Table 1). This number should incredibly increase in the future when the multiplicity of the secondary metabolites produced by both fungi and bacteria will be identified.

Antibiotics are well known for their lethal inhibitory activities. However, recent studies have demonstrated that these compounds can also have significant transcriptional activities at subinhibitory concentrations, which correspond to concentrations that are present in natural environments (Yim *et al.* 2007). A wide variety of response was observed depending on the microorganisms and the antibiotic studied (Table 2). Alteration of biofilm formation and of the frequencies of mutation were the effects the more frequently observed (Davies *et al.* 2006).

Auxofuran is the first ectomycorrhizal growth-promoting molecule produced by a mycorrhiza helper bacteria to be identified (Riedlinger *et al.* 2006). This compound, which exhibits structural similarities to the biologically inactive compound ulufuranol and to the benzopyran-type compound koninginin B, reproduces the effects of the helper strain *Streptomyces* sp. on both growth and transcriptional activities of *A. muscaria*. Interestingly, its synthesis is promoted in fungal-bacterial co-cultures and at acidic pH, which classically results from the growth of ectomycorrhizal fungi.

The last class of inducers of fungal-bacterial cross regulations are quorum sensing signal molecules (QS). QS is a widespread regulation mechanism, which permits to collectively regulate gene expression in bacteria according to cell density. Antibiotic production, motility or biofilm formation depend on quorum sensing regulation (Gonzalez & Keshavan, 2006). Two recent studies, the first concerning interactions between the opportunistic pathogens *C. albicans* and *P. aeruginosa* and the second concerning interactions between the ectomycorrhizal fungus *L. bicolor* S238N and the associated bacterial communities, have shown that some fungi perceive and react to N-HSL quorum sensing molecules (Hogan *et al.* 2004, Deveau *et al.*, unpublished data). Hogan *et al.* (2004) demonstrated that the 3,O-C12-HSL produced by the bacterium was sufficient to inhibit the fungal filamentation. They suggested that the 3,O-C12-HSL, which is structurally closely related to the *C. albicans* farnesol, hijacks the fungal hormone signaling pathway. Using transcriptomic tools, Deveau *et al.* showed that 3,O-C12-HSL induced the alteration of the transcription of about 200 genes

of the symbiotic fungus. It was hypothesized that HSL molecules may be used by the ectomycorrhizal fungus as an indication of a forthcoming nutritional competition with the surrounding bacterial community. A widespread mechanism of antagonism against QS producing bacteria is the secretion of interfering QS molecules, either by mimicking or degrading QS molecules. Interference has been retrieved in bacteria, mammalians, algae and higher plants. Recently, Rasmussen *et al.* (2005) showed the fungus *Penicillium* sp. can also produce inhibitors of *P. aeruginosa* QS system. All these results suggest QS molecules may play an important role in interaction mechanisms between fungi and bacteria in various ecosystems.

We can expect that much more signal and cross-regulations occur within the fungal-bacterial world. The previously described mechanisms of nutrient competition or cooperation, pH modifications, chemotaxis… should all lead to cross-regulations of gene expression and protein synthesis in fungal and bacterial cells. Genomic, transcriptomic, proteomic and metabolomic analyses combined to targeted mutagenesis should allow us to overcome more efficiently the bottlenecks in studying the mechanisms of fungal-bacterial interactions.

Conclusion

If a multiplicity of mechanisms has been proved to be involved in the interactions between bacteria and fungi, it should be not forgotten that these interactions generally result from a combination of processes. This is well illustrated by Hogan *et al.* (2004) for the interaction between *C. albicans* and *P. aeruginosa*. A spectrum of virulence factors including type IV pili, phospholipase C and phenazines were proved to control the antagonistic activity of the bacteria against the fungus. Furthermore, it should not be forgotten that 99 % of microorganisms are uncultivable. These species may play an important role in their ecosystems and interact with surrounding microorganisms among which fungi. So far, the mechanisms of these interactions have been completely neglected. The development of new techniques such as metagenomic and metatranscriptomic may provide new ways to analyze the black box of the interactions between uncultivable microorganisms. Among these cryptic microorganisms are the fungal intracellular bacteria (Schüßler *et al.* 2000, Bertaux *et al.* 2003, Partida-Martinez *et al.* 2005, Chamilos *et al.* 2007). They play a fundamental role in the functioning of non-pathogenic and pathogenic fungi during their association with plants and animals. Undoubtedly, any breakthrough in the understanding of the diversity and the role of these cryptic bacteria in their fungal hosts will greatly influence mycology.

II. La symbiose mycorhizienne et les bactéries auxiliaires de la mycorhization

II.1 La symbiose mycorhizienne

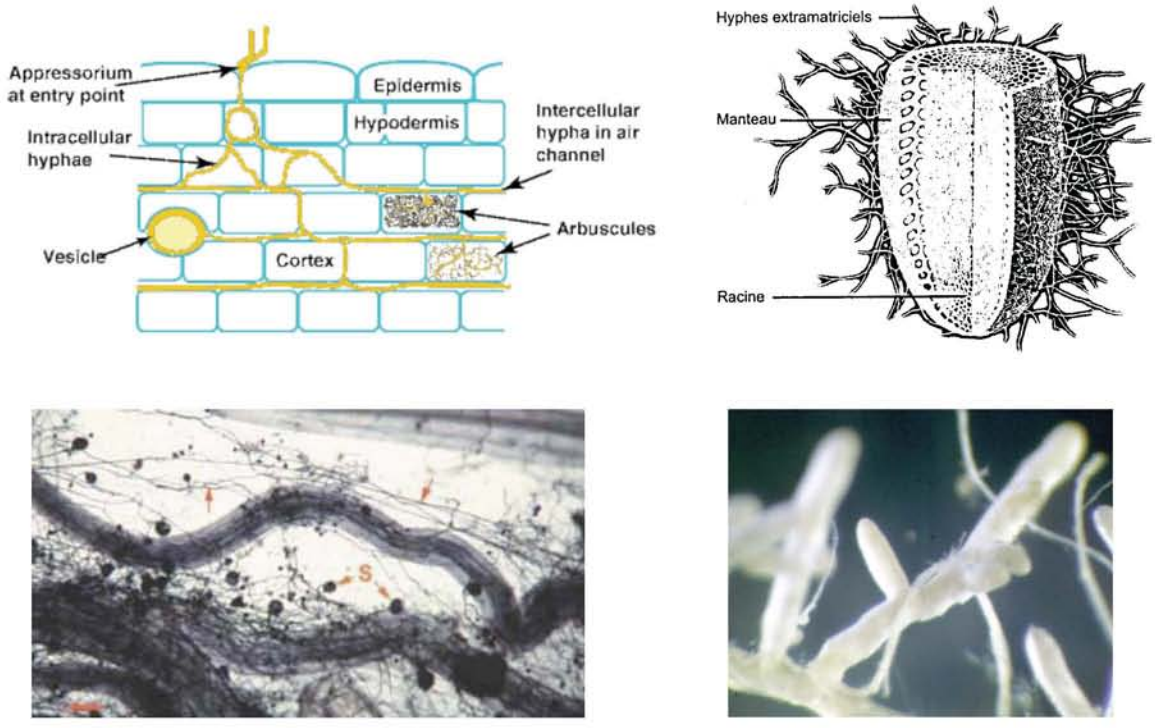
La symbiose mycorhizienne est une relation à bénéfice réciproque entre les racines d'une plante et le mycélium d'un champignon : tandis que la plante alimente le champignon hétérotrophe en carbone, le champignon fournit à son partenaire des minéraux et de l'eau qu'il puise dans le sol grâce à son dense réseau d'hyphes (Smith & Read, 1997). Les échanges de nutriments entre les deux symbiotes se produisent au niveau d'un organe mixte, la mycorhize. On considère que 95 % des végétaux sont mycorhizés. Cinq types d'associations mycorhiziennes ont été décrites sur la base de critères morphologiques et cytologiques : les ectomycorhizes, les endomycorhizes à vésicules et à arbuscules, les endomycorhizes à pelotons des orchidées, les mycorhizes éricoïdes et les ectendomycorhizes arbutoïdes. Toutefois, les symbioses endomycorhizes à vésicules et à arbuscules (deux tiers des plantes connues) et ectomycorhiziennes sont les plus répandues (environ 8000 espèces). Endo- et ectomycorhizes se différencient, d'une part, par l'anatomie de l'organe mixte et d'autre part, par l'identité taxonomique des deux partenaires. Les premières se rencontrent chez la majorité des espèces arbustives et herbacées et chez la plupart des arbres tropicaux et équatoriaux. Elle est due à des champignons inférieurs, les Zygomycètes, dont le mycélium pénètre dans les cellules du cortex de la plante hôte et forme des arbuscules sans perforer le plasmalemme (Figure I.1). La symbiose ectomycorhizienne, quant à elle, concerne la majorité des arbres des forêts boréales et tempérées. Elle peut être formée par une grande diversité de champignons appartenant aux phylla des Ascomycètes et des Basidiomycètes. Contrairement à la symbiose endomycorhizienne qui modifie peu la morphologie de la racine, l'établissement de la symbiose ectomycorhizienne aboutit à la formation d'un manteau fongique entourant les racines courtes des arbres puis s'insinuant entre les cellules du cortex (figure I.1). Ce réseau dense d'hyphes crée une très grande surface d'échange entre les cellules fongiques et racinaires.

II.2 La mycorhization contrôlée.

En raison de son rôle majeur dans la nutrition hydrominérale des arbres (cf. II.3) mais aussi de son effet protecteur vis-à-vis des agents pathogènes, la symbiose ectomycorhizienne accroit la résistance des arbres sur les sols pauvres occupés par les forêts.

Ces propriétés ont été mises à profit en sylviculture grâce au développement de la mycorhization contrôlée des semis d'arbres en pépinière. Cette technique qui consiste à inoculer des souches de champignons ectomycorhiziens sélectionnées, permet d'augmenter la croissance des arbres en pépinière comme en plantation (Le Tacon & Bouchard, 1986). Différentes études ont été menées de par le monde pour identifier les souches fongiques les plus adaptées à la pratique de la mycorhization contrôlée. Ainsi *Laccaria bicolor, Pisolithus tinctorius, Paxillus involutus, Scleroderma citrinum* ou *Cenococcum geophilum* ont montré de particulièrement bonnes aptitudes. Parallèlement, d'autres études ont été réalisées afin de déterminer les conditions biotiques et abiotiques les plus propices à l'établissement de cette symbiose. À cette occasion, il a été observé que les communautés bactériennes du sol pouvaient affecter le processus de mycorhization, soit positivement, soit négativement (Garbaye & Bowen 1989). De ces observations est né le concept de bactéries auxiliaires de la mycorhization (BAM) énoncé par Garbaye en 1994. Treize ans plus tard, si l'existence et le rôle joué par ces bactéries dans le processus de mycorhization ont été clairement démontrés (Tableau I.2)(Garbaye *et al.* 1990, Poole *et al.* 2001, Brûlé *et al.* 2001, Founoune *et al.* 2002, Maier *et al.* 2004), les mécanismes par lesquels les BAMs induisent une augmentation du taux de mycorhization restent peu documentés (Aspray *et al.* 2006, Schrey *et al.*, 2005 Riedlinger *et al.* 2006, Lehr *et al.* 2007). De nombreuses questions restent en suspens : signaux échangés, mécanismes moléculaires, spécificité des mécanismes… sont méconnus. L'article qui suit fait l'état des lieux des connaissances actuelles sur les BAMs et leurs mécanismes d'action.

Figure I.1. A. Schéma d'une coupe longitudinale d'une racine infectée par un champignon endomycorhizien à arbuscule et photographie d'une racine de poireau (*Allium porrum*) mycorrhizée par *Glomus mossae* (extrait de Brundrett *et al.* 1996). B. Schéma d'une racine infectée par un champignon ectomycorhizien (© H. Lagrange) et photographie d'une racine de chêne (*Quercus* sp.) mycorhizée par *Scleroderma citrinum* (© INRA).



A. Endomycorhize à arbuscule

B. Ectomycorhize

Table I.2. Quelques exemples de bactéries auxiliaires de la mycorhization et de leurs mécanismes d'action sur la formation de mycorhizes.

Champignons mycorhiziens	Souches de BAMs	Plante hôte	Mécanismes d'action	Références
Champignons ectomycorhiziens				
Amanita muscaria	*Streptomyces*	*Picea abies*	Augmentation de la croissance du mycélium pré-symbiotique et suppression des défenses de la plante	Schrey *et al.* 2005, Lehr *et al.* 2007
Pisolithus alba	*Pseudomonas monteilii*	*Acacia holosericea*	Augmentation de la croissance du mycélium pré-symbiotique	Founoune *et al.* 2002, Duponnois & Kisa 2006
Laccaria bicolor	*Pseudomonas fluorescens* BBc6R8	*Pseudotsuga menziensii*	Augmentation de la croissance et de la survie du mycélium pré-symbiotique	Duponnois & Garbaye 1991, Brulé *et al.* 2001
Lactarius rufus	Paenibacillus sp. EJP73	*Pinus sylvestris*	Augmentation de la ramification des racines	Poole *et al.* 2001, Aspray *et al.* 2006
Suillus luteus	Bacillus	*Pinus sylvestris*	Augmentation de la ramification des racines	Bending *et al.* 2002
Champignons endomycorhiziens				
Glomus fistulosum	*Pseudomonas putida*	*Zea mays, Solunom tuberosum*	Augmentation de la croissance du mycélium pré-symbiotique	Vosatka & Gryndler 1999
Glomus mosseae	*Bradyrrhizobium japonicum*	*Glycine max*	Induction de la sécrétion de flavonoïdes par les racines	Xie *et al.* 1995
Glomus mosseae	*Brevibacillus* sp.	*Trifolium pratense*	Résistance au stress hydrique	Vivas *et al.* 2003

Publication n°2. Coopération en sous-sol : quand les bactéries viennent à la rescousse de champignons symbiotiques des arbres

Des bactéries à la rescousse des champignons symbiotiques

Les champignons symbiotiques jouent un rôle majeur dans la nutrition des arbres. Cette symbiose est loin d'être une simple interaction bipartite entre plantes et champignons ; des bactéries interviennent en effet dans le processus de formation des mycorhizes. Si les mécanismes en jeu restent méconnus, le développement récent des techniques de génomique permet néanmoins d'espérer de nombreux progrès dans ce domaine.

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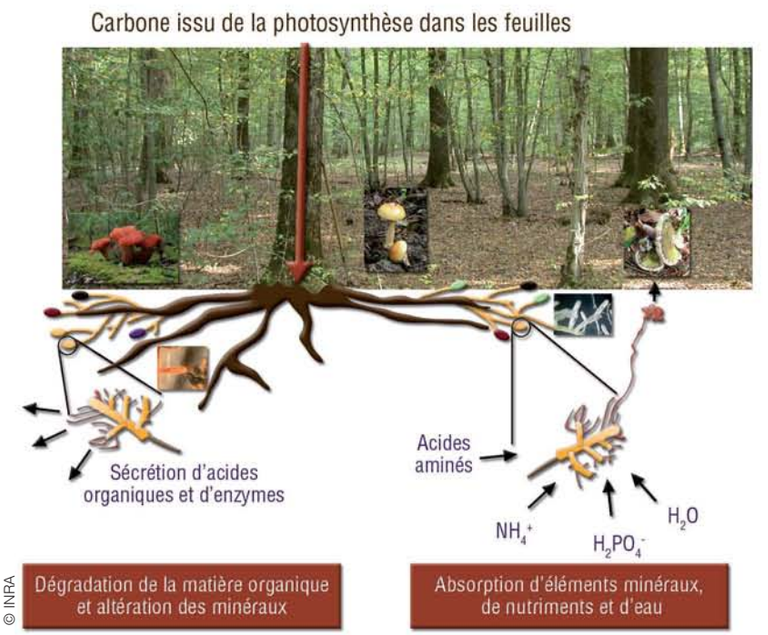
Les champignons symbiotiques ne font pas seulement le régal des gastronomes ; cèpes, truffes, lactaires et autres russules font aussi le bonheur des arbres. Les petits chapeaux qui peuplent les sous-bois de nos forêts (carpophores), sont en réalité les fructifications du champignon. Les organes végétatifs, non reproducteurs, se cachent à l'abri des regards, en sous-sol. Là, de vastes réseaux de mycélium fongique explorent des dizaines de mètres cubes de sol et s'associent aux racines des arbres dès que celles-ci croisent leur chemin.

Alors débute un dialogue moléculaire entre les deux partenaires. Après plusieurs jours de pourparlers naîtra un organe mixte : l'ectomycorhize. Il s'agit d'une structure d'échange entre la plante et le champignon : la plante, autotrophe, capable de fixer du carbone à partir du CO_2, de l'eau et de l'énergie lumineuse, fournit au champignon les sucres qu'il est incapable de synthétiser par lui-même **(figure 1)**. En échange, le champignon alimente la plante en eau et en éléments nutritifs tels que le phosphore ou l'azote. Ces nutriments sont peu accessibles aux racines des arbres car prisonniers dans les premiers centimètres du sol ou dans les minéraux.

Les champignons, quant à eux, sécrètent dans leur environnement proche (la mycorhizosphère), des enzymes et des acides qui libèrent ces éléments. Ils sont alors absorbés par le mycélium fongique, puis transférés à la plante au niveau de la mycorhize. Les champignons ectomycorhiziens jouent donc un rôle majeur dans la nutrition des arbres et ont de ce fait été l'objet de recherches approfondies au cours des vingt dernières années.

Physiologie, écologie et mécanismes des échanges nutritionnels entre le champignon et son hôte végétal sont aujourd'hui mieux compris. Ces propriétés ont d'ailleurs été mises à profit en sylviculture grâce au développement de la mycorhization contrôlée de semis d'arbres en pépinière. Cette technique consiste à inoculer des souches sélectionnées de champignons ectomycorhiziens et permet d'augmenter la croissance des arbres en pépinière comme en plantation **(1)**.



Échanges nutritionnels entre les champignons ectomycorhiziens et les arbres lors de la symbiose ectomycorhizienne

(1) Bertaux J *et al.* (2007) *Biofutur* 283, 35-8

Les bactéries auxiliaires : une nouvelle dimension pour la symbiose ectomycorhizienne

Plus récemment, un regard nouveau s'est posé sur le monde des mycorhizes : celui des bactériologistes. En milieu naturel, les mycorhizes sont entourées de communautés bactériennes complexes qui interagissent avec la plante et le champignon. Certaines souches bactériennes de la mycorhizosphère ont un effet délétère sur la formation des mycorhizes tandis que d'autres la favorisent. Ces dernières ont été baptisées « bactéries auxiliaires de la mycorhization » **(2, 3)**. Leur introduction dans un sol de pépinière forestière en compagnie de souches sélectionnées de champignons mycorhiziens augmente la croissance de jeunes plants d'arbre. Ces souches présentent donc un intérêt majeur pour la sylviculture dans le cadre des programmes de mycorhization contrôlée.

Bien que passées longtemps inaperçues, les bactéries auxiliaires de la mycorhization sont en fait répandues dans les sols. On soupçonne même certaines d'entre elles de se dissimuler à l'intérieur des cellules de champignon **(1)**. Des bactéries auxiliaires ont pu être isolées dans tous les habitats où elles ont été recherchées **(2)** : le sol associé aux racines, aussi appelé rhizosphère, la mycorhize et son environnement, les carpophores des champignons, les nodosités fixatrices d'azote de plantes légumineuses ou bien encore des galles.

Elles appartiennent à des groupes taxonomiques variés : des protéobactéries Gram négatives (*Agrobacterium*, *Azospirillum*, *Azotobacter*, *Burkholderia*, *Bradyrhizobium*, *Enterobacter*, *Pseudomonas*, *Klebsiella, Rhizobium*), des firmicutes (*Bacillus*, *Brevibacillus*, *Paenibacillus*) et des actinomycetes gram-positives (*Rhodococcus*, *Streptomyces*, *Arthrobacter*).

Des recherches supplémentaires seront nécessaires pour déterminer si des bactéries auxiliaires existent aussi dans des groupes que l'on sait difficilement cultivables (par exemple *Acidobacterium*), bien que très représentés dans les sols. La présence de bactéries auxiliaires de la mycorhization ne se restreint pas aux écosystèmes forestiers boréaux et tempérés. Dans les écosystèmes forestiers tropicaux, de telles bactéries ont également été isolées, laissant entrevoir la généricité du concept de bactéries auxiliaires de la mycorhization.

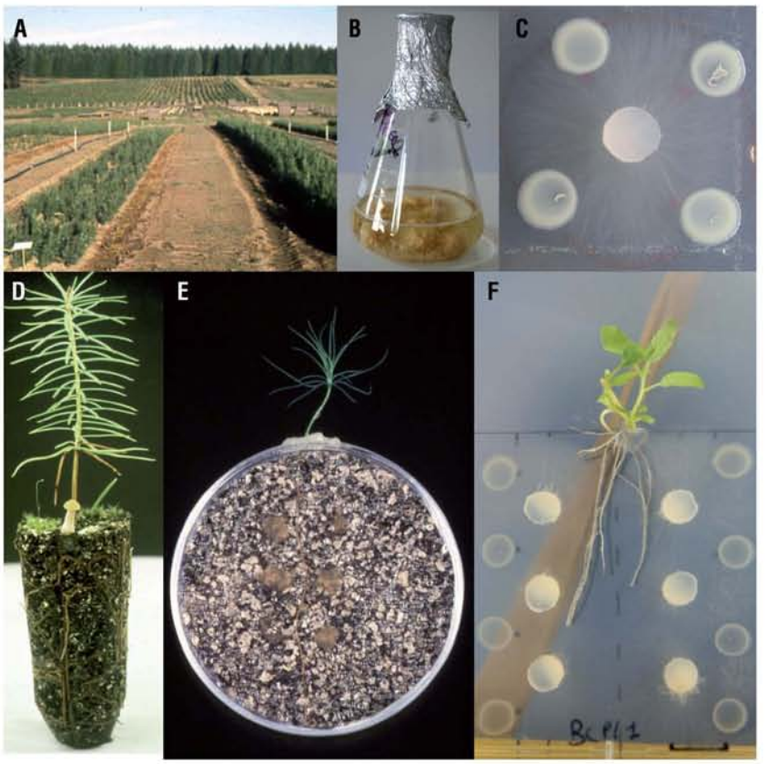
Comment venir en aide aux champignons ectomycorhiziens ?

Les bactéries auxiliaires sont susceptibles d'intervenir aux différentes étapes du cycle biologique des champignons ectomycorhiziens et du processus de mycorhization. Dans les sols, ces champignons sont présents sous trois formes principales : les spores, le mycélium saprophyte présymbiotique et le mycélium symbiotique. Toutes sont des cibles potentielles pour les bactéries auxiliaires. Plusieurs hypothèses ont été proposées dans le passé pour rendre compte des mécanismes de l'effet auxiliaire **(3)**.

Il a été montré que des bactéries sont capables de stimuler la germination des spores de champignons ectomycorhiziens **(4)**. D'autres améliorent la survie et la croissance du mycélium présymbiotique dans le sol, lorsque les conditions lui sont défavorables, augmentant ainsi la probabilité que le champignon rencontre une racine d'arbre compétente et s'y associe **(5)**. En effet, la croissance du mycélium présymbiotique dans le sol est fortement influencée par les paramètres du milieu, qu'ils soient abiotiques (pH, disponibilité en nutriments, présence de composés toxiques…) ou biotiques (compétition et antibiose avec les microflores résidentes). Les bactéries telluriques, de par le large éventail de leurs activités biologiques, peuvent également significativement modifier ces paramètres et ainsi favoriser indirectement l'établissement de la symbiose ectomycorhizienne.

Si certaines bactéries auxiliaires agissent sur la survie et la croissance présymbiotique des champignons ectomycorhiziens, d'autres ont un impact significatif sur les racines, augmentant par exemple leur ramification et le nombre de racines secondaires réceptives à la symbiose **(6)**. En outre, certaines bactéries auxiliaires pourraient indirectement favoriser la colonisation des racines par les champignons mycorhiziens, en induisant la synthèse de métabolites végétaux, tels que les flavonoïdes, responsables de l'attraction du mycélium vers les racines **(7)**.

Une autre étape clé du processus de mycorhization est la reconnaissance des deux partenaires plante-champignon. Il est en effet indispensable, pour sa survie, qu'une plante fasse la différence entre un sympathique mycélium de bolet et celui d'un quelconque pathogène qui va lente-



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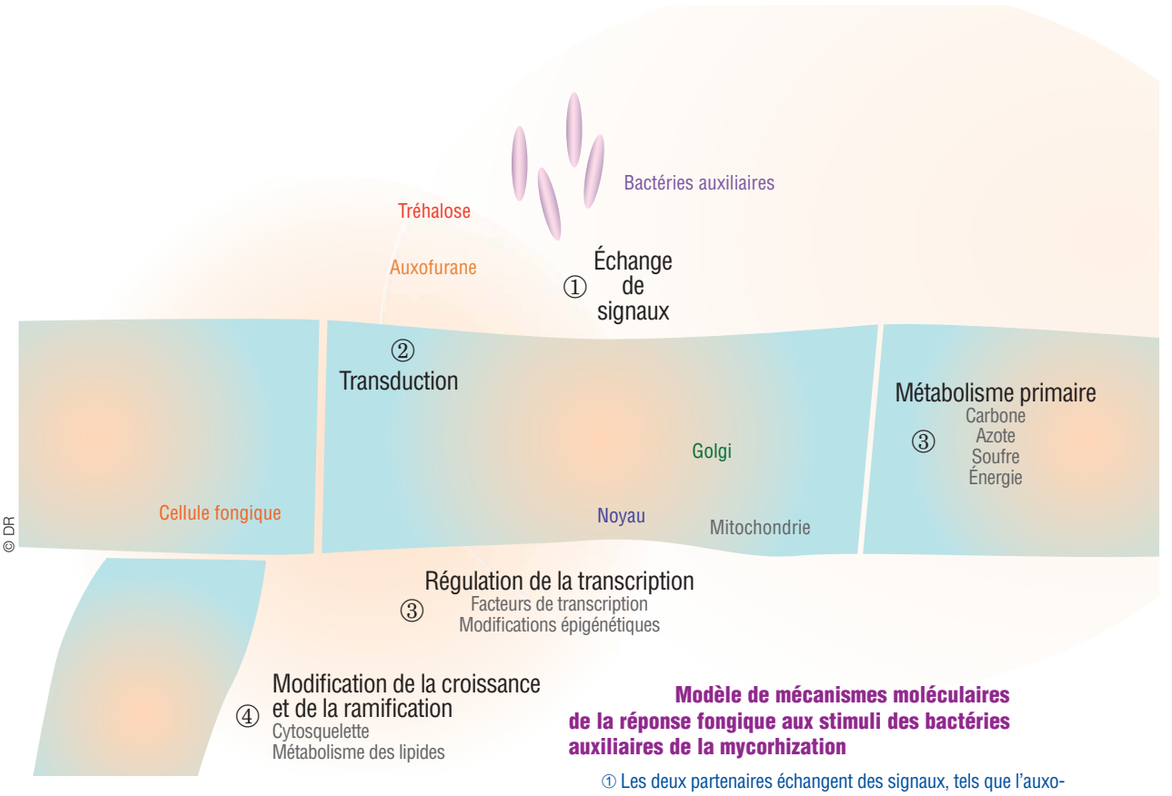
Modes d'expérimentation utilisés pour étudier les bactéries auxiliaires de la mycorhization

A. Inoculation contrôlée en pépinière
B. Co-culture bactérie-champignon en milieu liquide
C. Co-culture bactérie-champignon en milieu solide
D. Interaction tripartite en godet
E. Interaction tripartite sur tourbe-vermiculite en boîte de Petri
F. Interaction tripartite en milieu gélosé

(2) Frey-Klett P *et al.* (2007) *New Phytol* 176 (1), 22-36
(3) Garbaye J (1994) *New Phytol* 128, 197-210
(4) Ali AN, Jackson RM (1989) *Mycol Res* 93,182-6
(5) Brulé C *et al.* (2001) *Soil Biol Biochem* 33, 1683-94
(6) Poole EJ *et al.* (2001) *New Phytol* 151, 743-51
(7) Xie ZP *et al.* (1995) *Plant Physiol* 108, 1519-25



Modèle de mécanismes moléculaires de la réponse fongique aux stimuli des bactéries auxiliaires de la mycorhization

① Les deux partenaires échangent des signaux, tels que l'auxofurane bactérien ou le tréhalose fongique. ② Ces molécules signal sont reconnues par le champignon, activent des voies de transduction du signal et ③ induisent des modifications de la transcription de gènes fongiques, codant notamment pour des protéines impliquées dans le métabolisme primaire, la régulation de la transcription, le cytosquelette ou le métabolisme lipidique. ④ Ces modifications aboutissent à l'échelle macroscopique à une modification de la croissance et de la ramification du mycélium.

ment décomposer ses racines. Pour se défendre, la racine dispose d'un arsenal de moyens que le champignon ectomycorhizien doit désamorcer et/ou contourner pour que puisse s'établir la symbiose : enceinte protectrice pectinocellulolitique des cellules végétales, cortège de molécules enzymatiques ou oxydantes… Dans ce contexte, toute bactérie capable de produire des enzymes de dégradation des parois végétales **(8)** ou des métabolites inhibiteurs des réactions de défense de la plante **(9)** est une bactérie auxiliaire en puissance.

Après leur rencontre, leur reconnaissance et leur mariage, racines et champignons sont prêts pour une vie à deux, dans une demeure bâtie rien que pour eux, l'ectomycorhize. Mais sans les communautés bactériennes qui leur sont associées, les ectomycorhizes ne pourront pleinement accomplir les différentes tâches qui leur incombent. C'est ce que suggèrent des recherches récentes sur la mobilisation des nutriments dans les sols, la fixation de l'azote atmosphérique ou bien encore la protection des racines vis-à-vis des champignons phytopathogènes **(2)**.

Les connaissances actuelles sur les mécanismes d'action des bactéries auxiliaires

Une des difficultés de l'étude des mécanismes d'action des bactéries auxiliaires tient à la complexité biologique de l'écosystème sol dans lequel elles évoluent. Des dispositifs de culture simplifiés, aux paramètres contrôlés et associant un nombre limité de partenaires, ont été développés pour modéliser le plus finement possible les effets de ces bactéries sur les champignons ectomycorhiziens **(figure 2)**. Il a ainsi été montré que certaines bactéries auxiliaires induisent de fortes modifications de la morphologie des hyphes, semblables à celles observées dans les premières étapes de la mycorhization. Certaines stimulent aussi la croissance apicale du mycélium **(10)**. Cet effet est d'autant plus marqué que le milieu de croissance est carencé, expliquant ainsi l'effet promoteur de croissance observé lorsque le champignon se trouve dans des conditions de sol qui lui sont défavorables **(5)**.

À quelques exceptions près, les modifications de comportement du champignon se produisent alors même que les cellules bactériennes ne sont pas encore en contact physique avec leur partenaire. Ceci implique que des signaux soient émis par les bactéries et reconnus par les champignons. Peu de ces signaux ont jusqu'à présent été isolés mais les études préliminaires laissent à penser qu'ils sont de nature variable : certains sont volatils tandis que d'autres sont solubles. Le mieux caractérisé à l'heure actuelle est l'auxofurane, une molécule structurellement proche de l'auxine et produite par une souche bactérienne de *Streptomyces* sp. **(11)**. L'ajout de cette molécule à une culture mycélienne d'amanite tue-mouche (*Amanita muscaria*) permet de provoquer chez le champignon des modifications morphologiques identiques à celles occasionnées par la présence de la bactérie. Il s'agit d'un véritable dialogue entre les deux partenaires puisqu'en retour, le champignon multiplie par quatre la production d'auxofurane chez la bactérie.

D'autres métabolites secondaires ne jouent pas uniquement un rôle de signal. C'est le cas du tréhalose, un disaccharide accumulé par de nombreux champignons ectomycorhiziens et préférentiellement consommé par les bactéries de la mycorhizosphère de champignons accumulateurs. Il a été montré qu'une souche bactérienne de *Pseudomonas fluorescens* auxiliaire perçoit à distance la présence de ce sucre puis se déplace jusqu'à lui grâce à ses flagelles et s'en nourrit. Les évènements qui s'en suivent et qui sont à l'origine de l'effet auxiliaire restent mystérieux mais il a été démontré que la présence de tréhalose accroît l'effet promoteur de croissance du mycélium dans le cas d'une souche de *Pseudomonas monteilii*, elle aussi auxiliaire **(12)**.

(8) Mosse B (1962) *J Gen Microbiol* 27, 509-20
(9) Lehr NA *et al.* (2007) *New Phytol* 174, 892-903
(10) Deveau A *et al.* (2007) *New Phytol* 157, 743-55
(11) Riedlinger J *et al.* (2006) *Appl Environ Microbiol* 72, 3550-7
(12) Duponnois R, Kisa M (2006) *Can J Bot* 84, 1005-8

La régulation transcriptionnelle, une étape clé de l'interaction

Les modifications de la croissance et de la morphologie des hyphes induites par les bactéries auxiliaires, nécessitent des réarrangements du cytosquelette, le convoyage de métabolites utilisés pour la synthèse de nouvelles parois… Ces modifications sont mises en place suite à la perception de signaux, eux-mêmes transmis *via* des cascades de signalisation cellulaire. Comme nous venons de le voir, l'analyse de ces signaux, loin d'être connus dans le cas des interactions champignons ectomycorhiziens-bactéries auxiliaires, mériterait d'être approfondie. Une autre étape clé de la régulation des processus cellulaires est la transcription : l'expression de chacun des milliers de gènes d'une cellule est précisément contrôlée et modulée en fonction des besoins cellulaires et de l'environnement biotique et abiotique des cellules.

Divers outils permettent à l'heure actuelle de suivre la transcription de milliers voire de l'ensemble des gènes d'un organisme (PCR quantitative, puces à ADN, hybridation soustractive…), et donc d'identifier les gènes dont la transcription est modifiée en réponse à un signal. Ces technologies ont été utilisées chez deux champignons ectomycorhiziens cultivés en présence de bactéries auxiliaires **(10,11)**. Qu'il y ait contact physique ou non avec les bactéries auxiliaires, il a été montré que la transcription de gènes impliqués dans de multiples fonctions cellulaires est affectée par la présence de bactéries auxiliaires : synthèse lipidique et protéique, cytosquelette, voies de transduction du signal, régulation épigénétique, transport de métabolites… **(figure 3)**. Ces études transcriptomiques sont une première étape permettant d'identifier des gènes candidats impliqués dans la synthèse de molécules fongiques en réponse à la présence de bactéries auxiliaires. Il reste maintenant à démontrer leur rôle réel *via* leur inactivation par mutagenèse ou interférence à ARN. Ces technologies sont encore difficiles à mettre en œuvre chez certains champignons ectomycorhiziens mais devraient être disponibles d'ici quelques années.

Pour une analyse croisée des modèles d'interaction bactéries-champignons

Vingt ans après la découverte de l'existence des bactéries auxiliaires de la mycorhization, leurs mécanismes d'action commencent peu à peu à se dévoiler. Leur multiplicité s'explique peut-être par la diversité des espèces bactériennes et fongiques en jeu. Il est à noter que des mécanismes similaires ont été décrits dans des systèmes d'interactions bactéries-champignons très éloignés de la symbiose mycorhizienne. C'est par exemple le cas des interactions entre la levure pathogène de l'homme *Candida albicans* et la bactérie pathogène *Pseudomonas aeruginosa*, qui modifie la morphologie et la croissance de la levure au détriment de la santé humaine. Les microorganismes qui affectent notre santé, peuplent nos fromages ou bonifient nos vins prolifèrent naturellement dans un même environnement, le sol, où ils interagissent depuis des millions d'années. Il n'est donc pas si surprenant de retrouver des similitudes dans les mécanismes d'interactions entre ces différents modèles d'étude. Une comparaison approfondie de ces modèles serait des plus profitables pour mieux comprendre les mécanismes impliqués dans chaque interaction. ●

III. *Laccaria bicolor* S238N - *Pseudomonas fluorescens* BBc6R8, un couple modèle pour l'étude de l'effet auxiliaire de la mycorhization.

III.1 *Laccaria bicolor*, un champignon ectomycorhizien aux multiples facettes.

Origine de la souche

Laccaria bicolor est une espèce intermédiaire de *Laccaria laccata* et *Laccaria amethystina,* commune sous couvert de feuillus ou de résineux, en été comme en automne. Son chapeau fauve portant des lames violettes et la présence de mycélium pourpre à la base du pied lui ont valu la dénomination de « *bicolor* » (Bon, 2004). La souche S238 a été isolée par Molina et Trappe (Oregon, USA) à partir d'un carpophore en 1976 sous *Tsuga mentenziana* puis elle a été transférée au laboratoire de Microbiologie de Nancy en 1980. En 1996, elle a été rebaptisée S238N (N-Nancy) car la précocité de sa fructification, le nombre et la morphologie de ses carpophores la distinguent nettement de la souche d'origine (Di Battista *et al.* 1996).

Utilisation commerciale

Comparée à d'autres souches de champignons mycorhiziens, la souche S238N s'est révélée particulièrement performante pour augmenter la croissance des semis de Douglas (*Pseudotsuga menziensii*) en pépinière forestière (Le Tacon & Bouchard, 1986). La hauteur des plants est accrue de 86 % en fin de première année en comparaison du témoin non inoculé. L'inoculation de la souche S238N permet également d'augmenter la croissance de plants en forêt (Heinonsalo *et al.* 2004). Ces excellents résultats ont conduit l'INRA à déposer une licence d'exploitation exclusive avec label INRA de *L. bicolor* dans le cadre de la mycorhization contrôlée. En 2005-2006, 100 000 Douglas mycorhizés par *L. bicolor* S238N sous le label INRA sont sortis des pépinières françaises. Cela représente 1,25 % de la production française totale de Douglas (8 millions de plants). Toutes essences confondues, 62 millions de plants forestiers ont été produits en France pendant cette période (Ministère de l'Agriculture et de la Pêche).

Malgré les fortes performances de *L. bicolor* S238N, il arrive que la mycorhization contrôlée échoue car elle est étroitement dépendante de paramètres abiotiques (pH, humidité, fertilité) et biotiques (compétition, antagonisme, prédation) parfois difficiles à

contrôler. Des études ont donc été menées pour déterminer quels sont les paramètres biotiques et abiotiques les plus favorables à l'établissement de la symbiose ectomycorhizienne et quels sont les mécanismes impliqués dans la formation des ectomycorhizes.

Un modèle de laboratoire

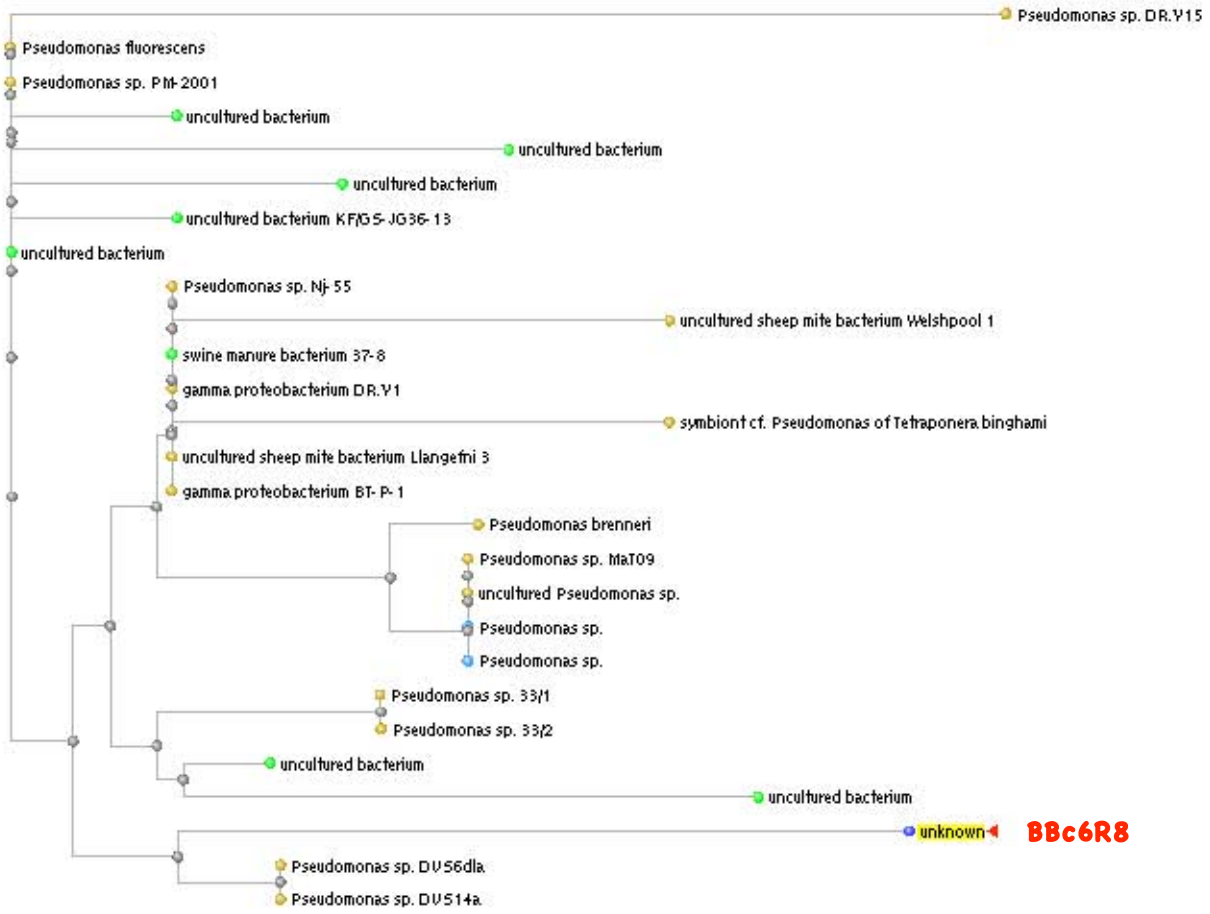
La croissance rapide de *L. bicolor* en culture pure et sa forte capacité à former des mycorhizes *in vitro* ont fait de cette espèce un modèle particulièrement attrayant pour l'étude des processus de mycorhization. Il est ainsi devenu le champignon de référence de nombreux laboratoires de recherche : plus de 150 articles portant sur *L. bicolor* ont été publiés dans des revues scientifiques depuis 1992 (ISI Web of Science, http://portal.isiknowledge.com/). De plus, la séquence du génome d'un monocaryon issu de la souche S238N a récemment été obtenue par le Join Genome Institute (Martin *et al.*, unpublished, Annexe 1). Jusqu'à présent aucun génome de champignon mycorhizien n'était disponible. Peu de temps auparavant, la séquence du génome du peuplier avait également été produite par le JGI (Tuskan *et al.* 2006). Or, il est relativement aisé d'obtenir des mycorrhizes de *L. bicolor* – Peuplier, tant en serre qu'*in vitro*. La connaissance de la séquence des génomes des deux partenaires symbiotiques en fait des modèles d'étude d'exception.

III.2 *Pseudomonas fluorescens* BBc6R8, une bactérie au service des champignons

Origine de la souche.

La souche *Pseudomonas fluorescens* BBc6 a été isolée d'un carpophore de *Laccaria bicolor* S238N, lui-même récolté dans une plantation de Douglas inoculée par le champignon, à Brouvelieures dans les Vosges (Garbaye *et al.* 1990). Il s'agit d'une bactérie flagellée de type Gram négatif. Elle a été classée au sein de l'espèce *P. fluorescens* d'après des critères phénotypiques à l'aide du système d'identification API (Duponnois & Garbaye 1991). Elle appartient au biovar I de l'espèce *P. fluorescens*, un biovar regroupant également les souches *P. fluorescens* associées aux mycorhizes de *L. bicolor* en pépinière forestière et caractérisées notamment par leur capacité à consommer le tréhalose (Frey *et al.* 1997). Le séquençage de la région 16S de l'ADN

Figure I.2. Arbre phylogénétique montrant le lien entre la souche BBc6R8 et le genre *Pseudomonas*, d'après le séquençage de la région 16S de l'ARN ribosomique.



ribosomique confirme l'appartenance de la souche BBc6R8 au genre *Pseudomonas* (Figure I.2, P. Frey-Klett, communication personnelle).

Effet auxiliaire de la mycorhization.

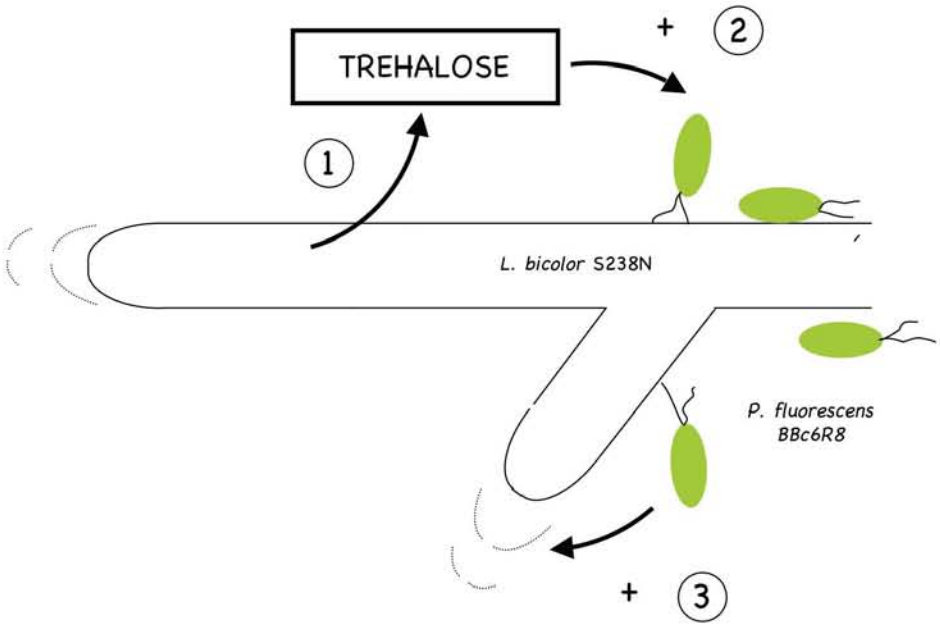
Cette souche stimule la mycorhization du Douglas par *L. bicolor* en conditions gnotobiotiques, en serre (Duponnois & Garbaye, 1991) et en pépinière forestière (Duponnois & Garbaye, 1992a). La grande régularité de l'effet de BBc6 sur la mycorhization a conduit l'INRA à déposer un brevet qui propose l'utilisation de cette souche comme moyen d'améliorer la croissance des plantes (Garbaye & Duponnois, 1992b).

La souche BBc6R8

La souche BBc6R8 est un mutant spontané résistant à la rifampicine, sélectionné par P. Frey-Klett en 1994 pour effectuer des suivis de la population bactérienne après inoculation dans l'environnement (Frey-Klett *et al.*, 1997). La mutation chromosomique, probablement située dans l'ARN polymérase, n'affecte pas le phénotype de la souche. A l'aide de ce mutant, il a pu être montré que l'effet auxiliaire se manifeste jusqu'aux faibles doses de 10 ufc/cm^3 en pépinière (Frey-Klett *et al.*, 1999). Toutes les études menées dans l'UMR1136 IAM sur l'effet auxiliaire de la mycorhization postérieurement à 1994 ont été réalisées avec la souche BBc6R8.

La souche BBc6R8 a par ailleurs été caractérisée du point de vue physiologique et écologique. Elle synthétise de l'acide indol acétique (Frey-Klett *et al.,* unpublished) et deux pyoverdines (Gamalero *et al.* 2003). Par contre elle ne produit pas de cyanure d'hydrogène et ne semble pas posséder les gènes de synthèse des antibiotiques classiques (phénazine, pyoluteorine, pyrrolnitrine et 2,4-diacetylphloroglucinol). Du point de vue écologique, la population bactérienne décroît rapidement dans le sol et la rhizosphère du Douglas après inoculation. En revanche, sa survie est significativement améliorée en présence de *L. bicolor* S238N et elle présente un tactisme vis-à-vis du tréhalose, un disaccharide fortement accumulé dans les hyphes de *L. bicolor* S238N. Sa localisation dans le sol reste méconnue : elle ne colonise préférentiellement ni la rhizosphère du Douglas ni les ectomycorhizes de *L. bicolor* S238N (Frey-Klett *et al.* 1999). Sen *et al.* (1996) ont démontré que la souche adhère sélectivement au mycélium

Figure I.4. Modèle de l'interaction entre *P. fluorescens* BBc6R8 et *L. bicolor* S238N.



extramatriciel de *L. laccata*, *in vitro.* Au vu de ces résultats, il a été suggéré que la bactérie pourrait être associée au mycélium libre de *L. laccata* dans le sol (Klett, 1996).

III.3 Etat actuel des connaissances concernant les mécanismes de l'effet auxiliaire de *P. fluorescens* BBc6R8 sur la mycorhization par *L. bicolor*

Garbaye (1994) avait proposé cinq mécanismes, non exclusifs les uns des autres, pouvant expliquer l'effet auxiliaire des bactéries : (1) une modification des propriétés physico-chimiques du sol favorable au mycélium fongique, (2) une augmentation de la germination des spores, (3) un accroissement des hyphes qui augmenterait la probabilité de rencontre avec les racines courtes, (4) une préparation des racines courtes à la symbiose et (5) un effet antagoniste contre des pathogènes du sol (Figure 3, II.3). A l'heure actuelle, les effets éventuels de BBc6R8 sur la germination des spores de *L. bicolor* et sur la compétence des racines courtes des hôtes du champignon sont inconnus. Par contre, cette souche bactérienne est antagoniste *in vitro* de certains champignons phytopathogènes (*Rhizoctonia*, *Fusarium*, Frey-Klett *et al.*, communication personnelle) mais montre un effet auxiliaire sur la croissance du champignon responsable du piétin échaudage *Gaeumannomyces graminis* (A. Sarniguet, communication personnelle). Enfin, Duponnois & Garbaye (1990) ont montré que la souche BBc6R8 produit *in vitro* de l'acide citrique qui est consommé par le champignon. Dans le sol, cette souche accroît la survie et la croissance du mycélium pré-symbiotique de *L. bicolor* S238N lors d'expériences réalisées en condition gnotobiotique (Brûlé *et al.* 2001). Elle présente également un effet positif sur la croissance des hyphes de *L. bicolor in vitro* (Duponnois & Garbaye, 1992a).

Au vue de ces observations, l'hypothèse d'une relation directe entre la bactérie et le champignon qui concernerait principalement la phase de survie et de croissance du mycélium pré-symbiotique est privilégiée (Figure I.4). La souche BBc6R8 serait attirée par des exudats fongiques tels que le tréhalose (1). La consommation de ces exudats lui serait bénéfique (2) et elle produirait en retour un ou plusieurs métabolites spécifiques qui seraient capables de stimuler la croissance et/ou la survie du champignon (3). Cependant, de nombreuses questions restent en suspens :

- par quels mécanismes la souche BBc6R8 stimule-t-elle la croissance et la survie de *L. bicolor* ?
- par quels mécanismes le champignon améliore-t-il la survie de la bactérie ?
- quelles sont les molécules signal échangées entre les deux organismes ?
- par quel processus le champignon distingue-t-il une bactérie auxiliaire d'une souche antagoniste ?

IV. Objectifs de la thèse et démarche expérimentale

L'objectif de cette thèse était de tenter d'apporter des éléments de réponse à ces différentes questions par deux approches complémentaires : l'une, sans a priori, consistant à décrire les modifications des transcriptomes des deux partenaires au cours de leur interaction et lors de confrontations avec d'autres micro-organismes rhizosphériques, à l'aide d'outils de type puces à ADN. La seconde approche, ciblée, consiste à examiner le rôle joué par certains métabolites tels que le tréhalose et la thiamine, ou l'implication du système de sécrétion bactérien de type III dans l'effet auxiliaire. Ces cibles ont été choisies suite à des études préliminaires prometteuses menées dans notre unité ou par d'autres laboratoires (équipe du Dr G. Preston, Université d'Exeter). Nous avons choisi de réaliser cette étude à partir d'un dispositif de confrontation bactérie-champignon *in vitro*, dans lequel l'ensemble des paramètres physico-chimiques est contrôlé. Il a été développé de façon à mimer l'effet bénéfique de la bactérie sur la croissance pré-symbiotique du champignon. Bien qu'entièrement artificiel ce type de dispositif semble plus adapté pour la recherche de signaux ou pour des études transcriptomiques que des systèmes en conditions semi-contrôlées on non-contrôlées en serre ou en pépinière. En effet, les problèmes techniques tels que l'extraction des ARN d'un seul organisme se posent dans des dispositifs semi-controlés ou non-contrôlés. De plus, la variabilité biologique est amplifiée par l'absence de maîtrise de paramètres biotiques et abiotiques et rend l'analyse des données difficile.

Chapter I

A gene profiling approach to explore the response of *L. bicolor* S238N to the mycorrhiza helper strain *P. fluorescens* BBc6R8

Chapitre I

Analyse transcriptomique des mécanismes moléculaires intervenant dans la réponse de *L. bicolor* à la souche bactérienne auxiliaire de la mycorhization *P. fluorescens* BBc6R8

Introduction

De l'intérêt des approches « globales ».

Les approches dites « globales » (i.e méthodes visant à étudier l'ensemble des gènes, protéines ou voies métaboliques) permettent d'obtenir une image à un instant *t* du statut transcriptomique, protéomique ou métabolique complet d'une cellule ou d'un groupe de cellules. Cette information est indispensable pour aborder le fonctionnement des cellules en terme de réseaux métaboliques dynamiques. On sait en effet aujourd'hui que l'étude des propriétés individuelles des molécules composant une cellule ne permet pas de comprendre le fonctionnement et les réponses de la cellule à des signaux extérieurs (Sweetlove & Fernie, 2005). Le fonctionnement cellulaire résulte d'interactions complexes entre une multitude de molécules. Il peut être décrit comme un jeu de réseaux moléculaires interdépendants et dynamiques : ces réseaux ne sont pas fixes comme le seraient des réseaux de voies ferrées mais ils constituent eux-mêmes des entités dynamiques dont la structure évolue en fonction des conditions environnantes.

Pour ces raisons, les mécanismes d'adaptation métabolique en réponse à un signal environnemental s'établissent à différentes échelles : modification de l'expression des gènes, de la traduction protéique et de l'activité de protéines cibles. Dès lors, seule la combinaison d'études transcriptomiques, protéomiques et enzymatiques permet d'avoir un aperçu complet de la réponse cellulaire. Toutefois la mise en œuvre d'études de cette ampleur s'avère souvent difficile du fait du temps nécessaire au développement des différentes approches et de leurs coûts. Ce genre de démarche est donc réservée à quelques organismes modèles tels que la levure, la drosophile, ou *Arabidopsis thaliana.* Néanmoins, les approches de transcriptomique n'en restent pas moins informatives car elles permettent d'identifier les réseaux de gènes qui jouent un rôle clé dans la réponse à un traitement donné.

Bien que la régulation transcriptomique ne soit pas considérée comme le système majeur de régulation cellulaire - principalement post-traductionnelle -, nous avons choisi d'utiliser cette approche pour explorer les mécanismes moléculaires menant à une augmentation de la croissance du mycélium et à une modification de la morphologie des hyphes de *L. bicolor* S238N en présence de la bactérie auxiliaire BBc6R8. En effet, il a été clairement démontré chez de nombreux organismes, tant procaryotes qu'eucaryotes,

que concernant les processus de développement et en réponse à de multiples stress, les régulations transcriptionnelles jouaient un rôle majeur. D'autre part, parmi les approches globales citées précédemment, la transcriptomique est aujourd'hui la méthode qui a connu les plus forts développements technologiques. De plus, les degrés de résolution obtenus aujourd'hui sont supérieurs à ceux obtenus en protéomique notamment : les électrophorèses en deux dimensions ne permettent pas de discriminer toutes les protéines d'une cellule et l'identification des spots n'est pas toujours aisée. Enfin mon laboratoire d'accueil dispose d'une expertise internationalement reconnue en transcriptomique végétale et fongique et dispose de tout l'équipement nécessaire pour la mise en œuvre de ce type d'expérience. Enfin, ma thèse porte sur le champignon ectomycorrhizien modèle *L. bicolor* S238N, dont le génome a été récemment séquencé à l'initiative de mon laboratoire d'accueil.

Objectifs et méthodes

Nous avons tenté de répondre à quatre questions par le biais de cette étude transcriptomique:

i. quels sont les transcrits impliqués dans l'augmentation de la croissance et la modification de la morphologie des hyphes en réponse à la présence de la bactérie auxiliaire BBc6R8 ?
ii. comment les mécanismes moléculaires induits en présence de la souche BBc6R8 se mettent-ils en place et évoluent au cours de l'interaction ?
iii. un contact physique entre les hyphes et les cellules bactériennes est-il nécessaire pour induire une réponse transcriptomique?
iv. quelle est la spécificité de la réponse moléculaire de *L. bicolor* S238N vis-à-vis de la souche *P. fluorescens* BBc6R8 ?

A cette fin, nous avons élaboré des membranes de nylon portant 4992 ADNc synthérisés à partir de banque d'EST de *L. bicolor* S238N. Elles ont été utilisées pour mesurer les variations de concentration des transcrits de *L. bicolor* S238N à différents temps de l'interaction avec *P. fluorescens* BBc6R8. Nous avons évalué le degré de spécificité de la réponse fongique en mesurant également l'expression de quelques transcrits régulés en présence de BBc6R8, lors de l'interaction avec cinq autres bactéries du sol. Dans un deuxième temps des puces à oligonucléotides (Nimblegen) comportant l'ensemble des

gènes du champignon ont été utilisées pour compléter les données précédemment acquises.

En parallèle, nous avons réalisé une description quantitative de l'évolution de la morphologie des hyphes fongiques aux différents temps de l'interaction afin d'établir des corrélations entre le phénotype macromoléculaire du champignon et les modifications d'expression des gènes. L'ensemble de cette étude a été effectuée à l'aide d'un dispositif de confrontation *in vitro*.

I.1. Development of the *in vitro* set up of confrontation between *L. bicolor* S238N and *P. fluorescens* BBc6R8.

The choice of the culture system is a key point when studying interactions between fungi and bacteria. A wide variety of systems of interaction has been designed depending on the objectives of the studies. Culture systems can be separated in two categories: *in vivo* systems such as field plantations, pot plantation or living organisms (plants, mammals…) and *in vitro* systems (liquid, semi-liquid and solid media). Theoretically, *in vivo* systems should be preferred because they mimic as much as possible the biological reality and they take into account the complex net of interactions that occur in natural systems. However, this advantage is also its major inconvenient as it is difficult to identify and to master all the biotic and abiotic parameters that have an effect on the interaction. As a consequence, the reproducibility of *in vivo* experiments is often low. Furthermore, the high complexity of the interactions that occur in natural environment is a difficulty to analyse them in details. For these reasons, researchers often use *in vitro* set up. Artificial systems permit to control and to modify independently abiotic and biotic parameters and experiments are highly reproducible. Moreover, *in vitro* experiments are generally more useful to study and to visualize microscopic events such as morphological modifications, chemotaxis and attachment processes, metabolite production… But it should always be reminded that the existence of mechanisms that are deduced from *in vitro* analyses must be verified *in vivo*.

In the present survey, the objective was to obtain a reproducible effect of the bacterial strain on the fungal growth in a controlled environment. Many factors have been tested (Table 1.1). Finally, it appeared that a reproducible effect was only obtained when the fungus was grown in limiting conditions. The utilization of a Pachlewski medium with low carbon (1 $g.L^{-1}$ of glucose) and no thiamine ($P20Th^{-}$), associated with a simultaneous inoculation of the bacteria and the fungus, and a temperature of incubation of 10°C gave the best results. In these conditions, a significant increase of the fungal growth was reproducibly observed after 12 days of co-culture. Interestingly, these conditions correspond to the ones encountered by the fungus in temperate forest soils: the year average temperature of soil is around 12°C and the carbon availability is low. Moreover, B. Palin showed the fungal growth-promoting effect of the bacteria was lost in summer.

Table 1.1. Impact of various parameters on the effect of *P. fluorescens* BBc6R8 on the growth of *L. bicolor* S238N *in vitro*. The effect of the time of inoculation (simultaneous – IS, or delayed –ID), the type of medium (P5 – 5g.L^{-1} glucose, 1g.L^{-1} maltose, thiamine ; P20 – 1 g.L^{-1} glucose, no maltose, thiamine ; P20Th^{-} 1 g.L^{-1} glucose, no maltose, no thiamine ; P20 1/10 – all components from P20 diluted 10x ; P20Th- 1/10 - all components from P20Th^{-} diluted 10x), the spotting of bacteria onto a cellulose circle and the temperature were tested. A negative, nulle or positive effect of the bacterial strain on the fungal growth is indicated by -, 0 or +, respectively. The time at which occurs the effect is also indicated. An arrow indicates that the effect occurs from the time written until the end of the experiment. A dot between two times indicates that the effect occurred between the time X and the time Y. For each treatment, seven replicates were performed. A one-way (time) ANOVA was performed to determine if the effect of the bacteria on the fungal growth was significant or not. nd : non determined.

Inoculation	Medium	Cellulose circle	Temperature (°C)	Effect	Time (days)
IS	P5	+	25	-	nd
IS	P20 1/10	+	25	-	7-26
IS	P5	-	25	-	14->
ID	P5	-	25	-	10->
IS	P20 1/10 Th-	+	25	-0	3-9
IS	P20 1/10	+	10	+0-	7-12,20->
IS	P20Th-	+	25	+0	5-7
IS	P20 1/10 Th-	+	25	+0	3-6
IS	P20 1/10	+	25	0	nd
IS	P20	+	25	0	-
IS	P20 1/10	+	25	0	-
IS	P20 1/10	-	25	0	-
IS	P20 1/10	+	10	0	-
IS	P20 1/10	+	25	0	-
IS	P20Th-	+	25	0	-
IS	P20Th-	-	25	0	-
IS	P5 1/10	+	25	0	-
IS	P20	+	25	+	nd
IS	P20 1/10	+	25	+	nd
IS	P20 1/10	+	25	+	4->
ID	P20 1/10	+	25	+	26->
IS	P20Th-	+	25	+	nd
IS	P20Th-	+	25	+	7->
IS	P20Th-	+	25	+	7->
IS	P20Th-	+	25	+	3->
IS	P20Th-	+	25	+	23->
IS	P20Th-	+	10	+	4->
IS	P20Th-	+	10	+	13->
IS	P20Th-	+	10	++	12->
IS	P20Th-	-	10	++	12->

At this time of the year, the growth of the free-living mycelium was naturally increased, maybe under the stimulation of the fungal internal clock.

Publication n°3. The mycorrhiza helper *Pseudomonas fluorescens* BBc6R8 has a specific priming effect on the growth, morphology and gene expression of the ectomycorrhizal fungus *Laccaria bicolor* S238N

New Phytologist

Research

The mycorrhiza helper *Pseudomonas fluorescens* BBc6R8 has a specific priming effect on the growth, morphology and gene expression of the ectomycorrhizal fungus *Laccaria bicolor* S238N

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Summary

• The mycorrhiza helper *Pseudomonas fluorescens* BBc6R8 promotes the presymbiotic survival and growth of the ectomycorrhizal fungus *Laccaria bicolor* S238N in the soil.

• An *in vitro* fungal–bacterial confrontation bioassay mimicking the promoting effects of the bacteria on fungal growth was set up to analyse the fungal morphological and transcriptional changes induced by the helper bacteria at three successive stages of the interaction. The specificity of the *P. fluorescens* BBc6R8 effect was assessed in comparison with six other rhizobacterial strains possessing mycorrhiza helper or pathogen antagonistic abilities.

• The helper BBc6R8 strain was the only strain to induce increases in the radial growth of the colony, hyphal apex density and branching angle. These morphological modifications were coupled with pleiotropic alterations of the fungal transcriptome, which varied throughout the interaction. Early stage-responsive genes were presumably involved in recognition processes and transcription regulation, while late stage-responsive genes encoded proteins of primary metabolism. Some of the responsive genes were partly specific to the interaction with *P. fluorescens* BBc6R8, whereas others were mutually regulated by different rhizobacteria.

• The results highlight the fact that the helper BBc6R8 strain has a specific priming effect on growth, morphology and gene expression of its fungal associate *L. bicolor* S238N.

Key words: fungal–bacterial interactions, *Laccaria bicolor* S238N, mycorrhiza helper bacteria, rhizobacteria, transcriptome.

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Introduction

The soil is probably one of the most complex ecosystems in which fungal–bacterial interactions operate. Complex deleterious, beneficial and commensal communities of fungi and bacteria surround plant roots. Among those, ectomycorrhizal fungi form symbiotic associations with tree roots and participate in tree nutrition (Smith & Read, 1997). They interact physically and functionally with soil bacterial communities, thus establishing the so-called 'multitrophic

ectomycorrhizal complex' which impacts gross production and nutrient (Frey-Klett & Garbaye, 2005). The formation of ectomycorrhizal symbiosis can be significantly improved by selected soil and mycorrhizosphere bacteria. These 'mycorrhiza helper bacteria' (MHB, Garbaye, 1994) belong to a wide range of genera (*Burkholderia*, *Paenibacillus*, Poole *et al.*, 2001; *Pseudomonas, Bacillus*, Duponnois & Garbaye, 1991, *Streptomyces*, Maier *et al.*, 2004). They have been described not only in temperate ecosystems, but also in tropical ones (Founoune *et al.*, 2002). Moreover, the 'mycorrhiza helper bacteria' concept is not restricted to ectomycorrhizal symbiosis, as some helper bacteria also promote the endomycorrhizal symbiosis (Duponnois & Plenchette, 2003).

The mechanisms by which helper bacteria stimulate mycorrhiza formation remain unclear. Five hypotheses have been proposed by Garbaye (1994): increase in the receptivity of the root towards mycorrhizal infection; improvement of the root–fungus recognition processes; stimulation of fungal growth during the presymbiotic stage; modifications of the physicochemical properties of the rhizosphere soil that improve mycorrhizal infection; and induction of fungal spore germination. So far, only the direct effects of helper bacteria on presymbiotic survival and growth of the mycorrhizal fungi in the soil have been well documented (Brulé *et al.*, 2001; Founoune *et al.*, 2002; Schrey *et al.*, 2005). However, little is known about the molecular mechanisms induced by helper bacteria concerning the fungal growth-promoting effect. Only changes in *Amanita muscaria* gene expression upon interaction with the helper bacteria *Streptomyces* sp. have been reported so far (Schrey *et al.*, 2005; Riedlinger *et al.*, 2006). Further studies involving other interaction models of mycorrhiza helper bacteria–ectomycorrhizal fungi are required to assess the specificity of the fungal gene regulations described to date (Bending, 2007).

The mycorrhiza helper *Pseudomonas fluorescens* strain BBc6R8 significantly promotes the establishment of ectomycorrhizal symbiosis between Douglas fir and the ectomycorrhizal fungus *Laccaria bicolor* S238N (Frey-Klett *et al.*, 1997). This promoting effect is related to the enhanced survival and growth of *L. bicolor* S238N mycelium during its presymbiotic phase in the soil, under unfavourable growth conditions (Brulé *et al.*, 2001). The aim of the present work was to elucidate the molecular mechanisms by which *P. fluorescens* BBc6R8 promotes *L. bicolor* S238N growth. For this purpose, an *in vitro* confrontation assay was first set up in which helper bacteria reproducibly improved mycelium growth. Hyphal morphology and growth were monitored along the interaction with *P. fluorescens* BBc6R8 and six other rhizospheric bacterial strains with mycorrhiza helper or pathogen antagonistic abilities. Using cDNA arrays, the impact of the helper *P. fluorescens* BBc6R8 on the fungal transcriptome was analysed at three key stages of the fungal–bacterial interaction: before, during and after physical contact. The specificity of the *P. fluorescens* BBc6R8 effect on gene expression was assessed by comparing the expression level of several target genes with those of the six other rhizobacterial strains.

Materials and Methods

Microorganisms and confrontation bioassays

The ectomycorrhizal basidiomycete *Laccaria bicolor* S238N (Maire P. D. Orton) was maintained on Pachlewski agar medium P5 (Di Battista *et al.*, 1996) at 25°C for 3 wk. The MHB *Pseudomonas fluorescens* strain BBc6R8 is a spontaneous rifampicin-resistant mutant. It phenotypically conforms to the parental strain BBc6 that was isolated from a *L. bicolor* fruiting body collected in a Douglas fir plantation (Duponnois & Garbaye, 1991; Frey-Klett *et al.*, 1997). The relevant characteristics of the six bacterial strains *Collimonas fungivorans* Ter331, *Paenibacillus* sp. EJP73, *Paenibacillus* sp. F2001L, *Bacillus subtilis* MB3, *Burkholderia* sp. EJP67 and *Pseudomonas fluorescens* Pf29A are listed in Table 1. All strains were maintained at –80°C in Luria-Bertani medium (Sambrook *et al.*,

Table 1 Bacterial strains used in this study

Strains	Relevant characteristics	Reference and origin
Pseudomonas fluorescens BBc6R8	Helper effect on the Douglas fir-*Laccaria bicolor* symbiosis	Frey-Klett *et al*. (1997) INRA-Nancy, France
Collimonas fungivorans Ter331	Chitinolytic and antagonistic effect on fungi in microcosms and on *L. bicolor* S238N-*P. sylvestris* symbiosis in glasshouse	de Boer *et al*. (2004) W. de Boer, NIOO-KNAW, the Netherlands
Paenibacillus sp. EJP73	Helper effect on the *Pinus sylvestris-Lactarius rufus* symbiosis	Poole *et al*. (2001); Aspray *et al*. (2006) G. Bending, Warwick HRI, England
Pseudomonas fluorescens Pf29A	Biocontrol agent against the wheat pathogen *Gaeumannomyces graminis* var *tritici*	Chapon *et al*. (2002) A. Sarniguet, INRA-Rennes, France
Bacillus subtilis MB3	*Bacillus subtilis*; helper bacteria of Douglas fir-*L. bicolor* mycorrhiza formation	Duponnois *et al*. (1991) INRA-Nancy, France
Burkholderia sp. EJP67	Helper effect on the *Pinus sylvestris-Lactarius rufus* symbiosis in microcosms	Poole *et al*. (2001) G. Bending, Warwick HRI, UK
Paenibacillus sp. F2001L	Presumed intracellular bacterial strain isolated from a liquid culture of *L. bicolor* S238N	Bertaux *et al*. (2003) INRA-Nancy, France

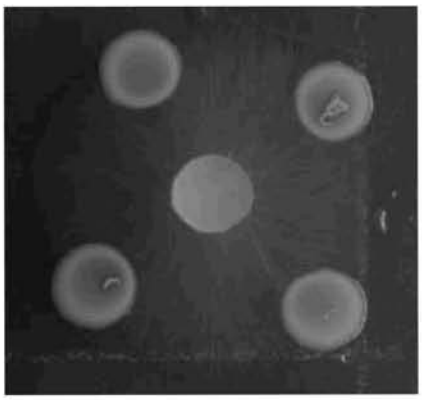

Fig. 1 Photograph of the *in vitro* bioassay after 21 d of dual growth.

1989) with 20% glycerol. In the present work, the bacterial strains were first grown on 10% TSA plates (3 g l^{-1} tryptic soy broth from Difco and 15 g l^{-1} of agar) at 25°C for 65 h to prepare the bacterial inoculum for the *in vitro* bioassay. Then, three to four colonies were picked and suspended in 2 ml of sterile deionized water before spreading on to 10% TSA medium. After 48 h of growth at 25°C, the bacteria were harvested and centrifuged at 3300 ***g*** for 10 min. The pellet was washed once, then resuspended in deionized water in order to obtain a suspension with $A600_{nm} \sim 0.7$ (*c.* 10^9 cfu ml^{-1}).

An *in vitro* confrontation bioassay was developed using 9-cm-diameter Petri dishes containing 20 ml per plate of a Pachlewski medium (Paschlewski & Pachlewska, 1974), which was modified as follows: 0.5 g Di-NH_4^+ tartrate, 1 g KH_2PO_4, 0.5 g $MgSO_4$, 1 g glucose, 1 ml 1/10 diluted Kanieltra micro-element solution and 20 g agar l^{-1} at pH 5.5 (P20Th-). A plug of *L. bicolor* S238N was cut out from the edge of a colony grown on P5 medium and transferred into the centre of a P20Th$^-$ plate (Fig. 1). Four 10 µl droplets of sterile deionized water (control treatment) or bacterial suspension (bacterial treatment) were distributed at 1.75 cm from the centre of the fungal plug. Plates were sealed with plastic tape and incubated at 10°C in the dark.

Mycelium growth and morphology measurements

The diameter of the fungal colony was measured every fourth day from day 12 to 40 after the addition of water or bacterial suspensions, with seven replicates per treatment. For the *P. fluorescens* BBc6R8-inoculated treatment only, the morphology of the fungal mycelium was analysed at three key steps of the interaction: before contact between the mycelium and the bacteria (14 d), at contact time (16 d) and after an extended contact period (21 d). For the other six bacterial strains, observations were made only at the precontact stage. Three biological replicates per treatment were performed. For each replicate, two photographs were taken using an Olympus BX41 microscope (×40 magnification) equipped with a ColorView System camera. The number of apices per microscopic field (3.5 mm^2), branching angles, branching densities (number of ramifications divided by the number of apices) and curvature of hyphae were measured on each photograph using AnalySIS software (Soft Imaging System, Olympus, Münster, Germany). The effect of the bacterial treatment on the growth and morphology of the fungal mycelium was determined using analysis of variance (ANOVA) at the threshold value of 0.05 and the Fisher test. The SuperANOVA 1.11 software (Abacus) was used for these statistical analyses.

cDNA array analysis cDNA libraries from pure culture of *L. bicolor* S238N mycelium and from three stages of *L. bicolor* S238N sporocarp development (Lb2 library: stipes and caps of 5–10 mm growing sporocarps; Lb3 library: caps of 30–40 mm mature sporocarps), collected under Douglas fir seedlings grown in a glasshouse, were constructed in the λTriplEx2 vector as previously described (Peter *et al.*, 2003). The cDNA inserts from bacterial clones were PCR-amplified and 4992 cDNAs were arrayed from 384-well microtitre plates on Nylon membranes, as described previously (Peter *et al.*, 2003).

RNA isolation for target preparation *L. bicolor* S238N mycelium from 50 plates was collected, frozen in liquid nitrogen and pooled. Mycelium was sampled in triplicate at three stages of the interaction: before contact between *L. bicolor* S238N and *P. fluorescens* BBc6R8 (14 d), at contact (16 d) and after an extended contact period (21 d). Mycelium was ground in a mortar with liquid nitrogen, and total RNA was extracted using Trizol as recommended by the manufacturer for small quantities of material (Invitrogen AB, Stockholm, Sweden). RNA was further purified using the RNA/DNA mini kit (Qiagen, Hilden, Germany). The quality of the RNA was checked via RNAse-free 1% agarose electrophoresis and by PCR amplification of four full-length cDNAs (mitochondrial fission-related protein, 60S ribosomal protein, cipC and profiline). To analyse the expression of target genes from *L. bicolor* S238N mycelium in interaction with the other rhizobacterial strains, the *in vitro* system described earlier was used, replacing drops of *P. fluorescens* BBc6R8 inoculum with suspensions of the other bacterial strains. Mycelium was collected and RNA was extracted using the RNeasy Plant mini-kit (Qiagen) following the manufacturer's recommendations. The quality of the RNA was checked by RNAse-free 1% agarose electrophoresis.

cDNA array hybridization Synthesis of complex cDNA probes, cDNA array hybridization and data analysis were performed as described by Peter *et al.* (2003) and Duplessis *et al.* (2005).

DNA sequencing cDNA clones encoding for up- and down-regulated transcripts were amplified by PCR and sequenced as previously described (Kohler *et al.*, 2003). Edited nucleotide

sequences were compared with the gene models predicted by the genome sequence assembly v.1.0 of *L. bicolor* S238N-H82 strain using the BlastN server on the JGI Laccaria Portal (http://genome.jgi-psf.org/Lacbi1/Lacbi1.home.html). Conserved domains of the predicted protein sequences were searched in Pfam, Smart and KOG databases (ftp://ftp.ncbi.nih.gov/pub/mmdb/cdd).

Quantitative PCR analysis To validate the cDNA array data, real-time quantitative PCR analyses were performed on seven up- or down-regulated genes selected for their potential biological relevance (Cipc, tectonin II, tra1, glutathione-S-transferase, hypothetical protein with yip1 domain, fumarate reductase and polyadenylate binding protein). Eight non-regulated genes were also used as controls and two of them (Lb17E10 and trehalose phosphorylase) were chosen for data normalization. The expression of genes encoding Tectonin II, Concanamycine-induced protein C (Cipc1), Transcription associated protein (Tra1), Gcn5, Spt3, Ada 3, peroxisomal hydratase dehydrogenase epimerase multiprotein (LbFOX2), Acetoacyl coenzyme A synthetase (LbAaCS) and cyclophiline 40 (LbCyp 40) was monitored by real-time PCR. The primer pairs (Supplementary material, Table S1) were designed using Primer 3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_http://www.cgi) and Amplify 3.1 (http://engels.genetics.wisc.edu/amplify). The following criteria were used: product size between 100 and 400 bp, melting temperature 60°C ± 1°C and GC% > 50%.

RNA samples were used for cDNA array analysis and for the real-time PCR measurements. For the analysis of BBc6R8-responsive genes during the interaction with other rhizobacteria, cDNA were synthesized from 0.5 mg of total RNA (iScript, Bio-Rad, Hercules, CA, USA). Each qPCR reaction (15 μl total volume) contained 2 μl of cDNA template, 300 nM of each primer and 1X SYBR green PCR master mix (Bio-Rad). Reactions were run using a MJ-opticon2 DNA real-time PCR system (Bio-Rad). The following cycling parameters were applied: 95°C for 3 min and then 40 cycles of 95°C for 30 s, 60°C for 1 min and 72°C for 30 s. A negative control was run for each primer pair. For data analysis, the geometric mean of the three biological replicates for each condition was calculated. The PCR efficiency was checked to be 100% and fold differences were calculated using the ΔΔCt method (Livak & Schmittgen, 2001).

Results

Mycelium growth and morphology

Pseudomonas fluorescens BBc6R8 significantly stimulated the extension of *L. bicolor* S238N mycelium (expressed as the diameter of the colony) as early as 14 d of dual cultivation (Fig. 2, Table 2). It also enhanced the hyphal branching angle and the hyphal branching density during the entire interaction (Fig. 3). Whereas the hyphal apex density was significantly enhanced before contact, it was significantly decreased at and after the time of contact. *Laccaria bicolor* S238N reacted differently to the presence of the six other helper and nonhelper rhizobacterial strains. Its growth was significantly enhanced by the MHB *Burkholderia* sp. EJP67, the presumed intracellular fungal *Paenibacillus* sp. F2001L, and later by the biocontrol *P. fluorescens* Pf29A. The MHB *B. subtilis* MB3 had no effect on fungal growth. By contrast, it was significantly decreased by the MHB strain *Paenibacillus* sp. EJP 73 and the chitinolytic *C. fungivorans* Ter331 (Fig. 2). *Pseudomonas fluorescens* BBc6R8 and *Burkholderia* sp. EJP67 were the only strains that had enhanced growth by the time mycelium was collected for transcript analysis and morphology measurements were performed (i.e. after 14 d of confrontation). At this time, the apex number was significantly reduced by *Burkholderia* sp. EJP67 (Table 2), while the branching angle was significantly enhanced by both *Burkholderia* sp. EJP67 and *C. fungivorans* Ter331. The branching density was affected by the four strains EJP67, Pf29A, MB3 and Ter 331. Finally, only *Burkholderia* sp. EJP67 induced a high amount of curvature in the hyphae (data not shown).

Fig. 2 Effect of bacterial strains *Pseudomonas fluorescens* BBc6R8 (black circles, dash points), *P. fuorescens* Pf29A (open triangles), *Collimonas fungivorans* Ter331 (black squares, dash points), *Paenibacillus* sp. EJP73 (black crosses, dot points), *Bacillus subtilis* MB3 (open circles), *Burkholderia* sp. EJP67 (black triangles, dash points) and *Paenibacillus* sp. F2001L (black diamonds, dot points) on the radial growth of *Laccaria bicolor* S238N (open squares) in the *in vitro* bioassay. Each point is the mean (± SD) of seven replicates. At the end of the kinetic, points with the same letters are not significantly different according to a one-factor (bacterial treatment) ANOVA ($P > 0.05$). The arrow indicates the precontact time at which mycelium was collected for *L. bicolor* S238N gene expression analysis.

Table 2 Effect of six rhizobacterial strains on *Laccaria bicolor* S238N hyphal extension and morphology after 14 d of dual culture

Bacterial treatment	Colony diameter (cm)	Apex number	Branching angle (°)	Branching density
Control	1.25 ± 0.02 a	38.5 ± 3.6 a	30.1 ± 1.3 a	22.5 ± 2.8 a
P. fluorescens BBc6R8	1.42 ± 0.02 b	52.3 ± 3.6 b	37.1 ± 1.8 b	31.3 ± 1.1 b
Burkholderia sp. EJP 67	1.40 ± 0.02 b	22.5 ± 2.3 c	58.1 ± 4.7 c	39.8 ± 2.7 bc
Paenibacillus sp. F2001L	1.26 ± 0.01 a	35.8 ± 2.4 a	30.3 ± 2.1 a	28.0 ± 1.4 a
P. fluorescens Pf29A	1.26 ± 0.02 a	33.7 ± 4.3 a	30.2 ± 1.6 a	34.7 ± 3.6 b
B. subtilis sp. MB3	1.26 ± 0.02 a	27.3 ± 4.2 a	30.1 ± 1.7 a	32.6 ± 3.2 b
Paenibacillus sp. EJP 73	0.99 ± 0.01 c	33.0 ± 5.8 a	34.4 ± 2.1 a	24.0 ± 5.4 a
C. fungivorans Ter331	0.91 ± 0.01 d	26.0 ± 7.7 a	41.1 ± 3.7 bc	43.5 ± 6.6 c

Hyphal extension, mean value of two perpendicular diameters of the fungal colony for seven biological replicates; apex number, the number of apices per microscopic field; branching density, the number of ramification divided by the number of apices. For the three variables (apex number, branching angle and branching density), each value corresponds to the mean value (± SE) of three biological replicates and two microscopic fields by replicate. In each column, mean values with the same letter are not significantly different according to a one-way ANOVA and the Fisher test ($P > 0.05$).

Transcriptional response of *L. bicolor* S238N during the interaction with the helper *P. fluorescens* BBc6R8

Transcripts of *L. bicolor* S238N showing an altered abundance in the presence of *P. fluorescens* BBc6R8 were identified by the following criteria: (i) a significant modification (Bayesian *t*-test) of gene expression was detected in at least two of the three replicates; and (ii) the average of fold changes (*P. fluorescens* BBc6R8 treatment vs control) in the expression level was > +2 or < –2 (Duplessis *et al.*, 2005). According to these criteria, we found 164 transcripts (3.2% of the total 4992 cDNAs) significantly regulated in least at one stage of interaction with *P. fluorescens* BBc6R8. The cDNA of 144 of these regulated transcripts were successfully sequenced; they encoded 104 different genes (Table S2). Among the regulated transcripts, 27 were up-regulated and none were significantly down-regulated during the early stage of interaction. At the contact stage, 18 transcripts were up-regulated and 11 were down-regulated. Finally, after a long contact period, 22 and 86 transcripts were up- and down-regulated, respectively. cDNA array results were confirmed by quantitative PCR measurements on six target genes (Fig. S1). MHB-responsive genes are involved in multiple cellular functions as showed by their distribution in various gene ontology (GO) categories (Fig. 4).

Some sets of transcripts belonging to the same GO showed concentration variation at only one step of the interaction. For instance, genes encoding protein involved in cell interaction (tectonin II), efflux processes (efflux protein of MFS family) and detoxification processes (two glutathione-S-transferases) were regulated at only the earliest stages (i.e. before or at contact time). Similarly, genes involved in protein synthesis, including 18 different ribosomal proteins, as well as those involved in the translation process (translation initiation factor SU1) and protein degradation (ubiquitin, ubiquitin extension protein, ubiquitin fusion protein) were only repressed after extended contact between mycelium and the bacterial cells. This repression of protein synthesis machinery was associated with a reduction in transcription of several genes involved in energy metabolism (i.e. ATP synthase subunits, cytochrome oxidase, ATP/ADP carrier protein, malic enzyme, PEP carboxylase and fumarate dehydrogenase).

By contrast, other sets of transcripts were affected at different times of the interaction (i.e. some genes before, and others at or after contact time). For example, the expression of genes encoding the histone H4-2, the tra1 subunit of the transcription regulatory SAGA complex (transcription regulation), and the splicing factor 3b (mRNA splicing) were enhanced before contact, while a gene encoding a transcription factor and *tra1* showed an increased expression at contact time. Finally, after extended contact, three genes involved in translation regulation (polyadenylate binding protein and U6, a small nuclear RNA-associated RNA binding protein) and the *tra1* gene showed decreased expression. Similarly, the expression of genes involved in growth and morphology was modified at different steps of the interaction: the gene encoding the concanamycin-induced protein C (CipC1), which could be involved in hyphal branching, was up-regulated two-fold before contact, while transcription of the gene encoding actin-1 was repressed after extended contact.

Analysis of transcription regulatory complex expression

Among the genes found regulated at all stages of the *L. bicolor* S238N–*P. fluorescens* BBc6R8 interaction, a gene encoding the Tra1 protein was identified. This protein is involved in four transcriptional regulatory complexes (SAGA, SALSA, SLIK, NuA4) in the yeast *S. cerevisiae* (Allard *et al.*, 1999; Sterner *et al.*, 1999; Wu *et al.*, 2004), all known for their histone acetyl transferase activity (HAT). Acetylation of

N-terminal histone tails reduces the affinity of nucleosomes to DNA and leads to an enhanced binding of transcription factors to their *cis*-regulatory DNA sites. The concentration of transcripts encoding for the histone H4, one target of HAT, was enhanced before contact (Table 3), suggesting that chromatin structure modifications take place in *L. bicolor* S238N in response to *P. fluorescens* BBc6R8. To know if the expression of other genes corresponding to key proteins of the precited transcriptional complexes was also regulated during the fungal–bacterial interaction, we searched for these genes in the annotated *L. bicolor* S238N-H82 genome and then measured their transcript concentrations by quantitative RT-PCR at the three stages of interaction. The SAGA complex (*S*pt-Ada-Gcn5–*a*cetyltransferase, Fig. 5) of *S. cerevisiae* is composed of 20 subunits; among those, 11 are shared with the SALSA complex (SAGA alterated complex, Sterner *et al.*, 2002) and 16 with the SLIK complex (SAGA like, Pray-Grant *et al.*, 2002). We identified the genes encoding 13 of these proteins in the *L. bicolor* S238N-H82 genome (Table 4). The Spt 20/Ada 5 and HFI1/ada 1 subunits, which are shared by the three complexes and are involved in the interaction with TATA box binding protein (TBP), were not found in the current assembly of the *L. bicolor* S238N-H82 genome. Similarly, the genes corresponding to the Sgf11, Sgf 29, Sus1 and Rtg2 subunits were not identified in the current genome assembly. By contrast, two components (Esa1 and Tra1) of NuA4 (*Nu*cleosome *a*cetyltransferase of histone H*4*; Allard *et al.*, 1999) were found. The concentration of the *gcn5* and *esa1* transcripts did not vary at any stage of the interaction, whereas the expression of *ada3* and *spt3* was significantly regulated before and at contact time (Table 4).

Expression analysis of BBc6R8-responsive genes during the interaction with other rhizobacteria

The expression of the genes *Cipc*, *tectonin2, fox2* and of four genes encoding proteins of the SAGA complex (*tra1*, *gcn5*, *spt3* and *ada3*) was investigated during the early stage of the interaction with the bacterial strains *P. fluorescens* Pf29A, *Paenibacillus* sp. F2001L, *B. subtilis* MB3 and *C. fungivorans* Ter331. No changes in the expression of the seven target genes was observed with the MHB *B. subtilis* strain MB3, nor with the presumed intrafungal *Paenibacillus* sp. F2001L (Table 4). By contrast, the expression of various BBc6R8-responsive genes was modified by the four other bacterial strains. The chitinolytic strain *C. fungivorans* Ter331 enhanced the expression of *tectonin2* and *ada3*, and decreased the expression of *Tra1*. The biocontrol strain *P. fluorescens* Pf29A induced an up-regulation of *tectonin2*, as well as *Tra1*, *gcn5* and *Cipc*. None of the tested strains induced modulation of *fox2* gene expression. Concerning the two MHB, *Burkholderia* sp. EJP67 and *Paenibacillus* sp. EJP73, the three replicates gave contradictory results (data not shown). Therefore we cannot conclude on their impact on the expression of the targeted genes.

Fig. 3 Effect of the helper bacterial strain *Pseudomonas fluorescens* BBc6R8 on the morphology of *Laccaria bicolor* S238N before contact (14 d of common growth), at contact time (16 d) and after contact (21 d). Open bars, control treatment with water; closed bars, *P. fluorescens* BBc6R8 treatment. Error bars denote standard error; *, significant differences with the control according to a *t*-test ($P < 0.05$) performed at each confrontation stage.

Fig. 4 Gene ontology to which up-regulated (white bar) or down-regulated (black bar) transcripts belonged. Grey bars, transcript categories that showed variable expression along the interaction. Data are expressed as a percentage of the total number of transcripts up-regulated, down-regulated or variably regulated at all stages of the interaction.

