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ACQUISITION AND TRANSFER OF SULFUR BY THE MODEL ARBUSCULAR MYCORRHIZAL FUNGUS *GLOMUS INTRARADICES*

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ACQUISITION AND TRANSFER OF SULFUR BY THE MODEL ARBUSCULAR MYCORRHIZAL FUNGUS *GLOMUS INTRARADICES*

By

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ABSTRACT

ACQUISITION AND TRANSFER OF SULFUR BY THE MODEL ARBUSCULAR MYCORRHIZAL FUNGUS *GLOMUS INTRARADICES*

By

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The amount of sulfur (S) in the environment is radically changing. Anthropogenic pollution has increased the annual worldwide terrestrial deposition of sulfur by more than 300 fold in the last 150 years. In recent decades, however, the legislated removal of S at the source has diminished S deposition by more than 6 fold in many areas of the United States, leading to increased reports of S deficiencies in crops. Technology in this area continues to simplify the process of source removal with the goal of achieving pre-industrial levels. Additionally, sulfate, the main form of S assimilated by plants, is highly mobile in soils and can leach into the water system at a rate equal to its addition to the soil surface depending on water addition. In response to these environmental changes, in recent years interest in plant S acquisition and assimilation has surged.

The arbuscular mycorrhizal (AM) fungal symbiosis has co-evolved with plants since their transition to terrestrial environments, and AM fungi are now known to associate with an overwhelming majority of land plants. The primary role of the symbiosis is to enhance the acquisition of nutrients in support of plant growth and homeostasis. Although the transfer of P, and, to a lesser extent, N, have been thoroughly analyzed, S acquisition by endo-mycorrhizal symbioses is a neglected area of research with often contradictory results. The availability of a monoxenic culture system and development of a model organism for mycorrhizal research, *Glomus intraradices*,

including a worldwide effort to sequence and annotate the genome, has opened the possibility to more definitively analyze S in relation to an AM fungal symbiosis. The goal of this thesis was to quantify and analyze the transfer of S in relation to plant demand, as well as pioneer research in the study of the regulation of S assimilation in AMF. This work complements the ultimate goal of mycorrhizal research: the use of AM fungi as a viable replacement for chemical fertilization in agriculture.

The model AMF *Glomus intraradices* transferred physiologically significant amounts of oxidized and reduced S forms to host roots, suggesting that S acquisition is a likely role of this fungus in natural ecosystems. The assimilation of sulfate by the symbiotic fungus was less regulated by reduced S sources than was the transfer to the host roots. Additionally, the simultaneous assimilation of sulfate and met or cys by pre-symbiotic mycelium is novel among studied fungi, whose sulfate assimilatory pathways are often completely diminished in response to reduced S sources. The regulation of sulfate acquisition, reduction, and assimilation was found to be greatly effected by ammonia assimilation and intracellular GSH concentration, and, unlike other fungi, less effected by reduced S forms cys and met. During germination, the addition of met led to very similar gene expression patterns compared to the addition of GSH, which did not correlate with uptake data. In general, changes in gene expression were not reflective of changes in S uptake with the exception of the down-regulation of a putative high affinity sulfate permease by cys addition in both the symbiotic and pre-symbiotic tissue. Unlike other fungi studied, transcriptional regulation does not appear to be the primary point of control for S acquisition by germinating spores of *G. intraradices*.

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TABLE OF CONTENTS

LIST OF FIGURES

Chapter 1

Introduction and Literature Review

Sulfate Assimilation in Plants and Fungi

Introduction

Associations with symbiotic fungi, which facilitate the collection of nutrients easily accessible to aquatic but not soil borne roots, may have made possible the transition of plants from aquatic to terrestrial environments. Independent estimations place the time which plants first colonized land at approximately 500 or 800 million years ago (Wellman *et al.*, 2003; Sanderson, 2003) while estimates of 600 to 1400 million years ago have been made for the divergence of the order Glomeromycota encompassing the arbuscular mycorrhizal fungi (AMF; Redecker *et al.*, 2000; Heckman *et al.*, 2001). Aside from vascular plants, the symbiosis also occurs between AMF and bryophytes, even cyanobacteria, suggesting that the symbiosis may have developed before plant roots (Read *et al.*, 2000; James *et al.*, 2006). In any case, the symbiosis is proven to have at least existed for the last 400 million years based on fossil evidence from the Rhynie chert in Scotland (Dotzler *et al.* 2009) and continues to be the dominant symbiosis on the planet (for a thorough review see Smith & Read, 2008).

In the United States, the active removal of sulfur (S) from coal and oil has dramatically decreased the rate of anthropogenic S environmental emissions for the last three decades (Baumgardner *et al.*, 2002; Lefohn *et al.*, 1999). This change has sparked numerous studies on plant S nutrition, especially as crops begin to display signs of S deficiency. Although the AM symbiosis is now accepted as a positive, sometimes definitive, contributor to phosphorus (P) and nitrogen (N) nutrition in plants, their contribution to S nutrition is still not characterized, which is the focus of this thesis.

Life Cycle

Arbuscular mycorrhizal fungi (AMF) are obligate, asexual plant symbionts involved in plant nutrient acquisition which function and complete their life cycles using photosynthetic carbohydrate (for a review, see Smith & Read, 2008). Initially, spores germinate producing germ tubes which explore the soil for a symbiotic partner. If unsuccessful, growth of the germ tube is arrested by an unknown programmed mechanism, and the cytosol retreats into the spore in preparation for re-germination (Logi *et al.*, 1998). Signals released by roots, specifically strigolactones, increase germ tube metabolism and growth (Akiyama *et al.*, 2005). This is followed by the development of an appresorial structure and the initiation of symbiosis at the root surface (Besserer *et al.*, 2008). Once the symbiosis is established, hyphae proliferate both intra- and extra-radically, forming two distinct metabolic environments (Shachar-Hill *et al.*, 1995) specialized in either the acquisition and radical distribution of photosynthetically-derived carbon substrates, or in hyphal growth through the soil matrix, acquisition, and transfer of nutrients to the plant (Smith & Read, 2008). Intra-radical mycelium (IRM) and extra-radical mycelium (ERM) can be equal in mass; however the IRM only occupies approximately 2% of symbiotic roots (Olsson & Johansen, 2000). Mechanism(s) triggering sporulation are not characterized, but appear to be connected to root senescence. Sporulation involves a massive transfer of storage lipids, proteins, and a large number of nuclei, hundreds to several thousands (Hosny *et al.*, 1998), into developing spores. After approximately three months of growth, 90% of the ERM mass is in the form of these large multinucleate spores (Olsson & Johansen, 2000).

The life cycle of AMF revolves around the conversion of plant photosynthate to triacylglycerols, which are the main nutritional form of carbon required for growth, sporulation, and germination. Glucose is imported from roots by IRM and converted to trehalose and glycogen, then to triacyl glycerides, the main storage form of C in AMF (ShacharHill *et al.*, 1995; Pfeffer *et al.*, 1999; Nagy *et al.*, 1980). Lipid bodies transport C from the IRM to the ERM and are utilized for mycelial growth and homeostasis through interconversions by the glyoxylate and gluconeogenic pathways (Bago *et al.*, 2002; Pfeffer *et al.*, 1999; Lammers *et al.*, 2001). ERM tissue is incapable of hexose uptake and is completely dependent on this transfer (Pfeffer *et al.*, 1999). In *G. intraradices*, the volume occupied by lipid bodies decreases from approximately 24% to 0.5% from host root to hyphal tip due to consumption along that length (Bago, 2000). Germinating spores and ERM can elongate and desaturate 16C fatty acids, but their synthesis is exclusive to IRM tissue (Trepanier *et al.*, 2005; Pfeffer *et al.*, 1999). The compartmentalization of fatty acid synthesis is a possible reason for the obligate nature of the symbiosis. Spores of *G. intarardices* are 20% neutral lipid by mass, 80% of which are 16C triacyl-glycerides (Olsson & Johansen, 2000), demonstrating the importance of this function for the completion of the fungal life cycle.

Morphology and Phylogeny

Gallaud (1905) was first to categorize the distinct structural classes of mycorrhizal fungi, which he termed the Arum, Paris, hepatic, and orchid types. Arum- and Paris-type endomycorrhiza predominate, and are characterized by the path of hyphal growth and morphology of absoption structures. Paris-type mycorrhizae develop entirely

intracellularly with irregularly coiled hyphae which form non-terminal coiled arbusculate structures. The endomycorrhizal fungus *Glomus intraradices* is an example of an Arum-type mycorrhizal fungus, which proliferates through intercellular air spaces and form tree-like, terminal arbuscules. These arbuscules envaginate root cortical cells, forming a periplastic space between the root cell and arbuscular membranes that is generally accepted as the site of nutrient transfer between the fungus and plant (Bago, 2000; vanAarle *et al.*, 2005). This is unlike Paris-type mycorrhizal interactions, where transfer of nutrients occurs at both the arbuscule and IRM (vanAarle *et al.*, 2005). While still unclear, the availability of intercellular air spaces may influence the dominant arbuscular mycorrhizal type (Dickson *et al.*, 2007). Surveys of plant/mycorrhizal interactions demonstrate that Arum-type fungi are mainly in symbiosis with fast-growing, high light plants, while Paris-type fungi are associated with slower growing low light or woody plants, possibly due to the presence of air spaces (Brundrett *et al*, 1990). A clear characterization of these types of AMF, however, is compromised by difficulties in discriminating between the structures, and therefore by possible misidentifications (Dickson, 2004).

Phylogenetic analyses of 28S rRNA, the large subunit of RNA polymerase II, 18S rRNA, and β-tubulin gene sequences (DaSilva *et al.*, 2006; Redecker & Raab, 2006; Shussler *et al.*, 2001; Msiska & Morton, 2009) currently place arbuscular mycorrhizal fungi (AMF) into ten families in the phylum *Glomeromycota*, order *Glomerales*, *Gigasporaceae, Glomaceae, Acaulosporaceae, Diversispora, Paraglomaceae, Geosiphoaceae, Ambisporaceae, Entrophosphosporaceae* and *Arcaesporaceae*. Within the phylum, *Glomus* is the largest genus, with more than 70 species characterized by

spores which bud from hyphal tips and germinate through the subtending hypha (Redecker & Raab, 2006). Lineages of *Glomus* are ubiquitous and often dominate AM fungal communities (Opik *et al.*, 2003), and several of them, chiefly *Glomus intraradices*, are commonly studied in laboratory settings (Smith & Read, 2008). This is the species whose genome is being sequenced (Martin *et al.*, 2008) and is the subject of the work presented here.

Plant benefits from AM symbioses

The main benefit of endomycorrhizal associations with plant roots is considered to be the improved inorganic nutrient acquisition. This is a consequence of the fungal extra-radical hyphae increasing the range of the rhizosphere, the surface area available for absorption of nutrients, and the ability to access micro-environments in soil normally unavailable to roots (Allen & Allen, 1990). However, arbuscular mycorrhizal fungal (AMF) symbioses also result in other ecologically significant benefits, including enhancement of drought tolerance and resistance to pathogenic fungal infection.

Drought Tolerance and Pathogen Resistance

As global climate change adjusts weather patterns, periods of drought are increasing in many areas of the world (Shein *et al.*, 2005) and represent a major abiotic cause of growth, fecundity, and yield limitations (Boyer, 1996). Mycorrhizal colonization has been shown to alleviate water stress (For a review see Ruiz-Lozano, 2003), possibly because hyphae radiating into the soil bridge air gaps that normally impede bulk water

flow (Allen, 2007). Colonization with *Glomus intraradices* or *Gigaspora margarita* increases stomatal conductance and drought tolerance in sorghum (*Sorghum bicolor)* and bean (*Phaseolus vulgaris*) plants independent of P nutrition (Cho *et al.,* 2006; Auge *et al.,* 2004a; 2004b).

Nutrient deficiency itself is an abiotic stress making plants more susceptible to pathogenic infections (Amtmann *et al.*, 2008), and AMF colonization enhances nutrient uptake thus enhancing the plant defensive ability. However, the symbiosis is also known to reduce pathogenic fungal frequency independent of nutritional effects (Yao *et al.*, 2002; Grandmaison *et al.,* 1993). Colonization by AMF is linked to the accumulation of specific phenolic compounds in the cell walls of *Allium cepa* roots which induce hyphal branching and control AMF development *in planta* (Grandmaison *et al.,* 1993). This effect, as well as the general resistance to fungal enzymatic cell wall degradation by phenolic compound deposition in root cell walls, provides a possible mechanism for the observed enhancement of the plant defense against invading fungi (Morandi *et al.,* 1996; Yao *et al.*, 2007).

Phosphorus Aquisition

Adsorption of phosphate is positively correlated with the concentration of Al, Mn, and Fe ions associated with clay particles (Lair *et al.*, 2009), and is the least mobile plant macronutrient in the soil (Gahoonia & Nielsen, 1991; Hinsinger, 2001). The endomycorrhizal symbiosis is postulated to have developed in response to the challenge plants faced with P nutrition during their transition from aquatic to terrestrial life (Karandashov & Bucher, 2005) based on the immobility of phosphate in terrestrial

environments as well as the clear benefits to plant P nutrition that the symbiosis confers (Hayman & Mosse, 1971; Bolan, 1991). The enhancement of P nutrition is therefore widely considered to be the major function of the endomycorrhizal symbiosis. Polyphosphates are synthesized and appear to be transported to the root depending on the availability of C for the fungus (Solaiman *et al.*, 1999; Bücking & Shachar-Hill, 2005), and root phosphate transporters have been shown to be specifically induced in several plant species by the mycorrhizal symbiosis (Javot *et al.*, 2007; Gomez *et al.*, 2009, Grace *et al.*, 2009; Nagy *et al.*, 2009; Glassop *et al.*, 2005). Recent evidence also exists which shows a connection between N and P nutrition, specifically that N fertilization may reduce colonization when P is non-limiting (Blanke *et al.*, 2005). The relationship between P and other nutrient transfer has been little studied and may be an important aspect of the function of endomycorrhizal symbioses.

Nitrogen and Sulfur Aquisition

To date the majority of studies done on endomycorrhizal symbioses examine their role in plant P nutrition (Smith & Read, 2008). However, recent evidence suggests that the biologically significant transfer of other macro- and micro-nutrients by AMF also takes place under natural conditions. While inorganic N is the preferred source for plant uptake, the majority of N in soils is often in organic form and substantial quantities of N can be transferred by AMF. Leigh and coworkers (2009) demonstrated that *Glomus intraradices* in association with *Plantago lanceolata* can transport enough N from labeled plant material to account for 20% of total plant N. The same fungus has been shown to increase the amount of N in mycorrhizal plants obtained from fertilizer by 38%, but only

under low N conditions (Azcon *et al.*, 2008). Compared to N, very little research has been done on S in relation to the endomycorrhizal symbiosis. Two studies showed little effect of AM fungi on the translocation of S (Morrison, 1962; Cooper & Tinker, 1978), while two others found significant increases in S levels in the plant (Rhodes & Gerdeman, 1978; Banjeree *et. al.*, 1999). Most dramatically, Rhodes & Gerdemann (1978) showed a 12 fold increase in the whole plant ^{35}S content in onion plants due to mycorrhizal transfer. Further research into the role of AMF in plant S nutrition is needed to re-evaluate past research and accurately assess the importance of this transfer.

Sulfate assimilation in plants

Owing to its multiple oxidation states, S is linked in some way to most plant cellular processes, including protein synthesis, redox balancing, transcriptional/post-translational regulation, central metabolism through cofactors, and defense/stress responses (Droux, 2004; Rausch & Wachter, 2005; Leustek & Saito, 1999). The cysteine component enables the efficient scavenging of oxidative metabolic byproducts by glutathione, which has been conserved since the initiation of aerobic life (Noctor *et al.*, 1998) and is necessary for the completion of the cell cycle in plants (May *et al.*, 1998). In a larger sense, the synthesis of cysteine and methionine by plants supports animal S metabolism, making plant S aquisition and assimilation an indispensible aspect of planetary animal life. The enzymatic pathway of S uptake, reduction, and assimilation, as well as the allosteric/transcriptional regulation and the role of compartmentalization in

the synthesis of the branch point of plant S metabolism, cysteine, are important aspects to the study of the role of mycorrhizal symbioses in plant S nutrition.

