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# Sulfur uptake in the ectomycorrhizal fungus *Laccaria bicolor* S238N

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**Abstract** The importance of the ectomycorrhiza symbiosis for plant acquisition of phosphorus and nitrogen is well established whereas its contribution to sulfur nutrition is only marginally understood. In a first step to investigate the role of ectomycorrhiza in plant sulfur nutrition, we characterized sulfate and glutathione uptake in *Laccaria bicolor*. By studying the regulation of sulfate uptake in this ectomycorrhizal fungus, we found that in contrast to bacteria, yeast, and plants, sulfate uptake in *L. bicolor* was not feedback-inhibited by glutathione. On the other hand, sulfate uptake was increased by sulfur starvation as in other organisms. The activity of 3′-phosphoadenosine 5′-phosphosulfate reductase, the key enzyme of the assimilatory sulfate reduction pathway in fungi, was increased by sulfur starvation and decreased after treatment with glutathione revealing an uncoupling of sulfate uptake and reduction in the presence of reduced sulfur compounds. These results support the hypothesis that *L. bicolor* increases sulfate supply to the plant by extended sulfate uptake and the plant provides the ectomycorrhizal fungus with reduced sulfur.

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## Introduction

Sulfur is essential for life. Some organisms, such as the metazoa or parasitic microorganisms, are dependent on the supply of organic sulfur in the form of amino acids, whereas plants, fungi, and most bacteria assimilate inorganic sulfur. The majority of these organisms take up sulfur in the form of sulfate, reduce it, and incorporate it into cysteine or homocysteine (Kopriva and Koprivova 2003; Leustek et al. 2000). For reduction, sulfate is first activated by ATP sulfurylase to adenosine 5′-phosphosulfate (APS). APS can either be directly reduced to sulfite by APS reductase (APR) in plants, algae, and many bacteria, or it is further phosphorylated to 3′-phosphoadenosine 5′-phosphosulfate (PAPS) by APS kinase in some enteric bacteria, cyanobacteria, yeast, and fungi (Kopriva et al. 2002). PAPS is then reduced to sulfite by PAPS reductase. Sulfite is reduced by sulfite reductase to sulfide which is incorporated into *O*-acetylserine by *O*-acetylserine (thiol) lyase to form cysteine (Kopriva and Koprivova 2003; Leustek et al. 2000). Sulfate uptake is a highly regulated process which has a high control over sulfur homeostasis. It is regulated by the nutritional status of the plant and the availability of external sulfate. Sulfur starvation results in an increased sulfate uptake in plants (Lappartient et al. 1999; Smith et al. 1997), bacteria (Kredich 1996), yeast (Ono et al. 1999), and filamentous fungi (Van de Kamp et al. 2000). Conversely, the uptake of sulfate diminishes when reduced sulfur compounds, such as glutathione, cysteine, or $H_2S$, are available (Herschbach and Rennenberg 1994; Lappartient et al. 1999; Van de Kamp et al. 2000; Westerman et al. 2001).

The importance of mycorrhiza for plant acquisition of phosphorus and nitrogen is well established (Aquino and Plassard 2004; Karandashov and Bucher 2005; Sarjala and Potila 2005; Tanaka and Yano 2005). On the other hand,

the role of mycorrhiza in sulfate uptake and metabolism is only marginally known (Rennenberg 1999). Formation of arbuscular mycorrhiza enhanced sulfate uptake in clover and maize (Banerjee et al. 1999; Gray and Gerdemann 1972). The ectomycorrhizal association, on the other hand, had no impact on the rate of sulfate uptake but influenced sulfate loading into the xylem of trees (Kreuzwieser and Rennenberg 1998; Seegmüller et al. 1996). However, the derepression of sulfate transport by sulfur deficiency observed in nonmycorrhizal beech roots was not observed in ectomycorrhizal roots, pointing to a significant contribution of the fungal partner in the regulation of sulfate uptake (Kreuzwieser et al. 1996; Kreuzwieser and Rennenberg 1998).