Table 3 Transcripts of *Laccaria bicolor* S238N regulated (*t*-test, ≥ 2.0, ≤ 2.0, in bold) upon the interaction with the helper strain *Pseudomonas fluorescens* BBc6R8

Accession number	Identity	Accession number of the best hit	Score	Before contact	Contact	After contact
Up-regulated before contact						
EL740045, EL740122	Cipc protein (*Emericella nidulans*)	CAC87272	4.00E–31	**3.2**	1.6	0.9
EL739265	Efflux protein, putative-MFS family (*Cryptococcus neoformans* var. *neoformans* JEC21)	CNB02040	1.00E–162	**6.4**	1.1	1.4
EL739689	Histone H4,2 (*Phanerochaete chrysosporium*)	P62792	4.00E–35	**2.1**	1.5	0.6
EL739275	Panthotenate kinase (*Cryptococcus neoformans* var. *neoformans* JEC21)	AAW42454	0.0	**2.1**	0.7	0.7
JGI_LbEX5106	Splicing factor 3b, subunit 4 (*Danio rerio*)	192318	3.00E–81	**8.7**	1.1	1.8
Up-regulated at the contact time						
EL739371	Ankyrin repeat protein (*Neosartorya fischeri NRRL 181*)	XP_001257449	9.00E–54	0.9	**2.2**	1.4
EL739436	Heat shock protein (*Schizosaccharomyces pombe*)	O14368	6.00E–73	1.9	**3.0**	1.8
Down-regulated at the contact time						
EL739383	Glutathione S-transferase (*Paxillus involutus*)	AAT91250	2.00E–78	0.9	**0.4**	1.5
JGI_LbEX8144	Glutathione S-transferase PM239 × 14 (GST class-phi) (*Arabidopsis thaliana*)	P42769	7.00E–81	1.0	**0.3**	1.0
Up-regulated after contact						
EL739467	Polyadenylate binding protein (*Cryptococcus neoformans* JEC21)	CNI01160	0.0	ns	ns	**5.8**
EL739886	Citrate synthase 2 (*Phanerochaete chrysosporium*)	jgi|Phchr1|38126|gww2.16.51.1	5.00E–163	0.9	0.6	**2.7**
Down-regulated after contact						
EL740070	Putative ubiquitin extension protein (*Oriza sativa*)	AAT93912	e-35	1.0	0.6	**0.4**
JGI_LbEX1896	Ubiquitin fusion protein (*Magnaporthe grisea*)	AAC13689	5.00E–65	1.0	0.8	**0.4**
JGI_LbEX876	Actin-1 (Beta-actin) (*Schizophyllum commune*)	Q9Y702	0.0	1.1	0.6	**0.4**

Table 3 *continued*

Accession number	Identity	Accession number of the best hit	Score	Before contact	Contact	After contact
JGI_LbEX2079	Histone H2b (*Agaricus bisporus*)	P78567	3.00E–14	1.0	1.0	**0.4**
EL739344	PEP carboxylase (*Cryptococcus neoformans* var. *neoformans* JEC21)	CNI03590	0.0	1.0	0.6	**0.4**
EL738956	LSM3 homologue, U6 small nuclear RNA-associated (*Saccharomyces cerevisiae*)	KEGG-27258	2.00E–40	1.2	0.7	**0.4**
EL739202	Vacuolar ATP synthase 16 kDa proteolipid subunit (*Neurospora crassa*)	P31413	3.00E–61	1.4	0.6	**0.4**
JGI_LbEX5683	Small heat shock protein (*Laccaria bicolor*)	AAM78595	6.00E–73	1.0	0.6	**0.4**
EL739415	Ras-related protein (*Laccaria bicolor*)	AAD01986		1.6	1.0	**0.4**
EL739129	NADP-dependent malic enzyme (*Flaveria pringlei*)	P36444	0.0	1.1	NS	**0.4**
EL739368, EL739199	Ubiquitin (*Phanerochaete chrysosporium*)	CAA80851	0.0	1.1	0.8	**0.3**
JGI_LbEX303	Dihydrolipoamide dehydrogenase precursor (*Aspergillus fumigatus* Af293)	EAL87307	8.00E–31	1.3	0.7	**0.3**
EL739358	Large subunit ribosomal protein L40e (*Eremothecium gossypii*) with ubiquitin domain IPR000626	KEGG-AFR285C	5.00E–33	0.8	0.8	**0.3**
EL739366	Eukaryotic translation initiation factor eIF-1 (protein translation factor SUI1) (*Saccharomyces cerevisiae*)	P32911	1.00E–50	1.1	1.2	**0.3**
EL739045	Probable RNA-binding protein C839.10 (*Homo sapiens*)	Q8WZK0	5.00E–09	1.2	0.9	**0.3**
JGI_LbEX2263	ADP, ATP carrier protein (ADP/ATP translocase) (*Neurospora crassa*)	P02723	0.0	0.7	0.7	**0.3**
JGI_LbEX5121	Integral membrane Yip1 family protein (*Arabidopsis thaliana*)	KEGG-At3g05280	1.00E–97	1.8	ns	**0.2**
EL740097	Fumarate reductase (NADH) (*Cryptococcus neoformans* var. *neoformans* JEC21)	AAW45754	0.0	1.3	0.8	**0.2**
JGI_LbEX4993	Glyceraldehyde-3-phosphate dehydrogenase (*Agaricus bisporus*)	AAA32634	1.00E–150	1.5	ns	**0.2**
Regulated all the time						
EL739455	Tra1 (*Phanerochaete chrysosporium*)	Phchr144967	0.0	**4.2**	**0.4**	**5.4**
Up-regulated before contact and at the contact time						
EL739445	Tectonin II (*Physarum polycephalum*)	AAC06201	6.00E–28	**3.1**	**2.4**	1.2
Up-regulated at the contact time and after contact						
EL739395	Heat shock protein	CNA02830	0.0	1.6	**2.2**	**2.8**
EL739391	Protein kinase (*Glycine max*)	AAA34002	6.00E–56	1.8	**2.9**	**2.8**
Down-regulated at the contact time and after contact						
EL739038	Mismatched base pair and cruciformDNA recognition protein (*Agaricus bisporus*)	CAB85690	2.00E–39	1.4	**0.5**	**0.4**
EL739050	ATP synthase alpha chain, mitochondrial precursor (*Cryptococcus neoformans var. neoformans* JEC21)	AAW44019	0.0	0.6	**0.2**	**0.4**
EL738997	F-type H^+-transporting ATPase f chain (*Eremothecium gossypii*)	KEGG-ACR203W	7.00E–43	1.1	**0.5**	**0.4**

ns, not significant.
cDNA clone ID, identity of the best BlastX, the GenBank accession number corresponding to the best hit and the E-value are given. The transcript ratio (*P. fluorescens* BBc6R8 treatment: *L. bicolor* S238N control) is given at the three times of the interaction: before contact, at the contact time and after contact.

Discussion

Growth and morphology modifications

Some previous studies showed that the effect of helper bacteria on mycorrhiza formation can be correlated with an increase in mycelium growth *in vitro* (Garbaye, 1994; Becker *et al.*, 1999; Maier *et al.*, 2004; Hildebrandt *et al.*, 2006). It has been suggested from these observations that the helper effect could result, in this case, from an increase in the growth and survival of the mycorrhizal mycelium in the soil, which would therefore increase the probability of

Table 4 Expression level of genes encoding key proteins of the SAGA and NuA4 regulatory complexes and of the marker genes *tectonin 2*, *Cipc* and *fox2* along the interaction between *Laccaria bicolor* and the helper strain *Pseudomonas fluorescens* BBc6R8, and only before the contact with the six other rhizobacterial strains

	P. fluorescens BBc6R8						
Transcript functions	Before contact	Contact time	After contact	*P. fluorescens* Pf29A	*Paenibacillus* sp. F2001L	*B. subtilis* MB3	*C. fungivorans* 331
SAGA complex							
Tra1	**4.5 ± 2.4**	*0.5* ± 0.3	**2.4 ± 0.4**	**2.6 ± 0.3**	1.5 ± 0.1	1.0 ± 0.1	*0.2* ± 0.1
Gcn5	1.1 ± 0.2	1.1 ± 0.5	0.8 ± 0.2	**3.1 ± 1.3**	1.0 ± 0.1	0.8 ± 0.1	**2.6 ± 0.8**
Ada3	*0.2 ± 0.02*	*0.3 ± 0.2*	*0.1 ± 0.1*	0.8 ± 0.2	1.1 ± 0.1	0.6 ± 0.1	0.5 ± 0.4
Spt3	0.7 ± 0.2	1.4 ± 0.5	0.7 ± 0.1	1.6 ± 0.4	1.5 ± 0.1	1.0 ± 0.1	**2.2 ± 0.3**
NuA4 complex							
Esa1	0.8 ± 0.1	*0.4 ± 0.1*	*0.5* ± 0.0	nd	nd	nd	nd
Recognition							
tectonin 2	**3.7 ± 0.6**	**3.4 ± 0.7**	nd	**9.2 ± 6.0**	1.1 ± 0.1	1.0 ± 0.2	**6.5 ± 1.2**
Morphology							
cipc2	**2.1 ± 0.1**	nd	nd	**3.4 ± 1.3**	1.1 ± 0.1	1.0 ± 0.1	1.4 ± 0.6
G. mosseae MHB responsive gene							
fox2	0.6 ± 0.1	*0.4* ± 0.1	1.2 ± 0.2	1.5 ± 0.4	1.1 ± 0.1	1.0 ± 0.1	nd
A. muscaria MHB responsive genes							
AaCS	1.3 ± 0.7	nd	0.6 ± 0.1	nd	nd	nd	nd
Cyp 40	*0.5 ± 0.1*	*0.3 ± 0.2*	0.9 ± 0.2	nd	nd	nd	nd

nd, nonmeasured value.
Measurements were performed by real-time PCR on *L. bicolor* S238N cDNAs from control (without bacteria) and bacterial treatments. Level of expression was calculated by the ΔΔCt method. Each value is the mean of three replicates. Data in bold and italic highlight expression rates up to 2.0 and down to 2.0, respectively.

Fig. 5 Scheme of the hypothetical SAGA structure and function in transcription (adapted from Sterner *et al*., 1999). Depicted is a hypothetical gene with an upstream activation sequence (UAS) and TATA box; the DNA is bound around nucleosomes (cylinders). The SAGA complex would interact with activators (A) while the histone acetyl transferase activity of the gcn5 subunit would acetylate (Ac = acyl groups) the amino-terminal tails of nucleosomal histone, providing more effective TATA binding of TATA binding protein (TBP). Further regulation would be provided by SAGA–TBP interactions through Spt3 and other factors.

root–fungus encounter and consequently, the number of mycorrhizas (Brulé *et al*., 2001). Here, we showed that the MHB *P. fluorescens* BBc6R8 not only enhanced the growth of *L. bicolor* mycelium, but also induced morphological changes of the mycelium *in vitro* (i.e. hyphal branching, apex density). The impact of bacteria on fungal morphology was previously reported in both antagonistic and mutualistic fungal–bacterial interactions (Bolwerk *et al*., 2003; Schrey *et al*., 2005; Ström *et al*., 2005). Interestingly, not all bacterial strains can modify fungal growth and morphology. The strain *B. subtilis* MB3, which promotes ectomycorrhizal symbiosis between *L. bicolor* and Douglas fir in glasshouses and nurseries (Duponnois & Garbaye, 1991), modified neither the growth nor the morphology of the *L. bicolor* colonies in our *in vitro* bioassay. On the contrary, the strain *Paenibacillus* sp. EJP73, which was isolated from a *Lactarius rufus* mycorrhiza and promoted the *L. rufus–Pinus sylvestris* symbiosis *in vitro* (Poole *et al*., 2001), as well as the *L. bicolor–P. sylvestris* symbiosis in the glasshouse (Aspray *et al*., 2006), significantly reduced the growth of *L. bicolor* colonies in our bioassay. This suggests that additional mechanisms not limited to growth increase are involved in the promotion of mycorrhiza formation by these two strains. The close presence of the plant could be required by EJP73 to exert its MHB activity (Aspray *et al*., 2006).

In opposition, the MHB effect of MB3 likely involves detoxification of the rhizospheric environment from autotoxic compounds produced by ectomycorrhizal fungi (Duponnois *et al.*, 1991).

Nonhelper rhizospheric bacteria, such as the biocontrol *P. fluorescens* Pf29A, were also able to induce alterations in growth and morphology of *L. bicolor* S238N. Nevertheless, *P. fluorescens* BBc6R8 was the only strain that enhanced both diametral growth of the colony, apex density and branching angle at the precontact stage. Interestingly, dramatic alterations in branching of hyphae occur in the earliest stages of root colonization by ectomycorrhizal fungi (Peterson & Bonfante, 1994; Martin *et al.*, 2001). In addition to its effect on the survival and growth of *L. bicolor*, the helper strain *P. fluorescens* BBc6R8 also induces hyphal morphological changes that could be beneficial to mycorrhizal infection of the host roots. Similar processes have been described in endomycorrhizal symbiosis where strigolactones from host plant root exudates induced growth and branching of germinating hyphae before root infection (Akiyama *et al.*, 2005; Besserer *et al.*, 2006).

Alteration in *L. bicolor* S238N transcriptome

Mycorrhiza helper bacteria and nonMHB specific alterations in the transcriptome have been poorly documented thus far (Schrey *et al.*, 2005; Hildebrandt *et al.*, 2006). Here, we analysed for the first time regulation of the fungal transcriptome at several stages of interaction with a helper bacterial strain (i.e. before contact, at contact time and after a prolonged contact, during which bacteria colonized the surface of fungal hyphae). Transcript concentration of *L. bicolor* S238N was significantly altered in response to bacterial stimuli. As previously observed by Schrey *et al.* (2005) on the *A. muscaria–Streptomyces* interaction, the presence of the MHB *P. fluorescens* BBc6R8 led to a moderate response of *L. bicolor* S238N at the transcriptome level, as only 3% of the analysed transcripts showed an altered concentration. We distinguished two separated phases in the fungal response. Before contact and after a few hours of direct interaction, BBc6R8-responsive fungal genes were mainly up-regulated; these genes code for proteins involved in recognition processes and transcriptional regulation. After prolonged contact with the MHB BBc6R8, the expression of > 100 genes was down-regulated; 50% of them encoded for proteins involved in protein synthesis and energy metabolism. Yeast orthologues of these genes were repressed when *Saccharomyces cerevisiae* was grown in starvation or stressful environmental growth conditions (Gasch & Werner-Washburne, 2002; Wu *et al.*, 2004). It was hypothesized that down-regulation of gene expression could help fungal cells to preserve energy while adapting to their growth environment. In our study, we also observed a down-regulation in the transcription of malic enzyme, PEP carboxylase and fumarate dehydrogenase, while citrate synthase (the enzyme involved in first step of the TCA cycle) was up-regulated. This suggests a shift in carbon use in the mycelium of *L. bicolor* after prolonged contact with *P. fluorescens* BBc6R8. Similarly, in *Neurospora crassa*, Xie *et al.* (2004) showed that glucose starvation induced genes of the TCA and glyoxalate cycle, while genes involved in glycolysis were down-regulated. Therefore, transcriptional modifications observed at this stage probably result from trophic competition occurring between *L. bicolor* S238N and *P. fluorescens* BBc6R8 after a long period of common growth in the *in vitro* assay. As a consequence, we will restrict discussion to the precontact stage, as it accurately mimicked the promoting effect of bacteria on the fungal growth as previously observed in soil (Brulé *et al.*, 2001).

As in most fungal transcriptome analyses, about half of the BBc6R8-responsive genes coded for hypothetical proteins. Interestingly, orthologues were not found in other sequenced genomes for most of these genes (72%), suggesting that they are unique to *L. bicolor*. Annotation of the *L. bicolor* S238N-H82 genome has shown that these expressed hypothetical proteins are abundant in *L. bicolor* (F. Martin *et al.*, unpublished). These orphan sequences should have rapidly evolved as they have no orthologues in the genome of *Coprinopsis cinerea*, an Agaricale saprotroph phylogenetically close to *Laccaria*; they may be related to specific adaptation of the fungus to its symbiotic lifestyle (Le Quéré *et al.*, 2006). Further studies will be necessary in order to clarify the role of the BBc6R8-responsive orphan sequences.

BBc6R8-responsive genes involved in bacterial recognition

We have identified a BBc6R8-responsive fungal gene (fourfold up-regulation) encoding for the tectonin II protein. This gene shares 54% similarity with the *tectonin II* of the amoebae *Physarum polycepharum* (Huh *et al.*, 1998). This protein participates in bacterial aggregation by the amoebae cells during the phagocytosis process. In *L. bicolor*, its expression levelled off to its constitutive amount after bacterial colonization of the mycelium. This gene appears to be specific to *L. bicolor* as no orthologue was found in the sequenced genome of *Coprinopsis cinerea*, *Phanerochaete chrysosporium*, *Cryptococcus neoformans* or *Neurospora crassa*. Further analysis on mycorrhizal fungi will be needed to confirm this hypothesis. This suggests that the tectonin orthologue of *L. bicolor* could play a role in cell recognition and/or fungal cell interaction with *P. fluorescens* BBc6R8, during the earliest phase of the interaction. Its expression was up-regulated not only by the helper *P. fluorescens* BBc6R8 strain and the biocontrol *P. fluorescens* Pf29A strain that promoted *L. bicolor* growth, but also by the antagonistic bacterial strain *C. fungivorans* Ter331. On the contrary, the helper strain *Burkholderia* sp. EJP67 that enhanced *L. bicolor* growth did not significantly induce expression of the tectonin gene. In consequence, transcription of the tectonin gene is not specifically

modulated by helper bacteria. However, up-regulation of this gene could be related to the ability of BBc6R8 to attach to *L. bicolor* mycelium. The parental strain *P. fluorescens* BBc6 (Sen *et al.*, 1994) and the rifampin mutant BBc6R8 (data not shown) are able to attach to *L. bicolor* mycelium. Further studies will be necessary to confirm this hypothesis with *P. fluorescens* Pf29A and *C. fungivorans* Ter 331.

Role of BBc6R8-responsive genes in fungal metabolism, growth, and morphology

Lipid metabolism was previously shown to be modulated in both endomycorrhizal– and ectomycorrhizal–MHB interactions (Requena *et al.*, 1999; Schrey *et al.*, 2005), as well as the early steps of mycorrhiza formation on pine roots by *L. bicolor* (Podila *et al.*, 2002). Here, we observed that the mRNA concentrations of pantothenate kinase, which catalyses the limiting step of coenzyme-A synthesis, and of *FOX2*, which encodes a multifunctional enzyme of the β-oxidation pathway, were both decreased during the precontact phase and at contact time, respectively. In the same way, its orthologue in *Glomus mosseae* was repressed at contact time with the MHB *B. subtilis* NR1 (Requena *et al.*, 1999). By contrast, no modification of the expression of the *A. muscaria*/*Streptomyces* AcH505 acetoacyl-CoA synthase regulated gene was found during the interaction between *L. bicolor* S238N and *P. fluorescens* BBc6R8. All these data strongly suggest that the response of mycorrhizal fungi to helper bacteria involves a modification of both fungal lipid anabolism and catabolism, which could result in an increase of lipid synthesis required for the enhanced fungal growth rate. However, the genes differentially regulated would depend on the fungal–bacterial systems.

The *cipc* gene was previously reported to correspond to expressed sequence tags from the two ectomycorrhizal fungi, *L. bicolor* S238N and *P. tinctorius* (Peter *et al.*, 2003). The early up-regulation of this gene could be linked to the modification of *L. bicolor* growth and morphology. In fact, this gene encodes for a protein of unknown function which was first identified when analysing changes in *Aspergillus nidulans* morphology in response to the antibiotic concanamycin A, a specific inhibitor of V-ATPase produced by *Streptomyces* bacterial species (Melin *et al.*, 2002). The concentration of this protein was enhanced in the fungal mycelium when the bacterial antibiotic was added to the culture medium. An orthologue of this gene was also suggested to be linked to changes in the growth and morphology of the ectomycorrhizal fungus *Paxillus involutus* in the early step of ectomycorrhiza formation with *Betula pendula* (Morel *et al.*, 2005). This gene could therefore be considered as a marker of the presymbiotic status of the fungus. Interestingly, the strain BBc6R8 was also proved to up-regulate expression of the *cipc* gene at the early stage of coculture with *L. bicolor*. Our results therefore suggest that *P. fluorescens* BBc6R8 could induce a shift in the mycelial physiology from a saprotrophic to a presymbiotic status, in addition to its effect on the presymbiotic survival and growth of the fungus (Brulé *et al.*, 2001). Further investigations will be needed to confirm this hypothesis.

Alteration of transcription machinery

Regulation of gene transcription involves recruitment of the RNA transcription polymerase II complex, the fixation of transcription factors on promoter sequences, and histone acetylation or methylation (for a review, see Cosma, 2002). In the yeast *S. cerevisiae*, the SAGA complex is required for the recruitment of the basal transcription machinery and regulates the expression of 10% of the genes (Wu *et al.*, 2004). Here, we demonstrate that expression of the largest subunit of the SAGA complex, the so-called Tra1, was regulated at all stages of the *L. bicolor* S238N–*P. fluorescens* BBc6R8 interaction. This protein plays a fundamental role in the functioning of the SAGA complex by interacting with transcriptional activators (Brown *et al.*, 2001). Several genes encoding for other SAGA subunits showed transcription changes in the presence of *P. fluorescens* BBc6R8 and *C. fungivorans* Ter331. Complex patterns of expression were observed as some genes were up-regulated, whereas others were down-regulated. But these proteins must play a major role in the response of *L. bicolor* S238N as the *Tra1* gene is one of the largest genes of the genome, with a size of 11 526 kb. Therefore, its increased transcription imposes a high energy cost for the cell. Moreover, some of the fungal transcripts regulated during the interaction with the helper *P. fluorescens* BBc6R8, such as FOX2, citrate synthase, PEP carboxylase and acetyl-CoA synthase, are homologous to *Saccharomyces* genes known to be under the control of two transcription factors, Adr1 (FOX2, citrate synthase) and Cat8 (PEP carboxylase, acetylCoA synthase), which themselves need to interact with histone acetyl transferase (Tachibana *et al.*, 2005). Interestingly, expression of the gene encoding histone H4 in *A. muscaria*, one target of HAT activities, was also stimulated by the MHB *Streptomyces* sp. AcH505 (Schrey *et al.*, 2005). As a consequence, these data suggest that at least part of the transcriptional response of ectomycorrhizal fungus to MHB involves epigenetic changes in the histone code.

Specificity of mycorrhizal fungus–MHB interactions

A key question arising from our study is whether the gene regulations involved are specific to our ectomycorrhizal fungus–MHB model. Interestingly, we identified several molecular determinants that had never been previously identified and thus appear to be specific to the *L. bicolor* S238N–*P. fluorescens* BBc6R8 interaction. Others were mutually regulated at the transcriptome level in the two distinguished ectomycorrhizal fungus–MHB pairs, *L. bicolor*–*P. fluorescens* and *A. muscaria*–*Streptomyces* sp. In addition, some responsive

genes identified in the *A. muscaria-Streptomyces* sp. and *Glomus mosseae-Bacillus subtilis* models were not regulated during the *L. bicolor–P. fluorescens* interaction (data not shown). We also observed that some genes overexpressed in the presence of the MHB *P. fluorescens* BBc6R8 were also regulated by other rhizobacteria. Consequently, our results highlight the fact that some fungal pathways are mutually regulated by different rhizobacteria, whereas others are specific to some MHB.

Our work emphasizes the importance of studying MHBs as models for genomic analysis of fungal–bacterial interactions. This should also benefit other research areas where fungal–bacterial interactions play a major role, such as plant protection and medicine (Frey-Klett & Garbaye, 2005; Bending, 2007).

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Supplementary Material

The following supplementary material is available for this article online:

Table S1 List of the primers used in this study and the gene model corresponding to each gene in *L. bicolor* H82 genome

Table S2 Composition of the transcription regulatory complexes SAGA, SALSA and SLIK in the yeast *S. cerevisiae* and gene model homologues in the genome of *L. bicolor* H82

Table S3 Transcripts of *L. bicolor* S238N regulated (*t*-test, $\leq \geq 2.0$, underlined in grey) upon the interaction with the helper strain *P. fluorescens* BBc6R8

Fig. S1 Validation of the cDNA-array data by real-time PCR analyses

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I.3 Analysis of the transcriptional response of *L. bicolor* to the presence of the helper bacterial strain *P. fluorescens* BBc6R8: a whole genome array approach.

The previous analysis was performed using a macroarray containing 4992 cDNA. The sequence of the *L. bicolor* H82-S238N genome revealed about 20.000 genes (Martin *et al*. unpublished, Annexe 1). Thus, less than a fourth of the transcriptome of the fungus was covered by the nylon arrays. A first generation of whole genome arrays was used to get a complete overview of the fungal transcriptome response to *P. fluorescens* BBc6R8, before physical contact. This time of the interaction was chosen as it reflected the priming effect of the bacteria on the fungus. Later on, we observed an artefactual trophic competition (cf. paragraph II.1).

I.3.1 Material and methods

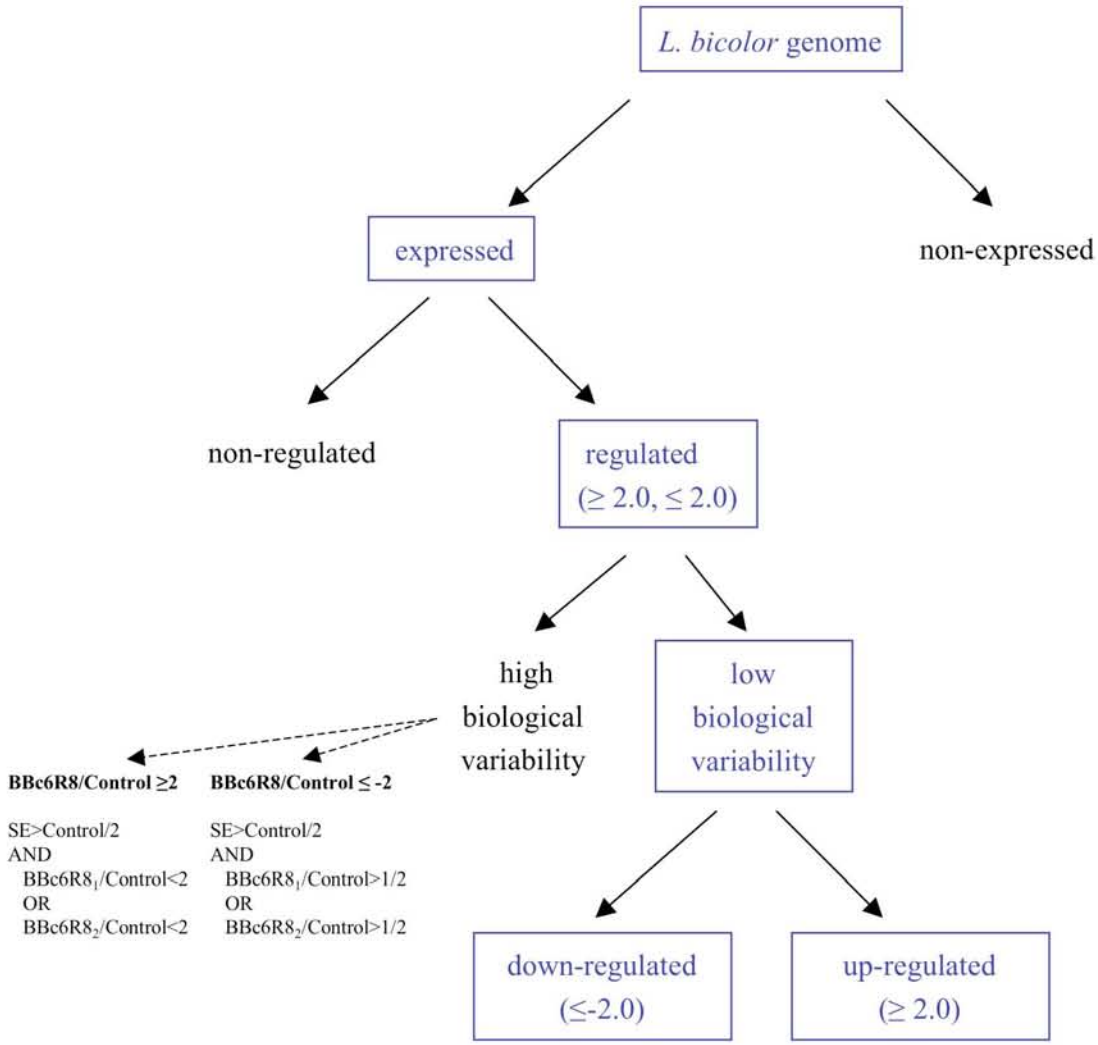
Bioassay and oligochips hybridization

The bioassay was prepared as described in Deveau *et al.* (2007). Probes were prepared using double strands cDNA from *L. bicolor* RNA previously used for cDNA macroarray experiments. Three oligochips were used: one was hybridized with a labelled cDNA synthesized from 14 days-old *L. bicolor* S238N RNA grown without bacteria and the two others were hybridized with labelled cDNA synthesized from 14 days-old *L. bicolor* S238N RNA grown in the presence of *P. fluorescens* BBc6R8. Hybridization were performed by Nimblegen (Madison, WI).

Oligochip

The *Laccaria* whole-genome expression array manufactured by NimbleGen (Madison, WI) contains in duplicates eight independent, non-identical, 60-mer probes per whole gene model. Included in the microarray are 20 614 annotated gene models (genome sequence v1.0), 1 680 additional predicted gene models, 30 000 random 60-mer control probes and labeling controls.

Figure 1.3. Procedure of the analysis of the raw data. Only data corresponding to the box criteria were selected for further analysis. "BBc6R8/Control" indicates the ratio of *L. bicolor* S238N transcripts concentration between the average of the *P. fluorescens* BBc6R8 treatments and the control treatment. "$BBc6R8_1$" and "$BBc6R8_2$" correspond to the *L. bicolor* S238N transcripts concentration in the first and in the second replicate. "Control" indicates the *L. bicolor* S238N transcripts concentration in the control treatment. SE : standard error



Data analysis

Data set was normalized by Nimblegen using the RMA method (Irizarry *et al.* 2003). The background value was calculated as the average value of the random probe intensities. The threshold between expressed and non-expressed genes was fixed at three times the background value. Analysis on regulation expression was only performed on genes showing intensities higher than the threshold expression value. The expression ratio was calculated as the ratio between the average intensity of the two biological treatments and the control treatment. No statistical test can be done on the data set because not enough biological replicates were used. This was a preliminary experiment to test the viability of the oligochips. The expensive price of the chips (1000 euros per chip) did not allow us to realise all the replicates needed in a first step. Nevertheless a filter based on standard error was applied on the data set to eliminate genes for which a high variability of intensities was observed between the two treatment replicates (figure 1.3). Genes responding to the following criteria were removed from the analysis:

For bacterial/control treatment ratio superior or equal to two, if the treatment intensities standard error was superior to the half of the control treatment intensity and if the intensity value from one of the two bacterial treatment intensities was inferior to two times the control intensity, then the data were removed. Similarly, for bacterial/control treatment ratio inferior or equal to minus two, if the treatment intensities standard error was superior to the half of the control treatment intensity and if one of the two bacterial treatment intensities was superior to the half of the control intensity, then the data were removed from the analysis.

The quality of the gene models from the JGI *Laccaria* annotation v1.0 corresponding to responsive genes remaining after the application of the filter was then manually checked on the jgi web site (http://genome.jgi-psf.org/Lacbi1/Lacbi1.home.html). According to qualitative quality criteria on the gene model (intron and UTR size) and on the BlastX results (E-value, length of the identical sequence, and of insertions), gene products were classified either into hypothetical proteins or proteins of known functions. The names that were given to the identified genes corresponded to the name of the best BlastX gene product result.

Figure 1.4. Level of regulation (BBc6R8 treatment/control treatment) of *L. bicolor* S238N significantly regulated transcripts before contact with *P. fluorescens* BBc6R8. Transcripts are classified in a decreasing way, from the most up- to the most down-regulated ones. Transcripts which are not regulated are not figured out.

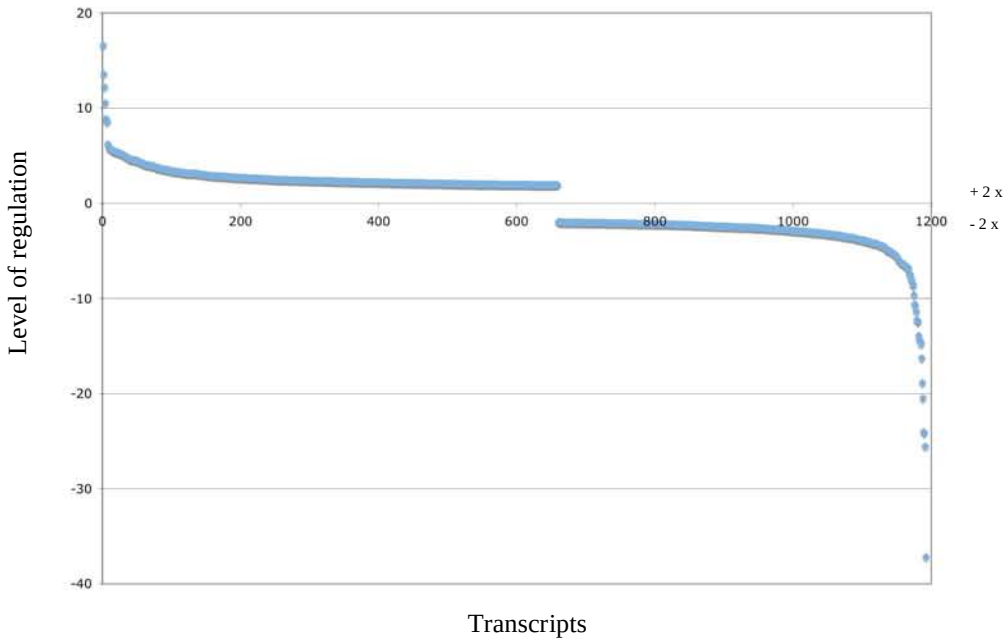
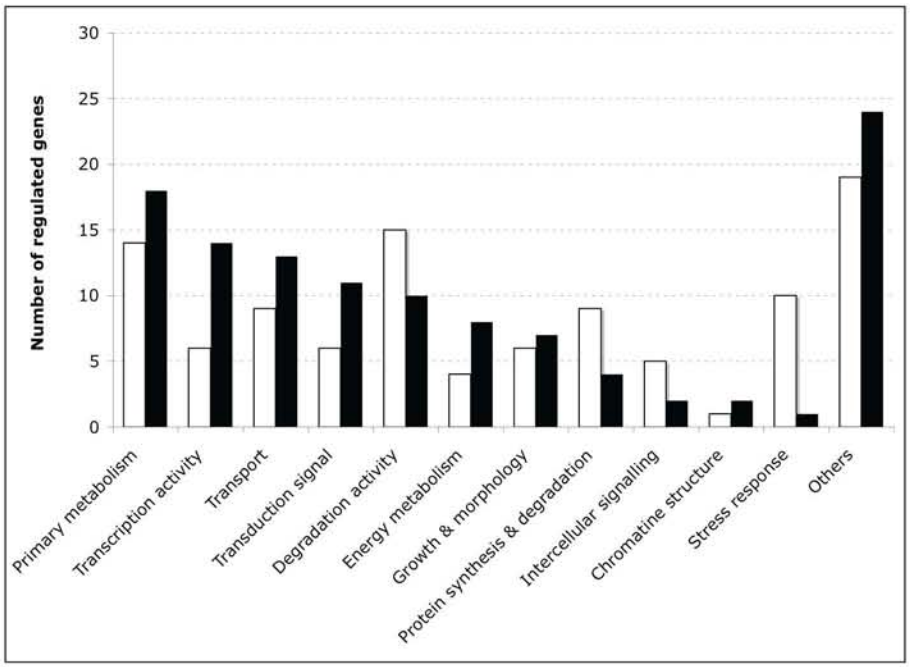


Figure 1.5. Gene ontology to which up- (white bar) and down-regulated (black bar) transcripts belonged.



I.3.2 Results

Applying the pre-cited criteria, 1193 transcripts showed modifications of their concentration among the 14.870 expressed genes (8 %). Among the regulated transcripts, 659 were up- and 535 were down-regulated, respectively. Responsive genes were scattered all along the genome; no hot spot of regulation was observed (data not shown). Level of regulation varied between plus 17 and minus 37 fold but 95 of the up- and 84 percent of the down-regulated genes had a level of expression varying between two and fivefold, respectively (Figure 1.4). Eighty percent of responsive-genes encode for hypothetical proteins (400 up- and 548 down-regulated, respectively, figure 1.5) among which 13 % hold a predicted secretion signal (SignalP, http://www.cbs.dtu.dk/services/SignalP/). Products encoded by BBc6R8-responsive genes are involved in multiple cellular functions as showed by the table 1.2 and the figure 1.5. The most highly up-regulated genes encoded for a lipase, a terpene synthase, a malate dehydrogenase and a monocarboxylate transporter from the MFS family. All the most highly repressed genes encoded for hypothetical proteins. Other gene products were involved into 11 main categories: primary metabolism, protein synthesis and degradation, energy metabolism, growth and morphology, transport, degradation activity, chromatine structure, transcription activity, transduction signal and intercellular signalling. Products from both up- and down-regulated genes belonged to each category. Genes involved in stress response were only up-regulated. The category "Others" include gene products for which an enzymatic function is known but the reaction catalyzed is too common to deduce a contribution to a specific cellular process (i.e kinase, phosphatase, oxidoreductase…). Similarly, ones should remind that many enzymes are implied in several cellular functions because of the high connexion between metabolic processes. As an example, acetylCoA enters in the Krebs cycle (respiration) but also in fatty acid degradation or in terpenoids synthesis. In the same way, an increased degradation of fatty acids could be linked either to cell wall modifications or to a shift in the carbon and energy metabolism.

Table 1.2. Transcripts of *L. bicolor* S238N regulated (≥ 2.0, ≤ -2.0) before contact with the helper strain *P. fluorescens* BBc6R8. Transcripts encoding for hypothetical proteins are given in Annexe 2. "M" and "S" indicate a mitochondrial and an extracellular localization, respectively.

Up-regulated genes					
SEQ_ID	NCBI Defintiion	Ratio T/C	protein ID	NCBI E value	Localization
Chromatine modification					
LACB00S00015929	acetyltransferase, GNAT family [*Colwellia psychrerythraea* 34H]	2,1	310437	2,00E-09	_
Energy metabolism					
LACB00S00001357	cytochrome P-450 cyp509A1 [*Cunninghamella elegans*]	2,5	243209	1,10E-52	M
LACB00S00003687	cytochrome P450 [*Lentinula edodes*]	2,5	311267	3,10E-97	S
LACB00S00016518	cytochrome P450 [*Lentinula edodes*]	2,4	312243	2,10E-86	S
LACB00S00001576	Chain D, Phenol Hydroxylase [*Trichosporon Cutaneum*]	2,0	188035	1,00E-127	_
Growth & morphology					
LACB00S00014430	Chitin deacetylase [*Cryptococcus neoformans var. neoformans*]	3,2	307596	5,10E-32	S
LACB00S00012188	actin-interacting protein 3 [*Yarrowia lipolytica*]	2,3	303602	9,10E-19	M
LACB00S00018422	PRY1 [*Saccharomyces cerevisiae*]	3,2	334862	4,10E-19	S
LACB00S00016993	Terpene synthase, metal-binding [*Rubrobacter xylanophilus* DSM 9941]	13,6	312850	6,10E-21	_
Intercellular signalling					
LACB00S00011031	membrane protein, [*Cryptococcus neoformans var. neoformans* JEC21]	2,6	300537	4,10E-36	S
LACB00S00006010	fasciclin I family protein, [*Aspergillus fumigatus*]	2,6	232955	1,10E-28	_
LACB00S00019382	ethylene-forming enzyme *[Neurospora crassa*]	2,4	256692	1,00E-107	_
LACB00S00006998	20 kDa protein having G-X-X-X-Q-X-W- motif [*Trichosporon mucoides*]	2,0	248441	1,10E-41	_
LACB00S00010666	hydrophobin [*Tricholoma terreum*]	2,2	251219	1,00E-19	S
Primary metabolism					
LACB00S00016535	hydroxymethylglutaryl-CoA synthase, [*Cryptococcusneoformans var. neoformans* JEC21]	2,8	239957	1,00E-133	_
LACB00S00007382	AFR440Cp [*Ashbya gossypii* ATCC 10895]	3,2	318937	1,00E-13	_
LACB00S00018104	L-aminoacid oxidase [*Hypocrea lixii*]	2,0	175400	2,10E-58	_
LACB00S00013286	TAL1 [*Saccharomyces bayanus*]	2,1	304919	2,00E-13	_
LACB00S00000059	CG10238-PA [*Drosophila melanogaster*]	2,1	300613	1,10E-25	_
LACB00S00002266	peroxisomal D3,D2-enoyl-CoA isomerase [*Aspergillus fumigatus* Af293]	2,0	305939	1,10E-40	_
LACB00S00009286	phospholipid metabolism-related protein, [*Cryptococcusneoformans var. neoformans* JEC21]	3,2	235655	7,10E-49	M
LACB00S00001312	phosphatidylserine decarboxylase precursor [*Lactobacillus acidophilus* NCFM]	2,8	192443	5,10E-73	_
LACB00S00000327	COG0167: Dihydroorotate dehydrogenase [*Haemophilus somnus* 129PT]	2,0	243747	2,10E-41	_
LACB00S00012951	galactinol synthase [*Brassica napus*]	4,6	295056	3,00E-15	_
LACB00S00015509	3(2),5 -bisphosphate nucleotidase HAL2 [*Cryptococcus neoformans* var.*neoformans* JEC21]	2,3	191792	4,10E-80	_
LACB00S00001045	alpha-ketoglutarate-dependent taurine dioxygenase [*Aspergillus fumigatus* Af293]	2,0	301702	7,10E-21	M
LACB00S00007694	lipase precursor-like protein [*Leishmania major*]	16,6	296287	1,00E-19	S
LACB00S00001638	malate dehydrogenase [*Aspergillus fumigatus* Af293]	12,3	305233	2,00E-16	S

Protein synthesis & degradation					
LACB00S00011448	Chaperone DnaJ1 [*Cryptococcus neoformans* var. *neoformans* JEC21]	2,9	191048	4,10E-33	_
LACB00S00017507	ATP-dependent chaperone, [*Trypanosoma brucei*]	2,7	295782	5,10E-48	_
LACB00S00007851	heat shock protein, [*Cryptococcus neoformans* var.neoformans JEC21]	2,0	190151	0.0	M
LACB00S00003561	NADH oxidoreductase complex I subunit [*Caenorhabditis elegans*]	2,0	292657	1,00E-06	_
LACB00S00012940	Pex3p [*Aspergillus fumigatus* Af293]	2,0	304499	5,10E-22	_
LACB00S00013840	Acidic ribosomal protein [*Schizosaccharomyces pombe*]	3,0	295176	6,00E-16	_
LACB00S00008971	ribonucleoprotein, [*Plasmodium falciparum* 3D7]	2,8	297858	1,00E-05	M
LACB00S00015187	eukaryotic translation initiation factor 3 subunit EifCa, putative[*Aspergillus fumigatus* Af293]	2,3	191687	1,00E-106	_
Saprophytic activity					
LACB00S00009333	exo-beta-1,3-glucanase [*Cryptococcus neoformans* var. neoformansJEC21]	5,1	250359	1,00E-139	S
LACB00S00014424	phage-related lysozyme [*Chromobacterium violaceum* ATCC12472]	3,4	307590	4,10E-21	S
LACB00S00006689	endoglucanase [*Irpex lacteus*]	2,0	319772	1,00E-137	S
LACB00S00000683	peptidyl-Lys metalloendopeptidase [*Grifola frondosa*]	2,3	301285	2,10E-94	S
LACB00S00005373	guanyl-specific ribonuclease U1 [*Ustilago maydis* 521]	2,4	189432	2,00E-15	S
LACB00S00003661	aryl-alcohol oxidase precursor [*Pleurotus eryngii*]	5,3	311240	5,10E-43	S
LACB00S00005177	endopeptidase, [*Cryptococcus neoformans* var. neoformansJEC21]	2,8	314168	2,10E-53	S
LACB00S00013769	metalloprotease [*Pleurotus ostreatus*]	2,1	295167	8,10E-72	S
LACB00S00005564	alginate lyase [*Haliotis discus hannai*]	2,0	293304	2,10E-25	S
Signal transduction					
LACB00S00014071	protein-histidine kinase, [*Cryptococcus neoformans* var.*neoformans* JEC21]	2,5	174796	0.0	_
LACB00S00019617	Cap3p [*Cryptococcus neoformans* var. neoformans]	2,2	307127	5,10E-22	_
LACB00S00016719	Rho1 GTP-binding protein [*Mucor rouxii*]	2,7	333548	1,00E-65	_
LACB00S00017666	Ras small GTPase Rho type [*Schizosaccharomyces pombe*]	2,3	240754	2,10E-56	_
LACB00S00017623	RHO1 [*Yarrowia lipolytica*]	2,2	153962	3,10E-44	_
LACB00S00017673	Ras small GTPase Rho type [*Schizosaccharomyces pombe*]	2,2	255472	3,10E-57	_
Stress response					
LACB00S00003147	Peroxiredoxin [*Synechococcus elongatus* PCC 7942]	2,5	308972	1,00E-18	_
LACB00S00003167	2-oxoacid-dependent oxidase [*Oryza sativa* (japonicacultivar-group)]	2,0	308995	2,10E-41	_
LACB00S00013522	thioredoxin, [*Aspergillus fumigatus* Af293]	2,0	295142	5,10E-28	_
LACB00S00006165	small heat shock protein [*Laccaria bicolor*]	2,6	248102	5,00E-16	_
LACB00S00019002	small heat shock protein [*Laccaria bicolor*]	2,5	186670	1,00E-77	_
LACB00S00009347	small heat shock protein [*Laccaria bicolor*]	2,3	190797	6,10E-82	_
LACB00S00003668	MGC89869 protein [*Xenopus tropicalis*]	2,0	292681	4,00E-08	_
Transcription regulation					
LACB00S00011094	transcription factor [*Aspergillus niger*]	2,2	302141	9,00E-16	M
LACB00S00001421	related to c-myb like protein [*Neurospora crassa*]	2,4	304993	2,00E-07	_
LACB00S00010995	ENSANGP00000012626 [*Anopheles gambiae* str. PEST]	5,2	150318	4,00E-12	M
LACB00S00014009	nucleus protein, [*Cryptococcus neoformans* var. neoformansJEC21]	2,1	307141	1,00E-82	_
LACB00S00009263	YlSIN3 [*Yarrowia lipolytica*]	2,0	298197	1,10E-22	_
Transport					

LACB00S00001307	Lysine-specific permease *[Photorhabdus luminescens* subsp. *laumondii* TTO1]	2,5	301980	2,10E-82	_
LACB00S00012131	gamma-aminobutyric acid transporter, [*Cryptococcus neoformans var. neoformans* JEC21]	2,8	185515	1,00E-111	S
LACB00S00018306	ammonium transporter [*Hebeloma cylindrosporum*]	2,1	315306	1,10E-55	_
LACB00S00003558	related to purine-cytosine permease [*Neurospora crassa*]	2,2	245872	5,10E-57	_
LACB00S00014066	COG0580: Glycerol uptake facilitator and related permeases (MajorIntrinsic Protein Family) [*Yersinia frederiksenii* ATCC33641]	2,2	307192	2,10E-20	_
LACB00S00013139	high-affinity hexose transporter [*Aspergillus fumigatus* Af293]	3,5	304755	1,00E-136	_
LACB00S00006474	MFS monocarboxylate transporter, [*Aspergillus fumigatus* Af293]	5,4	317337	2,00E-18	S
LACB00S00006478	MFS monocarboxylate transporter, [*Aspergillus fumigatus* Af293]	5,4	248030	3,10E-61	_
LACB00S00013210	amino acid permease [*Neurospora crassa*]	4,8	304842	1,00E-08	S
LACB00S00006061	related to monocarboxylate transporter [*Aspergillus fumigatus* Af293]	2,8	152320	2,10E-56	_
LACB00S00009274	efflux protein, [*Cryptococcus neoformans* var. *neoformans* JEC21]	2,6	298212	8,10E-77	_
LACB00S00009038	High-affinity nickel-transporter [Burkholderia cenocepacia HI2424]	2,2	297934	7,10E-28	S
LACB00S00009869	xenobiotic-transporting ATPase, [*Cryptococcus neoformans* var. *neoformans* JEC21]	2,0	299062	1,00E-13	S
Others					
LACB00S00007218	clitocypin analog [*Lepista nebularis*]	5,8	326016	4,00E-06	_
LACB00S00010094	PREDICTED: similar to TatD DNase domain containing 2 [*Canis familiaris*]	4,4	328338	2,10E-27	_
LACB00S00003556	short chain oxidoreductase [*Streptomyces coelicolor* A3(2)]	4,1	246562	1,10E-47	_
LACB00S00008627	protein serine/threonine kinase (Ran1), [*Aspergillus fumigatus* Af293]	4,0	250157	9,10E-51	_
LACB00S00009981	Aldo-keto reductase [*Aspergillus niger*]	3,7	236166	2,10E-91	_
LACB00S00014789	HIT family protein 1 [*Chaetomium globosum*]	3,2	191650	4,10E-36	_
LACB00S00006522	NACHT domain protein, [*Aspergillus fumigatus* Af293]	3,1	317387	2,00E-06	_
LACB00S00005752	cellulose-binding beta-glucosidase [*Phanerochaete chrysosporium*]	2,9	189477	1,00E-162	_
LACB00S00012511	GA14724-PA [*Drosophila pseudoobscura*]	2,7	303976	7,00E-18	_
LACB00S00007202	protein phosphatase 2A catalytic subunit [*Lycopersicon esculentum*]	2,7	248987	1,00E-06	_
LACB00S00010436	ATP binding / protein kinase/ protein serine/threonine kinase/protein-tyrosine kinase [*Arabidopsis thaliana*]	2,7	299799	8,10E-27	_
LACB00S00014462	PREDICTED: similar to TNNI3 interacting kinase isoform 1[*Strongylocentrotus purpuratus*]	2,6	307628	4,10E-21	_
LACB00S00000563	SPBC19G7.02 [*Schizosaccharomyces pombe*]	2,6	243106	8,10E-24	_
LACB00S00003589	aryl-alcohol oxidase precursor [*Pleurotus eryngii*]	2,6	311160	7,00E-09	_
LACB00S00003353	msh5 [*Coprinopsis cinerea*]	2,5	141362	0.0	_
LACB00S00005955	2-nitropropane dioxygenase, [*Cryptococcus neoformans* var.*neoformans* JEC21]	2,5	247855	1,00E-110	_
LACB00S00006515	elastinolytic metalloproteinase Mep [*Aspergillus fumigatus* Af293]	2,5	147315	8,10E-41	_
LACB00S00013366	Protein Kinase Sky1p [*Saccharomyces cerevisiae*]	2,5	306328	3,10E-36	_
LACB00S00012165	Protein kinase domain-containing protein [*Aspergillus fumigatus* Af293]	2,5	303582	1,10E-28	_
LACB00S00007302	ferric reductase transmembrane component, [*Cryptococcus neoformans var. neoformans*	2,4	184285	4,10E-93	_

	JEC21]				
LACB00S00004953	endopeptidase, [*Cryptococcus neoformans* var. *neoformans JEC21*]	2,4	144077	1,10E-60	_
LACB00S00013711	similar to protein carboxyl-o-methyltransferase [*Gallus gallus*]	2,4	295156	2,10E-55	_
LACB00S00011394	PREDICTED: similar to zinc finger protein 709 isoform 12 [*Mus musculus*]	2,4	302525	8,00E-08	_
LACB00S00003947	endochitinase [*Amanita muscaria*]	2,2	231399	8,00E-90	_
LACB00S00012162	IA-1=serine proteinase inhibitor *[Pleurotus ostreatus,* fruitingbodies, Peptide, 76 aa]	2,2	303579	7,00E-13	_
LACB00S00000975	protein kinase, [*Aspergillus fumigatus* Af293]	2,2	153763	1,00E-40	_
LACB00S00011427	chitinase 3 [*Coccidioides immitis*]	2,1	236861	3,10E-59	_
LACB00S00005470	TPR repeat:Peptidase, archaeal and bacterial C-terminal [*Trichodesmium erythraeum* IMS101]	2,1	151024	1,00E-142	_
LACB00S00012967	dual specificity protein phosphatase, [*Entamoe bahistolytica* HM-1:IMSS]	2,1	237909	5,00E-17	_
LACB00S00007217	clitocypin analog [*Lepista nebularis*]	2,1	326015	4,00E-05	_
LACB00S00006210	Ynd1p [*Cryptococcus neoformans* var. *neoformans*]	2,0	293572	7,00E-05	_

Down regulated genes

SEQ_ID	NCBI Defintion	Ratio T/C	protein ID	NCBI E value	Localization
Chromatine structure					
LACB00S00018974	mitotic chromosome condensation-related protein, putative[*Cryptococcus neoformans* var. *neoformans* JEC21]	-2,1	317541	1,00E-117	_
Energy metabolism					
LACB00S00019558	PREDICTED: similar to dehydrogenase E1 and transketolase domaincontaining protein 1 [*Bos taurus*]	-24,0	256909	0.0	M
LACB00S00014431	NADPH2 dehydrogenase chain OYE2 [*Neurospora crassa*]	-4,6	191617	2,10E-87	_
LACB00S00008720	NADH-dependent flavin oxidoreductase, [*Aspergillus fumigatus* Af293]	-4,2	291181	1,00E-131	_
LACB00S00011338	glyceraldehyde 3-phosphate dehydrogenase [*Armillariella tabescens*]	-2,9	163137	1,10E-34	_
LACB00S00019512	oxoglutarate dehydrogenase (succinyl-transferring), putative[*Cryptococcus neoformans* var. *neoformans* JEC21]	-2,4	141258	0.0	_
LACB00S00017625	Cytochrome P450, [*Cryptococcus neoformans* var. *neoformans* JEC21]	-2,3	314449	2,10E-68	S
LACB00S00012168	transaldolase, [*Cryptococcus neoformans* var. *neoformans* JEC21]	-2,2	303585	2,10E-39	_
LACB00S00010420	glyceraldehyde 3-phosphate dehydrogenase [*Armillariella tabescens*]	-2,0	160813	5,10E-56	_
LACB00S00006639	glyceraldehyde 3-phosphate dehydrogenase [*Armillariella tabescens*]	-2,0	160816	5,10E-76	_
Growth & morphology					
LACB00S00003032	N-acetylhexosaminidase [*Hypholoma fasciculare*]	-3,8	182604	1,00E-150	_
LACB00S00006604	cyclopropane fatty acid synthase [*Coprinopsis cinerea*]	-3,3	248368	0.0	M
LACB00S00015843	farnesyl-diphosphate farnesyltransferase, [*Cryptococcus neoformans* var. *neoformans* JEC21]	-3,1	310328	1,00E-05	_
LACB00S00000916	Dynactin, [*Cryptococcus neoformans* var. *neoformans* JEC21]	-2,9	301545	1,00E-12	_
LACB00S00015099	cytoplasmic dynein heavy chain [*Aspergillus oryzae*]	-2,7	309417	8,10E-55	_
LACB00S00003450	cytoplasmic dynein heavy chain [*Aspergillus oryzae*]	-2,5	309292	2,10E-55	S
LACB00S00016306	cyclopropane fatty acid synthase [*Coprinopsis cinerea*]	-2,5	254621	0.0	M

LACB00S00009098	cyclopropane fatty acid synthase [*Coprinopsis cinerea*]	-2,1	250402	0.0	M
LACB00S00013197	alpha-glucan synthase [*Cryptococcus neoformans* var. *neoformans*]	-2,1	304820	6,10E-19	_
Intercellular signalling					
LACB00S00019435	Cap64 protein [*Pleurotus ostreatus*]	-2,2	242010	9,10E-98	_
LACB00S00011982	Cap64 protein [*Pleurotus ostreatus*]	-2,1	157398	1,00E-158	S
Primary metabolism					
LACB00S00013630	uroporphyrin-III C-methyltransferase, [*Cryptococcus neoformans* var. *neoformans* JEC21]	-6,7	306663	3,00E-12	_
LACB00S00006331	multifunctional folic acid synthesis protein [*Neurospora crassa*]	-6,4	247939	3,10E-61	_
LACB00S00019869	ribonucleotide reductase large subunit (Rnr1), putative[*Aspergillus fumigatus* Af293]	-3,8	318621	1,10E-37	_
LACB00S00005319	sulfate adenylyltransferase [*Mucor circinelloides* f. *lusitanicus*]	-3,5	232595	4,10E-28	_
LACB00S00013633	uroporphyrin-III C-methyltransferase, [*Cryptococcus neoformans* var. *neoformans* JEC21]	-3,5	306667	6,00E-14	_
LACB00S00016950	uroporphyrin-III C-methyltransferase, [*Cryptococcus neoformans* var. *neoformans* JEC21]	-3,3	312806	9,00E-07	M
LACB00S00013632	uroporphyrin-III C-methyltransferase, [*Cryptococcus neoformans* var. *neoformans* JEC21]	-3,2	306666	6,00E-14	_
LACB00S00020209	uroporphyrin-III C-methyltransferase, [*Cryptococcus neoformans* var. *neoformans* JEC21]	-3,2	303005	6,00E-14	_
LACB00S00020606	purine nucleotide biosynthesis-related protein, putative[*Cryptococcus neoformans* var. *neoformans* JEC21]	-2,9	315043	8,00E-16	_
LACB00S00017575	uroporphyrin-III C-methyltransferase, [*Cryptococcus neoformans* var. *neoformans* JEC21]	-2,5	314396	1,00E-15	M
LACB00S00001203	sulfate adenyltransferase MET3 [*Cryptococcus neoformans* var.*grubii*]	-2,5	142989	0.0	_
LACB00S00004844	sulfate adenyltransferase MET3 [*Cryptococcus neoformans* var.*grubii*]	-2,2	183631	0.0	_
LACB00S00013512	aspartate carbamoyltransferase, [*Cryptococcus neoformans* var. *neoformans* JEC21]	-2,1	141163	0.0	_
LACB00S00016062	cystathionine gamma-lyase [*Neurospora crassa*]	-2,1	159481	1,10E-63	_
LACB00S00017838	glycogen metabolism-related protein, [*Cryptococcus neoformans* var. *neoformans* JEC21]	-2,1	295838	8,10E-24	_
LACB00S00019134	aspartate carbamoyltransferase, [*Cryptococcus neoformans* var. *neoformans* JEC21]	-2,0	140810	0.0	_
LACB00S00005570	acetate--CoA ligase [*Coprinopsis cinerea*]	-2,0	142473	1,00E-163	_
Protein synthesis & degradation					
LACB00S00007342	mitochondrial ribosome small subunit component RPS19 [*Pleurotus djamor*]	-14,3	248839	1,10E-39	M
LACB00S00002459	ubiquitin specific protease 48 isoform a [*Homo sapiens*]	-2,7	306135	1,10E-79	_
LACB00S00019618	eukaryotic initiation factor 4F subunit (eIF4F p130), putative[*Aspergillus fumigatus* Af293]	-2,0	242275	2,10E-59	_
Replication					
LACB00S00002441	mer3 [*Coprinopsis cinerea*]	-5,3	306118	0.0	_
LACB00S00011594	delta DNA polymerase, [*Cryptococcus neoformans* var.*neoformans* JEC21]	-2,3	251819	0.0	M
Saprophytic activity					
LACB00S00017619	endopeptidase, [*Cryptococcus neoformans* var. *neoformans JEC21*]	-3,8	314443	6,10E-37	S
LACB00S00012853	Ferroxidase pseudo-gene [*Auricularia polytricha*]	-3,1	304389	7,10E-56	S

LACB00S00014958	Ferroxidase [*Auricularia polytricha*]	-2,7	151539	0.0	S
LACB00S00005347	alpha-glucosidase B [*Aspergillus fumigatus* Af293]	-2,6	324539	0.0	S
LACB00S00010256	aryl-alcohol oxidase precursor [*Pleurotus eryngii*]	-2,6	299608	1,00E-169	S
LACB00S00012885	Ferroxidase [*Auricularia polytricha*]	-2,1	185642	0.0	S
Signal transduction					
LACB00S00013627	guanine nucleotide binding protein alpha subunit [*Lentinula edodes*]	-3,7	306661	3,00E-14	_
LACB00S00013611	heterotrimeric G protein alpha subunit B [*Schizophyllum commune*]	-3,5	145734	1,00E-153	_
LACB00S00013964	TBC domain protein, [*Aspergillus fumigatus* Af293]	-2,8	307084	4,10E-29	_
LACB00S00012729	GBA4.USTMA Guanine nucleotide-binding protein alpha-4 subunit[*Ustilago maydis* 521]	-2,6	304258	2,10E-23	_
LACB00S00013636	heterotrimeric G protein alpha subunit B [*Schizophyllum commune*]	-2,4	145822	1,00E-121	_
LACB00S00003692	Ras GTPase activator, [*Cryptococcus neoformans* var.*neoformans* JEC21]	-2,3	311272	0.0	M
LACB00S00013619	heterotrimeric G protein alpha subunit B [*Schizophyllum commune*]	-2,1	252895	3,10E-68	S
LACB00S00004669	guanine nucleotide exchange factor, [*Cryptococcus neoformans* var. *neoformans* JEC21]	-2,1	313621	1,00E-117	_
LACB00S00019139	GBA4.USTMA Guanine nucleotide-binding protein alpha-4 subunit[*Ustilago maydis* 521]	-2,1	335466	2,10E-24	_
LACB00S00013601	heterotrimeric G protein alpha subunit B [*Schizophyllum commune*]	-2,1	160465	1,00E-147	_
LACB00S00017592	heterotrimeric G protein alpha subunit B [*Schizophyllum commune*]	-2,0	145616	1,00E-125	_
LACB00S00001954	protein-histidine kinase, [*Cryptococcus neoformans* var.*neoformans* JEC21]	-2,0	305575	7,10E-35	S
LACB00S00016802	GBA4.USTMA Guanine nucleotide-binding protein alpha-4 subunit[*Ustilago maydis* 521]	-2,0	333606	5,10E-32	_
LACB00S00011020	calmodulin-dependent protein kinase I, [*Cryptococcus neoformans* var. *neoformans* JEC21]	-2,0	251340	4,10E-96	_
Transcription regulation					
LACB00S00001904	transcription factor protein [*Ciona intestinalis*]	-4,4	305525	3,00E-14	_
LACB00S00015874	regulatory protein cys-3 [*Neurospora crassa*]	-3,6	239654	4,00E-07	_
LACB00S00019340	pre-mRNA splicing factor, [*Cryptococcus neoformans* var.*neoformans* JEC21]	-3,2	164716	6,10E-20	_
LACB00S00018384	pre-mRNA splicing factor, [*Cryptococcus neoformans* var.*neoformans* JEC21]	-3,1	163323	6,10E-20	_
LACB00S00012935	transcriptional regulatory protein, [*Cryptococcus neoformans* var. *neoformans* JEC21]	-2,7	304495	1,10E-55	_
LACB00S00000449	related to helicase-DNA-binding protein [*Neurospora crassa*]	-2,7	301027	1,00E-145	_
LACB00S00001169	RSC complex subunit (Sth1), [*Aspergillus fumigatus* Af293]	-2,6	150762	0.0	_
LACB00S00013451	helicase, [*Cryptococcus neoformans* var. *neoformans* JEC21]	-2,5	306417	0.0	M
LACB00S00000616	ATP-dependent RNA helicase A, [*Cryptococcus neoformans* var. *neoformans* JEC21]	-2,4	243398	0.0	_
LACB00S00013099	pre-mRNA splicing factor, [*Cryptococcus neoformans* var. *neoformans* JEC21]	-2,4	304718	5,10E-57	_
LACB00S00015565	related to U4/U6 small nuclear ribonucleoprotein hPrp3 [*Neurospora crassa*]	-2,1	310000	8,10E-23	_
LACB00S00018468	RNA-directed RNA polymerase 1 [*Hordeum vulgare* subsp. *vulgare*]	-2,1	316322	5,10E-51	_
LACB00S00019055	pre-mRNA splicing factor RNA helicase (Prp43), putative[*Aspergillus fumigatus* Af293]	-2,0	148899	1,00E-12	_
LACB00S00007471	RNA polymerase III [*Schizosaccharomyces pombe*]	-2,0	190099	0.0	_
Transport					

LACB00S00007784	potassium transport protein, high-affinity, [*Cryptococcus neoformans* var. *neoformans* JEC21]	-3,4	296383	1,00E-116	_
LACB00S00019386	ATP-dependent permease, [*Cryptococcus neoformans* var. *neoformans* JEC21]	-3,1	256696	0.0	_
LACB00S00014396	ammonium transporter [*Hebeloma cylindrosporum*]	-3,1	331747	0.0	S
LACB00S00015042	pheromone transporter [*Cryptococcus neoformans* var. *neoformans*]	-3,0	308327	5,10E-39	_
LACB00S00019731	plasma membrane (H+) ATPase [*Uromyces viciae-fabae*]	-2,8	257178	2,10E-63	S
LACB00S00016194	plasma membrane H(+)-ATPase 1 [*Cryptococcus neoformans* var. *neoformans* JEC21]	-2,4	333132	1,00E-178	_
LACB00S00019676	nuclear pore complex protein, [*Cryptococcus neoformans* var. *neoformans* JEC21]	-2,1	309794	1,10E-74	_
LACB00S00002817	membrane transporter, [*Cryptococcus neoformans* var. *neoformans* JEC21]	-2,1	308613	1,00E-10	S
LACB00S00013920	hba2 [*Schizosaccharomyces pombe*]	-2,1	307042	0.0	_
LACB00S00008832	peptide transporter MTD1 [*Schizophyllum commune*]	-2,1	190652	0.0	_
LACB00S00014504	ATP-binding cassette (ABC) transporter, [*Cryptococcus neoformans* var. *neoformans* JEC21]	-2,1	155645	0.0	S
LACB00S00001318	Mg(2+) transport ATPase, P-type [*Bacillus cereus* ATCC 14579]	-2,1	301991	1,00E-156	_
LACB00S00013924	ABC transporter [*Schizosaccharomyces pombe*]	-2,0	253147	0.0	_
LACB00S00014265	phospholipid-translocating ATPase, [*Cryptococcus neoformans* var. *neoformans* JEC21]	-2,0	155569	0.0	_
LACB00S00000743	ABC transporter, [*Cryptococcus neoformans* var. *neoformans* JEC21]	-2,0	181553	0.0	_
Vacuole					
LACB00S00002180	retrograde transport, endosome to Golgi-related protein, putative[*Cryptococcus neoformans* var. *neoformans* JEC21]	-2,1	322083	1,00E-106	M
LACB00S00003642	vacuolar protein sorting 41, [*Cryptococcus neoformans* var. *neoformans* JEC21]	-2,2	311221	0.0	_
LACB00S00010767	vacuolar membrane protein, [*Cryptococcus neoformans* var. *neoformans* JEC21]	-3,0	251110	0.0	_
Others					
LACB00S00010461	Similar to protein kinase C, delta isoform 1 [*Bos taurus*]	-8,1	150425	5,00E-16	_
LACB00S00008824	ABL034Wp [*Ashbya gossypii* ATCC 10895]	-7,4	167414	9,10E-83	_
LACB00S00010320	Peptidase C14, caspase catalytic subunit p20 [*Anabaena variabilis* ATCC 29413]	-7,3	155578	1,00E-119	_
LACB00S00010302	Peptidase C14, caspase catalytic subunit p20 [*Anabaena variabilis* ATCC 29413]	-6,5	147433	7,10E-34	_
LACB00S00010277	Peptidase C14, caspase catalytic subunit p20 [*Anabaena variabilis* ATCC 29413]	-6,5	159530	2,10E-97	_
LACB00S00019599	Peptidase C14, caspase catalytic subunit p20 [*Anabaena variabilis* ATCC 29413]	-6,0	256967	5,10E-93	_
LACB00S00010354	Peptidase C14, caspase catalytic subunit p20 [*Anabaena variabilis* ATCC 29413]	-5,4	250987	2,10E-94	_
LACB00S00009433	ENSANGP00000020885 [*Anopheles gambiae* str. PEST]	-4,4	298457	7,00E-07	S
LACB00S00016090	laccase 2 [*Coprinopsis cinerea*]	-4,2	310622	0.0	M
LACB00S00006882	serine protein kinase (Sky1), [*Aspergillus fumigatus* Af293]	-3,5	166784	7,10E-27	_
LACB00S00019691	casein kinase I, [*Aspergillus fumigatus* Af293]	-3,3	164316	1,10E-57	_
LACB00S00011869	Exonuclease II, [*Cryptococcus neoformans* var. *neoformans* JEC21]	-2,8	303163	2,00E-14	M
LACB00S00007140	zinc-binding dehydrogenase, [*Cryptococcus neoformans* var. *neoformans* JEC21]	-2,7	248925	1,00E-127	_
LACB00S00010732	casein kinase I, [*Aspergillus fumigatus* Af293]	-2,7	160938	3,10E-53	_
LACB00S00005416	SPCC613.14 [*Schizosaccharomyces pombe*]	-2,6	157632	5,10E-50	_

LACB00S00007795	GH09355p [*Drosophila melanogaster*]	-2,6	296395	9,00E-12	M
LACB00S00010453	capsular associated protein, [*Aspergillus fumigatus* Af293]	-2,5	250899	4,10E-55	_
LACB00S00016578	casein kinase I, [*Aspergillus fumigatus* Af293]	-2,5	161095	1,10E-57	_
LACB00S00018868	oxysterol-binding protein, [*Cryptococcus neoformans* var. *neoformans* JEC21]	-2,4	335212	2,10E-58	M
LACB00S00007595	ATP binding / protein kinase/ protein serine/threonine kinase[*Arabidopsis thaliana*]	-2,4	319175	2,10E-38	_
LACB00S00017956	TPR repeat:Peptidase, archaeal and bacterial C-terminal[*Trichodesmium erythraeum* IMS101]	-2,4	150986	3,10E-78	_
LACB00S00013338	esterase/lipase/thioesterase family protein, [*Aspergillus fumigatus*]	-2,2	330882	1,00E-17	S
LACB00S00007276	casein kinase I [*Setosphaeria turcica*]	-2,2	153161	2,10E-52	_
LACB00S00001741	TPR repeat [*Methanosarcina barkeri* str. *fusaro*]	-2,2	305350	2,00E-06	S
LACB00S00001289	casein kinase [*Agaricus bisporus*]	-2,2	150213	1,00E-17	_
LACB00S00005035	casein kinase I, [*Aspergillus fumigatus* Af293]	-2,2	161077	4,10E-63	_
LACB00S00012529	Gcn2 [*Schizosaccharomyces pombe*]	-2,1	294998	1,00E-128	_
LACB00S00005354	alpha-glucosidase B [*Aspergillus fumigatus* Af293]	-2,1	151249	5,10E-98	_
LACB00S00003292	WD repeat domain 22 [*Mus musculus*]	-2,1	309123	3,10E-19	_
LACB00S00012993	Peptidase C14, caspase catalytic subunit p20 [*Anabaena variabilis* ATCC 29413]	-2,1	237865	1,00E-106	_
LACB00S00010489	isoamyl alcohol oxidase [*Aspergillus fumigatus* Af293]	-2,0	299855	3,10E-68	_
LACB00S00003646	Similar to Dual specificity protein phosphatase 2 (Dualspecificity protein phosphatase PAC-1) [*Bos taurus*]	-2,0	164161	4,00E-14	_
LACB00S00020242	Similar to TatD DNase domain containing 2 [*Bos taurus*]	-2,0	303664	7,10E-35	_
LACB00S00015525	Receptor-interacting serine-threonine kinase 2 [*Pantroglodytes*]	-2,0	309916	2,10E-22	_
LACB00S00005032	casein kinase I, [*Aspergillus fumigatus* Af293]	-2,0	146339	4,10E-63	_
LACB00S00019584	Exonuclease II, [*Cryptococcus neoformans* var. *neoformans* JEC21]	-2,0	304209	4,00E-11	_
LACB00S00017747	guanosine-diphosphatase, [*Cryptococcus neoformans* var. *neoformans* JEC21]	-2,0	334333	2,10E-72	M

I.3.3 Discussion

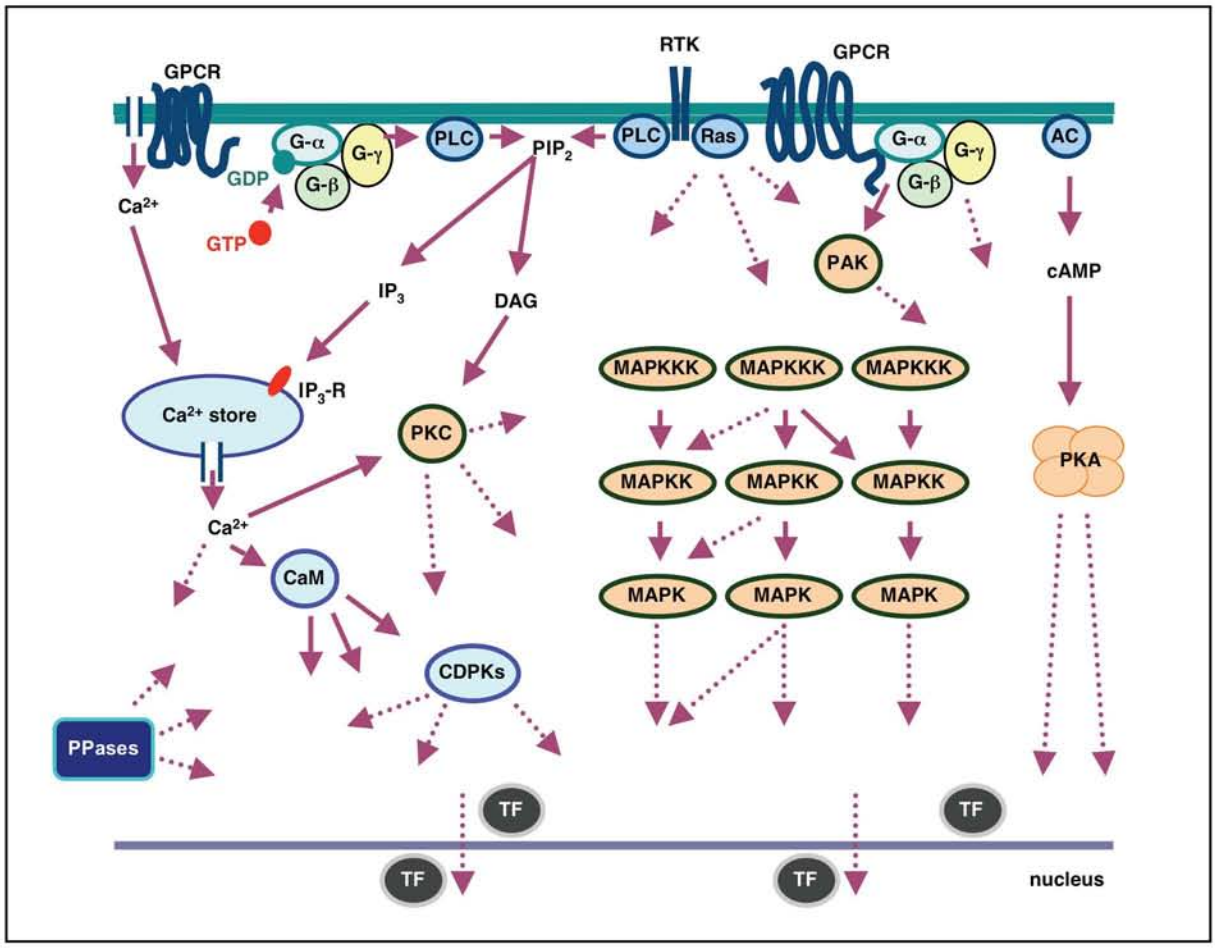
Validity of the oligochip results

This analysis was performed using the first generation of *L. bicolor* S238N whole genome arrays from Nimblegen. Data analysis of this first array design revealed two main distortions that we should keep in mind when interpreting the results. First, probes were designed on automatic computer annotated genes. None of the manually corrected genes was taken into account. This explains, for a part, why 80 % of the responsive genes fell down into the class of the hypothetical protein category. Forty percent of the responsive genes corresponded to genes that had an improbable topological pattern. It would be necessary for each of these genes to have a look at each probe intensity, then to identify which part of the gene model is expressed and regulated, and finally to try to make a new gene model. Secondly, about a half of the oligoprobes hybridizes with other genes than the expected ones (A. Kohler, personal communication). Therefore when one gene from a family is over-expressed, all the genes from this family seem to be regulated or conversely in the absence of concentration modification of the other members of the family the signal is diluted and the transcript is not identified as regulated. This second point, associated with the high biological variability, explains why part of the responsive genes previously identified using the cDNA nylon membrane was not found regulated in this data set.

Alteration of L. bicolor whole transcriptome at the early stage of interaction with P. fluorescens BBc6R8.

These whole genome arrays confirm the results already obtained using cDNA array: *P. fluorescens* BBc6R8 induced a pleiotropic reaction of *L. bicolor* S238N at the transcriptomic level with a low level of regulation, mainly between two- and fivefold. As previously described, epigenetic, lipid metabolism and cell wall modifications were also found regulated at the transcriptomic level. But this whole genome array analysis also revealed new features. First, it appeared that the transcriptomic response of the fungus affected more genes than we thought (less than 1% in the macroarray approach and at least eight percent here). This higher response is probably not linked to an artefact from the oligochips because a large part of the regulated transcripts does not correspond to multifamily genes. Secondly, new categories of responsive genes were discovered. Twenty-five genes encoding enzymes involved in the saprophytic activity of the fungus

Figure 1.6. Major signalling pathways described in fungi (from Duplessis, INRA Nancy, unpublished). The calmodulin pathway is underlined in blue, the MAP kinase pathway in green and the cAMP-protein kinase A pathway in orange. CaM: calmodulin, CDPK: calcium-dependant protein kinase, DAG: diacylglycerol, GPCR: G-protein coupled receptor, IP3: inositoltriphosphate, IP3-R: inositoltriphosphate receptor, MAPK : mitotic associated protein kinase, MAPKK: mitotic associated protein kinase kinase, MAPKKK: mitotic associated protein kinase kinase kinase, PAK: p21-activated kinase, PIP2: phosphatidyl inositol 4-5 biphosphate, PKA: protein kinase A, PKC: protein kinase C, PLC: phospholipase C, Ppases: phosphatases, RTK: receptor tyrosine kinase, TF: transcription factor.



(i.e cellulose, chitin, lignin, DNA degradation) were regulated. The expression of two-third of them was enhanced in the presence of the bacteria. The expression of extracellular hydrolysis enzymes is known to be repressed by the carbon catabolism in filamentous fungi (Ilmén *et al.* 1997, Ruijter *et al.* 1997, van Peij *et al.* 1998). The fungus and the bacteria grown together may have consumed most of the glucose from the medium and may have started to be starved and responded by over expressing genes encoding for enzymes that degrade organic matter. Fungal hydrolytic activities could also play a fundamental role in the pre-symbiotic life of the fungus in the soil as its only carbon resources available come from the reserve accumulated in the spore and in the soil. As a consequence, the over-expression of fungal hydrolyzing enzymes induced by the bacteria could explain the mycorrhiza helper effect of the strain BBc6R8, and notably the improvement of the pre-symbiotic survival in the soil.

The oligochips also revealed that several transcripts coding for signalling pathways elements were differentially accumulated before the contact with the helper bacteria. Two genes encoding a protein kinase A and a Cap3 protein, both components of the cAMP pathway, were up-regulated while two genes encoding a Gα protein and a Ras protein were down-regulated before any contact with the bacteria. This suggests that cAMP pathway could play a role in the transduction of putative bacterial signals perceived by the fungus. Up to now, several major signalling pathways have been extensively described in fungi, including the calmodulin dependant pathway, the MAP kinase and the cAMP pathway (figure 1.6). Gα and Ras are small GTP-binding proteins located at the vicinity of the plasma membrane where they are activated upon interaction with specific receptors known as G-protein coupled receptors. G-proteins trigger the first steps of the signalling pathways through the activation of various effectors and the generation of secondary messengers like cAMP (Koelle 2006). The cAMP pathway participates to many processes in fungi: conidiation, mating, morphogenesis, stress tolerance in *Neurospora crassa*, appressorium formation in *Magnaporthe grisea*, fruiting body formation in *Schizophyllum commune* or nutrient sensing in *Saccharomyces cerevisiae* (Lengeler *et al.* 2000, Rolland *et al.* 2002, Palmer & Horton 2006). Signal transduction is a very complex process involving frequent crosstalks between signalling cascades. Enzymes of these cascades are present in the cell to sense incoming signals and thus, are not necessarily falling under transcriptional activation. Therefore, transcriptional studies are not sufficient to explore the role and the functioning of signalling pathways in the helper

effect and knockout of responsive-genes will be necessary to gain further understanding. Several genes encoding proteins potentially involved in intercellular relationships were also found up-regulated. Three could be involved in adhesion process with bacterial cells: a lectin-like protein, a predicted protein containing a fascilin adhesion domain FAS1 and the hydrophobin LbHyd6. The lectin-like protein belongs to the family 13 of the CAZy Carbohydrate Binding Module (http://www.cazy.org/) and contains a ricin B domain that binds galactose residues. Lectins are carbohydrate-binding proteins generally involved in cell adhesion processes. Interestingly, some fungal lectins are suspected to be involved in recognition between ectomycorrhizal fungi and host roots (Giollant *et al.* 1993). Moreover Gram negative bacterial cell walls are rich in polysaccharides that can be recognized by lectin domains. However, numerous lectin proteins, among which the ricin, are glycoside hydrolases that are not involved in interaction processes. Thus, the presence of lectin domain in the sequence of a protein is not sufficient to conclude on its function. More studies are needed to determine if the *L. bicolor* lectin identified in this study is involved in the adhesion of bacteria or not. The second protein contains a fascilin adhesion domain that is though to physically interact with integrin domains of proteins inserted in cell membranes. Thus, this protein could also play a role in physical interaction with the helper bacteria. Finally, hydrophobins are small peptides that are cell wall located or excreted. They are involved in many steps of fungal biology where adhesion occurs (Linder *et al.* 2005). In ectomycorrhizal fungi, some hydrophobins are over expressed during root infection and their cell wall localization suggest a role in the mycorrhiza formation (Tagu *et al.* 2001). The *L. bicolor* genome contains 14 hydrophobin encoding genes that are differentially expressed depending on the tissue. Two are up-regulated in mycorrhiza but it is not the case of *LbHyd6* (Gibbon *et al.* unpublished results). Its expression was only altered in sporocarps (Gibbon *et al.* unpublished results) and in the mycelium before contact with the helper bacteria.

Hydrophobins and lectins have already been described in interaction processes between fungi and bacteria: hydrophobins were shown to protect fungal sporocarps against attack by bacterial parasites (Peñas *et al.* 1998) while lectin-type sugar receptors are frequently involved in fungi-bacteria aggregation by binding cell wall glycoproteins of the interacting cells (Peng *et al.* 2001). Interestingly, the helper bacterial strain *P. fluorescens* BBc6R8 can attach to *L. bicolor* mycelium in certain conditions (Sen *et al.* 1996). Sen *et al.* suggested a role of the bacterial flagellum in the adhesion process but

the fungal attachment molecules are unknown. The three proteins and the *tectonin-like* gene previously described (Deveau *et al.* 2007) are good candidates. Their over-expression before the physical contact is not inconsistent with this hypothesis. The transcription of these genes could be increased in preparation to the physical contact. In the case of the tectonin 2, we have previously observed that the gene was over expressed until the establishment of a physical contact (Deveau *et al.* 2007). Further analyses, notably on the binding capacity of the lectin-like, tectonin-like, fascilin-like and hydrophobin 6, are needed to determine what role they play in the interaction with the helper bacterial strain.

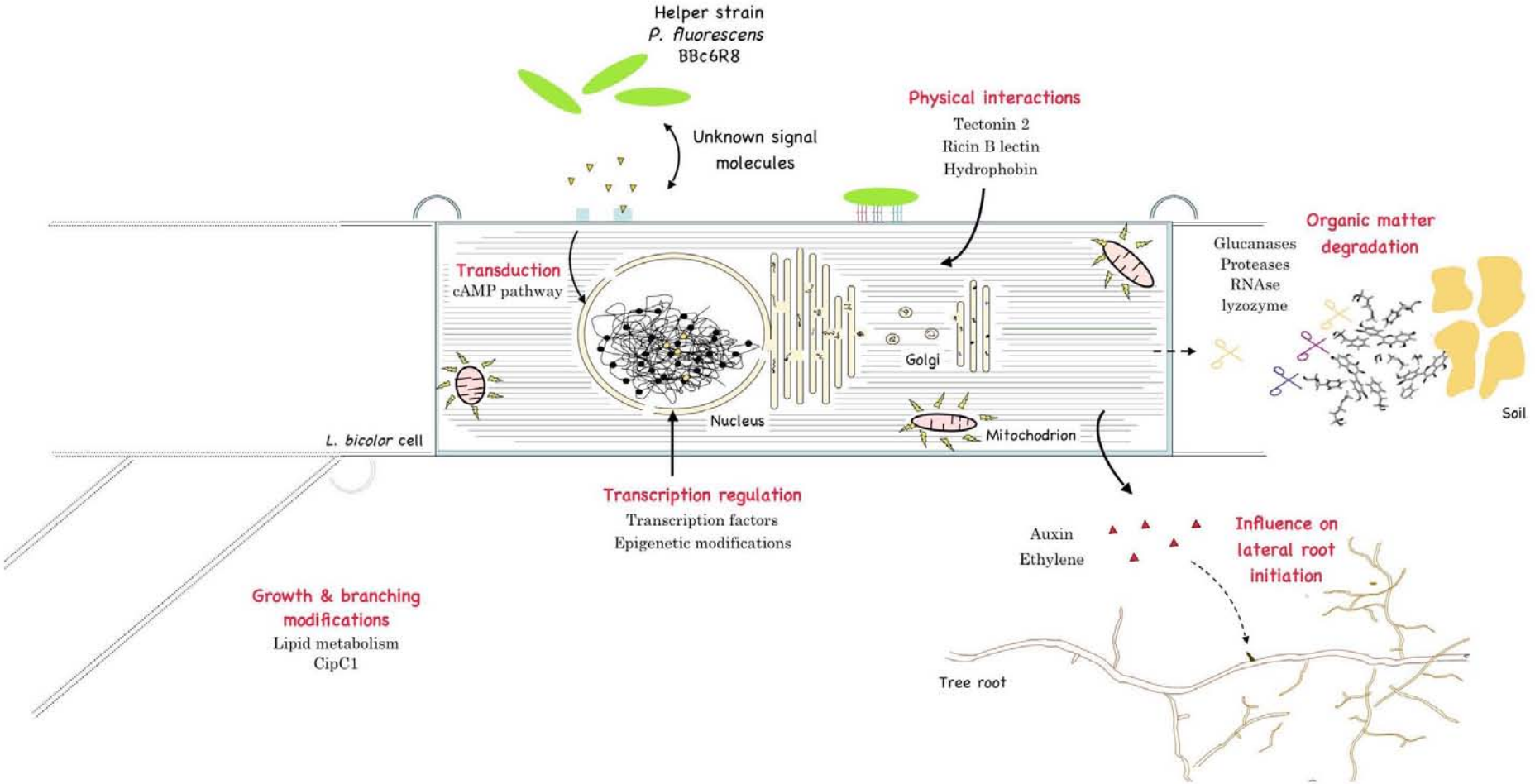
Finally, two genes encoding an auxin-efflux transporter and an enzyme involved in ethylene biosynthesis were over expressed. Interestingly, auxin modifies root architecture by inducing the proliferation of lateral roots, an essential step for mycorrhiza formation. The production of auxin by ectomycorrhizal fungi is suspected to be responsible for the alteration of root morphology (Kaska *et al.* 1999). Similarly, plant ethylene is known to regulate lateral root formation (Aloni *et al.* 2006). As a consequence, it is tempting to speculate that by inducing an overproduction of the fungal IAA and ethylene, the bacterial strain would improve the mycorrhizal establishment.

All together these data suggest that the helper effect of BBc6R8 is due to a combination of three main mechanisms: the increase of the pre-symbiotic growth and survival, the modification of the fungal morphology that prepares the mycelium to the symbiosis, and the secretion of plant hormones by the fungus. This transcriptomic approach revealed mechanisms that were unsuspected until today because they are difficult to experiment at the ecological level (i.e the increase of the fungal saprophytic activities and the over production of plant hormones).

However, we should keep in mind that microarrays are descriptive tools and that a correlation between transcription profiles and phenotypes does not demonstrate the contribution of these transcript products to the phenotype. Inactivation of the target genes highlighted by the microarray approach will be necessary to demonstrate the actual role of these genes in the helper mechanism. The knockout of targeted gene is not yet possible for *L. bicolor* S238N but the techniques are in development and should be available soon (Kemppainen *et al.* 2005).

Furthermore, the *in vitro* assay used in the two studies presented in this chapter mimics the primary interactions between the fungal mycelium and the bacteria but does not take into account the potential role of the plant in the helper effect. To answer this question we have started to develop a tripartite *in vitro* assay that mimics the interaction between *L. bicolor* S238N, *P. fluorescens* BBc6R8 and *Populus tremula* x *alba* 717-1-B4 (Annexe 3).

Figure 1.7. Schéma des différentes fonctions cellulaires de *L. bicolor* S238N répondant à l'échelle transcriptomique aux molécules élicitrices produites par *P. fluorescens* BBc6R8.



Conclusion

La bactérie auxiliaire BBc6R8 induit non seulement un accroissement des hyphes fongiques mais aussi des changements morphologiques : augmentation de la densité d'hyphes, de la densité de ramification et du degré de ramification. Parmi les sept souches bactériennes testées, seule BBc6R8 a induit des modifications de l'ensemble de ces traits morphologiques.

Ces modifications sont corrélées à des changements de la concentration d'environ huit pour cent des transcrits exprimés, et ce bien avant qu'un contact physique soit établi entre les deux partenaires. La bactérie auxiliaire produit donc des molécules élicitrices solubles et /ou volatiles qui sont perçues par le champignon. En revanche, après un contact physique prolongé entre la bactérie et le champignon dans le cadre du dispositif de confrontation *in vitro*, les deux micro-organismes semblent entrer en compétition trophique.

Les transcrits dont l'expression est régulée codent pour des protéines impliquées dans de nombreuses fonctions cellulaires. Les activités concernées suggèrent que l'effet auxiliaire résulterait de trois mécanismes majeurs (figure 1.7) :

i. une augmentation de la croissance et de la survie du mycélium liée à un accroissement de l'activité saprophyte du mycélium fongique. Une dégradation accrue de la matière organique du sol environnant fournirait d'avantage de nutriments à la fois au champignon ectomycorhizien et à la bactérie auxiliaire.
ii. une évolution du statut morphologique du mycélium fongique vers une forme ramifiée proche de l'état infectieux.
iii. une surproduction d'hormones végétales par le champignon qui pourrait aboutir à une production accrue de racines latérales par les arbres.

Ce modèle nécessite d'être confirmé par des études complémentaires notamment par l'inactivation des gènes pouvant jouer un rôle clé dans l'effet auxiliaire.