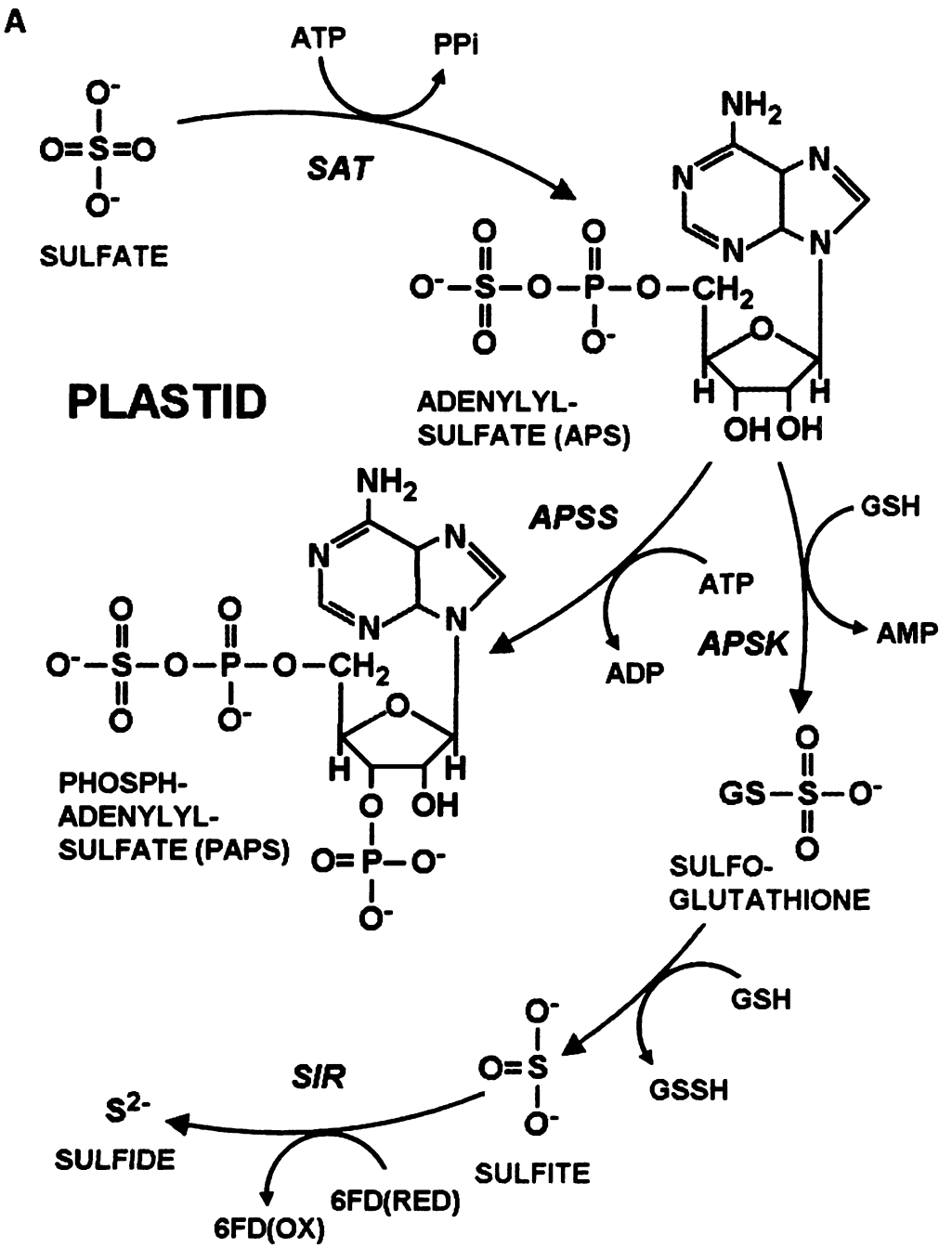
Sulfate uptake and reduction

Plant, fungal, and mammalian sulfate transporters share a common protein family and significant conservation on the amino acid level (Smith *et al.*, 1995; Vidmar *et al.*, 2000; Smith, 2003). S is imported by roots mostly as sulfate, moved around the plant with a variety of specialized transporters, and stored in vacuolar sap prior to reduction in the plastid (Ortiz-Lopez *et al.*, 2000; Leustek & Saito, 1999). The conversion of sulfate to sulfide is an energy intensive process requiring the equivalent of ten ATP (Hell & Jost, 1997). The initial activation step (Figure 1-1a), catalyzed by sulfate adenosyltransferase (SAT), produces a high energy sulfur-phosphate acid anhydride bond through the hydrolysis of pyrophosphate to form adenosyl-5'-phosphosulfate (APS; Phartiyal *et al.*, 2006). Further phosphorylation, catalyzed by APS kinase (APSK), forms the molecule 3'-phosphoadenosyl-5'-phosphosulfate (PAPS). This molecule is utilized by plants solely for direct sulfation reactions catalyzed by sulfotransferases, most notably producing glucosinolates which give mustard its distinct taste (Vauclare *et al.*, 2002). There is some debate over the mechanism by which activated sulfate is reduced to sulfite (Leustek & Saito, 1999). Based on sequence similarity with thioltransferases, it is likely that this reaction, catalyzed by APS sulfotransferase (APSS; Bick & Leustek, 1998), transfers sulfate to the cys moiety of glutathione (GSH) resulting in the spontaneous production of sulfite(Figure 1-1a). Free sulfite is further reduced through the enzyme sulfite reductase (SIR) by oxidation of either light-generated ferredoxin (Takahashi *et al*, 1996) or

glutathione (Prior *et al.*, 1999). Unlike other enzymes in the pathway which can have upwards of five isoforms, there is only one known gene encoding SIR in *A. thaliana*, and the localization of SIR to the plastid supports the exclusive reduction of sulfate in this compartment (Bork, 1998).

Figure 1-1 Model of sulfate assimilation in plant cells. Sulfate is imported into plant cell from the phloem and either sequestered in the vacuolar sap or transported into the plastid for reduction to sulfide. **(A)** In the plastid, sulfate is attached to adenosine through Sulfate Adenylyl Transferase (*SAT*) to form APS, which can be phosphorylated to PAPS by APS Kinase (*APSK*), or transfer the sulfate group to the cysteine moiety of glutathione through APS Sulfotransferase (*APSS*). PAPS production fuels direct sulfation reactions through a variety of SulfoTransferase enzymes. Sulfoglutathione reacts spontaneously with reduces glutathione to form oxidized glutathione and free sulfite, which is reduced by Sulfite Reductase (*SIR*) to sulfide. **(B)** *O*-acetylserine (OAS) is synthesized exclusively in the mitochondria by the cysteine synthase complex (*CSC*). Sulfide and OAS are transported to the cytosol by a specific transporter (open circle), where free OAS Thiol-Lyase (*OAS-TL*) enzyme catalyzes their condensation to form acetate and cysteine.

Figure 1-1


A
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PHOSPH-
ADENYLYL-
SULFATE (PAPS)
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6FD(OX)

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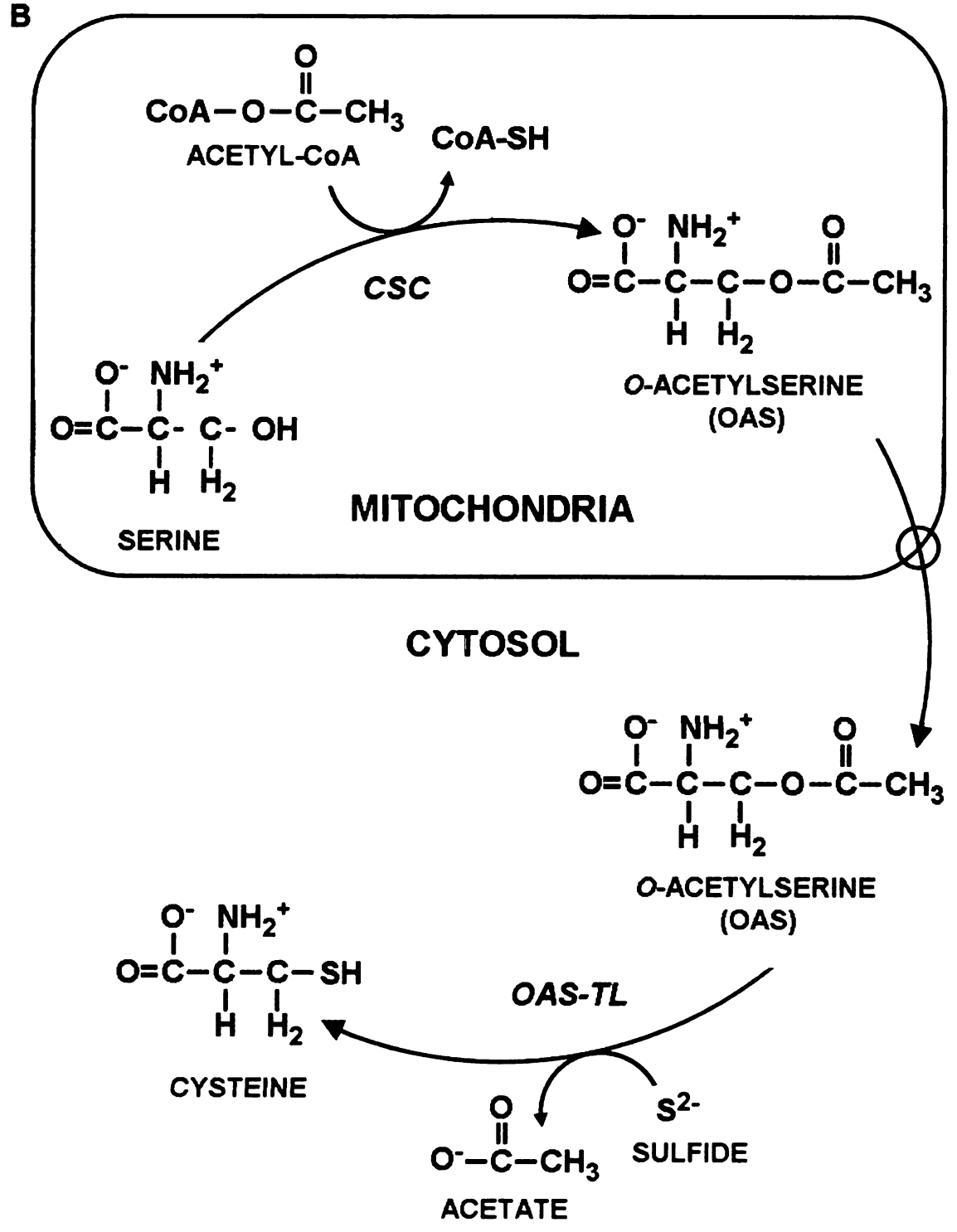


Synthesis of cysteine

Cysteine synthesis is the sole entry point of reduced S into plant central metabolism (Wirtz & Hell, 2006), and the allosteric regulation of its production differs significantly with fungi. The enzyme serine acetyltransferase (SERAT), catalyzing the formation of the C/N precursor molecule for cys synthesis, *O*-acetylserine (OAS), and *O*-acetylserine thiol lyase (OAS-TL), catalyzing the condensation of OAS and free sulfide to produce cys, together form a cysteine synthase complex (CSC; Droux, 2004). Specifically, one SERAT hexamer binds to the catalytic sites of two OAS-TL dimers to form the CSC (Feldman-Salit *et al.*, 2009), which consequently only catalyzes the formation of OAS (Wirtz and Hell, 2006; Figure 1-1b). Only free OAS-TL produces cys, but normally exhibits a 100-345 fold higher enzymatic activity than SERAT (Wirtz & Hell, 2006; Wirtz *et al.*, 2001; Wirtz *et al.*, 2004). The enzyme complex is dissociated by OAS and stabilized by sulfide *in vitro* (Berkowitz *et al.*, 2002). *In vivo* measurements in *A. thaliana* leaf cells have demonstrated that the cytosolic OAS/sulfide ratio in is too low under normal conditions to dissociate the CSC by at least ten fold (Krueger *et al.*, 2009), however, the allosteric regulation becomes important in the context of enzyme compartmentalization. In *A. thaliana*, three SERAT and nine OAS-TL isoforms have been localized to the cytosol, plastids, or mitochindria (Howarth *et al.*, 2003; Watanabe *et al.* 2008). Mutagenesis of SERAT isoforms have demonstrated a relative contribution of mitochondrial/cytosolic/plastidic OAS production of 8/2/0 (Haas *et al.*, 2008; Watanabe *et al.*, 2008). Likely due to its toxic effects on the respiratory electron transport chain (Nicholls, 1975; Truong *et al.*, 2006), sulfide is isolated from the mitochondria in *A. thaliana* and thus cys synthesis primarily occurs in the cytosol (Kreuger *et al.*, 2009).

Low sulfide and high OAS concentrations in the mitochondria allosterically control OAS synthesis by dissociation of the CSC. The high OAS-TL/SERAT ratio in the cytosol ensures cys synthesis, which is itself feed-back inhibited (Noji *et al.*, 1998).

Transcriptional regulation

Transcriptional regulation of the sulfate assimilation pathway is reflective of the toxicity of sulfite and sulfide to plant cells, controlling enzymatic steps prior to their formation. The constitutive expression of SERAT and OAS-TL (Maruyama-Nakashita *et al.*, 2003; Wirtz & Hell, 2006) suggests that transcriptional regulation may not be conducive to the homeostatic maintenance of cytosolic and plastidic sulfide concentrations. This may explain the complicated allosteric regulation of the CSC. Several analyses have revealed a significant down-regulation of only two principle enzymes, SAT and APSS (Kopriva, 2006). Of these two genes, APSS is under greater transcriptional control (Vauclare *et al.*, 2002), and is the only gene in the pathway up-regulated by S deprivation (Maruyama-Nakashita et al., 2003). It remains controversial if GSH or cys elicit transcriptional control of APSS (Vauclare *et al.*, 2002; Lee *et al.*, 2005). Recently a trans-acting positive transcriptional regulator of sulfate assimilation was discovered (SLIM1; Maruyama-Nakashita *et al.*, 2006). Although analogous to fungal transcriptional factors MET4/CYS3/METR, previous evidence does not yet support equally strict regulation of gene expression in plants.

Sulfur in agriculture

Intensive agricultural practices including tillage and phosphorus amendment, coupled with research showing the soil solution phosphorus concentration-dependent rejection of the AMF symbiosis by plant roots (Smith & Read, 2008), have led to the believe that the symbiosis is nonexistent in these types of agricultural systems. However, a recent survey of 90 agricultural sites in the Chinese Sichuan province revealed an abundance of AMF biomass and species diversity (Wang *et al.*, 2008), suggesting a possible role for AMF in current agricultural practices. Examining the relationship between plant S nutrition and endomycorrhizal symbioses is a necessary step in evaluating the use of AMF in agriculture for this purpose.

Sharp increases of S deficiency over the past two decades, especially in Western Europe, have made S nutrition an increasingly important topic in agricultural research. Fossil fuel combustion for the production of electricity is responsible for 63% of the total atmospheric sulfur dioxide emissions (Baumgardner *et al.*, 2002; Lefohn *et al.*, 1999). The reduction in industrial S emissions from coal combustion has had a dramatic effect on sulfur deposition. Additionally, changes in agricultural practices aimed at increasing the efficiency of fertilizer application, which previously and perhaps unknowingly relied on sulfur deposition as a source of fertilizer, now contribute to the S deficiency problem (Riley *et. al.*, 2000). Many crop yields have doubled over the last thirty years, increasing the demand on agricultural soils for all nutrients. Between 1974 and 1990, the amount of N utilized worldwide has roughly doubled associated with an equal rise in yields (Ceccotti, 1996). In comparison, S fertilization has remained the same or has decreased

due to the popular use of low-S fertilizers (*i.e.* urea) over S-containing fertilizers (*i.e.* ammonium sulfate) (Riley *et. al.*, 2000). Fertilization with N also leads to the activation of amino acid synthesis, increasing the demand for S, which if unavailable may lead to yield decreases on a regulatory level. Sulfur limitation in wheat results in a decrease in grain size (Zhao *et.al.*, 1999) while having little effect on vegetative growth.