Our aim is to elucidate the role of ectomycorrhiza in plant sulfur nutrition. Four scenarios may describe the effect of mycorrhization: (1) in contrast to phosphorus, nitrogen, and other nutrients, sulfur nutrition may not be affected in mycorrhizal plants; (2) in analogy with phosphorus nutrition, mycorrhization facilitates sulfate uptake but does not affect plant sulfate assimilation and fungal sulfur metabolism; (3) sulfate taken up by ectomycorrhizal fungi is reduced in mycelium and reduced sulfur compounds are transported into plants. These three scenarios seem unlikely, as sulfate uptake and xylem loading are clearly affected by mycorrhization (Gray and Gerdemann 1972; Banerjee et al. 1999; Seegmüller et al. 1996; Kreuzwieser and Rennenberg 1998), and no fungal-specific PAPS reductase activity was detected in mycorrhizal pine roots (Galli et al. 1993). The published data has however allowed us to envisage a fourth scenario assuming that ectomycorrhiza enhances the uptake of sulfate by increasing the area and number of entry points, and in turn the plant provides the fungus with reduced sulfur compounds. Plant sulfur nutrition would thus be improved by increased sulfate supply, and the fungus would be relieved from the energetically demanding reduction of sulfate connected with production of toxic intermediates. In this study, we describe the characterization of sulfate and glutathione transport in the free-living mycelium of the ectomycorrhizal fungus *Laccaria bicolor* and discuss possible effects of the mycorrhiza symbiosis on sulfate assimilation of both partners in the light of our hypothesis for sulfur nutrition in ectomycorrhizal plants.

## Materials and methods

### Growth conditions

*Laccaria bicolor* S238N (Maire) P.D. Orton (Di Battista et al. 1996) was obtained from the Collection of Ectomycorrhizal Fungi, INRA Nancy. The mycelium was cultivated in Erlenmeyer flasks containing 250 ml of Pachlewski medium (Pachlewski and Pachlewska 1974) at 22°C in the dark. For long-term experiments, the fungus was transferred into fresh media containing 2 mM sulfate, with or without the addition of 1 mM glutathione, or into sulfur-free medium, and was allowed to grow for 3 weeks. Sulfur-free medium was prepared by replacing all sulfate salts with the corresponding chlorides. For short-term experiments, the fungus was grown in Pachlewski nutrient solution for 3 weeks and transferred into the fresh media as above, where it was allowed to grow for another 4 days before analyses.

### Measurements of sulfate and glutathione uptake

To determine the rate of sulfate uptake, the free-living fungal mycelium was washed four times by filtration with transport buffer (5 mM *bis-tris* propane, 0.5 mM $CaCl_2$, pH 7) to remove the culture medium and divided to aliquots of ca. 600 mg. The aliquots were added to Erlenmeyer flasks containing 25 ml of transport buffer supplemented with different concentrations of $MgSO_4$ and [$^{35}$S]sulfate (Hartmann Analytic; specific activity 0.4–120 kBq/mmol) and incubated for 10 to 60 min at room temperature. Afterwards, the fungal material was washed four times with transport buffer to remove the [$^{35}$S]sulfate from the surface of the fungi. The material was weighed, transferred into a scintillation vial, and solubilized in 2 ml Soluene-350 tissue solubilizer (Canberra-Packard) for 24 h. Samples were treated with 200 µl isopropanol and bleached with 200 µl 35% $H_2O_2$ (v/v) at room temperature for 24 h to reduce quenching of radioactivity. Radioactivity in the samples was determined by liquid scintillation counting (Wallac System 1409, Wallac Oy) after addition of 10 ml scintillation fluid (OptiPhase HiSafe II, Wallac Oy) and vigorous shaking. For the analysis of regulation of sulfate transport, the fungal mycelium was transferred into the transport buffer supplemented with 0.2 mM $MgSO_4$ and [$^{35}$S]sulfate (specific activity 9.5 kBq/mmol) and the uptake was determined as above.

For characterization of glutathione (GSH) uptake in *L. bicolor*, the aliquots of free-living mycelium were transferred to Erlenmeyer flasks containing 25 ml of transport buffer with different concentrations of GSH and [$^{35}$S]GSH (NEN; specific activity 0.34–1450 kBq/mmol) and incubated for 60 min at room temperature. The samples were processed and analyzed as above. For competition experiments, the transport buffer was supplemented with 0.1 mM GSH, [$^{35}$S]GSH (specific activity 12 kBq/mmol), and either 1 mM glutamate or 0.1 mM leucine enkephaline (pentapeptide Tyr-Gly-Gly-Phe-Leu), and the uptake was determined as above (Zhang et al. 2004).