Par ailleurs, la comparaison de l'expression de plusieurs gènes cibles en présence des sept souches bactériennes suggère que le champignon serait capable de différencier les souches bactériennes et de réagir de façon spécifique à leur égard. Cette question fait l'objet du troisième chapitre.

Chapter II

Metabolic-mediated interactions between *L. bicolor* S238N and the helper bacterial strain *P. fluorescens* BBc6R8.

Chapitre II

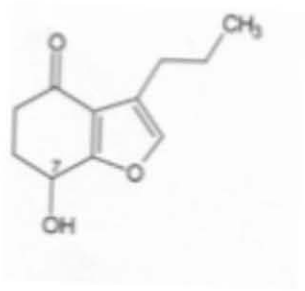
Échanges métaboliques au cours de l'interaction entre *L. bicolor* S238N et la bactérie auxiliaire de la mycorhization *P. fluorescens* BBc6R8

Introduction

L'identification des molécules actives est une étape clé dans la compréhension des mécanismes de l'interaction entre deux organismes. La réaction précoce de *L. bicolor* S238N, avant même qu'un contact physique ne soit établi avec la souche bactérienne *P. fluorescens* BBc6R8, indique que celle-ci produit des métabolites actifs, solubles ou volatiles, qui influencent le comportement du champignon. Ces molécules peuvent agir (1) directement en pénétrant dans les cellules fongiques ou en se fixant à des récepteurs fongiques, ou (2) indirectement en modifiant les propriétés de l'environnement (pH, nutriments, humidité, toxicité…). Dans le cas des interactions entre bactéries auxiliaires et champignons ectomycorrhiziens, les molécules actives sont principalement inconnues. Seul l'auxofuran produit par la souche bactérienne auxiliaire *Streptomyces* sp. a été identifié jusqu'à présent (Riedlinger *et al.* 2006). Il s'agit d'un composé aromatique (figure 2.1) dont la structure est proche de l'ulufuranol et de l'herbicide koninginin B. Son addition au milieu de culture dans des concentrations allant de 150 nM à 50 µM active la croissance d'*Amanita muscaria*. De plus cette molécule induit la surexpression des gènes codant pour l'acetyl-CoA synthetase, la cyclophilin 40 et une GABA perméase, trois gènes fongiques qui sont activés en présence de la souche AcH505 (Schrey *et al.* 2005, Riedlinger *et al.* 2006).

Des modifications de la croissance et de la morphologie des hyphes fongiques sont fréquemment observées lors d'interaction entre bactéries et champignons (Bolwerk *et al.* 2003, Hogan *et al.* 2004, Schrey *et al.* 2005, Ström *et al.* 2005, Vespermann *et al.* 2007). La nature des métabolites induisant ces changements morphologiques varie en fonction des modèles considérés. Il s'agit par exemple de la phenazine-1-carboxamide chez *Pseudomonas chlororaphis* (Bolwerk *et al.* 2003), de la molécule de quorum sensing 3,O-C12-HSL chez *P. aeruginosa* (Hogan *et al.* 2004), de l'acide phenyllactique, d'un dipeptide cyclique chez *Lactobacillus plantarum* MiLAB 393 (Ström *et al.* 2005) ou d'un cocktail de molécules volatiles chez des rhizobactéries (Kai *et al.* 2006, Vespermann *et al.* 2007). La plupart de ces molécules réduisent la croissance du mycélium en bloquant certains processus métaboliques. D'autres, tel l'auxofuran sécrété par la souche bactérienne *Streptomyces* sp. AcH505, la thiamine produite par *Bacillus sp.* TB-1, la dopamine par *Klebsiella aerogenes*, ou les strigolactones et les flavonoïdes produits par les racines, stimulent la croissance et la ramification de divers champignons (Riedlinger *et*

Figure 2.1. Structure moléculaire du 7-dehydroxy-auxofuran produit par la souche auxiliaire *Streptomyces* sp. AcH505 (d'après Riedlinger *et al.* 2006).



al. 2006, Rikhvanov *et al.* 1999, Frases *et al.* 2006, Akiyama *et al.* 2005, Bessérer *et al.* 2006).

Si les bactéries sont capables d'influencer fortement le développement des champignons qui partagent les mêmes niches, ceux-ci ne sont pas en reste. Ils produisent également une grande

variété de métabolites secondaires qui affectent positivement ou négativement l'établissement de populations bactériennes. Ainsi l'acide fusarique sécrété par les *Fusarium* et la pénicilline produite par les *Penicillium* montrent une forte activité antibactérienne et influencent la production de molécules antifongiques par les bactéries antagonistes (van Rij *et al.* 2005). A l'inverse des cas de coopération métabolique ont également été décrits (Chaucheyras-Durand & Fonty 2001, Alexandre *et al.* 2004, Uroz *et al.* 2007, cf. Introduction chapter). Des millions d'années de co-évolution entre champignons et bactéries ont conduit à la formation de mécanismes d'interactions extrêmement complexes faisant souvent intervenir plusieurs métabolites chez chacun des partenaires.

Dans le cas de l'interaction entre *L. bicolor* S238N et *P. fluorescens* BBc6R8, la nature des composés actifs produits par le champignon et la bactérie sont inconnus. Nous avons dans un premier temps cherché à savoir si les molécules étaient de nature volatile ou soluble. Puis nous avons étudié quels rôles pouvaient jouer deux métabolites d'intérêt, la thiamine et le tréhalose, dans l'interaction *L. bicolor* S238N – *P. fluorescens* BBc6R8.

II.1 The fungal *in vitro* growth promotion is due to a combination of soluble and volatile compounds.

The identity of both fungal and bacterial compounds that are involved in the interaction between *L. bicolor* S238N and *P. fluorescens* BBc6R8 is unknown. We have performed partitioning experiments to determine whether the metabolites involved in the growth promoting effect were soluble and/or volatile.

II.1.1. Material and methods.

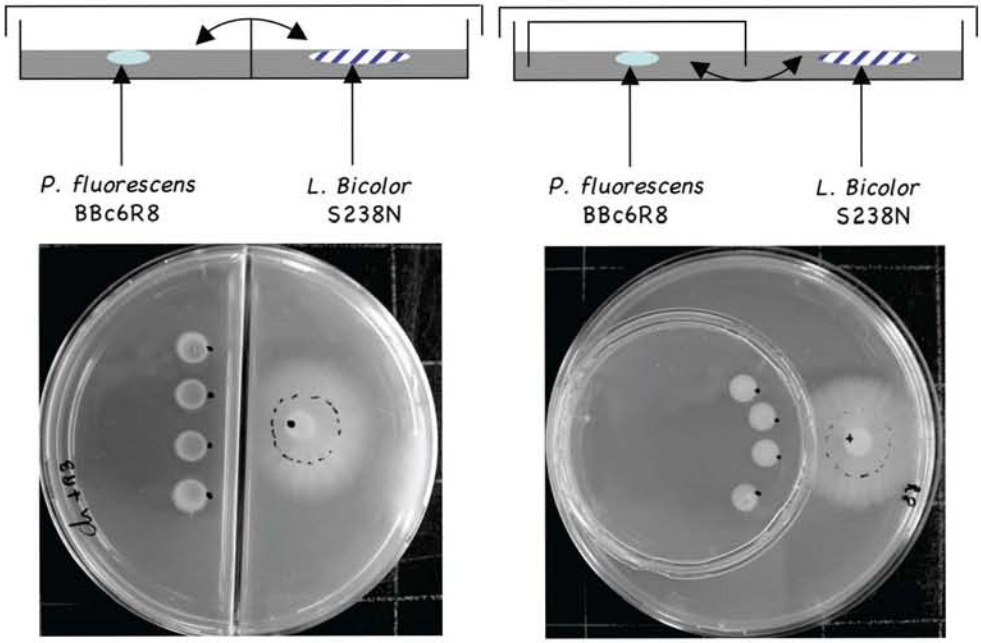
Partitioning experiments

The bioassay previously described (Deveau *et al.* 2007) was adapted to evaluate the role of both bacterial soluble and volatile compounds on the growth promotion of *L. bicolor* S238N. Partitioned 90-mm-diameter Petri dishes without soluble communication were used to measure the effect of bacterial volatile compounds (figure 2.2.A). To measure the effect of soluble compounds, bacterial drops were entrapped in a 55-mm-diameter Petri dish that impeded volatile communication (figure 2.2.B). In each case, the center of the fungal plug was put at 1.5 cm from the center of the two closest bacterial drops, as done in he bioassay previously described (Deveau *et al.* 2007). The bioassay without any restriction of communication described by Deveau *et al.* (2007), was also performed as a control, first to assess if there was an effect of the partitioning on the fungal growth and secondly to check the positive effect of the helper bacterial strain on the fungal growth. The diameter of the fungal colony was measured every five days from day 12 to day 41 after the addition of sterile water (control) or bacterial suspensions. Seven replicates per treatment were done and the whole experiment was performed two times (january-february 2005 and may-june 2005).

Effect of fungal and bacterial supernatants on L. bicolor S238N growth

One year-old mycelium from *L. bicolor* S238N maintained on Paschlewski P5 liquid medium was collected, washed with sterile water and cut in 100 mL of P20Th- medium with a Wassing Blender for 5 seconds. Two millilitres of cut mycelium were added to 100 mL of P20Th- in six sterile 250 mL Erlenmeyer flasks. In three Erlenmeyers, a bacterial suspension of *P. fluorescens* BBc6R8 was added in order to get a final bacterial **Figure**

2.2. Sketch and photographs of the experimental set up designed to evaluate the effect of bacterial volatile (type A) and soluble (type B) metabolites on the fungal growth. **A**. The 90-cm diameter Petri dish is partitioned in two compartments because of a plastic separation that does not allow soluble communication between bacterial colonies and the fungal mycelium (left). **B.** The bacterial colonies are entrapped into a 55-cm diameter Petri dish that is inserted in the agar medium. By the way, volatile communication is not impeded (right).



Type A Type B

concentration of 10^5 cfu.mL^{-1}. *Laccaria bicolor* S238N, *P. fluorescens* BBc6R8 and mixed *L. bicolor* S238N-*P.fluorescens* BBc6R8 were grown for 27 to 32 days in liquid P20Th$^-$ medium in the dark at 25°C. Supernatants were collected after centrifugation (6000 rpm, 10 min) and then used in the bioassay previously described (Deveau *et al.* 2007) instead of the bacterial drops. Two control treatments were performed: one with water drops and one with *P. fluorescens* BBc6R8 drops. Seven replicates were done per treatment. The diameter of the colony was measured after 12, 14 and 27 days of incubation at 10°C in the dark.

II.1.2 Results

Partitioning experiments

The comparison of the fungal extention in the bioassay without any restriction of communication and in the two partitioning experiments demonstrated that the plastic (Type A) and the Petri dish (Type B) partitions had no effect on the fungal growth. The bioassay without restriction of communication was also used to check the positive effect of *P. fluorescens* BBc6R8 on the fungal growth. Unexpectedly, the positive effect of the helper strain on the fungal growth was delayed in comparison to other experiments previously done (Table 2.1). A significant fungal growth increase in the presence of bacteria was obtained after 20, 24, 31 or 37 days of incubation, depending of the replicate (January, February, May or June). Concerning the partitioning experiments, the fungal growth promotion effect was absent in the two "type A" experiments and in one of the two "type B" experiments (Table 2.1). In the other one, a significant positive effect was measured but it was lower than the one induced by BBc6R8 without partitioning.

Effect of fungal and bacterial supernatants on L. bicolor S238N growth

L. bicolor S238N 27-days old growth supernatant had not positive effect on the fungal growth (Table 2.2). In contrast, both *P. fluorescens* BBc6R8 supernatant and mixed culture supernatant induced a fungal growth increase. Interestingly, the mixed culture stimulated growth earlier than the bacterial one.

Table 2.1. Effect of *P. fluorescens* BBc6R8 on *L. bicolor* S238N hyphal extension in non-partitioned or partitioned treatments. A plus sign indicates a significantly positive effect on the fungal growth, and a zero no effect. Numbers into brackets indicate at which time of the interaction the bacteria induced a positive effect on the fungal growth according to a one-way (bacterial treatment) ANOVA ($p>0.05$).

Time of the experiment	Control without restriction	Type A 	Type B 
January	+ (20)	0	
February	+ (31)	0	
May	+ (37)		+ (20)
June	+ (24)		0

Table 2.2. Effect of *P. fluorescens* BBc6R8, *L. bicolor* S238N 27 days-old supernatant, *P. fluorescens* BBc6R8 32 days-old supernatant, mixed *L. bicolor* S238N/*P. fluorescens* BBc6R8 27 days-old supernatant on *L. bicolor* S238N hyphal extension. Each value is the mean value (± SE) of seven replicates. In each colomn, mean values with the same letter are not significantly different according to a one-way (treatment) ANOVA ($p > 0.05$).

Treatment	Diameter of the fungal colony (cm)		
	12 days	14 days	27 days
L. bicolor S238N	1,3 ± 0.02 a	1.7 ± 0.04 a	3.2 ± 0.2 a
L. bicolor S238N + *P.fluorescens* BBc6R8	1.5 ± 0.03 b	1.9 ± 0.03 b	3.5 ± 0.03 b
L. bicolor S238N + *L. bicolor* S238N 27 days old supernatant	1.2 ± 0.04 c	1.6 ± 0.03 c	3.2 ± 0.05 a
L. bicolor S238N + BBc6R8 32 days old supernatant	1.3 ± 0.04 a	1.7 ± 0.04 a	3.3 ± 0.07 b
L. bicolor S238N + *L. bicolor* S238N / BBc6R8 27 days old supernatant	1.5 ± 0.01 b	1.8 ± 0.03 b	3.4 ± 0.05 b

II.1.3 Discussion

These data indicate that both volatile and soluble compounds are involved in the fungal growth-promoting effect. Moreover, volatile and soluble compounds were both necessary to induce a significant fungal growth increase. We think that the growth-promoting effect observed in one of the two replicates of the partitioning experiment of type B was due to a problem of air tightness in the assay. A third replicate experiment should be performed to confirm our interpretation of the results.

Two hypotheses can be proposed to explain why both aerial and soluble diffusions are required for observing the fungal growth-promoting effect: either the bacteria produce both volatile and soluble compounds that acted in combination on the fungal behaviour, either the fungus itself produces volatile and/or soluble metabolites that are necessary for the bacteria to exert their promoting effect through the production of volatile and/or soluble products. Complementary analyses on the effect of exudates from bacterial and mixed culture indicate that *P. fluorescens* BBc6R8 produces at least soluble inducer(s) and that their production is stimulated by *L. bicolor* S238N. A similar effect was observed in the case of the MHB model *A. muscaria* - *Streptomyces* sp.: the production of auxofuran was enhanced by the ectomycorrhizal fungus (Riedlinger *et al.* 2006).

II.2 Thiamine production by *P. fluorescens* BBc6R8: what role in the helper effect?

Thiamine pyrophosphate (TPP, B_1 vitamin) is an essential coenzyme required for the activity of several enzymes of the central carbon metabolism, such as transketolase, pyruvate dehydrogenae, pyruvate decarboxylase or α-ketoglutarate decarboxylase (Sohn *et al.* 2000). A TPP deficiency affects nucleic acid synthesis and energy metabolism. TPP can be synthesized *de novo* by most plants, bacteria and fungi. However, some fungi are impaired in this synthesis and require external source of thiamine or of one of its subunits to survive (Garraway & Evans, 1984). Others are able to synthesise the whole molecule but in suboptimal amounts. For this reason, many media which were optimized for the fungal growth contain the B_1 vitamin. Interestingly, *P. fluorescens* BBc6R8 exerted a reproducible growth-promoting effect on *L. bicolor* S238N on a modified

Pachlewski medium that lacks thiamine. Based on this knoweldge, we hypothezised that the ectomycorrhizal fungus is auxotroph for thiamine and that the bacterium provides the vitamin to the fungus. To test this hypothesis, we measured the effect of different concentrations of pure thiamine on the fungal growth, using the same bioassay as described before (Deveau *et al.* 2007). Then, we looked into the genome of the fungus for the genes involved in the thiamin biosynthesis and transport pathways. Finally, we studied the secretion of thiamine by the helper bacterial strain *P. fluorescens* BBc6R8.

II.2.1 Material & methods

Effect of thiamine on fungal growth.

The bioassay previously described (Deveau *et al.* 2007) was adapted to measure the effect of various concentrations of thiamine on fungal growth. Bacterial drops were replaced by 10μL drops of 0.2μm-filtrated thiamine solutions. According to the literature (Griffin, 1981) three concentrations were tested: 1.65, 16.5 and 165 μM. A control treatment with sterile water drops and a treatment with drops of *P. fluorescens* BBc6R8 were also performed. Seven replicates per treatment were done. The diameter of the fungal colony was measured after 12, 14 and 27 days of incubation at 10°C in the dark.

Computational analysis of L. bicolor S238N thiamine biosynthesis pathway

On the basis of the work of Rodriguez-Navarro *et al.* (2002) and Nosaka (2006) on thiamine biosynthesis pathway of *S. cerevisiae*, we searched for the presence of *THI2, THI3, THI4, THI5, THI6, THI10, THI11, THI12, THI13, THI20, THI21, THI80* genes into the genome of *L. bicolor* H82-S238N by doing BlastP comparison with *S. cerevisiae* proteins (THI2 CAA85203, THI3 Q07471, THI4 CAA97157, THI5 P43534, THI6 CAA97929, THI10 Q05998, THI11 P47183, THI12 P42883, THI13 Q07748, THI20 NP_014586, THI21 Q08975, THI80 CAA99346)

Detection of thiamine

Two complementary methods of thiamine detection were used to search for the presence of thiamine in bacterial and fungal supernatants: first, HPLC technique permited to detect a compound that co-eluted with thiamine. Secondly, the thiochrome method was used to confirm that this compound belonged to the thiamine family.

The HPLC method was adapted from Chatzminichalakis *et al.* (2004). Separation was done on a reverse phase RP C_{18} LiChrospher column (250 mm x 4.6 mm, 5μm), using a Beckman System GOLD HPLC. The mobile phase was delivered at a flow rate of 0.8 ml/min and consisted of the solvent A: 0.05 M CH_3COONH_4/CH_3OH (99/1, v/v) and B: H_2O/CH_3OH (50/50, v/v). A multi-steps gradient was used, starting at a A:B *v/v* composition of 99:1 and remaining isocratic for 4 min. Then the composition was changed linearly to reach 100% of solvent B after 18 min and finally elution was performed isocratically for 8 min. A 5-min equilibration time was observed between injections. Detection was performed with a photodiode array detector monitoring the eluent at 270 nm. The injection volume was 25 μL.

The thiochrome protocol was adapted from Batifoulier *et al.* (2005). Twenty μL of potassium hexacyanoferrate (30.4 mM $K_3Fe(CN)_6$ in NaOH 15%) were added to 200 μL of the solution to be tested solution, mixed by vortex 10s and left to stand exactly during one minute. Then 5 μL of NaOH 15% were added. Finally thiochrome fluorescence was measured at an emission wavelength of 460 nm and an excitation of 355 nm using a Victor3 microplate reader (Wallac Perkin-Elmer Life Sciences, Villebon-sur-Yvette, France). A standard curve of fluorescence was made using standard solutions of known thiamine concentrations, ranging from 3 to 600 μM. The curve was linear until 300 μM.

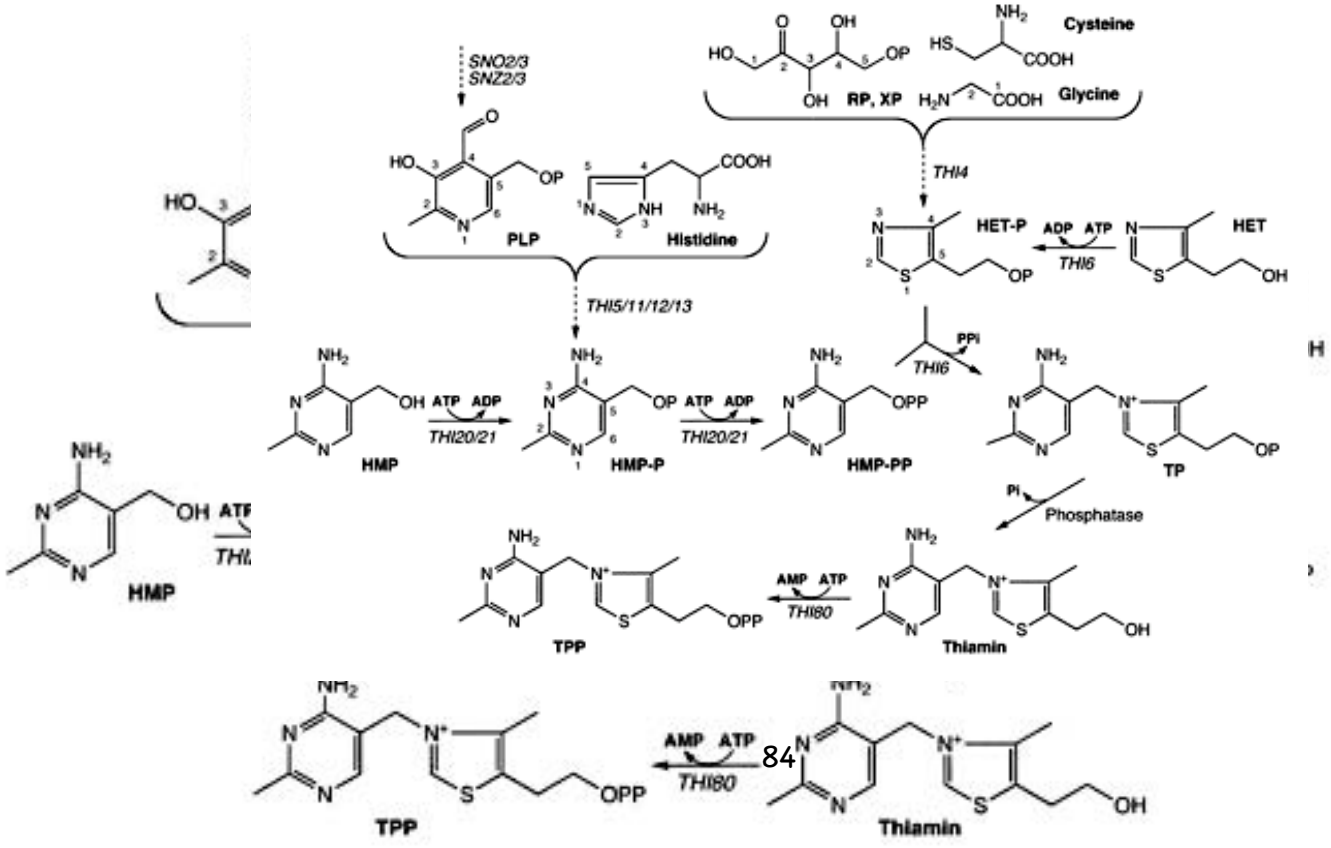
Thiamine production by the helper strain P. fluorescens BBc6R8

Pseudomonas fluorescens BBc6R8 was first grown on 10% TSA plates (3 $g.L^{-1}$ tryptic soy broth from Difco and 15 $g.L^{-1}$ of agar) at 25 °C for 65 h to prepare the bacterial inoculum. Then, three to four colonies were picked and suspended in 2 mL of sterile deionized water before spreading 120 μL on 10% TSA medium. After 48 h of growth at 25°C, the bacteria were harvested and centrifuged at 3300 g for 10 min. The pellet was washed twice, then resuspended in deionized water in order to obtain a suspension with $A600_{nm}$ of 0.7. Ten microlitres of this suspension were added to 10 mL of P20Th- medium and incubated at 25°C under 300 rpm rotating agitation. Two millilitres samples were collected after 48h, 72h and seven days of incubation. For each sample, the bacterial culture was centrifuged 20 min at 13.000 rpm and both the pellet and the supernatant were collected. The pellet was recovered with 500μL of sterile deionized water, then sonicated five minutes. Both the supernatants and the pellets were filtrated at 0.2 μm and concentrated ten times by evaporation with a speedvack. Twenty-five microlitres of

Table 2.3. Effect of *P. fluorescens* BBc6R8 and of thiamine on *L. bicolor* S238N hyphal extension after 12, 14 and 27 days of culture. Each value is the mean value (± SE) of seven replicates. In each column, mean values with the same letter are not significantly different according to a one-way (treatment) ANOVA ($P > 0.05$)

Treatment	Diameter of the fungal colony (cm)		
	12 days	14 days	27 days
L. bicolor S238N	1,3 ± 0.02 a	1.7 ± 0.04 a	3.2 ± 0.2 a
L. bicolor S238N + *P.fluorescens* BBc6R8	1.5 ± 0.03 b	1.9 ± 0.03 b	3.5 ± 0.03 b
L. bicolor S238N + thiamine 1.6 µM	1.3 ± 0.04 a	1.6 ± 0.04 a	3.4 ± 0.05 b
L. bicolor S238N + thiamine 16 µM	1,4 ± 0.01 b	1.7 ± 0.02 a	3.4 ± 0.03 b
L. bicolor S238N + thiamine 165 µM	1.1 ± 0.04 c	1.4 ± 0.05 c	3.2 ± 0.05 a

Figure 2.3. Thiamine pyrophosphate biosynthesis pathway in *S. cerevisiae*. Gene names are presented in italics. Solid arrows represent known reaction steps and dashed arrows indicate unknown reaction. HET: 4-methyl-5-ß-hydroxyethylthiazole, HMP: 2-methyl-4-amino-5-hydroxymethyl-pyrimidine, PLP: pyridoxal 5'-phosphate, RP: D-ribulose 5-phosphate, TP: thiamine phosphate, TPP: thiamine pyrophosphate, XP: D-xylulose 5-phosphate (from Nosaka, 2006).



each sample were injected into the HPLC. Parallely, the supernatant of a two-days old *L. bicolor* S238N liquid culture prepared as previously described (cf. II.1.1) in P20Th$^-$ medium was collected, prepared as described above and injected into the HPLC. The HPLC spectra of each sample were compared to the spectra obtained with a 40μM thiamine solution and with a P20Th$^-$ 10x concentrated medium.

Thiamine production by various soil bacterial strains

Pseudomonas fluorescens BBc6R8, *P. fluorescens* Pf29A, *Burkholeria* sp. EJP73 and *Paenibacillus* sp. EJP67 were cultured as described above. After 15 days of growth, solutions were centrifuged. The supernatants were collected, filtrated at 0.2 μm and concentrated four times by evaporation with a speedvack. Thiamine content of the concentrated solutions was determined using the thiochrome method.

Thiamine concentration in liquid L. bicolor S238N *culture and L. bicolor* S238N – *P. fluorescens* BBc6R8 *co-culture*

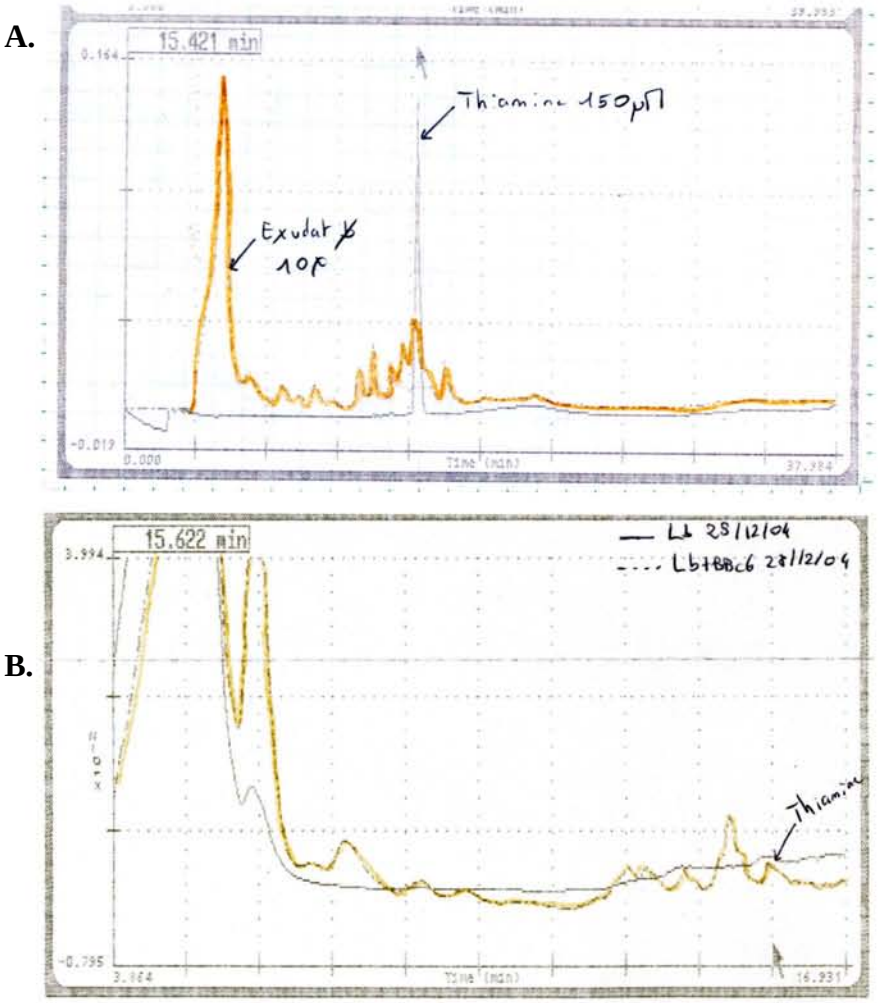
One year-old *L. bicolor* S238N maintained on Pachlewski P5 liquid medium was collected, washed with sterile water and cut in 100 mL of P20Th- medium with a Wassing Blender for 5s. Two millilitres of the cut mycelium were added to 100 mL of P20Th- in five sterile 250 mL Erlenmeyers. In two erlenmeyers, a bacterial suspension of *P. fluorescens* BBc6R8 was added in order to get a final bacterial concentration of 10^5 cfu.mL^{-1}. Each erlen was sealed with sterile aluminium paper and incubated with 130 rpm rotating agitation at 10°C. One-millilitre samples were collected in each replicates after 5, 7, 9, 14, 19, 26 and 34 days of culture. Each sample was centrifuge 10 min at 13.000 rpm, the supernatant was collected, filtrated at 0.2μm and conserved at -20°C. Concentration of thiamine in each sample was measured using the thiochrome method.

II.2.2 Results

Thiamine partially mimicked the fungal growth promoting effect of P. fluorescens BBc6R8

Both 1.6 μM (corresponding to 16 nmol) and 16 μM (160 nmol) concentrations of thiamine induced a significant (*t* test $P<0.05$) increase of the fungal growth after 27 days of incubation while the fungal growth-promoting effect in the treatment inoculated with *P. fluorescens* BBc6R8 was observed since 12 days. By contrast, the fungal growth was inhibited by the highest thiamine concentration (Table 2.3).

Figure 2.4. A. HPLC chromatogram of 7 days old *P. fluorescens* BBc6R8 supernatant (orange line) and 150μM thiamine (grey line). **B.** HPLC chromatogram of 7 days old *L. bicolor* S238N supernatant (grey line) and 7 days old co-culture of *L. bicolor* S238N and *P; fluorescens* BBc6R8 (orange line). The arrow indicates the time of elution of thiamine.



Laccaria bicolor S238N lacks the first steps of thiamine biosynthesis pathway

In yeast cells, biosynthesis of thiamine occurs via the condensation of thiazol and pyrimidine derivates (4-methyl-5-ß-hydroxyethylthiaole and 2-methyl-4-amino-5-hydroxymethylpyrimidine, Figure 2.3). The two ring structures are independently synthesised. Eleven genes involved in the synthesis of TPP have been identified in *S. cerevisiae* (Hohmann &Meacock, 1998). We have searched for their homologues in the genome of *L. bicolor* H82-S238N. Only homologues of THI6 (Protein ID 246751), THI20/21 (247597) and THI80 (294193, 146577-truncated gene) were found. By contrast, *L. bicolor* H82-S238N contains a gene encoding a transporter (151794) highly similar to the *THI10* thiamine transporter of *S. cerevisiae.*

P. fluorescens BBc6R8 *secretes thiamine*

No thiamine was detected by HPLC analysis either in P20Th$^-$ medium, *L. bicolor* S238N growth supernatant or in 48h-old and nor in 72h-old *P. fluorescens* BBc6R8 supernatant. In contrast, a peak that co-eluted with thiamine was observed in the 7 days-old bacterial supernatant and in bacterial pellets (Figure 2.4). The concentration of the thiamine-like compound was about 4μM.

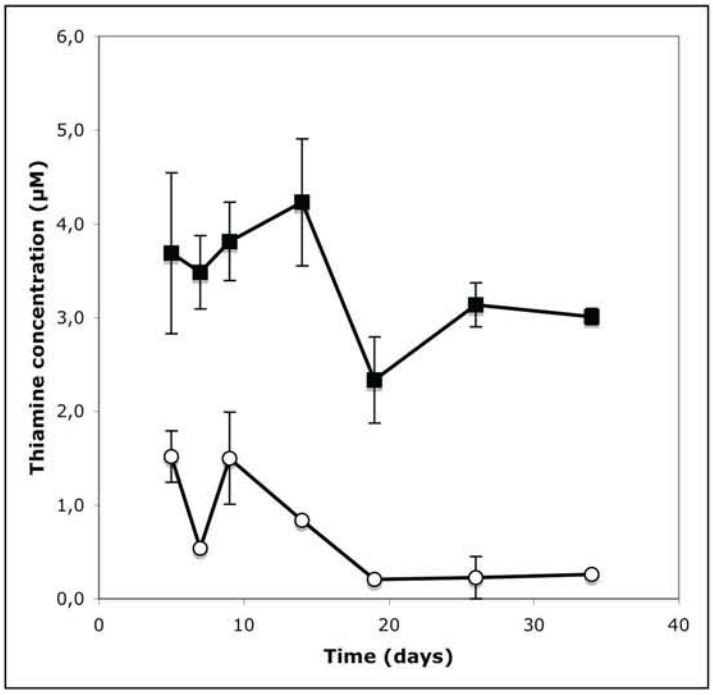
The presence of thiamine in the 7 day-old *P. fluorescens* BBc6R8 culture supernatant was confirmed by the thiochrome method. The oxidation of thiamine by $K_3Fe(CN)_6$ leads to the formation of thiochrome, a compound which fluoresces when excitated at 365 nm. After 15 days of culture, 15 μM of thiamine (1.6 nmol.μL^{-1}) were detected in the culture medium of *P. fluorescens* BBc6R8. Thiamine was also detected in the culture medium of *P. fluorescens* Pf29A, *Burkholderia* sp. EJP73 and *Paenibacillus* sp. EJP67 but in lower concentrations for two of them (Table 2.4).

Finally, we measured the concentration of thiamine in the culture medium of *L. bicolor* S238N grown in the presence or not of *P. fluorescens* BBc6R8 (Figure 2.5). A significantly higher concentration of thiamine was measured in the presence of the bacteria, according to a *t* test ($p < 0.05$).

Table 2.4. Production of thiamine by *P. fluorescens* BBc6R8, *P. fluorescens* Pf29A, *Paenibacillus* sp. EJP67 and *Burkholderia* sp. EJP73 after 15 days of growth. Measurements were made by the thiochrome method. Each value is the mean (± SE) of three replicates. In each column mean values with the same letter are not significantly different according to a one-way (bacterial treatment) ANOVA ($P > 0.05$)

Strains	Thiamine concentration (μM)
P. fluorescens BBc6R8	14.7 ± 1.1 a
P. fluorescens Pf29A	12.4 ± 1.2 a
Paenibacillus sp. EJP67	3.1 ± 0.5 b
Burkholderia sp. EJP73	6.6 ± 0.9 c

Figure 2.5. Concentration of thiamine measured by the thiochrome method in culture medium of *L. bicolor* S238N (white dots) or in co-culture of *L. bicolor* S238N and *P. fluorescens* BBc6R8. Each point is the mean value of two replicates (± SE).



II.2.3 Discussion

Because some fungi are known to be auxotroph for some vitamins and because the depletion of thiamine from the *in vitro* growth medium was necessary to obtain a reproducible growth-promoting effect of *P. fluorescens* BBc6R8 on *L. bicolor* S238N, we have hypothesized that bacterial thiamine could play a role in the fungal growth promotion. We have observed that thiamine concentrations of 1.6 and 16 μM induced an increase of the fungal growth that was similar to the one induced by the bacteria after three weeks of fungal-bacterial co-culture. Griffin (1981) reported that a concentration of thiamine ranging between 10^{-9} and 10^{-6} M was required for the growth of auxotrophic fungi, depending on the species. By contrast, high level of thiamine can reduce fungal growth (Garraway & Evans, 1984), probably because thiamine inhibits the transcription of TPP-dependent enzymes involved in the carbon metabolism: the carbon metabolism is impaired in the presence of high concentration of thiamine and thus, the growth is reduced (Hohmann & Meacock, 1998). This would explain the lower growth observed when adding 165 μM of thiamine. Genomic analysis indicated that *L. bicolor* S238N lacks the first steps of both pyrimidine and thiazole moieties biosynthesis pathways. But it possesses the enzymes that catalyze the condensation and the phosphorylation steps, and a putative thiamine transporter. Therefore the fungus should be able to use either subunits of TPP or either thiamine substrates to synthesize functional TPP. Interestingly, *P. fluorescens* BBc6R8 secreted thiamine in the range of concentration that we proved to induce a fungal growth increase. All together, these data indicate that the thiamine supplied by the helper bacteria could be one of the diffusible molecules responsible of the fungal growth promoting effect. However other metabolites should be also involved as the bacteria exerted an effect 15 days earlier than the vitamin alone. Moreover, thiamine secretion is not a specificity of the strain *P. fluorescens* BBc6R8 as we showed and as previously shown (Derylo & Skorupska 1993, Sierra *et al.* 1999). Nevertheless, thiamine could act in combination with other metabolites. To demonstrate the role of *P. fluorescens* BBc6R8 thiamine in the fungal growth-promoting effect, we have initiated the construction of a bacterial mutant that would be impaired in thiamine synthesis. We have not succeeded at the moment.

Interestingly, thiamine plays a fundamental role in other fungal-bacterial interactions: Rikhvanov *et al.* (1999) demonstrated that the thermophilic bacterium *Bacillus* sp. TB-1 excreted thiamine that not only promoted the growth of its niche-associated yeast

Debaryomyces vanriji but also increased the maximal temperature of the yeast growth. Conversely, the vitamin B1 is suspected to be involved in the growth stimulation of cellulolytic bacteria by *S. cerevsiae* in lamb rumen (Chaucheyras-Durand & Fonty, 2001). In another study, Dubuis *et al.* (2006) showed that thiamine was an important factor in the biological control of *P. fluorescens* CHA0 against root-pathogenic fungi. Thiamine may play a more complex role in fungi-bacterial interactions than thought up to now.

Publication n°4. Trehalose mediates the interactions between the ectomycorrhizal fungus *Laccaria bicolor* S238N and the mycorrhiza helper bacteria *Pseudomonas fluorescens* BBc6R8

II.3 Publication n°4. Trehalose mediates the interactions between the ectomycorrhizal fungus *Laccaria bicolor* and the mycorrhiza helper *Pseudomonas fluorescens* BBc6R8

Nota: most of the data reported in this article have been obtained by P. Frey-Klett, C. Brulé and M. Tanaka. My contribution to this work is restricted to transcriptomic analyses and the redaction of the manuscript.

Ecological significance of trehalose in the interaction between an ectomycorrhizal fungus and a mycorrhiza helper bacterium.

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Manuscript in preparation.

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Abstract

Because mycorrhiza helper bacteria improve mycorrhiza formation, their effect on the behaviour of mycorrhizal fungi has been extensively studied. By contrast, the respective effect of the mycorhizal fungi on the helper bacteria has been poorly documented. We demonstrated that the ectomycorrhizal fungus *Laccaria bicolor* S238N enhanced the survival of the helper strain *Pseudomonas fluorescens* BBc6R8 in the soil. Bacteria were attracted by fungal extracts as well as by trehalose, a disaccharide highly accumulated in the fungal mycelium. We hypothesize that the hyphosphere constitutes a niche in which the population of helper bacteria establishes and and then exert its helper effect.

Keywords. Trehalose, *L. bicolor*, mycorrhiza helper bacteria, ectomycorrhizal fungus, population dynamics

Introduction

Mycorrhiza Helper Bacteria (MHB) refers to a group of bacteria that improves the symbiotic association between mycorrhizal fungi and plant roots. Different mechanisms of the mycorrhiza helper effect have been reported (for a review, see Frey-Klett *et al.* 2007). For instance, mycorrhiza helper bacteria have been proved to influence the growth and survival of the ectomycorrhizal fungus during its pre-symbiotic live (Brulé *et al.* 2001, Founoune *et al.* 2002, Schrey *et al.* 2005), to induce its spore germination (Xavier & Germida 2003, Hildebrandt *et al.* 2006), to improve soil conduciveness (Vivas *et al.* 2005) or to reduce pathogens activity (Lehr *et al.* 2007). By contrast, little is known about the effects of the ectomycorrhizal fungi on the ecology and the behavior of the helper bacteria. Yet it was demonstrated that the ectomycorrhizal symbiosis affects both the genotypic and the functional diversity of the fungal-associated bacterial communities in soils (Frey-Klett *et al.* 2005, Offre *et al.* 2007, Uroz *et al.*, 2007). Because *Pseudomonas fluorescens* isolates from the Douglas-fir *L. bicolor* mycorrhizosphere preferentially use trehalose, the most abundant carbohydrate accumulated in the mycelium of *L. bicolor*, Frey *et al.* (1997) hypothesized that this fungus exerts a trehalose-mediated selection on the bacterial communities present in the vicinity of the ectomycorrhizas. In this context, the aim of this study was to monitor the effect of the ectomycorrhizal fungus *L. bicolor* S238N on the population of its associated-helper bacterial strain *Pseudomonas fluorescens* BBc6R8 and to elucidate the role of trehalose in the interaction between the ectomycorrhizal fungus and the bacteria. The helper strain BBc6R8 was isolated from a sporocarp of *L. laccata* S238N (renamed now as *L. bicolor*). It was proved to reproducibly improve the *L. bicolor*-Douglas-fir mycorrhiza formation both in glasshouse and nursery experiments (Duponnois & Garbaye, 1991). The highest promoting effect was obtained with the lowest dose of the bacterial inoculum (10^6 cells per m^2) even if the bacterial population rapidly decreased after inoculation (Frey-Klett *et al.* 1999). The bacteria are not preferentially located either in the vicinity of the ectmycorrhizae or in the sporocarp tissues (Frey-Klett *et al.* 1997) but are able to stick to *L. bicolor* hyphae (Sen *et al.* 1996). In this study, we analysed the effect of *L. bicolor* S238N on the growth and survival of *P. fluorescens* BBc6R8 in the soil. We also assessed the chemotactic effect of the mycelium and of fungal metabolites, such as trehalose, on the MHB strain. Finally, we monitored gene regulations of the fungal and bacterial trehalose metabolism during the *L. bicolor* BBc6R8-*P. fluorescens* BBc6R8 interaction *in vitro.*

Results and discussion

***L. bicolor* S238N increase the survival of *P. fluorescens* BBc6R8 in soil.**

The density of the *P. fluorescens* BBc6R8 population was monitored by dilution plating on a selective KB medium containing 100 mg.L^{-1} of rifampin (Frey-Klett *et al.*, 1997), in a nursery soil (three independent experiments) or in a peat-vermiculite mixture (three independent experiments), in the presence or in the absence of dead or alive mycelium of *L. bicolor* S238N. The mycelium was killed by irradiation with 20 Kgray of Cobalt 60. We revealed a significant and reproducible higher survival of the bacterial population in the presence of the ectomycorrhizal fungus since the first day after the bacterial inoculation (Figure 1). Therefore these data demonstrate that the ectomycorrhizal fungus improved the survival of the helper bacterial strain BBc6R8 in the soil. Such a beneficial effect of a mycorrhizal fungus on a MHB *P. fluorescens* strain was previously reported by Gamalero *et al.* (2004), who observed that the arbuscular mycorrhizal fungus *Glomus mosseae* improved the long-term survival of the strain *P. fluorescens* 92rk in the rhizosphere of tomato plants. Rhizosphere bacterial survival highly depends on the quality and the quantity of nutrient available. Bacteria preferentially colonize and rapidly grow on carbon resources like root exudates (Lugtenberg *et al.* 2001). Interestingly, *L. bicolor* S238N accumulates high quantities of trehalose in its hyphae. Trehalose is a disaccharide found in wide variety of organisms including bacteria, fungi, insects and plants, and it is highly accumulated in the mycelium of some fungi where it can reach 15 % of the dry weight (Elbein *et al.* 2003). Not all the soil bacteria are not able to use it as a carbon substrate. For instance, among the *P. fluorescens* species, only the strains belonging to Biovar I, to which the strain BBc6R8 also belongs, are able to consume it (Frey *et al.* 1997). Trehalose-accumulating hyphae would therefore constitute a niche for trehalose-degrading bacteria.

Trehalose mediates BBc6R8 growth and chemotaxis.

When growing the strain BBc6R8 on a minimal M9 medium containing different concentration of trehalose, the cell density was significantly enhanced by increasing trehalose concentration after 25 hours of growth (figure 2). The consumption of trehalose by the bacteria was confirmed by NMR measurements of trehalose concentration in the culture medium. No trehalose was detected in the culture medium after 30 hours of bacterial growth (initial concentration: 2.5mM, data not shown). We compared the chemotaxis of the strain BBc6R8 toward different carbon compounds known to be accumulated in ectomycorrhizal fungi, using the chemotaxis assay described by de Weert *et al.* (2003). Trehalose, and in a

lesser extent glutamate, were the only tested molecules that attracted the bacterial cells (Table 1). The chemotaxis effect of trehalose was reproducibly observed for concentrations ranging from 1 to 100 mM. In the case of glutamate, only one concentration (10 mM) induced the chemotaxis of strain BBc6R8. In contrast, glucose, arginine, γn- aminobutyric acid, citric acid, alanine, L-glutamine and mannitol had no effect on bacterial chemotaxis (data not shown). Interestingly, the chemotaxis of BBc6R8 was induced by crushed mycelium of *L. bicolor* with or without cell walls and in a lesser extent by the filtrated (0.2 μm) supernatant of the fungal culture medium (Table 1). The non-inoculated and the culture medium had no chemotactic effect.

Chemotaxis towards root exudates is a frequent mechanism involved in plant root colonization by plant growth promoting rhizobacteria (PGPR, Bais *et al.* 2006). It also plays an important role in the symbiotic interactions between rhizobia and their host plants (Currier & Strobel 1977). The impact of root exudates on bacterial chemotaxis has been frequently reported so far (Lugtenberg *et al.* 2001, Bais 2006). In contrast, chemotaxis towards fungi is much less documented. Yet bacteria are able to colonize all the fungal tissues: hyphae (Grewal & Rainey 1991, Artursson &Jansson 2003, Seneviratne & Jayasinghearachchi 2003, Toljander *et al.*, 2006), fruiting bodies (Garbaye & Bowen, 1989, Danell *et al.* 1993, Sbrana *et al.* 2000, Russo *et al.* 2003), spores (Roesti *et al.* 2005), mycorrhizae (Frey *et al.* 1997) and sclerotia (Arora *et al.* 1983). Here is the first report showing that ectomycorrhizal fungal extracts and metabolites are responsible for the chemotaxis of a MHB strain.

BBc6R8 concentrates around the fungal hyphae.

To check if besides chemotaxis cell-cell interaction between *P. fluorescens* BBc6R8 and *L. bicolor* S238N *in vitro* exists, we observed by microscopy the position of BBc6R8 cells co-cultured with *L. bicolor* S238N. For this, the bacterial strain was transformed with a mini-tn7 transposon to constitutively express green fluorescent protein without affecting the phenotype of the bacteria (Lambertsen *et al.* 2004). Bacteria were co-inoculated at a density of 10^8 cfu.mL^{-1} with a fungal plug on a glass side covered with a fine layer (<1 mm) of 0.3 % Sea kem GTG agarose (figure 3A). Mycelium was separated from the bacteria by 0.5 mm. After an incubation of 21 days at 10°C in the obscurity, we observed by microscopy that the BBc6R8 cells concentrated on the surface of the hyphae (figure 3B). It is noteworthy that previous observations from Sen *et al.* (1996) had revealed a specific attachment of BBc6, the parental strain which is not resistant to rifampin, to *L. bicolor* S238N hyphae. Colonization and adhesion to hyphae by bacteria has already been described in pathogenic interactions

(Sbrana *et al.* 2000, Singh & Arora 2001, Russo *et al.* 2003, de Weert *et al.* 2004) but also in the case of commensal interactions (Masaphy *et al*. 1987, Singh & Arora 2001). This colonization is generally correlated with a chemotactism of bacteria toward fungal exudates on which they feed. The hyphae then offer a niche for these soil bacteria.

Are the gene expressions of *L. bicolor* S238N enzymes involved in trehalose metabolism and of bacterial trehalase altered during the fungal-bacterial interaction ?

In the case of the antagonist relation between *Pythium debaryanum* and *P. fluorescens* ATCC 17400, Gaballa *et al.* (1997) demonstrated that a factor released by the fungus induced bacterial trehalase gene expression which was responsible of the antagonist activity. If trehalose-accumulating hyphae can constitute a niche for trehalose-degrading bacteria, we addressed the question of the impact of a possible impact of *L. bicolor* S238N on the expression of the bacterial trehalase gene. We used the *in vitro* confrontation assay described in Deveau *et al.* (2007) and collected the BBc6R8 cells and the *L. bicolor* S238N hyphae after 14 days of co-culture. The regulation of the bacterial gene expression was monitored by semi-quantitative RT-PCR (bacterial trehalase TreA, Sarniguet *et al.*). No modification of the concentration of the bacterial transcript was detected when the bacteria was grown in the presence of the fungus.

Conversely, microorganisms can regulate the transcription of targets of other microorganisms for their own benefit (Lutz *et al.* 2004, van Rij *et al.* 2005). Consequently, we looked for a possible impact of the trehalose-degrading bacteria on the regulation of the fungal trehalose metabolism. We monitored the expression of the fungal trehalose synthase TPS1, trehalose phosphatase TPS2, trehalose synthase regulatory subunit TSL1, trehalose phosphorylase, neutral and acid trehalase by quantitative PCR and with whole genome arrays. We used the same *in vitro* confrontation assay. No effect on the transcripts concentration of the fungus was detected in the presence of the bacteria.

Therefore, in our experimental assay, the two interacting microorganisms did not manipulate the trehalose metabolism of its partner, at the gene level. However, the analysis was performed after 14 days of co-culture. An effect may exist at another time of the interaction.

***L. bicolor* S238N does not actively exudate trehalose.**

To determine if *L. bicolor* S238N exudes trehalose, we used an overlay assay developed with a biosensor *P. fluorescens* strain that specifically detects trehalose up to a concentration of 1 mM (Sarniguet *et al.*). A positive signal was obtained when the fungal colony was grinded

before the addition of the overlay containing the biosensor bacterial strain (figure 4). This confirmed the presence of trehalose inside the mycelium of *L. bicolor* S238N, when grown on a modified CAA medium (Sarniguet *et al*) at 20°C during 15 days. In contrast, no signal was detected in the presence of either intact mycelium or five minutes water washings of *L. bicolor* colonies (figure 4). Two hypotheses can explain these results: either no trehalose is exuded by the fungus when grown on a solid medium or trehalose is exuded, but it is immediately degraded by a fungal extracellular trehalase. We recently demonstrated that *L. bicolor* possesses a transcribed gene encoding a protein having all the characteristics of such a trehalase (Deveau, unpublished data). If this second hypothesis is true, that would means that trehalose-degrading bacteria that live in the vicinity of the fungus in the soil, would have to compete with the fungal extracellular trehalase for their nutrition. Even though, exuded or not by the fungal hyphae, trehalose should be available for bacteria in soils via dead or broken hyphae (Gaballa *et al.* 1997, Corsetti *et al*. 2001). In the case of mycorrhizal fungi, hyphal turnover was estimated to be extremely rapid, between five and six days (Staddon *et al*. 2003) leading to the generation of significant quantity of dead hyphae. But trehalose is probably not the only molecule responsible for bacterial chemotaxis.

Our results highlight that the interaction between the ectomycorrhizal fungus *L. bicolor* S238N is not only beneficial to the fungus but also to the bacteria as its survival in soil is enhanced by the fungus. Bacteria were attracted by fungal extracts as well as by trehalose that is highly accumulated in the fungi. We suggest that the hyphosphere would constitute a niche in which the population of mycorrhiza helper bacteria survives and can exert a priming helper effect on the fungus (Brulé e*t al.* 2001, Deveau *et al.* 2007). Fungal trehalose would act as a chemotactic signal that attracts *P. fluorescens* BBc6R8 cells in the vicinity of the ectomycorrhizal fungus and potentially promote also the survival of the bacteria in this niche. Interestingly, Duponnois and Kisa (2006) recently demonstrated that trehalose mediates the growth-promoting effect of a MHB strain of *Pseudomonas monteilii* on the ectomycorrhizal fungus *Pisolithus albus* in a two compartment plate assay: *P. monteilii* significantly increased the fungal growth when cultivated on a minimal medium amended with trehalose, while none of the other seven organic compounds tested produced the same effect. Together with the data of Duponnois and Kisa (2006), our results highlight the ecological relevance of trehalose in the interactions between ectomycorrhizal fungi and mycorrhiza helper bacteria.

Acknowledgment.

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Figures

Figure 1. Population dynamics of *P. fluorescens* BBc6R8 per gram of dry matter in the presence (open circles) or in the absence of *L. bicolor* S238N mycelium in a nursery experiment. Each point represents the value of five replications. The two curves are significantly different on the basis of a two-factor (microbial treatment-time) ANOVA ($P<0.05$).

Figure 2. Kinetics of growth of *P. fluorescens* BBc8R8 on M9 minimal medium (6 $g.L^{-1}Na_2HPO_4$, 3 $g.L^{-1}$ KH_2PO_4, 1 $g.L^{-1}$ NH_4Cl, 0.25 $g.L^{-1}$ $MgSO_4$-$7H_2O$, 0.0147 $g.L^{-1}$ $CaCl_2$-H_2O, pH 7.8) containing different concentrations of trehalose. Each point represents the value of three replicates. At each sampling time each curve was significant different from others on the basis of a one-factor (trehalose concentration) ANOVA ($p<0.05$).

Figure 3. A. Scheme of the experimental set-up used to investigate attraction of *P. fluorescens* BBc6R8 toward *L. bicolor.* **B**. Visualization of the *in vitro* interaction between *gfp*-tagged *P. fluorescens* BBc6R8 and *L. bicolor*.). Bacteria were co-inoculated at a density of 10^8 $cfu.mL^{-1}$ with a 0.5 mm diameter mycelium plug on a glass side covered with a fine layer (<1 mm) of 0.3 % Sea kem GTG agarose. Photography was taken after 21 days of incubation at 10°C in the dark. Five days before microscopic observation, the initial plug of mycelium was cat and a cover glass was added.

Figure 4. Overlay assay of *L. bicolor* using 1 % HPMC. **A.** Pyt 5 strain grown on 10mM trehalose (positive control). **B.** Pyt 5 strain grown without trehalose (negative control). **C.** Pyt 5 strain grown on grinded mycelium. **D.** Pyt 5 grown on intact mycelium.

Table

Table 1. Chemotaxis assay of BBc6R8 strain towards culture fractions of *L. bicolor* S238N and different concentrations of trehalose. The signs + or – indicate the presence or the absence of chemotaxis towards the tested molecules, respectively. *L. bicolor* S238N was grown in liquid and on solid modified Paschlewski medium (2 $g.L^{-1}$ glucose, Paschlewski & Paschlewska, 1974). In each case, mycelium was washed in $MgSO_4$ 0.1M buffer two times and then centrifuge. Either pellet containing mycelium or supernantant were used for chemotaxis assay. The same results were obtained using tissues from liquid or solid culture. The chemotaxis assay was performed on agar medium as described by de Weert *et al.* (2003).

Substrates	Chemotaxis
Culture supernatant of *L. bicolor*	-/+
Centrifuged grinded mycelium	+++
Supernatant of centrifuged grinded mycelium	+++
Culture medium (P5)	-
Buffer ($MgSO_4$ 0.1M)	-
24 hours old BBc6R8 suspension	-
Trehalose 1mM	+
Trehalose 5 mM	+
Trehalose 10 mM	+
Trehalose 25 mM	+
Trehalose 50 mM	+
Trehalose 100 mM	+
Glutamate 10 mM	+

Figure 1 Deveau *et al.*

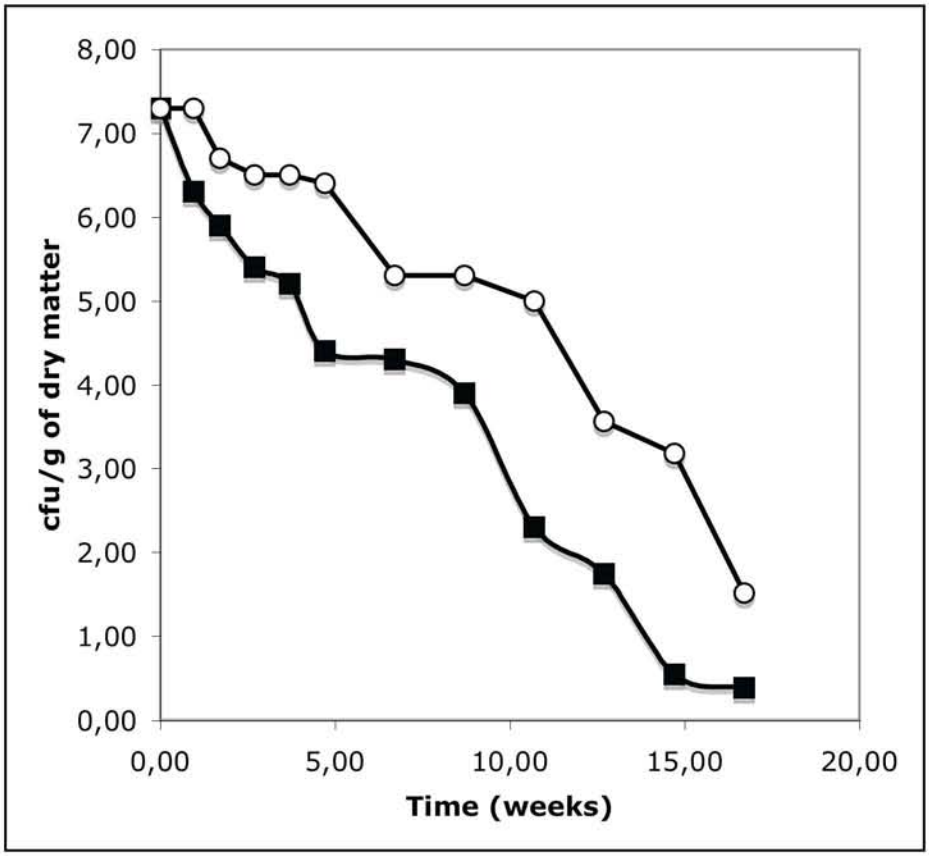


Figure 2 Deveau *et al.*

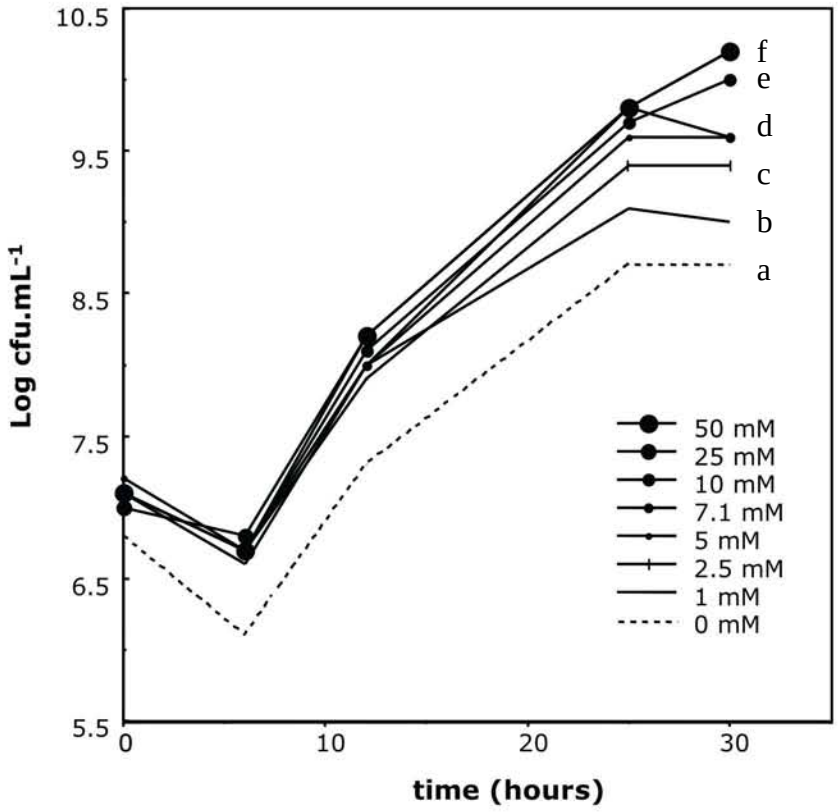
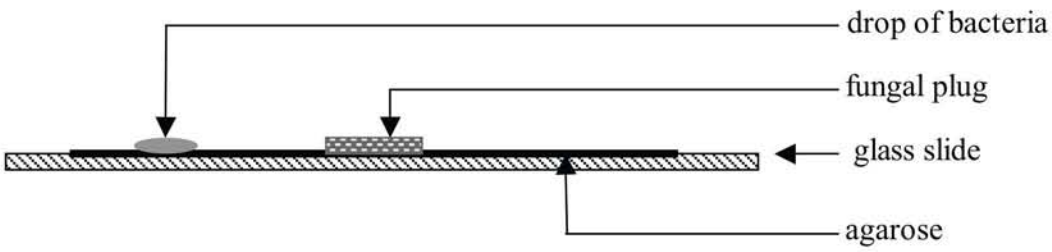


Figure 3 Deveau *et al.*

A.



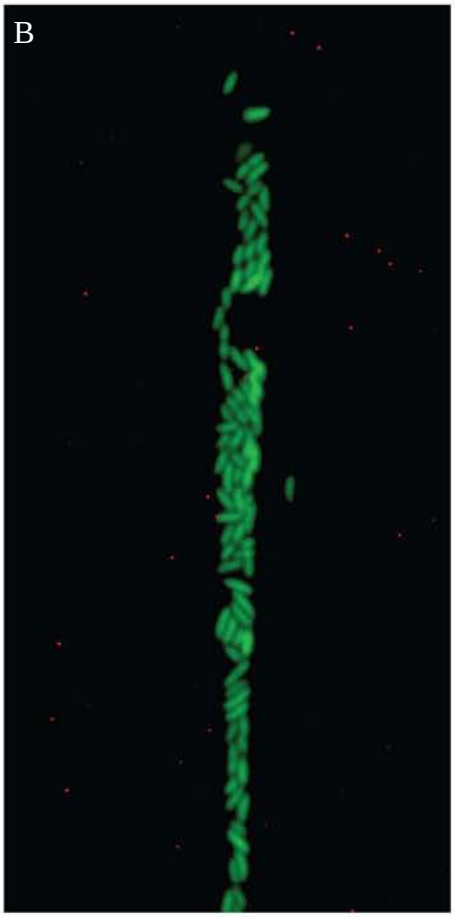
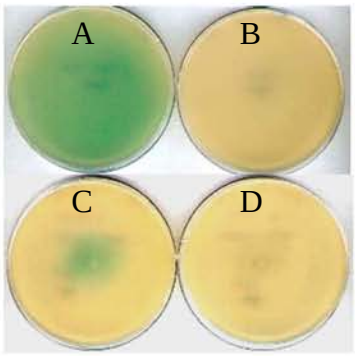


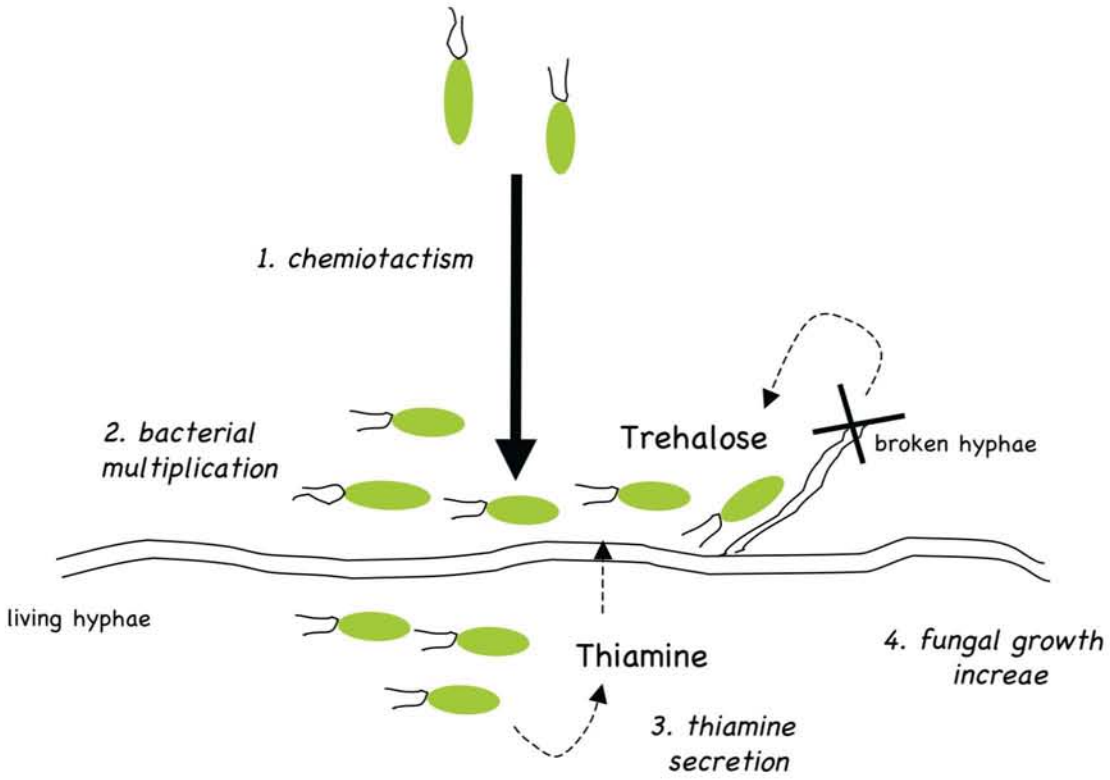
Figure 4 Deveau *et al.*



Conclusion

L'ensemble de nos résultats tend à montrer que l'interaction entre *L. bicolor* S238N et *P. fluorescens* BBc6R8 s'établit par l'intermédiaire la production et la perception d'un cocktail de métabolites solubles et volatiles produits par les deux partenaires. Nous proposons le modèle suivant (figure 2.6) : le tréhalose fongique attirerait la bactérie auxiliaire vers les hyphes (1) qui constitueraient une niche pour *P. fluorescens* BBc6R8. Des métabolites fongiques inconnus, dont peut-être le tréhalose, serviraient de substrat à la bactérie et amélioreraient sa survie (2). Parallèlement, la sécrétion de thiamine par la bactérie (3) concourrait à une augmentation de la survie et de la croissance pré-symbiotique du mycélium dans le sol (4). D'autres molécules jouent probablement un rôle important dans l'interaction. L'auxine et l'éthylène pourraient en faire partie (cf. chapitre 1). Des analyses complémentaires seront nécessaires, à la fois pour évaluer le rôle de ces deux molécules et pour identifier les autres composés sécrétés par les deux partenaires.

Figure 2.6. Schéma des échanges métaboliques entre *P. fluorescens* BBc6R8 et *L. bicolor* S238N potentiellement impliqués dans l'effet auxiliaire de la bactérie.



Chapter III

Perception and response of the ectomycorrhizal fungus *L. bicolor* S238N to soil bacteria : is there any specificity of the mechanisms ?

Chapitre III

Perception et réponse du champignon ectomycorhizien *L. bicolor* S238N aux bactéries : quel est le degré de spécificité des mécanismes moléculaires ?

Introduction

Les analyses précédemment décrites ont clairement démontré que *L. bicolor* S238N est capable de percevoir et de réagir à des molécules produites par la souche bactérienne auxiliaire de la mycorhization *P. fluorescens* BBc6R8. Ces études ont permis d'identifier un certain nombre de marqueurs moléculaires (gènes) et macromoléculaires (traits morphologiques) caractéristiques de la réponse de *L. bicolor* S238N à *P. fluorescens* BBc6R8 *in vitro*. Cependant, nous pouvons nous interroger quant à la spécificité de ces différents marqueurs. En effet, outre les arthropodes, nématodes et autres eucaryotes auxquels le champignon ectomycorhizien est confronté, le mycélium interagit dans les sols avec des centaines d'espèces bactériennes antagonistes, neutres, commensales ou mutualistes. Chacune modifie son environnement et sécrète des molécules signal de sorte que de multiples signaux sont produits à proximité du mycélium. Face à ces indicateurs, la réponse du champignon se traduit rarement par des bouleversements majeurs mais plus généralement par une combinaison de fins ajustements moléculaires. Différents indicateurs induisent donc probablement les mêmes marqueurs moléculaires. Ce phénomène a été clairement démontré chez les légumineuses où les facteurs Nod émis par les bactéries du genre *Rhizobium* induisent la même voie de signalisation que les champignons endomycorhiziens (Parniske 2007). Dans ce contexte, nous pouvons nous interroger sur la capacité des champignons ectomycorhiziens à percevoir leur environnement bactérien et à y répondre de manière discriminante. Différents niveaux de discrimination sont envisageables et la réponse transcriptomique de *L. bicolor* S238N à *P. fluorescens* BBc6R8 pourrait en réalité correspondre à la réponse du champignon face à :

i. des bactéries
ii. des bactéries Gram négatives
iii. des bactéries ayant un effet positif sur la croissance de *L. bicolor* S238N *in vitro*
iv. des bactéries auxiliaires
v. *P. fluorescens* BBc6R8

Dans le chapitre 1, nous avons montré que six gènes marqueurs de *L. bicolor* S238N (i.e régulés en présence de *P. fluorescens* BBc6R8) présentait des profils d'expression complexes lors d'interactions avec cinq bactéries rhizosphériques : chaque gène étudié

était régulé en réponse à la présence de plusieurs souches bactériennes sans que des corrélations entre les profils d'expression et les propriétés des bactéries (auxiliaire, biocontrôle, antagoniste) puissent être établies. Le nombre de gènes analysés dans cette étude était trop petit pour pouvoir identifier un ou des gènes spécifiquement régulés en réponse à la souche auxiliaire *P. fluorescens* BBc6R8 ou plus généralement en réponse à des bactéries auxiliaires.

Par ailleurs, les capacités de perception des signaux émis par les bactéries du sol par les champignons ectomycorhiziens et les réactions induites par ces éliciteurs chez ces champignons sont inconnus à l'heure actuelle.

Afin de répondre à ces deux questions, nous avons étudié la réponse transcriptomique du champignon *L. bicolor* S238N à la présence de trois bactéries rhizosphériques. L'une est fongivore et inhibe la croissance du champignon *in vitro* (*Collimonas fungivorans* Ter331), la seconde quant à elle accélère sa croissance *in vitro* sans pour autant être auxiliaire de la mycorhization (*P. fluorescens* Pf29A) et la dernière, bien qu'auxiliaire la mycorhization en serre (Aspray *et al.* 2006), réduit le développement du mycélium *in vitro*. Les études ont été réalisées avant contact entre le mycélium et les colonies bactériennes, comme précédemment décrit (Chapitre 1, Deveau *et al.* 2007). Les résultats ont été comparés à ceux obtenus lors de l'interaction avec *P. fluorescens* BBc6R8. Parallèlement nous avons analysé la réponse transcriptomique du champignon à deux molécules de quorum sensing. Ces petites molécules signal sont fréquemment produites par les bactéries Gram négatives qui les utilisent pour coordonner l'activité des cellules en fonction de leur densité. Or la mycorrhizosphère de *L. bicolor* abrite de nombreuses espèces de bactéries Gram négatives (Frey *et al.*) susceptibles de produire ces molécules de QS.

Publication n°5. A spy among soil bacteria : the ectomycorrhizal fungus *Laccaria bicolor* S238N perceives and reacts to quorum sensing signal molecules

III.1 Publication n°5. A spy among soil bacteria : the ectomycorrhizal fungus *Laccaria bicolor* S238N perceives and reacts to quorum sensing signal molecules.

A spy among rhizobacteria: the ectomycorrhizal fungus *Laccaria bicolor* S238N perceives and reacts to quorum sensing signal molecules.

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Abstract

Bacteria use small diffusible molecules to communicate and to coordinate population response by a cell density-depend mechanism named quorum sensing (QS). It was recently reported that QS signal molecules are also sensed by eukaryotic organisms thus acting as inter-kingdom signalling molecules. Although ectomycorrhizal fungi interact physically and metabolically with soil and rhizosphere bacteria, nothing is known about their possible response to QS molecules. Here, we detected the presence of QS-producing bacterial isolates in the mycorrhizosphere of *Laccaria bicolor* S238N. We demonstrated that the transcriptome of this symbiotic fungus differentially reacted to the presence of N-acyl homoserine lactones, C6-HSL and 3,O-C12-HSL. While C6-HSL induced no transcriptomic response, the concentration of 230 transcripts was altered in the presence of 3,O-C12-HSL. QS-responsive genes were mainly involved in primary metabolism, polarized growth process and oxidative stress. We suggest that *L. bicolor* uses long-chain QS molecules as a clue for the presence of antagonist or competitive soil bacteria and thus tunes its gene expression to prepare its metabolism for a forthcoming nutritional stress resulting from trophic competition.

Key words:

Quorum sensing, N-acyl homoserine lactone, ectomycorrhizal fungi, transcriptomic, fungal-bacterial interactions.

Introduction

In the rhizosphere, the high concentration of nutrient in root exudates attracts myriads of interacting soil microorganisms. Among those, ectomycorrhizal fungi (ECM) play a fundamental role in the hydro-mineral nutrition of trees by establishing a symbiotic association with tree roots. They provide water and minerals to their host plants which conversely feeds the fungus with photoassimilates (Smith & Read, 1997). The development and functioning of this symbiosis has been extensively studied using *in vitro* experiments (Martin *et al.* 2007), but the interactions of ECM with the complex bacterial communities that inhabit the rhizosphere and form biofilms on the hyphal surface (Nurmiaho-Lassila *et al.* 1997) have been neglected. Yet, several bacterial species, such as pseudomonads, can influence the formation and the functioning of the symbiosis (Frey-Klett & Garbaye, 2007). The so-called mycorrhiza helper bacteria (MHB), which promote mycorrhiza formation, may act through multiple mechanisms (Garbaye, 1994; Frey-Klett & Garbaye, 2007) such as the stimulation of spore germination, and the promotion of the presymbiotic hyphal growth (Duponnois & Plenchette 2003, Brulé *et al.* 2001, Schrey *et al.*, Deveau *et al.* 2007). Ectomycorrhizal fungi also perceive and react to other rhizobacteria (Deveau *et al.* 2007). Up to now, the signals triggering these fungal responses remain poorly documented. Auxofuran, a flavonoid-related compound produced by the MHB *Streptomyces* AcH505, is the only bacterial metabolite known to mediate a MHB effect (Riedlinger *et al.* 2006).

Many bacteria use small diffusible molecules to communicate and coordinate population response by a cell density-dependent mechanism named quorum sensing (QS). These molecules accumulate in the environment when bacterial cell densities increase such as in biofilms. When QS signal molecules reach a crucial threshold concentration, the bacteria detect and respond to them by synchronously altering gene expression. QS signal molecules include PQS (*Pseudomonas* quinolone signal, Diggle *et al.* 2006), furanosyl borate (AI-2,

Schauder *et al.* 2002), hydroxyl-palmitic methyl ester (Flavier *et al.* 1997) and N-acyl homoserine lactones produced by most gram negative bacteria (N-AHSL) (Fuqua *et al.*, 2001). These latter molecules are all composed of a homoserine lactone ring but differ with respect to the length and the C3 substitution of the N-linked acyl chain. N-AHSL are involved in bacterial pathogenicity and symbiosis with plants and animals (Swift *et al.*, 2001; Whitehead *et al.*, 2001; Von Bodman *et al.*, 2003). Interestingly, N-AHSL molecules are not only sensed by bacteria but also by some eukaryotic organisms (Shiner *et al.* 2005), among which the human yeast pathogen *Candida albicans*. The 3,O-C12-HSL produced by the opportunistic human bacterial pathogen, *Pseudomonas aeruginosa*, inhibits the *C. albicans* filamentation involved in the virulence of the fungus (Hogan *et al.* 2004). But it remains to understand how the information is interpreted on either side of the eukaryote/prokaryote divide.

Nothing is known so far about the suspected presence of bacterial QS signal molecules in the mycorrhizosphere and about the perception of such molecules by ectomycorrhizal fungi. Here we investigated the presence of QS-producing bacterial strains in the mycorrhizosphere of the ectomycorrhizal basidiomycete *L. bicolor* S238N. Then, we addressed the question of the effect of QS signal molecules on this eukaryotic organism. We tested the impact of a short (N-hexanoyl-L-homoserine lactone; C6-HSL) and a long (N-(3-oxo-dodecanoyl)-L-homoserine lactone; 3,O-C12-HSL) chain QS signal molecules on the transcriptome of *L. bicolor*.

RESULTS & DISCUSSION

Presence of bacteria producing N-AHSLs in the mycorrhizosphere of *L. bicolor* S238N.

The presence of bacteria producing N-AHSLs in the mycorrhizosphere of *L. bicolor* was first detected by plating six freshly excised Douglas fir -*L. bicolor* ectomycorrhizal tips on a LB

agar plate containing the biosensor strain *C. violaceum* CV026. The presence of purple spots demonstrated that short chain N-AHSL were released by ectomycorrhiza-associated bacteria. Based on these observations, a collection of 60 *P. fluorescens* isolates from bulk soil, mycorrhizae and mycorrhizosphere of Douglas fir-*L. bicolor* was then screened for N-AHSL production. Three *P. fluorescens* isolates from the bulk soil and nine from the mycorrhizosphere induced a positive signal with the biosensor *A. tumefaciens* NTL4 detecting long chain N-AHSLs. Therefore bacteria producing short- and long-chain N-AHSL co-inhabit with *L. bicolor* mycelium in mycorrhizae and mycorrhizosphere.

Response of *L. bicolor* S238N to the presence of N-AHSL signal molecules

Transcriptomic (cDNA arrays) and biochemical (GC-MS) analyses were performed to assess the response of free-living *L. bicolor* S238N mycelium to the presence of the two N-AHSL signal molecules, C6-HSL and 3,O-C12-HSL. Twenty-four and 179 unique C6-HSL- and 3,O-C12-HSL-responsive genes were identified by sequencing the 31 and 238 transcripts having an altered concentration in the presence of C6-HSL and 3,O-C12-HSL, respectively (Table 1, supplemental table S1). The change in transcript level induced by 3,O-C12-HSL and measured by the cDNA arrays was confirmed by quantitative PCR for the six target transcripts tested (Figure 2). In contrast, no modification of the concentration of the five target transcripts was detected by qPCR when adding C6-HSL in the growth medium, suggesting that C6-HSL induce no significant changes at the transcriptional level in *L. bicolor*. Comparative analyses of the effects of different N-AHSL molecules on a same eukaryotic organism have already given contrasted results. While human cells and the pathogenic fungus *C. albicans* only responded to 3,O-C12-HSL and to some of its derivates (Chhabra *et al.* 2003, Hogan *et al.* 2004), the plant *M. trunculata* and murine cells metabolically reacted to both short- and long chain molecules (Mathesius *et al* 2003,

Rumbaugh *et al*. 2007). Together, these data suggest that 3,O-C12-HSL receptor proteins are shared by numerous eukaryotic cells, including basidiomycetes as demonstrated for the first time in the present work. On the other hand, the sensing of short chain-HSLs appears to be specific for a restricted set of eukaryotes.

Among the 3,O-C12-HSL responsive genes, 73 % were down-regulated, with alteration levels ranging between two- and ten-fold excepted for few genes which were highly repressed (Table1). Two third of transcripts coded for hypothetical proteins. One third encoded proteins involved in four main processes (Figure 3, Table1): stress response and detoxification process (gluthatione-S-transferase, heat shock proteins), transcription regulation (transcription initiation factors, mismatched base pair and cruciform DNA recognition protein), primary carbon metabolism (lipid metabolism, protein synthesis and degradation) and mitochondrial respiration. Interestingly, the plant signal strigolactone that stimulates endomycorrhizal cell proliferation and branching also enhance mitochondrial respiration (Besserer *et al.*, 2006). Up- and down-regulated belonged to each of these categories, suggesting a shift in the physiological status of the cell due to the presence of 3,O-C12-HSL (Table 1). A similar pattern of gene regulation was already observed after an extended contact between *L. bicolor* and the mycorrhiza helper bacterial strain *P. fluorescens* BBc6R8 (Deveau *et al.* 2007), and before contact with the antagonistic strain *Collimonas fungivorans* Ter331 (data not shown). This kind of gene regulation is commonly observed in response to glucose starvation and environmental stresses in fungi (Gasch *et al.* 2002, Xie *et al.* 2004, Wu *et al.* 2004). It is assumed that this response help fungal cells to save energy and carbon skeletons while adapting to a novel growth environment. The release of QS signaling molecules by bacteria is tightly correlated to the bacterial cell density, the latter leading to a sharp decrease of soil nutrients. Therefore, the ectomycorrhizal fungus *L. bicolor* might sense QS signal molecules

as indicators of the forthcoming changes in the trophic status of its immediate environment. By sensing QS molecules, the fungus would prepare its metabolism for trophic competition with the bacterial communities colonizing the hyphosphere. This hypothesis would also explain why both GC-MS and biosensor analyses of the QS signal stability demonstrated that *L. bicolor* produced no enzymes that degrade C6- and 3,O-C12-HSL nor interfere with QS signals (data not shown). Such a use of QS signal molecules by eukaryotic cells as biosensors of the environment was already shown for the seawed *Enteromorpha* that uses bacterial N-AHSLs as a clue to select appropriate attachment sites (Joint *et al.* 2002, Diggle *et al.* 2007).

An alternative explanation of the fungal reaction to bacterial QS molecules relies on the strong structural similarities between the acyl chain of N-AHSL molecules and some eukaryotic hormones. According to Hogan *et al.* (2004), 3,O- C12-HSL from *P. aeruginosa* has enough structural similarity with farnesol, a fungal cell-density signal molecule, to mimic farnesol action in *C. albicans.* According this hypothesis, the 3,O-C12-HSL could hijack a *L. bicolor* signalling pathway. But it remains to be determined whether farnesol-like molecules exist in *L. bicolor*. Until today, farnesol-related molecules have been essentially found in ascomycete fungi (Nickerson *et al.* 2006). However an 11-mer peptide was recently reported to function as a cell-density autoregulatory molecule in the yeast basidiomycete *Cryptococcus neoformans* that causes life-threatening human diseases (Lee *et al.* 2007).

In other respects, it is known that QS controls the production of antibiotics against fungi by biocontrol *Pseudomonas* strains (Girard *et al.* 2006). Here, the 12 QS-producing *P. fluorescens* strains that were isolated from the bulk soil, the mycorrhizosphere or the mycorrhizas of *L. bicolor* were proved to be antagonistic *in vitro* to three to seven fungal phytopathogenic strains (Frey-Klett et al, 2005). As a consequence, *L. bicolor* could also sense N-AHSLs to induce defense reactions against deleterious bacteria. This hypothesis is supported by the fact that among the 3,O-C12-HSL up-regulated *L. bicolor* genes, some

126 encode for gluthatione-S-transferase and manganese superoxide dismutase involved in
127 reactive oxygen species (ROS) detoxification activated during pathogen attack (Sheehan *et al.*
128 2001).
129
130 To conclude, we showed that a basidiomycete is able to sense and react differentially to
131 some bacterial QS signals. We propose that bacterial QS molecules would play a role as
132 indicators of the presence of antagonistic and/or competitive bacteria which co-inhibit with
133 the ectomycorrhizal fungus in the rhizosphere of forest trees. It remains to be determined how
134 specific of the ectomycorrhizal fungus *L. bicolor* is this response.
135
136 **Material and methods**
137 **Bacterial strains.**
138 *Pseudomonas fluorescens* strains were isolated from *Laccaria bicolor* ectomycorrhizal root
139 tips of nine-monthes old Douglas fir (*Pseudotsuga menziesii*), ectomycorrhizosphere and bulk
140 soil in a forest nursery (Frey-Klett *et al.*, 2005). The biosensor strains *Chromobacterium*
141 *violaceum* CV026 (McClean *et al.*, 1997) and *Agrobacterium tumefaciens* NTL4 (pZLR4)
142 (Luo *et al.*, 2003) respond to short- and long-chain N-AHSLs, respectively. Except for the
143 biosensors, that were cultivated at 28°C in liquid LBm (Vaudequin-Dransart *et al.*, 1995) and
144 ABm (AB mannitol 4 $g.L^{-1}$; Chilton *et al.*, 1974) media respectively, all the bacterial strains
145 were cultivated on 1/10-strength tryptic soy agar (TSA) medium (3 $g.L^{-1}$ Tryptic Soy Broth
146 from Difco and 15 $g.L^{-1}$ agar) and incubated at 25°C.
147

QS-producing communities associated to *L. bicolor* ectomycorrhizae.

Six *L. bicolor* – Douglas mycorrhizae from one year-old nursery plants were collected. Prints of each mycorrhizal root tips were made on LB medium plates containing the *C. violaceum* biosensor. Presence of purple spots was observed after an overnight incubation at 28°C.

***Laccaria bicolor* growth and QS treatment.**

The ectomycorrhizal basidiomycete *L. bicolor* S238N (Maire P. D. Orton) was maintained on Pachlewski agar medium (P5, Di Battista *et al*. 1996) at 25°C during three weeks. Nine Petri dishes of 90 cm-diameter were filled with 12 mL of liquid P5 medium and glass beads, which were covered with a cellophane membrane previously boiled in EDTA (1 $g.L^{-1}$) during 20 minutes, washed four times with deionized water (18 MΩ) and autoclaved (20 min, 120°C). In each plate, a 6 mm-diameter plug of *L. bicolor* S238N was cut out from the edge of a colony grown on solid P5 medium and transferred on the center of the cellophane membrane. Plates were sealed with plastic tape and incubated at 25°C in the dark during 13 days. The cellophane membranes carrying the fungal colonies were then washed in sterile water and transferred in new Petri dishes containing glass beads and 12 ml of fresh liquid P5 medium. Depending on the treatment, N-hexanoyl-L-homoserine lactone (C6-HSL, Sigma) or N-(3-oxo-dodecanoyl)-L-homoserine lactone (3,O-C12-HSL, gift from Prs. S.R. Chhabra and P. Williams, Nottingham, UK) or ethyl acetate (control treatment) were added to the medium at a final concentration of 100 µM. Three replicates were done per treatment. After a 14-hour incubation time, the mycelium from the whole colony was sampled and frozen in liquid nitrogen. Incubation media were kept at -20°C prior to GC-MS analysis.

Stability of N-AHSL.

As *L. bicolor* might degrade N-AHSL, the stability of these molecules was investigated by incubating an aliquot of 100 µl of 0.22 µm filtrated liquid culture of *L. bicolor* S238N with C6-HSL (50µM final concentration in ABm buffered medium, pH 6.5). After 2-days incubation time at 25°C, N-AHSL stability was tested as described in Uroz *et al*. (2003).

GC-MS analysis of *L. bicolor* growth medium.

Twenty microliters of 0.22 µm filtrated *L. bicolor* liquid culture was transferred to microvials, dried in a Reacti-Therm Heating Module (Pierce, Rockford, IL, USA) and derivatized according to Javelle *et al*. (2003). Gas chromatography and mass spectrometry (GC-MS) analyses to detect any changes in *L. bicolor* secreted molecules were performed as described previously (Javelle *et al.*, 2003). Non-derivatized samples were also analyzed.

RNA isolation and cDNA array hybridization.

Laccaria bicolor RNA were extracted using the RNeasy Plant mini kit (Qiagen, Hilden, Germany) as recommended by the manufacturer for fungal material. The quality of the RNA was checked on RNAse free 1 % agarose electrophoresis. cDNA probes were synthesized and hybridized to cDNA arrays containing 4992 inserts from *L. bicolor* S238N mycelium and sporocarps cDNA libraries as described previously (Peter *et al.* 2003, Deveau *et al.* 2007). Data analysis were performed as described by Duplessis *et al.* (2005).

DNA sequencing.

cDNA clones encoding for AHSL-responsive transcripts were amplified by PCR and sequenced as previously described (Kohler *et al.* 2003). Edited nucleotide sequences were compared to the assembly v.1.0 sequence of the *L. bicolor* S238N-H82 genome using BlastN

196 server at the JGI Laccaria Portal (http://genome.jgi-psf.org/Lacbi1/Lacbi1.home.html).
197 Conserved domains of the predicted protein sequences were searched in Pfam, Smart and
198 KOG databases (ftp://ftp.ncbi.nih.gov/pub/mmdb/cdd).
199
200 **Quantitative PCR analysis.**
201 To validate the cDNA array data, real-time quantitative PCR analyses were performed on
202 eight up- or down-regulated genes, selected for their potential biological relevance or their
203 high regulation level (for C6-HSL treatment: Arp2/3, hypothetical Lb3F4B5 protein,
204 hypothetical Lb3F27F8 protein, hemolytic lectin, and gluthatione-S-transferase; for 3,O-C12
205 HSL treatment: ABC transporter, Arp2/3, Concanamycine-induced protein C, hemolytic
206 lectin, gluthatione-S-transferase and tectonin 2). Four non-regulated genes (hypothetical
207 Lb17E10 protein, hypothetical Lb2F9F9 protein, serine/threonine repetitive matrix 1,
208 trehalose phosphorylase) were also used as controls and two of them (hypothetical Lb17E10
209 protein and serine/threonine repetitive matrix 1) were chosen for data normalization. The
210 primer pairs (Table S1, web supplemental data) were designed using Primer 3
211 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and Amplify 3.1
212 (http://engels.genetics.wisc.edu/amplify). The following criteria were used: product size
213 between 100 and 400 bp, melting temperature 60°C ± 1°C and a GC % > 50 %. The same
214 RNA samples were used for cDNA array analysis and for real time PCR measurements.
215 Reactions were run using MJ-opticon2 DNA real-time PCR system (Bio-Rad, Hercules,
216 USA). The following cycling parameters were applied: 95°C for 3 min and then 40 cycles of
217 95°C for 30 s, 60°C for 1 min and 72°C for 30 s. A negative control was run for each primer
218 pair. For data analysis, the geometric mean of the three biological replicates for each
219 condition was calculated. The primer efficiency were between 90% and 110 %. Fold
220 differences were calculated by using the ΔΔCt method (Livak & Schmittgen 2001).