The role of microorganisms in plant S nutrition

Soil adsorption of sulfate ions is negatively correlated with the soil pH, and represents on average 2-5% of the total amount of S in the soil (Eriksen and Askegaard, 2000). At pH's >6.5, it is virtually non-existent due to the neutralization of pH dependent charges within the soil structure which otherwise bind anions, such as Al and Fe oxides (Scherer, 2001). The application of other anions, such as phosphate, lowers the adsorption and therefore increases the availability of sulfate to plants as well as to leaching effects. One effect of regular fertilization with phosphate is the decrease of sulfate in the soil by filling adsorption sites with phosphate anions, allowing sulfate ions to mobilize into unreachable areas for plant roots. Sulfate is highly soluble in water at normal pH ranges, resulting in efficient leaching from precipitation. The leaching of soil on an organic farm in Denmark was found to be 20 kg S ha^{-1} (Eriksen and Askegaard, 2000). This is likely the reason why approximately 98% of S found in soils is in an organically bound form (Scherer, 2001).

Microorganisms in the soil convert available sulfate to organic esters or carbon-bonded S. In laboratory-grown fungal cultures, sulfate concentration is directly

proportional to the synthesis of a common organic form of S, choline sulfate (Saggar *et al.*, 1981). Microbes re-mineralize organically bound sulfur when in need of carbon as an energy supply. The amount of sulfate present in soils is therefore governed by the relative rates of mineralization of organic S and the immobilization of free sulfate into organic compounds, which are both mainly the result of microbial activity. An average 6 to 39 kg S ha^{-1} of S is made available to plants in a variety of crop systems per year due to mineralization (Scherer, 2001). By contrast, Kühn and Weller (1977) conducted lysimeter experiments on fields with or without S fertilization, and found that sulfate additions simply leached out of the soil. It is likely that fertilization with sulfate fails to increase the total amount of S in soil over time due to leaching, making mineralization of organic S by soil microbes the most constant source of available S for plant nutrition.

Fungal sulfate assimilation

Compared with plants, fungi have similarities and differences in their uptake, reduction, and assimilation of sulfate (Figure 1-2a), most notably in their use of *O*-acetylhomoserine or *O*-acetylserine, or both, as final sulfide acceptors. In general, transcriptional regulation is coordinated among several assimilatory enzymes by a positive bZIP transcription factor. However, the regulation of this factor differs significantly between species, as does the allosteric regulation of the individual pathway enzymes. Most research in this area is done using one of three fungal model systems: *Saccharomyces cerevisiae*, *Aspergillus nidulans*, or *Neurospora crassa*. Aspects of S assimilation will be discussed in detail with respect to each of these fungi.

Saccharomyces cerevisiae

The reduction of sulfate to sulfide in *S. cerevisiae* only differs in one aspect from reduction by plants, although the two systems differ significantly in sulfide assimilation. Sulfate is imported through single high or low affinity sulfate transporters (SUL1;SUL2) and activated by attachment to the terminal phosphate group of ATP by the enzyme S-adenosyl transferase (MET3) forming 5'-adenylylsulfate (APS). APS is further phosphorylated by APS kinase (MET14) to 3'-phospho-5'-adenylylsulfate (PAPS). PAPS is considered toxic to cells (Thomas & Surdin-Kerjan, 1997), which may explain why in plants APS is further reduced to sulfide and PAPS is primarily used for direct sulfation. In fungi, sulfate reduction takes place through PAPS (Figure 1-2a), and the toxicity is likely countermanded by the action of a kinase which both returns PAPS to APS and is necessary for the hydrolysis of PAP to AMP (Murguía *et al.* 1996). Also unlike plants, in *S. cerevisiae* sulfide is assimilated by reaction with *O*-acetylhomoserine (OAH) through the enzyme homocysteine synthase (MET25), not with *O*-acetylserine through cysteine synthase (Figure 1-2b). Cysteine is then synthesized from homocysteine through the intermediate molecule cystathionine by a pathway that is absent in plants (Ono *et al.*, 1999), the reverse-transsulfuration or cystathionine pathway (CT pathway). In the CT pathway, homocysteine reacts with serine to form cystathionine through the enzyme cystathionine β-synthase (STR4). Cystathionine is then catabolized to α-ketobutyric acid, ammonia, and cysteine by the enzyme cystathionine γ-lyase (STR1). Homocysteine is also directly converted to methionine by homocysteine methyl transferase (MET6), making it the branch point of the pathway.

Figure 1-2 Model of sulfate assimilation in fungi. **(A)** Sulfate is imported by specialized high and low affinity sulfate permeases (*SUL*). Reduction to sulfide requires activation through adenylation by sulfate adenyly transferase (*SAT*) and further phosphrylation of the adenosyl moiety by adenyly phosphosulfate kinase (*APSK*). The sulfate moiety is attached to a cys residue of the phosphoadenyly phosphosulfate reductase enzyme (*PAPSR*) and reduced by two electrons. Sulfite is further reduced by thioredoxin and six electrons to sulfide by sulfide reductase (*SIR*). **(B)** Sulfide can enter fungal metabolism at cysteine synthase (*CS*) or *O*-acetylhomoserine thiol-lyase (*OAH-TL*), the latter requiring an active reverse transsulfuration pathway for the conversion of homocysteine to cysteine through cystathionine beta synthase (*CBS*), forming the intermediate cystathionine from homocysteine and serine, and cystathionine gamma lyase (*CGL*), which splits cystathionine to cysteine and homoserine.

Figure 1-2

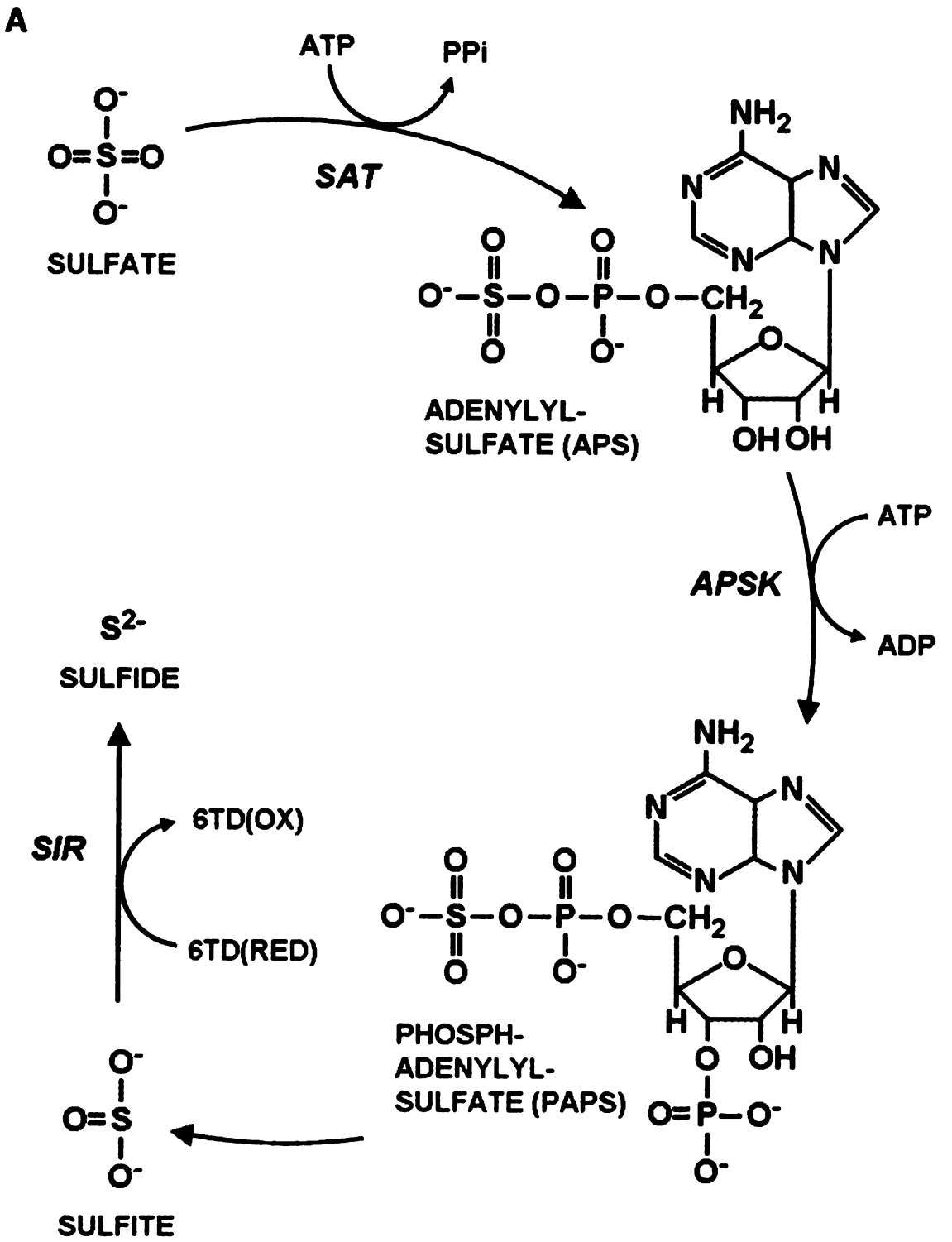
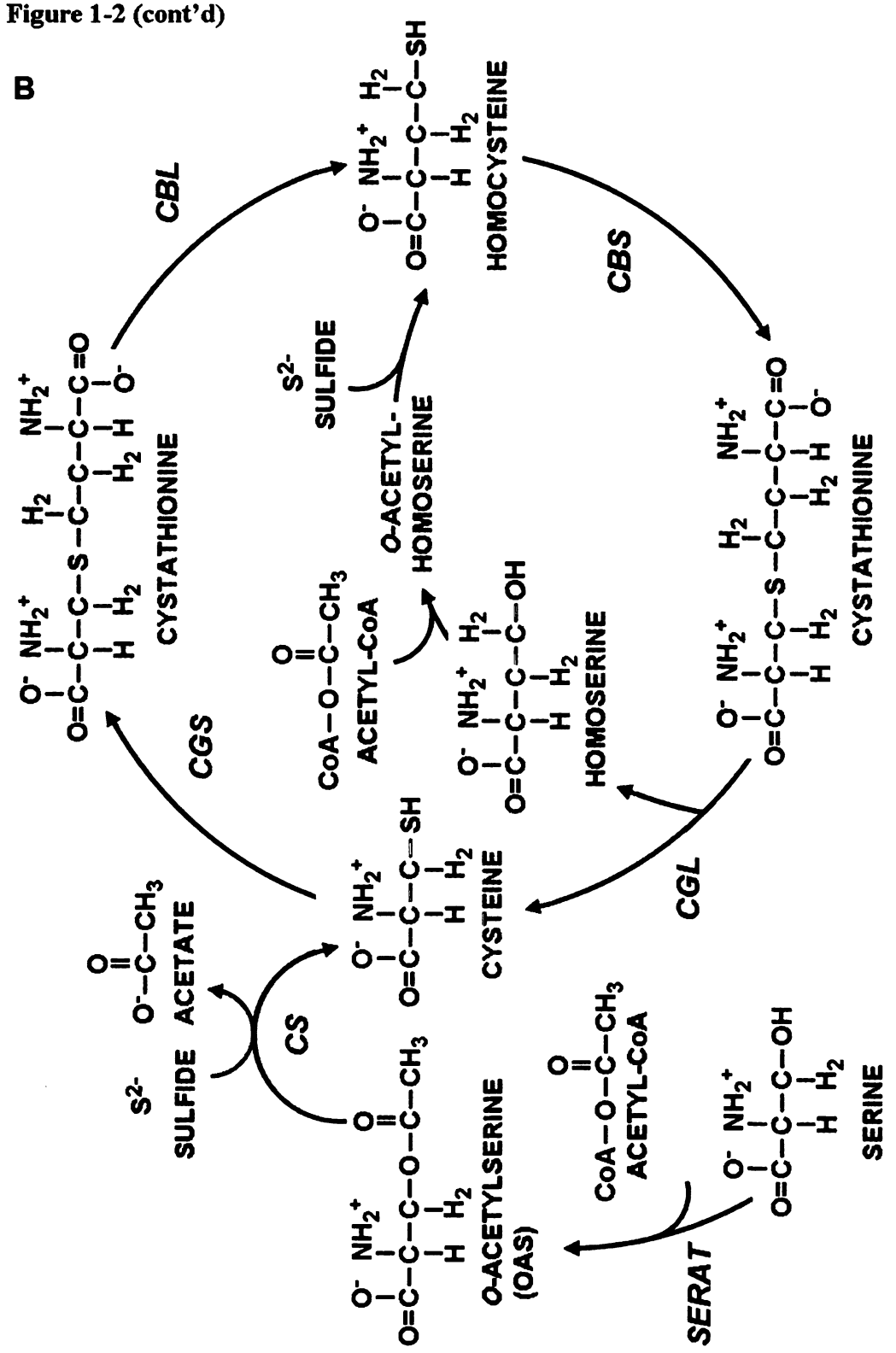

A
ATP
PPi
SAT
SULFATE
ADENYLYL-
SULFATE (APS)
ATP
APSK
ADP
S2-
SULFIDE
SIR
6TD(OX)
6TD(RED)
PHOSPH-
ADENYLYL-
SULFATE (PAPS)
SULFITE

Figure 1-2 (cont'd)



Sulfate reduction is a highly regulated process in *S. cerevisiae* involving feedback inhibition of enzymatic processes at multiple steps and strict control over the synthesis of new enzymes in the pathway (Marzluf, 1997). At the point of entry, the activity of both SUL1 and SUL2 is strongly inhibited by APS, and the synthesis of these two structural genes is completely suppressed by 0.8 mM methioine (met) and 0.2 mM 5'-adenosylmethionine (Adomet) (Breton and Surdin-Kerkan, 1977). SAT is a homohexamer *in vivo* (Ullrich, 2001), and each monomer can be separated into four domains. Of these domains, the SAT catalytic domain is linked to a domain with structural homology to APS kinase which does not bind PAPS and has no kinase activity, and is possible evidence of an evolutionary relationship with a bi-functional enzyme (Lalor *et al.*, 2003). The fungus *Penicillium chrysogenum* shares this APS kinase domain, which does bind to PAPS, decreasing the affinity of the catalytic domain for sulfate (MacRae *et al.*, 2002). Allosteric inhibition of SAT by PAPS therefore may have been lost in this organism. However, in *S. cerevisiae* the catalytic activity of SAT is strongly inhibited by the product (APS) as well as by sulfide to a lesser extent (deVito & Dreyfuss, 1964). The synthesis of SAT is suppressed by met and, interestingly, de-repressed by exogenous cys. In contrast, synthesis of sulfite reductase is most strongly suppressed by cys and only partially suppressed by met (deVito & Dreyfuss, 1964). Sulfite reductase activity is also strongly regulated by NADP+, which completely represses the synthesis of sulfide (Yoshimato & Sato, 1967). Exposure to met has also been shown to increase the synthesis of the CT pathway enzymes, cystathionine β-synthase and cystathionine γ-lyase (Paszewski & Grabski, 1974). The toxicity of PAPS

and the high energetic cost of sulfate reduction are likely reasons for the multi-level control of this pathway in *S. cerevisiae* and other fungi.