### Analysis of thiols

Low molecular weight thiols were analyzed in fungal material as described before (Herschbach et al. 2000). One hundred milligrams of free-living *L. bicolor* mycelium was ground in liquid nitrogen and extracted in 750 µl of 0.1 N HCl. After 10 min centrifugation, 120 µl of supernatant was mixed with 180 µl of 0.2 M CHES (*N*-cyclohexyl-2-aminoethanesulfonic acid) buffer, pH 9.3, and 30 µl of 15 mM dithiothreitol was added to reduce disulfides. After

1 h, 45 µl of 30 mM monobromobimane (ThiolyteR MB, Calbiochem) was added for derivatization of thiols, and the tubes were incubated for 15 min at room temperature in the dark. The reaction was stopped by adding 240 µl of 10% acetic acid to stabilize the conjugates. After 10 min centrifugation, 500 µl of samples was transferred to vials, and Bimane conjugates were separated by high-performance liquid chromatography (SUPELCOSIL TM LC-18, 25 cm×4.6 mm, 5 µm, Sigma-Aldrich) using 10% (v/v) methanol, 0.25% (v/v) acetic acid (pH 3.9) as solvent A and 90% (v/v) methanol, 0.25% (v/v) acetic acid (pH 3.9) as solvent B. The elution protocol employed a linear gradient from 96 to 82% A in 20 min, and the flow rate was kept constant at 1 ml/min. Bimane derivatives were detected fluorometrically (RF535, Shimadzu) with excitation at 390 nm and emission at 480 nm.

### PAPS reductase measurements

PAPS reductase activity was measured as the production of [$^{35}$S]sulfite, assayed as acid volatile radioactivity formed in the presence of 10 µM [$^{35}$S]PAPS (15–30 kBq/µmol) and 5 mM dithioerythritol according to the method described by Schmidt (1977). After treatments, aliquots of ca. 50 mg of the free-living *L. bicolor* mycelium were washed with water by filtration and homogenized in 500 µl of 50 mM $Na/KPO_4$ (pH 8) by sonication. Fifty microliters of the extract was added to the reaction mixture. The assays were incubated for 1 h at 37°C and stopped by adding 100 µl 1 M $Na_2SO_3$. The tubes were immediately transferred into scintillation vials containing 1 ml 1 M triethanolamine and 200 µl 1 M $H_2SO_4$ was added to the reaction mixtures. The vials were closed quickly and left overnight at room temperature to allow the quantitative transfer of [$^{35}$S]$SO_2$ from the assay into the triethanolamine solution. Radioactivity in the triethanolamine was determined by liquid scintillation counting after addition of 5 ml scintillation solution and vigorous shaking.

The protein concentration of the extracts was determined using the Bio–Rad reagent according to the manufacturer's instructions (Bio–Rad) with bovine serum albumin as a standard.

### Data analysis

Data shown are means (±SD) of four independent measurements. The mean values were compared for significance with a Student's *T* test. Regression analysis was performed with the sulfate and GSH uptake data after linearizations according to Lineweaver and Burk, Hanes, and Eadie and Hofstee. Results are shown only for the linearization method with the highest *R*-squared value of the best-fit line(s).

## Results

### Kinetic properties of sulfate uptake of *L. bicolor*

In the first step to address the role of mycorrhiza for plant sulfur nutrition, we analyzed sulfate uptake of the free-living mycorrhizal fungus *L. bicolor*. Aliquots of fungal mycelium were transferred into transport solutions with 20 different sulfate concentrations from 0.01 to 3 mM, and uptake of $^{35}$S-labeled sulfate was determined. With increasing sulfate concentrations, the rate of uptake showed a saturation curve (Fig. 1). The kinetic data were analyzed after linearizations according to Lineweaver–Burk, Hanes, and Eadie–Hofstee. The Eadie–Hofstee method produced best-fit lines with the highest *R*-squared values and revealed a presence of two transport systems with apparent $K_M$ values of 14.6 and 2,800 µM and apparent $V_{max}$ of 9.6 nmol $h^{-1}$ $g^{-1}$ FW and 180 nmol $h^{-1}$ $g^{-1}$ FW for the high- and low-affinity systems, respectively (Fig. 1b).