N-acyl homoserine extraction and identification.

For N-AHSL extraction, bacterial isolates were cultivated 24h in LBm medium buffered at pH 6.5 to prevent lactonolysis of N-AHSLs (Yates *et al*., 2002). Extraction was carried out essentially as described by Elasri *et al*. (2001). N-AHSLs were separated and visualized on TLC plates (C18-reverse phase) as described previously (Shaw *et al*. 1997). Saturated short chain N-AHSLs were detected on the TLC using the *C. violaceum* biosensor CV026 (McClean *et al*. 1997). Oxo- and hydroxy-derivatives, and long chain N-AHSLs were detected using the *A. tumefaciens* biosensor NTL4(pZLR4) (Luo *et al*. 2003). Gentamycin was used at 20 $\mu g.mL^{-1}$ final concentrations. X-Gal was included in the media at 40 $\mu g.mL^{-1}$. The presence of compounds stimulating or inhibiting the biosensor *C. violaceum* CV026 in the supernatant from *L. bicolor* S238N growth medium was checked after extraction with ethyl acetate by spotting organic and aqueous phases directly onto TLC (Shaw *et al* 1997).

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Legend of the figures:

Figure 1

Scheme of the experimental set-up used to investigate the effects of N-AHSL on *L. bicolor* mycelium. The fungus is grown on a cellophane membrane which lays on glass beads, immerged in P5 liquid medium containing N-AHL molecules.

Figure 2

Comparison of the expression ratio [3,O-C12-HSL-*L. bicolor* treatment/*L. bicolor* S238N control] measured by quantitative PCR (white) and cDNA array analysis (black). Values are the means of three replicates (± standard error).

Figure 3

Distribution of N-AHSL-responsive transcripts in Gene Ontology categories. Categories of transcripts showing an increased or decreased concentration in the presence of 3,O-C12-HSL are figured in black or white, respectively. Data are expressed in percent of the total number of transcripts 3,O-C12-HSL regulated.

Table 1. N-AHSL-responsive transcripts in *L. bicolor* S238N (t-test, ratio ≥ or ≤ 2.0) after 14 hours of incubation with 3,O-C12-HSL. cDNA clone ID, the NCBI database best match and the BlastX E. value are given. Values for the transcript regulation ratio [3,O-C12-HSL *L. bicolor* treatment/ *L. bicolor* S238N control] are the mean of three replicates.

V1.1 JGI protein identity	GeneBank Accession no	NCBI Database match	E-value	Expression ratio
3,O-C12 up-regulated genes				
Chromatine structure				
294940	JGI_LbEX216	Histone H1, gonadal [*Parechinus angulosus*]	1.E-17	3.7
185105	EL739209	histone H2A [*Agaricus bisporus*]	1.E-43	3.0
Detoxification processes				
184665	EL739210	glutathione S-transferase [*Paxillus involutus*]	7.E-81	3.9
296671	JGI_LbEX1156	glutathione S-transferase [*Paxillus involutus*]	5.E-20	2.6
Energy production & conversion				
183558	ES768865	NADH-ubiquinone oxidoreductase 20 kDa subunit [*Mus musculus*]	2.E-78	18.6
184667	EL739046	fumarate reductase (NADH) [*Cryptococcus neoformans var. neoformans JEC21*]	0.0	3.5
395215	EL740118	Cytochrome c oxydase [*Cryptococcus neoformans var. neoformans JEC21*]	3. E-29	3.2
Mito3634485		ATP synthase F0 subunit 9 [*Schizophyllum commune*]	e-22	2.1
Lipid metabolism				
305700	EL739449	acyl-CoA dehydrogenase, [*Cryptococcus neoformans var. neoformans* B-3501A]	0.0	3.2
305700	EL739449	Acyl-CoA dehydrogenase related protein S-adenosylmethionine-dependent [*Arabidopsis thaliana*]	7.E-41	2.5
295662	JGI_LbEX366	methyltransferase/cyclopropane-fatty-acyl-phospholipid synthase [*Arabidopsis thaliana*]	1.E-38	2.3
Proteases				
314443	JGI_LbEX2159	endopeptidase [*Cryptococcus neoformans var. neoformans* JEC21]	3.E-29	11.0

381567	EL739040	papalysine [*Homo sapiens*]	7.E-08	4.6
Transcription process				
396518	EL739219	Transcription initiation factor IIA small chain [*Schizosaccharomyces pombe*]	4.E-32	3.2
169021	JGI_LbEX1475	transcription initiation factor IId subunit [*Schizosaccharomyces pombe*]	2.E-30	2.7
Other				
187325	EL739208	peroxisomal biogenesis factor (PEX11) [*Aspergillus fumigatus* Af293]	5.E-98	5.4
309748	EL739391	protein kinase [*Leishmania major*]	2.E-03	3.4
324650	EL739415, EL739364	ras related protein [*Laccaria bicolor*]	2.E-26	3.3
384171	EL739214	Glutamic acid-rich protein precursor [*Plasmodium falciparum*]	4.E-10	2.9
399271	EL735445	tectonin II [*Physarum polycephalum*]	6.E-28	2.8
185358	JGI_LbEX1662	exo-beta-1,3-glucanase [*Lentinula edodes*]	1.E-52	2.1

3,O-C12 Down-regulated genes

Chromatine structure				
302873	EL739239	Chromatin-associated protein swi6 [*Schizosaccharomyces pombe*]	7.E-24	-10.2
190777	EL738671, EL739085, EL739331, EL739813, EL739644, EL739761	mismatched base pair and cruciform DNA recognition protein [*Agaricus bisporus*]	2.E-39	-4.1
Cytoskeleton & protein trafficking				
231695	EL739335	arp2/3 complex 21 kda subunit [*Cryptococcus neoformans var. neoformans* B-3501A]	8.E-78	-5.1
148581	EL738861	Autophagy-related protein 8 precursor (Autophagy-related ubiquitin-like modifier ATG8) [*Laccaria bicolor*]	2.E-74	-5.0
311622	EL738896	ADP-ribosylation factor [*Cryptococcus neoformans*]	2.E-94	-2.9
Energy production & conversion				
143231	JGI_LbEX1538	mitochondrial ATPase Afg1 [*Aspergillus fumigatus*	3.E-21	-10.9

		Af293]		
248838	EL739840	D-arabinono-1,4-lactone oxidase [*Cryptococcus neoformans var. neoformans* JEC21]	4.E-180	-5.1
315828	EL739457	FMN binding oxidoreductase [*Aspergillus fumigatus* Af293].	7.E-97	-4.2
305432	ES768861	ATP synthase alpha chain, mitochondrial precursor [*Cryptococcus neoformans var. neoformans* JEC21]	0.0	-2.7
Mitochondria	EL739475	ATP synthase 9	manual curation	-2.4
Proteases				
292906	EL739719, EL739344	Vacuolar aspartic protease precursor [*Candida albican*s]	0.0	-8.0
318727	EL739281	clitocypin analog [*Clitocybe nebularis*]	3. E-3	-4.2
Protein synthesis & maturation				
304792	ES768874	Srrm1_predicted; serine/arginine repetitive matrix 1 [*Rattus norvegicus*].	2.E-7	-8.0
292573	ES768887	40S ribosomal protein S21 [*Candida albicans*]	5.E-31	-7.0
291503	EL739604	Elongation factor 1-alpha [*Schizophyllum commune*]	0.0	-6.5
295367	ES768877	60S ribosomal protein L37-A [*Drosophila melanogaster*]	2.E-36	-6.1
165054	EL739461	40S ribosomalprotein S26 [*Schizophyllum commune*]	3.E-47	-5.4
172821	ES768889	Tryptophanyl-tRNA synthetase [*Schizosaccharomyces pombe*]	0.0	-5.0
191048	EL739835	chaperone regulator [*Cryptococcus neoformans var. neoformans B-3501A*]	5.E-63	-4.9
294111	EL739464	60 kDa chaperonin [*Thermus thermophilus HB27*]	2.E-14	-4.0
314121	EL738698	60S ribosomal protein L39 [*Candida albicans*]	1.E-17	-3.0
Stress response				
384583	ES768871	Heat shock protein 90 homolog [*Schizosaccharomyces pombe*]	0.0	-232712
300650	EL739652	HSP100 [*Pleurotus sajor-caju*].	0.0	-6.8
305899	ES768864	stress response RCI peptide, putative [*Aspergillus fumigatus* Af293]	3.E-22	-5.7
186670	EL739116	small heat shock protein [*Laccaria bicolor*]	5.E-77	-3.7
Transport				
297155	EL739477	Metabolite transport protein GIT1 [*Saccharomyces*	0.0	-5.1

		cerevisiae]		
187302	EL739072	ABC transporter [*Aspergillus fumigatus* Af293]	0.0	-4.6
295862	ES768860	Putative transmembrane protein, involved in the export of ammonia [*Candida albicans*]	2.E-49	-3.6
Others				
385500	EL739627	Chain A, Three dimensional structure of a novel pore-forming lectin [*Laetiporus sulphureus*]	5.E-34	-162148
318727	EL739623	laminarinase [*Phanerochaete chrysosporium*]	0.0	-11.0
399510	EL740045	CipC1 [*Paxillus involutus*]	4.E-31	-7.5
318163	EL739816	hemolytic lectin LSLa [*Laetiporus sulphureus*].	1.E-38	-5.5
244258	ES768884	26S proteasome regulatory complex, ATPase RPT4 [*Schizosaccharomyces pombe*]	0.0	-5.1
293277	EL739504, EL739810	ras related protein 2 [*Laccaria bicolor*]	1.E-08	-3.6
386769	EL738803	Proteoglycan-4 precursor (Lubricin) [*Homo sapiens*]	5.E-25	-3.0
293312	EL739290	carbonate dehydratase/ zinc ion binding [*Arabidopis thaliana*]	5.E-09	-2.9

Figure 1 Deveau *et al.*

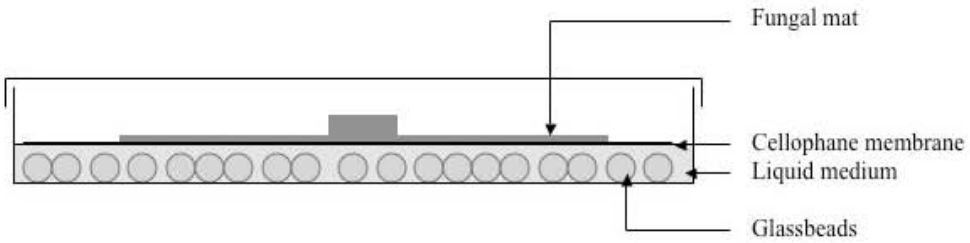


Figure 2 Deveau *et al*

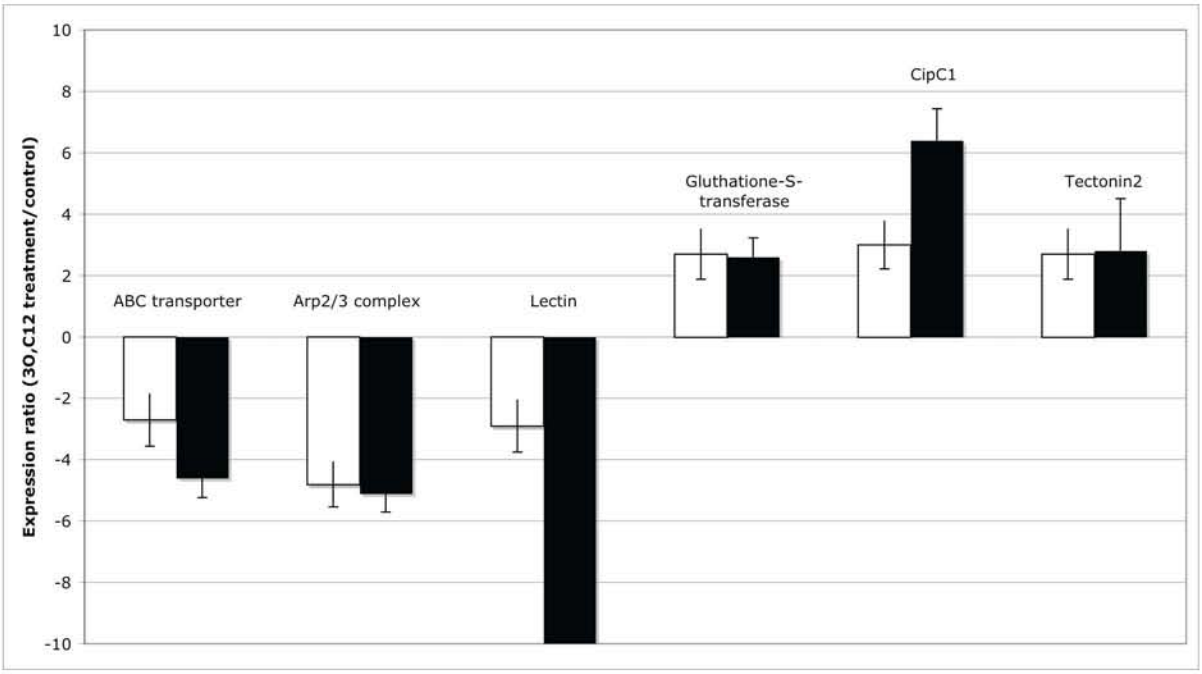
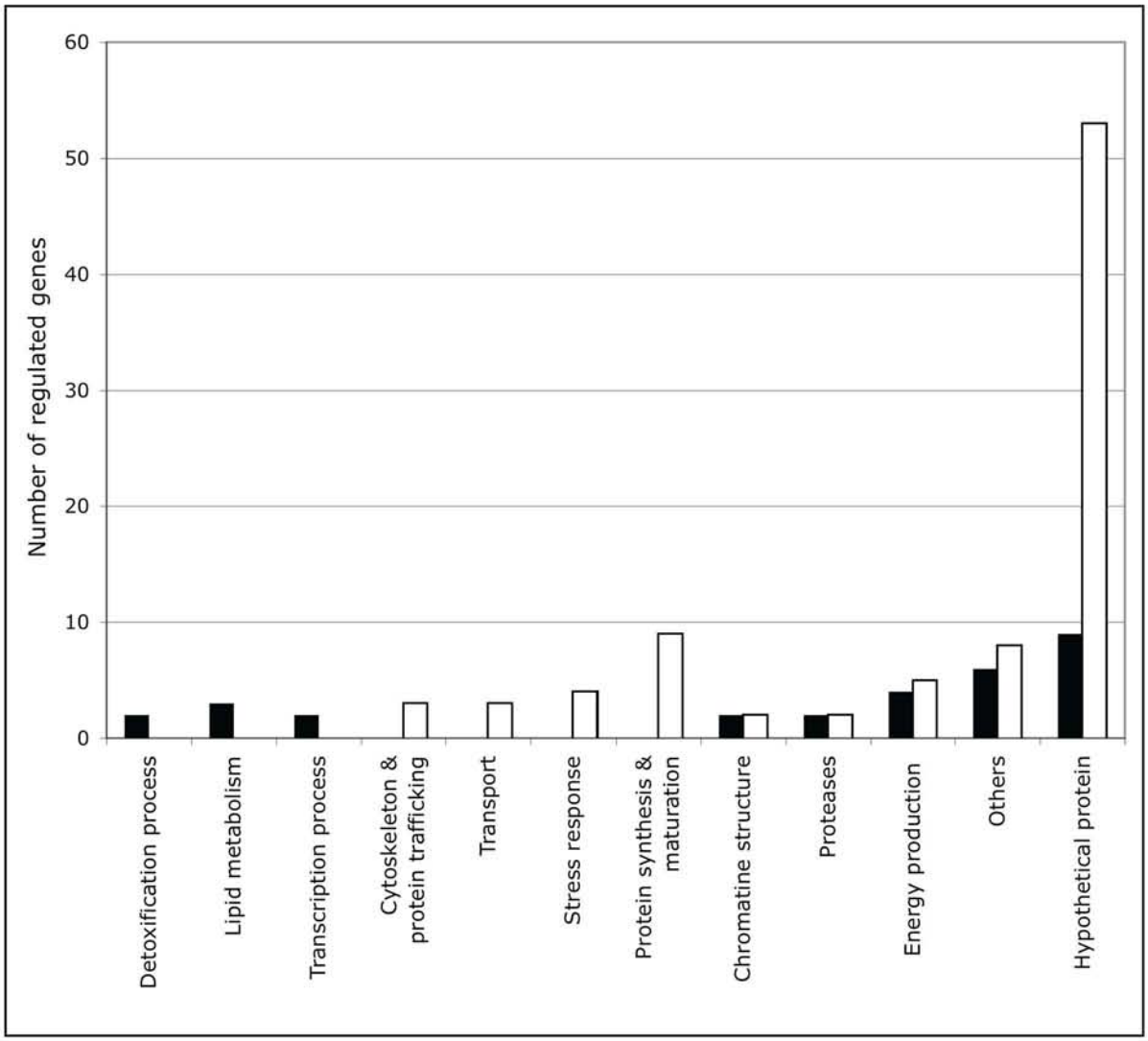


Figure 3 Deveau *et al.*



Publication n°6. Antagonistic effect of the chitinolytic bacterium *Collimonas fungivorans* Ter331 on the ectomycorrhizal fungus *Laccaria bicolor* S238N: a dual gene profiling approach

III.2 Perception and reaction of *L. bicolor* S238N to antagonistic *Collimonas* strains

III.2.1 Publication n°6. Antagonistic effect of the chitinolytic bacterium *Collimonas fungivorans* Ter331 on the ectomycorrhizal fungus *Laccaria bicolor* S238N: a dual gene profiling approach.

Antagonistic effect of the chitinolytic bacterium *Collimonas fungivorans* Ter331 on the ectomycorrhizal fungus *Laccaria bicolor* S238N: a dual gene profiling approach

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Abstract

Ectomycorrhizal fungi are surrounded by bacteria with which they interact physically and metabolically and which can affect positively or negatively mycorrhiza formation. Little is known about the mechanisms by which ectomycorrhizal fungi perceive and respond to antagonistic bacteria. In the present study, we analyzed the transcriptomic response of both the ectomycorrhizal fungus *Laccaria bicolor* S238N and the antagonistic strain *Collimonas fungivorans* Ter331 to each other's presence before cell-cell contact in an *in vitro* confrontation assay. The transcriptome of the antagonistic bacteria was only weakly affected by the presence of the fungus, as only fourteen genes were differentially expressed. By contrast, the fungus strongly reacted to the presence of the bacteria as shown by a strong growth reduction and by the downregulation of 112 genes which mainly encoded hypothetical proteins and proteins involved in primary metabolism. This shut down of the primary metabolism of the fungus induced by the bacteria results at least from deleterious modifications of the environment. In addition, our results suggest the existence of self defense cross-regulation between the two microorganisms: the ectomycorrhizal fungus induced a down-regulation of a bacterial chitinase-like gene while the bacteria reduced the transcription of fungal chitin deacetylase-like gene.

Key words: *Laccaria bicolor* S238N, *Collimonas fungivorans* Ter331, antagonism, transcriptome.

Introduction

Soil environments are colonized by myriads of organisms, from bacteria to springtails, from fungi to plant roots, all interacting together. All of them produce a wide variety of compounds, either toxic, neutral or beneficial to the surrounding macro- and microorganisms. The identification of these compounds and of their mechanisms of action is a corn stone for the understanding of the functioning of soil ecosystems.

In forest soils, ectomycorrhizal fungi form symbiotic associations with tree roots which play a major role in the hydro-mineral nutrition of the trees (Smith & Read, 1997). The formation and the functioning of the ectomycorrhizae can be positively or negatively affected by surrounding bacteria (Johansson *et al.* 2004, Frey-Klett *et al.* 2005). Special attention has been given to mycorrhiza helper bacteria (MHB) that enhance the symbiosis formation (for a review, see Frey-Klett *et al.* 2007). Recent progresses in understanding the mechanisms of the MHB effect have been made through the development of genomic approches (Schrey e*t al.* 2005, Riedlinger *et al.* 2006, Deveau *et al.* 2007). However, little is known about the mechanisms by which ectomycorrhizal fungi perceive and respond to bacteria that are antagonizing them, although such bacteria probably play an important role in the efficiency of the symbiosis and thus of the tree nutrition.

In this context, we have previously analysed the effect of several soil bacteria on the growth and the morphology of the mycelium of the ectomycorrhizal fungus *Laccaria bicolor* S238N (Deveau *et al.* 2007). Using an *in vitro* assay, we showed that the strain *Collimonas fungivorans* Ter331 strongly inhibited the hyphal growth and the apex density of the fungus before the establishment of physical contact between the two microorganisms. This bacterial strain has been isolated from a slightly acidic dune soil in the Netherlands (de Boer *et al.* 2004). However, the *Collimonas* genus is also common in forest soils and some strains have been found associated with ectomycorrhizae (Höppener-Ogawa *et al.* 2007, Uroz *et al.* 2007).

Interestingly, *C. fungivorans* Ter331 has a high chitinolytic activity and has the ability to grow at the expense of living fungal hyphae (de Boer *et al.* 2001). Furthermore, it exerts biocontrol activity against the fungal pathogen *Fusarium oxysporum* (Kamilova *et al.* 2007). Recently, the genomes of both the ectomycorrhizal fungus and the chitinolytic bacteria have been sequenced (Leveau *et al.* unpublished, Martin *et al. in press*) and transcriptomic analyses of the two organisms have been developed (Deveau *et al.* 2007, Leveau *et al.* unpublished). Microarray technology provides a powerful tool for giving an overview of cell responses to biotic interactions at the transcriptomic level. To our knowledge transcriptomic analyses have never been simultaneously performed onto two interacting fungi and bacteria. But it is a potent method to tackle the question of the molecular mechanisms of perception and of reaction of microorganisms during their interaction. Here, we analyzed the transcriptomic responses of both the ectomycorrhizal fungal strain and the antagonist bacterial strain after 14 days of co-culture. We used the Petri dish bioassay previously used to study the morphological response of the ectomycorrhizal fungus to the chitinolytic bacteria (Deveau *et al.* 2007). Analyses were focused on the first step of the interactions *ie* before the establishment of physical contact between the fungal and bacterial cells (Figure 1)

Results

Validation of *C. fungivorans* and *L. bicolor* expression array data

Sequences of the predicted open reading frames from *C. fungivorans* Ter331 (Leveau *et al.* unpublished) have been used by NimbleGen (Madison, WI, USA) to construct a 60-mer oligoarray for monitoring the *C. fungivorans* Ter331 transcriptome. Before analysing the response of the bacterial transcriptome to the fungus, we checked the validity of the *C. fungivorans* Ter331 expression array data by calculating the Pearson correlation coefficient, first between internal technical replicates and secondly between biological replicates

(Duplessis *et al.* 2005). Each array contained five replicates per probe. A Pearson correlation coefficient (*r*) of 0.99 between replicated spots indicated good agreement for signal intensity (Supplementary web data Figure S1a). The results showed good reproducibility between arrays: in all cases, the Pearson correlation coefficient of biological replicate array was > 0.95 (Figure S1b).

The description of the *L. bicolor* S238N cDNA array is provided in Deveau *et al.* (2007). The validity of data was checked by calculating the Pearson correlation coefficient between biological replicates. This coefficient was >0.95 (Figure S1c).

Alteration of *C. fungivorans* Ter331 transcriptome by *L. bicolor* S238N.

The presence of *L. bicolor* S238N had a very low impact on the transcriptome of *C. fungivorans* Ter331: the relative abundance of the transcripts corresponding to only fourteen bacterial genes significantly varied in the presence of the ectomycorrhizal hyphae, according to a Bayesian *t*-test and a Posterior Probability of gene Differential Expression (PPDE) analysis (Table 1). Four genes were up- and ten were down-regulated. The level of differential expression was fourfold at maximum. Up-regulated genes encoded for a 3-oxoadipate CoA-succinyl transferase catalyzing the conversion of acetoacetyl-CoA to acetoacetone and three hypothetical proteins (Table 1). Down-regulated genes encoded for an endochitinase B, a saccharopine dehydrogenase (amino acid metabolism), two short chain dehydrogenases, an HTH-transcriptional factor, an oxidoreductase and four hypothetical proteins. Three down-regulated genes (HTH-transcriptional factor, short chain dehydrogenase, oxidoreductase) were clustered together within less than 3 kb on the circular chromosome. Finally, it is interesting to note that more than a half of the responsive genes feature a signal peptide. The regulation of the endochitinase B, the zinc-binding oxidoreductase and the hypothetical protein 3082 encoding genes was confirmed by real-time quantitative PCR (Figure S2). By

contrast, the regulation of the short-chain dehydrogenase/reductase protein and the 3-oxoadipate CoA-succinyl transferase could not be confirmed.

Alteration of *L. bicolor* S238N transcriptome by *C. fungivorans* Ter331

One hundred and fifty four fungal transcripts (3% of the arrayed cDNAs) showed a significantly altered concentration in the presence *C. fungivorans* Ter331. The cDNA of 134 of these regulated transcripts were successfully sequenced; they encoded for 114 genes. Twenty six were up- and 88 were down-regulated. Two third of these genes corresponded to hypothetical proteins (22 up- and 52 down-regulated). The level of regulation varied between two- and 11-fold. The up-regulated transcripts encoded for two proteins involved in the replication process (DNA polymerase B and replication factor Rpa1) and one phosphoric monoester hydrolase implicated in transduction signal mechanisms (Table 2 & Table S1). Down-regulated transcripts encoded proteins mainly involved in stress response (gluthatione-S-transferases), protein synthesis (ribosomal proteins, elongation factor 1, splicing factor, small nuclear ribonucleoprotein), energy metabolism (cytochrome c oxidase, flavoprotein monooxygenase, ATP/ADP carrier proteins) and chromatine structure (Histones H1 and H2A, Figure 2). Interestingly, the concentration of a transcript encoding a chitin deacetylase was three times reduced in the presence of the bacteria.

Discussion

In a previous study we showed that *C. fungivorans* Ter331 strongly repressed the growth of *L. bicolor* S238N when co-cultured *in vitro* before any direct physical contact (Deveau *et al.* 2007). Here, we simultaneously analyzed the modifications of the fungal and bacterial transcriptomes to better understand the mechanisms by which fungal and bacterial cells perceive each other presence and respond to it.

The ectomycorrhizal fungus *L. bicolor* S238N dramatically reacted to the presence of *C. fungivorans* Ter331, mainly through the down-regulation of the expression of genes involved in mitochondrial activity, transcription and protein synthesis. We also observed the down-regulation of several genes encoding superoxide dismutases involved in cell protection against oxidative stress. This could be a reflection of the lower metabolic activity of the fungus as part of the oxidative stress is due to the primary metabolism of cells (Aguirre *et al.* 2005). All together, these data indicate that *L. bicolor* S238N react to the presence of *C. fungivorans* Ter331 through a shut down of its primary metabolic activity that is consistent with the significant decrease of the mycelium growth. Such a transcriptional shut down of the primary metabolism has been previously demonstrated in the case of *Saccharomyces* and *Neurospora* when they were grown in stressful environments or in starvation (Gasch & Werner-Washburne 2002, Xie *et al.* 2004).

Conversely, *C. fungivorans* Ter331 only weakly responded to the presence of *L. bicolor* S238N at the transcriptional level. Indeed, less than 15 bacterial transcripts (0.003 % of the genome) showed altered levels with little variation when the two microorganisms were co-cultured. Two hypotheses can be proposed to explain this result. First, the bacteria reacted to the presence of the fungus at an earlier step. This hypothesis is unlikely because the extent of the growing fungal colony is very small during the first 14 days of the experiment (about 1 cm of diameter). Therefore, the impact of the fungal biomass on the environment should be very low. Second, the antagonistic activity of *C. fungivorans* Ter331, which could be detected as early as eight days after the beginning of the co-culture, is mainly not fungal-induced. It has been frequently shown that the biocontrol activity of bacteria is positively autoregulated at the transcriptional level by the quorum sensing system (Haas & Defago, 2005). In our study, the bacterial density is high after 14 days of culture and a QS regulation could occur.

The antagonistic activity of the bacteria could result from various mechanisms. The first one is mycophagy (Leveau *et al.* 2008). Indeed, *C. fungivorans* is able to hydrolyze hyphae thanks to its chitinolytic activity (de Boer *et al.* 2001). However, we did not observe by microscopy any alteration of the integrity of the mycelium after an extended physical contact with the bacterial cells (data not shown). Antibiosis, competition for nutrient or deleterious alteration of the environment can also be involved (Whipps 2001). In our bioassay, when the fungus is co-cultivated with only two drops of bacterial colonies, the mycelium grows at a normal speed in the opposite direction of the bacterial colonies while its growth is strongly reduced in the vicinity of the bacteria (Figure 2). Thus, the antagonistic effect of *C. fungivorans* Ter331 is highly localized and is not due to the production of toxic volatile metabolites. Acidification of the environment by the bacteria may be involved in its deleterious effect against the fungus. Indeed, after 15 days of culture of *C. fungivorans* Ter331 in a P2OTh- liquid medium, the pH of the solution moved from 5.5 to 3. Furthermore, the growth of *L. bicolor* S238N is completely inhibited under pH 3 (J-L Churin, personal communication). Therefore the resulting high acidity may lead to deleterious physiological changes and growth of *L. bicolor* S238N. For instance, several authors have shown that enzyme activity and growth of different fungi could be affected by the acidification of the culture medium (Enokibara *et al*. 1993, Yamanaka, 2003). Conversely, the helper bacteria *P. fluorescens* BBc6R8 that stimulate the growth of the fungus slowly acidified the medium; these findings indicate that the pH of the culture medium could be a determinant for the developmental pattern of *L. bicolor* S238N. Finally, trophic competition is unlikely because of the small amount of fungal and bacterial biomass at the sampling time. This latter mechanism is thought to be involved in the biocontrol activity of *C. fungivorans* towards the phytopathogen *Fusarium oxysporum* f. sp. *radicis-lycopersici* (Kamilova *et al.* 2007). Therefore, it seems that *C. fungivorans* owns a broad spectrum of antifungal activities (mycophagy, antibiotic production, competition for

nutrients and niches, acidification…) that could be differentially expressed depending on the environment and on the interacting fungi. The factors that lead to the development of one strategy instead of another one and the importance of each mechanism on the bacterial growth and soil colonization have to be elucidated.

Our dual transcritpomic approach also provided a intriguing result: we observed the simultaneous down regulation of a bacterial and a fungal gene that are both linked to the chitin metabolism: the *C. fungivorans* Ter331 putative chitinase chiIII encoding gene and the *L. bicolor* S238N putative chitin deacetylase encoding gene. The bacterial chiIII product belongs to the glycoside hydrolase family 19 of the CAZy classification which includes chitinases (Coutinho & Henrissat 1999, Fritsche *et al.* submitted). Although the purified enzyme does not show activity towards chitin, the knock out of the gene suppresses the chitinolytic activity of the bacteria (Fritsche *et al.* submitted). Thus it may be involved in the chitinolytic process of *C. fungivorans* Ter331. Fungal chitin deacetylases hydrolyses the chitin of the fungal cell wall into chitosan, a deacetylated form of the chitin which is a poor substrate for chitinases. Indeed, chitinases require the presence of *N*-acetyl moieties for chitin recognition and catalysis (Blair *et al.* 2006). Deising & Siegrist (1995) and El Gueddari *et al.* (2002) suggested that the deacetylation of chitin of fungal phytopathogens would protect cell walls of the pathogenic hyphae from host chitinases. In this contexte, we speculate that the deacetylation of chitin protect *L. bicolor* S238N against the chitinolytic activity of *C. fungivorans* Ter331. Interestingly, the expression of chitin deacetylase encoding genes is also modulated during the assembly of cell wall yeast ascospore (Christodoulidou *et al.* 1999) and during ectomycorrhiza formation in *L. bicolor* S238N (F. Martin, personal communication). Thus, the regulation of the expression *L. bicolor* S238N chitin deacetylase gene in fungal-bacterial co-culture could also be linked to the morphological modifications of the fungal hyphae induced by the bacteria (Deveau *et al.* 2007). The cross-reduction of the concentration

of both bacterial chitinase chiIII and fungal chitin deacetylase transcripts is particularly noteworthy in a context of antagonistic interactions. It suggest that thee two microorganisms could counteract the activity of the other through negative signaling. Such a mechanism has already been reported in the case of three other antagonistic interactions between *Fusarium* sp. and two *Pseudomonas* strains (Notz *et al.* 2002, van Rij *et al.* 2005) and between *Fusarium* sp. and *Trichoderma atroviride* P1 (Lutz *et al.* 2003). In each case, a toxin produced by the one of the two microorganisms down-regulated the expression of antibiotic biosynthesis genes in the second microorganism.

Even if further research will be necessary to evaluate the relative importance of transcriptomic cross-regulations in the antagonistic interaction, microarray dual approach is a particularly adapted tool to detect this kind of mechanism. Up to now, this type of study was difficult to perform because genomic tools were rarely available for both interacting organisms. The recent massive sequencing of fungal and bacterial genomes simultaneously to the developing of whole genome arrays offer now great opportunities to explore the complex relationships between fungi and bacteria. It should not only benefit to the understanding of the functioning of ecosystems where fungal-bacterial interactions play a major role (Wargo & Hogan, 2006) but should also allow the discovery of new metabolites with pharmaceutical applications. Indeed, many secondary metabolites are probably produced only when microorganisms are interacting with others and are thus undetectable in pure culture (Furtado *et al.* 2002, de Boer *et al.* 2007).

Experimental procedures

Microorganisms and confrontation bioassay. The ectomycorrhizal basidiomycete *Laccaria bicolor* S238N (Maire P. D. Orton) was maintained on Pachlewski agar medium P5 (Di Battista *et al.* 1996) at 25°C during three weeks. *Collimonas fungivorans* Ter331 is

chitinolytic (de Boer *et al.* 2004) and antagonistic to *L. bicolor* S238N (Deveau *et al.* 2007). The bacterial strain was stored at -80°C in Luria-Bertani Medium (Sambrook *et al.* 1989) with 20% glycerol added. In the present work, the bacterial strain was first grown on 10 % TSA plates (3 $g.L^{-1}$ Tryptic Soy Broth from Difco and 15 $g.L^{-1}$ of agar) at 25°C for 65 hours to prepare the bacterial inoculum for the *in vitro* bioassay. The bioassay was performed as described by Deveau *et al.* (2007). Briefly, four droplets of bacterial solution (bacterial treatment) were distributed at 1.7 cm from the centre of a fungal plug. A control treatment was performed without the fungus. A second control treatment where bacteria were replaced by sterile water was performed to check the antagonistic activity of the bacteria on the fungal growth. Plates were then incubated at 10°C in the dark.

RNA isolation from bacteria and expression array hybridization.

After 14 days of incubation at 10°C and in the dark, the half part of the four bacterial drops which faces the fungal colony was collected from 10 plates, frozen in liquid nitrogen and then pooled (Figure 1). Two biological replicates per treatment were performed (2 times 10 plates). RNA was then extracted using Trizol (Invitrogen, Stockholm, Sweden) as recommended by the manufacturer. One hundred micrograms of total RNA were further purified using the RNeasy Plant mini kit (Qiagen, Hilden, Germany) as recommended by the manufacturer. Quality of RNA was checked by electrophoresis using the Experion laboratory chip automated electrophoresis system (Biorad, Saint Quentin, France). *C. fungivorans* Ter331 expression arrays were designed by NimbleGen Systems (Madison, WI, USA) as follows: 16 oligomers of 60 nucleotides long probes were designed for each of the 4529 predicted genes (4486 genes on the circular chromosome and 43 on the plasmid). It was not possible to design probes for six genes. For each of the 16 probes, three forward replicates and two reverse replicates were synthesised on the array (total of 80 probes per gene). 29012 random primers were also added and were used to quantify the background signal. cDNA synthesis and array

hybridization were performed by NimbleGen Systems (Madison Wi, USA) from 30 mg of total RNA. Data were normalized by NimbleGen using the Robust Multichip Average method (Irizarri *et al.* 2003). The normalized data set provided by NimbleGen contained the mean intensity value of the 16 probes per gene for each forward and reverse replicate (i.e. five intensity value per gene and per biological replicates). Data quality assessment was performed using analysis of variance (*t*-test) and a Bayesian statistical framework implemented in the Cyber-T web interface (Baldi & Long 2001) as described by Duplessis *et al.* (2005). A Posterior Probability of Differential Expression test (PPDE test) was used to determine the global false positive and negative levels inherent in high dimensional DNA array experiment. A PPDE value ranging between 0.98 and 1.0 indicates a high probability of true positive.

RNA isolation from *L. bicolor* S238N mycelium and cDNA array hybridization. Fungal and bacterial RNA were isolated as independent events. After 14 days of incubation in the dark at 10 °C, the whole mycelium from 50 plates was collected, frozen in liquid nitrogen, pooled and stored at -80°C. Mycelium was sampled in triplicates (3 times 50 plates). RNA from 50 mg of mycelium was then extracted using the RNeasy Plant mini kit (Qiagen, Hilden, Germany) as recommended by the manufacturer for fungal material. Quality of RNA was checked by RNAse-free 1 % agarose electrophoresis. Synthesis of complex cDNA probes from 400 ng of fungal RNA and cDNA hybridization on a Nylon membrane containing 4992 cDNA were performed as described by Peter *et al.* (2003). Data quality assessment was performed using analysis of variance (*t*-test) and a Bayesian statistical framework implemented in the Cyber-T web interface (Baldi & Long 2001) as described by Duplessis *et al.* (2005). Identification of cDNA clones corresponding to up- and down-regulated transcripts was done as described by Deveau *et al.* (2007).

Confirmation of cDNA array results by quantitative PCR. The validation of the bacterial NimbleGen arrays data was done by performing real-time quantitative PCR analyses on five

up- or down-regulated genes (short-chain dehydrogenase/reductase SDR, zinc-binding oxidoreductase, endochitinase B, 3-oxoadipate CoA-succinyl transferase and a hypothetical protein). Four non-regulated genes were also used as controls and three of them were chosen for data normalization (a DNA-3-methyladenine glycosidase and three hypothetical proteins). For the validation of fungal cDNA array data, real-time analyses were performed on three regulated target genes (Gluthatione-S-transferase, Tra1, Hypothetical protein Srrm1). Eight non-regulated genes were also used as controls and two of them (Hypothetical protein Lb17E10 and trehalose phosphorylase) were chosen for data normalization. Primer design and PCR amplification protocol are described in Deveau *et al.* (2007). The sequences of all primers are provided in Table S2 (Supplemental web data).

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Figures

Figure 1. Scheme of the *in vitro* confrontation assay and of the bacterial sampling for transcriptomic analyses.

Figure 2. Functional categories to which down-regulated fungal transcripts belonged. Data are expressed as a percentage of the total number of functionally identified down-regulated genes.

Figure 3. Antagonistic effect of *C. fungivorans* Ter331 on *L. bicolor* S238N mycelium growth after 45 days of dual culture. Only two droplets of *C. fungivorans* Ter331 solution (DO_{600nm} ~ 0.9) were distributed at 1.7 cm from the centre of a *L. bicolor* S238N plug, contrary to the standard bioassay used for transcriptomic and fungal growth analyses where four droplets were added.

Tables.

Table 1. Transcripts of *C. fungivorans* Ter 331 regulated (*t*-test, ≥ - 2.0, ≤1.8) in the presence of the ectomycorrhizal fungus *L. bicolor* S238N. Probe number on expression arrays, Gene ID, identity of the best blastp, the GenBank accession number corresponding to the best hit, the E-value the prediction of signal peptide (SignalP 3.0) and amino acid number are given. Finally, the fold corresponds to the ratio signal intensity between *L. bicolor* S238N and control treatments. The value of the PPDE test is also given.

Probe number	Gene ID	Best blast identity	E value	AC_Number	Signal peptide	Protein size (aa)	Fold	PPDE
COLLI000000001706	Colli20061116_1761	Hypothetical protein Bcep1808DRAFT_6388 [*Burkholderia vietnamiensis* G4]	4e-05	ZP_00421146	yes	122	2,2	1.000
COLLI000000003027	Colli20061116_3082	Hypothetical protein	no hit	-	yes	107	2,1	0.983
COLLI000000001614	Colli20061116_1669	3-oxoadipate CoA-succinyl transferase ScoA [*Burkholderia pseudomallei* 1710b]	2e-100	ABA50150	no	232	2,0	1.000
COLLI000000004086	Colli20061116_4141	Hypothetical protein [*Azoarcus sp.* EbN1]	2e-28	CAI06681	yes	152	2,0	0.986
COLLI000000001621	Colli20061116_1676	Endochitinase B [*Nicotiana tabacum*]	1e-80	P24091	no	216	-1,8	1.000
COLLI000000003211	Colli20061116_3266	Saccharopine dehydrogenase [*Hahella chejuensis* KCTC 2396]	2e-118	YP_432774	no	386	-2,0	1.000
COLLI000000003717	Colli20061116_3772	HTH-type transcriptional regulator [*Lactococcus lactis* subsp. *lactis*]	2e-63	P42097	no	206	-2,1	1.000
COLLI000000001624	Colli20061116_1679	Hypothetical protein [*Xanthomonas campestris* pv. *vesicatoria* str. 85-10]	3e-20	YP_363381	no	86	-2,2	1.000
COLLI000000002100	Colli20061116_2155	Short chain dehydrogenase [*Agrobacterium tumefaciens* str. C58]	2e-75	NP_396378	yes	262	-2,3	1.000
COLLI000000001534	Colli20061116_1589	Hypothetical protein	no hit	-	no	423	-2,4	1.000
COLLI000000001626	Colli20061116_1681	Hypothetical protein	no hit	-	yes	286	-2,4	1.000
COLLI000000003716	Colli20061116_3771	Short-chain dehydrogenase/reductase SDR [*Polaromonas* sp. JS666]	2e-92	YP_546970	yes	284	-2,6	1.000
COLLI000000003715	Colli20061116_3770	Zinc-binding oxidoreductase [*Rhizobium leguminosarum* bv. *viciae* 3841]	4e-117	YP_771542	no	333	-2,6	1.000
COLLI000000000098	Colli20061116_153	Hypothetical protein [*Bordetella bronchiseptica* RB50]	6e-15	NP_891025	yes	135	-4,0	1.000

Table 2. Transcripts of *L. bicolor* S238N regulated (*t*-test, ≥ - 2.0, ≤ 2.0) in the presence of the chitinolytic strain *C. fungivorans* Ter331. Protein ID, GenBank number of the corresponding EST, identity of the best blastX, and the E value of the BlastX are given. The fold corresponds to the ratio signal intensity between *C. fungivorans* Ter331 and the control treatment. The value of the PPDE test is also given. Complete data containing hypothetical proteins are given in Table S1 (web supplemental data).

Protein ID	EST AC Number	NCBI Best BlastX identity	BlastX E-value	Fold Change	PPDE test
Stress response					
184665	EL739982, EL739373	glutathione S-transferase [*Paxillus involutus*]	0	-3,3	9,88E-01
298606	EL739383, JGI_LbEX4932	glutathione S-transferase [*Coccidioides immitis* RS]	2E-69	-3,3	9,79E-01
296671	JGI_LbEX1156	Gluthatione S transferase [*Paxillus involutus*]	5E-20	-3,8	9,88E-01
Protein synthesis & transcription regulation					
127802	EL739167	Multiprotein-bridging factor 1 [*Kluyveromyces lactis*]	1,3E-41	-5,1	9,93E-01
125708	EL739481	40S ribosomal protein S20 [*Schizosaccharomyces pombe*]	0	-3	9,83E-01
183045	JGI_LbEX2069	40S ribosomal protein S3ae-a [*Cryptococcusneoformans* var. *neoformans* JEC21]	1E-100	-2,9	9,76E-01
304004	JGI_LbEX1164	60S ribosomal protein L7a [*Cryptococcusneoformans var. neoformans* JEC21]	2E-32	-3,7	9,75E-01
291503	EL739615 JGI_LbEX4956	Elongation factor 1-alpha [*Schizophyllum commune*]	0	-3,8	9,96E-01
304792	EL738654, EL739005, EL739211, EL740038 EL740039	Srrm1_predicted; serine/arginine repetitive matrix 1 [*Rattus norvegicus*].	1.9e-7	-3,9	9,85E-01
251796	JGI_LbEX1852	small nuclear ribonucleoprotein [*Arabidopsis thaliana*]	5E-25	-4,6	9,84E-01
300913	EL739054	small nuclear ribonucleoprotein E, putative [*Cryptococcus neoformans var. neoformans* JEC21]	2E-31	-4,5	9,93E-01
297290	JGI_LbEX2076	RNA binding protein [*Aspergillus fumigatus* Af293]	9E-13	-2,6	9,72E-01
Energy metabolism					
mito3634485.a1	JGI_LbEX266	cytochrome C oxidase subunit I [*Pseudotsuga menziesii*]	5E-22	-4,8	9,94E-01
173909	EL739225	cytochrome C oxidase polypeptide IV [*Schizosaccharomyces pombe*]	1E-40	-3,2	9,79E-01
genome traces	JGI_LbEX2019	cytochrome C oxidase subunit I	6,00E-05	-6,1	9,98E-01

genome traces	JGI_LbEX2161	cytochrome C oxidase subunit I [*Cryptococcus neoformans* var. *neoformans*]	4E-33	-5,3	9,96E-01
genome traces	JGI_LbEX256	cytochrome C oxidase subunit I [*Smittium culisetae*]	1E-38	-5	9,97E-01
192393	EL739466	ATP/ADP antiport [*Cryptococcus neoformans var. neoformans B-3501A*]	0	-4,3	9,87E-01
305503	EL739220	flavoprotein monooxygenase [*Paracoccus denitrificans PD1222*]	0	-3,4	9,84E-01
383670	EL739545	ATP/ADP carrier protein [*Candida albicans* SC5314]	0	-4,6	9,94E-01
Replication & chromatine structure					
150961	EL739367	DNA polymerase B *[Cryptococcus neoformans var. neoformans*]	0	5,1	9,77E-01
185105	JGI_LbEX447	histone H2a-1 [*Cryptococcus neoformans* var. *neoformans* JEC21]	3E-36	-3	9,74E-01
190640	JGI_LbEX5292	histone H2a [*Ustilago maydis* 521]	1E-36	-2,6	9,68E-01
246384	EL739455	Tra1 [*Cryptococcus neoformans var. neoformans*]	0	-2,7	9,12E-01
294940	EL739473	Histone H1 [*Parechinus angulosus]*	1E-17	-3	9,78E-01
182415	EL739378	damaged DNA binding protein [*Cryptococcus neoformans* var. *neoformans* JEC21]	8.6^{e}-05	5,9	9,77E-01
Signal transduction					
393186	EL740073	dual secificity protein kinase FUZ7 [*Ustilago maydis*]	0	-3,5	9,83E-01
297361	EL739376	Phosphoric monoesterase hydrolase [*Cryptococcus neoformans var. neoformans JEC21*]	0	4,5	9,76E-01
Other					
384171	EL740036	Glutamic acid-rich protein [*Plasmodium falciparum*]	3.7^{e}-10	-3,3	9,77E-01
313660	EL739476	Hypothetical protein	No hit	3,9	9,87E-01
229432	JGI_LbEX459	chitin deacetylase [*Schizophyllum commune*]	3E-76	-2,8	9,72E-01
383081	EL739037	hypothetical protein [*Cryptococcus neoformans var. neoformans JEC21*]	9.2^{e}-26	-4,1	9,89E-01
245383	JGI_LbEX469	Metalloprotease [*Gloeobacter violaceus* PCC 7421	4E-48	-4,4	9,90E-01
185826	EL739380	oxidoreductase, zinc-binding dehydrogenase family [*Aspergillus fumigatus* Af293]	1.5^{e}-44	-4,1	9,86E-01
301232	EL739275	Panthotenate kinase [*Cryptococcus neoformans var. neoformans JEC21*]	0	-3,2	9,80E-01
306303	EL739483	Protein kinase [*Glycine max*]	4.2^{e}-16	-3,7	9,78E-01

189031	JGI_LbEX2074	Synthase of the type 3 pneumococcal capsular polysaccharide [*Aspergillus fumigatus* Af293]	2E-45	-2,6	9,68E-01
216553	EL739468	cleft lip and palate associated transmembrane protein [*Cryptococcus neoformans var. neoformans JEC21*]	0	-5,7	9,89E-01

Figure 1.

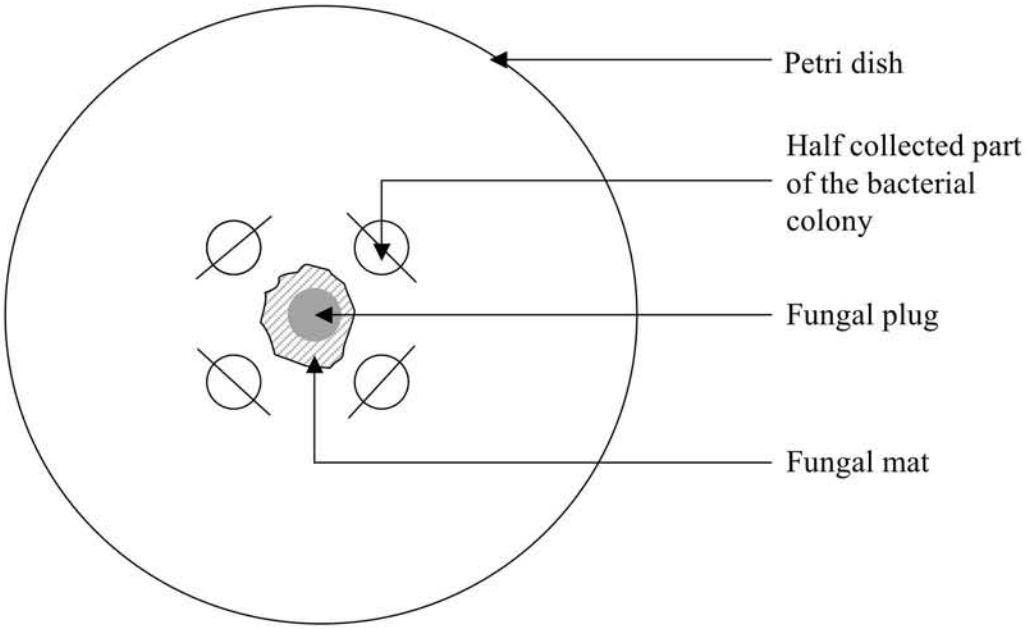

Petri dish
Half collected part of the bacterial colony
Fungal plug
Fungal mat

Figure 2.

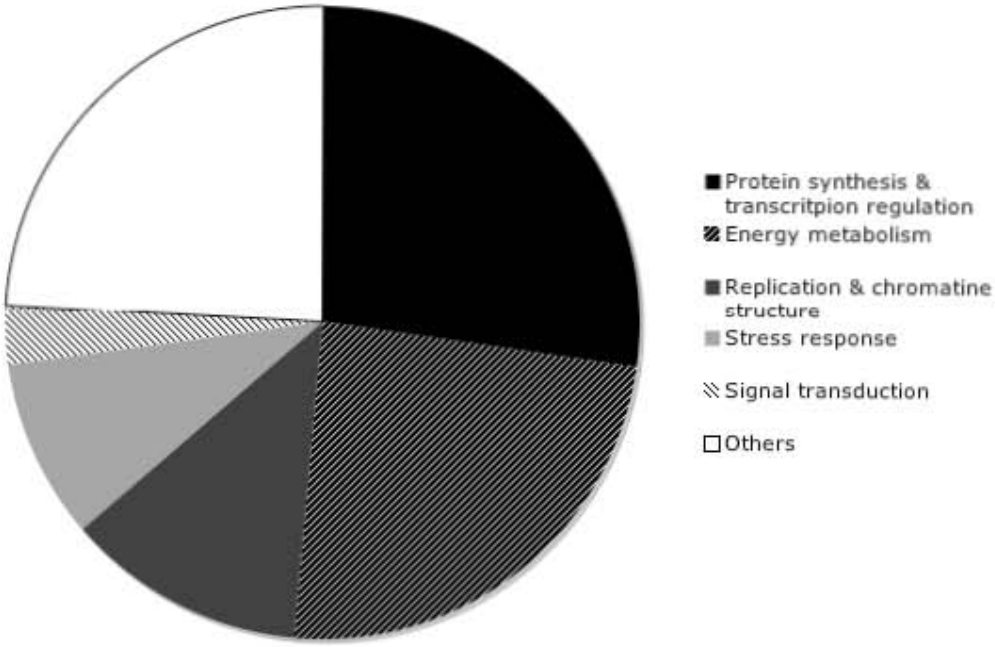
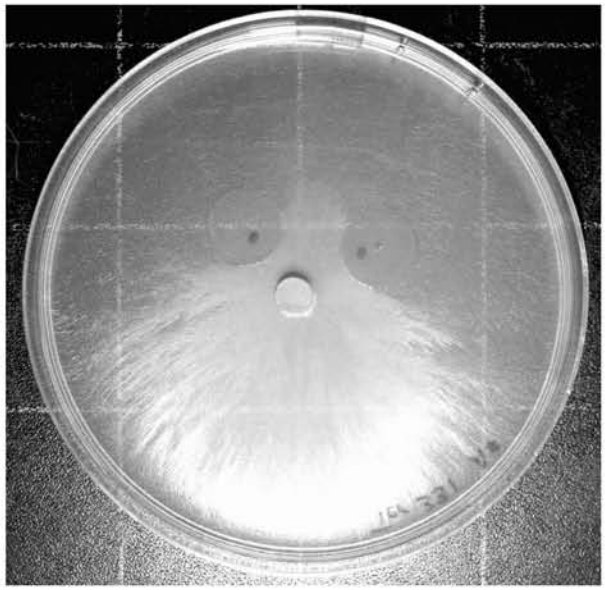

Protein synthesis & transcritpion regulation
Energy metabolism
Replication & chromatine structure
Stress response
Signal transduction
Others

Figure 3.



III.2.2 Impact of *Collimonas* sp. strains on *L. bicolor* S238N growth and morphology.

In the previous study, we have shown that the strain *C. fungivorans* Ter331 exerted a strong inhibition on *L. bicolor* S238N growth and induced modifications of the hyphal morphology. The bacterial strain has been isolated from sand soil (de Boer *et al.* 1998) but two studies have recently shown that *Collimonas* bacteria also inhabit forest and crop soils and can be closely associated with mycorrhizal fungi (Offre *et al.* 2007, Uroz *et al.* 2007). Uroz *et al.* (2007) demonstrated that the selected bacterial community associated with the ectomycorrhizal fungus *Scleroderma citrinum* harboured a high mineral weathering potential activity and suggested the existence of functional complementation between mycorrhizal fungi and bacteria in the tree nutrition. The *Collimonas* strains were among the most efficient weathering strains but were able to use very few carbon sources including trehalose, a disaccharide highly accumulated in many fungi. Data indicated that the *Collimonas* isolates could be fungi-dependant. They could feed on secreted fungal trehalose or they could hydrolyse hyphae to release it. In this context, we analyzed the effect of the *S. citrinum* associated *Collimonas* strains on the *L. bicolor* S238N growth and morphology, by using the set up previously described.

III.2.2.1 Material & methods

Biological strains.

The six *Collimonas* strains were isolated from the symbiotic mantle (PMB) or the mycorryzosphere (PML) of oak *Quercus petraea-Scleroderma citrinum* ectomycorrhizae (Uroz *et al.* 2007). Taxonomic identification was performed by sequencing the partial 16S rRNA gene and the *Rrs* gene (Uroz *et al.* 2007).

Bioassay experiment and morphology analysis.

The bioassay described by Deveau *et al.* (2007) was used. After 14 days of incubation at 10°C in the dark, the fungal colony diameter was measured and six photographs were taken using an Olympus BX41 microscope (x40 magnification) equipped with a ColorView system camera. The apex density, the branching density and the curvature of hyphae were measured on each photograph using the AnalySIS software (Soft Imaging System, Olympus, Münster, Germany).

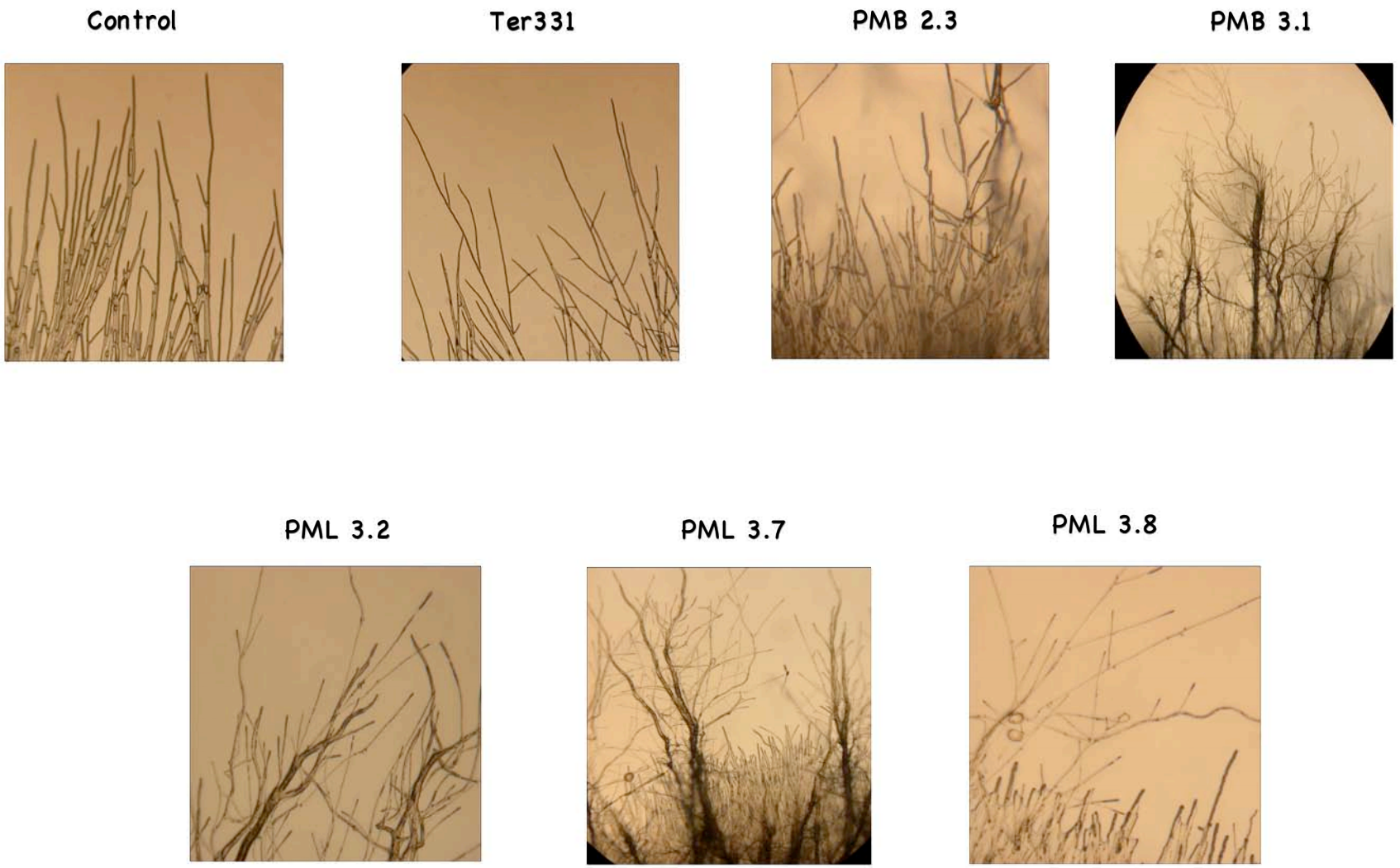
Table 3.1. Effect of six *Collimonas* strains isolated from the mycorrhizosphere of *Scleroderma citrinum* and of *Collimonas fungivorans* Ter331 on the *L. bicolor* S238N hyphal extension and morphology after 14 days of dual culture. A cross indicates a positive effect, a dash a negative effect and 0 no effect.

Strains	Diametral growth	Apex density	Branching	Curve	Rhizomorph structure
Ter331	-	-	+++	0	0
PMB 2.3	-	+	++	0	0
PMB 3.1	-	+++	+	++	+
PMB 3.2	-	+	+	+	+
PML 3.4	0	+	++	0	0
PML 3.7	-	++	++	++	+
PML 3.8	-	+++	+	+	0

III.2.2.2 Results & Discussion.

Except the strain PML 3.4, all the *Collimonas* strains induced a high inhibition of the *L. bicolor* S238N growth (Table 3.1). The reduction of growth was superior to the one induced by *C. fungivorans* Ter331. The six strains induced an increase of the apex and branching densities but at different intensities, depending on the strain. By contrast, only the strains PMB 3.1, PML 3.2, PML 3.7 induced rhizomorph structure formation (Figure 3.1) and hyphal curvature. Rhizomorph is a dense mass of hyphae forming a rootlike structure characteristic of many fungi, but not all are able to form it (Agerer 2001). Curiously, *L. bicolor* is not known to produce this kind of structure (F. Le Tacon, personal communication). It is generally admitted that rhizomorphs constitute conductive structures that allow the transport of water and minerals from the explorating mycelium towards the mycorrhizae. In the present case, we suggest that the *L. bicolor* rhizomorph-like would form defensive structure that could protect hyphae against *Collimonas* enzymatic attack. Indeed, *L. bicolor* S238N accumulates high quantities of trehalose inside its hyphae but does not secrete it (cf. chapter 2). Thus, it is not directly accessible to the bacteria, except in dead hyphae. Further microscopical analyses will be performed to see if a cell wall thickening is associated with this rhizomorph formation and if the *Collimonas* aggressive strains feed on the mycelium when no other carbon source is available. Moreover, the same experiment will be done with *Scleroderma citrinum* to determine whether the *Collimonas* strains that are associated to the mycorrhizosphere of this fungus form a complementary association or if they are aggressive against the fungus.

Figure 3.1. Microscope photographs (40x magnification) of *L. bicolor* S238N mycelium after 14 days of dual culture with different *Collimonas* strains or with water (control).



III.3 Meta analysis of the transcriptomic response of *L. bicolor* S238N to soil bacteria

III. 3.1 Response of *L. bicolor* S238N to *P. fluorescens* Pf29A and *Burkholderia* sp. EJP73

III.3.1.2 Material and methods.

Confrontation assay

We used the set up previously described (Deveau *et al.* 2007). For each treatment, three biological replicates were realized in may and june 2005 for *P. fluorescens* Pf29A and in june 2005 and march 2006 for *Burkholderia* sp. EJP73.

cDNA arrays

The protocol previously described was used (Deveau *et al.* 2007). Briefly, the mycelium was collected after 14 days of incubation in the presence or not of the two bacterial strains *P. fluorescens* Pf29A or *Burhholderia* sp. EJP73. cDNA probes were synthezised from RNA and were hybridized to 4992 cDNA nylon arrays.

Dotblot

Each responsive cDNA and four non responsive cDNA (Lb17E10, Lb01F08, a GTPase and a metalloprotease) were PCR amplified. The concentration of PCR products was measured on electrophoresis and then adjust at 5 ng.μL^{-1}. Fifty ng of each DNA sample was added to 20 μL of a denaturing solution (SSC 10X, 0.2N NaOH, 0.5% of xylene cyanol) and then spotted on a pre-treated nylon membrane as described by Voiblet *et al.* (2001). Each of the non responsive cDNA were spotted twice. The cDNA probes previously synthesized for cDNA arrays experiments were used for probes labelling and hybridization.

III.3.1.2 Results

Alteration of *L. bicolor* S238N by *P. fluorescens* Pf29A and *Burkholderia* sp. EJP73

No statistical differences between the bacterial and the control treatments were observed according to the PPDE test, in the presence of *P. fluorescens* Pf29A and of *Burkholderia* sp. EJP73. The PPDE test is a computational method performed after a

Table 3.2. Transcripts of *L. bicolor* S238N regulated in the presence of the strain *P. fluorescens* Pf29A. The cDNA clone ID, the identity of the best blastX result and the transcript ratio (*P. fluorescens* Pf29A treatment vs *L. bicolor* S238N control) are given. Nd : non determined, ns : non significant, hv : high variability.

Protein ID	Best Blast X identity	Clone n°	EST n°	Array	Dotblot	qPCR
126393	histone acyditransferase Tra1 [*Phanerochaete chrysosporium*]	LbIIf26A9	EL739455	3,8	2,1	2.6
190777	mismatched base pair and cruciform DNA recognition protein [*Agaricus bisporus*]	LbIIF20H10	EL739279 EL739280	431,8	2,2	nd
314494	GTP-binding protein rho1 [*Schizosaccharomyces pombe*]	LbIIIf21E11	EL740115	2,9	2,0	nd
312775	60S ribosomal protein L24 [*Cryptococcus neofrmeans*]	LbIIF25A2	EL739393 EL739394	2,8	3,8	nd
311269	Tectonin 2 [*Physarum polycephalum*]	LbIIF21A8	EL739294	4,1	2,5	9.2
192419	Hypothetical protein	Lb19E05	JGI_LbEX1670	2,2	2,1	nd
295051	Hypothetical protein	LbIIIf10A5	EL739867	2,9	2,4	nd
	Hypothetical protein	LbIIF21B8	-	2,6	2,7	nd
310896	Hypothetical protein	Lb13D03	JGI_LbEX329	2,5	3,0	nd
	Hypothetical protein	LbIIf22C3	-	3,8	7,0	nd
305825	Hypothetical protein	LbIIF13B7	EL739136	6,4	12,0	nd
		LbIIf17A9	EL739213	2,9	5,0	nd
308883	Hypothetical protein	LbIIIf05E8	EL740199	2,2	1,9	nd
388095	Hypothetical protein	LbIIF01A1	EL738647	2,4	hv	nd
		LbIIf15C7	EL739198	3,9	2,1	nd
		LbIIIf10H7	EL739919	9,2	2,4	nd
381807	Hypothetical protein	LbIIf04C9	EL738864	4,4	3,0	nd
		LbIIf22F5	EL739370	2,5	6,0	nd
		LbIIF09A1	EL739031	2,7	ns	2.2
		LbIIF12F9	EL739107	2,5	ns	nd
		LbIIf19B10	EL739227	2,2	2,1	nd
		LbIIIf20A10	EL740053	2,4	2,2	nd
		LbIIF16C11	EL739206	2,2	hv	nd
		LbIIF20H3	EL739282	2,3	hv	nd
		LbIIIf24G11	EL740126	2,6	hv	nd
		LbIIIf25A12	EL740135	3,0	2,7	nd
291363	Hypothetical protein	LbIIF13H4	EL739194	6,0	ns	nd
		LbIIF13H9	EL739195	30,5	2,3	nd
		LbIIF21A5	EL739287	4,0	3,5	nd
		LbIIIf21D4	EL740114	3,2	4,0	nd

Bayesian *t*-test for estimating experiment-wide false positive and negative levels based on the modeling of the Bayes p-value (Baldi & Long, 2001). We determined a list of 43 Pf29A-responsive cDNAs corresponding to 28 genes for which the Bayes p-value was under 0.01. The variation of concentration of these transcripts was controlled by dotblot, and by quantitative PCR for three targets (Tra 1, Tectonin 2 and a hypothetical protein). The significant variation of transcript concentrations was confirmed for 16 genes (Table 3.2). All were up-regulated and level of regulation varied between two- and tenfold. Eleven encoded hypothetical proteins. The five remaining genes encoded for the tectonin 2 (unknown function), a Rho GTP binding protein (signal transduction), the Tra1 subunit (transcription regulation), a mismatched base pair and cruciform DNA recognition protein (DNA binding and repair) and a 60S ribosomal protein (Protein translation). By contrast, the high variability between each biological replicate of the EJP73 treatment did not allow us to identify EJP73-responsive genes.
The results are discussed in the paragraph III.3.2

Variability between replicates

We have noticed a high variability of signal intensities between replicates, whatever the bacterial treatment used. As a consequence, many responsive genes were probably missed. It is particularly striking in the case of the EJP73 experiment. When each replicate was analyzed separately, about a hundred of genes were found regulated in the presence of the bacteria. This variability can come either from technical problems or either from the existence of several biological responses. The main step that could generate artificial variability is the mycelium sampling. We have noticed that some bacterial treatments induced structural modifications of the mycelium that made its sampling with a razor blade more difficult. Another explanation would be that the observed heterogeneity reflected a real biological diversity of response of the fungus depending on uncontrolled environmental parameters. We have already observed that the *in vitro* behaviour of the fungus was time-regulated: the helper bacteria have no or less promoting effect on the fungal growth during summer because the fungus itself grows very fast during this season even if it is kept at 10°C in the dark. The differences would result from changes in the physiological status of the fungus at the two times of sampling. Nevertheless, when all the biological replicates were performed simultaneously, the variability remained. This could suggest that several different responses would be

produced at the molecular level (Pouteau *et al.* 2007). Indeed, it has been shown that cells from a same tissue that have been submitted to the same treatment presented a tremendous variation in their transcriptomic response (Levsky *et al.* 2002). It implies that there is not a single deterministic response to a stimulus but that cells function as a chaotic model, mainly because of the Brown movements. Specific experiments will be needed to better understand the origin of the observed variability. The forthcoming acquisition of a microdissection laser microscope by our laboratory will allow us to test the different hypotheses because it will permit us to compare the response of different individual fungal cells to the bacterial stimuli.

III.3.2 Comparative response of *L. bicolor* S238N to the presence of different soil bacteria

In the previous parts (III.1, III.2, III.3.1), we have described the response of the ectomycorrhizal fungus to the presence of beneficial and antagonistic bacteria in its close environment and to two quorum sensing signal molecules. In a second time, we have compared all together the data sets that we have obtained with the three strains BBc6R8, Pf29A, Ter331 and the QS molecule 3,O-C12-HSL (Table 3.3, figure 3.2). Responsive genes were over expressed in the presence of the two growth-promoting bacterial *P. fluorescens* strains (BBc6R8 and Pf29A) while most of the responsive genes were down regulated in the presence of the antagonistic bacteria. A reduced fungal growth was linked to a decrease of the basal metabolic activity of the fungus. But with the exception of this correlation between the fungal growth and the type of fungal gene regulation, few shared responsive genes were identified: each bacterial strain and the 3,O-C12-HSL QS molecule induced a specific pattern of gene regulation. The *tectonin2* was the only responsive gene that did not depend on the treatment and *Tra1* was the only gene regulated in the presence of the three bacterial strains. Therefore, these two proteins probably play an important role in the interactions with bacteria. They appear to be good candidates as marker genes of fungal-bacterial interactions. Only 4 % of the responsive genes were regulated in at least two treatments (figure 3.2). Similarly, the regulated metabolic pathways were different depending of the bacterial treatment.

Table 3.3. Number of transcripts showing either a positive or a negative variation of concentration after 14 days of dual culture in the presence of *P. fluorescens* BBc6R8, *P. fluorescens* Pf29A, *C. fungivorans* Ter331, or after 14 hours of incubation with C6-HSL or 3,O-C12-HSL, in comparison to a control treatment. The effect of each bacterial strain on the fungal growth is given under. Finally, pie charts indicate the putative functions of the responsive transcripts. Data are expressed as a percentage of the total number of transcripts regulated in each treatment.

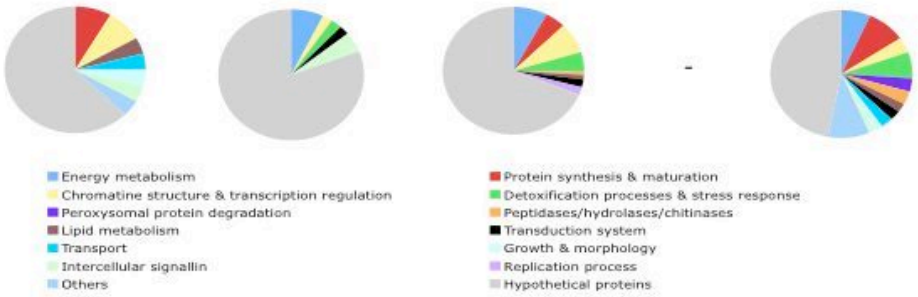
	P. fluorescens BBc6R8	*P. fluorescens* Pf29A	*C. fungivorans* Ter331	C6-HSL	3,O-C12-HSL
Significant variations of the transcript concentrations	38 ↗ 0 ↘	41 ↗ 2 ↘	24 ↗ 131 ↘	ns	54 ↗ 111 ↘
Effect on fungal growth	++	+	-	nd	nd
Putative functions of the genes products				-	

Figure 3.2. Wen diagram of *L. bicolor* S238N responsive-genes to the presence of different soil bacteria or QS signal molecule.

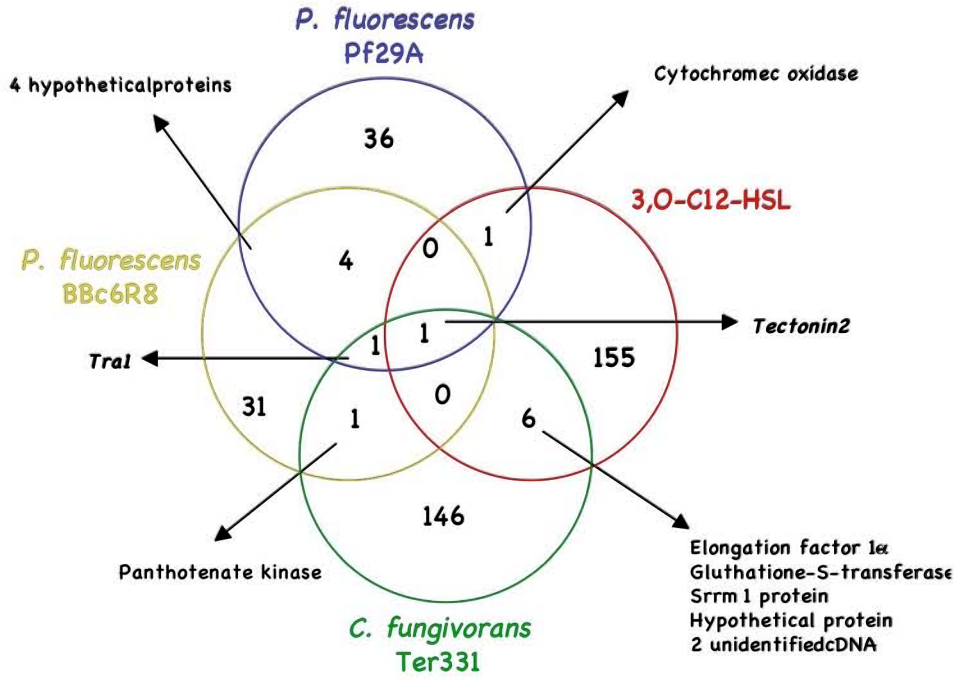


Table 3.4. Transcripts of *L. bicolor* S238N significantly regulated only in the presence of the helper strain *P. fluorescens* BBc6R8. The cDNA clone ID, the identity of the best blastX result and the transcript ratio (*P. fluorescens* BBc6R8 treatment vs *L. bicolor* S238N control) are given.

Clone number	Identity	Fold
Lb2F20F7	Efflux protein, putative-MFS family [*Cryptococcus neoformans var. neoformans* JEC21]	6.4
Lb2F25F1	Heat shock protein [*Schizosaccharomyces pombe*]	2.0
Lb3f05F10	Histone H4,2 [*Phanerochaete chrysosporium*]	2.1
Lb3f23G6	Meiotic recombination related protein [*Cryptococcus neoformans var. neoformans B-3501A*]	2.4
Lb01G22 Lb17G09	Splicing factor 3b, subunit 4 [*Danio rerio*]	8.7
Lb2F32B10	Hypothetical protein (no hit)	5.1
Lb2F08F2	Hypothetical protein (no hit)	3.8
Lb2F13C10, Lb3F06G1, Lb3F27C3	Hypothetical protein (no hit)	3.5
Lb2f17D12	Hypothetical protein (no hit)	3.5
Lb2F22B11	Hypothetical protein (no hit)	3.4
Lb3f16F12	Hypothetical protein (no hit)	3.3
Lb3f21C12	Hypothetical protein (no hit)	3.3
Lb2F18F10	Hypothetical protein (no hit)	3.1
Lb2F24H2, Lb3F01A1, Lb3F16F12, Lb3F10H7	Hypothetical protein (no hit)	3.1
Lb2F31E7	Hypothetical protein (no hit)	3.1
Lb2f15A7	Hypothetical protein (no hit)	3.0
Lb07F10	hypothetical protein (no hit)	2.6
Lb2F18G12	Hypothetical protein (no hit)	2.4
Lb03E13	hypothetical protein (no hit)	2.3

III.3.3 Towards the identification of "helper genes"

Through this comparative analysis, one should ask whether it is be possible to identify genes that would be specifically expressed or regulated in the presence of the helper bacterial strain *P. fluorescens* BBc6R8. These genes could then be used as indicators of the helper effect, notably to identify new mycorrhiza helper bacterial strains. The monitoring of their expressions could also be a useful tool for screening bacterial signal molecules or bacterial mutant libraries, for example (Riedlinger *et al.* 2006). Twenty responsive genes (31 cDNA) were specifically regulated in the presence of the helper strain and appeared to be good candidate genes of the helper effect of *P. fluorescens* BBc6R8 (Table 3.4). However their low level of regulation associated with their high variability of response make their use difficult. Moreover, so far, we have analyzed the effect of only a small set of bacteria that have a contrasted effect on the fungus. A comparative analysis of the expression of these candidate genes should be done using a larger set of bacterial strains and soil microorganisms. We can assume that each candidate gene can also be regulated in other conditions. Therefore, we more expect some gene networks to be specifically regulated in the presence of the helper bacteria rather than isolated genes.

These specific "helper genes" could also provide information about the mechanisms of the helper effect. The biocontrol Pf29A and the MHB BBc6R8 that both improve the *L. bicolor* growth *in vitro*, mainly induced different set of genes, indicating that different molecular pathways can lead to an *in vitro* growth increase. It confirmed that the improving of the fungal growth *in vitro* is not sufficient to induce a helper effect and that other mechanisms should be involved. The "helper genes" we identified so far encoded for a putative efflux protein of the MFS family, a splicing factor, a heat shock protein and several hypothetical proteins (Table 3.4). A functional characterization of these proteins as well as the knock out of these genes will be required in order to better understand the molecular mechanisms involved in the helper effect.

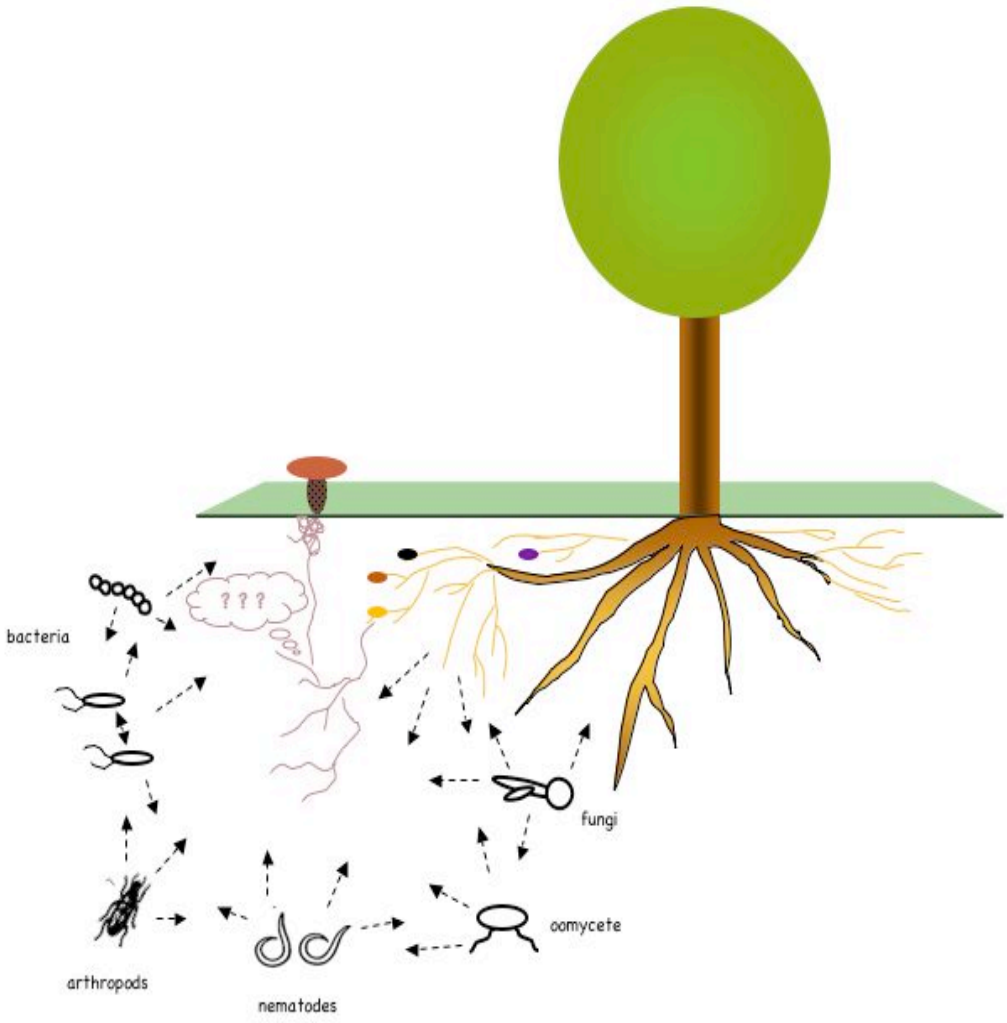
III.3.4 Fungal perception of surrounding bacterial activities

Up to now, only few informations are available concerning the mechanisms by which ectomycorrhizal fungi perceive and reliably react to modifications of their environment and more especially to their surrounding bacteria. Our studies have shown that *L. bicolor* S238N is able to perceive both antagonistic and non-antagonistic bacteria before a

physical contact and to react differently depending on the strain. Important differences in the responses were observed suggesting a high degree of specificity. In a comparative meta-analysis of the yeast responses to various environmental stresses, Gasch *et al.* (2002) demonstrated that a core set of genes was always regulated while others were more specific of each stress. In the present study, less than a third of the transcriptome of the fungus was investigated by using the cDNA arrays. Therefore, further experiments are needed to determine the level of specificity of the transcriptomic response of the fungus towards surrounding bacteria.

The remaining question to answer is do the bacteria produce molecules that are directly perceived by the fungus or does the fungus react to environmental modifications resulting from the metabolic activity of the bacteria. The two alternatives have been illustrated in the literature. It was proved that bacteria secrete a wide variety of secondary metabolites and signal molecules that directly interfere with eukaryotic cell functioning (cf. introduction chapter). But bacteria also modify their environment through pH modification, nutrient uptake, degradation activity, nitrification… Here, we showed that *L. bicolor* S238N is, at least, able to perceive a QS molecule. We hypothesize that the ectomycorrhizal fungus is able to sense the nutritional status from its surrounding environment through both chemical (pH, nutrient availability, humidity) and biological indicators (QS molecules).

Figure 3.3. Schéma des interactions complexes qui se déroulent entre les orgnanismes du sol.



Conclusion

La réponse induite par la souche auxiliaire de la mycorhization *P. fluorescens* BBc6R8 diffère nettement de celles provoquées par d'autres bactéries du sol. Plusieurs gènes surexprimés uniquement en présence de BBc6R8 ont été identifés.

Par ailleurs, notre analyse a montré que le champignon ectomycorhizien *L. bicolor* S238N est capable de percevoir la présence de différentes bactéries avant l'établissement d'un contact physique entre le mycélium et les cellules bactériennes et d'y réagir par des modifications transcriptomiques. A l'exception de la molécule bactérienne de QS 3,OC12-HSL dont nous avons montré l'effet sur le transcriptome fongique, les signaux biotiques ou abiotiques reconnus par les champignons ectomycorhiziens restent méconnus. Dans cette assemblée de bavards que constitue la microflore des sols, les mots employés et compris par chaque micro organisme forment une boîte noire qui reste à explorer (Figure 3.3). De plus en plus d'exemples montrent que des organismes eucaryotes sont capables de percevoir des signaux générés et utilisés par les organismes procaryotes ; *L. bicolor* S238N en fait partie. Il reste à déterminer s'il s'agit d'un véritable dialogue ou seulement d'espionnage.

Chapter IV

Focused analysis of some molecular mechanisms

Chapitre IV

Etude ciblée de quelques mécanismes moléculaires

Introduction

Parallèlement aux approches sans *a priori* de transcriptomique, nous avons mené des analyses avec *a priori* afin de mieux comprendre le rôle joué par certaines protéines dans l'effet auxiliaire. Notre attention s'est portée sur trois cibles: d'une part les protéines « tectonines-like », d'autre part quelques exoenzymes impliquées dans la mobilisation de la matière organique et enfin le système bactérien de sécrétion de type III.

Notre approche transcriptomique a montré que le transcrit d'une « tectonine-like » était accumulé dans les cellules de *L. bicolor* S238N lorsque celles-ci étaient en présence de diverses bactéries. Les tectonines appartiennent à la famille des tachylectines et ont été décrites pour la première fois chez le myxomycète *Physarum polycephalum* (Huh *et al.* 1998). Elles seraient impliquées dans la fixation des bactéries dont se nourrit le myxomycète, lors du processus de phagocytose. Dans ce contexte, la surexpression d'une « tectonine-like » par *L. bicolor* S238N en présence de bactéries est particulièrement intrigante, d'autant plus que cette protéine ne semble pas exister chez d'autres champignons. Nous avons donc cherché à savoir plus précisément quel rôle joue cette protéine dans les interactions entre *L. bicolor* S238N et les bactéries, et de quelle manière le champignon l'a acquise.

Un second point mis en avant par notre approche transcriptomique est l'accumulation de transcrits codant pour des exoenzymes présumées être impliquées dans l'activité saprophytique du champignon. Bien que l'essentiel du carbone consommé par les champignons ectomycorhiziens soit fourni par les plantes par l'intermédiaire des mycorhizes, les champignons ectomycorhiziens disposent d'une large gamme d'enzymes extracellulaires capables de dégrader la matière organique et donc de libérer des sources de carbone métabolisables par les cellules (Courty *et al.* 2005). Ces enzymes jouent, entre autres, probablement un rôle fondamental pour la survie du champignon lors de son développement pré-symbiotique. En effet, à ce stade, les seules sources nutritionnelles dont dispose le mycélium proviennent des réserves accumulées dans les spores. Or celles-ci sont principalement consommées lors de la germination. Dès lors, un accroissement de l'activité saprophytique du champignon pourrait s'avérer bénéfique pour sa survie. De même, les activités de mobilisation des nutriments développées par les bactéries environnantes pourraient améliorer la nutrition du champignon qu'elles colonisent. Nous avons donc entrepris une étude des capacités enzymatiques des deux partenaires.

Enfin, nous nous sommes intéressés au système de sécrétion de type III (TTSS) de *P. fluorescens* BBc6R8. Des études préliminaires menées par G. Preston *et al.* (Université d'Exeter, Royaume-Uni) ont montré que la souche BBc6R8 dispose de ce système de sécrétion. Pendant longtemps, ce système d'injection de facteurs de virulence au sein de cellules hôtes a été considéré comme une spécificité des bactéries pathogènes. Or des études récentes ont montré que le TTSS était répandu au sein des bactéries Gram négatives (Marie *et al.* 2001, Mazurier *et al.* 2004). Son rôle chez les bactéries non-pathogènes est à l'heure actuelle très discuté. Nous nous sommes donc interrogés sur son fonctionnement chez *P. fluorescens* BBc6R8 et plus particulièrement sur son implication dans l'interaction avec *L. bicolor* S238N.

IV. 1 Characterization of *L. bicolor* S238N tectonins-like proteins

Tectonins belong to the tachylectin-related proteins group of lectins (Mali *et al.* 2006). They have been discovered in the slime mold *Physarum polycephalum* in which they were proposed to facilitate the phagocytosis of bacteria by binding to bacterial cell wall components (Huh *et al.* 1998). Similarly, the tachylectins-related proteins bind bacterial lipopolysaccharides and seem to function in innate immunity (Saito *et al.* 1995, Nagai *et al.* 1999, Chen *et al.* 2001, Schröder *et al.* 2003, Galliano *et al.* 2003, Tsoi *et al.* 2004). In the horseshoe crab *Tachypleus*, tachylectins bind various components of bacterial cell walls and would be involved in the agglutination of bacteria colonizing the hemolymph of the crab (Saito *et al.* 1995). Similarly, tachylectins from carp eggs interact with both Gram negative and postive bacteria and are thought to be involved in fish immunity (Galliano *et al.* 2003) All of them are structurally characterized by having six tandem repeats of 31-37 amino acids in length, forming a ß-propeller structure (Fülöp & Jones, 1999). Their occurrence and function in innate immunity seem to be conserved in metazoan, at the exception of the *Hydractinia echinata* tachylectin that was proposed to play a role during neuronal development (Mali *et al.* 2006). Interestingly, this class of proteins has never been described in any fungus or plant.

Ectomycorrhizal fungi (ECM) play a fundamental role in the hydro-mineral nutrition of trees by establishing a symbiotic association with tree roots. They provide water and minerals to their host plants that conversely feed the fungi with photoassimilates (Smith & Read, 1997). The formation and the functioning of the symbiosis are influenced by interacting bacteria. Among those, the mycorrhiza helper bacteria (MHB) promote the mycorrhiza formation (Frey-Klett & Garbaye, 2007). While we were studying the molecular mechanisms of the interaction between the ECM *Laccaria bicolor* S238N and the MHB *Pseudomonas fluorescens* BBc6R8, we identified two transcripts that showed a high similarity with the *P. polycephalum* tectonins (Deveau *et al.* 2007). The gene expression of one of them was up regulated before contact between the mycelium and the MHB bacterial colony, and till few hours after the establishment of a physical contact. Further analysis showed that it was also overexpressed in the presence of other rhizobacteria such as the biocontrol strain *P. fluorescens* Pf29A and the mycophagous strain *Collimonas fungivorans* Ter331 (Deveau *et al.* 2007). It was also up-regulated in the presence of the 3,O-C12-HSL quorum sensing signal molecule (Deveau *et al.* 2007

unpublished). Because of the role of tachylectin-related proteins in metazoan interactions with bacteria, we hypothesised that the fungal tectonins could function in the physical interaction between ECM and bacteria. Interestingly, the tectonin-like genes could not be detected in the fungal genomes of the *Coprinopsis cinerea*, *Phanerochaete chrysosporium*, *Cryptococcus neoformans* and *Neurospora crassa*, suggesting that they may be specific of the ectomycorrhizal fungus of *L. bicolor* S238N among the fungal taxa. An hypothesis would be that the gene have been introduced in the fungal genome by an horizontal transfer mediated by a bacterial vector.