The transcription of genes involved in methionine biosynthesis is controlled by the intracellular concentration of Adomet sensed by a positive *trans*-acting basic leucine zipper transcriptional factor, Met4p (Thomas *et al.*, 1992; Cherest *et al.*, 1985), although methionyl-tRNA is also involved in regulation (Surdin-Kerjan *et al.* 1973). The regulatory function of Met4p depends on its association with a complex of proteins including a general transcription factor, centromere binding factor-1 (CBF-1), and a second S assimilation regulatory factor, Met28p, whose expression is itself controlled by Met4p (Kuras & Thomas, 1995). Under conditions of low met availability, the Met4p transcriptional activation complex associates with promoter regions of genes encoding S-adenosyl transferase (MET3), APS kinase (MET14), PAPS reductase (MET16), *O*-acetyl homoserine sulfydrolase (MET25), PAPS kinase (MET22), and the first enzyme for the synthesis of glutathione, glutamylcysteine synthetase (GSH1) (Kuras *et al.*, 2002; Wheeler *et al.*, 2002), activating transcription. The activity of the Met4p complex is controlled through the alteration or catabolism of Met4p by ubiquitylation (Kuras *et al.*, 2002). Ubiquitylation of Met4p is conducted by ubiquitin ligase complex associated with an F-box protein, Met30p (Rouillon *et al.*, 2000) whose activity is directly proportional to its concentration. Met30p transcription is positively regulated by Met4p when Adomet is abundant, so Met4p regulates its own breakdown (Smothers *et al.*, 2000). However, ubiquitylated Met4p is only degraded when *S. cerevisiae* is grown in minimal medium amended with met. In rich media, ubiquitylated Met4p is not only stable, but selectively activates genes encoding S-adenosyl methioine synthetic enzymes to supply one carbon

metabolism (Kuras *et al.*, 2002), thus serving as two distinct transcription factors. The complex between ubiquitin ligases and Met30p is stabilized by met, linking S metabolism to the regulation of S assimilation (Mountain *et al.*, 1993). Interestingly, the transcription of MET4 is not regulated by S source (Mountain *et al.*, 1993), but is regulated by another bZIP transcription factor, Gcn4p, which also regulates amino acid synthetic genes. Synthesis of met and cys is therefore coordinately regulated with general amino acid synthesis as well as the intracellular Adomet concentration.

Aspergillus nidulans

Sulfate reduction in *A. nidulans* is analogous to the *S. cerevisiae* pathway with few exceptions. Sulfate permease, while competitively inhibited by sulfate, accumulates intracellular sulfate up to a concentration of 0.04 M in a knock-out mutant of S-adenosyl transferase (sC), and a down-stream product is responsible for its allosteric inhibition (Bradfield *et al.*, 1970). S-adenosyl transferase is competitively inhibited by its product, APS, but unlike *S. cerevisiae*, it is allosterically inhibited by PAPS (Borgeswalmsley *et al.*, 1995; Renosto *et al.*, 1990). Growth in a high concentration of met (5 mM) completely abolishes sulfate assimilation measured by ^{35}S labeling (Paszewski *et al.*, 1984), however transcription of APS kinase (sD) and cysteine synthase (cysB) does not appear to be affected (Clarke *et al.* 1997; Topezewski *et al*, 1997). In *S. cerevisiae*, exogenously supplied met leads to the accumulation of met and Adomet, while in *A. nidulans* it results in cystathionine accumulation (Pieniazck *et al.*, 1973), which may explain the difference in transcriptional regulation by met between *Saccharomyces* and *Aspergillus*. The likely point of transcriptional regulation of sulfate

reduction is at the level of entry. Sulfate permease transcripts are reduced by at least four fold in high met (Grynberg *et al.*, 2001). An early study of pathway regulation indicates that sulfate permease, S-adenosyl transferase, and sulfite reductase protein levels are significantly reduced in cys, homocysteine, and met amended media (Paszewski & Grabski, 1974), which is antithetical to current gene expression data and has not since been repeated. These data led to the commonly held belief that the pathway is regulated by cys (Marzluf, 1997). Few studies to date have analyzed transcriptional regulation of sulfate reduction enzymes by cys, making this hypothesis difficult to evaluate. Unlike *S. cerevisiae*, *A. nidulans* possesses functional OAS and CT pathways. The CT pathway is now known to be the primary source of cys only when the OAS pathway is blocked (Paszewski & Grabski 1974; Pieniazck *et al.*, 1974), and the only biologically relevant pathway of cys synthesis under normal growth conditions in *A. nidulans* is through cysteine synthase (Brzywczy *et al.*, 2007).

Akin to *S. cerevisiae*, a positive acting bZIP regulatory protein was discovered for *A. nidulans*, MetRp, now known to be responsible for the transcriptional activation of homocysteine synthase (cysD), S-adenosyl transferase (sC), and cysteine synthase (cysB) (Natorff *et al.*, 2003). A knock-out mutation of MetR reduces incorporation of S from exogenous sulfate by 87% (Natorff *et al.*, 2003). Neither MetR nor Met4 are transcriptionally regulated by S sources themselves (Natorff *et al.*, 2003; Mountain *et al.*, 1993), but are regulated on the protein level apparently by similar mechanisms. Additionally, four negative regulatory proteins have also been discovered through mutagenesis studies, sulfur controller proteins (scon) A, B, C, and D (Natorff *et al.* 1993). The genetic sequence of sconB is homologous to met30 from *S. cerevisiae* and

contains an F-box motif for associating with ubiquitin ligase complexes as well (Natorff *et al*, 1998). Based on these similarities, it is likely, although not currently proven, that *S. cerevisiae* and *A. nidulans* contain similar ubiquitylation-based control systems for the regulation of sulfate assimilation.

Neurospora crassa

Although *N. crassa* was the model fungal system in which much of the research on global regulation of the sulfate assimilation pathway was first conducted, considerably less is known about regulation on the enzymatic level compared to *S. cerevisiae* or *A. nidulans*. Two sulfate permeases have been characterized, specific to either mycelial growth (cys-14) or germination (cys-13), and are highly expressed under S-limiting conditions (Ketter *et al.*, 1991). The synthesis of either isozyme is repressed completely by high external concentrations of met and feedback inhibited by sulfate up to a maximum of 60% (Marzluf, 1972). Interestingly, there is a time period in which there is no effect of met addition on the synthesis of cys-13 which lasts for approximately the first six hours of spore germination (Marzluf, 1972). This appears to be the only instance when germination-specific regulation of sulfate reduction was measured in fungi, and suggests that during this time transcriptional control of the pathway may differ from normal growth. Like *A. nidulans*, *P. chrysogenum*, and *P. duponti*, *N. crassa* sulfate adenylyl transferase enzyme is allosterically regulated by PAPS (Renosto *et al.*, 1990). Also similar to *A. nidulans*, *N. crassa* possessed functional CT and OAS pathways (Flavin & Slaughter, 1964; Marzluf, 1997), and cystathionine γ-lyase is strongly feedback regulated in by cysteine both fungi, as much as 92% in *N. crassa* (Paszewski & Grabski

1974; Flavin & Segal, 1964). The relative contribution of the two pathways has not been measured in *N. crassa* as it has in *A. nidulans*, but the overall similarity between the two fungal pathways suggests that the OAS pathway may be dominant in *N. crassa* as well.

The sulfate assimilatory pathway in *N. crassa* is transcriptionally regulated by a positive and negative acting control system, Cys3/Scon2, analogous to the Met3/Met30 system in *S. cerevisiae* and the MetR/SconB system in *A. nidulans* (Fu & Marzluf, 1990; Fu *et al.*, 1989; Paietta, 1990; Sizemore *et al.*, 2002; for a review see Marzluf, 1997). The F-box motif, now recognized as an important component of cell cycle regulation, was first discovered during the analysis of the negative-acting sulfur controller gene scon2 by Kumar and Paietta (1998), who also demonstrated that scon2 remains functional when its F-box motif is replaced with the analogous sequence from Met30. Scon2 is transcriptionally activated by the positive-acting transcriptional regulatory protein Cys3, analogous to the activation of Met30 by Met4 in *S. cerevisiae* (Paietta, 1990; Smothers *et al.* 2000). Another negative acting regulatory protein which associates with scon2 in the formation of a ubiquitin ligase complex, scon3, is homologous to sconC from *A. nidulans*, and like sconC and MetR, is not transcriptionally regulated by Cys3 (Sizemore *et al.*, 2002). Cys3 is necessary for the activation of genes encoding enzymes of the sulfate assimilatory pathway including cys14 and cys13 (Jarai & Marzluf, 1991; Marzluf & Metzenberg, 1968).

Conclusion and Rationale

The rise and fall of sulfur emissions is among the greatest anthropogenic environmental changes in history. According to ice core analysis, the amount of sulfate deposited from sea water per year has risen by 75% since 1850 (Mayaweski et al, 1990), resulting in the addition of approximately 4.5 billion metric tons globally (Lefohn et al, 1999). Although these emissions continue to increase on a global scale, the Clean Air Act has led to a striking decrease in S pollution in the US over the last three decades. In rural areas of the eastern part of the US, sulfate deposition fell by 29% between 1990 and 2003, a figure which correlates closely with the 33% emissions drop from power plants in the area over the same time period (Baumgardner *et al.*, 2002; Lefohn *et al.*, 1999). Based on current legislation, emissions are projected to decrease a further 11% by the year 2025 with nothing likely to prevent their future decline to near pre-Industrial Revolution levels.

The simplest solution to the impending necessity to replace the S now available to crop plants from atmospheric deposition is the fertilization of crops with sulfate. This is an inexpensive and readily available solution as the legislation requiring removal of S from coal and oil has led to a large excess of available sulfate. However, the soil mobility of sulfate can result in roughly equivalent rates of environmental input from soil leaching (Kühn & Weller, 1977). Therefore the use of sulfate to amend S deficiencies in crops will necessarily lead to the reintroduction of a pollutant into the water system, at least partially negating one of very few environmental success stories of the twentieth century. This thesis provides a foray into an alternative means of supplementing plant sulfate by

examining the transfer of S through a mycorrhizal symbiosis already known to enhance P and N nutrition in plants.

The role of AMF in plant S acquisition has been a largely neglected scientific problem. Previous studies produced inconclusive, often conflicting results (Morrison, 1962; Cooper & Tinker, 1978; Rhodes & Gerdeman, 1978a; 1978b; Banjeree *et. al.*, 1999). Recently, greater attention has been given to the role and regulation of S metabolism in plants (Kopriva, 2006; Rausch & Wachter, 2005; Bloem *et al.*, 2005) which, with environmental reasons already discussed, make investigations into S transfer through an AMF important. In response, three related studies were conducted of the quantification and regulation of S transfer using a monoxenic culture system (chapter 2), the regulation of S assimilation in the pre-symbiotic stage (chapter 3), and of connections between the regulation of N and S assimilation (chapter 4). The results of these studies demonstrate the ability of an AMF to import and assimilate both organic and inorganic S in the pre-symbiotic and symbiotic stages of development, as well as transfer physiologically relevant quantities of S to host roots.

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Chapter 2

Sulfur Transfer through an Arbuscular Mycorrhiza

The contents of this chapter are modified from an article published in Plant Physiology.

Allen JW, Shachar-Hill Y. 2009. Sulfur transfer through an arbuscular mycorrhiza. *Plant Physiology* **149**(1): 549-560.

Abstract

Despite the importance of sulfur for plant nutrition, the role of the arbuscular mycorrhizal symbiosis in sulfur uptake has received little attention. To address this issue, ^{35}S labeling experiments were performed on mycorrhizas of transformed carrot roots and *Glomus intraradices* grown monoxenically on bi-compartmental Petri dishes. The uptake and transfer of $^{35}SO_4^{2-}$ by the fungus and resulting ^{35}S partitioning into different metabolic pools in the host roots was analyzed when i) altering the sulfate concentration available to roots and ii) supplying the fungal compartment with cysteine, methionine or glutathione. Additonally, the uptake, transfer and partitioning of ^{35}S from the reduced S sources [^{35}S]cys and [^{35}S]met was determined. Sulfate was taken up by the fungus and transferred to mycorrhizal roots, increasing root S contents by 25% in a moderate (not growth limiting) concentration of sulfate. High sulfate levels in the mycorrhizal root compartment halved the uptake of $^{35}SO_4^{2-}$ from the fungal compartment. The addition of 1 mM methionine, cysteine, or glutathione to the fungal compartment reduced the transfer of sulfate by 26%, 45% and 80% respectively over one month. Similar quantities of ^{35}S were transferred to mycorrhizal roots whether $^{35}SO_4^{2-}$, [^{35}S]cys, or [^{35}S]met was supplied in the fungal compartment. Fungal transcripts for putative sulfur assimilatory genes were identified, indicating the presence of the trans-sulfuration pathway. The suppression of fungal sulfate transfer in the presence of cysteine coincided with a reduction in putative sulfate permease and not sulfate adenylyltransferase transcripts,

suggesting a role for fungal transcriptional regulation in S transfer to the host. A testable model is proposed describing root S acquisition through the AM symbiosis.

Introduction

Plants and arbuscular mycorrhizal (AM) fungi have been co-evolving since the Devonian period (Simon *et al.*, 1993; Brundrett, 2002). Today the AM symbiosis involves an estimated 80% of land plant species and 92% of plant families (Wang & Qiu, 2006). This symbiosis augments nutrient uptake by roots, which deplete the rhizosphere at a rate dependent on water availability, as well as the concentration and soil mobility of the nutrient (Bhat *et al.*, 1976; Li *et al.*, 1991; Gahoonia *et al.*, 1994; Wang *et al.*, 2004). Phosphorus is particularly immobile in soils (Gahoonia & Nielsen, 1991) and the AM symbiosis can increase plant growth in P deficient soils 19 fold (Hayman & Mosse, 1971). Although P acquisition is a major benefit of the symbiosis, a growing body of research points towards a more complex role for arbuscular mycorrhizas in nutrient uptake (Lambers *et al.*, 2008; Liu *et al.*, 2002). Nitrogen nutrition, for instance, is improved in a number of crop species (Perner *et al.*, 2008; Ortiz-Ceballos *et al.*, 2007) and a monoxenic culture system consisting of transformed roots in symbiosis with the mycorrhizal fungus *Glomus intraradices* has been used to demonstrate the capacity of this symbiosis to transfer N (Govindarajalu *et al.*, 2005; Jin *et al.* 2005).

Plants take up S primarily as the sulfate anion (Leustek, 1996), which is often found in low concentrations in the soil. The uptake and utilization of S by plants is reviewed by Leustek (2000) and Rennenberg *et al.* (2007). Sulfate is mobile and commonly lost through soil leaching (Eriksen & Askegaard, 2000). Because of this, typically 95% of the soil S is in organically bound forms such as sulfate esters (Scherer, 2001; Tabatabai, 1984) synthesized by soil microorganisms (Fitzgerald, 1976; Spencer &

Harada, 1960). Such forms of S are not thought to be significant sources for plants (Leustek, 1996). In the past, the effects of leaching have likely been masked by the high input of S from atmospheric pollution. However, the drastic reductions in S deposition in North America and Europe over the last decade have led to an equally dramatic rise in reported cases of S deficiency in crop species (Lefohn *et al.,* 1999; Baumgardner *et al.,* 2002; Riley *et al.,* 2000). Under conditions of low S availability, an arbuscular mycorrhizal symbiosis can increase the percentage of S in pot grown onions (Guo *et al.,* 2007) and maize (Banjeree *et al.,* 1999). The ability of mycorrhizal fungi to transfer N and P from organic compounds (Joner & Jakobsen, 1994; Hawkins *et al.,* 2000, Govindarajulu *et al.* 2005) also suggests the possibility that mycorrhizal plants might obtain S from organic sources.