### Regulation of sulfate uptake

To find out if sulfate uptake in *L. bicolor* is regulated in the same way as in yeast and other filamentous fungi, the uptake rates were measured in free-living fungal cultures adapted either to sulfur starvation or to presence of reduced sulfur source for 3 weeks. The GSH content of fungi was measured as an indicator of their organic sulfur status (Fig. 2b). As expected, sulfur starvation decreased cellular

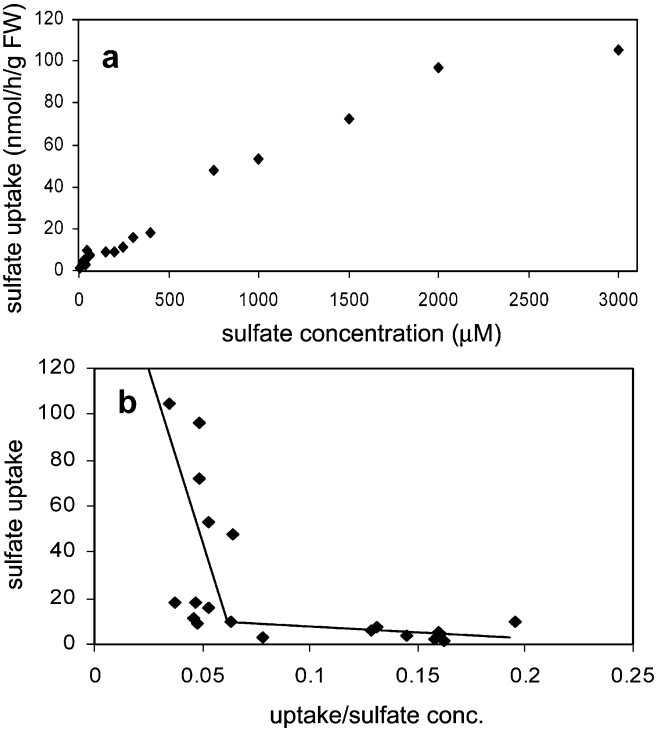


**Fig. 1** Sulfate uptake by mycelium of *Laccaria bicolor* determined using 20 different sulfate concentrations from 0.01 to 3 mM containing [$^{35}$S]$SO_4^{2-}$. **a** Concentration dependency of sulfate uptake rate. **b** Data linearized according to Eadie–Hofstee

GSH level, whereas treatment with GSH led to its high accumulation. Growth under sulfur starvation resulted in a significant increase of sulfate uptake capacity, measured after transfer of mycelium into 0.2 mM sulfate solution, compared to the control mycelium (Fig. 2a). Surprisingly, sulfate uptake was also slightly increased in fungi grown in the presence of GSH. These results suggest that sulfate uptake is derepressed in sulfur-starved *L. bicolor* similar to other organisms; however, the feedback repression of sulfate transport by GSH observed in plants, bacteria, and several filamentous fungi is overcome in this fungus.

To confirm that such regulation of sulfate uptake is not due only to long-term adaptation, we measured sulfate uptake in free-living fungal mycelium treated with sulfur deficiency and GSH for only 4 days. Similar to the long-term adaptation, after 4 days of sulfur starvation, the sulfate uptake was increased compared to control fungi (Fig. 3a). Significantly, GSH treatment again did not affect the sulfate transport rate. To test the possibility that GSH does not play any role in regulation of sulfur nutrition in *L. bicolor*, activity of the key enzyme of sulfate assimilation in fungi, the PAPS reductase, was determined. PAPS reductase activity was increased in sulfur-starved mycelium and decreased in mycelium exposed to external GSH (Fig. 3b), similar to findings in other organisms, e.g., *Escherichia coli* and yeast (Kredich 1993; Marzluf 1993). Therefore, it appears that in *L. bicolor*, reduced organic sulfur compounds still exert a feedback inhibition on sulfate reduction but, in contrast to other organisms, not on sulfate uptake.