In this context, we questioned more precisely the origin of the *L. bicolor* S238N tectonin-like genes and their role in the fungal physiology during its interaction with bacteria. We looked for the presence of tectonin-like genes in all the fungal and bacterial sequenced genomes and we analyzed the primary and secondary structures of the *L. bicolor* S238N sequences by comparison with eukaryotic closely related-ones. Then, we measured the tectonin-like transcript concentrations in various fungal tissues and the protein expression during the interaction with rhizobacteria.

IV. 1.1 Materials & methods

In silico analysis

Lbtect1 and *Lbtect2* gene and protein sequences (Protein ID 382412 and 399270) are available on the Join Genome Institute Laccaria Portal (http://genome.jgi-psf.org/Lacbi1/Lacbi1.home.html). The protein molecular mass was calculated with the Compute pI/MW tool of ExPASy (http://expasy.org). Signal peptide and transmembrane domain were searched using the SignalP 3.0 and THMM 2.0 servers (http://www.cbs.dtu.dk/services/).

A NCBI BLASTX search on protein databases was used to identify proteins with sequences similar to the *L. bicolor* tectonins. Sequences were aligned using the ClustalX 1.83.1 program. Phylogenetic tree was generated with the PAUP 4.0b10 program, using a Neighbor Joining algorithm. The TSL1 sequences of *Phanerochaete chrysosporium*, *Coprinopsis cinerea* and *Ustilago maydis* served as an out group.

A NCBI tBLASTN search on the fungal and microbe genome databases was used to identify similar sequences to the *L. bicolor* tectonins on the released sequenced of bacterial and fungal genomes.

Microorganisms and in vitro confrontation bioassay.

The ectomycorrhizal basidiomycete *Laccaria bicolor* S238N (Maire P. D. Orton) was maintained on Pachlewski agar medium P5 (Di Battista *et al.* 1996) at 25°C during three weeks. *Pseudomonas fluorescens* BBc6R8 and *Bacillus subtilis* MB3 are two helper bacterial strains (Duponnois & Garbaye 1991, Frey-Klett *et al.* 1997). *Pseudomonas fluorescens* Pf29A is a biocontrol against the wheat pathogen *Gaeumannomyces graminis* var *tritici* (Chapon *et al.* 2002). The three strains were maintained at -80°C in Luria-Bertani Medium (Sambroock *et al.* 1989) with 20% glycerol added. In the present work, the bacterial strains were first grown on 10 % TSA plates (Tryptic Soy Broth from Difco and 15 $g.L^{-1}$ of agar) at 25°C for 65 hours to prepare the bacterial inoculum for the *in vitro* bioassay. The bioassay was prepared as described by Deveau *et al.* (2007). Briefly, four droplets of the bacterial solution (bacterial treatment) were distributed at 1.7 cm from the centre of a fungal plug. A control treatment where bacteria were replaced by sterile water was performed. Plates were incubated at 10°C in the dark during 14 days.

Gene expression

The *Laccaria* whole-genome expression array manufactured by NimbleGen (Madison, WI) contains in duplicates eight independent, non-identical, 60-mer probes per whole gene model. Included in the microarray are 20 614 annotated gene models (genome sequence v1.0), 1 680 additional predicted gene models, 30 000 random 60-mer control probes and labeling controls. Free-living mycelium of *L. bicolor* S238N was grown onto cellophane-covered agar plates containing Pachlewski medium (Di Battista *et al.* 1996), and was grown for three weeks before harvesting the proliferating hyphal tips at the periphery. Ectomycorrhizae of *L. bicolor*/Douglas fir were synthesized by growing Douglas fir seedlings for nine month in polyethylene containers filled with a peat-vermiculite mix (1:1, v/v) and mixed with 2.5% (v/v) fungal inoculum. *L. bicolor*/Poplar ectomycorrhizae were synthesized either by growing cuttings of *Populus trichocarpa* for three month in pots containing Terragreen (Brenntag Lorraine, Toul, France) mixed with fungal inoculum in a peat-vermiculite mix (4:1, v/v) or by growing *in vitro Populus tremula x alba* (INRA clone 717-1B4) plantlets together with fungal inoculum for one month as described in (Handbook of Methods used in Rhizosphere research, 2006, editors Joerg Luster and Roger Finlay, p399, Swiss Federal Research Institute WSL). Fruiting bodies of *L. bicolor* S238N were collected below Douglas fir seedlings grown in a greenhouse and inoculated using *L.*

bicolor S238N as described (Di Battista *et al.* 1996). Tissues were immediately frozen in liquid nitrogen and RNA extraction was carried out using the RNeasy Plant Mini Kit (Qiagen). Total RNA preparations (two biological replicates for each sample) were amplified using the SMART PCR cDNA Synthesis Kit (Clontech) according to the manufacturer's instructions. Single dye labeling of samples, hybridization procedures, data acquisition, background correction and normalization were performed at the NimbleGen facilities (NimbleGen Systems, Reykjavik, Iceland) following their standard protocol (Irazzari *et al.* 2003). Average expression levels were calculated for each gene from the independent probes on the array and were used for further analysis. Array data from 3814 gene models were excluded from the analysis due to the absence of minimum one reliable oligo probe. Log2-transformed data were calculated and were subjected to the CyberT statistical framework (http://www.igb.uci.edu/servers/cybert/). It employs statistical analyses based on simple *t*-tests that use the observed variance of replicate gene measurements across replicate experiments, or regularized *t*-tests that use a Bayesian estimate of the variance among gene measurements within an experiment. Cyber-T also contains a computational method (PPDE) for estimating experiment-wide false positive and negative levels based on the modeling of *p*-value distributions. Transcripts with a PPDE above 0.95 and a more than 2.0 change in transcript level were considered as significantly differentially expressed in the *Laccaria* tissues. To estimate a cut-off level for expression, the mean intensity of the 30 000 random probes present on the microarray was calculated. Gene models with a threefold higher intensity were considered as transcribed. The complete expression dataset is available as series (accession number) at the Gene Expression Omnibus at NCBI (http://www.ncbi.nlm.nih.gov/geo/).

Protein extraction.

Forty milligrams of mycelium were grinded in a mortar with liquid nitrogen and soluble proteins were extracted as previously described by Dos Santos *et al.* (2005). Protein concentrations were measured with the BCA method (BioRad) following the protocol provided by the manufacturer and controlled by SDS-PAGE gel electrophoresis.

Antibodies preparation

Polyclonal antibodies against Lbtect2 peptides MPWKGISGSLSRISAC and GEIYRYTGDQGDPNH were prepared by Eurogentec (Liège, Belgium), following the Double XP program. Briefly, synthesised peptides were conjugated to carrier proteins. Then two rabbits were immunizated with a mix of the two coupled peptides in a 3-months protocol. After three months, total bleed was collected and the antisera were affinity purified.

Western blotting

Proteins were separated using a SDS-PAGE (15%, w/v) gel and electrotransfered onto a PVDF membrane (Immobilon-P membrane, Millipore), following the protocol recommended by the manufacturer. Then, membranes were incubated with anti-tectonin polyclonal antibodies diluted 1:500 in a TBST solution (8 g.L-1 NaCl, TriHCl 0.2M, 0.1% Tween 20, 10% p/v non fat milk) then washed four times in a TBST solution. The revelation was done using the Amplified alkaline phosphatase goat anti-rabbit immunoblot assay kit (BioRad), following the recommendation provided by the manufacturer.

IV. 1.2 Results

ORF and protein structure

Two tectonin-like genes (*LbTect* 1 and 2) were identified in the genome of *L. bicolor* H82-S238N. They are located on the scaffold 4 (position 616091-617941, *Lbtect* 2) and 5 (Position 1655832-1656841, *Lbtect* 1), and both contain an open reading frame (ORF) of 684 bp corresponding to 227 amino acid residues with a predicted molecular mass of 23,8 kDa. The two LbTect proteins share 85% of amino acids. No signal peptide neither transmembrane domain were detected. Similarly, no N-terminal galactose binding ricin site was found, contrary to the tectonin 2 from *P. polycephalum*. The GC% content of *Lbtect1* and *Lbtect2* sequences were 55 % and 56 %, respectively. By contrast, the average GC content of the assembled *Laccaria* genome is 46 %. The two tectonin-like proteins are entirely comprised of six ß-propeller TECPR (Inter Pro accession number IPR006624) tandem repeats of 34-36 amino acid residues joined by linkers of 1-5 amino acids (Figure 4.1).

Figure 4.1. Aligned repetitive sequences of LbTect1 (A), LbTect2 (B) and the TECPR motif (IPR006624).

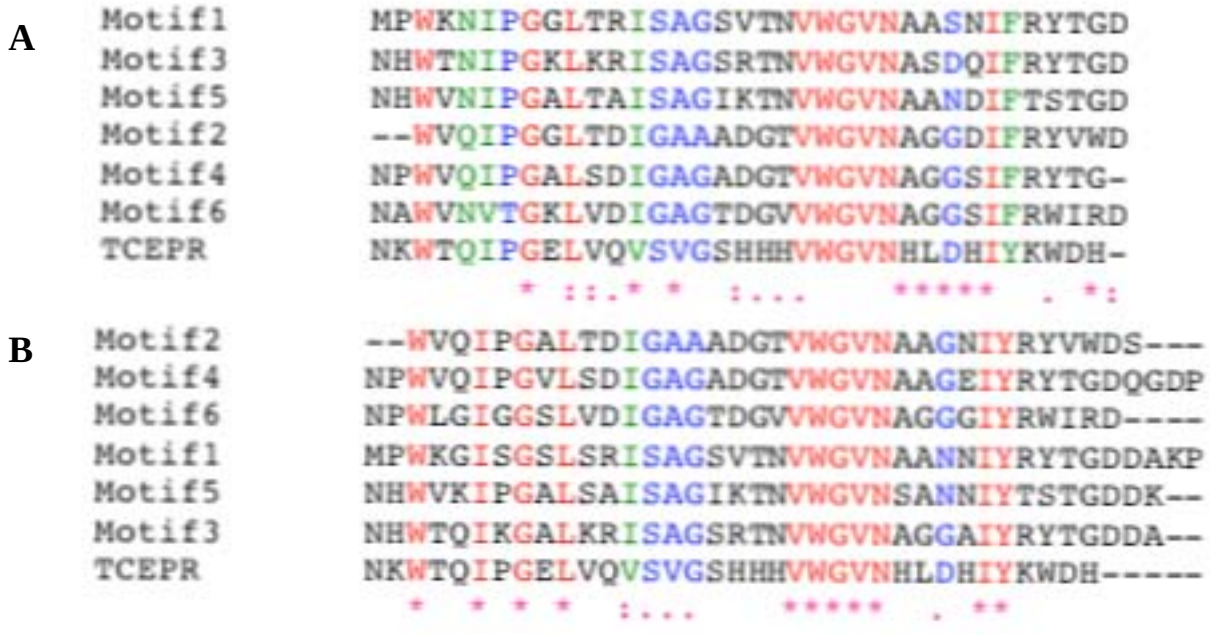


Figure 4.2. Secondary structures predicted for the LbTect1 and LbTect2. For each protein, the preference for each structure was determine using the GORIV algorithm (Garnier *et al.* 1996). "c" indicates a random coil, "e" a ß-sheet, and "h" an α-helix domain. The predicted secondary structure of *P. polycephalum* tectonins is also given.

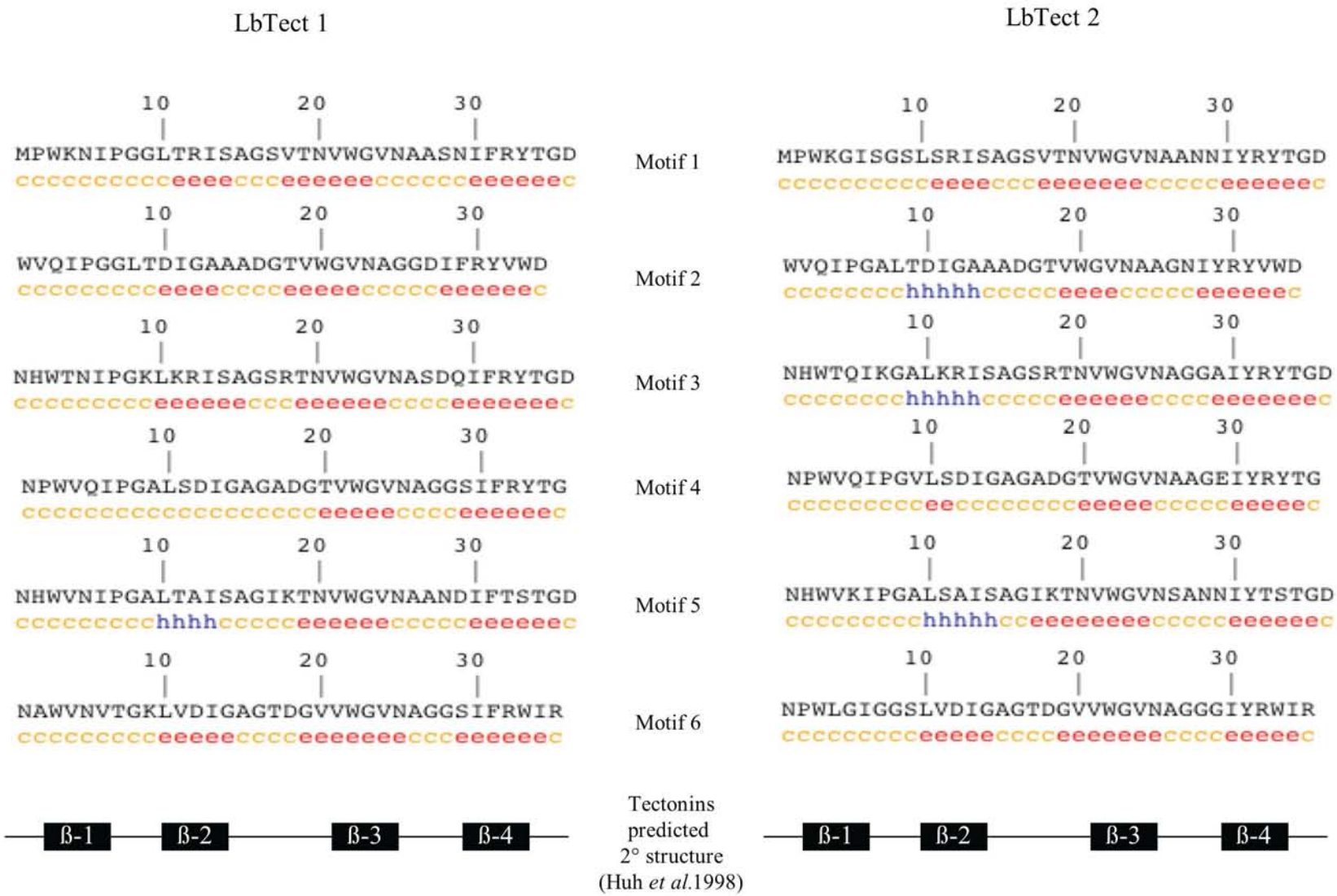
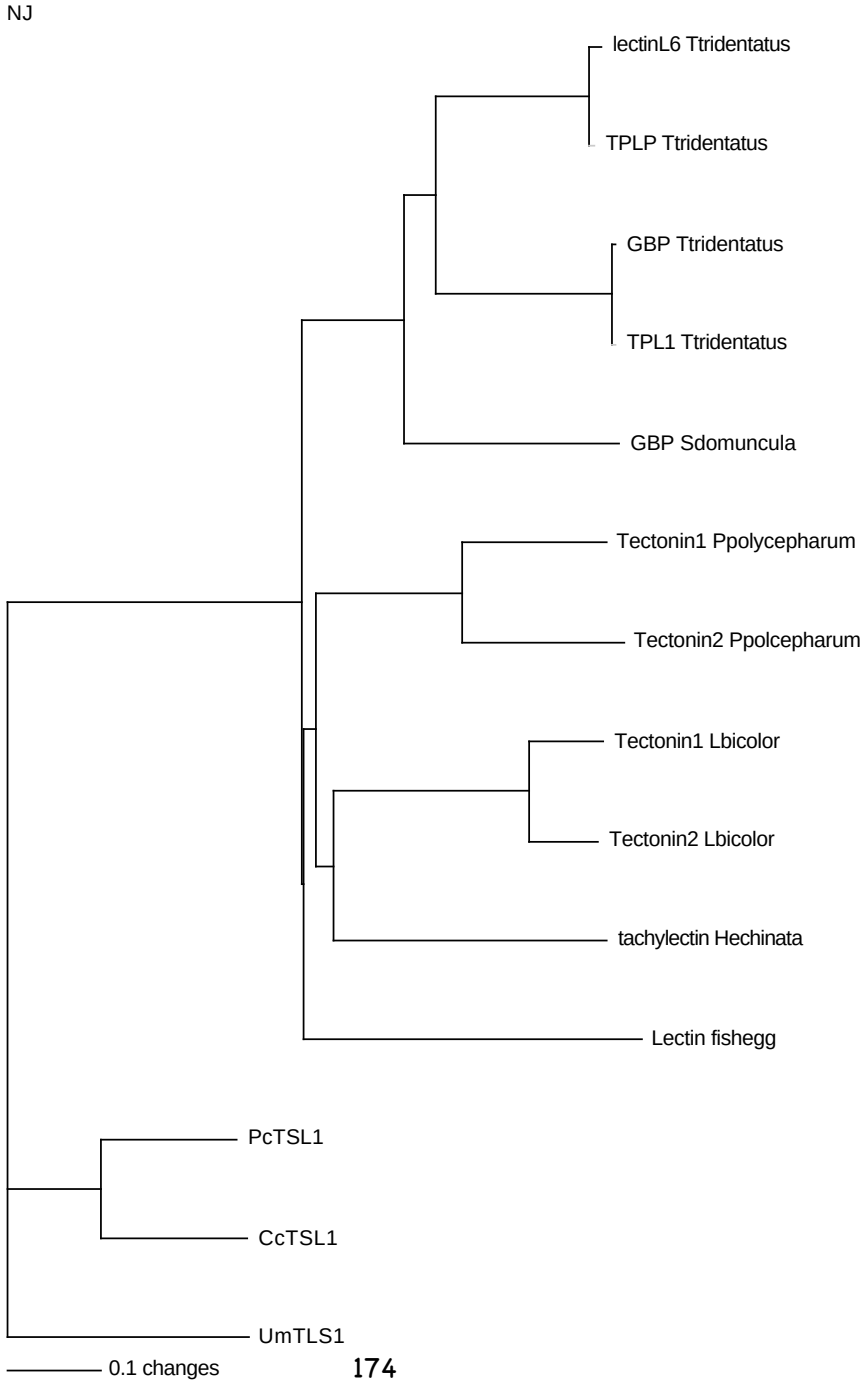


Figure 4.3. Neighbor-joining tree showing the phylogenetic relationship between *L. bicolor* tectonin 1 and 2 and identified tachylactins: Lectin L6 *Tachypleus tridentatus* P82151, TPLP Tachylectin-P *Tachypleus tridentatus* BAA88574, GBP *Tachypleus tridentatus* AAF74773, TPL *Tachypleus tridentatus* BAA88574, GBP *Suberites domuncula* CAD79378, Tectonin 1 *Physarum polycephalum* O61063, Tectonin 2 *Physarum polycephalum* O61064, tachylectin *Hydractinia echinata* CAI77215, lectin fish egg *Cyprinus carpio* P68512. The sequences of the TSL1 trehalose synthase frome *Ustilago maydis, Coprinopsis cinerea* and *Phanerochaete chrysosporium* were used as outgoup sequences.



Predicted secondary structure

Primary structures from tachylectin-like and tectonin proteins were proposed to form ß-propeller domains (Huh *et al.* 1998, Fülöp *et al.* 1999, Mali *et al.* 2006). In these proteins, each repeat appeared to form a four-strand ß-sheet. The GORV algorithm (Garnier *et al.* 1996) predicted the presence of 20 and 19 ß-strands separated by random coils in the LbTect1 and LbTect2 secondary structure, respectively (Figure 4.2). The presence of one and three α-helixes was also calculated, respectively. By comparison, the GORV algorithm predicted the existence of 20 ß-strands and three to two α-helixes in each *P. polycephalum* tectonins while the *H. echinata* tachylectin-like protein would contain 26 ß-strands and three α-helixes.

Phylogeny analysis

The amino acid sequence was the most similar to the two tectonins of the slime mold *P. polycephalum* and to the tachylectin-like of the cnidaria *Hydractinia echinata* (Figure 4.3). No similar sequence was found in the 67 sequenced fungal genomes, excepted in the genome of the yeast *Kluyveromyces waltii* NCYC 2644 (E value $1e^{-6}$, 26% of identity, 39 % of similarity). By contrast, a significant identity was found in eight bacterial genomes (Table 4.1). Furthermore, the TECPR domain that is characteristic of the lectin L6 (Saito *et al.* 1995), the tectonins and of tachylectins has not been found in any of the sequenced fungi (figure 4.4), according to the InterPro database (http://www.ebi.ac.uk/interpro). It is mainly found in metazoan. It was only detected in six bacteria (Table 4.2, *Shewanella woodyi* ATCC 51908, *Lyngbya* sp. PCC8106, *Nodularia spumigena* CCY9414, *Loktanella vestfoldensis* SKA53, *Stigmatella aurantiaca* DW4/3 and *Geobacter* sp. FRC-32) and two algae (*Ostreococcus lucimainus* and *tauri*).

Gene expression in fungal tissues

We have shown in previous studies that the *Lbtect2* gene was over expressed in mycelium during the interaction with several rhizobacteria and in the presence of the 3,O-C12-HSL quorum sensing molecule (Deveau *et al.* 2007, Deveau *et al.* unpublished). In a second time, we have measured the expression level of the two

Table 4.1. Identity of the bacterial strains which have in their genome a sequence similar to the *L. bicolor* Tectonin 2. The accession number of the contig, the E-value and the environment from which the strains have been isolated are provided. A point of interrogation indicates that the species is frequently found in this type of environment but we were not able to find the information about the strain.

Best TBLASTN identity	AC Number	E-value	Environment
Shewanella woodyi ATCC 51908	NZ_AAUC01000014	2^{e}-20	Marine
Methylobacterium sp.	NZ_ABAY1000024	6^{e}-15	Soil ?
Burkholderia ubonensis	NZ_ABBE01000900	2^{e}-12	Soil
Stigmatella aurantiaca	NZ_AAMD01000132	7^{e}-8	Rotted wood
Frankia sp. EAN1pec	NZ_AAII01000036	1^{e}-6	Nodules ?
Pseudomonas aeruginosa PA7	NC_009656	2^{e}-5	Clinical isolate
Methylobacterium extorquens	NZ_ABEC01000001	1^{e}-4	Soil ?
Methylobacterium chloromethanicum	NZ_ABEX01000007	4^{e}-4	Petrochemical factory soil
Geobacter bemidjiensis	NZ_AAZA01000062	4^{e}-4	Aquifer subsurface sediment

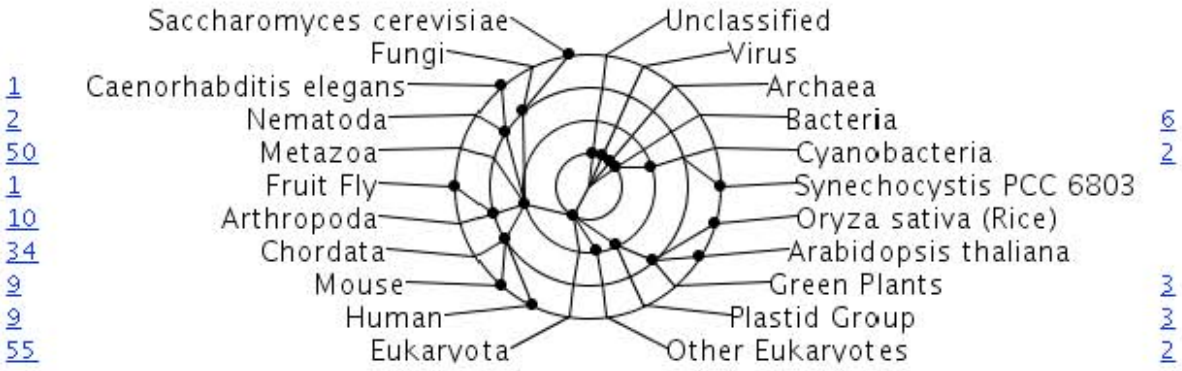
Table 4.2. List of the archea and eubacterial strains that harbour in their genomes predicted genes that encode protein containing at least one TECPR domain, according to the InterPro database. The taxa to which they belong (C : cyanobacteria, P : proteobacteria), the putative protein function, the number of TECPR domain in the protein and the environment from which the strains have been isolated are given.

Strain	Taxa	Protein function	Nb of TECPR domain	Environment
Shewanella woodyi ATCC 51908	P	β-propeller TECPR precursor	5	Marine
Lyngbya sp. PCC8106	C	Uncharacterized protein	4	Marine
Nodularia spumigena CCY9414	C	Hemagglutinin related protein	8	Marine
Loktanella vestfoldensis SKA53	P	Type II DNA modification enzyme	1	Marine
Stigmatella aurantiaca DW4/3	P	Uncharacterized protein	8	Rooted wood
Geobacter sp. FRC-32	P	β-propeller TECPR protein	4	Water

Table 4.3. Expression level of the *Lbtect1* and *Lbtect2* genes in *L. bicolor* S238N Douglas fir mycorrhizae, *L. bicolor* S238N poplar mycorrhizae, *L. bicolor* S238N sporocarps and in co-culture of *L. bicolor* S238N and the helper bacteria *P. fluorescens* BBc6R8, relatively to *L. bicolor* S238N free-living mycelium. Measurements were performed using whole genome oligochips, except the expression of *Lbtect2* in the *P. fluorescens* BBc6R8 treatment which was measured by real-time PCR. Each value is the mean of two replicates (± SE), except the expression of *Lbtect2* in the *P. fluorescens* BBc6R8 treatment which is the mean value of four replicates and the expression of *Lbtect1* in the *P. fluorescens* BBc6R8 treatment for which only one replicate has been performed.

	Douglas fir mycorrhizae	**Poplar mycorrhizae**	**Sporocarps**	***P. fluorescens* BBc6R8**
LbTect 1	1.2 ± 0	3.2 ± 0.01	2 .7 ± 0.01	1.0
LbTect 2	6.5 ± 0.05	8.1 ± 0.07	8.3 ± 0.07	3.7 ± 0.6*

Figure 4.4. View of the taxonomic range of sequences in which were identified at least one TECPR motif (from Interpro database, http://www.ebi.ac.uk/interpro/). The numbers indicate how many organisms of the taxa harbour a TECPR motif.



transcripts in Douglas fir and poplar mycorrhizae, in sporocarps and in free-living mycelium by using Nimblegen whole genome arrays. The *Lbtect 2* transcript was six to eight times more abundant in mycorrhizae and sporocarps than in sterile free-living mycelium (Table 4.3). *Lbtect 1* was overexpressed in Poplar mycorrhizae (3.2 times) and sporocarps (2.7 times) in comparison to the mycelium.

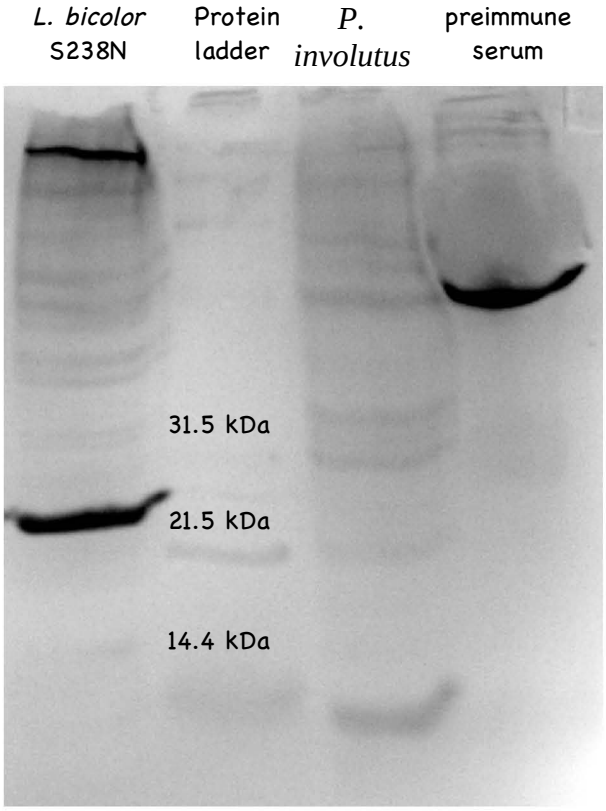
Protein expression in L. bicolor S238N

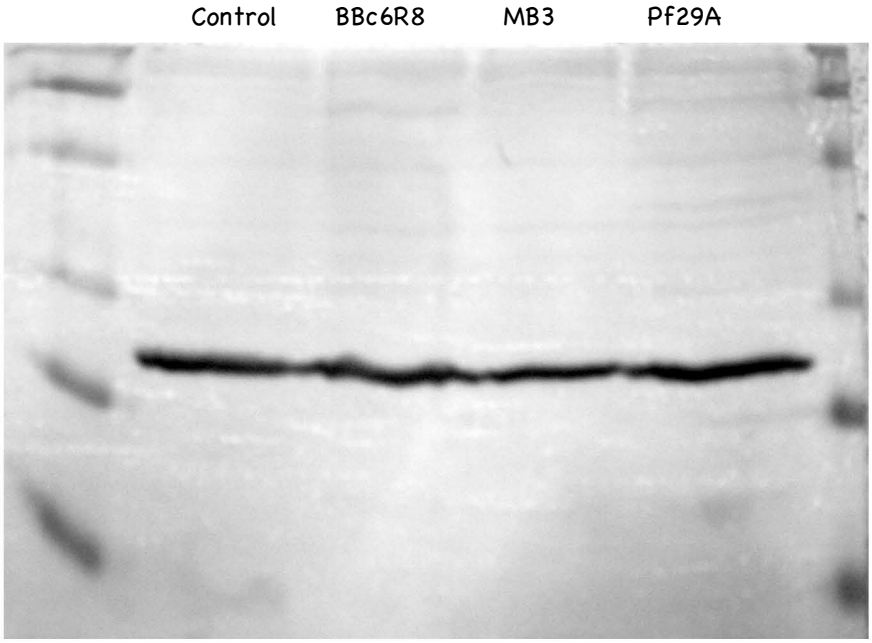
The anti-LbTect2 sera recognized a 23-kDa protein in the soluble protein extract of *L. bicolor* S238N. By contrast, no 23-kDa protein was detected neither in soluble protein extract of the other ectomycorrhizal fungus *Paxillus involutus* nor in pre-immune rabbit serum (Figure 4.5A). No difference in Lbtect2 protein concentration in the fungal extract was observed between the *L. bicolor* S238N-bacteria treatments (*P. fluorescens* BBc6R8, *Bacillus* sp. MB3, *P. fluorescens* Pf29A) and *L. bicolor* S238N (Figure 4.5B).

IV.1.3 Discussion

We have discovered in the genome of the ectomycorrhizal fungus *L. bicolor* S238N two genes that are closely related to the tachylectin gene family. Strikingly, these genes are completely absent from other fungal known genomes. By contrast, they were found in many metazoan organisms, from cnidarian to vertebrates where they seem to mainly function in the innate immunity of these organisms. Most of these organisms are aquatic, except the slime mold *Physarum polycephalum* to which the *Lbtect* is the most similar. Interestingly, this myxomycete colonizes dead organic matter in temperate and tropical forests. Thus *L.* bicolor S238N and *P. polycephalum* share the same environment. The plasmodia engulf bacteria, myxomycete amoebae and other microbes. The tectonins are thought to facilitate the phagocytosis of bacteria by binding to bacterial cell wall components (Huh *et al.* 1998). Because of the complete absence of tectonin-like sequences in other fungal genomes and the relatively high degree of similarity between *P. polycephalum* tectonins and *L. bicolor* S238N, it is improbable that the fungal genes were inherited vertically from an ancient common ancestor and lost in all other

Figure 4.5 A. Western blot of *L. bicolor* S238N, *P. involutus* protein soluble total extracts from mycelium and rabbit pre-immune serum using Lbtect polyclonal antibodies. **B.** Western blot of protein soluble extracts from 14 days old *L. bicolor* mycelium (control) or 14 days-old *L. bicolor* mycelium co-cultured with *P. fluorescens* BBc6R8, *Bacillus subtilis* MB3 or *Pseudomonas fluorescens* Pf29A .





fungi. The horizontal gene transfer (HGT) of a tectonin gene by *L. bicolor* S238N is a more parsimonious hypothesis. The gene would have been duplicated later on in the fungal genome. This hypothesis is supported by the very high degree of identity between the two fungal sequences. This transfer could have been mediated by bacteria. Indeed, among the bacteria that harbour proteins with TECPR domains or have a significant similarity with the *Lbtect*, two are frequently found in soils (*Stigmatella aurantiaca* DW4/3, Silakowski *et al.* 1999; *Pseudomonas aeruginosa*, Green *et al.* 1974).

A direct or indirect HGT event between a myxomycete and a basidiomycete has never been demonstrated so far. But several examples of HGT from prokaryotes toward fungi (Klotz & Loewen, 2003), plants (Richards *et al.* 2006a) and protists (Andersson *et al.* 2007) have been described. Conversely, intra eukaryotic HGT are thought to be rare events (Archibald *et al.* 2003). However, Richards *et al.* (2006b) have recently illustrated a case of HGT between ascomycetes and oomycetes. Some recent evidences indicate that HGT between protists could have occurred through a prokaryotic vector (Andersson *et al.* 2007). Interestingly, Brown *et al.* (2003) suggested that the intracellular colonization of eukaryotes by bacteria could increase the occurrence of HGT. Recent studies have demonstrated that *L. bicolor* S238N harbours endobacteria that appear to be environmentally acquired (Bertaux *et al.* 2005, chapter V.2). These bacteria may have play a role in the transfer of the tectonin like genes.

The remaining question is the function of these two fungal tectonin-like proteins in the fungal physiology and in the interaction between *L. bicolor* S238N and fungal-associated bacteria. Most of the tachylectins-like proteins described are involved in physical interaction with bacteria. However, Mali *et al.* (2006) recently demonstrated that a tachylectin-like protein from the cnidaria *Hydractinia* was not an immune molecule but was more probably involved in neuronal development processes. The primary and secondary structure of the tachylectin pattern was conserved in this protein but it held a signal peptide. In contrast, in the case of the tectonin-like proteins of *L. bicolor* S238N, the primary and secondary structure of the *P. polyphalum* tectonin was conserved but we did not detect any signal peptide. *Lbtect1 and LbTect 2* genes were expressed in all the fungal tissues and

overexpressed in mycorrhizae, sporocarps and mycelium grown in the presence of bacteria. Interestingly, both mycorhiza and sporocarps contains many bacteria. All together, it is tempting to speculate that the *L. bicolor* tectonins-like play a role in the interactions with surrounding bacteria. *L. bicolor* S238N is known to harbour intracellular bacteria (Bertaux *et al.* 2003, see also chapter 5 section 5.II) but the mechanism by which they are internalized is unknown. As in the myxomycete *P. polycephalum*, the LbTect may play a role in bacterial endocytosis. The immunolocalization experiments that will be performed in the next weeks may provide an answer.

Figure 4.5. Enzymatic activities of *P. fluorescens* BBc6R8 after 0, 3, 15, 22 and 27 days of growth in P20Th- medium. Each value is the mean of 2 replicates (± SE). Close circles on solid line, acid phosphatase ; close squares on solid line, chitinase ; open circles on dash line, cellulase ; black diamonds on solid line, ß-glucosidase ; open squares on dash line, glucuronidase.

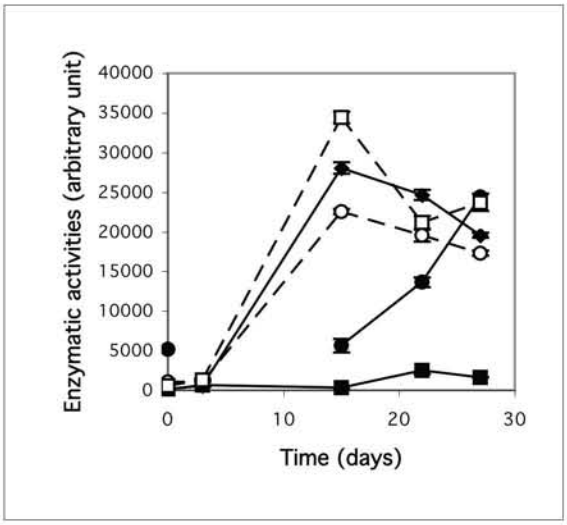
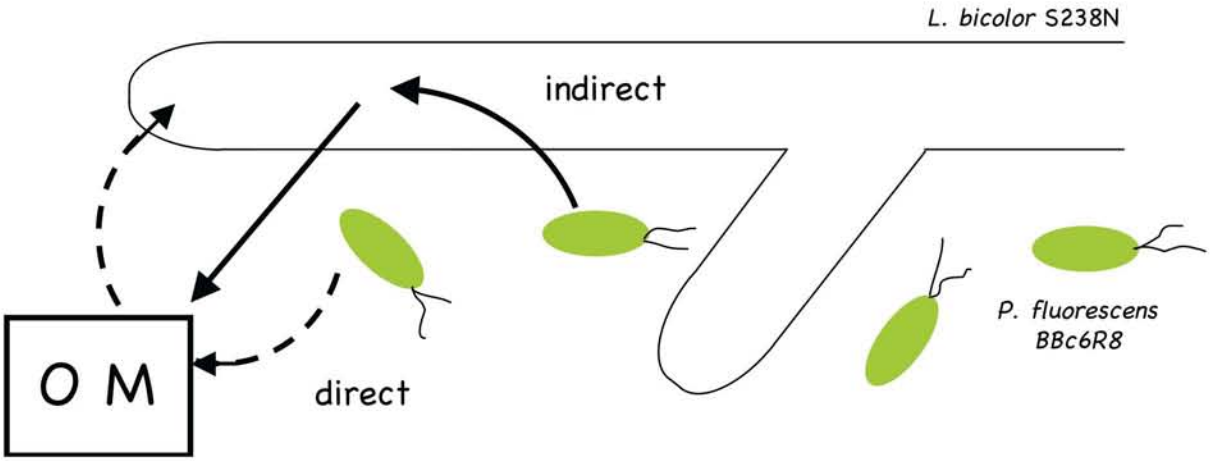


Figure 4.6. Effects of *P. fluorescens* BBc6R8 on *L. bicolor* S238N nutrition. The bacteria can directly degradate organic matter (OM) from the soil or stimulate the saprotrophic activities of the fungus.



IV.2 Enzymatic activities of *P. fluorescens* BBc6R8

IV.2.1 Material & method

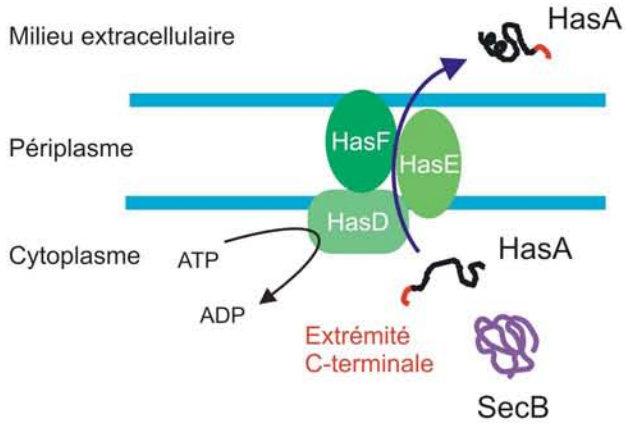
Pseudomonas fluorescens BBc6R8 was first grown on 10% TSA plates at 25°C for 65 h to prepare the bacterial inoculum. Then, three colonies were picked and suspended in 2 mL of sterile deionized water before spreading 120 µL on 10 % TSA medium. After 48 h of growth at 25°C, the bacteria were harvested and centrifuged at 3300 g for 10 min. The pellet was washed twice in 1 mL of sterile deionized water and then resuspended in water to obtain a suspension with $A600_{nm}$ of 0.9. Twenty microlitres of this bacterial suspension was added to 200 mL of P20Th$^-$ liquid medium and incubated at 25°C in the dark under a 300 rpm rotating agitation. After 0, 3, 15, 22 and 27 days of incubation, 100 µL of bacterial culture were harvested and diluted 10 times in fresh P20Th$^-$ liquid medium. The protocol described by Courty *et al.* (2005) to measure the acid phosphatase, chitinase, ß-glucosidase, cellulase and glucuronidase activities of ectomycorrhizal tips was adapted to bacterial suspensions as following. The buffer solution was replaced by one hundred microlitres of the diluted bacterial suspension. The P20Th$^-$ liquid medium was used as a negative control. The auto fluorescence of the *P. fluorescens* BBc6R8 suspension was also measured and subtracted to the fluorescence produced by enzymatic activities.

IV.2.2 Results & discussion

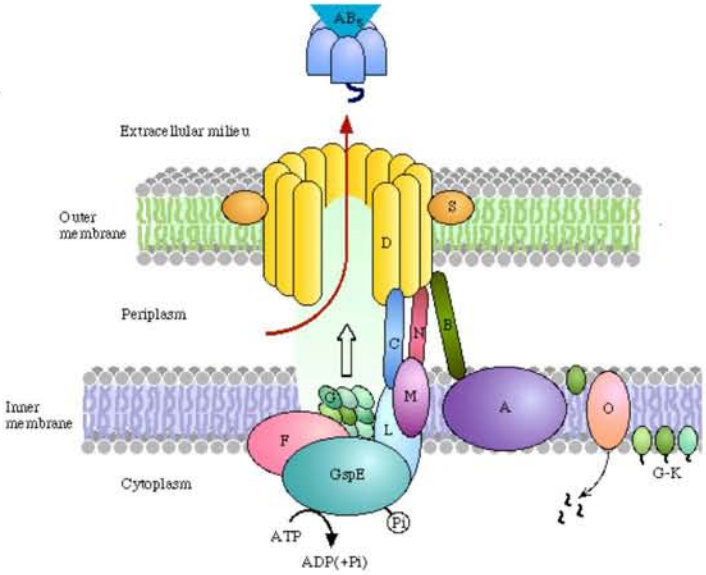
No enzymatic activities was measured before incubation and after three days of incubation, except for the acid phosphatase (figure 4.5). The chitinase activity remained low at each time of the kinetic. Conversely, the glucosidase, ß-glucuronidase and cellulase activities increased after 3 days of culture. The acid phosphatase activity increased after 15 days. To explain the time before the appearance of the first enzymatic we speculate that the bacteria first consumed the nutritive resources from their growth medium before inducing their enzymatic activities in order to liberate new nutritional components from their environment. During the pre-symbiotic stage of their life, ectomycorrhizal fungi depend on the resources they are able to up-take from their surrounding environment. Thus, the

Figure 4.7. Schematic overview of the type I, II, III and IV secretion systems as illustrated by *E. coli* (© Institut Pasteur, Bacterial membrane unit), *P. syringae* (© KEGG database), *P. aeruginosa* (© KEGG database) and *P. syringae* (© KEGG database).

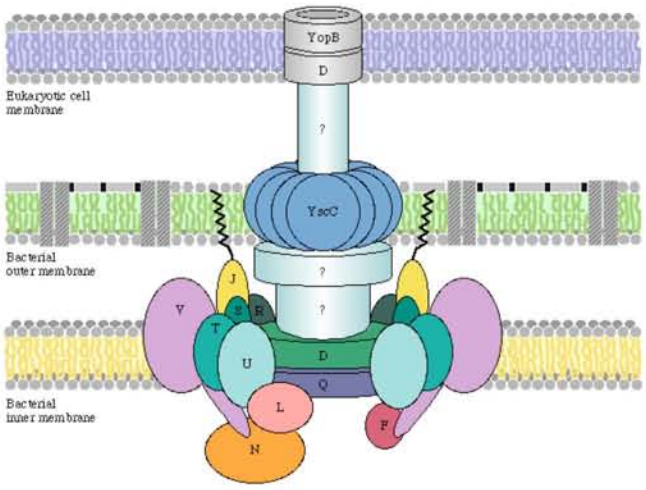
Type I



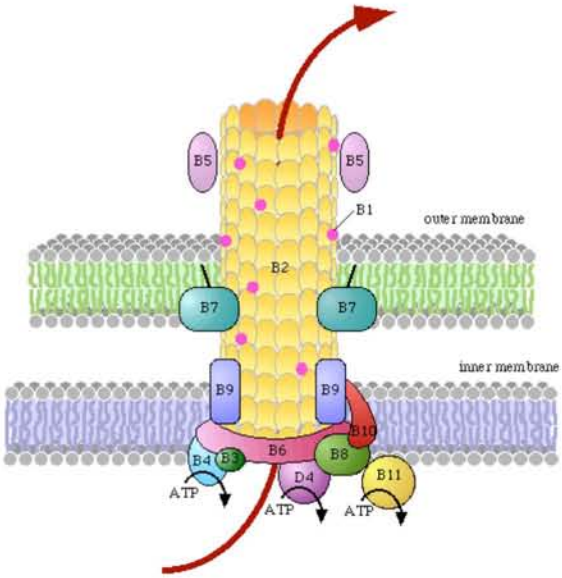
Type II



Type III



Type IV



bacterial saprotrophic activities could be beneficial to the fungus. Conversely, fungi and bacteria can also compete for the same resources. Numerous examples of nutritional competition between fungi and bacteria have been described, notably in soil and rhizosphere environments (Whipps *et al.* 2001). It is one of the main mechanisms of the biocontrol effect of rhizobacteria towards phytopathogenic fungi. But conversely, nutritional cooperation between microbes is also a common phenomenon (cf. General Introduction, chap1).

Moreover, our transcriptomic analyses indicated that *P. fluorescens* BBc6R8 induced an over expression of genes encoding for fungal extracellular degradation enzymes (cf. Chapter 1.III). We have hypothesized that this could lead to a better nutrition of the fungus and thus to a higher pre-symbiotic growth (Figure 4.6). We will use the same kind of enzymatic test to measure the effect of the bacteria on the fungal enzymatic activities.

IV.3 Characterization and role of the *P. fluorescens* BBc6R8 type III secretion system in the helper effect.

Four principal types of secretion systems are present in Gram negative bacteria (figure 4.7). The type I and II are dedicated to the excretion of molecules into the extra cellular medium while the types III and IV are implicated in cell-to-cell secretion (Hueck *et al.* 1998). The type I secretion system, also called ABC transporter (for ATP Binding Cassette) is responsible of the export of extracellular enzymes like the hemolysin for *Escherichia coli*, the adenylate cyclase for *Bordetella pertussis* or proteases for *Pseudomonas aeruginosa*. Contrary to the type I and III in which the secretion is direct, the excretion via the type II is performed in two steps: proteins are first translocated into the periplasmic space where they are folded and then in a second time they are secreted through the external membrane. The type IV system plays a major role in plasmid exchange during the conjugation but also in the virulence of some bacteria. It transports both proteins and nucleoproteins complexes. Finally, the type III secretion system (TTSS) can be compared to a molecular syringe that introduces virulence factors

directly into eukaryotic host cells. The introduced factors are then able to subvert host cell functions in a way that is beneficial to the invading bacteria. It is widely distributed among proteobacterial pathogens of plants, animals and human. For a long time, it has been thought that this machinery was specific to bacterial pathogens. But recently, several studies have demonstrated that TTSS systems were also present in non-pathogenics bacteria like symbiotic rhizobium or saprophytic fluorescent pseudomonads (Marie *et al.* 2001, Mazurier *et al.* 2004). In these cases, the TTSS functioned as an export system but it seems also that, in certain bacteria, it has evolved toward other functions (Jackson *et al.* 2005).
Little is known about the TTSS system of *P. fluorescens* BBc6R8. By using degenerated primers, Preston *et al.* have successfully amplified several genes homolog to *P. fluorescens* SBW25 (G. Preston, personal communication). But the BBc6R8 TTSS system has never been described in its entirety and its role in the bacterial physiology and in interactions with other microorganisms is unknown. In this context, we have engaged preliminary analyses of the BBc6R8 TTSS system.

IV.3.1 Material & methods

In silico analysis of the TTSS cluster of P. fluorescens BBc6R8

On the basis of the work of Preston *et al.* (2001) on the type III secretion system of *P. fluorescens* SBW25, we searched for the presence of the component of the rsp cluster in the genome of *P. fluorescens* BBc6R8 by doing TBLASTN comparison with the *P. fluorescens* SBW25 and *P. syringae* pv. s*yringae* 61 genes.

Overexpression of RspL and RspR in P. fluorescens BBc6R8.

The rspL and rspR genes were cloned into the broad host range plasmid pML121, downstream of the constitutive *nptII* promoter by Preston *et al.* (2001). The resulting plasmids and the empty vector were introduced into *P. fluorescens* BBc6R8 by conjugation. Donor, acceptor and helper strains were grown on LB (± Kanamycin 50 μg. μL^{-1}) overnight at 25°C. Bacteria were then collected, washed with sterile deionized water twice and DO_{600} was adjusted to 0.8 with sterile deionized water. A 1/5/5 mix of acceptor/donor/helper was done and drops of 10 μL of this mix was put on LB medium. After 4 hours of incubation at 25°C, drops

Table 4.4. Comparison of the *P. fluorescens* BBc6R8 TTSS cluster to the *P. fluorescens* SBW25 and *P. syringae* pv. *syringae* 61 ones. Accession numbers of the *P. fluorescens* SBW25 (Rsc/Rsp) and *P. syringae* pv. *syringae* 61 (Hrc/Hrp), start and stop position of the corresponding genes in *P. fluorescens* BBc6R8, E-value of the TBlastN and percentage of similarity with *P. fluorescens* SBW25 (Rsc/Rsp) and *P. syringae* pv. *syringae* 61 (Hrc/Hrp) proteins are given. The predicted function and properties of each protein is also given (from Preston *et al.* 2001). IM : Inner Membrane associated, OM : Outer Membrane associated.

ORFs	AC Number	Start position	Stop position	E-value	Percentage of similarity	Predicted function
RspL	AAK81917	2413	1874	1,00E-84	90	ECF sigma factor
RspO	AAK81919	8326	8595	3,00E-27	80	Accessory protein ?
RspP	AAK81920	8547	9062	2,00E-35	55	Accessory protein ?
RscQA	AAK81921	9071	9619	1,00E-62	73	IM (FliY/FliM)
RscQB	AAK81922	9719	10081	8,00E-52	84	IM ? (FliY/FliM)
RscR	AAK81923	10081	10731	3,00E-93	86	IM (FliP)
RscS	AAK81924	10742	11002	2,00E-25	68	IM (FliQ)
RscT	AAK81925	11016	11744	1,00E-103	81	IM (FliR)
RscU	AAK81926	11800	12891	1,00E-148	88	IM (FlhB)
RspV	AAK81927	13247	12897	1,00E-28	65	Negative regulator of *hrp/rsp* expression
RspT	AAK81928	13435	13262	2,00E-26	91	Accessory protein
RscC	AAK81929	15591	13453	0,00E+00	87	OM protein
rspG	AAK81930	15999	15610	1,00E-26	59	Accessory protein
RspF	AAK81931	-	-	-	-	Unknown
RspE	AAK81932	16913	16359	3,00E-27	54	FliH
RspD	AAK81933	17478	16945	2,00E-25	49	Unknown
RscJ	AAK81934	18220	17525	1,00E-94	83	IM/OM lipoprotein (FliF)
RspB	AAK81935	-	-	-	-	Unknown
RspA	AAK81936	-	-	-	-	Structural component of pilus
RspR	AAK81937	20083	19172	1,00E-121	81	sigma 54-dependent protein
HrpZ	AAQ92359	-	-	-	-	Accessory protein
HrcN	ABQ88354	6858	8191	5,00E-163	72	
HrpQ	ABQ88353	5935	6846	3,00E-52	50	IM (FliG)
HrcV	ABQ88352	3786	5822	0,00E+00	75	IM (FlhA)
HrpJ	ABQ88351	2773	3774	2,00E-36	48	Accessory protein?

were collected, the DO_{600} was adjusted to 0.8 and bacterial suspensions were spread on LB kanamycin plates. After an overnight growth at 25°C two bacterial colonies corresponding to BBc6R8 transformant were collected. It was checked that the strain BBc6R8 was not able to grow on LB kanamycin plates.

Effect of BBc6R8-RspL and -RspR overexpressing strains on L. bicolor S238N growth

The bioassay previously described (Deveau *et al.* 2007) was used to evaluate the effect of the overexpression of *rspL* and *rspR* genes from *P. fluorescens* SBW25 in *P. fluorescens* BBc6R8, on the growth of *L. bicolor* S238N. Two control experiments were performed to first check the positive effect of the non-transformed strain *P. fluorescens* BBc6R8 on the fungal growth and secondly to verify that the transformation of *P. fluorescens* BBc6R8 with the empty vector pML121 had no consequence on the fungal growth-promoting effect of BBc6R8.

IV.3.2 Results

In silico analysis of the P. fluorescens BBc6R8 TTSS system

A total of 22 open reading frames (ORF) were identified. The same gene names used by Preston *et al.* (2001) were assigned to ORFs that displayed a significant degree of similarity with *P. fluorescens* SBW25 or *P. syringae* pv. *syringae* 61 TTSS genes. The size, the position and the similarity to *P. fluorescens* SBW25 or *P. syringae* pv. s*yringae* 61, TTSS genes are listed in Table 4.4. Predicted function of putative proteins encoded by the TTSS cluster are also mentionned. The transcriptional organization of the TTSS cluster, as predicted by sequence analysis and homology with *P. fluorescens* SBW25 and *P. syringae* pv. s*yringae* 61, is shown in figure 4.8. The operon organization of the cluster is the same than the *P. fluorescens* SBW25 but there are three principal differences: (i) *P. fluorescens* SBW25 cluster contains the hrpV operon (hrpJ, hrcV and hrpQ) which is lacking in the *P. fluorescens* SBW25 TTSS cluster. (ii) *P. fluorescens* BBc6R8 TTSS cluster lacks the homologues of *hrpA* and B in the *hrpJ* operon of *P. fluorescens* SBW25 and of *rspF* in the rspC operon of *P. fluorescens* SBW25. (iii) no homologue of the

Figure 4.8. The TTSS gene cluster of *P. fluorescens* BBc6R8 and comparison with *P. fluorescens* SBW25 and *P. syringae* pv. *syringae* 61. **A**. Schematic representation of *P. fluorescens* BBc6R8 TTSS operons cluster. **B**. Comparative organization of ORFs in the TTSS gene cluster of *P. fluorescens* BBc6R8 (BBc6R8) , the rsp gene cluster of *P. fluorescens* SBW25 (SBW25) and the *hrp* gene cluster of *P. syringae* pv. *syringae* 61 (syringae). The arrangement of conserved sequences between of *P. fluorescens* BBc6R8, *P. fluorescens* SBW25 and *P. syringae* pv. *syringae* 61 is indicated by shading. The putative organization of the clusters is shown by arrows (adapted from Preston *et al.* 2001).

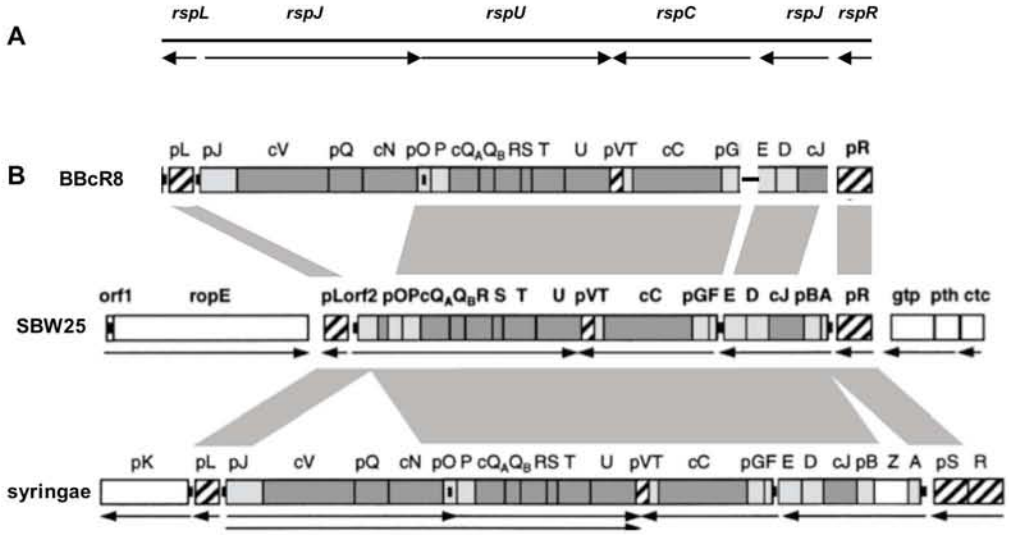


Table 4.5. Effect of wild type or transformed cells of *P. fluorescens* BBc6R8 on the growth of *L. bicolor* S238N. Each value is the mean value (± SE) of seven replicates. In each column, mean values with the same letter are not significantly different according to a one-way (treatment) ANOVA ($p > 0.05$)

Treatment	Fungal colony diameter (cm)				
	9 days	15 days	20 days	30 days	44 days
Control	1.0 ± 0.010a	2.1 ± 0.024a	2.8 ± 0.056a	4.5 ± 0.069a	6.6 ± 0.070a
P. fluorescens BBc6R8	0.9 ± 0.017a	2.1 ± 0.030a	3.1 ± 0.029a	5.0 ± 0.049b	7.8 ± 0.088b
P. fluorescens BBc6R8-pML121	0.6 ± 0b	1.3 ± 0.024b	2.3 ± 0.041b	4.2 ± 0.041a	7.1 ± 0.051a
P. fluorescens BBc6R8-rspL	0.6 ± 0.012b	1.3 ± 0.030b	2.0 ± 0.044b	4.0 ± 0.054c	6.7 ± 0.073a
P. fluorescens BBc6R8-rspR	0.8 ± 0.06b1	1.8 ± 0.135b	2.6 ± 0.166b	4.7 ± 0.182a	7.4 ± 0.220b

P. fluorescens SBW25 *ropE* is present in the vicinity of the *P. fluorescens* BBc6R8 cluster.

Effect of the overexpression of the TTSS regulatory genes rspL and rspR on the interaction between P. fluorescens BBc6R8 and L. bicolor S238N

Unexpectidly, the positive effect of the wild type helper strain BBc6R8 on the fungal growth was delayed (Table 4.5): a significant increase of growth was obtained after 30 days while it normally occurs after 12 days. The experiment was performed during the summer 2007. We recurrently observed that the growth-promoting effect of *P. fluorescens* BBc6R8 on *L. bicolor* S238N is delayed during summer because the fungus alone grows faster at this time of the year, maybe because of an internal clock. Moreover, we observed that the strain transformed with the empty vector pML121 had either a negative (9, 15 and 20 days of incubation) or no (30 and 44 days of incubation) promoting effect on the growth of *L. bicolor*. Similarly, the strain that was transformed with the *rspL* gene had either a negative (9, 15, 20, 30 days) or no promoting effect (44 days) on *L. bicolor* S238N growth. Finally, the strain transformed with the *rspR* gene had no positive effect on fungal growth, except after 44 days of incubation where it has a positive effect. The experiment should be performed another time to verify if in condition of a normal wild type growth-promoting effect (i.e after 12 days of incubation) on *L. bicolor* S238N, strain transformed with the empty vector still have a negative effect on the fungal growth.

IV.3.3 Discussion

According to the *in silico* analysis, *P. fluorescens* BBc6R8 possesses a TTSS system that could be functional. Indeed, the missing genes encode for non-structural proteins. This is a new example of TTSS system in a non-pathogenic bacterial species (Mazurier *et al.* 2004). A more extended comparison with other TTSS systems from pathogenic and non-pathogenic strains will be performed to better characterize the BBc6R8 system.

The second question we asked was whether the BBc6R8 TTSS system was involved or not in the interaction between *L. bicolor* S238N and *P. fluorescens* BBc6R8. The primary study we performed did not allow us to conclude. Indeed, the transformation of the bacterial strain by the empty vector led to a modification of the behaviour of the helper bacteria. The vector or the genes contained in the vector may interfere with a native

plasmid (G. Preston, personal communication). At the present time, we do not know if the bacteria have or not any plasmid. Thanks to the genome sequence of *P. fluorescens* BBc6R8, we should be able soon to monitor the expression of the different genes involved in the TTSS system and to knock out the key genes.

Conclusion

Les études préliminaires présentées dans ce chapitre ne sont pas encore assez avancées pour permettre de conclure quant au rôle précis joué par les activités de dégradation de la matière organique, les tectonines fongiques et le système de sécrétion de type III dans l'effet auxiliaire de BBc6R8. Toutefois les premiers résultats ne contredisent pas leur implication et des études complémentaires vont être menées dans les mois qui viennent pour approfondir les pistes engagées.

Chapter V

Curation and analysis of the genome *L. bicolor* H82-S238N

Chapitre V

Annotation et analyse du génome de *L. bicolor* H82-S238N

Introduction

Lorsque cette thèse a été débutée en novembre 2004, ni le génome de *L. bicolor* H82-S238N ni celui de *P. fluorescens* BBc6R8 n'étaient disponibles. La séquence assemblée de *L. bicolor* H82-S238N a été fournie par le JGI à la communauté des annotateurs au printemps 2005. Une grande part de mon travail s'est alors appuyée sur ce jeu de données. En effet, la connaissance de la séquence complète d'un génome facilite grandement les analyses de biologie moléculaire : il est plus aisé d'identifier les protéines codées par les ADNc déposés sur les membranes de nylon. D'autre part, la connaissance quasi-immédiate de la séquence complète d'un gène à partir du séquençage d'un fragment évite de passer plusieurs semaines à rechercher par amplification cette même séquence. De même, quelques jours seulement sont nécessaires pour reconstituer une voie métabolique complète *in silico*, alors qu'il faut plusieurs semaines pour obtenir les mêmes résultats de manière expérimentale. Enfin l'utilisation de puces de type génome entier n'aurait pas été possible sans un séquençage préalable. De multiples applications peuvent être tirées de la connaissance de la séquence d'un génome. Toutefois, il n'en demeure pas moins nécessaire de vérifier expérimentalement les informations prédites *in silico*.
Pour ma part, j'ai participé à l'annotation du génome de *L. bicolor* H82-S238N, en m'attachant à décrire dans un premier temps le métabolisme carboné du champignon puis en explorant les séquences à haut GC% à la recherche d'une potentielle bactérie endocellulaire.

Publication n°7. The major pathways of the carbohydrate metabolism in the ectomycorrhizal basidiomycete *Laccaria bicolor* S238N

The major pathways of carbohydrate metabolism in the ectomycorrhizal basidiomycete *Laccaria bicolor* S238N

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Abstract

- The primary carbohydrate metabolism of an ectomycorrhizal fungus and its transcriptional regulation had never been characterized at the genome scale while it plays a fundamental role in the functioning of the symbiosis.
- In this study, the genome sequence of the ectomycorrhizal basidiomycete *Laccaria bicolor* S238N-H82 was explored to construct a comprehensive genome-wide inventory of pathways involved in primary carbohydrate metabolism.
- Several genes and gene families were annotated, including those of the glycolysis, pentose phosphate pathway, tricarboxylic acid cycle, and trehalose and mannitol metabolism.
- The transcriptional regulation of these pathways was studied using whole-genome expression oligoarrays and quantitative PCR in free living mycelium, ectomycorrhizae and fruiting bodies.
- Pathways of carbohydrate biosynthesis and catabolism are identical in *L. bicolor* compared with other sequenced saprotrophic basidiomycetes.
- Ectomycorrhiza formation induced a shift in carbohydrate metabolism which is partly regulated at transcriptional level.

Keywords: ectomycorrhiza, *Laccaria bicolor*, carbon, mannitol, trehalose, annotation, transcriptome

Introduction

In the mutualistic ectomycorrhizal symbiosis, the nutritional relationships between the plant-fungus partners rely on a bi-directional flux of nutrients. The mycobiont hyphal networks radiating into the soil and litter absorb soil nutrients that are translocated throughout strands and rhizomorphs to the host root. The absorption, translocation and assimilation of mineral ions by hyphae require carbon skeletons, ATP and reducing power, as NAD(P)H which are generated by carbohydrate oxidative pathways. Although ectomycorrhizal fungi are facultative saprotrophs, the analysis of the *Laccaria bicolor* genome has revealed that this ectomycorrhizal basidiomycete is poorly adapted for efficient degradation of soil carbon-rich lignocellulose, which likely reflects a reliance on host-supplied photoassimilates (but see Koide *et al.*, 2008). Up to 30 % of these assimilates, mainly as sucrose, can be transferred to the associated fungus (Finlay & Söderström, 1992). Sucrose downloaded into the symbiotic apoplastic interface is then hydrolyzed into fructose and glucose via the action of the plant sucrose invertase (Nehls *et al.* 2007). The resulting glucose (and fructose) are actively taken up by the fungal hyphae where they feed the carbohydrate metabolism, leading to the synthesis of trehalose, polyols and other storage compounds (glycogen, fatty acids) (Martin *et al.*, 1998). Carbohydrate catabolism also provides energy for hyphal growth and supplies carbon skeleton to other metabolisms (notably the amino acid biosynthesis). Storage carbohydrates fulfil multiple functions in ectomycorrhizae; they not only constitute a source of carbon and energy but also protect mycorrhiza against a variety of environmental stresses such as dessication and frost (Elbein, 2003). Furthermore, the conversion of host hexoses into fungus-specific storage carbohydrates, such as polyols and trehalose, creates a strong driving force for plant carbon allocation to symbiotic tissues (Martin *et al.* 1998, Nehls *et al.* 2001, López *et al.* 2007). Polyols may be the compatible solutes responsible for generating the hydrostatic pressure used by the hyphae to break the root surface and penetrates between epidermal cells to initiate the Hartig net (Martin *et al.* 1998). Both mannitol and trehalose play a key role in the regulation of glucose metabolism and carbon storage (Wiemken *et al.* 2007), but biosynthesis and degradation pathways of these carbohydrates have not been comprehensively described in ectomycorrhizal fungi and it remains to be determined whether they are fully operative.

There is evidence that the development and functioning of ectomycorrhizal symbiosis bring about dramatic modification of carbon metabolism in the host roots and in the mycobiont forming the mutualistic association (Martin *et al.* 1987, Hampp & Schaeffer 1995, Martin *et al.* 1998). The utilization patterns of [1–^{13}C]glucose by *Eucalyptus globulus* seedlings and *Pisolithus microcarpus* mycelium was

influenced by mycorrhizal colonization, with a greater allocation of carbon to short chain polyols, arabitol and erythritol and to trehalose in the mycelium and a suppression of sucrose synthesis in colonized roots (Martin *et al*., 1998). It appears that fungal metabolism dominates the assimilation of exogenous carbohydrates into symbiotic tissues. Several *P. microcarpus* transcripts coding for enzymes in the glycolysis, tricarboxylic acid (TCA) cycle and the mitochondrial electron transport chain were upregulated in symbiotic tissues 7 to 12 days after contact (Duplessis *et al*., 2005) confirming a general stimulation of the glucose respiration pathways. Transcript profiling confirmed this shift in carbon metabolism in the *Paxillus involutus–Betula pendula* ectomycorrhiza (Johansson *et al*., 2004; Le Quéré *et al*., 2005).

So far, the primary carbohydrate metabolism of an ectomycorrhizal fungus has not been characterized at a genome-scale and it is not known if symbiotic fungi have gained or lost specific pathways in comparison to saprotrophic fungi. Here, we characterize the complete set of genes involved in primary carbohydrate metabolism in the recently sequenced *Laccaria bicolor* genome (Martin *et al*., 2008). This includes cataloguing predicted carbohydrate metabolism proteins, surveying their level of transcripts in various tissues and conducting phylogenetic analyses on enzymes of trehalose and mannitol metabolism.

Materiel & methods

Growth of L. bicolor S238N, mycorrhiza synthesis, sampling and RNA extraction

Free-living mycelium of *L. bicolor* S238N was grown onto cellophane-covered agar plates containing Pachlewski medium (Di Battista *et al.* 1996) for three weeks before harvesting the proliferating hyphal tips at the colony edge. Ectomycorrhizas of *L. bicolor*/*Pseudotsuga menziesii* were synthesized by growing Douglas fir seedlings for nine months in polyethylene containers filled with a peat-vermiculite mix (1:1, v/v) and mixed with 2.5% (v/v) fungal inoculum as described previously (Frey-Klett *et al.*, 1997). Ectomycorrhizas of *L. bicolor*/*Populus trichocarpa* were synthesized either by growing cuttings of *P. trichocarpa* for three months in pots containing Terragreen (Brenntag Lorraine, Toul, France) mixed with fungal inoculum in a peat-vermiculite mix (4:1, v/v). *In vitro P. tremula x alba* (INRA clone 717-1B4) plantlets inoculated with *L. bicolor* S238N were produced as described in Luster & Finlay (2006) and sampled one month after contact. Ectomycorrhizal root tips of *L. bicolor* were identified under the dissection microscope after harvesting and stored in liquid nitrogen. Fruiting bodies of *L. bicolor* S238N were collected beneath Douglas fir seedlings grown in a greenhouse and

inoculated using *L. bicolor* S238N as described (Di Battista *et al.* 1996). Tissues were immediately frozen in liquid nitrogen and RNA extraction was carried out using the RNeasy Plant Mini Kit (Qiagen).

In silico genome automatic annotation and manual curation

Using the Blast, Advanced Search and Gene Ontology tools at the JGI *Laccaria* Genome database (http://genome.jgi-psf.org/ Lacbi1/Lacbi1.home.html), we identified gene models involved in the glycolysis, pentose phosphate pathway, gluconeogenesis, glycogen, trehalose and mannitol metabolism in the draft genome of *L. bicolor* S238N-H82. Gene prediction at JGI was performed using four methods: GENEWISE, FGENESH, GRAILEXP6, and EUGENE, and gene models were selected by the JGI annotation pipeline (Martin *et al.*, 2008). Selection of the models was based on EST support, completeness, and homology to a curated set of proteins. All detected gene models of the carbohydrate metabolism were inspected manually, and the automatically selected best gene model of the JGI *Laccaria* genome database was modified if necessary.

Additionally, searches were performed with the use of a range of sequences of carbohydrate metabolism proteins and genes available from fungi at NCBI GenBank (http://www.ncbi.nlm.nih.gov/) and UNIPROT (http://expasy.org/) to probe the *Laccaria* genome database using the BLASTN, TBLASTN, and BLASTP algorithms as incorporated in the JGI accession page and the INRA *Laccaria* DB (http://mycor.nancy.inra.fr/IMGC/LaccariaGenome/). The putative homologues that were detected were characterized based on conserved domains, identities, and E-values. *L. bicolor* gene models were corrected when necessary. Manual annotation was carried out using the ARTEMIS software (http://www.sanger.ac.uk/Software/Artemis/). The manually annotated gene sequences were aligned and verified using the programmes ClustalX (version 1.83.1) (Jeanmougin *et al.* 1998). Each curated homologue was further used for BLAST search at the JGI, YeastDB (http://www.yeastgenome.org/) and Broad-MIT Institute (http://www.broad.mit.edu/) databases to check for similar genes in other fungi, including *Aspergillus nidulans, Coprinopsis cinerea, C. neoformans, N. crassa, Ustilago maydis*, *Phaenerochaete chrysosporum* and *S. cerevisiae.*

Subcellular localization of putative proteins was predicted using TargetP 1.1 (http://www.cbs.dtu.dk/services/TargetP/) and WoLF PSORT (http://wolfpsort.seq.cbrc.jp/) prediction algorithms. *L. bicolor* S238N-H82-derived sequences were used for BLAST analysis of the EST database available at INRA *Laccaria* DB (Kohler *et al.*, 2008, this issue).

Sequence alignment and phylogenetic analysis

Predicted protein sequences from the present genome survey were aligned using the programme ClustalX using default settings. The aligned sequences were exported to a NEXUS file and Neighbour Joining (NJ) trees were generated with the PAUP 4.0b10 program (Swofford, 1999), using the NJ algorithm using default settings.

Transcript profiling

Total RNA preparations (two biological replicates for each sample) were amplified using the SMART PCR cDNA Synthesis Kit (Clontech) according to the manufacturer's instructions. Single dye labeling of samples, hybridization procedures, data acquisition, background correction and normalization were performed at the NimbleGen Systems facilities (NimbleGen Systems, Reykjavik, Iceland) following their standard protocol. The *L. bicolor* whole-genome expression oligoarray (NimbleGen Systems) contains in duplicate eight independent, non-identical, 60-mer probes per whole gene model. Included in the microarray are 20,614 JGI annotated gene models (genome annotation v1.0), 1,680 additional EUGENE predicted gene models, 30 000 random 60-mer control probes and labeling controls. To estimate a cut-off level for expression, the mean intensity of the 30,000 random probes present on the microarray was calculated. Gene models with a signal intensity three-fold higher than the calculated cut-off were considered as transcribed. Log_2-transformed data were subjected to the CyberT statistical analysis (http://www.igb.uci.edu/servers/cybert/) as described in Martin *et al.* (2008). Transcript concentration in ectomycorrhizas and fruiting bodies were compared with those measured in the free-living mycelium. Changes in transcript ratio with a PPDE ≥ 0.95 and Bayesian Ln$p \leq 0.05$ were considered as being statistically significant.

Validation of array data by quantitative PCR

The validation of array data was done by performing real-time quantitative PCR analyses on ten transcripts involved in the metabolism of trehalose [trehalose phosphorylase (E.C. 2.4.1.64), trehalose phosphate synthase (E.C.2.4.1.15), trehalose phosphatase (E.C.3.1.3.12), trehalose synthase regulatory subunit, uridine phosphoglucose pyrophosphorylase (E.C.2.7.7.9), acid trehalase (E.C.3.2.1.28)], and mannitol [mannitol dehydrogenase (E.C. 1.1.1.38), medium chain dehydrogenase/reductase 1 and 2] and in glycolysis [(fructose-1,6-bisphosphate aldolase (E.C. 4.1.2.13)]. Three transcripts with a constitutive expression were also used for data normalization (Elongation Factor 3, GTPase,

Metalloprotease). Primer design and PCR amplification protocol are described in Deveau *et al.* (2007). The sequences of all primers are provided in Table S1 (Supplemental web data).

^{13}C Nuclear magnetic resonance spectroscopy

Extraction of soluble compounds was performed as described by Martin & Canet (1986). Neutral carbohydrates were purified on Dowex 50WX8-200 ion-exchange resine (Sigma) and NMR analysis was carried out as described previously Martin *et al.* (1998).

Results

The major source of carbon used by most ectomycorrhizal hyphae comes from the sucrose provided by the host plant (Nehls *et al.* 2007), altough several species are capable of obtaining carbon saprotrophically (Koide *et al*., 2008). No sucrose invertase (E.C. 3.2.1.26) was found in the genome *L. bicolor* (Martin *et al.*, 2008) confirming that sucrose is converted into fructose and glucose into the symbiosis apoplastic space by the plant invertase. Then, glucose (and to a lesser extent fructose) are taken by mycobiont monosaccharide transporters (Nehls *et al.* 2008). Another substantial source of carbon is the anaplerotic fixation of CO_2 leading to the synthesis of oxaloacetate/malate from pyruvate (Martin *et al.* 1986, 1998). This carboxylation step is catalyzed by pyruvate carboxylase (PYC, E.C.6.4.1.1). A gene encoding for this enzyme was identified in the genome of *L. bicolor* H82-S238N (Figure 1).

Hexose catabolism.

Hexose catabolism starts by the glycolysis, which is the process whereby sugars are metabolized into pyruvate before oxydation by TCA cycle or the ethanolic fermentation (Figure 1). Three glycolytic pathways have been described: the Embden-Meyerhof pathway (EM), which utilizes NAD as electron acceptor, the pentose phosphate pathway (PPP), which uses NADP, and the Entner Doudoroff pathway (ED). The genes coding for the enzymes of the EM, PPP, and glycolysis pathways were all identified in *L. bicolor* genome (Figure 1). Similarly, all genes encoding proteins involved in TCA cycle and ethanol fermentation were identified. Genes encoding for cytosolic isoforms of citrate synthase (CS, E.C. 2.3.3.1) and aconitase (ACO, E.C. 4.2.1.3) involved in the glyoxylate shunt, and for malate dehydrogenase (MDH, E.C.1.1.1.37) involved in the gluconeogenesis were also identified in the H82 haploid genome. Finally, four genes encoding for isocitrate dehydrogenases (IDH, E.C.1.1.1.42) were

found. Two encode for the mitochondrial NAD-dependent subunits of the IDH involved in TCA cycle (JGI protein number: 311842, 311861), and another gene model encodes for NAD-dependent isoform involved in lysine biosynthesis (protein number: 229977). The fourth encode for the mitochondrial NADP-dependent isoenzyme that does not participate to the TCA cycle (protein number: 317084).

The ED pathway is widely distributed among prokaryotes and may also occur in some filamentous fungi, notably in *Aspergillus* species (Elzainy *et al*. 1973). However the gene encoding for the key enzyme KDG aldolase that converts D-2-keto-3-deoxygluconate (KDG) into D-glyceraldehyde and pyruvate has never been characterized (J. Nielsen, personal communication) and was not found in *L. bicolor* genome.

Mannitol metabolism

Several pathways are involved in mannitol synthesis in fungi. In Ascomycetes, mannitol is produced via fructose 6-P and mannitol 1-P by the consecutive action of hexokinase (E.C 2.7.1.1) and NAD-dependent mannitol 1-phosphate 5-dehydrogenase (M1PDH, EC 1.1.1.17), followed by a dephosphorylation step catalyzed by the mannitol 1-phosphatase (M1Pase, EC 3.1.3.22) resulting in mannitol formation. The polyol is then reconverted into fructose by NADP-mannitol dehydrogenase (MtDH, EC 1.1.1.138) generating NADPH through the so-called mannitol cycle (Hult & Gatenbeck, 1978). In most basidiomycetes, there is no M1PDH and mannitol is most likely formed by direct reduction of fructose through a mannitol 2-dehydrogenase using either NAD^+ (E.C.1.1.67) or $NADP^+$ (E.C.1.1.1.138) as a cofactor (Hult *et al.* 1980, Voegele *et al.* 2005). *L. bicolor* harbours a single of MtDH-encoding gene. Two genes that are highly similar to *C. neoformans* genes were annotated as M1PDH encoding genes in the genome of *L. bicolor*. These genes were also annotated in other sequenced basidiomycetes genomes (Figure 2). The proteins encoded by these genes have however all the features of medium-chain dehydrogenase/reductases (MDR, Ceccaroli *et al.* 2007); they harbour the coenzyme-binding motif Gly-Xaa-Gly-Xaa-Xaa-Gly and are 350 residues-long.

Trehalose metabolism

At least five different pathways of trehalose biosynthesis have been described (Avonce *et al.* 2006). The most widely reported in fungi is the one involving the enzyme trehalose-phosphate synthase (TPS1) that catalyzes the transfer of a glucosyl-residue from uridine-diphospho-glucose to glucose-6-P. The resulting trehalose-6-P is subsequently dephosphorylated by the trehalose P-phosphatase (TPP) to

yield trehalose (Figure 1). In *S. cerevisiae*, trehalose synthesis is mediated by a multi-enzymes complex made up of four subunits (Bell *et al.* 1998): the two enzymes TPS (called TPS1) and TPP (TPS2), and two regulatory sub-units (TSL1 and TPS3). *L. bicolor* S238N harbours all the genes encoding the enzymes of the TPS pathway (Figure 1). A putative regulatory subunit showing 50 % of similarity with the TSL1 subunit of *S. cerevisiae* was also found. In contrast, the second regulatory subunit TPS3 was not present in the genome of *L. bicolor* S238N. Both TPS2 and TSL1 contain a glycosyl transferase (GT20) and a trehalose phosphatase domain, while TPS1 only hold a GT20 domain. The deduced protein sequences of TPS1, TPS2 and TSL1 showed the highest identity with thoses of *C. cinerea* (Figure 3).

A second pathway involving trehalose phosphorylase (TP) was described in a few fungi (Kitamoto *et al.* 1998, Saito *et al.* 1998, Wannet *et al.* 1998, Eis & Nidetzky 1999, Han *et al.* 2003, López *et al.* 2007). The enzyme may catalyse the reversible hydrolysis of trehalose into α- or β-glucose-1-P and glucose. In *L. bicolor* S238N, a single gene encoding TP is present in the genome as previously mentioned by López *et al.* (2007).

The three other pathways using maltose (TS pathway), maltodextrines (TreY/TreZ pathway) or ADP-glucose (TreT pathway) as substrates are only found in Eubacteria and Archaeabacteria (DeSmet *et al.* 2000, Avonce *et al.* 2006). No sequence was identified when maltooligosyltrehalose synthase and maltooligosyltrehalose hydrolase (TreY/TreZ; Genbank accession number Q53237, Q53238) bacterial protein sequences were used as BLAST query against *L. bicolor* genome. In contrast, a gene model with 35 % of sequence identity with *Pimelobacter sp.* maltose α-D-glucosyltransferase (TS pathway) was identified in the *L. bicolor* genome (Prot ID: 133065). However, this gene have the highest identity with a bacterial oligo-1,6-glucosidase (E.C. 3.2.1.10) which is involved in the hydrolysis of 1,6-α-D-glucosidic linkages in some oligosaccharides.

The catabolism of trehalose takes place mainly by the action of trehalases that specifically and irreversibly catalyse the hydrolysis of trehalose into glucose (Jorge *et al.* 1997, Parrou *et al.* 2005). Most fungi possess two types of trehalose hydrolases, referred as “neutral” and “acid” trehalases in respect to their optimal pH activity. Neutral trehalase is cytosolic while the acid enzyme is located at the cell surface. *L. bicolor* S238N harbours the genes encoding for these acid (AT) and neutral trehalases (NT). The acid trehalase contains both a neutral (Pfam 01204) and an acid trehalase domains (COG 1626). The subcellular localization algorithm SignalP identified a signal peptide in N-terminal position (amino acid 1 to 19) and predicted an extracellular localization. The neutral trehalase identified in the genome belongs to the glycoside hydrolase family 37. A Ca^{2+}-binding sequence was identified in the

N-terminal part (position 90 to 119) while no cAMP-dependent protein phosphorylation sites was found using InterProScan (Zdobnov & Apweiler, 2001) and Smart 4.0 (Letunic *et al.* 2004) programmes.

Finally, we searched for the presence of trehalose transporters in the *L. bicolor* genome. Two were described in *S. cerevisiae*: a high-affinity H^+-trehalose symporter (Agt1, AAY99642.1) and a low-affinity transporter system (Mal21, CAB46745.1). Both proteins also transport maltose with an opposite affinity (Stambuk & de Araujo, 2001). We queried (using BlastP) *L. bicolor* predicted gene models using Agt1 and Mal21 sequences and identified two genes encoding for transporters of the MFS superfamily (see also Nehls *et al.* 2008). The two predicted proteins showed a low sequence identity with the yeast transporters, but a high similarity (73 % and 72 %) with the *Amanita muscaria* Mst-1 transporter which is involved in specific uptake of monosacharides (Wiese *et al.* 2001). Therefore, *L. bicolor* probably lacks a specific trehalose transporter.

Transcriptional regulation of carbohydrate metabolism

The expression of genes involved in the carbohydrate metabolism was analyzed using whole genome expression oligoarrays (Kohler *et al.* 2008) and quantitative PCR. Transcript profiling was carried out using ectomycorrhizal root tips of *L. bicolor*/*P. trichocarpa*, *L. bicolor*/*P. menziensii*, *L. bicolor*/*P. tremula x P. alba*, *L. bicolor* fruiting bodies and free-living mycelium grown on a glucose-rich agar-medium. Transcripts were detected for all the genes analyzed indicating that all the genes involved in the primary carbohydrate metabolism were expressed whatever the fungal tissues considered. All the duplicated genes were similarly transcribed in all tissues, excepted for *MDR2* and *GAPDH1* that were expressed at a higher level in fruiting body than in mycelium and ectomycorrhizas (Figure 4). Changes in gene expression was analysed upon key developmental processes, i.e, ectomycorrhiza and fruiting body *vs.* free-living mycelium (Figure 4). Observed alterations in transcript concentrations were low and ranged between two- and five-fold, except for *MtdH*, *MDR2* and *GAPDH1* whose expression was upregulated more than ten fold.

Overall, the transcription pattern of genes involved in carbohydrate primary metabolism in Douglas fir and *P. trichocarpa* ectomycorrhizas was similar when the plantlets were grown in the glasshouse. In contrast, we observed slight differences with their pattern of expression in *P. tremula x alba* mycorrhizas synthesised *in vitro* (Figure 4).

The transcription of genes involved in PPP and TCA cycle was not significantly altered either in fruiting body or in mycorrhiza. The transcription of genes involved in EM glycolysis (*GK*, *HK*),

trehalose (*TPS*, *TP* and *AT*) and glycogen (*GP*) metabolism was up-regulated in mycorrhizas from plantlets grown in glasshouse. Conversely, the transcription of *MtDH*, *MDR* and *GAPDH1* (EM glycolysis) was enhanced in fruiting body. Data were validated by quantitative PCR except for the acid trehalase for which array and quantitative PCR results were not congruent (Figure S1). Levels of regulation measured by quantitative PCR were generally higher than those measured with oligoarrays.

^{13}C NMR

To identify the major soluble carbohydrate accumulated in *L. bicolor* mycelium and fruiting bodies, the soluble neutral carbohydrates were analyzed by ^{13}C natural abundance NMR as described previously (Martin & Canet, 1986; Martin *et al.*, 1985, 1998). Trehalose was the only soluble carbohydrate detected in fruiting body tissues (Fig. 5) and free-living mycelium (data not shown). The concentration of trehalose was ~ 4mM in both tissues.

Discussion

Although the extramatrical hyphae of *L. bicolor* may have a significant saprotrophic ability, as revealed by the abundance of proteases, glucanases and carbohydrate-active enzymes acting on animal and bacterial polysaccharides in its genome (Cullen, 2008; Martin *et al.*, 2008). However, *L. bicolor* has only a single gene encoding an endoglucanase with a cellulose-binding domain, and no genes for exocellobiohydrolases. There is also little evidence of the oxidative systems necessary for lignin degradation, such as lignin-depolymerizing peroxidases. The hyphae forming the Hartig net in colonized roots are likely biotrophic and rely on the host sucrose for their carbon metabolism. Carbohydrate exchanges between plant roots and *L. bicolor* mycelium is the cornerstone of the mycorrhizal symbiosis. Interestingly, enzymatic activities measurements and NMR analyses performed on various ectomycorrhizal fungi suggested that the primary carbohydrate metabolism of these symbionts does not differ from the one of non symbiotic fungal species (Martin *et al.* 1985, Ramstedt *et al.* 1989, Martin *et al.* 1998, Bago *et al.* 1999, Rangel-Castro *et al.* 2002). This is confirmed by the present annotation of *L. bicolor* genome: all the common glycolytic and storage pathways have been identified and seem to be functional as they are transcribed. The evolution toward mycorrhizal symbiosis did not lead to the loss or to the expansion of gene families involved in the primary carbon metabolism as it is often observed in obligatory symbiosis (Moran 2007).

The recent sequencing of the genomes of five basidiomycetes [*C. neoformans* (Loftus *et al.* 2005), *C. cinerea*, *P. chrysosporium* (Martinez *et al.* 2004), *U. maydis* (Kämper *et al.* 2006) and *L. bicolor* (Martin *et al.* 2008)] allowed a better characterization of trehalose and mannitol metabolism. Trehalose and mannitol are the main carbohydrates accumulated in fungi, including ectomycorrhizal species, where they can contribute up to 30% of the mycelium dry weight (Martin *et al.* 1985, Ramstedt *et al.* 1989, Martin *et al.* 1998, Stoop & Mooibroek 1998, Parrou *et al.* 2005). Both metabolites can serve as a storehouse of glucose and for synthesis of cellular components. Mannitol is also involved in osmotic stabilization of hyphae and may also play an important role in the recycling of reductants (NADPH and NADP). By contrast, trehalose could act in fungi as a stabilizer of cellular membranes and proteins. Our genome analysis provide new insights on trehalose and mannitol metabolism in *L. bicolor*. Concerning trehalose metabolism, we showed that trehalose phosphorylase (TP), oftenly described as a secondary enzyme present in a limited number of fungi (Elbein 2003, Parrou *et al.* 2005, Avonce *et al.* 2006), is found in all sequenced genomes. Homologs are present in *A. muscaria* (López *et al.* 2007), *P. chrysoporium*, *C. cinerea*, *C. neoformans*, *A. fumigatus*, *M. grisea* and *N. crassa* genomes (Figure 3). By contrast, no homolog was found either in the basidiomycete *U. maydis* or in the ascomycete *S. cerevisiae*. Therefore, this enzyme is present in species from the Aphyllophoromycetidae subclass and in some other basidiomycete and ascomycete species. It remains to determine whether the hydrolytic activity of the TP is reversible. While the degradation activity has been demonstrated (Kitamoto *et al.* 1998, Han *et al.* 2003), its anabolic activity has only been established *in vitro* (Saito *et al.* 1998, Wannet *et al.* 1998). But recent observations indicated that the enzyme could also work in this way *in vivo* (Han *et al.* 2003, López *et al.* 2007)

Our genomic survey also provided new insights on acid trehalase classification. Parrou *et al.* (2005) established that acid trehalases can be clustered into two groups depending on the presence of a signal peptide or a N-terminal transmembrane domain. A third category was established for trehalases from *M. grisea*, *N. crassa* and *G.* zeae that harboured a non-canonical structure with dual characteristics of both neutral and acid trehalases. The acid trehalase of *L. bicolor* belongs to this latter category. Actually this class of extracellular enzymes may contain many acid trehalases from filamentous fungi as it was identified in the genome of *L. bicolor* S238N, but also in all the sequenced genomes of filamentous fungi.