The small number of reports on the effects of AM colonization on the uptake of sulfur, which is present in amount equal to P in plants, have presented differing results (Gray & Gerdemann, 1973; Rhodes & Gerdemann, 1978a; Cooper & Tinker, 1978). The pioneering study by Gray & Gerdemann (1973) showing that mycorrhizal colonization in clover and maize increased ^{35}S uptake compared to non-mycorrhizal plants was repeated by Rhodes & Gerdemann (1978a) in onion, but shown to be heavily influenced by P nutritional benefits. Rhodes & Gerdemann (1978b) were also the first to examine the transfer of $^{35}SO_4^{2-}$ through a mycorrhizal fungus exposed to an isolated source unavailable to the host plant. In a study published the same year, mycorrhizal clover grown in sterile bi-compartmental soil/agar plates failed to show a significant amount of transfer of isolated S through the fungus (Cooper & Tinker, 1978). More recent research on ectomycorrhizas is more definitive on the subject (For a review see Rennenberg,

1999). In the association between Beech seedlings and the ectomycorrhizal fungi *Laccaria laccata* or *Paxillus involutus*, only *L. laccata* was shown to increase S uptake in S deficient conditions, and only when N was supplied (Kreuzwieser & Rennenberg, 1998). Interestingly, an analysis of free-living *Laccaria bicolor* showed that in contrast with the results from other filamentous fungi and yeast, sulfate uptake was unaffected by the presence of sulfur metabolites (Mansouri-Bauly *et al.*, 2006). This may be an adaptation enabling the fungus to continue supplying sulfate to the host after its own needs are met (Mansouri-Bauly *et al.*, 2006).

Though little is known about the mechanism of S assimilation and its regulation in mycorrhizal fungi, these pathways have been extensively characterized in other fungal species (For a review see Marzluf, 1997). Cysteine (cys) is the major entry point of reduced S into metabolism, and is formed by the reaction of sulfide (made by the successive reduction of sulfate to sulfite and of sulfite to sulfide) with *O*-acetylserine catalyzed by cysteine synthase. However, through the reverse trans-sulfuration pathway, both *Aspergillus* and *Neurospora* species are able to efficiently convert methionine (met) to cys (Marzluf, 1993), making met an efficient sulfur source for these fungi. This pathway is crucial in *Saccharomyces cerevisiae*, where sulfide is assimilated not though cysteine synthase, but through the sulfhydrolation of *O*-acetylhomoserine to form homocysteine from which met is made (Ono *et al.*, 1999; Thomas & Surdin-Kerjan, 1997). By contrast, *Schizosaccharomyces pombe* has been shown to be deficient in reverse trans-sulfuration pathway enzymes making it dependent on cysteine synthase for S assimilation (Brzywczy *et al.*, 2002). This results in a reduced regulatory role for met in *S. pombe* (Brzywczy *et al.*, 2002). There is also evidence that the S assimilation enzymes

are transcriptionally regulated in *S. cerevisiae* specifically by levels of cys (Hansen & Johannesen, 2000). Both positive and negative global regulatory proteins have been discovered in *N. crassa*, *A. nidulans*, and *S. cerevisiae* that share homology at the protein level (Marzluf, 1997). In *N. crassa* and *S. cerevisiae*, the expression of the S assimilatory genes is almost completely absent in the presence cys or met, and is greatly reduced by glutathione (gsh) addition (Ono *et al.*, 1999; Ono *et al.*, 1991; Marzluf, 1997). These same enzymes in *Aspergillus nidulans*, on the other hand, are not down regulated in response to sulfur metabolites (Natorff *et al.*, 2003; Sienko & Paszewski, 1999; Clark *et al.*, 1997). The transcription of sulfate permeases is suppressed in all species studied by low concentrations of S metabolites (Grynberg *et al.*, 2001; Pilsyk *et al.*, 2007; Bradfield *et al.*, 1970; Cherest *et al.*, 1997; Ketter & Marzluf, 1988; Ketter *et al.*, 1991; Marzluf, 1993; 1997). A putative cysteine synthase transcript has been identified from the AM fungus *Glomus intraradices* (http://darwin.nmsu.edu/~plammers/glomus/), which appears to be the only report on the identity or regulation of AM fungal genes related to S metabolism, transport, or regulation.

This study was aimed at determining the forms of S taken up, metabolized, and transferred to its host roots by the fungal partner in the AM symbiosis. A second goal was to identify effectors of S transfer to host roots, and to begin to identify AM fungal genes of S handling and their regulation. Using an AM symbiosis of monoxenic cultured roots the transfer of S was shown to be nutritionally significant and to be regulated by S availability to the host and by S metabolite availability to the extraradical mycelia. The uptake and transfer of reduced S through the symbiosis was also demonstrated. Putative homologs of sulfur uptake and metabolism genes were also identified and their

transcriptional regulation examined, indicating that sulfate uptake is transcriptionally regulated at the level of sulfate permease. These findings allow the development of a testable model of S uptake and transfer in the AM symbiosis.

Experimental Procedures

Chemicals and reagents

Gel-gro gellan (MP Biomedical, Solon, OH) was used for the solidification of growth media. Radioactively labeled sulfate was obtained as $Na_2{}^{35}SO_4$ from MP Biomedicals (Solon, OH). Labeled cys and met were separated from a crude mixture of ^{35}S-labeled compounds (TRAN^{35}S-LABEL^{TM}; MP Biomedicals, Solon, OH) containing equal to or less than 70% [^{35}S]-met and 15% [^{35}S]-cys. The compounds were separated using an amino acid analyzer (Hitachi L8800). Retention times for the amino acids were determined using post-column ninhydrin bonding and UV absorption. The radioactive compounds were then collected in a ninhydrin-free environment by manual collection of the appropriate fractions.

Growth media

All experimental procedures used M medium (Fortin *et al.*, 2002) with modifications to reduce the sulfate content. This was autoclaved and, for culture plates, solidified with 3.5 gL^{-1} Gel Gro gellan (MP Biomedicals, Solon, OH). The 3 mM $MgSO_4$ normally present in M medium was replaced with 0.1 mM $MgCl_2$. An additional 2 mM of calcium was added as $CaCl_2$ to replace the divalent cation concentration needed for the solidification of Gel-gro. Sulfate was supplied as Na_2SO_4 at different concentrations as indicated. Normal microelement concentrations for M media containing

0.02 mM sulfate were used for each experiment. These were included in all experiments with the exception of the measurement of non-mycorrhizal root growth (Figure 1a.). The medium in the root- but not fungal-compartments contained 10 gL^{-1} (29.2 mM) sucrose. Na_2SO_4 was added to the fungal compartments at the time of labeling as 0.2 mL of concentrated solution.

Growth of roots and mycorrhizas

Uncolonized Ri T-DNA transformed roots of *Daucus carrota* (DC2; Diop *et al.*, 1992) were grown at 25°C with 0.02, 0.12, 0.5, or 3 mM Na_2SO_4 in modified liquid M media. Roots were inoculated at plate inception as previously described (Pfeffer *et al.*, 1999). The roots and fungus were allowed to proliferate on both sides of bi-compartmented Petri plates at 25°C until the fungal extraradical mycelium was well developed (~six weeks). The colonized roots and media in each compartment were transferred to empty compartments of new plates in which the other compartment contained new medium. At the time of transfer, the media in which the mycorrhizal roots were growing was supplemented with one quarter of the original nutrient contents including sucrose and phosphorus, but excluding $CaCl_2$, added as ~0.5-1mL of a sterile solution. Root growth over the barrier after transplantation was prevented by pruning, and the fungal ERM typically grew over the barrier within one week of the transfer, colonizing the empty compartment. Fungal compartments were labeled when ERM had growth into at least half of the media, or within two weeks of crossing the barrier, whichever was first.

Collection of roots and ERM

Root material was collected with forceps and rinsed for 5 min in deionized water to remove external- and reduce apoplastic- ^{35}S. The collected roots were rinsed again with deionized water in a separate container, blotted dry, frozen in liquid nitrogen, and lyophilized. ERM was collected by blending the solidified medium at high speed in 10 mM sodium citrate buffer (pH 6.0) at an approximate gel: buffer ratio of 1:2.5 for 2 min, which dissolves the gellan (Pfeffer *et al.,* 1999). Tissue was collected on a sieve which was rinsed with four 30 mL aliquots of cold 10 mM Na_2SO_4, followed by an equal quantity of cold deionized water. The final flow through was checked for radioactivity, which was not detectable by scintillation counting in a 0.5 mL aliquot. To collect ERM from root compartments, the roots were first removed from the media under a dissecting microscope. Fungal mycelium collected from the sieve was blotted briefly, and frozen in liquid nitrogen. Tissue was weighed after overnight lyophilization.

Extraction procedure

For biochemical fractionation, lyophilized fungal mycelium was pulverized with two 3 mm stainless steel beads using a bead mill (Retsch MM301). Due to the high lipid content, 0.1 mL of cold methanol:water (70:30) was added to aid in disruption. The samples were shaken at 30 Hz for 4 min, and 2 µL samples were analyzed by dissecting microscope to ensure that hyphae and any spores had been broken. After disruption, 0.9 mL of cold methanol:water (70:30) was added and the sample was vortexed for 5 min. Samples were then centrifuged and supernatants collected. The cold aqueous methanol

extraction was repeated twice more using 1 mL each time, and the supernatants pooled. 0.5 mL of supernatant solution was scintillation counted after adding to 5 mL of BioSafe II (MP Biomedicals) scintillation cocktail. In order to determine the amount of sulfate in the samples, the sulfate was precipitated from an aliquot of the aqueous alcohol extract by adding 0.1 mL of 100 mM Na_2SO_4 and 0.3 mL of 10 mM HCl solution to 0.5 mL of the sample followed by vortexing and the addition of 0.1 mL of 100 mM $BaCl_2$. Samples were then incubated for 30 min at 100°C. The resulting barium sulfate precipitate was removed by centrifugation, and the sulfate-free supernatant was scintillation counted. The amount of sulfate in each sample was determined by the subtraction of these counts from those obtained from an aliquot of the original aqueous alcohol extract. Tests with standards showed that 99.8 +/- 0.1 % of sulfate was removed by this procedure (three trials with three samples each) and that 96.4 +/- 1.3 % of ^{35}S-Cys and 95.6 +/- 1.6 % of ^{35}S-Met remained in solution.

Residues after aqueous ethanol extraction were extracted using 1mL of protein extraction buffer containing 9 M urea, 1 % SDS, 25 mM Tris-HCl pH 6.8, 1 mM EDTA, and 0.7 M 2-mercaptoethanol as described by Osherov and May (1998). After vortexing and centrifugation at 10,000 rpm for 5 minutes, 0.5 mL of the supernatant was added to 5 ml of scintillation cocktail and counted. The remaining cellular debris was transferred to sealed glass vials containing 0.5 mL TS-2 tissue solubilizer (MP Biomedicals) and incubated at 70°C for 1-2 weeks until solubilized. The solubilized solution was mixed with 5 mL of scintillation fluid and titrated to pH 7 with HCl. This solution was diluted ten fold to diminish color quenching and analyzed by scintillation counting.

Dried roots were pulverized in 15 mL centrifuge tubes with ten 5 mm metal beads for 30 min using a paint shaker. Five mL of cold methanol:water (70:30) was added to the powder and the tubes were vortexed for five minutes. While keeping particulates suspended, a 1 mL aliquot of the solution was transferred to a microcentrifuge tube and centrifuged. Subsequent steps were as for fungal samples.

Measurements of ^{35}S in media

Three 1 cm diameter wells were excavated in the gel at the time of labeling with a sterilized cork borer as far from one another as possible. 0.1-0.2 mL of media of the same composition as that in the compartment being analyzed was added to each well, and this volume was maintained through weekly refilling as needed. At the start of the experiment, label was added to one of the wells, and ^{35}S in each of the three wells were measured after one week to ensure efficient diffusion of the radioactivity through the plate. ^{35}S contents were measured by adding 50 µl of liquid media to 5 mL of scintillation fluid, followed by scintillation counting.

Non-mycorrhizal root measurements

Roots were grown on solidified M media containing 0.12 mM S as Na_2SO_4 for four weeks and ~5cm root segments were aseptically removed, weighed, and added to 20mL of liquid M media without S ($ZnSO_4$* $7H_2O$ and $CuSO_4$* $5H_2O$ replaced by 1.2 mg of ZnCl and 0.5 mg of $CuCl_2$) for measurements of sulfur-mediated growth

limitation. For each sulfate concentration (0, 0.005, 0.01, 0.02, 0.05, 0.1, 0.5, and 3 mM), roots were weighed then placed on 20 mL of solidified media per side of three bi-compartmental petri plates and allowed to grow for three months. The roots were collected, dried at 70°C overnight, and weighed. To measure S uptake, roots were grown at 0.12 mM sulfate as above and transferred into 20mL of S- media solidified with gellan containing 0.02, 0.12, or 3mM sulfate labeled with 11.5 μCi of $Na_2[^{35}SO_4]$. After four weeks, the roots were removed from the media, rinsed several times in deionized water to remove external radioactivity, lyophilized, and weighed. The extraction procedure for the radio-labeled tissue was simplified to include only the aqueous alcohol and solubilization steps.

Addition of S metabolic regulators

Cys, met, and gsh were added to a final concentration of 1 mM (as 0.2-0.5 ml of sterile aqueous solutions) to the fungal compartments of split plates with ten week old roots growing with 0.52 mM Na_2SO_4. The uptake of 37.4 μCi of $Na_2[^{35}SO_4]$, which was added to fungal compartments simultaneously with metabolic regulators and 0.12 mM Na_2SO_4, was monitored by analyzing aliquots of media from a liquid filled well as described above. The plates were collected at 2, 4, and 6 weeks. The fungal mycelium was collected by dissolving the gellan in a blender with 10 mM sodium citrate (pH 6), then sieving the blended solution. The roots were extracted from the media using forceps and rinsed in deionized water for five minutes before freezing. The fungal and root tissues were extracted as described above.

Using cultures grown as for the above experiment, 1 mM of cys labeled with 25.7 μCi of [^{35}S]cys or 1 mM met labeled with 54.2 μCi of [^{35}S]met was applied to the fungal sides of 15 plates as the sole S source. The plates were incubated at 25°C for one month prior to collecting and extracting the biochemical fractions as described above.

RNA extraction and putative gene fragment isolation

Sequences of putative sulfur gene fragments were identified from an EST database (Jun *et al.*, 2002) and from additional high throughput sequencing data (PJ Lammers, Shachar-Hill and coworkers unpublished results) from cDNA made from extraradical mycelium. To confirm their identity, total RNA was extracted using the RNeasy Plant Mini Kit (Qiagen, Valencia, CA) from frozen *G. intraradices* germinating spore tissue, disrupted as already described using a bead mill, followed by DNA removal using RNase-free DNase (Turbo DNA-free; Ambion, Austin, TX). Samples were split into two aliquots following quantification by absorbance for the synthesis of cDNA and a control without reverse transcriptase. cDNA was synthesized using SuperScript II reverse transcriptase (Invitrogen corp., Carlsbad, CA). Primer sets were developed from EST and high throughput sequencing data as follows: Putative sulfate permease forward primer 5'-TAGCAATAATTACAAGAATACCAG-3', and reverse primer 5'-GGGATTCTTATCTTGGAA-3'; putative cystathionine β-synthase forward primer 5'-CTAGCCACTCCTGTAATTGTTCCTC -3', and reverse primer 5-GAGAAAGCAGGAATACTTAAACCAGG -3'; putative cystathionine γ-lyase forward primer 5'- GGTGGAATGTTAAGTTTTAGGATTAAGG -3', and reverse primer 5'-CATCAACATCTTCGATACCGATG-3'. PCR products were separated by and isolated

from agarose gel using the QIAquick Gel Extraction Kit (QIAGEN GmbH, Germany) and sequenced by an ABI PRISM® 3100 Genetic Analyzer. (Applied Biosystems, CA, USA). Resulting sequences showed single open reading frames. The sequences studied were submitted to GenBank and given the following accession numbers: Putative high affinity sulfate permease (FJ161947); putative sulfate adenylyltransferase (FJ161948); putative cystathionine γ-lyase (FJ161949); putative cystathionine β-synthase (FJ161950).