## Glutathione uptake by *L. bicolor*

Sulfur is present in soil not only as sulfate. A great proportion of sulfur in soils occurs as organic sulfur compounds. Our previous experiments indicated that free-living *L. bicolor* is capable of GSH uptake. We therefore investigated the kinetics of the GSH transport in *L. bicolor* grown in liquid culture. With increasing GSH concentrations (0.002–0.6 mM), the rate of uptake showed a saturation curve (Fig. 4a). The kinetic data were analyzed after linearizations according to Lineweaver–Burk, Hanes, and Eadie–Hofstee. The Lineweaver–Burk method produced best-fit line with the highest *R*-squared values (Fig. 4b) revealing presence of a single transport system with apparent $K_M$ value of

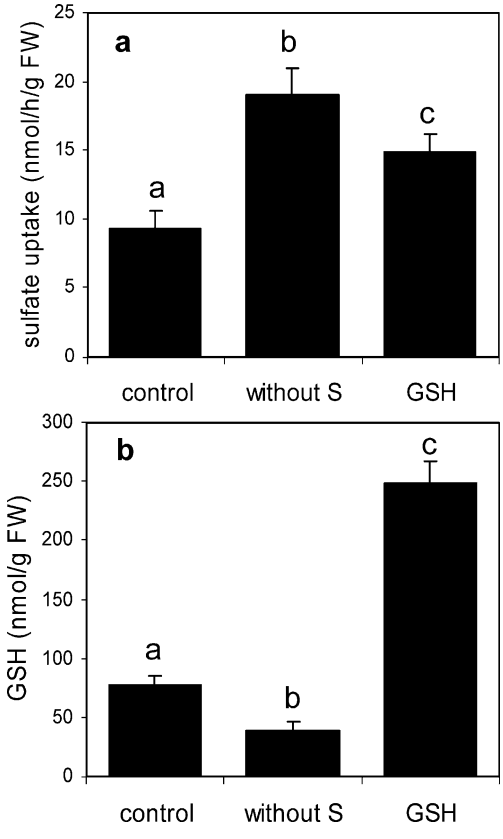


**Fig. 2** Regulation of sulfate uptake by sulfur deficiency and glutathione in *Laccaria bicolor*. Free-living fungal mycelium was grown for 3 weeks in control nutrient solution (containing 2 mM sulfate) (control), in the control solution supplemented with 1 mM glutathione (GSH), or in sulfur-free solution where all sulfate salts were exchanged for chlorides (without S). The sulfate uptake rate (**a**) and concentration of GSH (**b**) were determined. Data presented are means±SD of four independent measurements. *Different letters* indicate values significantly different with $p \leq 0.05$

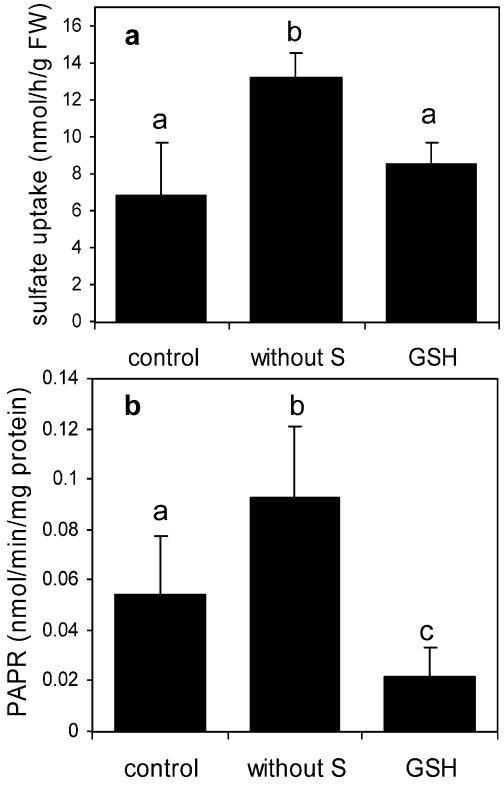


**Fig. 3** Uncoupling of the regulation of sulfate uptake and sulfate reduction in *Laccaria bicolor*. Free-living fungal mycelium was grown for 4 days in control nutrient solution (containing 2 mM sulfate) (control), in the control solution supplemented with 1 mM glutathione (GSH), or in sulfur-free solution where all sulfate salts were exchanged for chlorides (without S). The sulfate uptake rate (**a**) and activity of PAPS reductase (**b**) were determined. Data presented are means±SD of four independent measurements. *Different letters* indicate values significantly different with $p \leq 0.05$

188 μM and apparent $V_{max}$ of 58.6 nmol $h^{-1}$ $g^{-1}$ FW. This is the first demonstration of GSH uptake by a mycorrhizal fungus. The kinetic characteristics of the GSH uptake in free-living *L. bicolor* differ significantly from those of yeast which possess a high-affinity GSH transporter with $K_M$ of 54 $\mu$M (Bourbouloux et al. 2000). Addition of 1 mM glutamate or 0.1 mM pentapeptide leucine enkephaline did not affect the uptake of GSH (data not shown), revealing that the uptake is catalyzed by a specific GSH transporter and not by amino acid or peptide transporters, respectively.