In basidiomycetes, mannitol synthesis is thought to occur through MtDH. Indeed, no M1PDH activity has ever been measured in any basidiomycetes (Hult *et al.* 1980). Two genes have been annotated as encoding M1PDH enzymes in *C. neoformans*. But the enzymatic activities of the

corresponding proteins have not been measured. Orthologs of these genes are present in all the sequenced basidiomycetous genome, including *L. bicolor*. But they are more closely related to alcool dehydrogenase than to mannitol dehydrogenase according to the phylogenetic analysis (Figure 2). Furthermore, the two MDR transcripts from *L. bicolor* are transcribed in free-living mycelium, while no mannitol was detected in hyphae by NMR. Conversely, MtDH transcript was barely detectable in free-living mycelium. Altogether, these results suggest that these genes do not encode for M1PDH.

Transcriptional regulation of carbohydrate metabolism in ectomycorrhizae and fruiting bodies

The ectomycorrhizal symbiosis leads to dramatic changes in carbon metabolism in the mycobiont forming the association (Martin *et al.* 1987, Hampp & Schaeffer 1995, Martin *et al.* 1998, López *et al.* 2007). Trehalose, mannitol and various small polyols have been reported to accumulate during mycorrhiza formation (Ineichen & Wiemken 1992, Martin *et al* 1998, Nehls *et al*. 2001). This shift in fungal metabolism was correlated with an alteration of the transcription of genes encoding proteins involved in glucose respiratory pathways (Voiblet *et al.* 2001, Johansson *et al*. 2004, Duplessis *et al.* 2005). A single gene encoding hexokinase (HK) was found up-regulated whatever the basidiomycetous species analyzed so far, *e.g. P. microcarpus* and *P. involutus*. In *L. bicolor*, glucokinase and hexokinase encoding genes showed a weak increased transcription in both poplar and Douglas fir mycorrhizas. In *S. cerevisiae*, the *HXK2* gene, encoding for a hexokinase, plays a pivotal role in the control of the expression of primary carbon metabolism genes, including it own transcription (Moreno & Herrero, 2002). The ectomycorrhizal hexokinase may also participate to carbon metabolism regulation during the symbiosis establishment as already suggested in the ascomycete *T. borchii* (Ceccaroli *et al.* 1999). This ectomycorrhizal fungus harbours three distinct enzymatic forms of hexokinases which are differentially expressed during mycelium growth.

Another striking alteration in *L. bicolor* carbohydrate metabolism is the up-regulation of all the genes encoding proteins of the trehalose synthase complex in symbiotic tissues, indicating that the accumulation of trehalose in *L. bicolor* mycorrhizas is controlled at the transcriptional level. In contrast, we observed the repression of the genes encoding trehalose phosphorylase and neutral trehalase, and a strong up-regulation of mannitol dehydrogenase genes in fruiting body. This suggests that a metabolic shift is likely to occur during *L. bicolor* fruiting body formation. However, mannitol was not detected in the fruiting bodies of *L. bicolor* using natural abundance ^{13}C NMR (Fig. 5). This suggests that if mannitol synthesis occurs the turn-over of the polyol pool is so high that it does not accumulate. In the ectomycorrhizal ascomycetous fungi, *C. geophilum* and *S. brunnea*, the synthesised

mannitol is immediately consumed as demonstrated by the high isotopic scrambling observed in ^{13}C-NMR experiment (Martin *et al.* 1985, 1988). Although trehalose and mannitol are the most commonly carbohydrate accumulated in fungi, patterns of accumulation of these compounds highly differed between species of ectomycorrhizal fungi: in *C. geophilum* (Martin *et al.* 1985), *T. borchii* (Ceccaroli *et al.* 2003) and *P. tinctorius* (Martin *et al.* 1998), the main carbohydrate detected by NMR in free-living mycelium is mannitol. Conversely, *L. bicolor* and *P. croceum* accumulate only trehalose (Ramstedt *et al.* 1989, present study), while both trehalose and mannitol were found in *C. cibarius* mycelium (Rangel-Castro *et al.* 2002). The cause of these various metabolic patterns remains to be determined.

The present *in silico* metabolic reconstruction of the central carbon metabolism in *L. bicolor* showed that the carbohydrate metabolism in this symbiotic fungus does not differ from saprophytic fungi and that ectomycorrhiza formation induces a carbon metabolic shift that is controlled at the transcriptional level.

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Figure Legends

Figure 1. Map of the central carbohydrate metabolism in *Laccaria bicolor* S238N-H82. The glycolysis, pentose phosphate pathway and tricarboxylic acid cycle, and the metabolism of glycogen, trehalose, glycerol and ethanol are depicted. Gene products contributing to these pathways are indicated. Numbers correspond to the protein ID of each enzyme in the JGI annotation v.1.0. ID numbers in italic are for proteins that showed their highest similarity with bacterial sequences. Name into brackets indicates multicomplex enzymes and numbers below corresponds to their subunits. GLYCOLYSIS: GK, glucokinase; HK, hexokinase; PGM, phosphoglucomutase; GPI, glucose-6-phosphate isomerase; PFK, phosphofructokinase; FBA, fructose bisphosphate aldolase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PGK, phosphoglycerate kinase; PGAM, phosphoglycerate mutase; ENO, enolase; PYK, pyruvate kinase; TPI, triose phosphate isomerase. PENTOSE PHOSPHATE PATHWAY: GPDH, glucose-6-phosphate 1-dehydrogenase; PGL, 6-phosphogluconolactonase; PGD, 6-phosphogluconate dehydrogenase; RPE, ribulose-5-phosphate 3-epimerase; RPI, ribulose 5-phosphate isomerase; TK, transketolase; TA, transaldolase. GLUCONEOGENESIS: PYCK, phosphoenolpyruvate carboxykinase; FBP, fructose bisphosphatase. ETHANOL PATHWAY: PDC, pyruvate decarboxylase. GLYCOGEN METABOLISM: GSI, glycogen synthase initiator; GP, glycogen phosphorylase. TREHALOSE METABOLISM: TPS1, trehalose 6-phosphate synthase; TPS2, trehalose 6-phosphate phosphatase; TP, trehalose phosphorylase; NT, neutral trehalase; AT, acid trehalase. TCA Cycle: PDH, pyruvate dehydrogenase; CS, citrate synthase; ACO, aconitase; IDH, isocitrate dehydrogenase; OGDH, a-ketoglutarate dehydrogenase; SAS, succinyl CoA synthase; SDH, succinate dehydrogenase; FH, fumarase; MDH, malate dehydrogenase; PYC, pyruvate carboxylase. MANNITOL METABOLISM: MtDH, NADP-dependent mannitol dehydrogenase.

Figure 2. Neighbour-Joining tree of *Laccaria bicolor* NADP-dependent mannitol dehydrogenase (MtDH), medium-chain dehydrogenase/reductases (MDR), alcool dehydrogenase (ADH) and mannitol-1-phosphate 5-dehydrogenase (M1PDH). The GenBank accession number for each protein sequence is given in Table S2.

Figure 3. Neighbour-Joining tree of *Laccaria bicolor* trehalose-6-P-synthase (TPS1), trehalose phosphate-phosphatase (TPS2), trehalose-6-P-synthase regulatory subunit (TSL1) or trehalose phosphorylase (TP). The GenBank accession number for each protein sequence is given in Table S3.

Figure 4. Relative expression level of *L. bicolor* S238N genes involved in the major pathways of the carbohydrate metabolism in ectomycorrhizal root tips of *L. bicolor*/*Populus trichocarpa* associations grown in pots (red bars), of *L. bicolor*/*Populus tremula x alba* associations grown *in vitro* (yellow bars), of *L. bicolor*/*Pseudosuga menziensii* associations grown in pots (green bars) and in fruiting bodies (blue bars). Transcription level of each gene was measured using NimbleGen whole-genome expression oligoarrays (Kohler *et al.* 2008) and compared to the level in free-livingmycelium grown on P5 agar medium. Two biological replicates were performed for the mycelium, the Douglas fir mycorrhiza and the fruiting bodies (the values indicated are the mean value).

Figure 5. Trehalose accumulation in *L. bicolor* fruiting body. NMR spectra (100.62 Mhz) of fruiting body methanolic extracts of *L. bicolor* S238N. The spectrum represents the time average of 16,000 scans of 2-s pulse intervals. (T), trehalose.

Figure 1 Deveau *et al.*

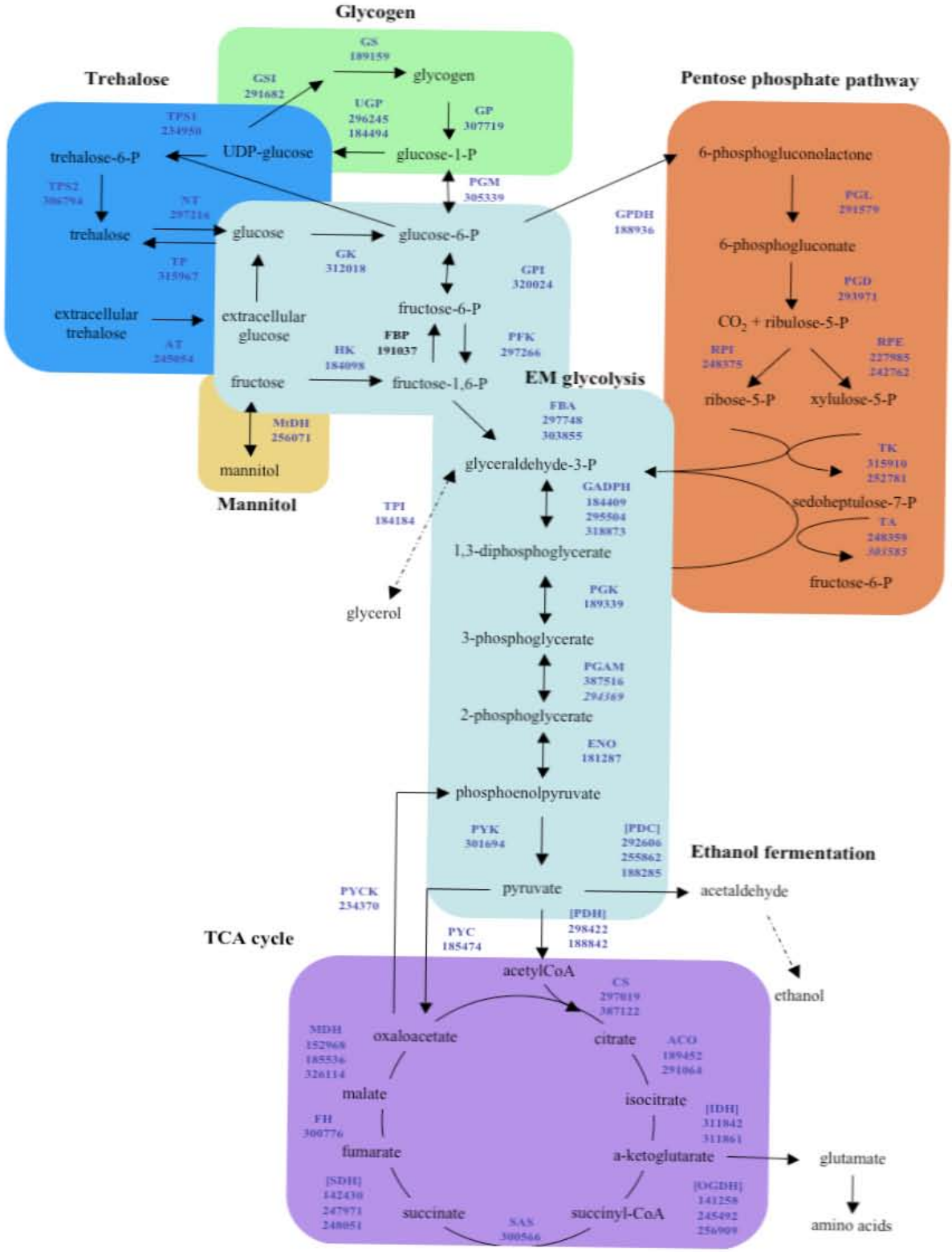


Figure 2 Deveau *et al.*

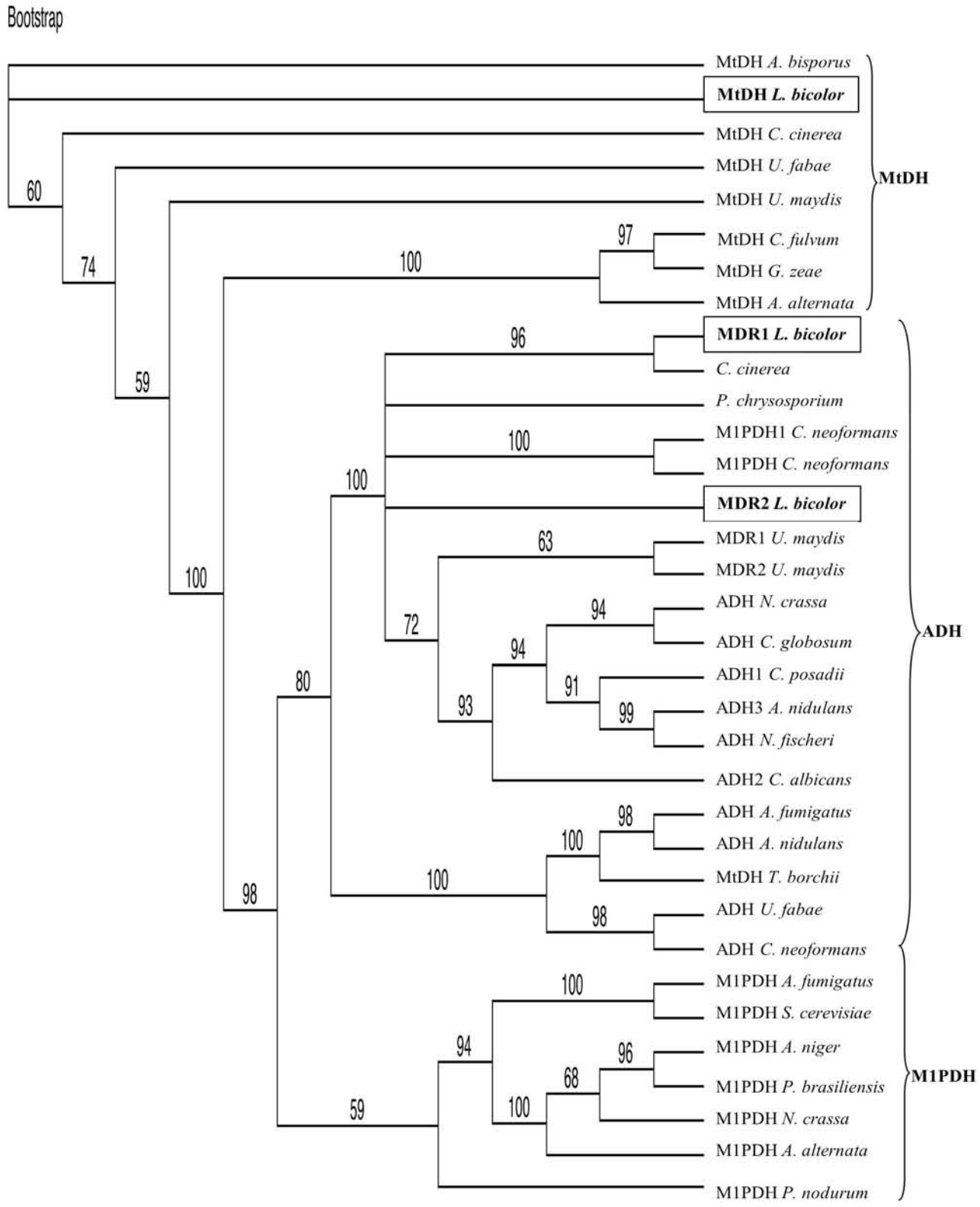


Figure 3 Deveau *et al.*

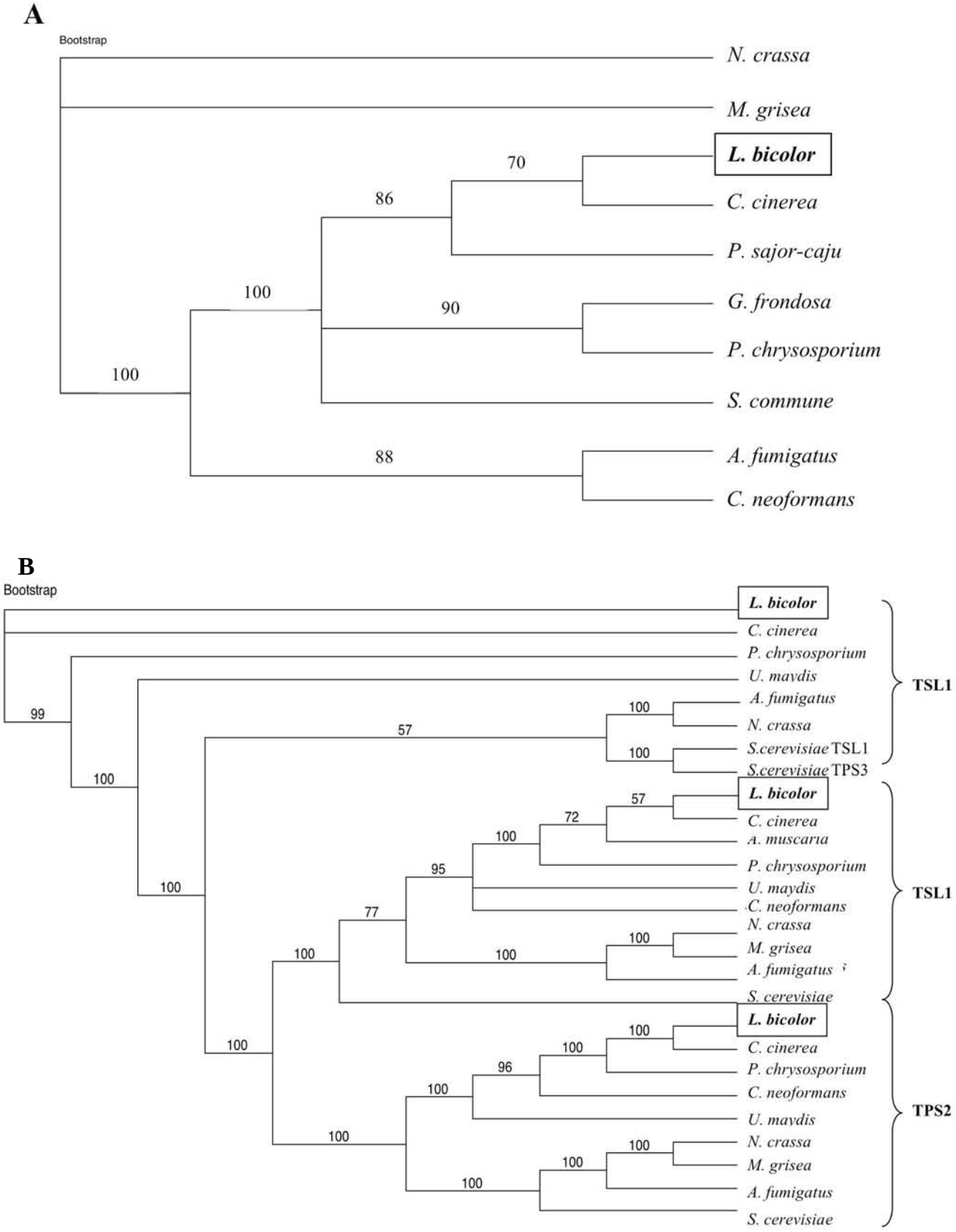


Figure 4 Deveau *et al.*

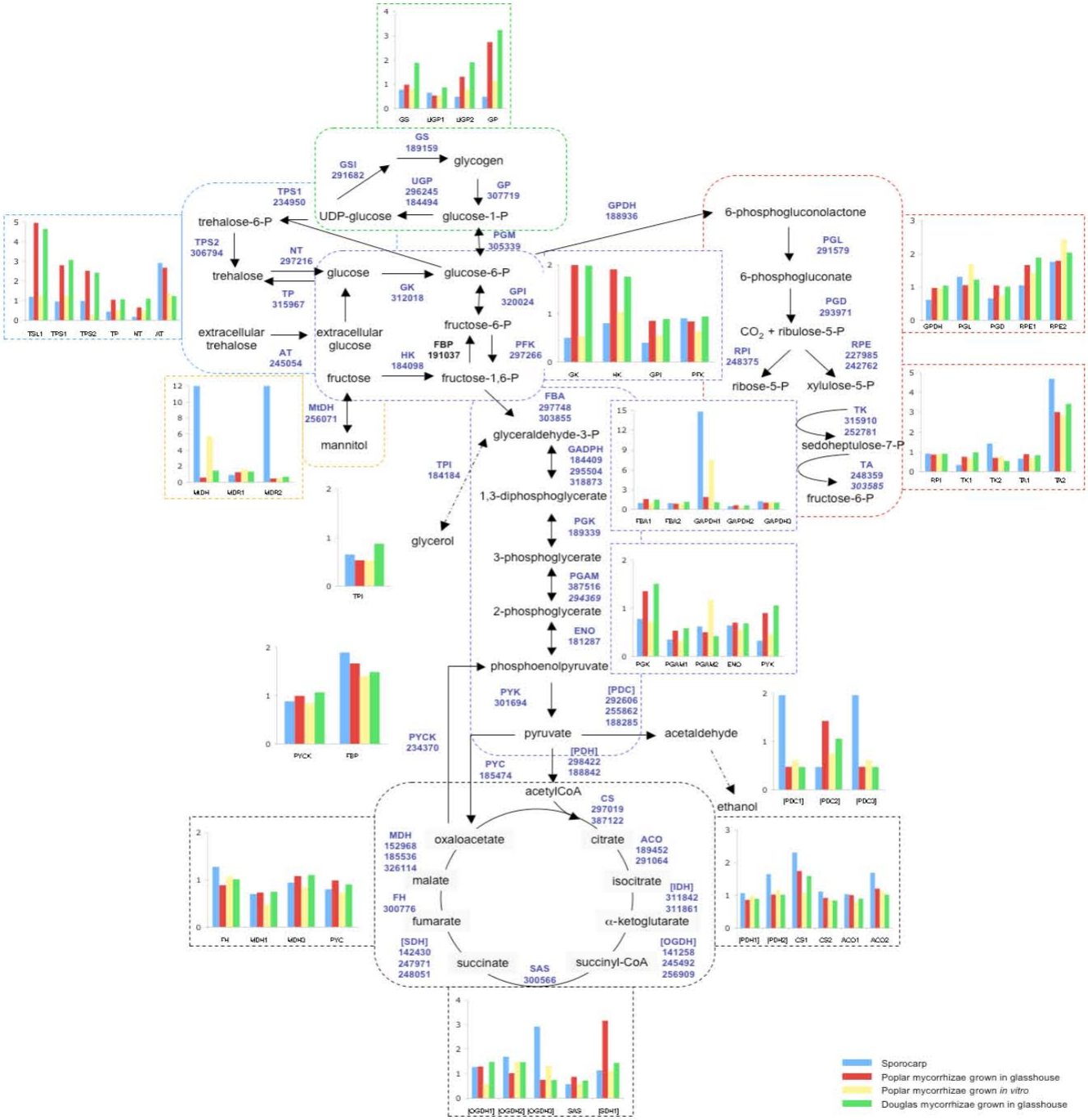
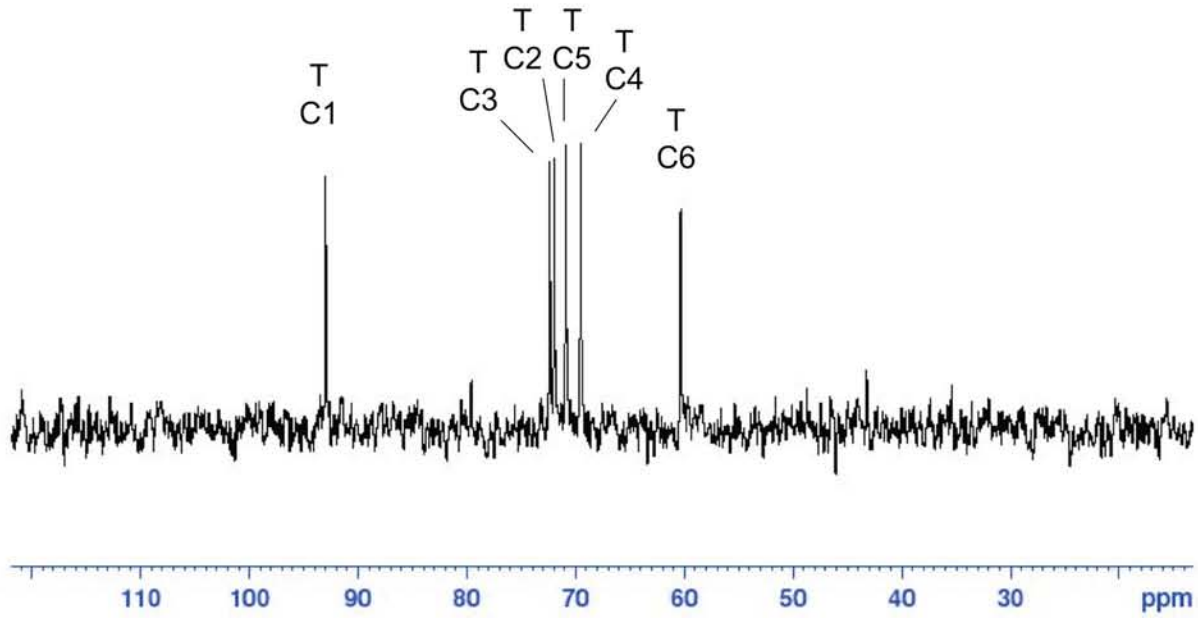


Figure 5 Deveau *et al.*



Supplemental Web data.

Table S1. List of primers used in this study.

Table S2. List of the GenBank accession numbers used for the construction of the mannitol enzyme NJ tree.

Table S3. List of the GenBank accession numbers used for the construction of the trehalose enzyme NJ trees.

Figure S1. Validation of the oligo-array data by real-time PCR analyses.

V.2 Does *L. bicolor* S238N-H82 conceal endobacteria ?

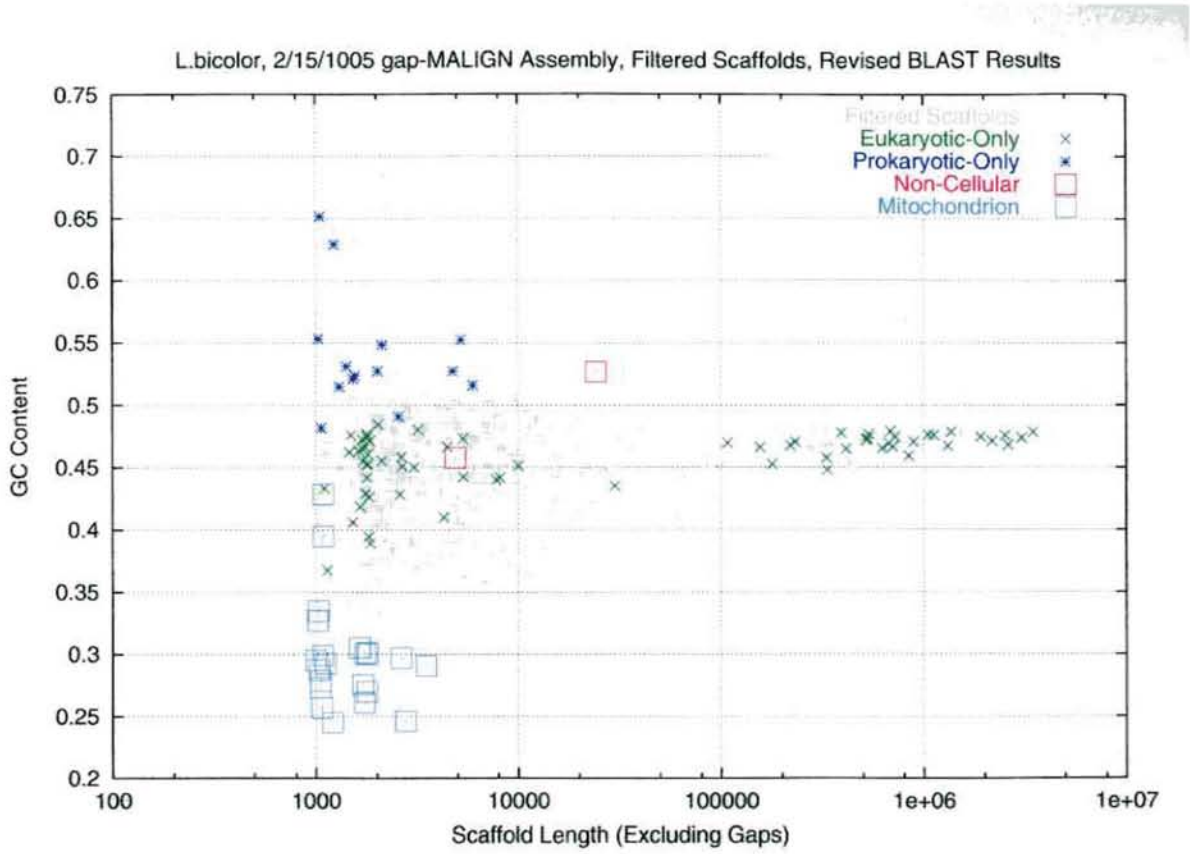
Fungal genomes are classically composed of two types of sequences characterized by their GC content: a first group of sequences with GC% ranging from 45 % to 50 % which corresponds to the fungal genome, and a second group of about 30 kb which contains the mitochondrial genome with a GC% ranging between 25 and 35 % (Figure 5.1). In the case of *L. bicolor* H82-S238N however, the GC diagram revealed a third unexpected group with a high GC content (60 to 65%), typical of bacterial genomes. The presence of intracellular bacteria in *L. bicolor* was suspected since a long time. Indeed, bacterial proliferations have recurrently been observed for the past 15 years in fermentor cultures of *L. bicolor* strain S238N, suggesting the presence of cryptic bacteria in the axenic cultures of the fungus. Bertaux *et al.* (2003) previously detected the presence of intracellular bacteria in different sub-culture of *L. bicolor* S238N by using fluorescence *in situ* hybridization (FISH) in combination with confocal laser scanning microscopy. The bacteria, identified as a *Paenibacillus* spp., were rare and heterogeneously distributed in the mycelium. The sequenced strain *L. bicolor* H82-S238N is a monokaryotic strain germinated from a spore from a sporophore of *L. bicolor* S238N (Martin *et al.*, unpublished, Annexe 1). The sporophore was collected associated to a seedling of *Pseudotsuga menziesii* inoculated with *L. bicolor* strain S238N in a glasshouse. The spores were collected and germinated according to Fries (1983). In this context, we have analyzed the high GC% sequences isolated in the genome of *L. bicolor* H82-S238N. First we have compared these sequences to those present in the NCBI genome database. Secondly, we have designed primers from these high GC sequences and we have tried to amplify by PCR fragments of *L. bicolor* H82-S238N DNA using these primers. Finally we have sequenced the amplified fragments and compared their sequences to the high GC ones.

V.2.1 Material & methods

In silico analysis

A NCBI BLASTN search in nucleotides databases was used to identify genes with sequences similar to the 32 scaffolds with high GC%. The BLASTN was performed against

Figure 5.1. Representation of the *L. bicolor* H82-S238N scaffolds length depending on their GC content.



the 918 bacterial and 47 archeal genomes which are available on the microbe genome database in the NCBI (http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi).

Sterile DNA extraction

DNA extraction of *L. bicolor* H82-S238N were performed in sterile conditions under a vertical laminary flow. DNA was extracted from a fungal culture grown on P5 solid medium (Pachlewski & Pachlewska, 1974) by using two DNA extraction kits (Instagene & Qiagen), as recommended by the manufacturers. All buffers were sterilized by autoclaving followed by a 0.2μm filtration before performing the extraction. As a negative control (C1), a DNA extraction from sterile deionized water was performed.

PCR amplification

Primers for PCR amplification of portions of the scaffold 1120 (5' GTCAACGGCAAGGAAGAGAA 3'; 5' CCTTACTCCGGATGGAAACA 3'), 1161 (5' CCACCACGACATCTACTCCATC 3'; 5' CACGCCCACTTCGGACA 3'), 130 (5' ACTTCAGCGCAGGAACAAAG 3'; 5' GGCGATATCACCTACCTGGAC 3') and 134 (5' GGCACAGCAGGTGAGGAAT 3'; 5' AAGGACATCGTGGGCAATC 3') were designed with the Amplify 3.1.4 program.

Extracted DNA was diluted 10 times and then amplified by quantitative PCR (qPCR). Each qPCR reaction (15 μL total volume) contained 2 μL of DNA template, 300 nM of each primer and 1X SYBR green PCR master mix (Bio-Rad). The following cycling parameters were applied: 95 °C for 3 min and then 40 cycles of 95°C for 30 s, 60°C for 1 min and 72°C for 30s. Two negative controls were performed for each primer pair using sterile deionised water (C2) and C1 as templates. After the qPCR, products were migrated into a 2 % agarose gel electrophoresis and visualised under UV.

Purification, cloning and sequencing of qPCR products

Each PCR product was extracted from the agarose gel and purified with GFX PCR DNA gel Band purification kit (Amersham). Purified products were cloned into bacteria using the TOPO cloning kit as recommended by the manufacturer (Invitrogen). A ß-gal blue/white screening was used to identify positive clones. White bacteria were collected and a PCR amplification of the inserts was performed using the M13 universal primers. The size of the PCR products was checked on 1% agarose gel electrophoresis, then

Table 5.1. Characteristics of the *L. bicolor* H82-S238N high GC % scaffolds. The size of the scaffolds (i.e assembled sequences), the best BlastN results, the E-value of the best blastN, the function encoded by genes inside best BlastN sequence and the accession number of the best blastN results are provided.

Scaffold name	Size (bp)	Best BLASTN result	E-value	Function	AC number
scaffold_1120 1053	1053	*Pseudomonas* sp. K82	0,00E+00	4-carboxy-2-hydroxymuconate-6-semialdehyde dehydrogenase	ABD84014.1
scaffold_1125 1045	1045	*Polaromonas* sp. JS666	4,00E-16	Twin-arginine translocation pathway signal	ABD69745
scaffold_1161 1025	1025	*Polaromonas* sp. JS666	4,00E-124	Glutamate syntase (ferredoxin)	ABE42747.1
scaffold_129 35667	2246	*Ralstonia eutropha* JMP134	2,00E-51	L-carnitine dehydratase/bile acid-inducible protein F	AAZ62268.1
		Pseudomonas sp. K82 pca	4,00E-49	protocatechuate 4,5-dioxygenase alpha subunit	ABD84012.1
		Polaromonas sp. JS666	5,00E-85	acetylglutamate kinase	ABE45876.1
scaffold_130 35797	2519	*Polaromonas* sp. JS666	3,00E-18	two component transcriptional regulator, winged helix family	ABE45875.1
		Polaromonas sp. JS666	3,00E-15	extracellular solute-binding protein, family 3	ABE45800.1
scaffold_131 35589	2284	*Polaromonas* sp. JS666	2,00E-48	protein of unknown function UPF0054	ABE45989.1
		Polaromonas sp. JS666	2,00E-32	AMP-dependent synthetase and ligase	ABE46117.1
scaffold_132 35537	2143	*Ralstonia eutropha* JMP134	2,00E-05	Uncharacterized protein UPF0065	AAZ60562.1
scaffold_134 35252	2116	*Rhodoferax ferrireducens* T118	0,00E+00	cell divisionFtsK/SpoIIIE	ABD70887.1
		Rhodoferax ferrireducens T118	6,00E-17	Twin-arginine translocation pathway signal	ABD69745
scaffold_135 35171	1507	*Rhodoferax ferrireducens* DSM 15236	6,00E-10	Uncharacterized protein UPF0065	ABD71009.1
scaffold_136 35111	***2397***	*Pseudomonas* sp. ATCC 19121	0,00E+00	L-aspartate 4-carboxylyase	AAQ07948.1
scaffold_139 35033	2697	No hit	NS	No hit	
scaffold_140 35009	1593	*Burkholderia thailandensis* E264	6,00E-04	phage terminase, large subunit, putative	ABC35656.1
scaffold_141 35000	1753	*Bordetella bronchiseptica* strain RB50	0,00E+00	cytochrome ubiquinol oxidase subunit I	CAE31807.1
scaffold_926 1360	1360	*Polaromonas* sp. JS666	3,00E-39	CheA signal transduction histidine kinases	ABE43092.1
scaffold_970 1262	1262	*Polaromonas* sp. JS666	2,00E-31	heat schock protein Hsp90	ABE42574.1
		Pseudomonas fluorescens PfO-1	2,00E-27	aquaporin	ABA73169.1
scaffold_977 1252	1252	No hit	NS	No hit	
scaffold_979 1249	1249	*Rhodoferax ferrireducens* DSM 15236	4,00E-26	flagellar M-ring protein FliF	ABD68301.1
scaffold_983 1237	1237	*Polaromonas* sp. JS666	0,00E+00	ATP synthase F1, beta subunit	ABE42292.1
scaffold_986 1232	1232	*Neisseria meningitidis* MC58	2,00E-15	putative TonB-dependent receptor	AAF40744.1
scaffold_416 5946	5946	*Exiguobacterium sibiricum* 255-15	0,00E+00	molybdopterin biosynthesis protein B, molybdopterin biosynthesis protein C	NZ_AADW02000034
scaffold_441 5201	5201	*Escherichia coli* W3110	0,00E+00	predicted transporter subunit	AC_000091
scaffold_454 4737	4737	*Escherichia coli* W3110	0,00E+00	predicted transferase with NAD(P)-binding Rossmann-fold domain	AC_000091
scaffold_710 2553	2553	*Escherichia coli* W3110	0,00E+00	enterobactin synthase multienzyme complex component	AC_000091
scaffold_753 2123	2123	*Thermoanaerobacter ethanolicus* X514	0,00E+00	predicted membrane fusion protein component of efflux pump, membrane anchor	NZ_AATV01000052
scaffold_773 2019	2019	*Thermoanaerobacter ethanolicus* X514	0,00E+00	cardiolipin synthase 2	NZ_AATV01000052
scaffold_859 1563	1563	*Escherichia coli* W3110	0,00E+00	RNA helicase	AC_000091
scaffold_866 1531	1531	*Escherichia coli* W3110	0,00E+00	Sugar kinase	AC_000091
scaffold_905 1420	1420	*Escherichia coli* B	0,00E+00	iron-enterobactin transporter subunit	NZ_AAWW01000031
scaffold_949 1311	1311	*Escherichia coli* W3110	0,00E+00	magnesium trasnporter	AC_000091
scaffold_1108 1066	1066	*Escherichia coli* W3110	0,00E+00	DNA-bindign transcriptional regulator	AC_000091
scaffold_1146 1034	1034	*Escherichia coli* W3110	0,00E+00	enterobactin synthase multienzyme complex component	AC_000091

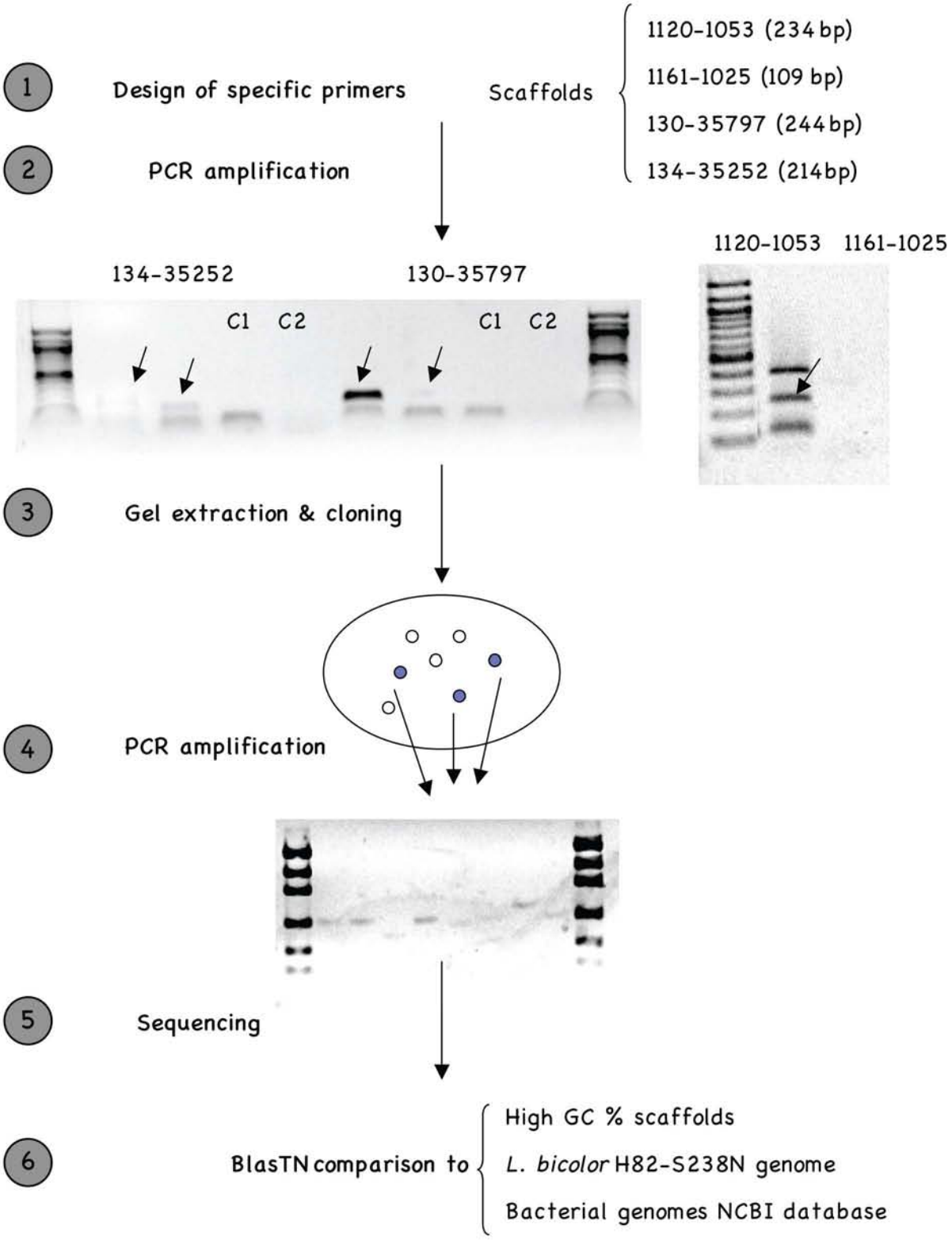
purified with the gel band purification kit (Amersham). Purified products were sequenced as described by Kohler *et al.* (2003). Edited nucleotide sequences were compared to the putative bacterial scaffolds sequences and to the nucleotide database of the NCBI with the BlastN algorithm.

V.2.2 Results

Thirty-two scaffolds (i.e assembled sequences) showing a GC% higher than 60 % were identified (Table 5.1). The size of the scaffolds varied between 1025 and 5946 bases, for a total number of 62 474 bases. Thirteen scaffolds were identical to sequences from *Escherichia coli* strains. Two had no identity with known sequences and 17 showed high level of identity with sequences from other bacterial genomes. Into these 17 scaffolds, 23 genes were identified. They had high level of similarity with *Polaromonas* sp. JS666 (10 genes), *Rhodoferax ferrireducens* (4 genes), *Ralstonia eutropha* JMP134 (2 genes), *Pseudomonas* sp. K82 (2 genes), *Pseudomonas* sp. ATCCC 19121 (1 gene), *Burkholderia thailandensis* E264 (1 gene), *Bordetellla bronchiseptica* RB50 (1 gene), *Pseudomonas fluorescens* PfO-1 (1 gene) and *Neisseria meningitidis* MC58 (1 gene). All these bacterial strains belong to the ß-proteobaceria group, except the *Pseudomonas* strains that belong to the γ-proteobacteria group.

In a second step, we have amplified by PCR four sequences coming from the scaffolds 1120-1053, 1161-1025, 130-35797 and 134-35252 (Figure 5.2). The four sequences were chosen because they showed the highest degree of similarity with bacterial sequences (1). Several PCR products were obtained with each primer pair (2). In each case a PCR product of the expected this was obtain, except for the 1161-1025 amplification. An amplification was also performed on DNA extracted from sterilized water used for DNA elution and PCR mastermix preparation. No PCR product of the expected size was obtained in this control treatment. Therefore the PCR products observed after amplification of the DNA from *L. bicolor* H82-S238N with primer pairs designed on high GC sequences were not coming from contaminants (3). The PCR products of the expected size were cloned. Seven inserts of different size coming from the 130-35797 and 134-35252 PCR products were obtained (4). We failed to clone the PCR product obtained with the 1120-1053 primer pair. Inserts of the expected size were sequenced (5). Four sequences had 93 % of identity with the sequence of the scaffold 134-35252 (6). They had no identity with the assembled genome of *L. bicolor* H82-S238N (sequence with a GC

Figure 5-2. Procedure used to amplify, clone, sequence and identify PCR products from *L. bicolor* S238N PCR products.



% between 45% and 50 %). Three sequences had no or few identity either with the 130-35797 or the 134-35252 scaffolds. But they were found in the fungal genome.

V.2.3 Discussion

Thirteen of the high GC % sequences identified in the genome of *L. bicolor* H82-S238N corresponded to contaminants that were probably coming from the DNA libraries at any step of the JGI sequencing procedure (*E. coli* sequences). Indeed, for the genome sequencing, the cosmids, in which the fragments of *L. bicolor* H82-S238N genomic DNA were inserted, were used to transform *E. coli* strain. But the other half of high GC% sequences corresponded to either ß-proteobacteria or γ-proteobacteria. The presence of these sequences cannot be explained by contaminations resulting from another sequencing project performed at the JGI because they were spread among the plates of sequencing (F. Martin, personal communication). Furthermore these bacteria are not used as a tool for molecular biology as it is the case for *E. coli* strains. We successfully amplified a part of one bacterial scaffold using a template from a DNA sample that we had extracted from an axenic culture of *L. bicolor* H82-S238N. This suggests that the hypothetical bacterial sequences present in the genome of *L. bicolor* H82-S238N did not come from a contamination that would have occurred during the extraction of the DNA used for the genome sequencing. We can thus conclude that the bacteria are present in the pure culture of *L. bicolor* H82-S238N either inside or outside the hyphae. We were not able to see any bacterial cells using classical methods of microscopy. Fluorescence *in situ* hybridization experiments will be necessary to precisely determine their localization. Using the same approach, Bertaux *et al.* (2005) previously demonstrated that axenic culture of *L. bicolor* S238N harboured intracellular *Paenibacillus* sp. cells. In the present case, complementary analyses will be necessary to elucidate the taxonomic identity of the bacteria.

Intracellular bacteria were found in many eukaryotic organisms: aphids (*Schizaphis graminum*, Baumann *et al.* 1995), bivalves (*Bankia setacea*, Distel *et al.* 1991), plants (*Gunnera* spp., Rai *et al.* 2000) and in few fungi (*Rhizopus microsporus*, Partida-Martinez & Hertweck 2005). In mycorrhizal fungi, the examples of endobacteria which have been reported so far concern species belonging to the *Glomeromycota* group (Schüßler *et al.* 2001, Bianciotto *et al.* 2000). Barbieri *et al.* (2000) and Bertaux *et al.* (2005) were the first to suggest the presence of endobacteria in ectomycorrhizal fungi. The endobacteria

which were identified in the dikaryotic strain *L. bicolor* S238N belonged to the Firmicutes, a group taxonomically distant from the Proteobacteria one. However, the *L. bicolor* H82-S238N strain has been isolated from a spore coming from a sporocarp produced in non sterile conditions in a glasshouse. Bertaux *et al.* (2005) suggested that the endobacteria of *L. bicolor* S238N were environmentally acquired. In this hypothesis, the H82-S238N endobacteria would have penetrated into the hyphae after the inoculation of *L. bicolor* S238N in the soil. But this hypothesis implies that the endobacteria was then vertically transmitted into the spores. Vertical transmission of intracellular bacteria has already been described in the endomycorrhizal fungus *Gigaspora margarita* (Bianciotto *et al.* 2004) but remains to be demonstrated in ectomycorrhizal fungi. A second point that needs to be investigated is the role of these endobacteria in the fungal physiology and its impact on the ectomycorrhizal symbiosis. Finally, one should address the question of the involvement of these endobacteria in horizontal gene transfer between bacteria and ectomycorrhizal fungi.

Conclusion.

La combinaison d'outils bioinformatiques et de méthodes de biologie moléculaire plus classiques nous a permis de montrer que le métabolisme primaire du carbone du champignon ectomycorhizien *L. bicolor* ne diffère pas de celui de champignons saprophytiques. De plus, il est apparu que la mycorhization et la fructification conduisent à des modifications de la transcription des gènes impliqués dans le métabolisme carboné. Ces modifications transcriptionnelles aboutissent probablement à l'évolution du statut métabolique de la cellule : tandis que les mycorhizes de *L. bicolor* se caractériseraient par un fort métabolisme du tréhalose, les carpophores accumuleraient de grandes quantités de mannitol.

Par ailleurs, les analyses *in silico* ont confirmé la présence d'une ou plusieurs souches bactériennes véhiculées par les hyphes de *L. bicolor*. Il reste maintenant à déterminer la localisation (endocellulaire ou associée aux hyphes) et l'identité précise des souches.

Au cours du mois de juillet 2007, le génome de la souche auxiliaire de la mycorhization *P. fluorescens* BBc6R8 a également été séquencé et assemblé. Une analyse du contenu et des caractéristiques de ce génome va être réalisée dans les mois à venir. De grandes avancées dans la compréhension des mécanismes d'interaction entre *L. bicolor* S238N et *P. fluorescens* BBc6R8 devraient être faites grâce à la connaissance de ces deux génomes.

General Conclusion

Conclusion Générale

Final discussion, conclusion and future prospects

About the methods.

Advantages and disadvantages of an in vitro approach.

During my PhD, all the experiments have been performed with an *in vitro* confrontation assay, which has been designed to reduce biological variations that would be due to uncontrolled biotic and abiotic parameters. The use of an *in vitro* assay also facilitates the sampling of the biological material and permits to perform a precise description of the interaction during the time. Thanks to this *in vitro* confrontation assay we have been able to examine the early step of the interaction before physical contact between the two micro organisms and to demonstrate that the response of the fungus was early initiated. This would have been difficult to show in a natural environment where microorganisms are heterogeneously localised and where cell metabolisms are not synchronised.

However, if this simplified *in vitro* assay is technically advantageous, its simplicity is also its main disadvantage because the interactions with other organisms that occur in a natural environment, notably with tree roots, are absent. In the present case, the absence of the plant is justified by the fact that we were focusing on the pre-symbiotic life of the fungus. Indeed, Brulé *et al.* (2001) have demonstrated that the helper strain *P. fluorescens* BBc6R8 induces an increase of the survival and the growth of the mycelium of *L. bicolor* S238N before physical and molecular contact with roots. Interestingly, our experiments have revealed the existence of other unexpected mechanisms. First, *P. fluorescens* BBc6R8 induced an over expression of several fungal genes involved in the biosynthesis of plant hormones. This suggests that the bacteria could indirectly prepare the roots to the symbiosis. Secondly, the increased branching of the mycelium which was correlated with an over expression of some genes also over expressed during root infection by ectomycorrhizal fungi indicates that the bacteria could also prepare the fungal hyphae to the root infection. Now, it is necessary to demonstrate that these modifications of the fungal transcriptome correlate with modifications of the fine root branching and the mycorhization rate. For this purpose, we have started to design a tripartite *in vitro* assay including a poplar plant partner. During the first experiment that we have performed, we have obtained mycorrhizae between *L.*

bicolor S238N and Poplar but the inoculation of *P. fluorescens* BBc6R8 has reduced the total number of mycorrhizae. This reduction was observed in all the following treatments: roots pre-incubated with the bacteria, fungus pre-incubated with bacteria and three partners inoculated at the same time. More experiments are needed to adjust the experimental design to obtain an *in vitro* promoting effect of the bacterial strain on mycorrhiza formation.

Advantages and disadvantages of the transcriptomic approach.

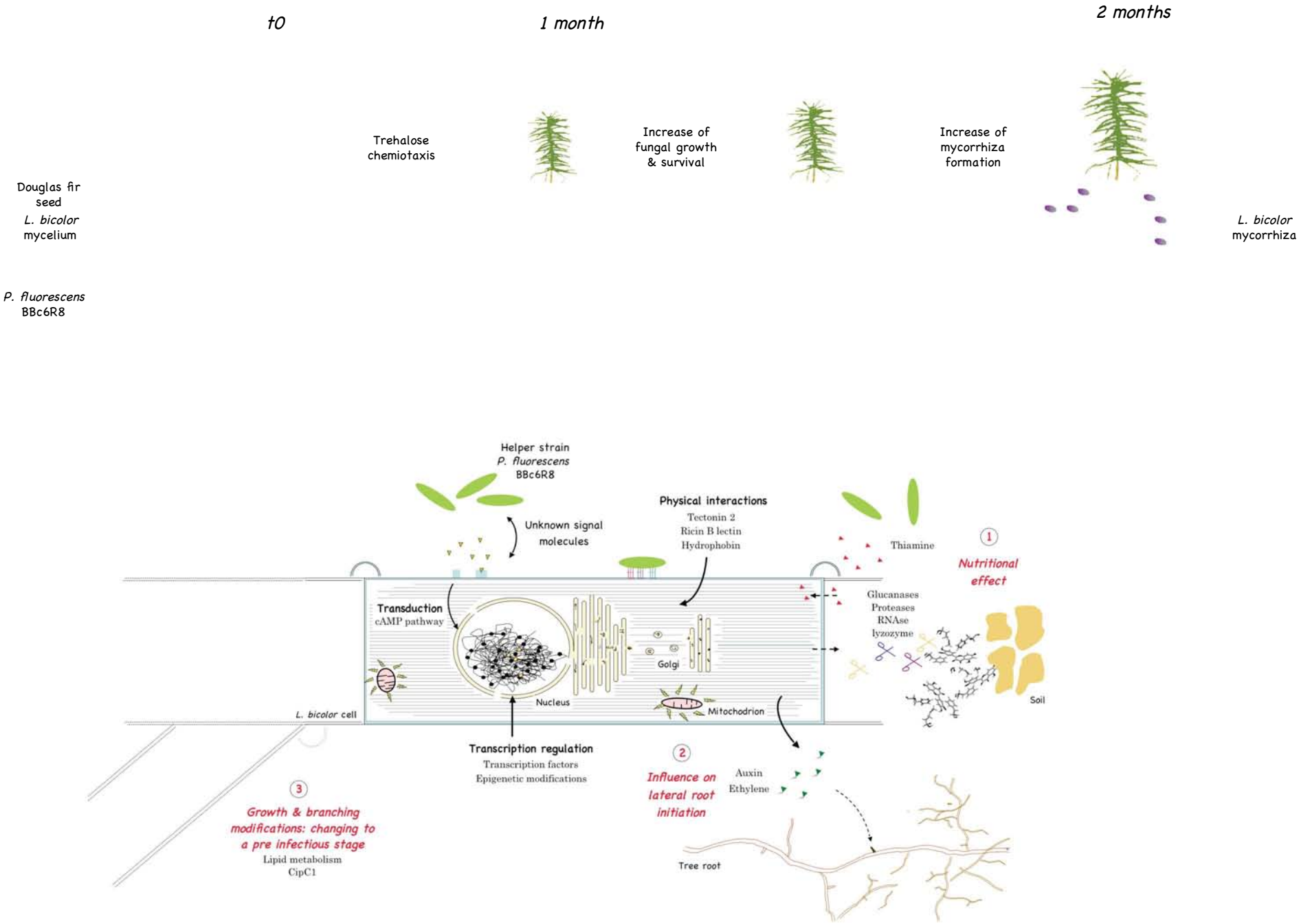
When I have started my PhD, the involvement of transcriptomic regulations in the mycorrhiza helper effect was unknown. Thanks to the knowledge of the full sequence of the genome of *L. bicolor* H82-S238N and to the creation of oligochips that covered the whole genome, we have been able to demonstrate not only that transcriptomic regulations occur during the interactions between the ectomycorrhizal fungus *L. bicolor* S238N and soil bacteria but also that unsuspected mechanisms control the helper effect of *P. fluorescens* BBc6R8 (saprophytic activity & over production of plant hormones, cf previous paragraph). These mechanisms have not been described so far because of the difficulty to set up dedicated experiments. The recent sequencing of the genome of the helper strain *P. fluorescens* BBc6R8 should allow us to perform soon the same kind of experiment on the bacteria in order to identify the genes which are involved in the helper effect and to determine whether these genes are constitutively expressed or induced in the presence of the fungus.

Although the transcriptomic approach is powerful, it should not be forgotten that it is a descriptive method which only refers to gene expression. Variations of transcripts concentration are not always followed by modifications of protein concentrations or enzymatic activities. Thus, our work should be considered as a prospective analyses that should be followed by demonstrative studies in the future. As a consequence, the mechanisms of the helper effect which are proposed below will need to be confirmed.

Conceptual model of the mycorrhiza helper effect of *P. fluorescens* BBc6R8.

The conceptual model we propose integrate all the data acquired during this thesis and previously generated by R. Duponnois (1992), P. Frey-Klett (1996) and C. Brulé (2001)(figure C1).

Figure C1. Model of the mechanisms of the helper effect of *P. fluorescens* BBc6R8



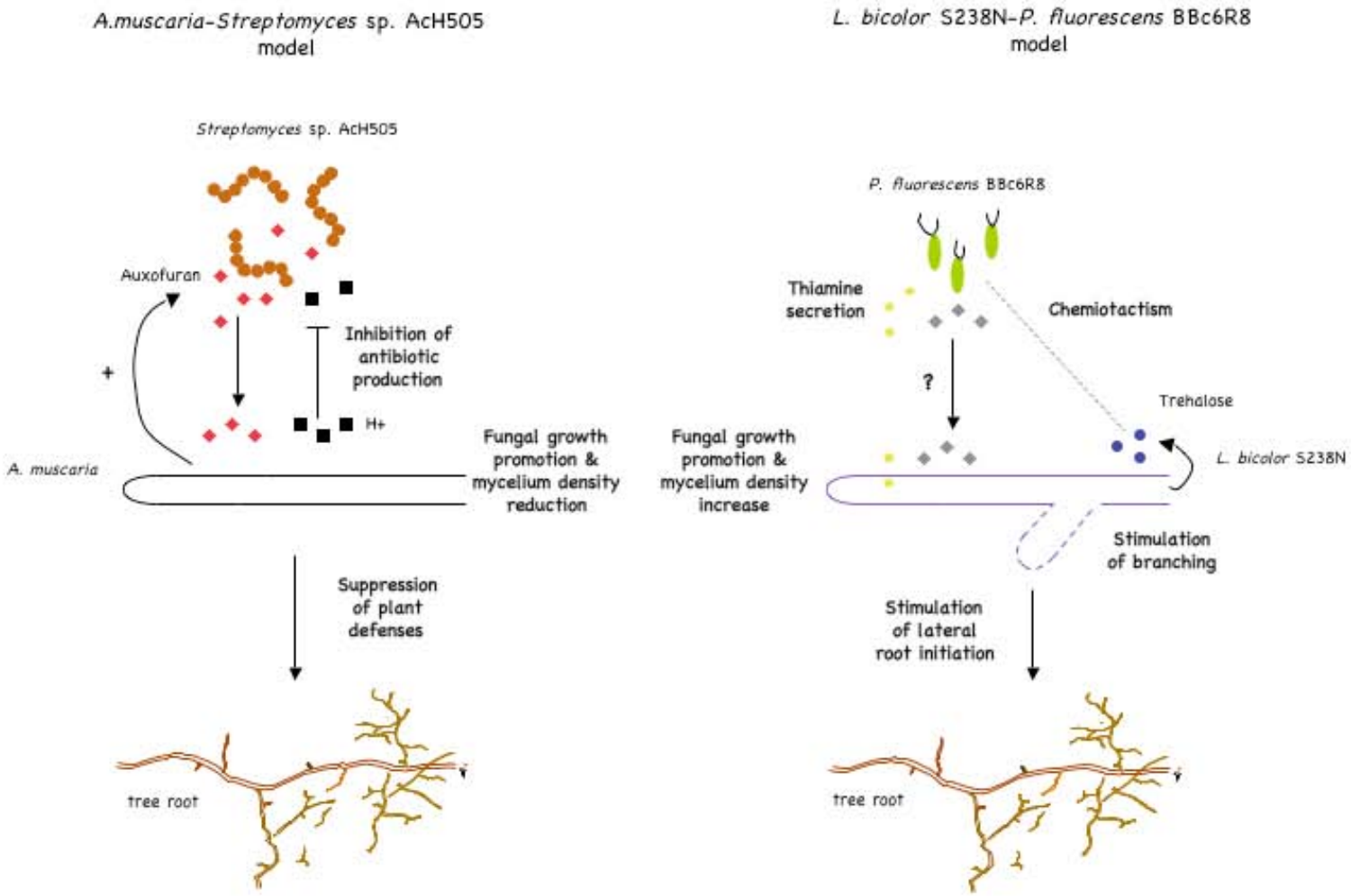
Trehalose that is concentrated in the hyphae of *L. bicolor* S238N attracts the bacterial cells in the vicinity of the mycelium. The consumption of the disaccharide by the bacteria increases the survival of the bacterial population. The sugar is released through broken or dead hyphae. Then, the bacteria exert their benefit effect through a combination of mechanisms that would imply modifications of several primary metabolisms :

1. an improvement of the nutritional status of the fungus due to (i) a bacterial production of thiamine, a vitamin that the ectomycorrhizal fungus is unable to synthesise *de novo*, (ii) a stimulation of both fungal and bacterial saprophytic activities that leads to an enhanced nutrient availability in soil. This improvement of the nutritional status of the fungus leads to an increase of the survival and the growth of the pre-symbiotic mycelium.
2. an induction of an over production of plant hormones by the fungus that leads to an enhanced production of fine roots by the host plant and thus to higher number of mycorrhizae formation.
3. an induction of morphological modifications of the fungal mycelium that prepares the fungus to the root infection.

What is the specificity of the molecular mechanisms of the helper effect of *P. fluorescens* BBc6R8 ?

While I was performing my PhD, other research teams have performed similar analyses on the MHB models *A. muscaria- Streptomyces* sp. AcH 505 (M. Tarkka *et al.*), *Lactarius rufus*-BAMs-*Pinus sylvestris* (Bending *et al.*) et *Pisolithus albus* IR110 – *Pseudomonas monteilii* HR13 (Duponnois *et al.*, IRD Dakar, Sénégal). Tarkka *et al.* and Duponnois *et al.* have also analyzed mechanisms that are involved in the improvement of the presymbiotic growth of the fungus (Maier 2004, Schrey *et al.* 2005, Riedlinger *et al.* 2006, Lehr *et al.* 2007, Duponnois & Kisa 2006) while Bending *et al.* have studied the effects of helper bacteria on the morphology and the receptivity of roots (Aspray *et al.* 2006). Interestingly, molecular mechanisms of the helper effect that have been described differ, depending on the model studied (figure C2). In the case of the pair *A. muscaria* – *Streptomyces* sp. AcH505, the helper strain produces auxofuran, a molecule that induces modifications of the transcription of genes involved in the primary metabolism, the growth and the cellular proliferation of the fungus, and induces a cytoskeleton

Figure C2. Comparison of the helper mechanisms in the models *A.muscaria-Streptomyces* sp. AcH505 and *L. bicolor* S238N-*P. fluorescens* BBc6R8.



rearrangement. All these modifications lead to an increase of the fungal growth and a reduction of its hyphal density and its biomass. The production of auxofuran is stimulated by the fungus while the synthesis of antibiotics by *Streptomyces* sp. is inhibited thanks to the secretion of protons and organic acids that reduce the pH of the environment (Riedlinger *et al.* 2006). Furthermore, the helper strain *Streptomyces* sp. AcH505 seems to suppress plant defence reactions. Thus, the helper effect of *Streptomyces* sp. AcH505 appear to be the result of a combination of mechanisms, as in the case of *P. fluorescens* BBc6R8. But these mechanisms are different. Concerning the model *Pisolithus albus* IR110 – *Pseudomonas monteilii* HR13, the growth-promotion effect of the bacteria would depend on the presence of trehalose on the environment.

The comparison of these three models suggest that if mycorrhiza helper mmycorrhiza are common and depend neither on the type of symbiosis (endo- or ectomycorrhizae) nor on the host plant (Frey-Klett *et al.* 2007), the molecular mechanisms of the MHB effect appear to be specific of the bacterial strains and of the fungus. By contrast, some of these mechanisms have been previously described as being involved in other fungi-bacteria interactions (cf. General Introduction, Chap1). As an example, the secretion of thiamine is also involved in the improvement of the growth of the yeast *Debaryomyces vanriji* by the bacterial strain *Bacillus* sp. TB-1 (Rikhvanov *et al.* 1999) and in the interaction between *S. cerevisiae* and cellulolytic bacteria from the lamb rumen (Chaucheyras-Durand & Fonty, 2001). Similarly, interactions between bacteria and fungi frequently lead to modifications of the morphology of hyphae (Bolwerk *et al.* 2003, Hogan *et al.* 2004, Ström *et al.* 2005, Hildebrandt *et al.* 2006) that are sometimes correlated with lipid metabolism modifications (Requena *et al.* 1999, Melin *et al.* 2002, Lee *et al.* 2000).

Towards an integrative analysis of the interaction between ectomycorrhizal fungi and their environment.

Soil is probably one of the most complex ecosystem because of the high number and the diversity of soil organisms that create dense and interconnected networks of interactions. However, the mechanisms and the consequences of these interactions are poorly understood so far, notably in the case of the ectomycorrhizal fungi. It is now well established that the mycorrhizal symbiosis cannot be considered as a simple bipartite interaction between mycorrhizal fungi and roots. Similarly, thinking that ectomycorrhizal

fungi and helper bacteria are only interacting together would be a mistake. Indeed, both *L. bicolor* S238N and *P. fluorescens* BBc6R8 are continuously interacting with other bacterial and fungal populations, and with predators (amoebae, nematodes, collembolans…) in natural conditions. The high complexity of these interactions is a limitation for their study *in vivo*. That is why it is necessary to use simplified and controlled *in vitro* bioassay. The *in vitro* assay that we have designed for the analysis of the helper effect can also be used to analyse interactions between ectomycorrhizal fungi and various microorganisms. Thanks to this bioassay, we have demonstrated that *L. bicolor* S238N reacts differently to the presence of various bacteria from the soil and is also able to perceive quorum sensing signal molecules. This assay was also recently used to study the effect of mycophageous collembolans on the fungal transcriptome. Preliminary results have suggested that the fungus do not respond to the grazing activity of the insects (preliminary results from a collaboration with Pr Scheu team, University of Darmstadt, Germany).

All these results confirm and highlight the importance of the biotic environment on the fungal behaviour. Complementary analyses are necessary to better understand which are the signal molecules produced and perceived by the ectomycorrhizal fungus and how they interfere all together. Nevertheless, the datasets we have acquired could contribute to the modelling of the interaction between ectomycorrhizal fungi and their environment through the use of mathematical models and informatics tools. Indeed, modelling is the only way to grasp these high levels of complexity.

Perspectives.

Apart from our trancriptomic data that need to be confirmed, some steps of the model of interaction between *L. bicolor* S238N and *P. fluorescens* BBc6R8 need to be clarified. First, one should identify the effectors that are produced by the helper strain. Indeed, the thiamine is not sufficient by its own to induce a growth-promoting effect since 12 days. It seems that a cocktail of unknown volatile and soluble compounds is necessary. Similarly, the role of the trehalose in the interaction remains quite mysterious: the absence of secretion of the disasccharide indicates that the fungus probably exudates other chemiotaxic molecules. Therefore signal molecules produced by both the bacteria and the fungus remain to be discovered. Many tools are now available to isolate and to chemically identify such molecules. As an example, Barbieri *et al.* (2005) have identified

65 volatile compounds produced by *Staphylococcus pasteuri* that inhibit the development of *Tuber borchii*. In this purpose, they used solid-phase microextraction and gas chromatography-mass spectrometry. The same method could be used to identify the molecules that are produced by *P. fluorescens* BBc6R8 and *L. bicolor* S238N along their interaction.

The ecological niche of *P. fluorescens* BBc6R8 remains also to be determined. Because of the bacterial chemotaxis towards fungal extracts, the attachment of the bacteria to the hyphae and the formation of biofilm-like structures along the mycelium, we have suggested that the hyphae of *L. bicolor* S238N could be the ecological niche of the helper bacteria. However, all these data have been obtained *in vitro* and it is necessary to confirm them *in vivo.* Furthermore, it is still to be determined whether the bacteria are randomly distributed along the hyphae or if they concentrate in particular spots.

Moreover, some points have not been studied during this thesis. For example, the impact of the pH on the helper effect of BBc6R8 has not been analysed whereas it plays a key role in many fungal-bacterial interactions (cf. General Introduction, Chap1), notably in the case of the *A. muscaria-Streptomyces* AcH505 model. Interestingly, recent studies performed by P. Frey-Klett and B. Palin in our laboratory have shown that the effect of BBc6R8 is enhanced *in vitro* at neutral pH. All the studies that have been performed previously were done at pH 5.5.

Finally, the presence of intracellular bacteria in *L. bicolor* S238N hyphae is particularly intriguing. These cryptic bacteria seem to be acquired from the environment of the fungus and occasionally vertically transmitted (Bertaux *et al.* 2005). The role they play in the physiology of the fungus and in the process of mycorhiza formation is unknown so far. However, we have observed that the presumably endobacterial strain *Paenibacillus* sp. F2001L enhances the growth of the ectomycorrhizal fungus *in vitro* and produces thiamine. It is tempting to speculate that the strain could be beneficial to the growth and survival of the fungus when it is intracellular. Several studies have demonstrated that endobacteria play a crucial role in the biological cycle of some fungi ((Schüßler *et al.* 2001, Partida-Martinez *et al.* 2007). It may be also the case of the endobacteria for *L. bicolor* S238N. In the future, understanding the interaction between endobacteria and ectomycorrhizal fungi could be very profitable to the research on MHB and more generally on interactions between fungi and their biotic environment.

Discussion générale, Conclusion et Perspectives

Choix des méthodes

Atouts et inconvénients d'une démarche focalisée sur un dispositif d'interaction in vitro.

L'ensemble des données acquises au cours de ma thèse ont été obtenues à partir d'un dispositif d'interaction *in vitro*. Ce dispositif a été mis au point afin de minimiser les variations du comportement des deux micro-organismes qui seraient dues à des paramètres biotiques et abiotiques non contrôlés en conditions naturelles. De plus, le système de confrontation *in vitro* utilisé facilite l'échantillonnage des deux partenaires (mycélium et bactérie) et permet d'effectuer un suivi précis au cours du temps, tant du point de vue morphologique que transcriptomique. Ainsi, nous avons montré que la réponse fongique était initiée à un stade précoce, avant qu'un contact physique soit établi entre le mycélium et les cellules bactériennes. Cet aspect cinétique est difficile à maîtriser en conditions naturelles en raison de l'hétérogénéité de la localisation des micro-organismes et de l'absence de synchronisation des cellules.

Toutefois, si la simplification des interactions à l'aide d'un dispositif *in vitro* comporte de nombreux avantages techniques, cette simplification constitue en même temps son inconvénient majeur car les multiples interactions qui ont lieu *in vivo* avec d'autres organismes, notamment les arbres, ne sont pas prises en compte. L'utilisation d'un dispositif dépourvu de la plante se justifie par le fait que nous nous soyons focalisés sur les mécanismes moléculaires responsables de l'effet des bactéries sur la croissance pré-symbiotique du champignon ectomycorhizien. Nos recherches étaient motivées par les résultats de Brulé *et al.* (2001) qui avaient démontré que la bactérie induit une augmentation de la survie et de la croissance du mycélium au stade pré-symbiotique, avant que tout contact physique et moléculaire ne soit établi avec les racines des arbres. Au final, le dispositif expérimental initialement conçu pour étudier cette phase de l'interaction a révélé l'existence d'autres mécanismes insoupçonnés. D'une part, le fait que la souche *P. fluorescens* BBc6R8 induise une surexpression de certains gènes fongiques impliqués dans la synthèse d'hormones végétales laisse à penser que la bactérie pourrait préparer le système racinaire de la plante à la symbiose, par l'intermédiaire du champignon (Chapitre 1). D'autre part, la ramification du mycélium en présence de la bactérie et la surexpression de gènes fongiques par ailleurs connus pour être induits en

présence des racines lors des stades précoces de la symbiose ectomycorhizienne suggèrent que la bactérie auxiliaire préparerait également la physiologie du champignon à l'infection des racines. Il reste à démontrer que les modifications transcriptionnelles mises en évidence affectent effectivement l'apparition des racines courtes et le processus de mycorhization. Dans cette optique, nous avons entrepris la mise au point d'un dispositif d'interaction *in vitro* à trois partenaires dans lequel serait reproduit de manière contrôlée l'effet auxiliaire de la souche bactérienne sur le taux de mycorhization des racines de peuplier par *L. bicolor* S238N. Si les premiers résultats obtenus sont encourageants concernant la possibilité de mycorhizer les racines de peuplier par *L. bicolor* S238N en milieu gélosé, le protocole reste à adapter pour obtenir un effet positif de *P. fluorescens* BBc6R8 sur la mycorhization. Jusqu'à présent, nous n'avons obtenu qu'un effet délétère. Ni la pré-incubation des racines en présence de la souche auxiliaire, ni la pré-incubation du champignon avec BBc6R8, ni l'incubation simultanée des trois partenaires n'ont permis de reproduire l'effet auxiliaire attendu.

Atouts et inconvénients de l'approche transcriptomique

Lorsque ce travail a été entrepris, le rôle joué par les régulations transcriptomiques dans l'effet auxiliaire de la mycorhization n'avait jamais été étudié. Grâce à la connaissance de la séquence complète du génome de *L. bicolor* H82-S238N et de la création de micropuces à oligonucléotides couvrant l'ensemble des transcrits de l'organisme, notre approche sans *a priori* a non seulement permis de montrer l'existence de régulation transcriptomique lors d'interactions entre un champignon ectomycorhizien et des bactéries du sol, mais elle a aussi dévoilé des mécanismes jusqu'alors insoupçonnés, car difficiles à expérimenter *in vivo* (cf. paragraphe précédant). Le séquençage récent du génome de la bactérie auxiliaire de la mycorhization *P. fluorescens* BBc6R8 permet d'envisager d'utiliser la même démarche de transcriptomique dans un avenir proche afin d'apprécier l'impact du champignon sur le comportement de la bactérie. Nous n'avons en effet pour l'instant que peu de données concernant la réponse de la bactérie au cours de l'interaction avec *L. bicolor* S238N. Cette étude devrait notamment permettre de savoir si les gènes responsables de l'effet auxiliaire de la bactérie sont exprimés de manière constitutive ou s'ils sont induits par le champignon ectomycorhizien, et quelle est leur spécificité.

Malgré les atouts de la méthode transcriptomique évoqués ci-dessus, il convient de ne pas oublier qu'il s'agit d'une méthode descriptive dont les résultats nécessitent d'être confirmés par d'autres approches. En effet, des variations de concentration de transcrits ne se traduisent pas forcément par des variations de concentration des protéines ou par des variations de l'activité enzymatique totale d'une protéine. Les études réalisées au cours de cette thèse sont donc de nature prospective et les résultats obtenus doivent servir de base à des analyses ciblées qui ne seront cette fois plus uniquement descriptives mais démonstratives. De ce fait, les différents mécanismes proposés nécessitent d'être confirmés et le modèle décrit ci-dessous constitue une base de travail pour de futures études.

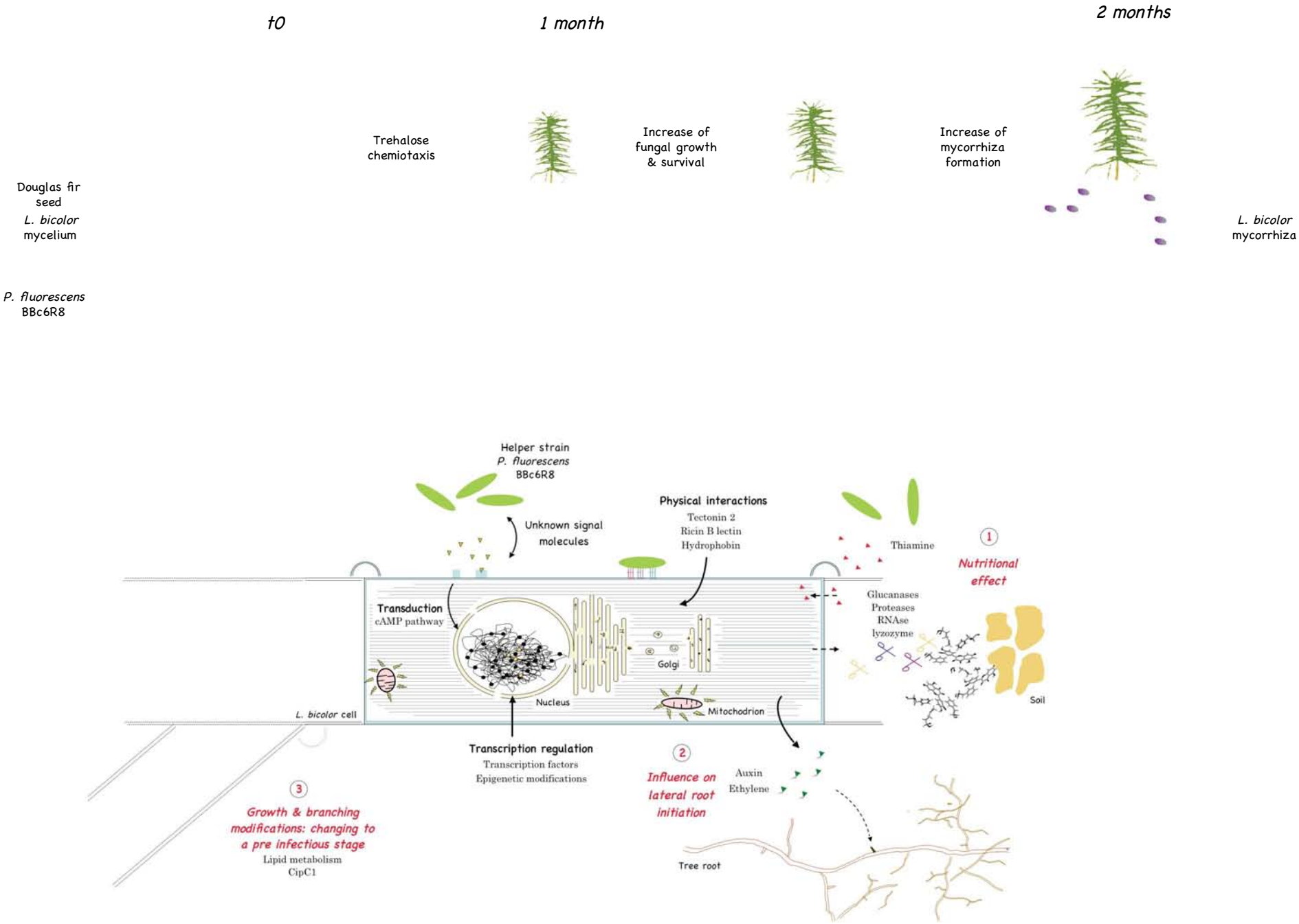
Un modèle des mécanismes de l'effet auxiliaire de *P. fluorescens* BBc6R8.

Nous proposons ici un modèle d'interaction entre *L. bicolor* et *P. fluorescens* BBc6R8 élaboré à partir des différents résultats obtenus au cours de ce travail de thèse et des données précédemment acquises par R. Duponnois (1992), P. Frey-Klett (1996) et C. Brulé (2001) (figure C1).

Le tréhalose accumulé dans le mycélium fongique attire les cellules bactériennes auprès des hyphes. La consommation du disaccharide, accessible aux bactéries au niveau des hyphes brisés et/ou morts, améliore la survie de la population bactérienne au voisinage du mycélium. L'effet bénéfique de la souche bactérienne sur l'établissement de mycorhize par *L. bicolor* S238N s'opère ensuite par l'intermédiaire d'une combinaison de mécanismes qui sont régulés au moins à l'échelle transcriptomique et font intervenir l'ajustement de plusieurs métabolismes primaires :

1. Une amélioration du statut nutritionnel du mycélium due (i) à une production de thiamine par la bactérie, une vitamine que *L. bicolor* est incapable de synthétiser *de novo*, (ii) à une stimulation des activités saprophytiques fongiques et bactériennes qui mène à une augmentation de la quantité de nutriments disponibles dans le sol pour les deux micro-organismes. Cette amélioration du statut nutritionnel du champignon est responsable de la meilleure survie et de la croissance accrue du mycélium pré-symbiotique
2. L'induction d'une surproduction d'hormones végétales par le champignon qui aboutit à une production accrue de racines latérales par l'hôte et donc un taux de mycorhization supérieur.

Figure C1. Modèle des mécanismes de l'effet auxiliaire de *P. fluorescens* BBc6R88

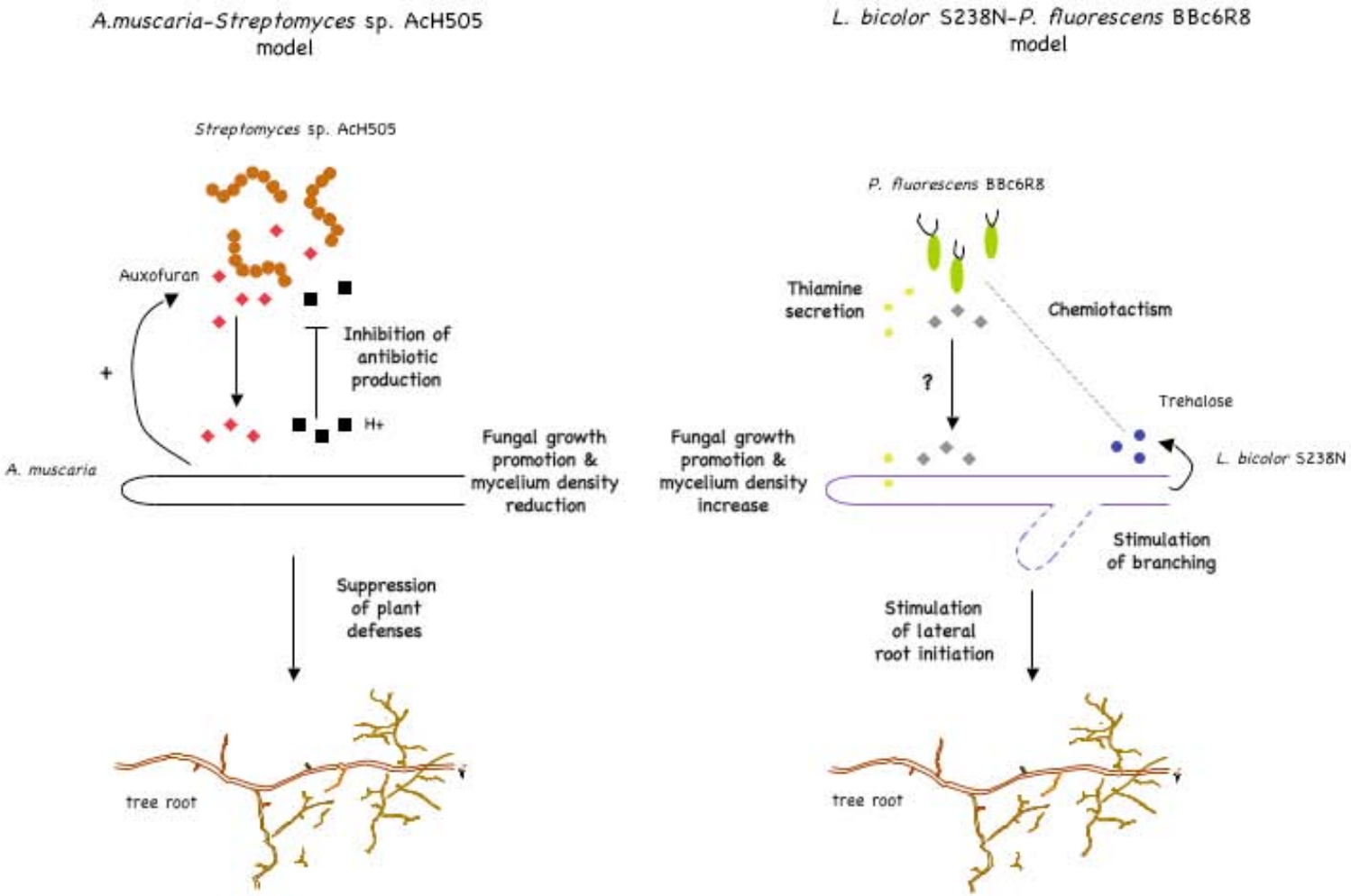


1. L'induction de modifications morphologiques du mycélium fongique proches de celles de l'état infectieux qui réduit le temps nécessaire à l'établissement de la mycorhization.

Quelle est la spécificité des mécanismes moléculaires de l'effet auxiliaire ?

Pendant que se déroulait ce projet, d'autres équipes de recherche ont mené des études comparables sur les couples modèles *A. muscaria- Streptomyces* sp. AcH 505 (M. Tarkka *et al.*), *Lactarius rufus*-BAMs-*Pinus sylvestris* (Bending *et al.*) et *Pisolithus albus* IR110 – *Pseudomonas monteilii* HR13 (Duponnois *et al.*, IRD Dakar, Sénégal). Tarkka *et al.* et Duponnois *et al.* se sont également penchés sur les mécanismes aboutissant à une augmentation de la croissance du mycélium en phase pré-symbiotique (Maier 2004, Schrey *et al.* 2005, Riedlinger *et al.* 2006, Lehr *et al.* 2007, Duponnois & Kisa 2006) tandis que Bending *et al.* ont étudié l'effet des bactéries sur la morphologie et la réceptivité des racines (Aspray *et al.* 2006). Il est intéressant de noter que les mécanismes moléculaires de l'effet auxiliaire sont relativement différents d'un modèle à l'autre (Figure C2). Ainsi, dans le cas du couple *A. muscaria-Streptomyces* sp. AcH 505, pour lequel l'analyse moléculaire des mécanismes a été la plus poussée, la souche auxiliaire produit de l'auxofuran, une molécule qui induit des modifications de la transcription de gènes impliqués dans le métabolisme primaire, dans la croissance et la prolifération cellulaire du champignon, et une réorganisation de son cytosquelette. L'ensemble de ces mécanismes aboutit à une augmentation de la croissance en longueur du mycélium et à une réduction de sa densité et de sa biomasse. La production d'auxofuran par la bactérie est stimulée par le champignon. A l'inverse, ce dernier inhibe la production d'antibiotiques bactériens délétères pour la croissance du champignon, grâce à la sécrétion d'acides organiques et de protons qui abaissent le pH de l'environnement (Riedlinger *et al.* 2006). Par ailleurs, la souche auxiliaire *Streptomyces* sp. AcH505 induit une suppression des réactions de défense de la plante, ce qui permettrait une infection plus rapide des racines courtes par le champignon ectomycorhizien (Lehr *et al.* 2007). L'effet auxiliaire de *Streptomyces* sp. AcH505 serait donc la résultante d'une combinaison de mécanismes, comme dans le cas de la souche *P. fluorescens* BBc6R8 mais la nature de ces mécanismes serait différente. Concernant le modèle *Pisolithus albus* IR110 – *Pseudomonas monteilii* HR13, l'induction de la croissance du champignon par la bactérie serait dépendante de la présence de tréhalose dans le milieu.

Figure C2. Comparaison des mécanismes de l'effet auxiliaire chez les organismes modèles *A.muscaria-Streptomyces* sp. AcH505 and *L. bicolor* S238N-*P. fluorescens* BBc6R8.



Si l'existence de bactéries auxiliaires de la mycorhization est confirmée dans de nombreux environnements et ne dépend ni du type de symbiose (endo- ou ectomycorhizienne) ni de la plante hôte (Frey-Klett *et al.* 2007), il semble que les mécanismes d'action de ces bactéries soient relativement spécifiques. Il sera nécessaire d'élargir l'étude moléculaire des mécanismes d'interaction à d'autres modèles de bactéries auxiliaires de la mycorhization pour confirmer cette spécificité. En revanche, il est important de signaler que certains mécanismes identifiés au cours de ce travail de thèse ont aussi été décrits dans d'autres interactions entre bactéries et champignons (cf. Chap 1 Introduction Générale). Par exemple, la sécrétion de thiamine participe à l'amélioration de la croissance de la levure *Debaryomyces vanriji* par la souche bactérienne *Bacillus* sp. TB-1 dans les sources chaudes de la région Baïkal (Rikhvanov *et al.* 1999). Elle est aussi impliquée dans l'interaction entre *S. cerevisiae* et les bactéries cellulolitiques du rumen de l'agneau (Chaucheyras-Durand & Fonty, 2001). De même, l'interaction entre bactéries et champignons filamenteux, qu'elle soit bénéfique ou délétère pour le champignon, aboutit fréquemment à des modifications de la morphologie et de la croissances des hyphes (Bolwerk *et al.* 2003, Hogan *et al.* 2004, Ström *et al.* 2005, Hildebrandt *et al.* 2006), qui sont parfois corrélées à des modifications du métabolisme lipidique (Requena *et al.* 1999, Melin *et al.* 2002, Lee *et al.* 2000).

Vers une approche intégrative des interactions entre le champignon ectomycorhizien et son environnement.

Le sol est probablement l'un des écosystèmes les plus complexes en raison de la quantité et de la diversité d'organismes qui le peuplent et qui génèrent des réseaux d'interactions denses et interconnectés. Les mécanismes et les conséquences de ces interactions sont particulièrement méconnus, notamment en ce qui concerne les champignons ectomycorhizens. De même que l'on sait maintenant que l'on ne peut pas réduire la mycorhize à une interaction bipartite entre le champignon et la racine, considérer que les champignons ectomycorhiziens et les bactéries auxiliaires n'interagissent qu'entre eux serait une erreur. En effet, en conditions naturelles ils sont en interaction permanente avec d'autres populations bactériennes et fongiques, ainsi qu'avec des prédateurs (amibes, nématodes, collemboles). La complexité des interactions en jeu limite leur étude *in vivo* et il est indispensable d'avoir recours à des dispositifs simplifiés et contrôlés. Le dispositif d'interaction *in vitro* que nous avons mis au point dans le cadre de l'étude de l'effet

auxiliaire de la mycorhization présente l'avantage de pouvoir être utilisé pour étudier plus généralement les mécanismes moléculaires par lesquels un champignon ectomycorhizien perçoit son environnement biotique et y réagit. Grâce à ce dispositif, nous avons pu montrer que *L. bicolor* S238N réagit à la présence de diverses bactéries de manière contrastée et qu'il est capable de percevoir des molécules signal de quorum sensing, destinées à l'origine au dialogue entre cellules bactériennes. Ce dispositif a également pu être utilisé pour étudier l'impact de collemboles mycophages sur le champignon à l'échelle transcriptomique. Les premiers résultats obtenus indiquent une absence de réponse fongique à l'activité mycophage des collemboles (résultats préliminaires d'une collaboration avec l'équipe du Pr. Scheu, Université de Darmstadt, Allemagne).