Quantitative real time PCR measurements

Mycorrhizal split plates were grown until the fungal compartment was approximately half colonized. To the fungal compartment 1 mL of a filter sterilized solution containing either sodium sulfate or sodium sulfate and cys was applied to give a final concentration of 0.12 mM sulfate and 1 mM cys. Plates were incubated for 24 hrs before tissue from five to seven plates was collected as described above and immediately frozen in liquid nitrogen. RNA was extracted and converted to cDNA as described above. The initial qRT-PCR reaction mixture containing primers at a concentration of 300 nM and 1 ng of cDNA template was made to 0.015 mL in a 96 well plate, 0.01 mL of which was transferred to a 384 well plate and the PCR reactions were monitored using an ABI Prism 7900 HT Sequence Detection System (Applied Biosystems, Austin, TX) with the following cycling program: 50°C for 2 min, 95°C for 10 min followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. Power SYBR Green 2-Step Master Mix (Applied Biosystems, Austin, TX) was used for all real time PCR assays. The $\Delta\Delta C_T$, and comparative C_T, methods were utilized for the determination of relative gene expression (Livak and Schmittgen, 2001). The expression of an S4 ribosomal protein was used to

normalize relative gene expression data as described by Govindarajalu *et al.* (2005). Other primer sequences (IDT, Syntegen, Skokie, IL) for QRT-PCR were designed using Primer Express software from Applied Biosystems (Austin, TX) as follows: Putative high affinity sulfate permease forward 5'-TTGGATCATTCTTTCATGCGTATC-3', reverse 5'- GACACCGGCTAATGGAGTACGT-3'; putative S-adenosyl transferase forward 5'-CCGGAGTTGATGATCCTTACG-3', reverse 5'-ACTGACACACTGTTTACTAACATCAACAA-3'; putative cystathionine β-synthase forward 5'-TGCTTCAGTTGGTGTACGAACAA-3', reverse 5'-AAATGAGCCAGGAAAAGGTTGA-3'; putative cystathionine γ-synthase forward 5'-GACCAGCGTGAGTCATTTTAGAAG-3', reverse 5'-AGCTGAATCTTTGGGTGGTGTT-3'. Experimental samples were measured using six technical replicates for each of three biological replicates per condition, and errors were expressed as standard error of the mean.

Results

Growth and sulfate uptake by non-mycorrhizal roots

To establish conditions for studying S transfer between host roots and AM fungus, the relationship between sulfate availability, root growth, and sulfate utilization was investigated in uncolonized roots (Figure 2-1). In the absence of available sulfate there was a three fold decrease in root growth compared to growth under saturating sulfate levels (3 mM; Figure 2-1A). Final dry weights were not significantly different when compared singly at sulfate concentrations above 0.005 mM, however there was a consistent trend of increasing dry weight with increased S availability. Root growth was detected even in roots exposed to medium containing no S, probably due to internal S stores within the initial root segments. A much greater difference was seen in the uptake and incorporation of sulfate when roots were labeled with $^{35}SO_4^{-2}$ at high (3 mM), low (0.014 mM), or intermediate (0.114 mM) sulfate levels (Figure 1B). There was a close to four fold increase in both aqueous alcohol-soluble and –insoluble S content from low to intermediate S conditions, and an eleven fold increase between low and high S. A reduction in the root compartment sulfate concentration from 3 mM to 0.12 mM resulted in a reduction in uptake of 64% (Figure 2-1B), with little or no reduction (27% difference in mean values, not statistically significant at the 95% confidence level) in growth (Figure 2-1A). Lowering the sulfate available to mycorrhizal roots to less than 0.05 mM had a negative impact on the symbiosis, reducing the number of plates where the fungal ERM grew over the barrier from greater than 80% to less than 20% (data not shown). There was no such reduction when 0.12 mM sulfate was utilized.

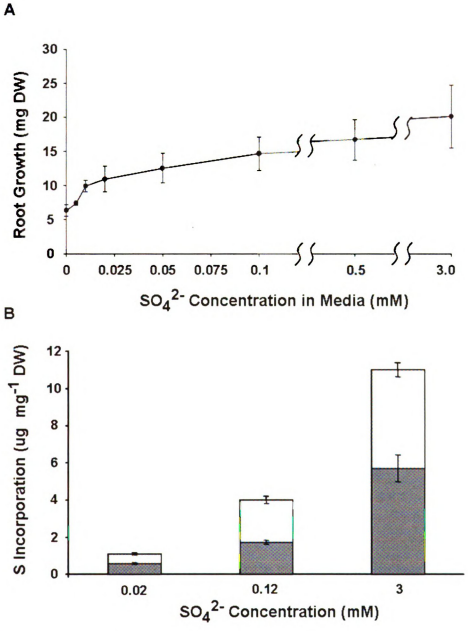

Figure 2-1 The effect of sulfate concentration on the growth and sulfur incorporation of non-mycorrhizal transformed carrot roots. (A) Root growth measured as dry weight increase at different concentrations of Na_2SO_4 in 20mL of modified liquid M media (St.Arnaud et.al., 1996). (B) The incorporation of S from solid media containing 0.02, 0.12, or 3 mM Na_2SO_4 labeled with 11.5 μCi of $Na_2[^{35}SO_4]$ was measured in roots after four weeks of labeled growth. Labeled root compounds were extracted with cold MeOH:H_2O (70:30) (white bars) before the cellular residue was solubilized in tissue solubilizer (gray bars). The fractions were analyzed by liquid scintillation counting. Error is expressed as standard error of the mean.

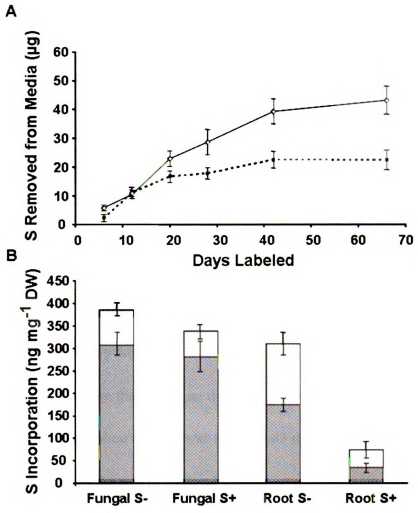

Figure 2-2 Increased sulfate availability in the root compartments of split plates reduces transfer of S through the distal fungal ERM. (A) The uptake of S by ERM from plates labeled with 30 µCi of $Na_2[^{35}SO_4]$ in 0.12 mM Na_2SO_4 in the fungal compartments was measured as the removal of radioactivity from the media when the root compartments were supplied with moderate S (0.12 mM Na_2SO_4; solid line) or high S (3mM Na_2SO_4; broken line). (B) Measurements of S incorporated into the roots (Root S+/-) and distal fungal (Fungal S+/-) ERM from the above experiment after 66 days of labeling; S+ refers to high sulfate levels (3mM) in root compartments and S- to moderate levels (0.12mM). Radioactivity was measured by scintillation counting after a crude separation by cold MeOH:H_2O (70:30) extraction (white bars) followed by solubilization of the remaining tissue (gray bars). The aqueous alcohol extraction dissolves sulfate, sulfite, S amino acids, and potentially also unidentified sulfated esters while the remainder includes proteins, sulfolipids, and other unknown products. Values are presented as mean +/- s.e.m ($n \geq 5$).

Fungal uptake and transfer of sulfate is influenced by root access to sulfate

The extraradical mycelium of *Glomus intraradices* takes up sulfate at significant rates (Figure 2-2A) and transfers most of it to the mycorrhizal roots (Figure 2-2B). The total amount of ^{35}S in ERM tissue was less than 5% of the amount found in the root tissue, owing to the greater than twenty-fold increase in biomass between the two (tissue mass data not shown). The availability of S in the root compartment strongly influences fungal uptake in the distal ERM compartment. More than twice as much sulfate was removed from the fungal compartment when the corresponding root compartment was exposed to 0.12 mM versus 3 mM S (Figure 2-2A). When roots were grown in 0.12 mM sulfate, uptake by the fungus was linear up to day 40 ($R^2 = 0.98$). The fungus removed 0.98 μg of S from the media per day during this time. Fungal uptake of S when 3 mM sulfate was supplied to roots was linear (0.83 μg of S per day, $R^2 = 0.92$) only for the first 20 days before the rate of removal slowed.

The total amount of S transferred from the fungal compartment to roots grown at high sulfate levels was about one quarter of that in roots exposed to moderate sulfate levels (Figure 2-2B). In contrast, there was no significant effect of sulfate levels in the root compartment on the incorporation of S by the distal ERM. Additionally, in roots growing at moderate S levels the fraction containing sulfate and the sulfur amino acids contained approximately twice as much ^{35}S as did the same fraction in the ERM.

The amount of ^{35}S in the media of the colonized root compartments was monitored throughout the experiment (see methods) and found to be low. The movement into the root compartment media from the fungal compartment was linear ($R^2 = 0.98$) at

63 ng S per day when the root compartment media contained 0.12 mM sulfate. This represents 6.4% of the amount transfer to the roots. The movement into the high S root media was also linear ($R^2 = 0.99$) with 55 ng S per day being moved across the barrier, representing 6.6% of the amount transferred to the roots.

Figure 2-3 Transfer of $^{35}SO_4^{2-}$ through the fungus versus direct root uptake. **(A)** The uptake of S over 104 days from root and fungal compartment media. The root (proximal) compartments of split plates were labeled with 17.3 μCi of $Na_2[^{35}SO4]$ in 0.2 mM Na_2SO_4 (171 μg S). Half of the fungal (distal) compartments were labeled with 17.3 μCi of $Na_2[^{35}SO_4]$ in 0.2 mM Na_2SO_4 (171 μg S, open diamonds, solid line), while the others contained 0.02 mM sulfate without ^{35}S (closed squares, broken line). Distal uptake was monitored from labeled plates (closed triangles, solid line). A straight broken line denotes the total initial S content of the proximal compartment media. **(B)** Incorporation of ^{35}S by roots when the fungal side was supplied with low unlabeled (0.02 mM) or moderate ^{35}S-labeled (0.2 mM) sulfate was measured after 110 days of labeling by chemical fractionation into sulfate- (white bars), amino acid- (light gray bars), and protein-containing (medium gray bars) pools, and solubilized remaining tissue (dark gray bars) pools measured by liquid scintillation counting. **(C)** Incorporation of ^{35}S into different pools of ERM collected from the root or fungal side compartments of split plates when the fungal compartments contained 0.02mM unlabeled sulfate (S-) or 0.2mM of ^{35}S labeled (S+); as above the root side compartments of all plates contained 0.2mM ^{35}S labeled sulfate . Fractions are depicted as in panel A. Values represent means +/- s.e.m. ($n \geq 4$).

Figure 2-3

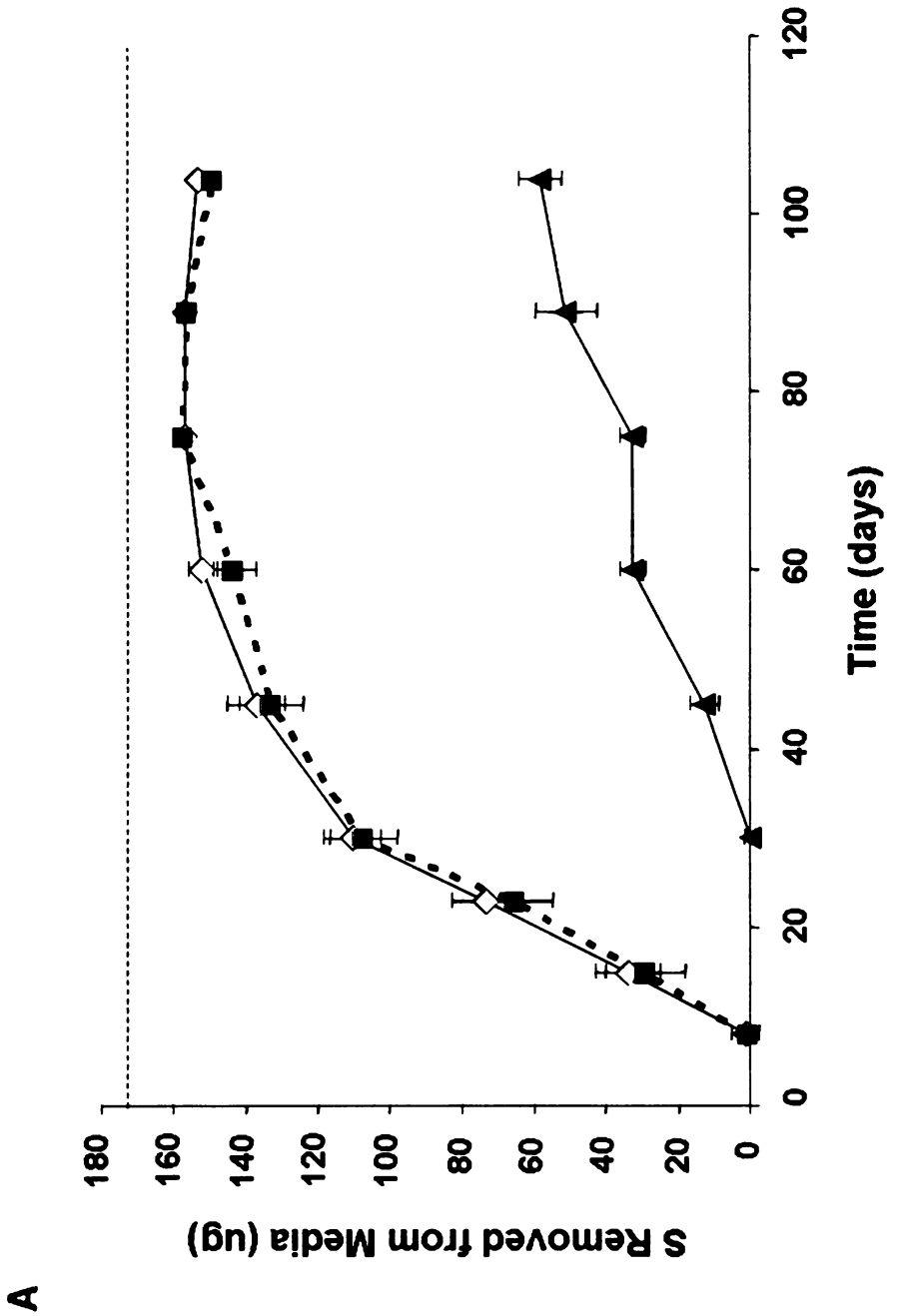
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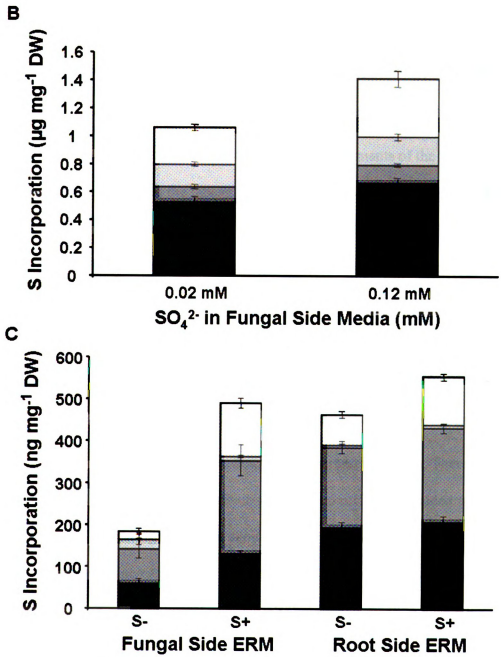
Figure 2-3 (cont'd)



Fungal transfer of s to the mycorrhizal roots versus direct uptake by roots

The relative contribution of S obtained from the fungal ERM to total root S was determined at intermediate S levels (0.22 mM) by comparing fungal derived ^{35}S in roots with ^{35}S directly absorbed by the roots themselves. Plates were labeled with $^{35}SO_4^{2-}$ in the root compartment to determine the amount of S incorporated by direct root uptake. Half of these plates were also labeled in the fungal compartment with $^{35}SO_4^{2-}$ at 0.22 mM sulfate while no S was added to the fungal compartments of the remaining plates. The roots took up most of the labeled sulfate initially provided within 8 weeks (Figure 2-3A). This uptake was not affected by the presence of sulfate in the fungal compartments. The uptake of S from the fungal compartment media began when the fungus crossed the barrier (~30 days after inoculation) and was comparable to direct root uptake rates in the next few weeks (Figure 2-3A). S incorporation by the root and fungal tissue after ~10 weeks of growth is shown in figures 3B and 3C. When the fungal compartment contained $^{35}SO_4^{2-}$, the total incorporation of S by the roots increased by ~25% (Figure 2-3B). The largest change was in the sulfate fraction, which increased by 60%. There were no significant differences in root growth observed when sulfate was added to the fungal compartments (root weight data not shown), which is consistent with the lack of significant growth increase between moderate and high sulfate growth conditions (Figure 2-1). When the fungal side was labeled, total incorporation of ^{35}S by the fungus was similar in ERM collected from the fungal and root compartments when the fungal side was labeled (Figure 2-3C), which is consistent with the absence of any known physiological differences between the ERM in the two compartments, and showing that

the availability of S to the distal ERM did not affect incorporation by the ERM in the root compartment. ^{35}S accumulated in the fungal ERM from the unlabeled fungal compartment showing that in a low S environment (0.02 mM) S can be moved by the fungus from the root compartment to the distal ERM (Figure 2-3C).