## Discussion

In the first step to investigate the role of mycorrhiza in plant sulfur nutrition, we characterized sulfate uptake and its regulation in the ectomycorrhizal fungus *L. bicolor*. Our analysis revealed the presence of two sulfate uptake systems of high and low affinity in the free-living mycelium, similar to those in the yeast *Saccharomyces cerevisiae* (Breton and Surdin-Kerjan 1977). Only one uptake system, with a $K_M$ intermediate between those for high- and low-affinity systems, has been described for nonmycorrhizal filamentous fungi (Bradfield et al. 1970).

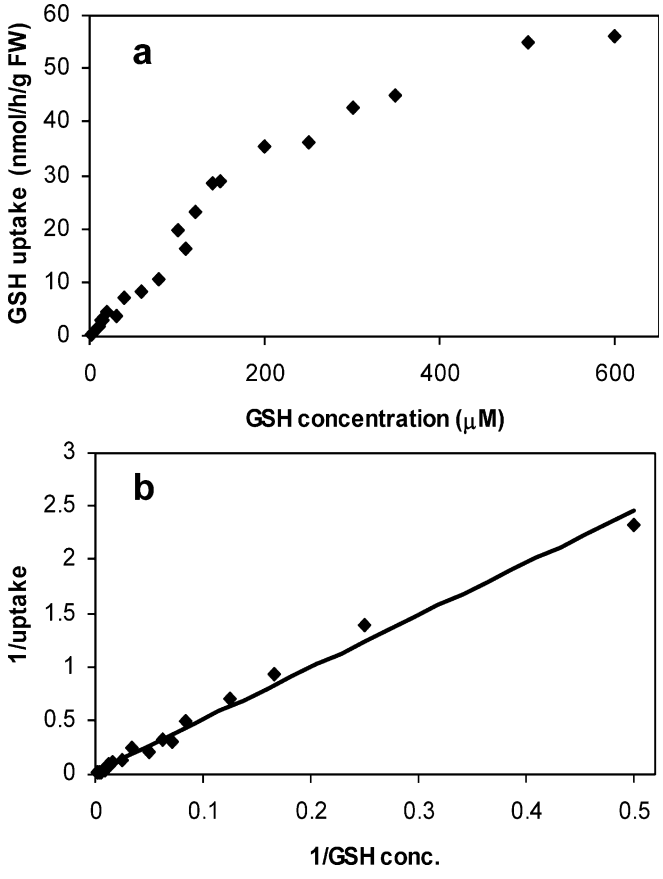


**Fig. 4** Glutathione uptake by *Laccaria bicolor* determined using 20 different GSH concentrations from 0.002 to 0.6 mM containing [$^{35}$S]GSH. **a** Concentration dependency of GSH uptake rate. **b** Data linearized according to Lineweaver–Burk

The apparent $K_M$ for sulfate of the high-affinity uptake system of free-living *L. bicolor* (14.6 $\mu$M) agrees very well with the corresponding $K_M$ of 15 $\mu$M previously reported for both mycorrhizal and nonmycorrhizal beech roots, whereas the apparent $V_{max}$ in the free-living mycelium is about twofold lower (9.6 nmol $h^{-1}$ $g^{-1}$ FW) than in mycorrhizal roots (19 nmol $h^{-1}$ $g^{-1}$ FW) or nonmycorrhizal roots (22 nmol $h^{-1}$ $g^{-1}$ FW) (Kreuzwieser et al. 1996; Kreuzwieser and Rennenberg 1998). On the other hand, the apparent $K_M$ of the low-affinity sulfate uptake systems of free-living *Laccaria* mycelium (2.8 mM) and nonmycorrhizal beech roots (10 mM) (Kreuzwieser et al. 1996) is much higher than the $K_M$ of the low-affinity transporter in mycorrhizal roots (0.16 mM; Kreuzwieser and Rennenberg 1998). This discrepancy can be explained either by changes in the kinetic parameters of the plant and/or fungal transporters in mycorrhiza, or by the existence of a new transport system, induced in the fungus or in the plant during mycorrhization.