L'ensemble de ces résultats confirment l'importance de l'environnement biotique sur le comportement du champignon. Des études complémentaires seront nécessaires pour comprendre quels sont les signaux produits et perçus par le champignon et comment ils interfèrent les uns avec les autres. Néanmoins l'ensemble des données acquises en conditions contrôlées *in vitro* pourra servir de base pour modéliser les interactions entre le champignon ectomycorhizien et son environnement à l'aide de modèles mathématiques et d'outils informatiques. Seule la modélisation permet en effet d'appréhender de hauts niveaux de complexité.

Perspectives

Outre la confirmation nécessaire des hypothèses formulées à partir des données de transcriptomique, le modèle d'interaction entre *L. bicolor* S238N et *P. fluorescens* BBc6R8 que nous proposons à l'issue de cette thèse présente plusieurs zones d'ombre. La première se situe au niveau des molécules effectrices produites par la souche auxiliaire. Si la thiamine sécrétée par BBc6R8 a un effet bénéfique sur la croissance *in vitro* du champignon, cette molécule n'est pas suffisante à elle seule pour reproduire l'effet de promotion de croissance induit par la bactérie dès 12 jours. Un cocktail de molécules diffusibles et volatiles, dont la nature reste à déterminer, semble nécessaire. De même, le rôle du tréhalose dans l'interaction reste flou : l'absence d'exudation du disaccharide par les hyphes fongiques laisse supposer que le champignon sécrète d'autres molécules chimiotactiques. De plus, la présence de tréhalose dans le milieu ne semble pas indispensable pour que la bactérie exerce son effet auxiliaire. Les signaux produits par la

bactérie et le champignon restent donc majoritairement inconnus. De nombreux outils sont aujourd'hui disponibles pour isoler des molécules et identifier leur nature chimique. Ainsi, Barbieri *et al.* (2005) ont identifié 65 composés volatiles produits par la bactérie *Staphylococcus pasteuri* et impliqués dans son antagonisme vis-à-vis du champignon ectomycorhizien *Tuber borchii,* en utilisant la technique de chromatographie en phase gazeuse couplée à la spectrométrie de masse. La même méthode permettrait peut-être d'identifier les molécules sécrétées par *P. fluorescens* BBc6R8 et *L.bicolor* S238N au cours de leur interaction.

La niche écologique de la bactérie dans le sol reste elle aussi à éclaircir. L'hypothèse selon laquelle les hyphes de *L. bicolor* S238N constitueraient une niche pour la souche *P. fluorescens* BBc6R8 est étayée par les données de chimiotactisme, d'attachement de la bactérie aux hyphes et par l'observation de structure de type biofilm le long des hyphes de *L. bicolor* S238N. Mais ces observations ont été obtenues *in vitro* et sont à confirmer *in vivo.* D'autre part il reste à déterminer si les bactéries sont distribuées de manière aléatoire le long des hyphes ou bien si elles s'agrègent en des lieux spécifiques où des métabolites fongiques seraient sécrétés.

Par ailleurs, un certain nombre de questions n'ont pas été abordées au cours de cette thèse. C'est par exemple le cas du rôle du pH dans l'effet auxiliaire. Il est fréquemment impliqué dans les interactions bactéries-champignons (cf. Chap 1 Introduction Générale) et joue un rôle majeur dans le modèle *A. muscaria-Streptomyces* AcH505. Des études récemment menées par P. Frey-Klett et B. Palin au sein de notre équipe ont montré que l'effet de la souche BBc6R8 sur la croissance de *L. bicolor* S238N *in vitro* était amplifié à pH neutre par rapport à l'effet mesuré à pH 5,5 (P. Frey-klett, communication personnelle).

Enfin, la présence de bactéries intracellulaires dans les hyphes de *L. bicolor* est particulièrement intrigante. Ces bactéries, longtemps restées cryptiques, semblent provenir de l'environnement direct du champignon et pouvoir être occasionnellement transmises par les spores. Le rôle précis qu'elles jouent dans la physiologie du champignon et dans le processus de mycorhization est pour le moment inconnu. Toutefois, nous avons observé que la souche *Paenibacillus* sp. F2001L, tout comme *P. fluorescens* BBc6R8, induit une augmentation de la croissance du mycélium de *L. bicolor* S238N *in vitro* et produit de la thiamine. Cette souche pourrait donc être favorable au développement et à la survie du mycélium lorsqu'elle est intracellulaire. Plusieurs études

ont mis en évidence le rôle clé joué par des bactéries intracellulaires dans la biologie des champignons (Schüßler *et al.* 2001, Lumini *et al.* 2007, Partida-Martinez & Hertweck 2005, Partida-Martinez *et al.* 2007). Elucider le rôle et le fonctionnement des interactions entre les champignons mycorhiziens et les bactéries intracellulaires associées pourrait constituer une avancée majeure dans la compréhension des mécanismes de l'effet auxiliaire et des relations entre champignons et bactéries.

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ANNEXES

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The genome of *Laccaria bicolor* provides insights into mycorrhizal symbiosis

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Mycorrhizal symbioses—the union of roots and soil fungi—are universal in terrestrial ecosystems and may have been fundamental to land colonization by plants[1,2]. Boreal, temperate and montane forests all depend on ectomycorrhizae[1]. Identification of the primary factors that regulate symbiotic development and metabolic activity will therefore open the door to understanding the role of ectomycorrhizae in plant development and physiology, allowing the full ecological significance of this symbiosis to be explored. Here we report the genome sequence of the ectomycorrhizal basidiomycete *Laccaria bicolor* (Fig. 1) and highlight gene sets involved in rhizosphere colonization and symbiosis. This 65-megabase genome assembly contains 20,000 predicted protein-encoding genes and a very large number of transposons and repeated sequences. We detected unexpected genomic features, most notably a battery of effector-type small secreted proteins (SSPs) with unknown function, several of which are only expressed in symbiotic tissues. The most highly expressed SSP accumulates in the proliferating hyphae colonizing the host root. The ectomycorrhizae-specific SSPs probably have a decisive role in the establishment of the symbiosis. The unexpected observation that the genome of *L. bicolor* lacks carbohydrate-active enzymes involved in degradation of plant cell walls, but maintains the ability to degrade non-plant cell wall polysaccharides, reveals the dual saprotrophic and biotrophic lifestyle of the mycorrhizal fungus that enables it to grow within both soil and living plant roots. The predicted gene inventory of the *L. bicolor* genome, therefore, points to previously unknown mechanisms of symbiosis operating in biotrophic mycorrhizal fungi. The availability of this genome provides an unparalleled opportunity to develop a deeper understanding of the processes by which symbionts interact with plants within their ecosystem to perform vital functions in the carbon and nitrogen cycles that are fundamental to sustainable plant productivity.

The 65-megabase genome of *Laccaria bicolor* (Maire) P. D. Orton is the largest sequenced fungal genome published so far[3–7] (Table 1). Although no evidence for large-scale duplications was observed within the *L. bicolor* genome, tandem duplication occurred within multigene families (Supplementary Fig. 4). Transposable elements comprised a higher proportion (21%) than that identified in the other sequenced fungal genomes and may therefore account for the relatively large genome of *L. bicolor* (Supplementary Table 3). Approximately 20,000 protein-coding genes were identified by combined gene predictions (Supplementary Information Section 2). Expression of nearly 80% (~16,000) of the predicted genes was detected in free-living mycelium, ectomycorrhizal root tips or fruiting bodies (Supplementary Table 4) using NimbleGen custom-oligoarrays (Supplementary Information Section 9). Most genes are activated in almost all tissues, whereas other more specialized genes are only activated in some specific developmental stages, such as the free-living mycelium, ectomycorrhizae or the fruiting body (Supplementary Table 5).

Only 14,464 *L. bicolor* proteins (70%) showed sequence similarity (BLASTX, cut-off *e*-value >0.001) to documented proteins. Most homologues were found in the sequenced basidiomycetes *Phanerochaete chrysosporium*[4], *Cryptococcus neoformans*[5], *Ustilago maydis*[6] and *Coprinopsis cinerea*[7] (Supplementary Table 6). The percentage of proteins found in multigene families was related to genome size and was the largest in *L. bicolor* (Fig. 2). This was mainly owing to the expansion of protein family size, but was also because of the larger number of protein families in *L. bicolor* compared to the other basidiomycetes (Supplementary Table 7). Expansion of protein family sizes in *L. bicolor* was prominent in the lineage-specific multigene families. Marked gene family expansions occurred in those genes predicted to have roles in protein–protein interactions (for example, WD40-domain-containing proteins) and in signal transduction

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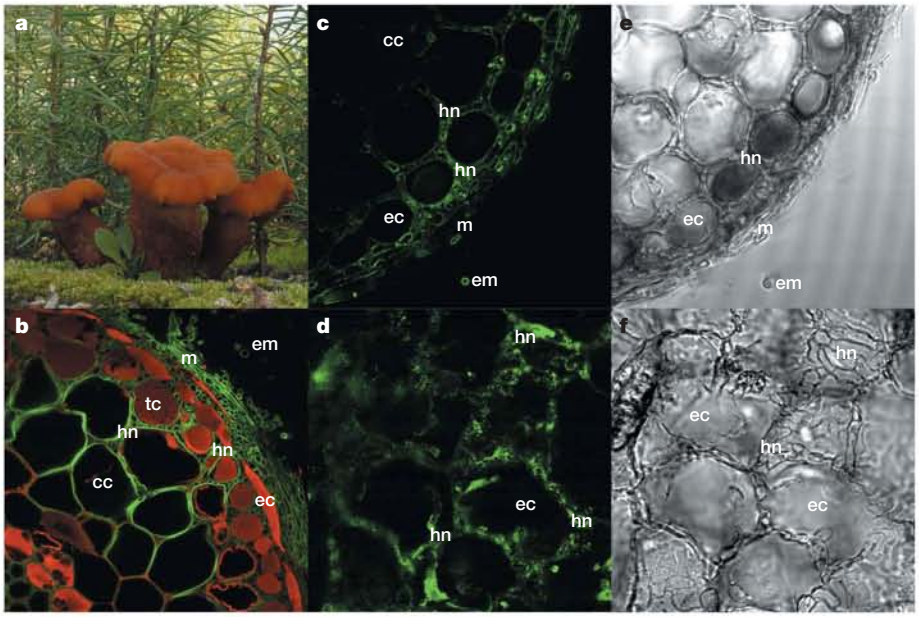

Figure 1 | Ectomycorrhizal symbiosis and the localization of the SSP MISSP7. **a**, Fruiting bodies of *L. bicolor* colonizing seedlings of Douglas fir (photograph courtesy of D. Vairelles). **b**, Laser-scanning confocal microscopy image of a transverse section of *Pseudotsuga menziesii–L. bicolor* ectomycorrhizae showing extramatrical mycelium (em), the mantle (m) and hyphae (hn) between epidermal (ec), tannin (tc) and cortical (cc) root cells. Scale, 10 μm. **c–f**, Immunofluorescent localization of MISSP7. Transverse (**c**, **e**) and longitudinal (**d**, **f**) sections of *P. trichocarpa–L. bicolor* ectomycorrhizae. MISSP7 was detected in the hyphae of the mantle (m) and the Hartig net (hn) ensheathing epidermal cells (ec). Rectangles in **d** and **f** show the finger-like, labyrinthine hyphal system accumulating a large amount of MISSP7. **e**, **f**, Phase contrast images. Scale, 10 μm.

mechanisms (Supplementary Table 7). Two new classes of GTPase α genes were found and may be candidates for the complex communication that must occur between the mycobiont and its host plant during mycorrhizae establishment (Supplementary Table 8). Several transcripts coding for expanded and lineage-specific gene families were upregulated in symbiotic and fruiting body tissues, suggesting a role in tissue differentiation (Supplementary Tables 5 and 9).

In our analysis of annotated genes, in particular that of paralogous gene families, we highlighted processes that may be related to the biotrophic and saprotrophic lifestyles of *L. bicolor*. Twelve predicted proteins showed a similarity to known haustoria-expressed secreted proteins of the basidiomycetous rusts *Uromyces fabae*[8] and *Melampsora lini*[9], which are involved in pathogenesis (Supplementary Table 10). Out of the 2,931 proteins predicted to be secreted by *L. bicolor*, most (67%) cannot be ascribed a function, and 82% of these predicted proteins are specific to *L. bicolor*. Within this set, we found a large number of genes that encode cysteine-rich products that have a predicted size of <300 amino acids. Of these 278 SSPs, 69% belong to multigene families, but only nine groups comprising a total of 33 SSPs co-localized in the genome (Supplementary Fig. 5). The structure of two of these clusters is shown in Supplementary Fig. 6. Other SSPs are scattered all over the genome, and we found no correlation between SSP and transposable element genome localization (Supplementary Fig. 5). Transcript profiling revealed that the expression of several SSP genes is specifically induced in the symbiotic interaction (Table 2 and Supplementary Fig. 10). Five of the 20 most highly upregulated fungal transcripts in ectomycorrhizal root tips code for SSPs (Supplementary Table 5). These mycorrhiza-induced cysteine-rich SSPs (MISSPs) belong to *L. bicolor*-specific orphan gene families. Within the MISSPs, we found a family of secreted proteins with a CFEM domain (INTERPRO IPR014005) (Supplementary Figs 7 and 8), as previously identified in the plant pathogenic fungi *M. lini*[9] and *Magnaporthea grisea*[10] (Supplementary Table 10), and proteins with a gonadotropin (IPR0001545) or snake-toxin-like (SSF57302) domains related to the cysteine-knot domain. Expression of several SSPs was downregulated in ectomycorrhizal root tips (cluster E in Supplementary Fig. 10), suggesting a complex interplay between these secreted proteins in the symbiosis interaction.

The rich assortment of MISSPs may therefore act as effector proteins to manipulate host cell signalling or to suppress defence pathways during infection, as suggested for pathogenic rusts[8,9], smuts[6] (*U. maydis*) and *Phytophthora*[11] species. To have a role in symbiosis development, MISSPs should be expressed in *L. bicolor* hyphae colonizing the root tips. To test this assertion, we determined the tissue distribution of the mycorrhiza-induced cysteine-rich SSPof 7 kDa (MISSP7) (JGI identification number 298595) showing the highest induction in ectomycorrhizal tips (Table 2 and Supplementary Table 5). Two peptides, one of which is located in the amino-terminal and the other in the carboxy-terminal part of the mature protein, were selected as antigens for the production of anti-MISSP7 antibodies. The selected peptides were not found in the deduced protein sequences of other *L. bicolor* gene models, nor in the *Populus trichocarpa* genome[12]. MISSP7

Table 1 | Genome characteristics of *L. bicolor* and other basidiomycetes

Genome characteristics	*L. bicolor*	*C. cinerea*[7]	*P. chrysosporium*[4]	*C. neoformans*[5]	*U. maydis*[6]
Strain	S238N-H82	Okayama7#130	RP78	H99	521
Sequencing institution	JGI	Broad	JGI	Broad	Broad
Genome assembly (Mb)	64.9	37.5	35.1	19.5	19.7
GC content (%)	46.6	51.6	53.2	48.2	54
Number of protein-coding genes	20,614	13,544	10,048	7,302	6,522
Coding sequence <300 bp	2,191	838	163	313	58
Average gene length (bp)	1,533	1,679	1,667	1,828	1,935
Average coding sequence length (bp)	1,134	1,352	1,366	1,502	1,840
Average exon length (nt)	210.1	251	232	253	1,051
Average intron length (nt)	92.7	75	117	66	127

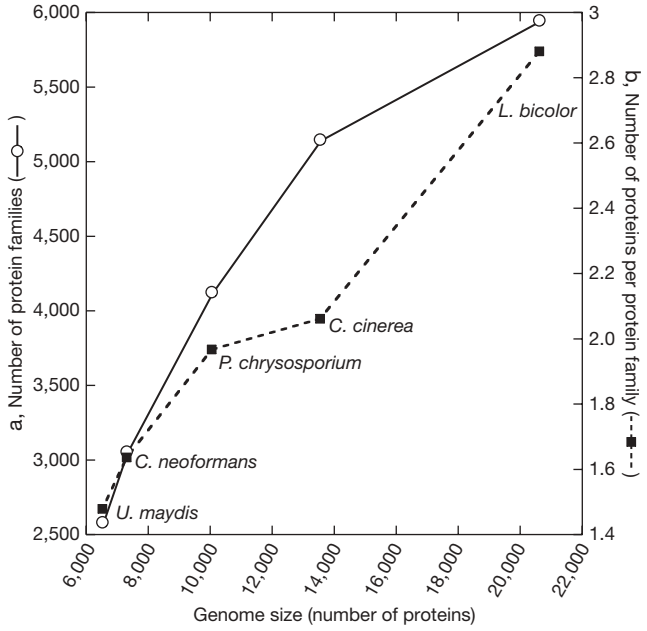

Figure 2 | Expansion of protein families in *L. bicolor*. **a**, Relationship between genome size and the number of protein families. **b**, Relationship between genome size and protein family sizes in five sequenced basidiomycetes. Protein sequences predicted from the genome sequences of *L. bicolor*, *C. cinerea*, *P. chrysosporium*, *C. neoformans* and *U. maydis* were clustered into families using the TRIBE-MCL algorithm (see Supplementary Information Section 5 for details).

localization in *L. bicolour–P. trichocarpa* ectomycorrhizal root tips by indirect immunofluorescence is illustrated in Fig. 1 and Supplementary Fig. 11. Control images in which the ectomycorrhizae sections were obtained by replacing primary anti-MISSP7 antibodies with pre-immune IgG are shown in Supplementary Fig. 12. For cases in which ectomycorrhizae were treated with anti-MISSP7 antibody followed by fluorescent-labelled secondary antibody, fluorescence was localized in the hyphae colonizing short roots (Fig. 1 and Supplementary Fig. 11) and was not detected in the free-living mycelium (Supplementary Fig. 12). Although MISSP7 was detected in the hyphal mantle layers ensheating the root tips, the protein mainly accumulated in the finger-like, labyrinthine branch hyphal system (Hartig net), which provides a very large area of contact between cells of the two symbionts. It accumulated in the cytosol and cell wall of the fungal cells. The MISSP7 protein could therefore interact with the plant components after secretion. MISSP7 shares no sequence similarity or protein motif with other SSPs.

Comparison of the MISSP sequences did not reveal a specific conserved motif that could potentially contribute to their function or to targeting to the host cell, such as the RXLR motif[11] of phytopathogenic *Phytophthora* or the malaria parasite. SSPs with upregulated expression in fruiting bodies (Supplementary Table 5 and Supplementary Fig. 10) may have a role in the differentiation of the sexual tissues and/or the aggregation of sporophore tissues. Interestingly, there is a large set of SSP genes showing significant changes in gene expression in both ectomycorrhizal root tips and fruiting bodies (cluster A in Supplementary Fig. 10), suggesting that both developmental processes recruit similar gene networks (for example, those involved in hyphal aggregation).

Host trees are able to harness the formidable web of mycorrhizal hyphae (which permeates the soil and decaying leaf litter) for their nutritional benefit. A process that is pivotal to the success of ectomycorrhizal interactions is therefore the equitable exchange of nutrients between the symbiont and its host plant[1,2,13]. A comparison with other basidiomycetes (Supplementary Table 12) revealed that the total number of predicted transporters is larger in *L. bicolor* compared to *C. cinerea* and *P. chrysosporium*. Interestingly, *L. bicolor* has multiple ammonia transporters, although it encodes a single nitrate permease. Ammonia is arguably the most important inorganic nitrogen source for ectomycorrhizal fungi[14]. One of the ammonia transporters (*AMT2.2*), for instance, is greatly upregulated in ectomycorrhizae (Supplementary Table 5). Therefore, *L. bicolor* shows an increased genetic potential in terms of nitrogen uptake compared to other basidiomycetes. These capabilities are consistent with *L. bicolor* being exposed to a range of nitrogen sources from the decay of organic matter[15].

Although the *L. bicolor* genome contains numerous genes coding for key hydrolytic enzymes, such as proteases and lipases, we observed an extreme reduction in the number of enzymes involved in the degradation of plant cell wall (PCW) oligosaccharides and polysaccharides. Glycoside hydrolases, glycosyltransferases, polysaccharide lyases, carbohydrate esterases and their ancillary carbohydrate-binding modules were identified using the carbohydrate-active enzyme (CAZyme) classification (http://www.cazy.org/). A comparison of the *L. bicolor* candidate CAZymes with fungal phytopathogens

Table 2 | Changes in expression of transcripts coding for MISSPs

Protein identification (JGI *Laccaria* database)	Family size	Length (amino acids)	Transcript concentration (FLM)	*P. menziesii* ECM/FLM ratio (fold)	*P. trichocarpa* ECM/FLM ratio (fold)	Features
298595	sc	68	ND	21,877	12,913	MISSP7
333839	5	129	ND	7,844	1,931	Glycosyl phosphatidylinositol (GPI)-anchored
298667	2	70	ND	1,906	1,407	
332226	8	181	43	847	780	CFEM domain (INTERPRO IPR014005)
311468	2	59	ND	191	ND	
295737	8	288	131	171	252	
334759	sc	101	ND	109	18	
395403	4	121	24	103	93	
333423	9	120	6	102	72	Gonadotropin domain (IPR0001545)
312262	4	106	85	69	53	
295625	4	199	325	66	48	
325402	8	238	310	49	74	Snake toxin-like (SSF57302)
316998	sc	56	137	29	57	
333197	3	148	266	17	8	
327918	2	154	763	13	4	Homologue in *C. cinerea*
307956	sc	74	336	13	90	Whey acidic domain (IPR008197)
327246	sc	194	1,025	10	18	Homologue in *C. cinerea*
303550	5	98	1,365	10	14	
300377	2	291	5,499	10	8	
293250	sc	224	127	9	10	Homologue in *C. cinerea*
298648	sc	64	1,108	8	12	
298646	2	73	1,028	7	14	
293729	3	210	3,000	7	7	

Transcript profiling was performed on free-living mycelium (FLM) and ectomycorrhizal root tips (ECM) of poplar (*P. trichocarpa*) and Douglas fir (*P. menziesii*). See Supplementary Information Section 9 for details. Abbreviations: ND, not detected; sc, single copy.

confirms the adaptation of its enzyme repertoire to symbiosis and reveals the strategy used for the interaction with the host (Supplementary Tables 13 and 14). The reduction in PCW CAZymes affects almost all glycoside hydrolase families, culminating in the complete absence of several key families. For instance, there is only one candidate cellulase (glycoside hydrolase 5, GH5) appended to the sole fungal carbohydrate-binding module (CBM1) found in the genome, and no cellulases from families GH6 and GH7 (Supplementary Table 14). Similar reductions or loss of hemicellulose- and pectin-degrading enzymes were also noted. These observations suggest that the inventory of *L. bicolor* PCW-degrading enzymes underwent massive gene loss as a result of its adaptation to a symbiotic lifestyle, and that this species is now unable to use many PCW polysaccharides as a carbon source, including those found in soil and leaf litter. The remaining small set of secreted CAZymes with potential action on plant polysaccharides (for example, GH28 polygalacturonases) is probably required for cell wall remodelling during fungal tissue differentiation because their expression was upregulated in both fruiting bodies and ectomycorrhizae (Supplementary Table 15 and Supplementary Fig. 13). In contrast, transcripts coding for proteins with an expansin domain were only induced in ectomycorrhizae, suggesting they may be used by *L. bicolor* for penetrating into the root apoplastic space.

To survive before its mycorrhizal association with its host, *L. bicolor* seems to have developed a capacity to degrade non-plant (for example, animal and bacterial) oligosaccharides and polysaccharides; this is suggested by retention of CAZymes from families GH79, polysaccharide lyase 8 (PL8), PL14 and GH88 (Supplementary Table 14). Interestingly, there is no invertase gene in the *L. bicolor* genome, implying that this fungus is unable to use sucrose directly from the plant. This is consistent with earlier observations[16] that *L. bicolor* depends on its host plant to provide glucose in exchange for nitrogen. We also noticed an expansion of CAZymes involved in the fungal cell wall biosynthesis and rearrangement, almost entirely owing to an increased number of putative chitin synthases and enzymes acting on β-glucans (Supplementary Table 14). Several of the corresponding genes are up- or down-regulated in developmental processes requiring cell wall alterations such as formation of fruiting bodies or mycorrhizae (Supplementary Table 15 and Supplementary Fig. 13).

Ectomycorrhizal fungi have an important role in mobilizing nitrogen from well-decomposed organic matter[2,15]. The hyphal network permeating the soil might therefore be expected to express a wide diversity of proteolytic enzymes. The total number of secreted proteases (116 members) identified (Supplementary Fig. 14) is relatively large compared with that in other sequenced saprotrophic basidiomycetes, such as *C. cinerea* and *P. chrysosporium*. Secreted aspartyl-, metallo- and serine-proteases may have a role in degradation of decomposing litter[15], confirming that *L. bicolor* has also the ability to use nitrogen of animal origin, as suggested previously[17]. They may also have a role in developmental processes because the expression of several secreted proteases is up- or down-regulated in fruiting bodies and ectomycorrhizal root tips (Supplementary Table 16). Mycelial mats formed by *L. bicolor* hyphae colonizing organic matter therefore possess the ability to degrade proteins from decomposing leaf litter.

Our analysis of the gene space reveals a multi-faceted mutualistic biotroph equipped to take advantage of transient occurrences of high-nutrient niches (living host roots and decaying soil organic matter) within a heterogeneous, low-nutrient environment. The availability of genomes from mutualistic, saprotrophic[4] and pathogenic[6] fungi, as well as from the mycorrhizal tree *P. trichocarpa*[12], now provides an unparalleled opportunity to develop a deeper understanding of the processes by which fungi colonize wood and soil litter, and also interact with living plants within their ecosystem, to perform vital functions in the carbon and nitrogen cycles[2] that are fundamental to sustainable plant productivity.

METHODS SUMMARY

Genomic sequence. Scaffolds and assemblies for all genomic sequences generated by this project are also available from the Joint Genome Institute (JGI) portal (http://genome.jgi-psf.org/Lacbi1/Lacbi1.download.ftp.html). A genome browser is available from JGI (http://www.jgi.doe.gov/laccaria). BLAST search of the genome is available at JGI (http://www.jgi.doe.gov/laccaria) and INRA LaccariaDB (http://mycor.nancy.inra.fr/IMGC/LaccariaGenome/).

Predicted gene models. Consensus gene predictions, produced by combining several different gene predictors, are available from JGI (http://www.jgi.doe.gov/laccaria) as General Feature Format (GFF) files. These gene models can also be accessed from the Genome Browser in the JGI *L. bicolor* portal (http://www.jgi.doe.gov/laccaria).

Gene annotations. Tables compiling KEGG, PFAM, KOG and best BLAST hits for predicted gene models, transposable element and CAZyme data, as well as Tribe-MCL gene families, are available from INRA LaccariaDB (http://mycor.nancy.inra.fr/IMGC/LaccariaGenome/).

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Contributions A.A., D.A., A.B., E.J.J.D., F.D., J.G., A. K., E.L., V.P., A.S., H.J.S. and J.W. contributed equally to this work as second authors. M.C., B.H., U.K., S.L., Y.V.d.P., G.K.P., A.P., P.J.P., P.M.R., P.R., I.R.S., J.E.S., A.T., G.T. and I.V.G. contributed equally to this work as senior authors. Individual contributions were as follows. For the sequencing project, F.M. and G.T. initiated the sequencing project in the wake of the *Populus* genome project. F.M., I.V.G. and P.M.R. developed and coordinated

the sequencing, annotation and transcriptome projects. S.L. and sequencing staff at JGI performed the shotgun assembly. H.J.S. and his staff at JGI performed the JAZZ assembly of the nuclear and mitochondrial genome. J.G. and J.S. performed the Arachne assembly, closed up repetitive gaps and fixed missasembled regions. A.A., A.S., J.W., M.M., P.R., Y.V.P. and I.V.G. did the *ab initio* annotation of protein-coding gene models. A.K., E.L., P.B., C.D., J.C.D., M.P., G.K.P., A.T. and F.M. provided expressed sequence tag/cDNA information for the *ab initio* and manual annotation. Genome statistics was performed by D.A., F.D., J.W., P.R., I.V.G. and F.M. A.A. and Y.V.G., and F.D. and M.P.O.-L.S. were responsible for database design and maintenance at JGI and INRA, respectively. For genome analysis, D.C., M.P. and G.K.P. were responsible for DNA extraction and purification; D.A., F.D., Y.C.L., B.R., Y.V.P., P.R., J.E.S., A.T., I.V.G. and F.M. for comparative genome analysis; B.C., D.A. and A.T. for genome synteny; J.L., T.Y., G.T., F.M. and F.L.T. for construction of the genetic map; S.D.F. for single nucleotide polymorphisms; A.K., F.D. and F.M. for DNA arrays; A.D., B.C. and F.M. for high-GC sequences; J.W., P.R. and F.M. for tRNA, snRNA and rDNA; E.G.J.D., P.M.C., B.H. for carbohydrate active enzymes; A.D. and P.F.-K. for carbohydrate metabolism; J.G., P.H., U.K. and F.M. for cell wall proteins and secretome; L.F.-T., G.G., D.M. and R.M. for cytoskeleton and motor proteins; M.R., I.F. and A.P. for lipid metabolism; and H.N.-H., U.K. and I.R.S. for mating type genes. F.M. was responsible for genome analysis of the mitochondrion; P.E.C., P.H., M.B., S.K. and U.K. for the multi-copper oxidases; M.B., P.E.C. and F.M. for the proteases; N.R. for the redox genes; S.D., P.J. and G.K.P. for the signal transduction pathway; A.B., D.B., C.F., E.L., B.M., U.N., V.P. and M.C. for transporters; J.L., J.W., P.R., F.M. and H.Q. for transposable elements; V.P., J.G., A.B. and V.L. for immunolocalization of SSP; and J.E.S. and P.J.P. for the *Coprinopsis* genome. F.M.organized co-ordination between different groups. F.M. wrote and edited the paper with input from I.V.G., A.T., D.A., P.R., M.C. and B.H.

Author Information The whole-genome shotgun project has been deposited at GenBank/EMBL/DDBJ under project accession number ABFE00000000. The version described in this paper including assembly and annotation is the first version, ABFE01000000. The complete expression data set is available as a series under accession number GSE9784 at the Gene Expression Omnibus at NCBI (http://www.ncbi.nlm.nih.gov/geo/). Reprints and permissions information is available at www.nature.com/reprints. This paper is distributed under the terms of the Creative Commons Attribution-Non-Commercial-Share Alike licence, and is freely available to all readers at www.nature.com/nature. Correspondence and requests for materials should be addressed to F.M. (fmartin@nancy.inra.fr).

METHODS

Genome sequencing. The haploid genome of the strain S238N-H82 from *L. bicolor* (Maire) P. D. Orton was sequenced with the use of a whole-genome shotgun strategy. All data were generated by paired-end sequencing of cloned inserts using Sanger technology on ABI3730xl sequencers. Supplementary Table 1 gives the number of reads obtained per library.

Genome assembly. The data were assembled using release 1.0.1b of JAZZ, a JGI whole-genome shotgun assembler. On the basis of the number of alignments per read, the main genome scaffolds were at a depth of 9.88. The amount of sequence in the unplaced reads was 6.5 Mb, which is sufficient to cover the main-genome gaps to a mean depth of 9.9. A total of 64.9 Mb are captured in the scaffold assembly (Supplementary Table 2).

Genome annotation. Gene models were predicted using FgenesH[17], homology-based FgenesH$^+$ (ref. 18) and Genewise[19], as well as EuGène[20] and TwinScan[21], and alignments of several complementary DNA resources (Supplementary Information Section 3). The JGI pipeline selected a best representative gene model for each locus on the basis of expressed sequence tag support and similarity to known proteins from other organisms, and predicted 20,614 protein-coding gene models. All predicted genes were annotated using Gene Ontology[21], eukaryotic clusters of orthologous groups[22] and KEGG pathways[23]. Protein domains were predicted using InterProScan[24]. Signal peptides were predicted in 2,931 *L. bicolor* proteins by both the hidden Markov and the neural network algorithms of SignalP[25]. After eliminating predicted transmembrane proteins and removal of transposable element fragments, we selected 278 cysteine-rich secreted proteins with a size of $<$300 amino acids. Gene families were built from proteins in *L. bicolor*, *C. cinerea*, *P. chrysosporium*, *C. neoformans* and *U. maydis* using Tribe-MCL tools[26] with default settings.

Indirect immunofluorescent localization of MISSP7. The peptides LRALGQASQGGDLHR and GPIPNAVFRRVPEPNF located in the N-terminal and C-terminal parts of the MISSP7 sequence (without the signal peptide) were synthesized and used as antigens for the generation of antibodies in rabbits according to the manufacturer's procedures (Eurogentec). The anti-MISSP7 immunoglobulin (Ig)G fraction was purified using the MAbTrap kit (GE Healthcare) according to the manufacturer's recommendations. Subsequently, the IgG-containing fraction was desalted using a HiTrap desalting column (GE Healthcare). The concentration of purified IgG from pre-immune serum was determined by Bradford assay using a Bio-Rad protein assay. The final concentration of anti-MISSP7 IgG was 0.16 mg ml^{-1}. Immunolocalization was performed essentially as described in refs 27 and 28, with slight modifications (Supplementary Section 10).

Gene expression. Average expression levels of genes in different tissues and conditions were analysed using CyberT statistical framework (http://www.igb.uci.edu/servers/cybert/) and hierarchical clustering with EPCLUST (http://ep.ebi.ac.uk/EP/EPCLUST/) (Supplementary Information Section 8).

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Annexe 2

Transcripts encoding for hypothetical proteins of *L. bicolor* S238N regulated (≥ 2.0, ≤ -2.0) before contact with the helper strain *P. fluorescens* BBc6R8.

Up-regulated genes

SEQ_ID	NCBI Defintiion	Ratio T/C	protein ID	NCBI E value	Localization
Hypothetical protein					
LACB00S00003815	Gag-like protein [Tricholoma matsutake]	5,7	323351	1,00E-08	_
LACB00S00018835	Hypothetical protein CBG16621 [Caenorhabditis briggsae]	10,6	335185	3,00E-05	_
LACB00S00016299	No hit found	8,9	295580		_
LACB00S00002627	No hit found	8,7	292386		_
LACB00S00009074	No hit found	8,6	297969		S
LACB00S00001636	No hit found	6,2	321666		_
LACB00S00019083	No hit found	6,0	335401		S
LACB00S00015664	No hit found	5,7	310127		M
LACB00S00011897	No hit found	5,6	303209		_
LACB00S00014042	No hit found	5,6	307172		_
LACB00S00001423	ergot alkaloid biosynthetic protein A [Claviceps purpurea]	5,6	304995	2,10E-37	M
LACB00S00003211	No hit found	5,6	309040		_
LACB00S00015236	hypothetical protein MG03440.4 [Magnaporthe grisea 70-15]	5,5	148472	8,10E-24	_
LACB00S00005371	No hit found	5,5	293254		M
LACB00S00017804	No hit found	5,5	295829		_
LACB00S00018801	hypothetical protein AN1580.2 [Aspergillus nidulans FGSC A4]	5,4	241577	2,10E-64	_
LACB00S00010093	No hit found	5,4	299361		S
LACB00S00014840	No hit found	5,3	295379		_
LACB00S00012626	No hit found	5,3	304113		_
LACB00S00017811	No hit found	5,2	334383		_
LACB00S00002643	beta-galactosidase fused to beta-N-acetylhexosaminidase[Propionibacterium acnes KPA171202]	5,2	322448	6,00E-05	_
LACB00S00017484	hypothetical protein MG03030.4 [Magnaporthe grisea 70-15]	5,1	334148	1,10E-24	_
LACB00S00015582	hypothetical protein UM00262.1 [Ustilago maydis 521]	5,0	310024	3,00E-11	S
LACB00S00014298	No hit found	5,0	331669		M
LACB00S00010793	No hit found	4,9	300267		_
LACB00S00012952	No hit found	4,8	295057		S
LACB00S00017482	No hit found	4,7	313396		_
LACB00S00010251	No hit found	4,7	294592		M
LACB00S00014870	No hit found	4,6	308102		_
LACB00S00016460	No hit found	4,6	312166		_
LACB00S00010037	No hit found	4,6	328298		_
LACB00S00000964	No hit found	4,6	301607		_
LACB00S00010643	No hit found	4,6	300071		_
LACB00S00014017	No hit found	4,6	331435		_
LACB00S00011988	No hit found	4,6	329824		_
LACB00S00017068	No hit found	4,6	295696		_
LACB00S00002146	No hit found	4,5	322057		_
LACB00S00001027	No hit found	4,5	301681		M
LACB00S00014255	No hit found	4,4	295282		_
LACB00S00017049	No hit found	4,4	312908		M
LACB00S00019268	No hit found	4,3	296052		M
LACB00S00000965	hypothetical protein FG05203.1 [Gibberella zeae PH-1]	4,3	321144	4,10E-63	M
LACB00S00006063	No hit found	4,3	316896		_

LACB00S00009213	hypothetical protein FG03449.1 [Gibberella zeae PH-1]	4,3	294476	1,10E-29	_
LACB00S00015778	No hit found	4,3	310249		M
LACB00S00008751	No hit found	4,1	297623		M
LACB00S00016076	No hit found	4,1	333052		_
LACB00S00010010	No hit found	4,1	299271		S
LACB00S00000988	No hit found	4,1	301635		_
LACB00S00020243	No hit found	4,1	303665		S
LACB00S00017037	No hit found	4,1	312897		S
LACB00S00007215	hypothetical protein UM02390.1 [Ustilago maydis 521]	4,0	318760	2,00E-14	M
LACB00S00012427	No hit found	4,0	303865		S
LACB00S00002379	No hit found	4,0	306051		_
LACB00S00011206	No hit found	4,0	294743		_
LACB00S00000687	No hit found	4,0	301289		M
LACB00S00017485	hypothetical protein FG11144.1 [Gibberella zeae PH-1]	3,9	313400	2,10E-24	M
LACB00S00009462	No hit found	3,9	298489		M
LACB00S00004779	hypothetical protein UM02110.1 [Ustilago maydis 521]	3,9	232209	2,10E-54	_
LACB00S00013420	cAMP-independent regulatory protein, [Cryptococcusneoformans var. neoformans JEC21]	3,8	306386	3,10E-40	_
LACB00S00019406	No hit found	3,8	298343		M
LACB00S00018682	hypothetical protein Franean1DRAFT_2300 [Frankia sp. EAN1pec]	3,8	316607	1,00E-05	_
LACB00S00007232	No hit found	3,8	326027		_
LACB00S00000752	hypothetical protein AN4107.2 [Aspergillus nidulans FGSC A4]	3,8	242814	1,00E-155	_
LACB00S00008096	No hit found	3,7	296720		S
LACB00S00005356	predicted protein [Gibberella zeae PH-1]	3,7	315381	3,00E-12	_
LACB00S00015136	No hit found	3,7	295416		_
LACB00S00012937	No hit found	3,7	330571		_
LACB00S00006345	No hit found	3,7	325348		_
LACB00S00003667	No hit found	3,6	311245		S
LACB00S00012144	conserved hypothetical protein [Cryptococcus neoformans var.neoformans JEC21]	3,6	174464	3,10E-71	_
LACB00S00018874	No hit found	3,6	335215		_
LACB00S00008094	No hit found	3,6	296716		S
LACB00S00008092	No hit found	3,6	296711		S
LACB00S00013425	No hit found	3,6	330954		_
LACB00S00014856	No hit found	3,6	295380		S
LACB00S00012333	No hit found	3,5	330098		M
LACB00S00014256	No hit found	3,5	307414		M
LACB00S00013341	No hit found	3,5	295103		_
LACB00S00017065	No hit found	3,5	295694		M
LACB00S00000232	No hit found	3,5	300794		_
LACB00S00017654	hypothetical protein FG03872.1 [Gibberella zeae PH-1]	3,4	314483	5,10E-90	_
LACB00S00015473	hypothetical protein UM00262.1 [Ustilago maydis 521]	3,4	309859	6,00E-14	S
LACB00S00005353	No hit found	3,4	315377		M
LACB00S00011819	No hit found	3,4	329702		M
LACB00S00012784	No hit found	3,4	304316		_
LACB00S00005331	No hit found	3,4	315352		S
LACB00S00010616	hypothetical protein AN3660.2 [Aspergillus nidulans FGSC A4]	3,4	300036	2,00E-14	S
LACB00S00005823	No hit found	3,3	315926		_
LACB00S00005211	No hit found	3,3	324439		_
LACB00S00015995	mismatched base pair and cruciform DNA recognition protein[Agaricus bisporus]	3,3	295545	7,10E-26	_
LACB00S00009461	No hit found	3,3	298488		M
LACB00S00003616	No hit found	3,3	311195		_
LACB00S00001291	No hit found	3,3	321400		S
LACB00S00019295	No hit found	3,3	296130		M

LACB00S00014838	No hit found	3,3	295378		_
LACB00S00016272	No hit found	3,3	333197		S
LACB00S00017647	hypothetical protein UM05122.1 [Ustilago maydis 521]	3,3	143646	4,10E-91	S
LACB00S00009677	No hit found	3,3	298749		_
LACB00S00008755	No hit found	3,2	297627		M
LACB00S00012311	conserved hypothetical protein [Cryptococcus neoformans var.neoformans JEC21]	3,2	303749	0.0	_
LACB00S00019692	hypothetical protein FG04972.1 [Gibberella zeae PH-1]	3,2	336290	7,10E-27	_
LACB00S00003738	conserved hypothetical protein [Cryptococcus neoformans var.neoformans JEC21]	3,2	292701	4,00E-16	_
LACB00S00005096	tudor domain containing 1 isoform 2 [Mus musculus]	3,2	314078	3,00E-05	_
LACB00S00013233	No hit found	3,2	330798		_
LACB00S00001506	No hit found	3,2	305096		_
LACB00S00013234	hypothetical protein [Coprinopsis cinerea]	3,2	330799	1,00E-07	S
LACB00S00009783	No hit found	3,2	294551		_
LACB00S00001987	No hit found	3,2	305621		M
LACB00S00010858	No hit found	3,2	328946		_
LACB00S00016399	No hit found	3,2	310999		M
LACB00S00019554	No hit found	3,2	303416		_
LACB00S00008238	hypothetical protein CNBN0640 [Cryptococcus neoformans var.neoformans B-3501A]	3,2	296997	2,10E-81	_
LACB00S00009902	No hit found	3,2	299096		_
LACB00S00008724	hypothetical protein UM04085.1 [Ustilago maydis 521]	3,1	297593	3,00E-08	S
LACB00S00013773	No hit found	3,1	306852		S
LACB00S00012023	No hit found	3,1	303381		S
LACB00S00010246	No hit found	3,1	294590		S
LACB00S00003702	No hit found	3,1	323260		_
LACB00S00017669	No hit found	3,1	314500		_
LACB00S00020406	No hit found	3,1	336247		_
LACB00S00011889	No hit found	3,1	303193		_
LACB00S00019145	No hit found	3,0	318410		M
LACB00S00008064	No hit found	3,0	326666		M
LACB00S00002134	No hit found	3,0	305798		M
LACB00S00006617	No hit found	3,0	325554		S
LACB00S00013002	No hit found	3,0	304571		_
LACB00S00017935	hypothetical protein AN5539.2 [Aspergillus nidulans FGSC A4]	2,9	314841	8,00E-13	_
LACB00S00020154	No hit found	2,9	335969		_
LACB00S00017072	No hit found	2,9	333814		_
LACB00S00007034	No hit found	2,9	318344		S
LACB00S00017465	related to glyoxal oxidase precursor [Neurospora crassa]	2,9	313372	5,10E-33	_
LACB00S00010805	No hit found	2,9	328907		M
LACB00S00010637	No hit found	2,9	300066		M
LACB00S00017010	No hit found	2,9	333763		_
LACB00S00009671	helicase-like protein [Oryza sativa (japonica cultivar-group)]	2,9	298744	1,00E-11	S
LACB00S00005880	No hit found	2,9	315988		S
LACB00S00006136	No hit found	2,9	325174		_
LACB00S00010867	No hit found	2,9	300347		_
LACB00S00011689	basidiospore development protein [Coprinopsis cinerea]	2,9	302906	7,10E-53	_
LACB00S00008626	hypothetical protein CNBA1650 [Cryptococcus neoformans var.neoformans B-3501A]	2,9	294306	3,00E-12	S
LACB00S00012954	No hit found	2,9	304512		_
LACB00S00015356	No hit found	2,9	309721		M
LACB00S00003630	Yel023cp [Saccharomyces cerevisiae]	2,9	311208	8,10E-34	_
LACB00S00017676	No hit found	2,9	314508		S
LACB00S00009552	No hit found	2,8	298604		M
LACB00S00011788	No hit found	2,8	303049		_

LACB00S00014881	hypothetical protein AN8615.2 [Aspergillus nidulans FGSC A4]	2,8	253742	6,10E-69	S
LACB00S00006146	No hit found	2,8	316982		_
LACB00S00019477	No hit found	2,8	335888		S
LACB00S00005147	unnamed protein product [Kluyveromyces lactis NRRL Y-1140]	2,8	314132	2,00E-05	_
LACB00S00009576	No hit found	2,8	298634		_
LACB00S00007776	hypothetical protein FG03872.1 [Gibberella zeae PH-1]	2,8	184655	7,00E-94	M
LACB00S00016451	expressed protein [Cryptococcus neoformans var. neoformans JEC21]	2,8	312155	1,10E-24	_
LACB00S00016603	No hit found	2,8	333462		_
LACB00S00010055	No hit found	2,8	328310		_
LACB00S00009960	No hit found	2,8	299211		M
LACB00S00014613	No hit found	2,8	307792		M
LACB00S00010550	No hit found	2,8	328707		M
LACB00S00006738	hypothetical protein MG08572.4 [Magnaporthe grisea 70-15]	2,8	318017	3,00E-17	S
LACB00S00009212	No hit found	2,8	298127		M
LACB00S00005332	No hit found	2,7	315353		S
LACB00S00008759	No hit found	2,7	297631		_
LACB00S00019150	No hit found	2,7	335476		_
LACB00S00019933	No hit found	2,7	296840		S
LACB00S00010460	hypothetical protein Afu2g17630 [Aspergillus fumigatus Af293]	2,7	328635	5,00E-08	S
LACB00S00013449	No hit found	2,7	306414		M
LACB00S00017987	hypothetical protein MG11080.4 [Magnaporthe grisea 70-15]	2,7	334538	1,00E-09	_
LACB00S00007383	hypothetical protein CNK01790 [Cryptococcus neoformans var.neoformans JEC21]	2,7	318938	4,00E-08	M
LACB00S00003571	No hit found	2,7	311135		_
LACB00S00012905	No hit found	2,7	295048		_
LACB00S00016537	No hit found	2,7	312263		M
LACB00S00015930	unknown [Nosema locustae]	2,7	310438	3,00E-19	_
LACB00S00001976	No hit found	2,7	305605		S
LACB00S00004890	No hit found	2,7	313856		M
LACB00S00008198	No hit found	2,7	296954		_
LACB00S00018834	No hit found	2,7	335184		_
LACB00S00000256	No hit found	2,7	320618		S
LACB00S00017062	No hit found	2,7	312924		S
LACB00S00015957	No hit found	2,7	332960		_
LACB00S00009381	No hit found	2,7	298402		_
LACB00S00006346	No hit found	2,7	325349		_
LACB00S00019764	No hit found	2,7	317679		_
LACB00S00009282	No hit found	2,7	327678		_
LACB00S00003457	No hit found	2,6	323078		_
LACB00S00012140	No hit found	2,6	303550		S
LACB00S00014484	No hit found	2,6	307651		_
LACB00S00016996	No hit found	2,6	333751		_
LACB00S00005194	No hit found	2,6	314190		_
LACB00S00015370	unnamed protein product [Aspergillus oryzae]	2,6	332507	2,10E-39	_
LACB00S00011225	hypothetical protein Afu6g03790 [Aspergillus fumigatus Af293]	2,6	302288	2,00E-06	S
LACB00S00012670	No hit found	2,6	304161		M
LACB00S00005599	No hit found	2,6	315674		S
LACB00S00019595	No hit found	2,6	306253		S
LACB00S00019755	No hit found	2,6	317666		_
LACB00S00007155	No hit found	2,6	318702		_
LACB00S00017802	No hit found	2,6	295828		S
LACB00S00012130	hypothetical protein FG05681.1 [Gibberella zeae PH-1]	2,6	303541	4,00E-05	_
LACB00S00018750	No hit found	2,5	316689		_
LACB00S00016509	expressed protein [Cryptococcus neoformans var. neoformans JEC21]	2,5	333394	9,00E-16	_

LACB00S00000944	No hit found	2,5	301577		_
LACB00S00004415	No hit found	2,5	312046		S
LACB00S00007818	meu13 [Schizosaccharomyces pombe]	2,5	296418	2,00E-14	_
LACB00S00007364	hypothetical protein UM02449.1 [Ustilago maydis 521]	2,5	318917	3,00E-17	_
LACB00S00006548	PREDICTED: similar to late embryogenesis abundant protein-like [Pantroglodytes]	2,5	325496	6,00E-13	M
LACB00S00002984	No hit found	2,5	292440		_
LACB00S00011787	No hit found	2,5	303048		_
LACB00S00013729	hypothetical protein FG11167.1 [Gibberella zeae PH-1]	2,5	153882	3,10E-45	_
LACB00S00006688	No hit found	2,5	317968		M
LACB00S00006533	hypothetical protein CNBD3750 [Cryptococcus neoformans var.neoformans B-3501A]	2,5	325488	5,00E-05	_
LACB00S00006948	No hit found	2,5	318246		_
LACB00S00010038	No hit found	2,5	299309		_
LACB00S00009959	No hit found	2,5	299210		_
LACB00S00006621	No hit found	2,5	317894		S
LACB00S00006693	No hit found	2,5	317974		M
LACB00S00002173	No hit found	2,5	305838		_
LACB00S00017047	No hit found	2,5	333789		_
LACB00S00007425	alr2472 [Nostoc sp. PCC 7120]	2,5	318986	4,00E-05	_
LACB00S00017951	No hit found	2,5	334504		M
LACB00S00007977	No hit found	2,5	294101		S
LACB00S00001543	No hit found	2,5	321600		S
LACB00S00019805	No hit found	2,5	296060		M
LACB00S00016499	No hit found	2,5	312222		_
LACB00S00010984	No hit found	2,5	300477		_
LACB00S00016346	No hit found	2,5	310923		_
LACB00S00017169	related to calcium-independent phospholipase A2 [Neurospora crassa]	2,5	295706	1,00E-15	M
LACB00S00013170	No hit found	2,5	304790		_
LACB00S00007320	No hit found	2,5	318869		_
LACB00S00003411	No hit found	2,5	292626		M
LACB00S00003187	alcohol dehydrogenase, zinc-containing [Aspergillus fumigatusAf293]	2,4	244943	9,00E-60	_
LACB00S00007260	No hit found	2,4	318809		S
LACB00S00012043	No hit found	2,4	329860		_
LACB00S00016630	No hit found	2,4	312375		_
LACB00S00017979	No hit found	2,4	314915		_
LACB00S00012610	No hit found	2,4	304087		M
LACB00S00018340	No hit found	2,4	316181		M
LACB00S00014473	No hit found	2,4	307642		S
LACB00S00012625	No hit found	2,4	304112		M
LACB00S00015787	No hit found	2,4	332824		M
LACB00S00006069	f-box protein pof6, [Cryptococcus neoformans var. neoformansJEC21]	2,4	248167	1,00E-130	_
LACB00S00014736	No hit found	2,4	307960		S
LACB00S00011865	No hit found	2,4	329729		_
LACB00S00000779	hypothetical protein MG10699.4 [Magnaporthe grisea 70-15]	2,4	161044	1,10E-31	_
LACB00S00009008	No hit found	2,4	297896		_
LACB00S00020397	No hit found	2,4	309951		S
LACB00S00009549	No hit found	2,4	327912		M
LACB00S00010298	No hit found	2,4	299648		S
LACB00S00014090	No hit found	2,4	331500		_
LACB00S00000773	No hit found	2,4	301385		_
LACB00S00015301	No hit found	2,4	309659		_
LACB00S00016850	No hit found	2,4	312687		M
LACB00S00010646	No hit found	2,4	294650		_
LACB00S00010378	NACHT domain protein, [Aspergillus fumigatus Af293]	2,4	328567	8,10E-33	_
LACB00S00017594	No hit found	2,4	334221		_
LACB00S00011850	No hit found	2,4	303134		M

LACB00S00001975	No hit found	2,4	305602		_
LACB00S00003794	hypothetical protein FG04539.1 [Gibberella zeae PH-1]	2,4	311386	1,00E-06	_
LACB00S00005915	No hit found	2,4	316025		_
LACB00S00011851	No hit found	2,4	303135		_
LACB00S00009588	No hit found	2,4	298646		S
LACB00S00001512	hypothetical protein FG08952.1 [Gibberella zeae PH-1]	2,4	305103	2,10E-40	_
LACB00S00012593	hypothetical protein FG01793.1 [Gibberella zeae PH-1]	2,4	295007	5,10E-58	_
LACB00S00007151	No hit found	2,4	325968		_
LACB00S00013358	alpha-NAC, muscle-specific form gp220 [Mus musculus]	2,4	306320	2,00E-07	_
LACB00S00017933	No hit found	2,4	295845		_
LACB00S00007216	No hit found	2,4	318761		M
LACB00S00020558	No hit found	2,4	309590		S
LACB00S00014833	No hit found	2,4	308064		_
LACB00S00019750	No hit found	2,4	296030		_
LACB00S00005855	hypothetical protein UM04183.1 [Ustilago maydis 521]	2,4	315961	2,00E-12	_
LACB00S00016309	No hit found	2,3	310879		_
LACB00S00008445	No hit found	2,3	297221		_
LACB00S00003531	No hit found	2,3	311093		_
LACB00S00011653	No hit found	2,3	329586		_
LACB00S00009344	No hit found	2,3	298284		_
LACB00S00014035	hypothetical protein UM03880.1 [Ustilago maydis 521]	2,3	160038	5,10E-53	M
LACB00S00005568	No hit found	2,3	315634		S
LACB00S00011546	No hit found	2,3	329498		_
LACB00S00006629	No hit found	2,3	293679		_
LACB00S00004085	hypothetical protein UM01629.1 [Ustilago maydis 521]	2,3	323571	2,00E-19	S
LACB00S00019207	No hit found	2,3	335546		S
LACB00S00006514	No hit found	2,3	293663		_
LACB00S00000088	No hit found	2,3	300644		M
LACB00S00006893	related to calcium-independent phospholipase A2 [Neurospora crassa]	2,3	318179	6,00E-11	_
LACB00S00016220	hypothetical protein BF2971 [Bacteroides fragilis YCH46]	2,3	291363	7,00E-09	_
LACB00S00009615	No hit found	2,3	298671		_
LACB00S00016376	No hit found	2,3	310974		_
LACB00S00009966	No hit found	2,3	299219		_
LACB00S00012424	hypothetical protein MG05855.4 [Magnaporthe grisea 70-15]	2,3	303861	2,10E-89	M
LACB00S00000937	No hit found	2,3	301568		_
LACB00S00000985	No hit found	2,3	301632		_
LACB00S00000994	hypothetical protein CNBG3050 [Cryptococcus neoformans var.neoformans B-3501A]	2,3	301641	3,00E-07	_
LACB00S00018659	No hit found	2,3	316583		M
LACB00S00003653	No hit found	2,3	311231		_
LACB00S00017450	No hit found	2,3	334118		_
LACB00S00001661	hypothetical protein FG08952.1 [Gibberella zeae PH-1]	2,3	305257	2,10E-37	_
LACB00S00016743	No hit found	2,3	312517		_
LACB00S00020063	No hit found	2,3	335862		M
LACB00S00005414	No hit found	2,3	324594		_
LACB00S00010623	No hit found	2,3	328766		_
LACB00S00003736	No hit found	2,3	323288		_
LACB00S00002952	No hit found	2,3	308758		_
LACB00S00006938	No hit found	2,3	318235		M
LACB00S00016846	No hit found	2,3	312682		M
LACB00S00018139	Cap3p [Cryptococcus neoformans var. neoformans]	2,3	295875	7,10E-75	S
LACB00S00014885	No hit found	2,3	295385		M

LACB00S00018190	hypothetical protein AN6361.2 [Aspergillus nidulans FGSC A4]	2,3	241237	4,10E-31	_
LACB00S00002882	No hit found	2,3	308683		S
LACB00S00011723	No hit found	2,3	302943		_
LACB00S00005623	hypothetical protein CNA03290 [Cryptococcus neoformans var.neoformans JEC21]	2,3	324765	2,00E-07	_
LACB00S00016310	No hit found	2,3	295584		_
LACB00S00010500	expressed protein [Cryptococcus neoformans var. neoformans JEC21]	2,3	299866	2,00E-16	_
LACB00S00014839	No hit found	2,3	308069		_
LACB00S00005565	No hit found	2,3	293305		S
LACB00S00019269	No hit found	2,3	335605		_
LACB00S00009408	No hit found	2,3	327788		M
LACB00S00019363	No hit found	2,3	296790		_
LACB00S00006097	No hit found	2,3	316931		M
LACB00S00016586	No hit found	2,3	333452		S
LACB00S00017080	No hit found	2,3	333821		_
LACB00S00002133	No hit found	2,3	305797		M
LACB00S00003849	ubiquitin-protein ligase, [Cryptococcus neoformans var.neoformans JEC21]	2,3	311453	6,00E-11	S
LACB00S00011651	No hit found	2,3	302864		_
LACB00S00013697	No hit found	2,3	306774		M
LACB00S00007146	No hit found	2,3	325964		M
LACB00S00018875	No hit found	2,3	335216		_
LACB00S00008419	DNA binding protein Ncp1, [Cryptococcus neoformans var.neoformans JEC21]	2,3	297193	2,00E-15	_
LACB00S00010880	hypothetical protein AN6463.2 [Aspergillus nidulans FGSC A4]	2,3	328962	3,00E-11	_
LACB00S00012370	No hit found	2,3	303813		M
LACB00S00003068	No hit found	2,2	308889		_
LACB00S00015293	No hit found	2,2	309651		M
LACB00S00009238	hypothetical protein UM04689.1 [Ustilago maydis 521]	2,2	294481	1,00E-14	_
LACB00S00005188	No hit found	2,2	314179		_
LACB00S00009460	No hit found	2,2	327831		_
LACB00S00011847	No hit found	2,2	303131		_
LACB00S00018108	hypothetical protein AN6760.2 [Aspergillus nidulans FGSC A4]	2,2	168601	8,10E-23	_
LACB00S00017618	No hit found	2,2	295801		_
LACB00S00013526	histidine acid phosphatase, [Aspergillus fumigatus Af293]	2,2	306538	2,00E-06	_
LACB00S00007661	No hit found	2,2	293995		_
LACB00S00010795	PREDICTED: similar to late embryogenesis abundant protein-like [Pantroglodytes]	2,2	300269	2,00E-10	M
LACB00S00010092	No hit found	2,2	299360		S
LACB00S00010502	No hit found	2,2	299868		S
LACB00S00009009	No hit found	2,2	297897		_
LACB00S00016127	No hit found	2,2	310663		_
LACB00S00013683	No hit found	2,2	295151		M
LACB00S00018353	No hit found	2,2	316194		M
LACB00S00011776	No hit found	2,2	294852		_
LACB00S00014751	hypothetical protein UM03900.1 [Ustilago maydis 521]	2,2	295364	2,10E-40	_
LACB00S00012989	No hit found	2,2	304555		M
LACB00S00010755	No hit found	2,2	300193		S
LACB00S00008474	No hit found	2,2	327003		_
LACB00S00010904	No hit found	2,2	294690		_
LACB00S00009453	No hit found	2,2	327825		_
LACB00S00016139	No hit found	2,2	333099		_
LACB00S00003741	hypothetical protein UM01098.1 [Ustilago maydis 521]	2,2	311330	5,10E-39	_
LACB00S00007865	No hit found	2,2	296468		M
LACB00S00008692	conserved hypothetical protein [Neurospora crassa]	2,2	294334	1,00E-14	_

LACB00S00012401	No hit found	2,2	330150		S
LACB00S00010372	No hit found	2,2	299725		_
LACB00S00014826	No hit found	2,2	332089		_
LACB00S00004704	No hit found	2,2	313660		_
LACB00S00010921	expressed protein [Cryptococcus neoformans var. neoformans JEC21]	2,2	300413	2,00E-09	_
LACB00S00009838	No hit found	2,2	299017		_
LACB00S00016770	No hit found	2,2	312562		M
LACB00S00005730	hypothetical protein UM04920.1 [Ustilago maydis 521]	2,2	315821	6,00E-15	_
LACB00S00013313	No hit found	2,2	330860		M
LACB00S00006311	No hit found	2,2	293600		_
LACB00S00019084	No hit found	2,2	317712		_
LACB00S00013370	No hit found	2,2	330907		_
LACB00S00012127	No hit found	2,2	303536		S
LACB00S00006627	conserved hypothetical protein [Frankia sp. EAN1pec]	2,2	233630	3,10E-95	_
LACB00S00020089	No hit found	2,2	299542		M
LACB00S00010159	No hit found	2,2	299442		M
LACB00S00013293	No hit found	2,2	304926		M
LACB00S00017263	No hit found	2,2	295735		_
LACB00S00010888	No hit found	2,2	300377		S
LACB00S00011241	No hit found	2,2	302309		M
LACB00S00016703	histidine acid phosphatase, [Aspergillus fumigatus Af293]	2,2	312463	2,00E-06	_
LACB00S00003463	No hit found	2,2	323083		M
LACB00S00014312	centromeric DNA binding protein, [Cryptococcus neoformansvar. neoformans JEC21]	2,2	307469	2,10E-33	M
LACB00S00004432	hypothetical protein MG02709.4 [Magnaporthe grisea 70-15]	2,2	246464	1,10E-57	_
LACB00S00006706	No hit found	2,2	317986		S
LACB00S00012201	No hit found	2,2	329994		_
LACB00S00008433	PREDICTED: similar to C42C1.13 [Bos taurus]	2,2	297208	1,00E-05	_
LACB00S00000603	conserved hypothetical protein [Shewanella sp. PV-4]	2,2	301196	4,00E-12	_
LACB00S00018069	No hit found	2,2	334592		S
LACB00S00009310	No hit found	2,2	298252		M
LACB00S00000753	No hit found	2,1	320972		_
LACB00S00016878	No hit found	2,1	295648		_
LACB00S00001437	No hit found	2,1	305011		S
LACB00S00013218	hypothetical protein AN2445.2 [Aspergillus nidulans FGSC A4]	2,1	330785	1,00E-05	S
LACB00S00015920	No hit found	2,1	310429		_
LACB00S00020152	No hit found	2,1	302362		S
LACB00S00010558	No hit found	2,1	328712		_
LACB00S00013283	No hit found	2,1	304916		_
LACB00S00001892	No hit found	2,1	305512		S
LACB00S00007971	No hit found	2,1	296577		S
LACB00S00016092	No hit found	2,1	310624		_
LACB00S00010513	No hit found	2,1	328676		M
LACB00S00013996	No hit found	2,1	307112		M
LACB00S00006843	No hit found	2,1	293734		_
LACB00S00011799	No hit found	2,1	303062		_
LACB00S00011311	No hit found	2,1	329307		_
LACB00S00009811	No hit found	2,1	328103		_
LACB00S00007954	No hit found	2,1	296560		_
LACB00S00003900	No hit found	2,1	311507		_
LACB00S00016449	hypothetical protein CNBN0230 [Cryptococcus neoformans var.neoformans B-3501A]	2,1	312153	1,10E-30	_
LACB00S00010656	expressed protein [Cryptococcus neoformans var. neoformans JEC21]	2,1	300092	1,00E-10	_
LACB00S00020578	hypothetical protein Afu3g07390 [Aspergillus fumigatus Af293]	2,1	312533	6,00E-16	_

LACB00S00012439	conserved hypothetical protein [Aspergillus fumigatus Af293]	2,1	294969	6,10E-21	M
LACB00S00005410	conserved hypothetical protein [Aspergillus fumigatus Af293]	2,1	315443	3,10E-48	M
LACB00S00012976	No hit found	2,1	304540		_
LACB00S00008398	PREDICTED: similar to CG6282-PA, isoform A [Danio rerio]	2,1	297170	3,10E-40	S
LACB00S00005729	No hit found	2,1	315819		_
LACB00S00019775	No hit found	2,1	318478		_
LACB00S00007007	expressed protein [Cryptococcus neoformans var. neoformans JEC21]	2,1	318310	1,00E-09	_
LACB00S00001320	No hit found	2,1	301993		_
LACB00S00000873	No hit found	2,1	301495		_
LACB00S00017939	unknown [Nosema locustae]	2,1	334492	3,00E-10	M
LACB00S00005119	CG6066-PA [Drosophila melanogaster]	2,1	314103	3,10E-25	M
LACB00S00004648	No hit found	2,1	313600		_
LACB00S00008090	No hit found	2,1	326686		M
LACB00S00002891	No hit found	2,1	308695		_
LACB00S00009485	hypothetical protein [Schizophyllum commune]	2,1	327859	4,10E-21	S
LACB00S00009046	No hit found	2,1	327488		_
LACB00S00014808	No hit found	2,1	308038		M
LACB00S00011848	No hit found	2,1	303132		S
LACB00S00005135	No hit found	2,1	324374		_
LACB00S00019210	No hit found	2,1	318542		_
LACB00S00013448	No hit found	2,1	330974		_
LACB00S00006344	No hit found	2,1	325347		_
LACB00S00002132	No hit found	2,1	322045		_
LACB00S00001196	No hit found	2,1	291919		M
LACB00S00012912	predicted protein [Neurospora crassa]	2,1	304467	2,00E-07	_
LACB00S00016126	No hit found	2,1	310662		S
LACB00S00011573	No hit found	2,1	302784		_
LACB00S00006727	No hit found	2,1	293710		_
LACB00S00010444	No hit found	2,1	299807		S
LACB00S00012582	No hit found	2,1	330302		_
LACB00S00016885	No hit found	2,1	333662		_
LACB00S00000110	No hit found	2,1	300670		M
LACB00S00018336	SPCC1739.05 [Schizosaccharomyces pombe]	2,1	334800	3,00E-12	_
LACB00S00019978	No hit found	2,1	298769		_
LACB00S00017196	motor, [Cryptococcus neoformans var. neoformans JEC21]	2,1	313077	2,00E-07	_
LACB00S00016232	No hit found	2,1	310797		_
LACB00S00003108	No hit found	2,1	322797		_
LACB00S00011286	No hit found	2,1	302403		_
LACB00S00016256	No hit found	2,0	295570		S
LACB00S00000691	No hit found	2,0	301293		S
LACB00S00017522	No hit found	2,0	334173		M
LACB00S00019829	No hit found	2,0	310119		_
LACB00S00003034	No hit found	2,0	308846		_
LACB00S00017614	No hit found	2,0	295799		_
LACB00S00001505	No hit found	2,0	305095		_
LACB00S00000238	hypothetical protein CNBA1470 [Cryptococcus neoformans var.neoformans B-3501A]	2,0	242982	3,10E-64	_
LACB00S00001643	hypothetical protein UM05369.1 [Ustilago maydis 521]	2,0	305240	1,00E-111	_
LACB00S00017718	No hit found	2,0	295822		_
LACB00S00002834	No hit found	2,0	292403		_
LACB00S00010725	No hit found	2,0	300162		M
LACB00S00016718	No hit found	2,0	333547		_
LACB00S00013173	No hit found	2,0	304793		S
LACB00S00018550	No hit found	2,0	316424		M
LACB00S00016665	No hit found	2,0	312429		_
LACB00S00007047	No hit found	2,0	318358		S
LACB00S00014299	No hit found	2,0	331670		S
LACB00S00010385	No hit found	2,0	299737		M

LACB00S00001592	KH domain protein [Aspergillus fumigatus Af293]	2,0	305185	7,10E-31	_
LACB00S00013706	No hit found	2,0	306783		M
LACB00S00009602	No hit found	2,0	327951		M
LACB00S00014429	hypothetical protein AN7953.2 [Aspergillus nidulans FGSC A4]	2,0	307595	6,10E-72	_
LACB00S00018514	NACHT domain protein, [Aspergillus fumigatus Af293]	2,0	316379	1,10E-27	_
LACB00S00005746	No hit found	2,0	324866		_
LACB00S00011855	No hit found	2,0	303139		M
LACB00S00015266	No hit found	2,0	309623		_
LACB00S00019023	No hit found	2,0	335334		_
LACB00S00000369	No hit found	2,0	300941		_
LACB00S00016709	No hit found	2,0	333543		M
LACB00S00012145	No hit found	2,0	329947		S
LACB00S00006316	No hit found	2,0	317168		S
LACB00S00008051	No hit found	2,0	296664		_
LACB00S00016244	hypothetical protein Afu4g04140 [Aspergillus fumigatus Af293]	2,0	333174	1,00E-101	_
LACB00S00010787	No hit found	2,0	300260		S
LACB00S00011678	expressed protein [Cryptococcus neoformans var. neoformans JEC21]	2,0	294839	6,00E-06	_
LACB00S00005669	hypothetical protein UM04540.1 [Ustilago maydis 521]	2,0	293346	4,10E-29	_
LACB00S00014791	SirH [Leptosphaeria maculans]	2,0	308023	2,10E-20	S
LACB00S00013529	No hit found	2,0	306541		M
LACB00S00010402	No hit found	2,0	299753		_
LACB00S00003636	hypothetical protein FG07607.1 [Gibberella zeae PH-1]	2,0	311215	1,00E-10	_
LACB00S00016322	No hit found	2,0	333237		_
LACB00S00010359	No hit found	2,0	299712		_
LACB00S00014246	No hit found	2,0	307401		_
LACB00S00002258	hypothetical protein UM01269.1 [Ustilago maydis 521]	2,0	305931	1,00E-139	M
LACB00S00009330	No hit found	2,0	294497		_
LACB00S00012254	No hit found	2,0	303692		S
LACB00S00000735	hypothetical protein FG06190.1 [Gibberella zeae PH-1]	2,0	301343	1,00E-15	S
LACB00S00017133	No hit found	2,0	312996		_
LACB00S00011102	No hit found	2,0	294721		M
LACB00S00017947	No hit found	2,0	314858		_
LACB00S00005751	conserved hypothetical protein [Aspergillus fumigatus Af293]	2,0	233103	1,00E-116	_
LACB00S00006079	No hit found	2,0	316911		_
LACB00S00006164	No hit found	2,0	316998		S
LACB00S00012053	No hit found	2,0	329870		_
LACB00S00018469	COG3172: Predicted ATPase/kinase involved in NAD metabolism[Lactococcus lactis subsp. cremoris SK11]	2,0	334902	3,00E-05	_
LACB00S00017630	No hit found	2,0	314454		_
LACB00S00007416	filament-specific protein UFU57 [Ustilago maydis]	2,0	318974	2,00E-07	S
LACB00S00018295	tryptophan 2,3-dioxygenase, [Cryptococcus neoformans var.neoformans JEC21]	2,0	315290	1,00E-13	_
LACB00S00001923	No hit found	2,0	292116		M
LACB00S00001341	No hit found	2,0	291942		_
LACB00S00013185	TPR repeat:TPR repeat [Crocosphaera watsonii WH 8501]	2,0	304805	2,10E-36	_
LACB00S00018698	No hit found	2,0	316621		_
LACB00S00009673	No hit found	2,0	328002		_
LACB00S00001280	No hit found	2,0	321388		_
LACB00S00016386	No hit found	2,0	310985		_
LACB00S00008350	No hit found	2,0	297114		S
LACB00S00012917	No hit found	2,0	330556		_
LACB00S00012792	No hit found	2,0	304324		_
LACB00S00003733	No hit found	2,0	323285		M
LACB00S00017672	No hit found	2,0	314504		S

LACB00S00015059	No hit found	2,0	308354		_
LACB00S00001244	No hit found	2,0	301919		_
LACB00S00015246	No hit found	2,0	332409		_
LACB00S00012105	No hit found	2,0	329917		_
LACB00S00013249	No hit found	2,0	330810		S
LACB00S00002511	hypothetical protein FG08686.1 [Gibberella zeae PH-1]	2,0	306191	2,10E-22	_
LACB00S00014857	No hit found	2,0	332113		S
LACB00S00008372	hypothetical protein UM05359.1 [Ustilago maydis 521]	2,0	297142	3,00E-11	S
LACB00S00002909	No hit found	2,0	322637		_
LACB00S00012677	No hit found	2,0	295017		_
LACB00S00003477	No hit found	2,0	323090		_
LACB00S00008097	No hit found	2,0	296721		_
LACB00S00019429	tryptophan 2,3-dioxygenase, [Cryptococcus neoformans var.neoformans JEC21]	2,0	298376	2,00E-14	_
LACB00S00010472	No hit found	2,0	294625		_
LACB00S00012617	No hit found	2,0	330330		M
LACB00S00017759	No hit found	2,0	314625		_
LACB00S00001604	predicted protein [Neurospora crassa]	2,0	305198	2,00E-06	_
LACB00S00006817	No hit found	2,0	318096		_
LACB00S00014675	No hit found	2,0	331976		M
LACB00S00016254	No hit found	2,0	333182		M
LACB00S00005487	No hit found	2,0	293276		_
LACB00S00003742	elongation factor-2 kinase, [Entamoeba histolyticaHM-1:IMSS]	2,0	311331	4,10E-20	_
LACB00S00019366	hypothetical protein CNBJ2110 [Cryptococcus neoformans var.neoformans B-3501A]	2,0	256686	4,10E-79	M
LACB00S00018362	No hit found	2,0	316203		S
LACB00S00009536	No hit found	2,0	327902		_
LACB00S00011412	No hit found	2,0	294770		_
LACB00S00008872	No hit found	2,0	294403		_
LACB00S00020046	No hit found	2,0	299169		_
LACB00S00014421	No hit found	2,0	331770		S
LACB00S00015249	No hit found	2,0	309606		_
LACB00S00016051	No hit found	2,0	333033		_
LACB00S00005569	solute carrier family 41 member 1, [Cryptococcusneoformans var. neoformans JEC21]	2,1	315635	4,00E-12	_
LACB00S00007810	conserved hypothetical protein [Cryptococcus neoformans var.neoformans JEC21]	4,9	296410	1,00E-117	_
LACB00S00006540	NACHT domain protein, [Aspergillus fumigatus Af293]	4,7	167149	1,00E-79	_

Down regulated genes

SEQ_ID	NCBI Defintiion	Ratio T/C	protein ID	NCBI E value	Localization
Hypothetical proteins					
LACB00S00008779	No hit found	-37,1	153756		_
LACB00S00016087	No hit found	-25,5	310619		_
LACB00S00012271	No hit found	-24,1	330053		_
LACB00S00016790	cytoplasm protein, [Cryptococcus neoformans var.neoformans JEC21]	-20,4	254960	1,00E-121	_
LACB00S00014788	No hit found	-18,8	295368		_
LACB00S00015402	No hit found	-16,2	309770		S
LACB00S00006020	No hit found	-14,8	325085		M
LACB00S00010488	No hit found	-14,4	299854		_
LACB00S00006750	No hit found	-13,9	325664		M
LACB00S00007013	No hit found	-12,4	318320		S
LACB00S00013427	WSC domain protein, [Aspergillus fumigatus Af293]	-12,4	306393	5,10E-58	S
LACB00S00011621	No hit found	-12,2	329556		_
LACB00S00016088	No hit found	-11,4	310620		M
LACB00S00018325	hypothetical protein CNF02680 [Cryptococcus neoformans var.neoformans JEC21]	-10,6	334792	3,10E-20	M

LACB00S00019030	SPAC6B12.16 [Schizosaccharomyces pombe]	-10,6	317614	1,10E-21	_
LACB00S00016255	hypothetical protein CNL06480 [Cryptococcus neoformans var.neoformans JEC21]	-9,6	310824	2,10E-22	_
LACB00S00017572	No hit found	-8,6	334206		_
LACB00S00017981	No hit found	-8,5	314917		_
LACB00S00014436	hypothetical protein CNBF1690 [Cryptococcus neoformans var.neoformans B-3501A]	-8,0	295326	2,10E-25	_
LACB00S00006620	No hit found	-7,6	325558		_
LACB00S00009688	No hit found	-6,8	298856		_
LACB00S00010557	hypothetical protein UM00962.1 [Ustilago maydis 521]	-6,7	185287	1,00E-107	_
LACB00S00013392	salicylate hydroxylase [Aspergillus fumigatus Af293]	-6,7	306354	6,10E-27	S
LACB00S00010111	No hit found	-6,5	299384		_
LACB00S00005016	No hit found	-6,3	324286		S
LACB00S00002156	No hit found	-6,3	305821		M
LACB00S00016305	No hit found	-6,3	333223		S
LACB00S00011381	No hit found	-6,1	302510		_
LACB00S00009099	No hit found	-6,1	327529		M
LACB00S00011875	hAT family dimerisation domain, [Oryza sativa (japonicacultivar-group)]	-5,9	303168	1,00E-05	_
LACB00S00015923	2OG-Fe(II) oxygenase superfamily [Frankia sp. EAN1pec]	-5,9	310432	1,00E-05	_
LACB00S00005346	No hit found	-5,6	315365		_
LACB00S00001266	No hit found	-5,5	321374		_
LACB00S00000757	No hit found	-5,4	320976		_
LACB00S00014534	No hit found	-5,4	307711		M
LACB00S00005459	Moloney leukemia virus 10 [Mus musculus]	-5,3	315499	3,00E-16	_
LACB00S00010380	No hit found	-5,2	328569		_
LACB00S00000857	hypothetical protein AN8085.2 [Aspergillus nidulans FGSC A4]	-5,1	301472	4,10E-29	_
LACB00S00008052	motor, [Cryptococcus neoformans var. neoformans JEC21]	-5,1	296665	1,00E-14	_
LACB00S00011870	Exonuclease II, [Cryptococcus neoformans var. neoformansJEC21]	-5,0	303164	3,00E-07	_
LACB00S00007278	No hit found	-5,0	326066		S
LACB00S00018806	No hit found	-5,0	316748		M
LACB00S00013276	ferric reductase [Candida albicans SC5314]	-4,9	304908	7,00E-06	_
LACB00S00001988	No hit found	-4,9	321931		_
LACB00S00008307	No hit found	-4,9	326859		M
LACB00S00009500	No hit found	-4,8	298543		_
LACB00S00017854	alcohol dehydrogenase, zinc-containing, [Aspergillusfumigatus Af293]	-4,7	314747	8,00E-11	S
LACB00S00011871	Exonuclease II, [Cryptococcus neoformans var. neoformansJEC21]	-4,6	303165	2,10E-55	_
LACB00S00014991	No hit found	-4,5	332213		_
LACB00S00006296	Origin recognition complex, subunit 3-like protein [Danio rerio]	-4,5	317138	3,10E-21	_
LACB00S00016010	AE016780 membrane protein, putative, [Cryptococcusneoformans var. neoformans JEC21]	-4,4	186194	3,10E-66	_
LACB00S00001633	hypothetical protein UM02256.1 [Ustilago maydis 521]	-4,4	305228	1,10E-35	_
LACB00S00008320	No hit found	-4,4	326870		_
LACB00S00013492	No hit found	-4,4	331023		_
LACB00S00002868	SNF1-related kinase complex anchoring protein SIP1, putative[Cryptococcus neoformans var. neoformans JEC21]	-4,3	308668	1,00E-10	_
LACB00S00017511	No hit found	-4,2	314324		_
LACB00S00018136	LOC397739 protein [Xenopus laevis]	-4,2	315111	4,00E-05	_
LACB00S00009061	No hit found	-4,2	327501		_
LACB00S00011046	hypothetical protein AN3543.2 [Aspergillus nidulans FGSC A4]	-4,2	148596	2,00E-19	_
LACB00S00017328	No hit found	-4,2	334021		_
LACB00S00014533	No hit found	-4,1	331859		M

LACB00S00019770	No hit found	-4,1	318447		_
LACB00S00009275	No hit found	-4,1	327672		_
LACB00S00011965	signal sequence binding protein, [Cryptococcus neoformansvar. neoformans JEC21]	-4,1	303295	3,00E-07	_
LACB00S00001369	No hit found	-4,0	302048		M
LACB00S00013896	RING finger protein, [Aspergillus fumigatus Af293]	-4,0	307015	3,10E-47	_
LACB00S00003212	Medusa [Aspergillus fumigatus Af293]	-4,0	168394	2,10E-37	_
LACB00S00014435	No hit found	-4,0	331782		_
LACB00S00018404	SPBC56F2.04 [Schizosaccharomyces pombe]	-4,0	256076	0.0	_
LACB00S00009799	No hit found	-3,9	328095		M
LACB00S00017512	No hit found	-3,9	314325		M
LACB00S00014453	No hit found	-3,9	331791		M
LACB00S00020256	No hit found	-3,9	336075		_
LACB00S00016398	Chain A, Crystal Structure Of Aspartic Proteinase From IrpexLacteus	-3,8	310997	9,10E-97	S
LACB00S00009667	PREDICTED: similar to dehydrogenase E1 and transketolase domaincontaining protein 1 [Canis familiaris]	-3,8	298735	9,10E-79	_
LACB00S00014561	hypothetical protein FG07413.1 [Gibberella zeae PH-1]	-3,8	307739	3,10E-20	M
LACB00S00016422	No hit found	-3,8	333321		_
LACB00S00001769	No hit found	-3,8	305379		_
LACB00S00016487	No hit found	-3,7	312198		S
LACB00S00009754	No hit found	-3,7	298925		_
LACB00S00017318	No hit found	-3,7	313215		M
LACB00S00013595	hypothetical protein CaO19_5894 [Candida albicans SC5314]	-3,7	306617	2,00E-14	_
LACB00S00004842	No hit found	-3,6	313798		_
LACB00S00015369	No hit found	-3,6	309736		M
LACB00S00018451	hypothetical protein UM04092.1 [Ustilago maydis 521]	-3,6	295948	6,10E-42	_
LACB00S00016935	AMP-binding domain containing protein, [Aspergillusfumigatus Af293]	-3,6	312776	5,10E-86	_
LACB00S00009694	signal sequence binding protein, [Cryptococcus neoformansvar. neoformans JEC21]	-3,6	298863	3,00E-07	_
LACB00S00019942	No hit found	-3,5	297411		_
LACB00S00002256	No hit found	-3,5	305929		M
LACB00S00016209	PREDICTED: similar to ATP-binding cassette, sub-family B (MDR/TAP),member 11 [Canis familiaris]	-3,5	310758	2,00E-06	S
LACB00S00004927	related to cytochrome P450 [Neurospora crassa]	-3,5	293156	2,00E-06	_
LACB00S00005085	No hit found	-3,5	314068		S
LACB00S00019553	hypothetical protein MG10221.4 [Magnaporthe grisea 70-15]	-3,5	303415	4,00E-09	_
LACB00S00011008	hypothetical protein UM03156.1 [Ustilago maydis 521]	-3,4	300507	1,00E-11	_
LACB00S00017024	hypothetical protein CNC02420 [Cryptococcus neoformans var.neoformans JEC21]	-3,4	312882	1,10E-62	S
LACB00S00000949	No hit found	-3,4	301586		_
LACB00S00013913	No hit found	-3,4	331348		S
LACB00S00016976	RNA lariat debranching enzyme, [Cryptococcus neoformansvar. neoformans JEC21]	-3,4	312833	9,00E-10	_
LACB00S00013330	No hit found	-3,4	295100		_
LACB00S00012476	No hit found	-3,3	303915		S
LACB00S00013558	hypothetical protein UM00854.1 [Ustilago maydis 521]	-3,3	148696	6,10E-44	_
LACB00S00009659	Dehydrogenase E1 and transketolase domain containing protein 1[Homo sapiens]	-3,3	298724	7,10E-36	M
LACB00S00004124	PREDICTED: similar to Transcription factor SOX-6 isoform 2 [Canisfamiliaris]	-3,3	311738	2,00E-10	_
LACB00S00018713	No hit found	-3,3	316639		_
LACB00S00019300	hypothetical protein FG03324.1 [Gibberella zeae PH-1]	-3,3	296138	5,00E-13	_
LACB00S00000322	No hit found	-3,3	300890		_
LACB00S00014758	No hit found	-3,3	307987		_

LACB00S00017752	No hit found	-3,3	334337		M
LACB00S00012085	hypothetical protein UM01066.1 [Ustilago maydis 521]	-3,2	303489	1,10E-22	_
LACB00S00000950	No hit found	-3,2	301588		_
LACB00S00008630	Taz1-interacting factor 1 (TAF1), [Aspergillus fumigatusAf293]	-3,2	327145	3,10E-34	_
LACB00S00011989	hypothetical protein CNBJ1220 [Cryptococcus neoformans var.neoformans B-3501A]	-3,2	164081	2,00E-13	_
LACB00S00015144	xylulokinase, [Cryptococcus neoformans var. neoformansJEC21]	-3,1	309466	2,10E-42	S
LACB00S00011664	No hit found	-3,1	302878		_
LACB00S00012481	No hit found	-3,1	303921		_
LACB00S00002743	hypothetical protein AN1725.2 [Aspergillus nidulans FGSC A4]	-3,1	308533	4,10E-31	_
LACB00S00003775	No hit found	-3,1	311363		_
LACB00S00013226	No hit found	-3,1	330791		_
LACB00S00001295	No hit found	-3,1	301969		M
LACB00S00009714	vacuolar ATP synthase subunit e, [Cryptococcusneoformans var. neoformans JEC21]	-3,1	298889	2,00E-06	_
LACB00S00003594	No hit found	-3,1	323181		_
LACB00S00010123	No hit found	-3,0	299401		S
LACB00S00015940	No hit found	-3,0	310448		_
LACB00S00012545	hypothetical protein [Neurospora crassa]	-3,0	304017	4,00E-07	S
LACB00S00011207	No hit found	-3,0	329224		_
LACB00S00016164	No hit found	-3,0	310705		_
LACB00S00001366	No hit found	-3,0	302044		_
LACB00S00000432	vacuolar acidification-related protein, [Cryptococcusneoformans var. neoformans JEC21]	-3,0	301010	0.0	_
LACB00S00010362	No hit found	-3,0	294600		_
LACB00S00001017	No hit found	-3,0	321188		M
LACB00S00016372	No hit found	-3,0	333279		_
LACB00S00010842	No hit found	-3,0	300317		M
LACB00S00013384	No hit found	-2,9	330921		_
LACB00S00016216	No hit found	-2,9	333150		S
LACB00S00011575	No hit found	-2,9	302786		_
LACB00S00019493	cohesin complex subunit psm1, [Cryptococcus neoformansvar. neoformans JEC21]	-2,9	335903	2,00E-19	_
LACB00S00004309	No hit found	-2,9	311934		_
LACB00S00016735	No hit found	-2,9	312507		M
LACB00S00007497	No hit found	-2,9	319064		_
LACB00S00009915	No hit found	-2,9	328203		_
LACB00S00006569	protein binding / ubiquitin-protein ligase/ zinc ion binding[Arabidopsis thaliana]	-2,9	317831	7,00E-05	_
LACB00S00015708	No hit found	-2,9	332760		M
LACB00S00015100	cohesin complex subunit psm1, [Cryptococcus neoformansvar. neoformans JEC21]	-2,9	332296	3,10E-19	_
LACB00S00013760	No hit found	-2,9	238351		_
LACB00S00014798	No hit found	-2,9	332066		_
LACB00S00015636	proteophosphoglycan 5 [Leishmania major]	-2,9	239538	9,10E-22	_
LACB00S00017561	circumsporozoite protein [Plasmodium chabaudi]	-2,9	295790	6,00E-11	_
LACB00S00009872	hypothetical protein UM03281.1 [Ustilago maydis 521]	-2,8	328164	2,00E-07	_
LACB00S00013762	No hit found	-2,8	306844		_
LACB00S00017850	No hit found	-2,8	314742		_
LACB00S00005970	hypothetical protein UM02123.1 [Ustilago maydis 521]	-2,8	316084	1,00E-174	_
LACB00S00015051	No hit found	-2,8	332261		_
LACB00S00012076	No hit found	-2,8	329890		_
LACB00S00004888	hypothetical protein UM03907.1 [Ustilago maydis 521]	-2,8	313854	4,00E-08	M
LACB00S00005976	DNA topoisomerase IV subunit A, [Aspergillus fumigatusAf293]	-2,8	325049	1,00E-04	_