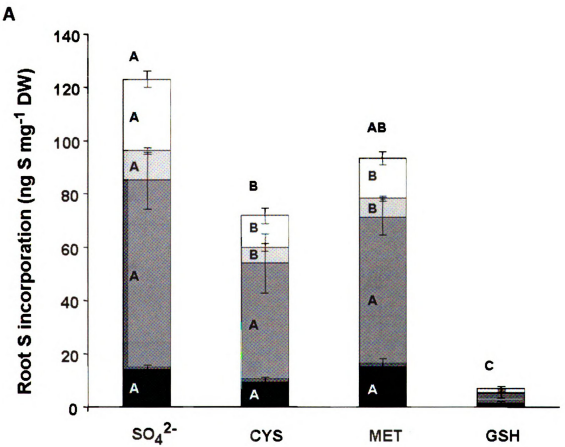


Figure 2-4 The effect of common sulfur metabolite repressors cysteine, methionine, and glutathione on the transfer and allocation of ^{35}S in an arbuscular mycorrhiza. The incorporation of ^{35}S into roots and fungal ERM was analyzed after labeling the distal compartments with 37.4 μCi of $Na_2[^{35}SO_4]$ in 0.12 mM Na_2SO_4 (91 μg S) alone (SO_4^{2-}), or with 1mM cysteine (CYS), 1mM methionine (MET), or 1mM reduced glutathione (GSH). Root (panels **A-C**) and fungal (panels **D-F**) was analyzed after two (**A**,**D**), four (**B**,**E**), and six (**C**,**F**) weeks. Fungal ERM was collected from the distal compartments of same plates as those from which root tissue was collected from the proximal compartments. Tissue was analyzed by chemical fractionation into pools containing sulfate (white bars), amino acids (light gray bars), proteins (medium gray bars), and the tissue solubilized biomass remaining after extraction (dark gray bars). Fractions were analyzed by liquid scintillation counting. Letters within the bars represent ANOVA single factor analyses between bars of that type. Letters above the complete bar graphs are the analyses of total S uptake. Values are reported as mean +/- s.e.m. ($n \geq 5$).

Figure 2-4 (cont'd)

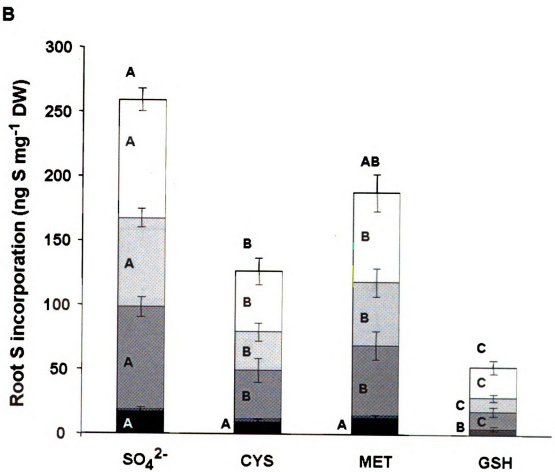


Figure 2-4 (cont'd)

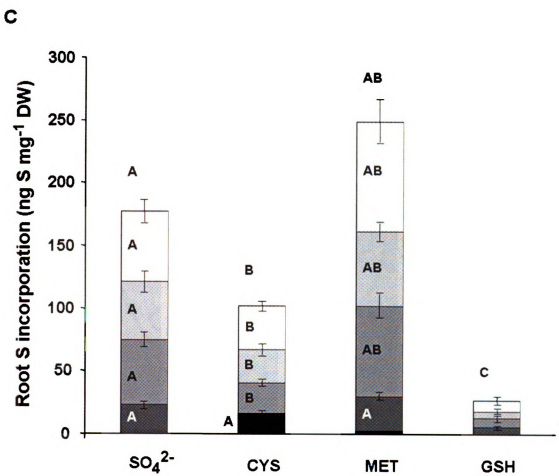


Figure 2-4 (cont'd)

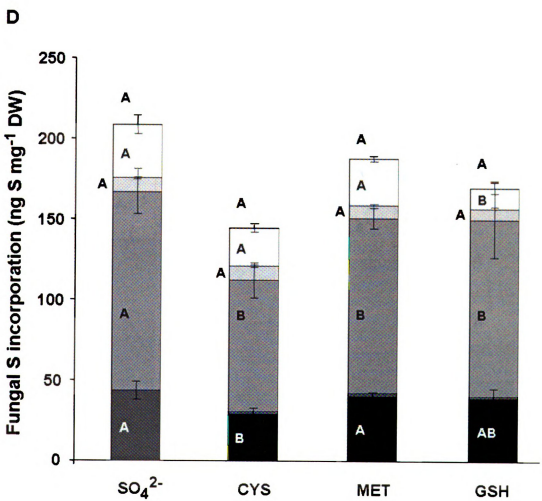


Figure 2-4 (cont'd)

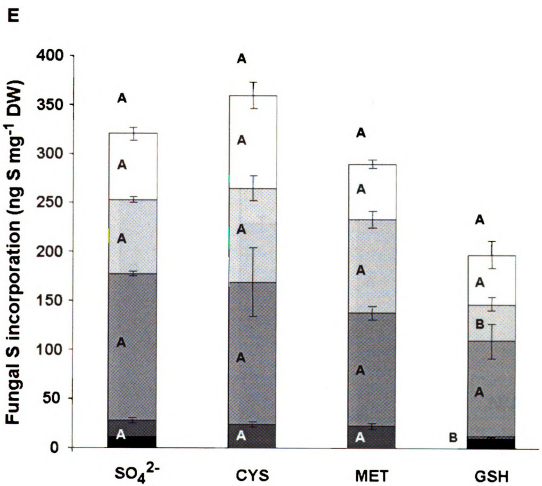
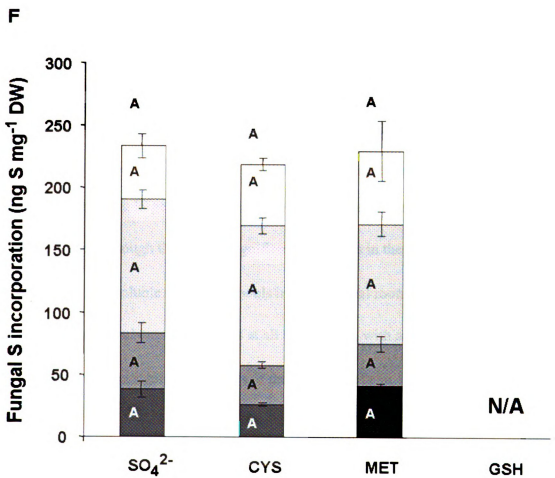


Figure 2-4 (cont'd)



The effect of sulfur metabolites on sulfate uptake and transfer through the mycorrhizal symbiosis

Radio-labeled sulfate was added to the fungal compartments of split plates together with methionine (met), cysteine (cys), and glutathione (gsh) in amounts previously shown to suppress sulfate assimilation in other fungi (Kuras & Thomas, 1995; Ono *et al.*, 1999; Ketter & Marzluf, 1988; Ketter *et al.*, 1991; Grynberg *et al.*, 2001). The distribution of ^{35}S in root and fungal tissues was then measured after two, four, and six weeks. Substantial quantities of S were transferred to the roots over the course of the experiment despite the presence of 0.5 mM sulfate in the root compartments (Figure 2-4A to C). The presence of cys decreased S transfer to the mycorrhizal roots by an average of 45% throughout the experiment, with the sulfate- and amino acid- containing pools of S in the roots showing larger fractional decreases. Methionine had no significant effect on total S transfer although there were significant decreases in the ^{35}S transferred to the low molecular weight soluble metabolite pools in mycorrhizal roots. Glutathione dramatically reduced ^{35}S accumulation in the roots at all time points, with an average reduction of 80%. There were no significant effects of the presence of cys, met or gsh on total S incorporation from labeled sulfate by the distal ERM and little or no effect on the partitioning of S within this tissue (Figures 2-4D to F).

Glutathione greatly reduced the growth of fungal ERM in the distal compartment though not the root growth (not shown) so it is unclear whether the reduction in the transfer of S by gsh treated ERM is due to the regulation of sulfate uptake or simply to reduced fungal growth. Neither cys nor met significantly affected the growth of fungal

ERM or roots, with ERM biomass approximately doubling between weeks two and four (data not shown). Thus the effect of cys on S transfer to mycorrhizal roots does not appear to be due to growth inhibition.

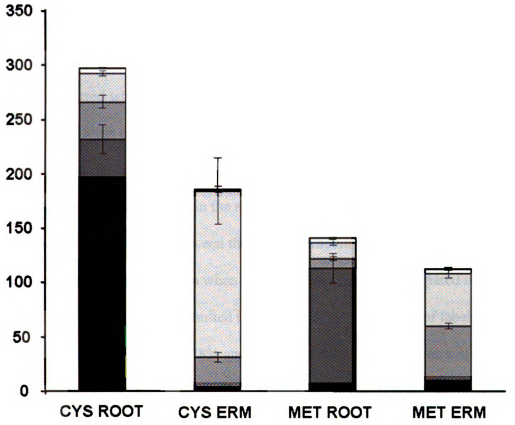


Figure 2-5 Transfer of reduced S through a mycorrhizal symbiosis. Mycorrhizal roots were grown in 0.5 mM Na_2SO_4 and the fungal partner allowed to grow into distal compartments containing either 1mM cysteine (CYS) labeled with 25.7 μCi [^{35}S]-Cys or 1mM methionine (MET) labeled with 54.2 μCi of [^{35}S]-Met as the sole S source. Roots and fungal distal ERM were collected after four weeks of labeling and the radioactive counts split into different biochemical fractions. Fractions included SO_4^{2-} (white bars), amino acids (light gray bars), proteins (medium gray bars), and the remaining solubilized cellular material (dark gray bars) and were obtained through scintillation counting. Values are reported as mean +/- s.e.m. (n=9).

Fungal uptake and transfer to host roots of reduced s

Fungal ERM imports and transfers substantial amounts of reduced S supplied as cys or met (Figure 2-5). The amount of S transferred when ^{35}S-labeled cys is the sole source of S in the fungal compartment is comparable to the S transfer when plates are labeled with sulfate (Figures 2-2B, 2-3B, and 2-4A to C). Roughly half of this amount was transferred when met was provided (Figure 2-5). The incorporation of S by the fungal mycelium per mg dry weight when labeled cys is supplied to the fungal compartment is less than the level found in the roots by about 40%. Utilizing met as an S source led to a 46% increase in the amino acid fraction in the ERM (Figure 2-5). There were striking differences between the relative amount of S in the solubilized fractions in both roots and fungal mycelia when reduced S was supplied compared to sulfate. Labeling with cys and met resulted in a much higher percentage of labeling in the solubilized root tissue pool (78% and 88%, respectively) than when sulfate was supplied as the primary S source (7-21%; Figures 2-4A to D). The opposite relationship was found in fungal mycelia, where applying cys or met as primary sources of S led to solubilized tissue fractions that were at most 8% of total S incorporated (Figure 2-5). When labeled sulfate was the primary S source, 5-22% of the total S incorporation is found in the tissue solubilized fraction (Figures 2-4D to F).

Gene expression measurements

The expression of fungal transcripts encoding putative genes involved in sulfate assimilation was analyzed in relation to the application of cys to the distal ERM. Partial sequences of transcripts representing putative sulfur assimilatory genes were identified by high throughput 454 sequencing of cDNA from fungal ERM grown in split plates in M

medium (3mM sulfate). These included a 234 bp sequence with 67% identity at the amino acid level to SUL1 from *S. cerevisiae*, and a 247 bp sequence with 67 % and 70% identities at the amino acid level to sulfate adenylyltransferase from *A. nidulans* and *A. terreus*, respectively. Sequences with homology to enzymes defining the reverse trans-sulfuration pathway were also identified. These included a 403 bp sequence with 79% and 71% identities at the amino acid level to cystathionine β-synthase from *A. fumigatus* and CYS4 from *S. cerevisiae*. Additionally, a 220 bp sequence was identified with 58% and 69% amino acid identities to CYS3 from *S. cerevisiae* and cystathionine γ-lyase from *A. fumigatus*. The presence of transcripts for these putative sulfur genes is are consistent with sulfate uptake and assimilation via the usual assimilation pathway, and also strongly suggests the presence of the reverse trans-sulfuration pathway in AM fungi. The expression of the sulfate permease and reverse trans-sulfuration enzyme candidates were analyzed in ERM after exposure to 0.12 mM sulfate, with or without the presence of 1 mM cys. The conditions were chosen to enable the comparison between gene expression changes and the uptake and transfer of radio-labeled sulfate with cys addition (Figure 2-4A to C). There was a reduction in the expression of the candidate high affinity sulfate permease sequence by a factor of 3.15 +/- 1.15. The expression of sequences with homology to sulfate adenylyltransferase and cystathionine γ-lyase was not significantly different between treatments. The expression with cys addition of the putative cystathionine γ-lyase sequence studied was significantly different. However, the difference was small (less than two fold).

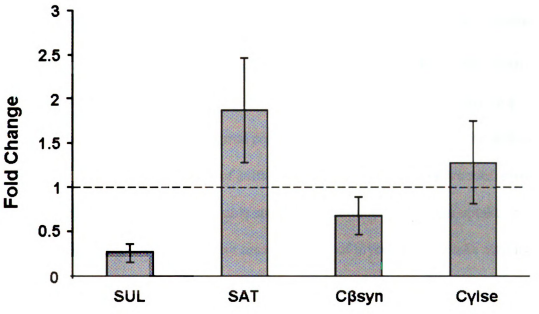


Figure 2-6 Fungal gene expression changes in response to the addition of 1 mM cysteine to ERM. Expression relative to control plates of sequences with homology to high affinity sulfate permease (SUL), S-adenosyl transferase (SAT), cystathionine β-synthase (Cβsyn), and cystathionine γ-lyase (Cγlse) was analyzed by quantitative real-time PCR using a ribosomal protein sequence as a control as previously reported (Govindarajulu *et al.*, 2005; see methods). RNA was extracted from ERM tissue collected after 24 hr incubation with 0.12 mM SO_4^{2-}, and with or without 1 mM cys added to the distal compartments of five split plates per sample. Values are reported as mean +/- s.e.m. (n=3 biological replicates each consisting of tissue from 5 to 7 plates).