In contrast to plants, yeast, or filamentous fungi, no information has been available until now on the regulation of sulfate uptake in mycorrhizal fungi. Similar to other organisms (Kredich 1996; Lappartient et al. 1999; Ono et al. 1999; Smith et al. 1997; Van de Kamp et al. 2000; Vauclare et al. 2002), the rate of sulfate uptake in free-living *L. bicolor* increased both after a short-term exposure and a long-term adaptation to sulfur starvation. According to a model of demand-driven control of sulfate assimilation, similar to plants, bacteria, or filamentous fungi, it was expected that the presence of a reduced sulfur compound (GSH) would repress sulfate uptake by *L. bicolor*. This, however, was not the case as the sulfate uptake was not diminished by growth of the fungus in the presence of 1 mM GSH. Thiol measurements showed that fungal GSH concentration was high under this condition so that the lack of the sulfate uptake regulation cannot be attributed to an inability to take up GSH.

These results could be explained by a general inability of GSH to regulate sulfur metabolism in *L. bicolor*. However, this is clearly not the case. In other organisms, GSH inhibits not only sulfate uptake but also its reduction (Herschbach and Rennenberg 1994; Lappartient et al. 1999; Vauclare et al. 2002; Westerman et al. 2001), and, indeed, PAPS reductase activity was reduced in GSH-treated *L. bicolor* although sulfate uptake was not. Thus, in contrast to bacteria, yeast, or plants, the feedback inhibition of sulfate reduction by GSH in *L. bicolor* seems to be uncoupled from the regulation of sulfate uptake. On the other hand, sulfur starvation affects both sulfate uptake and reduction in *L. bicolor* as in other organisms. It is clear that more work is necessary to understand the control of sulfate assimilation in mycorrhizal fungi.

High-affinity glutathione transporters have been described for yeast and plants (Bogs et al. 2003; Bourbouloux et al. 2000; Zhang et al. 2004) but not before for mycorrhizal fungi. In this study, we have shown that *L. bicolor* is able to take up GSH and that it possesses a specific GSH transporter. As in yeast and plants, the uptake rate of GSH by *L. bicolor* was not reduced due to

competition with small peptides, even though the GSH transporter belongs to the family of oligopeptide transporters (Bogs et al. 2003; Bourbouloux et al. 2000; Zhang et al. 2004). GSH transport has already been characterized in oak roots colonized by *L. bicolor*, and the kinetic parameters of GSH uptake were remarkably similar to the catalytic properties of glutamine uptake suggesting a similar transport system (Seegmüller and Rennenberg 2002). The fact that GSH transport in free-living *L. bicolor* is not inhibited by glutamate further indicates that GSH transport is specific and independent also of amino acid transport. As for sulfate uptake, the kinetic parameters of GSH transport in free-living *L. bicolor* differ from those reported for mycorrhizal roots associated with this fungus. The $K_M$ for GSH in free-living *L. bicolor* (0.19 mM) is comparable to the $K_M$ of 0.14 mM for the low-affinity uptake system of mycorrhizal roots reported by Seegmüller and Rennenberg (2002), and the $V_{max}$ is much lower in the free-living fungus (59 nmol $h^{-1}$ $g^{-1}$ FW) compared to mycorrhizal roots (360 nmol $h^{-1}$ $g^{-1}$ FW). However, the two sets of data might be difficult to compare as the incubation time with labeled GSH was 7 h for the mycorrhizal roots which is much longer than that of the experiments described in this study (1 h), so that the uptake rates calculated by Seegmüller and Rennenberg (2002) could be influenced by induction of synthesis and/or degradation of new transporters.

In conclusion, we have characterized sulfate and glutathione transport in free-living *L. bicolor* and shown that, in contrast to other organisms, sulfate uptake is not feedback-repressed by reduced sulfur compounds in this fungus. These data support our hypothesis for sulfur nutrition in ectomycorrhizal plants, where the fungus increases sulfate supply to the plant by extended sulfate uptake and the plant provides the fungus with reduced sulfur. However, further information concerning fluxes of sulfur compounds between plant and fungi is necessary to fully understand the role of mycorrhiza in plant sulfur nutrition.

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