LACB00S00002926	PREDICTED: similar to Nucleolar phosphoprotein p130 (Nucleolar 130kDa protein) (140 kDa nucleolar phosphoprotein) (Nopp140)(Nucleolar and coiled-body phosphoprotein 1)[Strongylocentrotus purpuratus]	-2,8	322652	1,00E-09	_
LACB00S00008705	No hit found	-2,7	297573		_
LACB00S00005499	No hit found	-2,7	315548		_
LACB00S00009933	No hit found	-2,7	294575		_
LACB00S00015451	inositolphosphorylceramide synthase, [Cryptococcusneoformans var. neoformans JEC21]	-2,7	332565	4,10E-26	_
LACB00S00016171	No hit found	-2,7	333118		S
LACB00S00008135	Hypothetical protein ZK1055.6b [Caenorhabditis elegans]	-2,7	296878	3,00E-14	_
LACB00S00009836	No hit found	-2,7	328130		M
LACB00S00013222	No hit found	-2,7	304852		_
LACB00S00004506	CG3047-PA [Drosophila melanogaster]	-2,7	313429	5,00E-10	_
LACB00S00005675	all2423 [Nostoc sp. PCC 7120]	-2,7	315761	1,00E-09	S
LACB00S00012559	No hit found	-2,7	304035		_
LACB00S00018723	No hit found	-2,7	316648		_
LACB00S00008802	No hit found	-2,7	327287		M
LACB00S00011083	hypothetical protein AN3543.2 [Aspergillus nidulans FGSC A4]	-2,7	302131	6,10E-36	_
LACB00S00016296	No hit found	-2,7	310868		_
LACB00S00001254	No hit found	-2,7	291923		_
LACB00S00019943	No hit found	-2,6	297412		_
LACB00S00015648	No hit found	-2,6	332712		_
LACB00S00018707	hypothetical protein AN3376.2 [Aspergillus nidulans FGSC A4]	-2,6	316630	1,00E-05	_
LACB00S00018300	No hit found	-2,6	315295		_
LACB00S00016652	No hit found	-2,6	333496		_
LACB00S00016280	hypothetical protein CNH02380 [Cryptococcus neoformans var.neoformans JEC21]	-2,6	310848	1,10E-19	M
LACB00S00003451	cohesin complex subunit psm1, [Cryptococcus neoformansvar. neoformans JEC21]	-2,6	309293	1,10E-41	_
LACB00S00017103	No hit found	-2,6	333837		M
LACB00S00004647	proteophosphoglycan 5 [Leishmania major]	-2,6	293041	4,00E-06	_
LACB00S00009631	No hit found	-2,6	327976		_
LACB00S00018727	No hit found	-2,6	316657		S
LACB00S00017963	No hit found	-2,6	334515		_
LACB00S00010750	hypothetical protein CNBC4810 [Cryptococcus neoformans var.neoformans B-3501A]	-2,5	300187	6,10E-22	_
LACB00S00018614	No hit found	-2,5	316518		_
LACB00S00016479	expressed protein [Cryptococcus neoformans var. neoformans JEC21]	-2,5	333369	4,00E-05	_
LACB00S00010316	motor, [Cryptococcus neoformans var. neoformans JEC21]	-2,5	299671	2,00E-19	_
LACB00S00016135	phosphatidylserine decarboxylase, [Cryptococcus neoformansvar. neoformans JEC21]	-2,5	310670	1,10E-34	_
LACB00S00017124	hypothetical protein CNBA6410 [Cryptococcus neoformans var.neoformans B-3501A]	-2,5	312988	2,00E-09	_
LACB00S00011626	No hit found	-2,5	302838		M
LACB00S00008884	No hit found	-2,5	297766		S
LACB00S00018427	No hit found	-2,5	316274		S
LACB00S00007117	No hit found	-2,5	325938		_
LACB00S00016440	No hit found	-2,5	311044		_
LACB00S00006626	No hit found	-2,5	325561		S
LACB00S00003449	motor, [Cryptococcus neoformans var. neoformans JEC21]	-2,5	309291	1,00E-11	_
LACB00S00005048	response to drug-related protein, [Cryptococcus neoformansvar. neoformans JEC21]	-2,5	314025	1,10E-77	M
LACB00S00009490	No hit found	-2,5	327868		_
LACB00S00007098	hypothetical protein CNBH2360 [Cryptococcus neoformans var.neoformans B-3501A]	-2,5	318651	1,10E-20	_
LACB00S00014486	No hit found	-2,5	331817		M

LACB00S00011084	hypothetical protein AN3543.2 [Aspergillus nidulans FGSC A4]	-2,5	163955	2,10E-20	_
LACB00S00016199	No hit found	-2,5	310749		_
LACB00S00008127	No hit found	-2,5	326720		_
LACB00S00019307	No hit found	-2,5	296147		_
LACB00S00001251	No hit found	-2,5	321360		M
LACB00S00014703	No hit found	-2,4	307895		_
LACB00S00016203	No hit found	-2,4	310753		M
LACB00S00004565	No hit found	-2,4	313502		_
LACB00S00015447	hypothetical protein AN3543.2 [Aspergillus nidulans FGSC A4]	-2,4	147296	1,10E-29	_
LACB00S00001696	hypothetical protein UM03335.1 [Ustilago maydis 521]	-2,4	292026	7,10E-25	_
LACB00S00018452	No hit found	-2,4	334887		_
LACB00S00011985	No hit found	-2,4	303330		_
LACB00S00000876	conserved hypothetical protein [Cryptococcus neoformans var.neoformans JEC21]	-2,4	227808	1,00E-119	_
LACB00S00013372	hus1 [Schizosaccharomyces pombe]	-2,4	306335	3,00E-10	_
LACB00S00001194	No hit found	-2,4	301868		_
LACB00S00014369	hypothetical protein UM05660.1 [Ustilago maydis 521]	-2,4	331725	5,00E-13	M
LACB00S00014662	hypothetical protein UM00962.1 [Ustilago maydis 521]	-2,4	253660	1,00E-112	_
LACB00S00014655	hypothetical protein UM00962.1 [Ustilago maydis 521]	-2,4	191633	1,00E-113	_
LACB00S00009662	No hit found	-2,4	298727		_
LACB00S00016219	No hit found	-2,4	295563		_
LACB00S00011973	No hit found	-2,4	303306		S
LACB00S00012902	conserved hypothetical protein [Aspergillus fumigatus Af293]	-2,4	304456	3,10E-32	M
LACB00S00003988	hypothetical protein UM01378.1 [Ustilago maydis 521]	-2,4	311599	1,10E-38	_
LACB00S00010618	No hit found	-2,4	300038		_
LACB00S00019852	No hit found	-2,4	335407		M
LACB00S00002414	No hit found	-2,4	306087		_
LACB00S00014159	hypothetical protein CNL05030 [Cryptococcus neoformans var.neoformans JEC21]	-2,4	143989	1,00E-109	_
LACB00S00019357	No hit found	-2,4	296057		S
LACB00S00005222	hypothetical protein CNBD1090 [Cryptococcus neoformans var.neoformans B-3501A]	-2,4	232388	7,10E-29	_
LACB00S00017971	No hit found	-2,4	314894		M
LACB00S00016816	No hit found	-2,4	333619		_
LACB00S00008266	No hit found	-2,4	297027		M
LACB00S00002869	No hit found	-2,4	322606		S
LACB00S00013025	NAD-specific glutamate dehydrogenase [Agaricus bisporus]	-2,4	304596	6,10E-40	M
LACB00S00011692	oxidoreductase, short chain dehydrogenase/reductase family[Aspergillus fumigatus Af293]	-2,4	302910	3,00E-07	_
LACB00S00011249	No hit found	-2,3	302318		M
LACB00S00019415	No hit found	-2,3	298362		_
LACB00S00009695	No hit found	-2,3	298864		_
LACB00S00008342	hypothetical protein CNBG4520 [Cryptococcus neoformans var.neoformans B-3501A]	-2,3	249927	1,10E-94	_
LACB00S00018374	No hit found	-2,3	334827		_
LACB00S00011350	hypothetical protein FG03449.1 [Gibberella zeae PH-1]	-2,3	329339	6,00E-17	S
LACB00S00013509	No hit found	-2,3	306515		_
LACB00S00011557	No hit found	-2,3	302766		_
LACB00S00004405	No hit found	-2,3	312034		_
LACB00S00002325	hypothetical protein UM02736.1 [Ustilago maydis 521]	-2,3	305997	2,10E-91	_
LACB00S00000722	No hit found	-2,3	301329		S
LACB00S00019281	No hit found	-2,3	296109		M

LACB00S00004874	COG0644: Dehydrogenases (flavoproteins) [Burkholderia pseudomallei668]	-2,3	247116	7,10E-33	_
LACB00S00012042	predicted protein [Gibberella zeae PH-1]	-2,3	303440	4,00E-06	_
LACB00S00009122	No hit found	-2,3	327546		_
LACB00S00010337	No hit found	-2,3	299693		_
LACB00S00017329	phospholipid binding protein, [Cryptococcus neoformansvar. neoformans JEC21]	-2,3	334022	3,00E-12	_
LACB00S00015820	hypothetical protein FG03324.1 [Gibberella zeae PH-1]	-2,3	332852	2,00E-13	_
LACB00S00005627	No hit found	-2,3	315704		_
LACB00S00016713	predicted protein [Neurospora crassa]	-2,3	312475	2,10E-21	S
LACB00S00019005	No hit found	-2,3	317587		_
LACB00S00010132	No hit found	-2,3	328370		_
LACB00S00016095	hypothetical protein AN3074.2 [Aspergillus nidulans FGSC A4]	-2,3	310627	4,00E-08	M
LACB00S00013434	hypothetical protein [Yarrowia lipolytica]	-2,3	306399	5,10E-22	S
LACB00S00017998	No hit found	-2,3	314933		_
LACB00S00009829	No hit found	-2,2	328123		M
LACB00S00000456	hypothetical protein CNBL1680 [Cryptococcus neoformans var.neoformans B-3501A]	-2,2	301034	0.0	M
LACB00S00003501	No hit found	-2,2	323109		M
LACB00S00019100	No hit found	-2,2	296035		_
LACB00S00017833	unnamed protein product [Debaryomyces hansenii CBS767]	-2,2	168989	6,00E-18	_
LACB00S00009198	No hit found	-2,2	294472		_
LACB00S00000973	No hit found	-2,2	301618		_
LACB00S00010617	conserved hypothetical protein [Neurospora crassa]	-2,2	300037	3,10E-27	_
LACB00S00013903	No hit found	-2,2	331341		M
LACB00S00000859	hypothetical protein UM02227.1 [Ustilago maydis 521]	-2,2	301475	3,10E-22	S
LACB00S00005005	hypothetical protein AN3325.2 [Aspergillus nidulans FGSC A4]	-2,2	232120	4,10E-97	_
LACB00S00019663	No hit found	-2,2	309346		S
LACB00S00015164	bromodomain protein, [Entamoeba histolytica HM-1:IMSS]	-2,2	149883	6,00E-13	_
LACB00S00006970	hypothetical protein UM00287.1 [Ustilago maydis 521]	-2,2	318272	6,10E-35	_
LACB00S00012152	No hit found	-2,2	294881		M
LACB00S00006029	No hit found	-2,2	325092		_
LACB00S00011105	SPCC576.05 [Schizosaccharomyces pombe]	-2,2	329137	9,10E-33	_
LACB00S00006098	No hit found	-2,2	325142		_
LACB00S00005058	No hit found	-2,2	324319		M
LACB00S00006834	hypothetical protein UM02976.1 [Ustilago maydis 521]	-2,2	318113	9,10E-64	M
LACB00S00013395	hypothetical protein CNBD5190 [Cryptococcus neoformans var.neoformans B-3501A]	-2,2	146215	1,10E-72	_
LACB00S00011069	hypothetical protein AN3267.2 [Aspergillus nidulans FGSC A4]	-2,2	251522	1,10E-33	_
LACB00S00016769	hypothetical protein FG05871.1 [Gibberella zeae PH-1]	-2,2	312561	9,10E-44	_
LACB00S00012835	Theileria-specific sub-telomeric protein, SVSP family, putative[Theileria annulata]	-2,2	304371	4,00E-05	_
LACB00S00000796	No hit found	-2,2	321007		M
LACB00S00011846	No hit found	-2,2	303130		_
LACB00S00015151	No hit found	-2,2	332334		_
LACB00S00015835	No hit found	-2,2	332865		_
LACB00S00003897	hypothetical protein CNBI2790 [Cryptococcus neoformans var.neoformans B-3501A]	-2,2	246146	7,10E-85	_
LACB00S00007183	conserved hypothetical protein [Aspergillus fumigatus Af293]	-2,2	318730	1,00E-07	_
LACB00S00015403	No hit found	-2,2	309771		_
LACB00S00010371	No hit found	-2,2	328563		_
LACB00S00002030	hypothetical protein UM04545.1 [Ustilago maydis 521]	-2,2	305678	1,10E-83	_

LACB00S00001371	splicing factor 3a, subunit 1 [Danio rerio]	-2,1	302050	8,00E-16	_
LACB00S00009936	No hit found	-2,1	299125		_
LACB00S00013305	No hit found	-2,1	330853		_
LACB00S00004625	SH3 and Ded_cyto domain protein, [Aspergillus fumigatusAf293]	-2,1	140780	0.0	_
LACB00S00009022	No hit found	-2,1	327471		_
LACB00S00004579	No hit found	-2,1	323963		_
LACB00S00008309	No hit found	-2,1	326861		S
LACB00S00011485	No hit found	-2,1	329451		_
LACB00S00008133	chloroplast Toc64-1 [Physcomitrella patens]	-2,1	296876	4,00E-08	_
LACB00S00000591	CRO1 protein [Aspergillus fumigatus Af293]	-2,1	301184	3,10E-36	_
LACB00S00010011	No hit found	-2,1	299272		M
LACB00S00011750	No hit found	-2,1	329660		_
LACB00S00018575	nucleus protein, [Cryptococcus neoformans var. neoformansJEC21]	-2,1	316453	8,10E-70	_
LACB00S00018477	No hit found	-2,1	316331		M
LACB00S00018268	No hit found	-2,1	315254		S
LACB00S00012024	No hit found	-2,1	303382		_
LACB00S00011694	No hit found	-2,1	302914		_
LACB00S00014366	nonsense-mediated mRNA decay protein (Nmd5), [Aspergillusfumigatus Af293]	-2,1	307524	5,00E-06	_
LACB00S00006064	No hit found	-2,1	316897		_
LACB00S00015409	No hit found	-2,1	309780		M
LACB00S00006221	No hit found	-2,1	317059		_
LACB00S00004763	No hit found	-2,1	324099		_
LACB00S00002951	No hit found	-2,1	308757		_
LACB00S00010160	hypothetical protein CNBJ2820 [Cryptococcus neoformans var.neoformans B-3501A]	-2,1	294583	3,00E-13	_
LACB00S00002645	hypothetical protein CNBG3530 [Cryptococcus neoformans var.neoformans B-3501A]	-2,1	308456	9,10E-52	_
LACB00S00001496	hypothetical protein UM05383.1 [Ustilago maydis 521]	-2,1	305084	1,00E-142	M
LACB00S00011071	hypothetical protein AN3543.2 [Aspergillus nidulans FGSC A4]	-2,1	302118	7,10E-62	_
LACB00S00016515	No hit found	-2,1	333398		M
LACB00S00019557	No hit found	-2,1	303639		S
LACB00S00009223	No hit found	-2,1	298144		_
LACB00S00019968	No hit found	-2,1	335747		_
LACB00S00012403	novel protein [Danio rerio]	-2,1	303842	3,10E-21	_
LACB00S00019209	No hit found	-2,1	318538		_
LACB00S00001551	No hit found	-2,1	305145		_
LACB00S00006570	PREDICTED: similar to Sacsin [Danio rerio]	-2,1	317832	1,10E-42	_
LACB00S00015098	motor, [Cryptococcus neoformans var. neoformans JEC21]	-2,1	309416	3,10E-71	_
LACB00S00007870	PREDICTED: similar to polycystic kidney disease 1-like 3 [Rattusnorvegicus]	-2,1	326525	3,00E-05	_
LACB00S00004154	hypothetical protein CNBG1440 [Cryptococcus neoformans var.neoformans B-3501A]	-2,1	292878	8,00E-07	S
LACB00S00007947	conserved hypothetical protein [Aspergillus fumigatus Af293]	-2,1	249411	1,00E-122	_
LACB00S00017559	No hit found	-2,0	314380		M
LACB00S00005396	PREDICTED: similar to eukaryotic translation initiation factor 2C,1 [Danio rerio]	-2,0	315430	2,00E-09	_
LACB00S00011818	No hit found	-2,0	329701		S
LACB00S00019191	B1015E06.2 [Oryza sativa (japonica cultivar-group)]	-2,0	241872	1,10E-42	_
LACB00S00004753	Unknown (protein for MGC:85638) [Danio rerio]	-2,0	313710	6,10E-25	_
LACB00S00015833	No hit found	-2,0	332863		_
LACB00S00003578	unnamed protein product [Aspergillus oryzae]	-2,0	323171	1,10E-46	S
LACB00S00019384	No hit found	-2,0	296820		_
LACB00S00016268	No hit found	-2,0	333193		S
LACB00S00014584	No hit found	-2,0	331905		M
LACB00S00015623	No hit found	-2,0	332700		_

LACB00S00018911	hypothetical protein AN8085.2 [Aspergillus nidulans FGSC A4]	-2,0	164386	7,10E-27	_
LACB00S00003782	hypothetical protein FG06312.1 [Gibberella zeae PH-1]	-2,0	311371	1,00E-128	_
LACB00S00000943	predicted protein [Neurospora crassa]	-2,0	301576	3,10E-35	_
LACB00S00014112	unnamed protein product [Aspergillus oryzae]	-2,0	331516	1,10E-27	_
LACB00S00020094	hypothetical protein CNJ01590 [Cryptococcus neoformans var.neoformans JEC21]	-2,0	299891	1,00E-14	S
LACB00S00004212	histone acetyltransferase, [Cryptococcus neoformans var.neoformans JEC21]	-2,0	246384	0.0	_
LACB00S00003960	hypothetical protein CNBB0220 [Cryptococcus neoformans var.neoformans B-3501A]	-2,0	311569	5,10E-54	M
LACB00S00013661	No hit found	-2,0	306701		M
LACB00S00018522	No hit found	-2,0	334943		S
LACB00S00006785	No hit found	-2,0	293727		M
LACB00S00001550	hypothetical protein UM05408.1 [Ustilago maydis 521]	-2,0	305144	5,00E-07	_
LACB00S00014208	No hit found	-2,0	307363		_
LACB00S00008423	No hit found	-2,0	297197		_
LACB00S00005656	PREDICTED: similar to Fraser syndrome 1 isoform 1, extracellularmatrix protein [Gallus gallus]	-2,0	293338	2,00E-16	_
LACB00S00019303	No hit found	-2,0	335633		_
LACB00S00003593	hypothetical protein AN3543.2 [Aspergillus nidulans FGSC A4]	-2,0	162339	2,10E-28	_
LACB00S00016099	No hit found	-2,0	333069		_
LACB00S00015694	No hit found	-2,0	332747		_
LACB00S00005741	No hit found	-2,0	315832		_
LACB00S00016124	No hit found	-2,0	310660		S
LACB00S00013967	No hit found	-2,0	331397		_
LACB00S00007631	signal transducer, [Cryptococcus neoformans var. neoformansJEC21]	-2,0	296216	1,00E-14	_
LACB00S00003625	No hit found	-2,0	311203		M
LACB00S00010100	No hit found	-2,0	328343		_
LACB00S00008116	No hit found	-2,0	326710		_
LACB00S00020043	No hit found	-2,0	335845		_
LACB00S00006740	No hit found	-2,0	318019		_
LACB00S00010183	unnamed protein product [Macaca fascicularis]	-2,0	299469	2,10E-37	_
LACB00S00007391	No hit found	-2,0	318946		_
LACB00S00000388	expressed protein [Cryptococcus neoformans var. neoformans JEC21]	-2,0	300963	1,10E-46	_
LACB00S00015832	hypothetical protein FG03324.1 [Gibberella zeae PH-1]	-2,0	332862	8,00E-06	_
LACB00S00011313	RNA polymerase III transcription factor, [Cryptococcusneoformans var. neoformans JEC21]	-2,0	329309	8,10E-28	_
LACB00S00017292	hypothetical protein FG03449.1 [Gibberella zeae PH-1]	-2,0	313185	5,10E-30	_
LACB00S00018967	No hit found	-2,0	317534		_
LACB00S00001629	hypothetical protein FG08252.1 [Gibberella zeae PH-1]	-2,0	305223	3,00E-18	_
LACB00S00007939	conserved hypothetical protein [Aspergillus fumigatus Af293]	-2,0	296546	3,10E-32	M
LACB00S00016830	No hit found	-2,0	333626		_
LACB00S00016761	No hit found	-2,0	333579		M
LACB00S00005204	No hit found	-2,0	314198		M
LACB00S00010806	No hit found	-2,0	300281		M
LACB00S00015127	ATPase, [Cryptococcus neoformans var. neoformans JEC21]	-2,0	239167	1,00E-108	_
LACB00S00014036	No hit found	-2,0	307166		M
LACB00S00009964	cleavage and polyadenylation specific protein, [Cryptococcusneoformans var. neoformans JEC21]	-2,0	190892	0.0	_
LACB00S00007367	No hit found	-2,0	326145		_

LACB00S00019202	hypothetical protein AN3376.2 [Aspergillus nidulans FGSC A4]	-2,0	318530	1,00E-05	_
LACB00S00011529	No hit found	-2,0	329486		_
LACB00S00000768	hypothetical protein UM02919.1 [Ustilago maydis 521]	-2,0	228793	8,10E-78	M
LACB00S00015030	No hit found	-2,0	308314		_
LACB00S00015813	No hit found	-2,0	332845		_
LACB00S00015064	No hit found	-2,0	332269		_
LACB00S00018654	similar to Uncharacterized protein conserved in bacteria [Frankiasp. EAN1pec]	-2,0	335047	9,10E-54	_
LACB00S00004152	mRNA-nucleus export-related protein, [Cryptococcusneoformans var. neoformans JEC21]	-2,0	292877	1,00E-09	_
LACB00S00018440	capsular associated protein [Cryptococcus neoformans var.neoformans JEC21]	-2,0	316290	3,00E-08	_
LACB00S00011503	No hit found	-2,0	302666		M
LACB00S00011502	No hit found	-2,0	329467		_
LACB00S00002889	No hit found	-2,0	308693		S
LACB00S00004522	inositolphosphorylceramide synthase, IPC1p [Filobasidiellaneoformans]	-2,0	313445	3,00E-07	S

Annexe 3.

In vitro « sandwich » assay of interactions between Poplar, L. bicolor S238N and P. fluorescens BBc6R8 on Petri dishes.

Adapted from the in vitro "sandwich" culture system on Petri dishes for poplar plants designed by J. Richter (INRA-Nancy)

Summary.

This assay has been developed to mimic the promoting effect of the MHB *P. fluorescens* BBc6R8 on the establishment of poplar-*L. bicolor* S238N ectomycorrhizal symbiosis. Briefly, poplar plants are grown on MS medium in Petri dishes during two months. Then they are transferred on P20Th$^-$ medium with or without the ectomycorrhizal fungus and the helper bacterial strain.

1. Required material

Material	Provider	Reference
Petri dishes 12x12 cm	CML BP1205J	MBPET05
Cellophane membrane	Hutchinson	
Cotton sticks (dental rolls, n°2)	Helvemed Switzerland	F01SU152
Sterile 50 ml Falcon tubes (for vitamin stock solution)		
0,22 um sterile filters and a syringe	Millipore	SLGP033RS
Sterile forceps and blades		
Parafilm		
Electrical isolation tape		
Black paper basket bags		

2. Required chemicals

Product	Provider	Advice	Reference
Microelements	Sigma Aldrich	Keep at 4°C	M 0529
Macroelements	Sigma Aldrich	Keep at 4°C	M 0654
L-Glutamin	Sigma Aldrich		G 3126
Myoinositol	Sigma Aldrich		M 5125
D+-Glucose	Sigma Aldrich		G 7520
Nicotinic acid	Sigma Aldrich		N 0765
Pyridoxidine	Sigma Aldrich		P 8666
Thiamine hydrochloride	Sigma Aldrich		T 4625
Calcium Panthothenate	Prolabo		22 395 137

L-Cysteine chlorhydrate	Sigma Aldrich		C4820
Agar Agar	Prolabo		VWR Prolabo 20768 292
Biotine	Sigma Aldrich	Keep at 4°C	B 3399
Di-NH4 tartrate	VWR Prolabo		21 355 268
KH_2PO_4	Fisher Scientific		P/4760/60
$MgSO_4$ * $7H_2O$	Labosi		A4836501
Kanieltra	-	Keep away from light and store at 4°C	-
KOH	Normapur		26 657 298
EDTA	Sigma Aldrich		

3. Composition of stock solutions

(only the first one is to prepare, the others are bought ready to use)

- **100x vitamin stock solution for MS plant medium**
  (make 50 ml sterile filtered aliquots and keep them at -20°C)

Product	Concentration in the vit-stock solution (mg/l)	Mass to take for 500 ml 100x vitamins stock solution (mg)
Nicotinic acid	100	50
Pyroxidine	100	50
Thiamine	100	50
Calcium Panthothenate	100	50
L-cysteine Chlorhytdrate	100	50
Biotin stock solution 0.1 mg/ ml	1	5 ml

- **Composition of Sigma Micro- and Macroelements 10x Stock-Solutions**

Component (mg/L)	Macroelements	Microelements
Ammonium nitrate	1650	-
Boric acid	-	6,2
Calcium chloride anhydrous	332.2	-
Cobalt chloride * $6H_2O$	-	0.025
Cupric sulphate * $5H_2O$	-	0.025
Na_2-EDTA	-	37.3
Ferrous sulphate * 7 H_2O	-	27.8
Magnesium sulphate	180.7	-
Manganese sulphate * H_2O	-	16.9
Molybdic acid (sodium salt) * $2H_2O$	-	0.25
Potassium iodide	-	0.83
Potassium nitrate	1900	-
Potassium phosphate monobasic	170	-
Zinc sulphate * 7 H_2O	-	8.6

❖ Composition of Kanieltra 1000x stock solution for fungal P20Th- medium

Product	Concentration in the stock solution (g/l)
Iron (in cheated form)	6
Molybdenum	0.27
B_2O_3	8.45
Manganese	5
Cupper	0.625
Zinc	2.27

4. Detailed description of the method

Preparation of poplar plants on MS

❖ Cellophane membranes (for plants and fungi)

Cut the cellophane membranes at the size of the Petri dish and cut them into two halves. Put the membranes for 20 minutes into boiling water containing 1g/l EDTA in order to permeabilize the membrane. Rinse the membranes 4x in a big recipient with ultra pure water and autoclave them once or twice.

❖ MS medium

MS medium is prepared in the following way:

For 1 litre

Macroelements	50 ml
Microelements	100 ml
Myoinositol	100 mg
L-Glutamine	200 mg
Glucose	1 g

Mix all these ingredients and adjust the pH with KOH 0.25N to 5.9-6.

Add 12 g agar-agar per litre and autoclave. Defreeze meanwhile the vitamin stock solution.

Let the medium cool down to 50-60°C after autoclaving and add 10 ml/L of Vitamin stock solution. Make around 18 petri dishes (12x12cm) with 1L medium.

❖ Planting poplar explants

Before making poplar explants you have to put a half cellophane membrane on each MS petri dish (see figure 1). Use forceps to do so and avoid air bubbles under the cellophane membrane.

Use then approx. 10 cm tall poplar plants in tubes. Take them out of the tube and put them (working under the laminar flow bench) on a sterilized (autoclaved) tile. Cut all leaves off and cut between the internodes to make explants of around 1 cm. Put these explants on the petri dish at the limit of the cellophane membrane. Take care to put them in such a way that roots will grow on the cellophane and not under the cellophane in the medium. Take a small cube of MS agar

medium and lay it on the bottom end of the plant in order to stick the plant to the cellophane membrane. In the end put a cotton stick (dental roll) at the bottom of the Petri dish, close it with parafilm and the bottom part additionally with the electrical isolation tape.

A small bag which covers the bottom half of the petri dish (where the roots will develop) is made from a cut, black paper bin bag and bond. Put the Petri dishes in vertical position and let plants develop in a growth chamber at 24°C during 6 weeks. When the stems have reached the top of the Petri dish, cut the stem with a sterile scalpel above the first internode. Let plants develop again in a growth chamber at 24°C during 2 weeks.

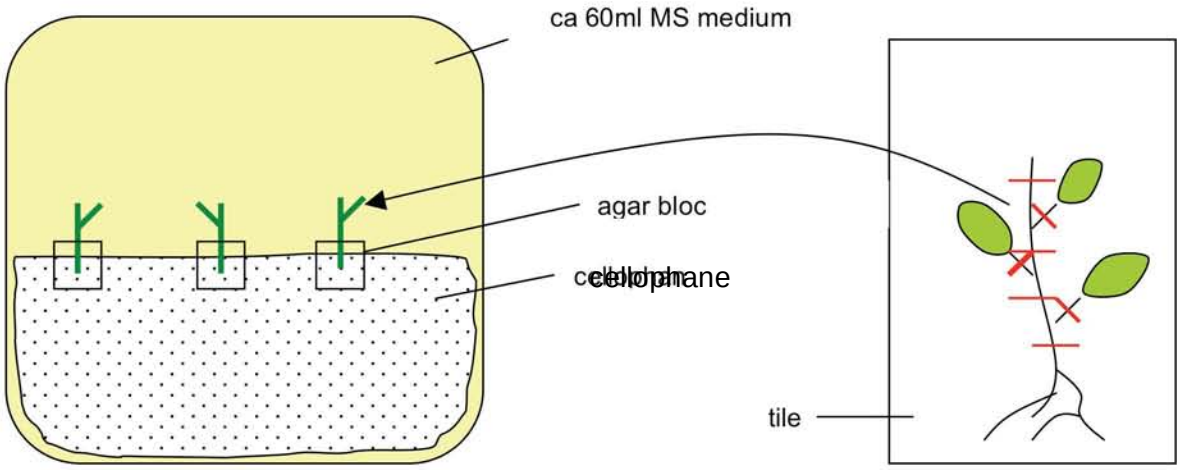


Figure 1: Arrangement of plants and cellophane membrane in petri dishes

Preparation of the interaction assay

❖ P20Th- medium

P20Th- medium is prepared in the following way:

For 1 Litre:

Di-NH_4-tartrate	0.5 g
KH_2PO_4	1 g
$MgSO_4$*$7H_2O$	0.5 g
Glucose D+	1 g
Kanieltra solution 1000x	1 ml

Add 20g/L agar-agar to the solution and autoclave.

Pour the medium into squared petri dishes of 12x12cm (about 18/L medium).

Control assay:

❖ Inoculation of *Laccaria bicolor* S238N

14 days before poplar plants are ready to be used, six plugs of 3 weeks old *Laccaria* growing on P5 medium are put on the P20Th- plates. Dishes are closed with electrical isolation tape and put into culture chamber in a horizontal position at 10°C. After 14 days of culture, *Populus tremula* x *alba* 717-1-B4 are transferred in the Petri dishes where the mycelium was growing. The roots are put between the two straits of mycelium (Figure 2, 3). Dishes are closed with electrical isolation tape and put into culture chamber in a 45° axis position at 10°C, with 16h per day of white light.

❖ Bacterial inoculum preparation

P. fluorescens BBc6R8 is first grown on 10 % TSA plates (3 $g.L^{-1}$ tryptic soy broth from Difco and 15 $g.L^{-1}$) at 25°C for 65 h. Then, three to four colonies were picked and suspended in 2 mL of sterile deionized water before spreading onto 10% TSA medium. After 48 h of growth at 25°C, the bacteria were harvested and centrifuged at 3300 g for 10 min. The pellet was washed once, then resuspended in deionized water in order to obtain a suspension with $A600_{nm}$ of 0.7.

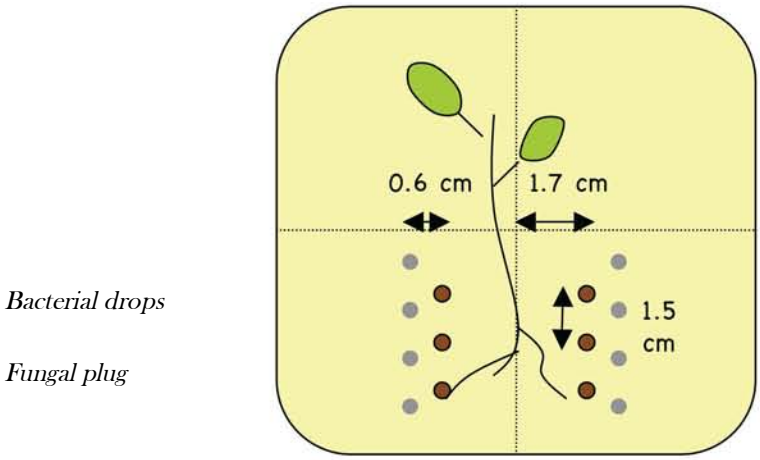


Figure 2. General view of the bioassay

Treatment 1: effect of the helper bacteria on the interaction between the ectomycorrhizal fungus and poplar roots.

14 days before poplar plants are ready to be used, six plugs of 3 weeks old *Laccaria* growing on P5 medium are put on the P20Th- plates. Dishes are closed with electrical isolation tape and put into culture chamber in a horizontal position at 10°C. After 14 days of culture, plants are transferred in the Petri dishes where the mycelium was growing. The roots are put between the the mycelium (Figure 2, 3). Dishes are closed with electrical isolation tape and put into culture chamber in a 45° axis position at 10°C, with 16h per day of white light. One day later, eight drops of bacterial solution prepared as described above are put. Dishes are closed with electrical isolation tape and put into culture chamber in a 45° axis position at 10°C, with 16h per day of white light.

Treatment 2: effect of the helper bacteria on the pre symbiotic fungal growth

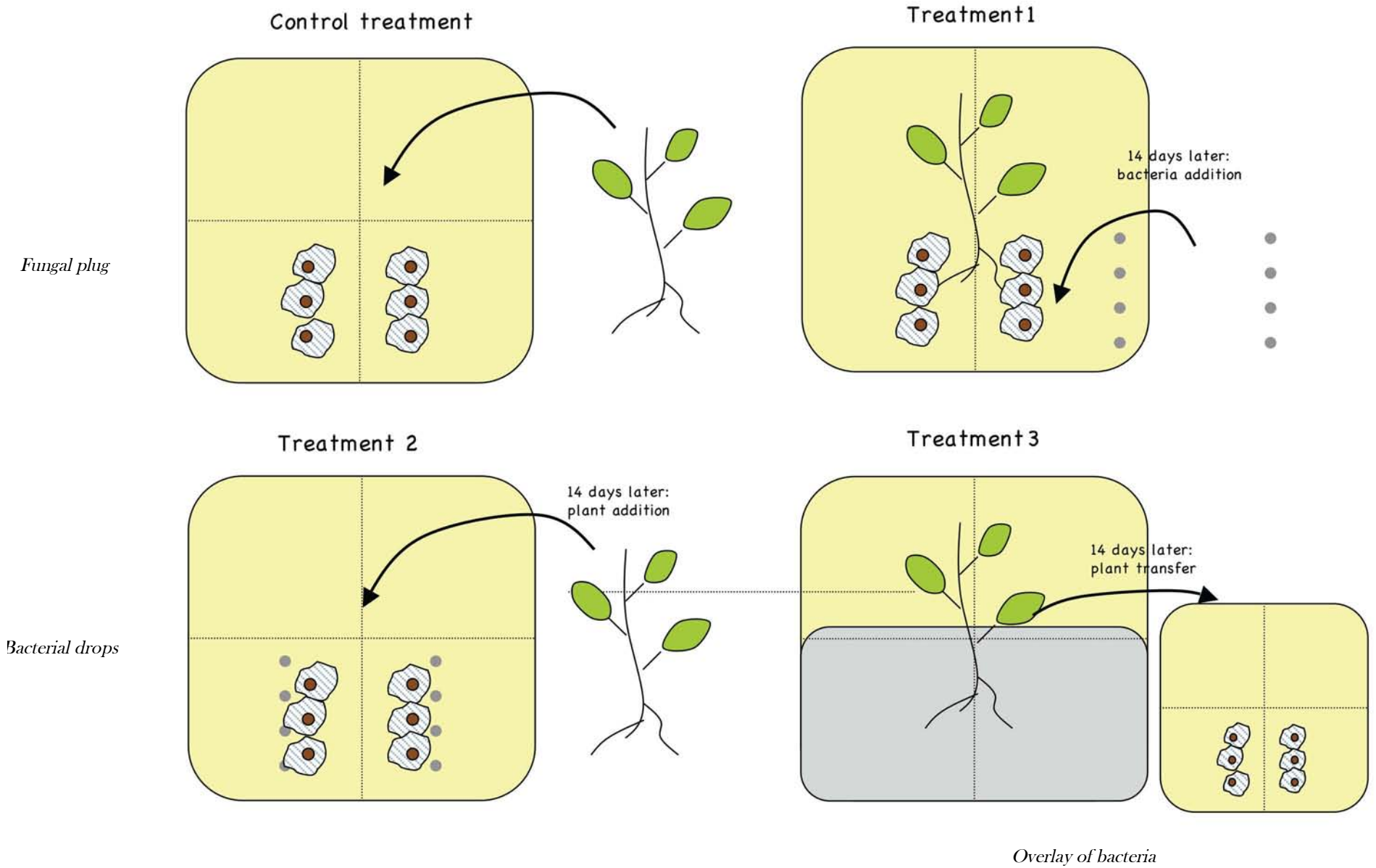
14 days before poplar plants are ready to be used, 8 bacerial drops of 10 µL bacteria and 6 fungal plugs cut out from the edge of a colony grown on P5 medium are put onto P20Th- medium as showed in figure 3. Dishes are closed with electrical isolation tape and put into culture chamber in a horizontal position at 10°C. After 14 days of culture, plants are transferred in the Petri dishes where the mycelium and the bacteria were growing. The roots are put between the two straits of mycelium (Figure 2, 3). . Dishes are closed with electrical isolation tape and put into culture chamber in a 45° axis position at 10°C, with 16h per day of white light.

Treatment 3: effect of the helper bacteria on roots

14 days before poplar plants are ready to be used, fungal plug are put onto P20Th- medium. In the same time a P20Th- medium in which are added 100µL of a 1/100 diluted suspention of $A600_{nm}$ ≈ 0.64 helper bacteria to prepare a bacterial overlay. Plant are added on this solid medium and grown for 14 days in a culture chamber in a 45° axis position at 10°C, with 16h per day of white light. After 14 days, plants are transferred onto fungal Petri dishes.

Annexe 3

Figure 3.



Annexe 4.

Supplemental web data, publication n°5 : A spy among soil bacteria : the ectomycorrhizal fungus Laccaria bicolor S238N perceives and reacts to quorum sensing signal molecules.

Table S1. N-AHSL-responsive transcripts in *L. bicolor* S238N (t-test, ratio ≥ or ≤ 2.0) after 14 hours of incubation with C6-HSL or 3,O-C12-HSL. cDNA clone ID, the NCBI database best match and the BlastX E. value are given. Values for the transcript regulation ratio [3,O-C12-HSL *L. bicolor* treatment/ *L. bicolor* S238N control] and [C6-HSL *L. bicolor* treatment/ *L. bicolor* S238N control] are the mean of three replicates.

V1.1 JGI protein identity	GeneBank Accession no	NCBI Database match	E-value	Expression ratio
3,O-C12 up-regulated genes				
Energy metabolism				
183558	ES768865	NADH-ubiquinone oxidoreductase 20 kDa subunit [Mus musculus]	2.E-78	18.6
387122	ES768893	Citrate synthase, mitochondrial precursor [*Aspergillus niger*]	0.0	3.5
184667	EL739046	fumarate reductase (NADH) [*Cryptococcus neoformans var. neoformans JEC21*]	0.0	3.5
395215	EL740118	Cytochrome c oxydase [*Cryptococcus neoformans var. neoformans JEC21*]	3. E-29	3.2
185358	JGI_LbEX1662	exo-beta-1,3-glucanase [*Lentinula edodes*]	1.E-52	2.1
mito3634485.a1		ATP synthase F0 subunit 9 [*Schizophyllum commune*]	e-22	2.1
Protein turnover				
314443	JGI_LbEX2159	endopeptidase [*Cryptococcus neoformans var. neoformans* JEC21]	3.E-29	11.0
292906	EL740042	aspartic endopeptidase [*Candida albicans*]	0.0	9.5
187325	EL739208	peroxisomal biogenesis factor (PEX11) [*Aspergillus fumigatus* Af293]	5.E-98	5.4
192623	EL739487	Ubiquitin [*Phanerocheate chrysosporium*]	0.0	3.6
Detoxification processes				
183753	EL739400	hydroxyacylglutathione hydrolase [*Cryptococcus neoformans var. neoformans* B-3501A]	6.E-97	4.4
184665	EL739210	glutathione S-transferase [*Paxillus involutus*]	7.E-81	3.9
296671	JGI_LbEX1156	glutathione S-transferase [*Paxillus involutus*]	5.E-20	2.6
Chromatine structure & transcription regulation				
294940	JGI_LbEX216	Histone H1, gonadal [*Parechinus angulosus*]	1.E-17	3.7

396518	EL739219	probable transcription initiation factor IIA small chain [*Schizosaccharomyces pombe*]	4.E-32	3.2
185105	EL739209	histone H2A [*Agaricus bisporus*]	1.E-43	3.0
169021	JGI_LbEX1475	transcription initiation factor IId subunit [*Schizosaccharomyces pombe*]	2.E-30	2.7
Protein synthesis				
192547	ES768863	ribosomal protein S18 [*Coprinopsis cinerea okayama7#130*]	1.E-18	4.0
191248	EL739426	large subunit ribosomal protein L8e [*Ashbya gossypii* ATCC 10895]	0.0	3.4
293350	EL739863	elongation factor 3 [*Schizosaccharomyces pombe*]	0.0	3.3
382027		40S ribosomal protein S20 [*Schizosaccharomyces pombe*]	3.E-54	2.7
Lipid metabolism				
305700	EL739449	acyl-CoA dehydrogenase, [*Cryptococcus neoformans var. neoformans* B-3501A]	0.0	3.2
305700	EL739449	Acyl-CoA dehydrogenase related protein S-adenosylmethionine-dependent [*Arabidopsis thaliana*]	7.E-41	2.5
190245	JGI_LbEX1103	Acyl-CoA dehydrogenase related protein [*Arabidopsis thaliana*]	7,00E-41	2,5
295662	JGI_LbEX366	methyltransferase/cyclopropane-fatty-acyl-phospholipid synthase [*Arabidopsis thaliana*]	1.E-38	2.3
Signalisation				
324650	EL739415, EL739364	ras related protein [*Laccaria bicolor*]	2.E-26	3.3
Other				
314443	JGI_LbEX2159	endopeptidase [*Cryptococcus neoformans* var. *neoformans* JEC21]	3,00E-29	11,0
381567	EL739040	papalysine [*Homo sapiens*]	7.E-08	4.6
309748	EL739391	protein kinase [*Leishmania major*]	2.E-03	3.4
384171	EL739214	Glutamic acid-rich protein precursor [*Plasmodium falciparum*]	4.E-10	2.9
399271	EL735445	tectonin II [*Physarum polycephalum*]	6.E-28	2.8
Hypothetical proteins				
379879		hypothetical protein [*Cryptococcus neoformans* var. *neoformans*]	4,00E-55	4,2
314071		unknown protein [*Arabidopsis thaliana*]	0.06	3,8
295544		UPF0337 protein bsl2407 [*Bradyrhizobium japonicum*]	1,00E-05	3,5
294063	EL739269	Expressed protein [*Cryptococcus neoformans* var. *neoformans* JEC21]	9,00E-21	3,5
300911	JGI_LbEX2008	hypothetical protein UM06032.1 [*Ustilago maydis* 521]	9,00E-11	2,6
305825	EL739369	no hit	-	2,6
no hit	JGI_LbEX1942	no hit	-	2,5
315144	EL739204	hypothetical protein [*Phanerochaete chrysosporium*]	4,80E-31	2,3
380431	EL739458	no hit	-	2,2
3,O-C12 Down-regulated genes				
Protein degradation via peroxysome				
244258	ES768884	26S proteasome regulatory complex, ATPase RPT4 [*Schizosaccharomyces pombe*]	0.0	-5.1
389514	EL739641	peroxin19 Pex19p [*Cryptococcus neoformans var.neoformans JEC21]*	1.9e-16	-125152
192623	EL739199	ubiquitin [*Phanerochaete chrysosporium*]	0.0	-2.2
Protein synthesis & maturation				

292573	ES768887	40S ribosomal protein S21 [*Candida albicans*]	5.E-31	-7.0
165054	EL739461	40S ribosomalprotein S26 [*Schizophyllum commune*]	3.E-47	-5.4
294111	EL739464	60 kDa chaperonin [*Thermus thermophilus HB27*]	2.E-14	-4.0
191048	EL739835	chaperone regulator [*Cryptococcus neoformans var. neoformans B-3501A*]	5.E-63	-4.9
291503	EL739604	Elongation factor 1-alpha [*Schizophyllum commune*]	0.0	-6.5
295367	ES768877	60S ribosomal protein L37-A [*Drosophila melanogaster*]	2.E-36	-6.1
304792	ES768874	Srrm1_predicted; serine/arginine repetitive matrix 1 [*Rattus norvegicus*].	2.E-7	-8.0
172821	ES768889	Tryptophanyl-tRNA synthetase [*Schizosaccharomyces pombe*]	0.0	-5.0
314121	EL738698	60S ribosomal protein L39 [*Candida albicans*]	1.E-17	-3.0
Transport				
187302	EL739072	ABC transporter [*Aspergillus fumigatus* Af293]	0.0	-4.6
297155	EL739477	Metabolite transport protein GIT1 [*Saccharomyces cerevisiae*]	0.0	-5.1
295862	ES768860	Putative transmembrane protein, involved in the export of ammonia [*Candida albicans*]	2.E-49	-3.6
Cytoskeleton & protein trafficking				
311622	EL738896	ADP-ribosylation factor [*Cryptococcus neoformans*]	2.E-94	-2.9
148581	EL738861	Autophagy-related protein 8 precursor (Autophagy-related ubiquitin-like modifier ATG8) [*Laccaria bicolor*]	2.E-74	-5.0
231695	EL739335	arp2/3 complex 21 kda subunit [*Cryptococcus neoformans var. neoformans* B-3501A]	8.E-78	-5.1
Energy metabolism				
143231	JGI_LbEX1538	mitochondrial ATPase Afg1 [*Aspergillus fumigatus* Af293]	3.E-21	-10.9
305432	ES768861	ATP synthase alpha chain, mitochondrial precursor [*Cryptococcus neoformans var. neoformans* JEC21]	0.0	-2.7
315828	EL739457	FMN binding oxidoreductase [*Aspergillus fumigatus* Af293].	7.E-97	-4.2
Mitochondria	EL739475	ATP synthase 9	manual curation	-2.4
Proteases				
292906	EL739719, EL739344	Vacuolar aspartic protease precursor [*Candida albicans*]	0.0	-8.0
318727	EL739281	clitocypin analog [*Clitocybe nebularis*]	3. E-3	-4.2
Lipid metabolism				
312243	EL739629	cytochrome P450 [*Lentinula edodes*]	3.E-151	-127063
Detoxification process & stress response				
248838	EL739840	D-arabinono-1,4-lactone oxidase [*Cryptococcus neoformans* var. *neoformans* JEC21]	4.E-180	-5.1
384583	ES768871	Heat shock protein 90 homolog [*Schizosaccharomyces pombe*]	0.0	-232712
300650	EL739652	HSP100 [*Pleurotus sajor-caju*].	0.0	-6.8
186670	EL739116	small heat shock protein [*Laccaria bicolor*]	5.E-77	-3.7
305899	ES768864	stress response RCI peptide, putative [*Aspergillus fumigatus* Af293]	3.E-22	-5.7

Chromatine structure & transcription regulation				
190777	EL738671, EL739085, EL739331, EL739813, EL739644, EL739761	mismatched base pair and cruciform DNA recognition protein [*Agaricus bisporus*]	2.E-39	-4.1
302873	EL739239	Chromatin-associated protein swi6 [*Schizosaccharomyces pombe*]	7.E-24	-10.2
178653	EL739761	Peroxiredoxin DOT5 (Thioredoxin reductase) [*Saccharomyces cerevisiae*]	1.5 e-27	-21127
Others				
318163	EL739816	hemolytic lectin LSLa [*Laetiporus sulphureus*].	1.E-38	-5.5
293277	EL739504, EL739810	ras related protein 2 [*Laccaria bicolor*]	1.E-08	-3.6
318727	EL739623	laminarinase [*Phanerochaete chrysosporium*]	0.0	-11.0
293312	EL739290	carbonate dehydratase/ zinc ion binding [*Arabidopis thaliana*]	5.E-09	-2.9
386769	EL738803	Proteoglycan-4 precursor (Lubricin) [*Homo sapiens*]	5.E-25	-3.0
399510	EL740045	CipC1 [*Paxillus involutus*]	4.E-31	-7.5
295299	EL739595	g1/s-specific cyclin pcl1 (cyclin hcs26) [*Mus musculus*]	3.E-14	-121854
385500	EL739627	Chain A, Three dimensional structure of a novel pore-forming lectin [Laetiporus sulphureus]	5.E-34	-162148
Hypothetical proteins				
301642	EL739270	no hit	-	-225818
no model	ES768873	no hit	-	-179919
306030	EL739649	conserved hypothetical protein [*Cryptococcus neoformans* var. *neoformans* JEC21]	2,00E-45	-143903
385192	ES768875	Hypothetical protein C32A11.02c in chromosome I [*Schizosaccharomyces pombe*]	2,00E-41	-120070
332942	ES768872	hypothetical protein UM00431.1 [*Ustilago maydis* 521]	9,00E-13	-114862
no model	ES768876	no hit	-	-112049
no model	ES768870	no hit	-	-108026
317829	EL739524	hypothetical protein AN3627.2 [*Aspergillus nidulans* FGSC A4].	3,00E-28	-107775
no hit	EL739588	no hit	-	-105501
232261	EL739480	hypothetical protein UM01569.1 [*Ustilago maydis* 521]	1,00E-133	-95217
no model	EL739158	no hit	-	-16544
genome traces	EL739462	no hit	-	-11433
388083	ES768886	no hit	-	-6439
no model	ES768869	no hit	-	-306
no hit	ES768879	no hit	-	-237
no hit	ES768881	no hit	-	-221
325204	EL739650	hypothetical protein CNBJ0800 [*Cryptococcus neoformans var. neoformans* B3501A]	6,00E-11	-85
	EL739717	hypothetical protein UM02950.1 [*Ustilago maydis 521*]		-11
326764	EL739230	proteoglycan 4 [*Homo sapiens*]	1,00E-24	-11
no model	ES768890	no hit	-	-9
387232	ES768888	hypothetical protein CNI00680 [*Cryptococcus neoformans* JEC21]	6,00E-45	-9
397352	EL739253	no hit	-	-8

305130	EL739841	Hypothetical protein with domain BTP/Poz [Phanerochaete chrysosporium]	5,00E-30	-8
311423	ES768867	hypothetical protein UM01150.1 [*Ustilago maydis* 521]	5,00E-09	-7
331792	EL739329	no hit	-	-7
381791	EL739605, ES768885	no hit	-	-6
genome traces	EL739463	no hit	-	-6
no model	EL739097	no hit	-	-6
no hit	EL739811	no hit	-	-6
311710	EL739238	not hit	-	-5
no hit	EL739850	no hit	-	-5
no hit	EL739033	no hit	-	-5
295545	ES768891	UPF0337 protein bsl2407 [*Bradyrhizobium japonicum*]	1,00E-05	-5
384275	EL739459	Hypothetical proline-rich protein [*Owenia fusiformis*]	1,00E-08	-4
391051	EL739250, ES768883	Histone H1 [*Parechinus angulosus*]	1,00E-17	-4
327435	EL739234	hypothetical protein XP_534218 [*Canis familiaris*]	1,00E-25	-4
249936	EL739827	hypothetical protein CNH03650 [*Cryptococcus neoformans var. neoformans* JEC21]	4,00E-13	-4
no hit	EL740110	no hit	-	-4
308882	EL738888	no hit	-	-4
no hit	EL739843	no hit	-	-4
387341	EL739351	no hit	-	-4
646505	EL739347	expressed protein [*Cryptococcus neoformans* var. *neoformans* JEC21]	9,00E-28	-3
no model	ES768880	no hit	-	-3
no hit	ES768868	no hit	-	-3
293775	ES768882	Conserved hypothetical protein [*Candida albicans*]	2,00E-12	-3
379822	EL739471	Hypothetical protein [*Schizophyllum commune*]	1,00E-94	-3
no model	EL739482	no hit	-	-3
305544	JGI_LbEX2148	no hit	-	-3
no hit	ES768878	no hit	-	-3
genome traces	ES768866	no hit	-	-3
256175	JGI_LbEX1535	hypothetical protein ybr269cp [*Saccharomyces cerevisiae*]	7,00E-07	-3
no model	EL739231	no hit	-	-2
397352	EL739052	no hit	-	-2

V1.1 JGI protein identity	GeneBank Accession no	NCBI Database match	E-value	Expression ratio
C6-HSL up-regulated genes				
Detoxification process				
296671	JGI_LbEX1156	glutathione S-transferase [*Paxillus involutus*]	5,00E-20	2,4
Hypothetical proteins				
no hit	no hit	no hit	-	5,8
384843	ES768894	60S ribosomal protein L41 [*Agaricus bisporus*]	1,00E-08	2,7
305825	EL739369	no hit	-	2,0
318799	EL739047	no hit	-	2,0
C6-HSL down-regulated genes				
Cytoskeleton & protein trafficing				

311622	EL738896	ADP-ribosylation factor [*Cryptococcus neoformans*]	2,00E-94	-2,7
Energy metabolism				
143231	JGI_LbEX1538	mitochondrial ATPase (Afg1) [*Aspergillus fumigatus* Af293]	3,00E-21	-2,9
315828	EL739457	FMN binding oxidoreductase, putative [*Aspergillus fumigatus* Af293].	7,00E-97	-3,8
185536	ES768892	malate dehydrogenase, NAD-dependent *[Aspergillus fumigatus* Af293]	0	-6,1
Amino acid metabolism				
381012	EL739460	aspartate aminotransferase mitochondrial precursor [*Cryptococcus neoformans* JEC21]	0	-3,5
Transcritpion regulation				
190777	EL739813	mismatched base pair and cruciform DNA recognition protein [*Agaricus bisporus*]	2,00E-39	-4,3
301049	JGI_LbEX144	Med7p [*Saccharomyces cerevisiae*]	4,00E-10	-4,7
Transport				
295862	ES768860	Putative transmembrane protein, involved in the export of ammonia. [*Candida albicans*]	2,00E-49	-4,0
Protein synthesis				
192404	EL740189	ribosomal protein L13A [*Xanthophyllomyces dendrorhous*]	9,00E-106	-18,8
Others				
385500	EL739627	Chain A, Three Dimensional Structure Of A Novel Pore-Forming Lectin [*Laetiporus sulphureus*]		-239864,8
Hypothetical proteins				
no hit	EL739615	no hit	-	-16,9
no hit	EL739646	no hit	-	-16,8
no hit	EL740196	Hypothetical protein	-	-7,9
no hit	EL740053	Hypothetical protein_Pf29A_2	-	-4,7
no hit	EL739722	no hit	-	-4,6
no hit	EL739465	no hit	-	-4,3
no hit	EL739850	hypothetical protein UM04013.1 [Ustilago maydis 521]	-	-4,1
no hit	EL739463	no hit	-	-3,6
305130	EL739841	Hypothetical protein with domain BTP/Poz [Phanerochaete chrysosporium]	5,00E-30	-3,6

Annexe 5.

Supplemental web data. Publication n°6, Molecular mechanisms of the antagonism between the ectomycorrhizal fungus Laccaria bicolor S238N and the chitinolytic bacteria Collimonas fungivorans Ter331 : a dual gene profiling approach.

Table S1. Transcripts of *L. bicolor* S238N regulated (*t*-test, ≥ - 2.0, ≤ 2.0) in the presence of the chitinolytic strain *C. fungivorans* Ter331. Protein ID, GenBank number of the corresponding EST, identity of the best blastX, and the E value of the BlastX are given. The fold corresponds to the ratio signal intensity between *C. fungivorans* Ter331 and the control treatment. The value of the PPDE test is also given.

Protein ID	EST AC Number	NCBI Best BlastX identity	BlastX E-value	Fold Change	PPDE test
Stress response					
184665	EL739982, EL739373	glutathione S-transferase [*Paxillus involutus*]	0	-3,3	9,88E-01
298606	EL739383, JGI_LbEX4932	glutathione S-transferase [*Coccidioides immitis* RS]	2E-69	-3,3	9,79E-01
296671	JGI_LbEX1156	Gluthatione S transferase [*Paxillus involutus*]	5E-20	-3,8	9,88E-01
Protein synthesis & transcription regulation					
127802	EL739167	Multiprotein-bridging factor 1 [*Kluyveromyces lactis*]	1,3E-41	-5,1	9,93E-01
125708	EL739481	40S ribosomal protein S20 [*Schizosaccharomyces pombe*]	0	-3	9,83E-01
183045	JGI_LbEX2069	40S ribosomal protein S3ae-a [*Cryptococcusneoformans* var. *neoformans* JEC21]	1E-100	-2,9	9,76E-01
304004	JGI_LbEX1164	60S ribosomal protein L7a [C*ryptococcusneoformans var. neoformans* JEC21]	2E-32	-3,7	9,75E-01
291503	EL739615 JGI_LbEX4956	Elongation factor 1-alpha [*Schizophyllum commune*]	0	-3,8	9,96E-01
304792	EL738654, EL739005, EL739211, EL740038 EL740039	Srrm1_predicted; serine/arginine repetitive matrix 1 [*Rattus norvegicus*].	1.9e-7	-3,9	9,85E-01
251796	JGI_LbEX1852	small nuclear ribonucleoprotein [*Arabidopsis thaliana*]	5E-25	-4,6	9,84E-01
300913	EL739054	small nuclear ribonucleoprotein E, putative [*Cryptococcus neoformans var. neoformans* JEC21]	2E-31	-4,5	9,93E-01
297290	JGI_LbEX2076	RNA binding protein [*Aspergillus fumigatus* Af293]	9E-13	-2,6	9,72E-01
Energy metabolism					

mito3634485.a1	JGI_LbEX266	cytochrome C oxidase subunit I [*Pseudotsuga menziesii*]	5E-22	-4,8	9,94E-01
173909	EL739225	cytochrome C oxidase polypeptide IV [*Schizosaccharomyces pombe*]	1E-40	-3,2	9,79E-01
genome traces	JGI_LbEX2019	cytochrome C oxidase subunit I	6,00E-05	-6,1	9,98E-01
genome traces	JGI_LbEX2161	cytochrome C oxidase subunit I [*Cryptococcus neoformans* var. *neoformans*]	4E-33	-5,3	9,96E-01
genome traces	JGI_LbEX256	cytochrome C oxidase subunit I [*Smittium culisetae*]	1E-38	-5	9,97E-01
192393	EL739466	ATP/ADP antiport [*Cryptococcus neoformans var. neoformans B-3501A*]	0	-4,3	9,87E-01
305503	EL739220	flavoprotein monooxygenase [*Paracoccus denitrificans PD1222*]	0	-3,4	9,84E-01
383670	EL739545	ATP/ADP carrier protein [*Candida albicans* SC5314]	0	-4,6	9,94E-01
Replication & chromatine structure					
150961	EL739367	DNA polymerase B *[Cryptococcus neoformans var. neoformans*]	0	5,1	9,77E-01
185105	JGI_LbEX447	histone H2a-1 [*Cryptococcus neoformans* var. *neoformans* JEC21]	3E-36	-3	9,74E-01
190640	JGI_LbEX5292	histone H2a [*Ustilago maydis* 521]	1E-36	-2,6	9,68E-01
246384	EL739455	Tra1 [*Cryptococcus neoformans var. neoformans*]	0	-2,7	9,12E-01
294940	EL739473	Histone H1 [*Parechinus angulosus]*	1E-17	-3	9,78E-01
182415	EL739378	damaged DNA binding protein [*Cryptococcus neoformans* var. *neoformans* JEC21]	8.6^{e}-05	5,9	9,77E-01
Signal transduction					
393186	EL740073	dual secificity protein kinase FUZ7 [*Ustilago maydis*]	0	-3,5	9,83E-01
297361	EL739376	Phosphoric monoesterase hydrolase [*Cryptococcus neoformans var. neoformans JEC21*]	0	4,5	9,76E-01
Other					
384171	EL740036	Glutamic acid-rich protein [*Plasmodium falciparum*]	3.7^{e}-10	-3,3	9,77E-01
229432	JGI_LbEX459	chitin deacetylase [*Schizophyllum commune*]	3E-76	-2,8	9,72E-01
245383	JGI_LbEX469	Metalloprotease [*Gloeobacter violaceus* PCC 7421	4E-48	-4,4	9,90E-01
185826	EL739380	oxidoreductase, zinc-binding dehydrogenase family [*Aspergillus fumigatus* Af293]	1.5^{e}-44	-4,1	9,86E-01
301232	EL739275	Panthotenate kinase [*Cryptococcus neoformans var. neoformans JEC21*]	0	-3,2	9,80E-01
306303	EL739483	Protein kinase [*Glycine max*]	4.2^{e}-16	-3,7	9,78E-01

189031	JGI_LbEX2074	Synthase of the type 3 pneumococcal capsular polysaccharide [*Aspergillus fumigatus* Af293]	2E-45	-2,6	9,68E-01
216553	EL739468	cleft lip and palate associated transmembrane protein [*Cryptococcus neoformans var. neoformans JEC21*]	0	-5,7	9,89E-01
Hypothetical proteins					
318749	EL739384	clitocypin cysteine proteinase inhibitor (manual curation) [*Clitocybe nebularis*]	No hit	11	9,92E-01
386412	EL739602	Hypothetical protein with GIT helical motif [*Cryptococcus neoformans var. neoformans JEC21*]	0	9,1	9,86E-01
307960	EL739212	Hypothetical protein	No hit	8,2	9,76E-01
385359	EL739479	Hypothetical protein [*Cryptococcus neoformans var. neoformans JEC21*]	3.2^{e}-10	6,7	9,84E-01
		Hypothetical protein		6,3	9,89E-01
384719	EL739205	No hit	No hit	5,9	9,71E-01
318727	EL739472	Hypothetical protein	No hit	5,8	9,73E-01
296533	EL739207	Hypothetical protein [*Coprinopsis cinerea*]	No hit	5,8	9,71E-01
No predicted model	EL739908	No hit	No hit	5,5	9,80E-01
295355	EL739119	No hit	No hit	5,4	9,72E-01
309620	EL739190	expressed protein [*Phanerochaete chrysosporium*]	0	5	9,72E-01
		Hypothetical protein	No hit	4,8	9,86E-01
393598	EL739375	Hypothetical protein [*Coprinopsis cinerea*]	No hit	4,7	9,80E-01
383392	EL739474	Hypothetical protein [*Coprinopsis cinerea*]	No hit	4,7	9,82E-01
No predicted model	EL739478	No hit	No hit	4,3	9,85E-01
315836	EL739203	hypothetical protein [*Phanerochaete chrysosporium*]	5E-31	4,1	9,76E-01
313660	EL739476	Hypothetical protein	No hit	3,9	9,87E-01
313608	EL739149	Hypothetical protein [*Ustilago maydis* 521]	1.1^{e}-6	3,6	9,76E-01
		Hypothetical protein	No hit	3,6	9,71E-01
308883	EL740043 EL738888 EL739223 EL739047 EL739379 EL739226 EL740113 EL740125 EL738671 EL739726	No hit	No hit	-3,1	9,65E-01
No hit	JGI_LbEX1944	no hit	No hit	-2,6	9,71E-01

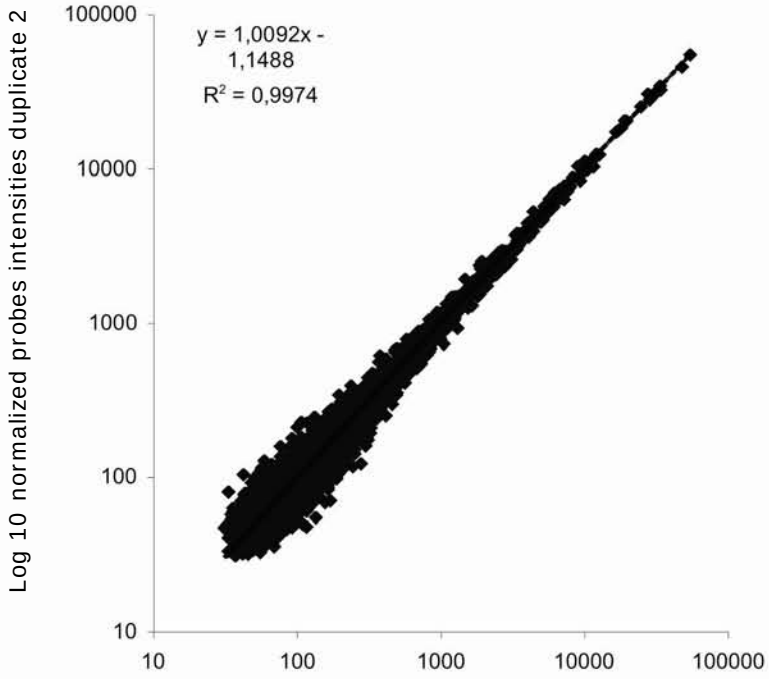
292014	EL739102 EL739372	No hit	No hit	-2,6	9,71E-01
304278	EL739032 JGI_LbEX1344	hypothetical protein		-2,7	9,73E-01
no hit	JGI_LbEX1148	hypothetical protein	No hit	-2,8	9,72E-01
291441	JGI_LbEX4947	hypothetical protein		-2,8	9,79E-01
No hit	JGI_LbEX5317	hypothetical protein	No hit	-2,9	9,79E-01
306212	EL739374	hypothetical protein CNBL2250 [Cryptococcus neoformans var. neoformans B-3501A]	2,00E-12	-2,9	9,70E-01
295386	EL738941	Hypothetical protein	No hit	-3	9,83E-01
No hit	JGI_LbEX5196	hypothetical protein	no hit	-3,1	9,77E-01
319082	JGI_LbEX2265	hypothetical protein UM03468.1 [Ustilago maydis 521]	1,00E-07	-3,1	9,73E-01
249253	JGI_LbEX1010	hypothetical protein [*Aspergillus fumigatus* Af293]	2E-12	-3,1	9,73E-01
300726	EL740123	Hypothetical protein UM00199_1 [Ustilago maydis 521]	0	-3,1	9,69E-01
332923	JGI_LbEX1793	hypothetical protein	No hit	-3,1	9,86E-01
308883	EL739605	Hypothetical protein	No hit	-3,1	9,82E-01
326534	EL739548	hypothetical protein [Phanerochaete chrysosporium]	9,5E-17	-3,2	9,78E-01
388095	EL739469	Hypothetical protein spe Lac	No hit	-3,2	9,71E-01
305371	EL739048	Hypothetical protein [*Phanerochaete chrysosporium]*	0	-3,3	9,78E-01
308883	EL740043	Hypothetical protein	No hit	-3,3	9,89E-01
297146	EL739941	Hypothetical protein [*Phanerochaete chrysosporium*]	0	-3,4	9,80E-01
311796	JGI_LbEX2244	hypothetical protein	No hit	-3,4	9,87E-01
292159	EL739193	Secreted proline-rich protein; similarity to predicted protein of Coprinopsis cinerea. The most highly expressed transcript in free-living mycelium of L. bicolor S238N. Single copy gene.	0	-3,4	9,76E-01
311407	EL739042	hypothetical protein [Cryptococcus neoformans var. neoformans JEC21]	5E-17	-3,5	9,83E-01
295051	EL740196 EL740110	Hypothetical protein	No hit	-3,9	9,90E-01 9,72E-01
319140		hypothetical protein	No hit	-3,7	9,89E-01
No model	EL739486	Hypothetical protein	No hit	-3,7	9,87E-01
318970	EL739377	Hypothetical protein	No hit	-3,7	9,82E-01
No hit		hypothetical protein	No hit	-3,7	9,85E-01
294111	EL739053	hypothetical protein with domain chaperonin Cnp60 [*Thermus Thermophilus*]	3E-18	-3,8	9,82E-01
No hit		hypothetical protein	No hit	-3,9	9,91E-01
388083	EL740116	Hypothetical protein	No hit	-3,9	9,90E-01

233623	EL739387	Hypothetical protein [*Homo sapiens*]	8,00E-27	-3,9	9,85E-01
293605	EL739488	Hypothetical protein with Complex 1 LYR protein domain [*Saccharomyces cerevisiae]*	5,00E-16	-4	9,88E-01
315776	EL738665	Hypothetical protein	No hit	-4	9,83E-01
293300	EL738927	Hypothetical protein	No hit	-4	9,91E-01
383081	EL739037	hypothetical protein [*Cryptococcus neoformans var. neoformans JEC21*]	9.2^{e}-26	-4,1	9,89E-01
304792		hypothetical protein	No hit	-4,1	9,82E-01
No hit	EL739328	No hit	No hit	-4,1	9,93E-01
No model	EL739215	Hypothetical protein	No hit	-4,2	9,89E-01
No hit		hypothetical protein	No hit	-6,6	9,98E-01
314444	EL740044	hypothetical protein [Phanerochaete chrysosporium]	3,30E-41	-4,3	9,94E-01
291511	EL739352	syntenic homolog of Saccharomyces cerevisiae YPL004C [*Eremothecium gossypii*]	2.6^{e}-20	-4,8	9,97E-01

Table S2. List of the primers used in this study and the gene model corresponding to each gene in *L. bicolor* H82 genome.

Gene name	Primer sequences
tra 1	5'AGGGCAATACACTGAGGACAA 3' 5' TAGAGGGTGATCCGTTTCCA 3'
gluthatione S transferase	5' CCTTTCGAGTTCCACAGCAT 3' 5' GATTAGGCCCGGTGTACCTT 3'
Hypothetical protein Srrm1	5' CCCGAGCCTTTCTCTCTCTT 3' 5' TGTGCTTGTGGAGTCTGAGG 3'
Short-chain dehydrogenase/reductase	5' GCTCGGTATTGGGTTTTGTG 3' 5' CTTCCTTCACGCGTAAGTCC 3'
Zinc-binding oxidoreductase	5' ATTCATCGCAATCAGGGAAG 3' 5' CGAGCACCAGGTCGTAGTTT 3'
Endochitinase B	5' GAAAGGCCTTGGAACTGGAT 3' 5' AGGATCTGCATCGCCTTGT 3'
3-oxoadipate CoA-succinyl transferase	5' GATGGAACGTTCGCTGGTAG 3' 5' CTCGACCAGATGCTCGACTT 3'
Hypothetical protein 3082	5' CACCCAGAGTTCCTGCAAAC 3' 5' ACTTTTGCTCACGGTTCAGG 3'
DNA-3-methyladenine glycosidase	5' GTCTGTCCCAAGACGACTCC 3' 5' GTCCATCTCCATCCACTGCT 3'
Hypothetical protein 251	5' ACCTTGACGAACTGTCGCAT 3' 5' CGTTGTCGTAGCTCGATTCA 3'
Hypothetical protein 1059	5' GATGTCGAACAACGAGCAGA 3' 5' ACCACATCGCTGGCTTTATC 3'
Hypothetical protein 1748	5' ACAGCCTGACCATTTTCACC 3' 5' GCCAGCTGATGTCCTGTTTT 3'

Figure S1. Array quality and variation within and between experiments. (a) Scatter plots comparing the signal intensity of duplicate spots on the same array. (b) Scatterplot comparing the sinal intensity of pairs of arrays hybridized with two biological probes replicates.



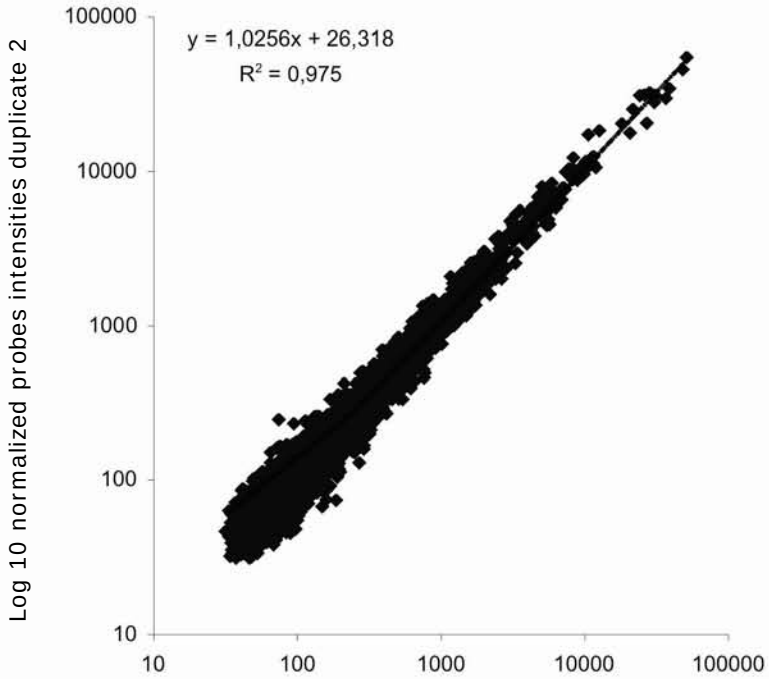
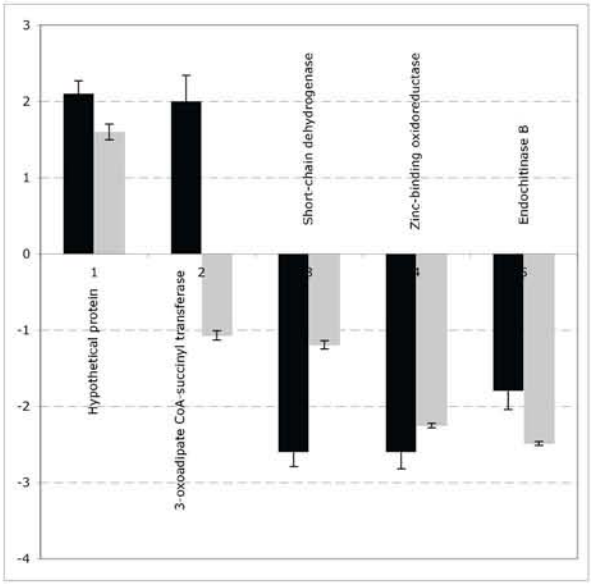
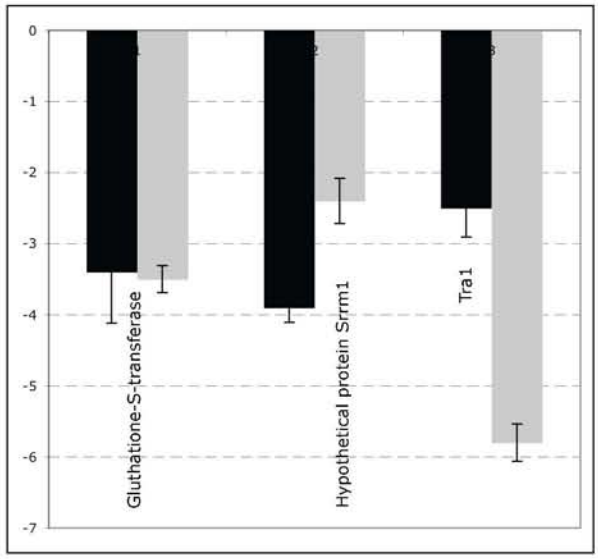


Figure S2. Validation of *C. fungivorans* Ter331 whole genome array (A) and *L. bicolor* S238N cDNA array. Comparative expression ration [*L. bicolor* S238N treatment/ control treatment] of *L. bicolor*-responsive transcripts (A) and [*C. fungivorans* Ter 331 treatment/ control treatment] of *C. fungivorans* Ter331-responsive transcripts measured by array (black) or quantitative PCR (grey). Standard error is presented.

A



B



Annexe 6.

Supplemental web data. Publication n°7. The major pathways of carbohydrate metabolism in the ectomycorrhizal basidiomycete *Laccaria bicolor* S238N.

Table S1. List of primers used in this study.

Gene name	Primer sequences
TP	5’ ACCCGACTCTGGCTGGAC 3’ 5’ AGTACGGGGTATGGGGAGAC 3’
TPS1	5’ GTTTGGCACTGTCGAGTTCA 3’ 5’ GTGCCTGCTGACAAGCA 3’
TPS2	5’ AGGTGCCTGCAGATGCT 3’ 5’ CGGGATTGATTCTCGGA 3’
TSL1	5’ CTACGAAGGGCTCAACATCG 3’ 5’ AGTTGATCCGTGACACCACA 3’
UGP1	5’ CCAAGCCAAAGGATACCAAG 3’ 5’ ATCGGGACTGATTGAAGGTG 3’
AT	5’ CCTGTGGACCGTCTATCTCG 3’ 5’ TTCTCTGGCAGAACCTGGAC 3’
MtDH	5’ GCCGTGTCTCCTGGTTATGT 3’ GACCGCCGTCAACAAAGTAT 3’
MDR1	5’ GGGTATGTGGGAGGTGTGAG 3’ 5’ AACTCGCAGACCCATAGCAG 3’
MDR2	5’ GATGCCTGGATCGACTACACT 3’ 5’ ATAGGAACCGTGGATGGTGA 3’
EF3	5’ CTCATCCTTCCTGCTCTTCTTCACG 3’ 5’ CGGTGGCTTTTGTAAGTGAATCACG 3’
GTPase ß subunit	5’ ATGATTCTTACGGATACCCTAAACG 3’ 5’ AGTGTTAAGATCCCAAAGACGAAG 3’
Metalloprotease	5’ ATCAAACGATGGTGTTGTTATTCTT 3’ 5’ GTCGTCGCTATAATCCATAAAGTTG 3’
fructose-1,6-bisphosphate aldolase	5’ CCGAGGACGTGTATGATGTG 3’ 5’ GACCCACTCTCCTTCTCCTG 3’

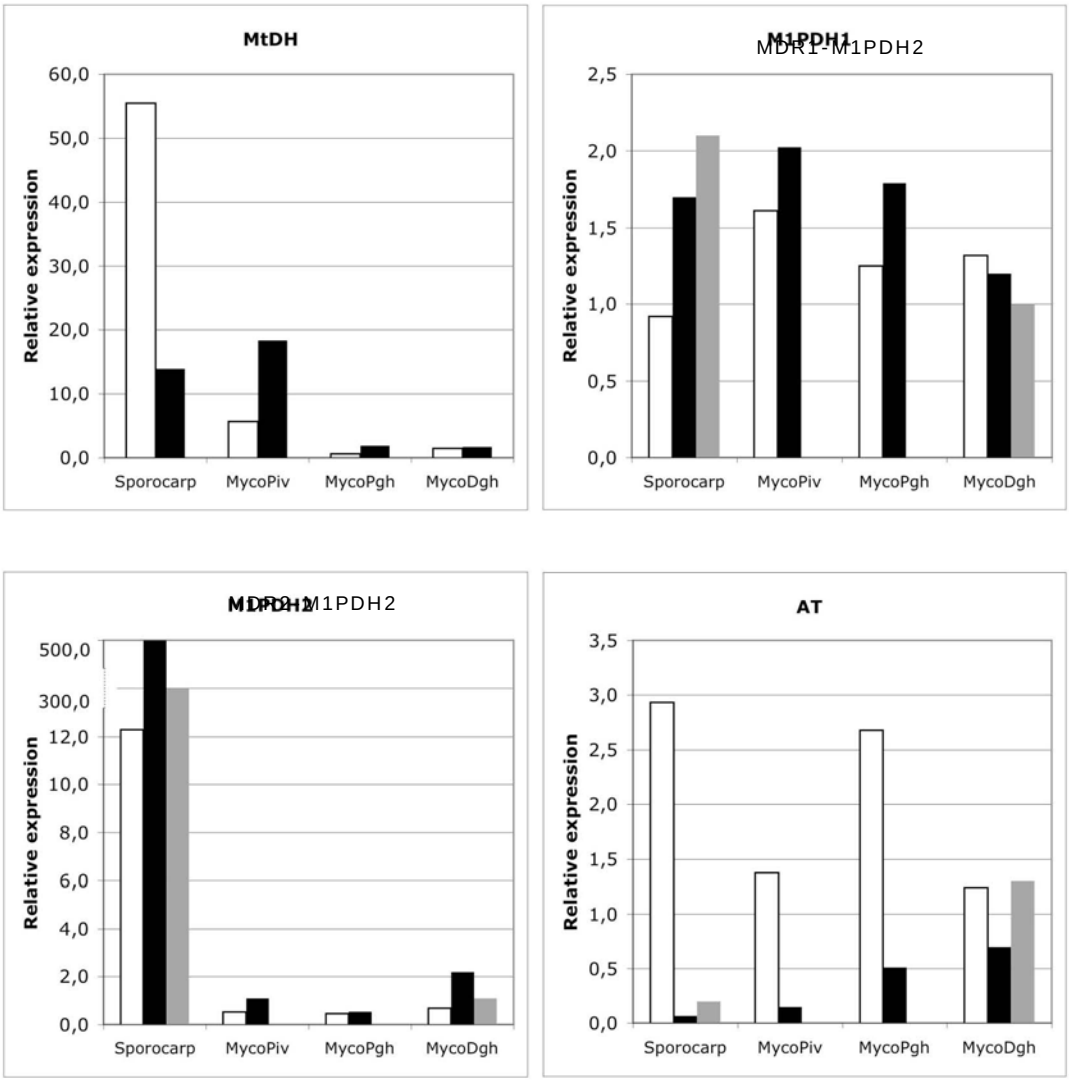
Table S2. GenBank accession numbers of sequences coding for enzymes of mannitol metabolism used for the construction of the NJ tree. Protein sequences indicated by a star have been retrieved from the *Phanerochaete chrysosporium* JGI web portal (http://genome.jgi-psf.org/Phchr1/Phchr1.home.html). M1PDH: Mannitol 1-phosphate 5-dehydrogenase; MtDH: mannitol dehydrogenase; MDR: medium-chain dehydrogenase/reductase; ADH: alcohol dehydrogenase.

Protein	**Species**	**Accession number**
M1PDH	*Alternaria alternata*	AAQ63948
M1PDH	*Aspergillus fumigatus*	EAL89350
M1PDH	*Aspergillus niger*	AAL89587
M1PDH1	*Cryptococcus neoformans*	XP_571772
M1PDH2	*Cryptococcus neoformans*	XP570793
M1PDH	*Neurospora crassa*	EAA33240
M1PDH	*Paracoccidioides brasiliensis*	AAO47089
M1PDH	*Phaeosphaeria nodorum*	AAT84078
M1PDH	*Saccharomyces cerevisae*	NP_010844
MDR	*Coprinopsis cinerea*	EAU8492
MDR	*Phanerochaete chrysosporium*	JGI ID 4796*
MDR1	*Ustilago maydis*	EAK82646
MDR2	*Ustilago maydis*	XP_758937
MtdH	*Agaricus bisporus*	AAC79985
MtdH	*Alternaria alternata*	AAO91800
MtdH	*Cladosporium fulvum*	AAK67169
MtdH	*Coprinopsis cinerea*	EAU83808
MtdH	*Gibberella zeae*	AAP33281
MtdH	*Uromyces fabae*	AAB39878
MtdH	*Ustilago maydis*	XP_761863
ADH	*Aspergillus fumigatus*	XP_750211
ADH	*Aspergillus nidulans*	XP_658047
ADH3	*Aspergillus nidulans*	XP_659890
ADH2	*Candida albicans*	XP_717649
ADH1	*Chaetomium globosum*	EAQ83781
ADH1	*Coccidioides posadasii*	ABH10637
ADH	*Cryptococcus neoformans*	XP_569885
ADH1	*Neurospora crassa*	XP_957177
ADH	*Uromyces fabae*	CAH10835

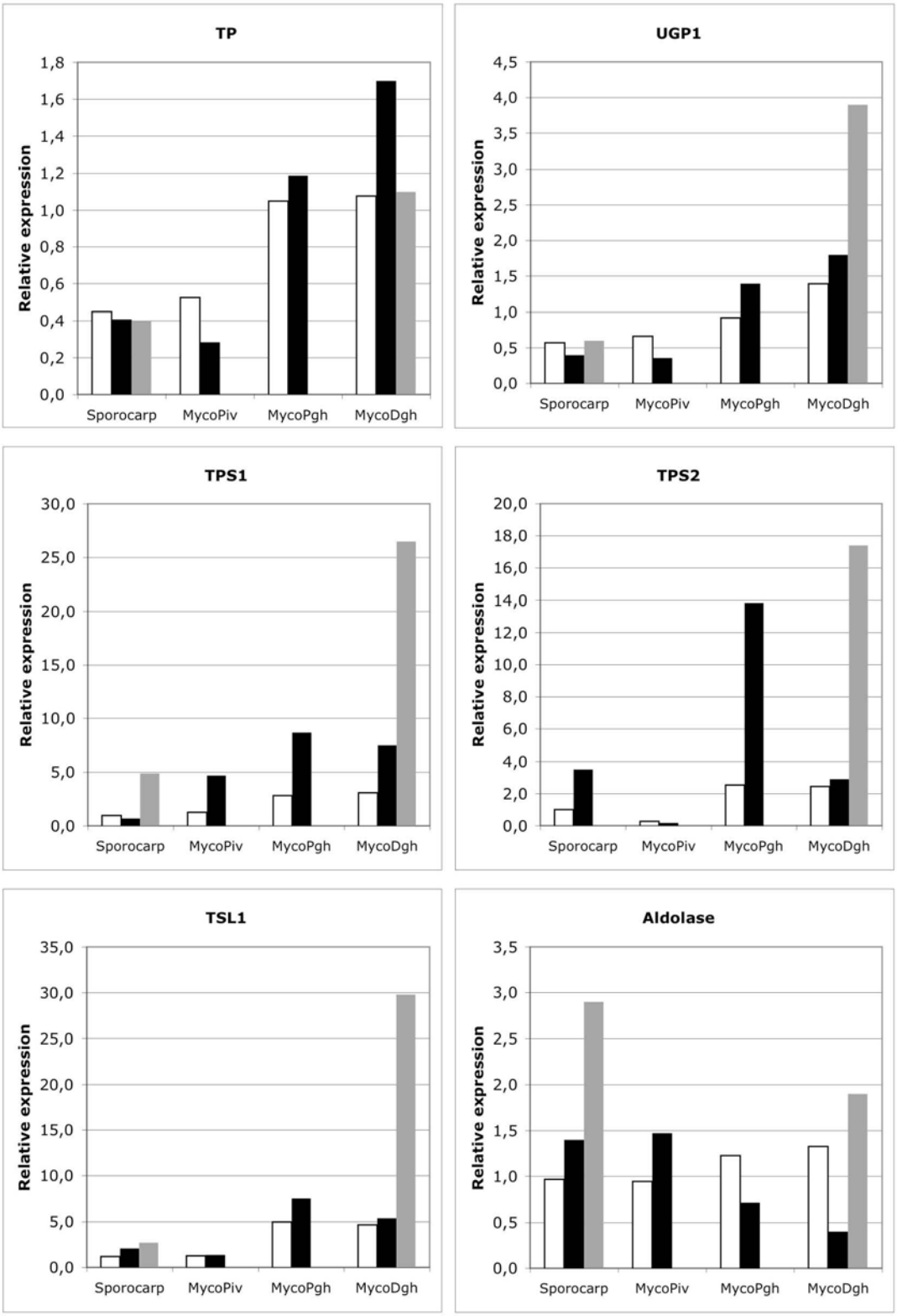
Table S3. GenBank accession numbers of sequences coding for enzymes of trehalose metabolism used for the construction of the NJ tree. Protein sequences indicated by a star have been retrieved from the *Phanerochaete chrysosporium* JGI web portal (http://genome.jgi-psf.org/Phchr1/Phchr1.home.html).

Protein	Species	Accession number
TP	*Aspergillus fumigatus*	XP_754411
TP	*Coprinopsis cinerea*	EAU88234
TP	*Cryptococcus neoformans*	XP_572131
TP	*Grifola frondosa*	BAA31349
TP	*Magnaporthe grisea*	XP_369049
TP	*Neurospora crassa*	AAC64285
TP	*Phanerochaete chrysosporium*	JGI ID 122462*
TP	*Pleurothus sajor-caju*	AAF22230
TP	*Schizophyllum commune*	ABC84380
TPS1	*Amanita muscaria*	CAC42133
TPS1	*Aspergillus fumigatus*	EAL89135
TPS1	*Coprinopsis cinerea*	EAU85301
TPS1	*Cryptococcus neoformans*	AAW45200
TPS1	*Magnaporthe grisea*	AAN46744
TPS1	*Neurospora crassa*	XP_958954
TPS1	*Phanerochaete chrysosporium*	JGI ID 130257*
TPS1	*Saccharomyces cerevisae*	Q00764
TPS1	*Ustilago maydis*	XP_756576
TPS2	*Aspergillus fumigatus*	EAL92998
TPS2	*Coprinopsis cinerea*	EAU89762
TPS2	*Cryptococcus neoformans*	AAW41876
TPS2	*Magnaporthe grisea*	XP_360898
TPS2	*Neurospora crassa*	XP_956349
TPS2	*Phanerochaete chrysosporium*	136294
TPS2	*Saccharomyces cerevisae*	P31688
TPS2	*Ustilago maydis*	XP_758537
TPS3	*Aspergillus fumigatus*	EAL87076
TPS3	*Saccharomyces cerevisae*	P38426
TSL1	*Coprinopsis cinerea*	EAU87507
TSL1	*Magnaporthe grisea*	XP_363123
TSL1	*Neurospora crassa*	EAA3573
TSL1	*Phanerochaete chrysosporium*	JGI ID 122165*
TSL1	*Saccharomyces cerevisae*	P38427
TSL1	*Ustilago maydis*	XP_759392

Figure S1. Validation of oligoarray data by real-time quantitative PCR analyses. Comparative expression ratio for [sporocarp/mycelium], [mycorrhiza of poplar grown *in vitro* (MycoPiv)/mycelium], [mycorrhiza of poplar grown in greenhouse (MycoPgh)/mycelium], [mycorrhiza of Douglas grown in pot (MycoDgh)/mycelium] of *L. bicolor* S238N MtdH, MDR1-M1PDH1, MDR2-M1PDH2, AT, TP, UGP1, TPS1, TPS2, TSL1 and 1,6-bisphosphate aldolase transcripts measured by oligoarrays (white) and quantitative PCR (black and grey). Two biological replicates were performed on cDNA from fruit body and Douglas mycorrhizae for quantitative PCR. Values for the first (black bar) and second (grey bar) replicate are shown.



Annexe 6


TP
Relative expression
1,8
1,6
1,4
1,2
1,0
0,8
0,6
0,4
0,2
0,0
Sporocarp
MycoPiv
MycoPgh
MycoDgh
UGP1
Relative expression
4,5
4,0
3,5
3,0
2,5
2,0
1,5
1,0
0,5
0,0
Sporocarp
MycoPiv
MycoPgh
MycoDgh
TPS1
Relative expression
30,0
25,0
20,0
15,0
10,0
5,0
0,0
Sporocarp
MycoPiv
MycoPgh
MycoDgh
TPS2
Relative expression
20,0
18,0
16,0
14,0
12,0
10,0
8,0
6,0
4,0
2,0
0,0
Sporocarp
MycoPiv
MycoPgh
MycoDgh
TSL1
Relative expression
35,0
30,0
25,0
20,0
15,0
10,0
5,0
0,0
Sporocarp
MycoPiv
MycoPgh
MycoDgh
Aldolase
Relative expression
3,5
3,0
2,5
2,0
1,5
1,0
0,5
0,0
Sporocarp
MycoPiv
MycoPgh
MycoDgh