Discussion

The uptake of ^{35}S by non-mycorrhizal roots was dependent on the external sulfate concentration (Figure 2-1B), as has been reported for tomato seedlings and carrot storage root sections (Lopez *et al.,* 2002; Cram, 1983). In carrot, Cram (1983) showed a proportional increase up to an external concentration of 50 mM, leading the authors to conclude that feedback inhibition of sulfate uptake is absent. Vegetative root growth was less affected by alterations in available sulfate concentrations than was uptake or reduction (Figure 2-1A). Subsequent measurements of mycorrhizal uptake activity were conducted using sulfate concentrations that increased potential differences in root uptake and contents and minimized the limitation of growth by S.

Bi-compartmental Petri plate cultures are a well established model mycorrhizal system (St. Arnaud *et al.* 1996) used extensively in studies of mycorrhizal metabolism and nutrient transfer (e.g. Govindarajulu *et al.,* 2005). This system made possible the use of defined media and the isolation of the majority of extra-radical mycelium (ERM) from the rhizosphere, allowing S movement through the ERM to be quantified. With moderate S levels available to the roots (0.12 mM), uptake and transfer by the fungus increased the amount of S in the roots by 25% (Figure 2-3B). The movement of ^{35}S into roots was also observed in all the experiments regardless of the S-source utilized in quantities much higher than those found in fungal ERM. Fungal uptake and transfer of S was influenced by the concentration of sulfate available to the host roots. Increasing the sulfate concentration in the root compartment from 0.12 mM to 3 mM resulted in a halving of fungal uptake (Figure 2-2A), while approximately one fifth of the amount of transferred S

was found in roots grown in 3 mM sulfate compared to the amount found in roots grown in 0.12 mM sulfate (Figure 2-2B). These reductions may be due to host signaling and/or to changes in the concentration gradient of S at the root:fungal interface.

After supplying ^{35}S-sulfate to the fungal compartment under a range of conditions, labeled sulfate was invariably found in roots in amounts that dwarfed the total quantities in the fungal mycelium. For example, after six weeks of labeling the fungal compartment with $^{35}SO_4^{2-}$, total sulfate in the fungus was 5.1% of the amount in the roots (Figures 2-4C and F). Roots in that experiment were grown in 0.5 mM sulfate and the distal ERM was supplied with 0.12 mM, and the same was true (5.3%) when the roots were grown in 0.22 mM sulfate and the fungus was also labeled with 0.22 mM sulfate (Figure 2-3B and C). Based on this evidence one may conclude that the sulfate anion is transferred by *G. intraradices* to host roots. However, as shown in Figure 5, the fungus is clearly also capable of the uptake and transfer of reduced forms of S at rates comparable to sulfate. Thus the ERM can supply the host plant with organic forms of S from the soil. Since an estimated 95% of the S in soils is in organic forms, the ability of mycorrhizal plants to access S from this source is potentially important for plant nutrition.

The first steps towards understanding the regulation of sulfate uptake by a mycorrhizal fungus in the symbiotic state were taken by analyzing the effect of common sulfur metabolite repressors cys, met, and gsh on the uptake and transfer of radio-labeled sulfate through the fungus. The addition of 1 mM met was shown to suppress the expression of sulfur assimilation pathway genes in yeast (Kuras & Thomas, 1995) and the complete suppression of uptake has been demonstrated in the presence of ten times lower levels (Breton & Surdin Kerjan, 1977). This finding was later refined by mutant analyses

and the suppression shown to be due to the synthesis of cys through the reverse trans-sulfuration pathway (Ono *et al.*, 1999). The sulfate permeases sB in *A. nidulans* and Cys14 in *N. crassa* are also strongly suppressed by met (Ketter & Marzluf, 1988; Ketter *et al.*, 1991; Grynberg *et al.*, 2001). In contrast to these organisms, the addition of 1 mM met to the fungal compartments of mycorrhizal split plates had no significant effect on total sulfate uptake and transfer (Figure 2-4).

By contrast the application of 1 mM cys to the ERM resulted in S transfer from fungus to mycorrhizal roots being approximately halved, (Figures 2-4A to C). The amount by which sulfate transfer is reduced in the presence of cys (Figure 2-4) is similar to the level of S that is taken up as cys and transferred to the roots (Figure 2-5). Labeled gsh has been shown to be imported by ectomycorrhizal oak trees at rates comparable to sulfate (Seegmüller & Rennenberg, 2002). However, the addition of 1 mM gsh to endomycorrhizal distal ERM resulted in a suppression of the growth of this tissue. While the transfer of sulfate continued and the assimilation by the ERM was largely unaffected on a per mg basis, S transfer was greatly diminished. Since gsh also suppressed the expression of the putative sulfate permease (data not shown), it is unclear how much of the effect of gsh was due to regulation of transport and how much to repression of ERM growth. Thus the regulation of S uptake and metabolism in *G. intraradices* is different from that in other filamentous fungi studied to date.

The changes in expression of genes putatively involved in sulfate transport and assimilation indicates a role for transcriptional regulation of sulfate permease in the reduction in sulfate transport in the presence of cys. The reduction in S transfer of 45% with the application of 1 mM cys to distal ERM (Figure 2-4C) correlated with a 3.8 +/-

1.2 fold reduction in the expression of mRNA encoding a putative sulfate permease. The lack of a significant change in the expression of a cDNA sequence with high homology to S-adenosyl transferases suggests that the reduction in S transfer is a function of reduced uptake rather than sulfate reduction. However, the analysis of more S assimilation pathway genes and compartment-specific sulfate permease isozymes is needed before concluding that the transcriptional regulation of this sulfate permease gene explains the reduction in S transfer with the addition of cys. Sequences encoding putative genes with homology to cystathionine β-synthase and cystathionine γ-lyase were also identified. Their expression is indicative of a functional reverse trans-sulfuration pathway in *Glomus intraradices*, akin to *A. nidulans* and *S. saccharomyces*, but unlike *S. pombe* (Ono *et al.*, 1992a, 1992b; Paszewski and Grabski, 1975; Brzywczy *et al.*, 2002). The putative cystathionine β-synthase sequence was slightly down-regulated (1.49 +/- 0.41 fold) in the presence of cys while the sequence with homology to cystathionine γ-lyase showed no significant change.

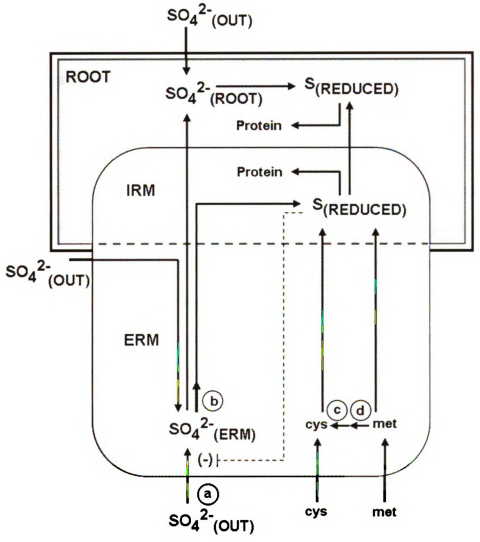


Figure 2-7 Model depicting S transfer through an endomycorrhizal symbiosis. Roots and fungal mycelium both import SO_4^{2-} from external sources, and fungal uptake can supply isolated mycelium. The transfer of SO_4^{2-} through the mycorrhizal symbiosis is inversely proportional to root uptake. Cys and met are imported by the fungus, resulting in a reduction of fungal uptake of SO_4^{2-} (dashed line) and the transfer of a reduced form of S to the root ($S_{(REDUCED)}$). The reduction of SO_4^{2-} and uptake of reduced S leads to incorporation in the protein pools in both roots and fungus. Putative steps in the assimilation pathway based on sequence data are depicted as gray arrows. Steps involving putative sulfur assimilation genes are labeled in gray letters as follows: (a) high affinity sulfate permease; (b) sulfate adenylyltransferase; (c) γ-cystathionine lyase; (d) β-cystathionine synthetase. IRM, intraradical mycelium; ERM, extraradical mycelium.

Figure 2-7 is a working model consistent with the findings presented. We have demonstrated uptake, assimilation, and transfer of sulfate (Figures 2-3B and 2-4A to C) and of reduced S (Figure 2-5) by the fungal partner to the host roots in physiologically significant quantities, along with bidirectional transport of sulfate within the fungal ERM (Figure 2-3C). The inverse relationship between the rhizospheric sulfate concentration and the amount of sulfate uptake from the fungal compartment (Figures 2-2A to B) suggests that an increase in root intracellular or apoplastic sulfate can suppress both the transfer of sulfate from the intraradical hyphae to the host and the uptake by the extraradical hyphae. This relationship is depicted in the model as the negative regulation of sulfate transfer across the root:hyphal interface by root sulfate levels, which subsequently results in an increase in the fungal intracellular sulfate concentration and suppression of uptake in the distal ERM. The relationship between the transfer of a reduced form of S when cys or met are imported by the fungus and the uptake of sulfate in the presence of cys and met suggest a simultaneous transfer of sulfate and reduced S and/or a regulatory response. Relative real-time PCR measurements of expression for a putative high affinity sulfate permease gene revealed a ~73% reduction in transcript levels when the ERM was exposed to 1 mM cys, indicating a regulatory effect on sulfate uptake (Figures 2-6 and 2-7). The working model proposed is a testable interpretation of the findings presented that invites future experiments to verify gene identities, localize expression within the host roots and analyze regulation in mycorrhizas of whole plants.

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Chapter 3

Uptake and Assimilation of S during Presymbiotic Growth of an Arbuscular Mycorrhizal Fungus

Abstract

Arbuscular mycorrhizal fungal spores germinate in pure water yet readily import C and N. The biological significance of this uptake is unknown and the use of other macronutrients including S is unexplored. The uptake of $^{35}SO_4^{2-}$, [^{35}S]cys, and [^{35}S]met, and the incorporation of ^{35}S into different metabolic pools during the presymbiotic growth of *Glomus intraradices* were measured by scintillation counting. Sulfate assimilation was analyzed in relation to sulfate concentration and exposure to unlabeled and labeled cys and met, glutathione (GSH) and buthionine sulfoximine (BSO). Corresponding changes in relative gene expression of putative sulfate assimilation pathway genes were measured by quantitative real-time PCR. *Glomus intraradices* showed little preference for reduced S sources over sulfate. Exposure to cys or GSH initially reduced sulfate uptake by ^{35}% and 63% respectively. Met addition did not decrease sulfate uptake while gene expression measurements suggested the presence of a functional cystathionine pathway. Gene expression measurements revealed a two fold decrease in S-related genes after cys addition while GSH or met more than doubled the expression of all genes analyzed. S acquisition by germinating spores of *G. intraradices* appears to be regulated post-transcriptionally and to a lesser extent than in other fungi studied.

Introduction

The arbuscular mycorrhizal (AM) fungal symbiosis evolved more than 400 million years ago (Dotzler *et al.*, 2006) and remains the most widespread underground symbiosis. AM fungi can be found in association with more than 90% of plant families in all biomes filled by land plants excluding arctic tundra (Wang & Qiu, 2006). Although the AM symbiosis helps plants take up mineral nutrients, especially phosphorus, from the soil (Cooper & Tinker, 1978; Li *et al.*, 1991; Gahoonia *et al.*, 1994; Wang *et al.*, 2004; Hepper & Warner, 1983), the application of AM fungi to agriculture has been limited by their obligate biotrophic nature and resulting difficulties in efficient large scale propagation of inoculum (Douds *et al.*, 2006). It is therefore a goal of mycorrhizal research to increase the practical use of AM fungi by determining a culturing method that enables their propagation in the absence of host roots.

The transition from pre-symbiotic to symbiotic growth was an early focus of efforts to identify a method of root-less propagation of AM fungi (Hepper, 1984; Carr *et al.,* 1985). Fungal spores germinate without external mineral or carbon sources although they are imported when available (Bücking *et al.*, 2008; Smith & Read, 2008; Gachomo *et al.*, 2009), and germ tubes severed from the spore rely on them to extend normally (Hepper, 1983). Germinating spores are limited in the uptake and use of exogenous carbon sources and lack some primary metabolic pathways including de novo fatty acid synthesis (Bago *et al.*, 1999). Recently, chemicals in root exudates including an isolated signaling molecule, strigolactone, have been shown to alter the metabolism and gene expression of AM fungal germinating spores in the absence of a host (Besserer *et al.*,

2008). The addition of root exudates alters gene expression in *Gigaspora rosea*, inducing changes in physiological state in preparation for root penetration (Tamasloukht *et al.*, 2003). A better understanding of the regulation of metabolic functions and nutrient allocation during pre-symbiotic growth may thus reveal signaling pathways that govern the developmental stages of the fungus. This study examined the regulation of sulfur assimilation in germinating spores of *Glomus intraradices* as part of the ongoing effort to achieve axenic propagation of AM fungi.

Direct reduction of sulfate with NADPH is a highly endergonic reaction, and is bypassed in fungi through adenylation and further phosphorylation of the adenosyl group (Lowe, 1991; Marzluf, 1997). These steps are carried out by S-adenylyl-transferase (SAT) and adenylyl-sulfate kinase (APSK), respectively, and are followed by the two step reduction of the activated sulfate moiety to sulfide. The initial reduction of the sulfate moiety by thioredoxin oxidation is mediated by the enzyme 3'-phosphoadenosine-5'-phosphosulfate reductase (PAPSR); sulfite reductase is responsible for further reduction to sulfide. Sulfide is incorporated into either *O*-acetylserine (OAS) or *O*-acetylhomoserine (OAH), forming cysteine or homocysteine, respectively. Fungi and archaebacteria are the only organisms known to possess both assimilation pathways (Marzluf, 1997; Zhou and White, 1991).

The dominant path of sulfide incorporation varies among fungi studied (Marzluf, 1997). The assimilation of sulfide through OAH requires the conversion of homocysteine to cysteine by a specific two-step enzymatic pathway, the Cystathionine (CT) pathway, which involves the conversion of homocysteine and serine to cystathionine by the enzyme cystathionine β-synthase (CBS) followed by the cleavage of cystathionine to α-

ketobutyric acid, ammonia, and cysteine by cystathionine γ-lyase (CGL). In *Saccharomyces cerevisiae*, cysteine synthesis occurs solely through the CT pathway (Cherest & Surdin-Kerjan, 1992; Ono *et al.*, 1999), while the pathway is absent in the closely related yeast *Candida valida* (Piotrowska, 1993). This is also true for *Schizosaccharomyces pombe* (Brzywczy *et al.*, 2002), although the possession of both pathways is the norm. *Aspergillus nidulans* and *Neurospora crassa* contain the enzymes to assimilate sulfide into either cysteine or homocysteine, as do the yeast *Yarrowia lipolytica*, *Cephalosporium acremonium*, and ***Trichosporon cutaneum*** (Brzywczy & Paszewski, 1993; Paszewski & Grabski, 1974; Morzycka & Paszewski, 1979; Jackobsen & Metzenberg, 1977; Lewandowska & Paszewski, 1987), although in *A. nidulans* the synthesis of cysteine is dominant (Brzywczy *et al.*, 2007). Other fungi may have similar biases.

Regulation of S assimilation in fungi is mediated by the transcription and allosteric control of S assimilation enzymes (Marzluf, 1997; Foster *et al.*, 1994; Renosto *et al.*, 1990; Bradfield *et al.*, 1970). A homologous positive regulator controlling a host of S assimilation genes has been identified for *Neurospora crassa* (CYS3; Marzluf and Metzenberg, 1968), *Aspergillus nidulans* (METR; Natorff *et al.*, 2003), and *Saccharomyces cerevisiae* (MET4; Thomas *et al.*, 1992). Repression of sulfate assimilation by the addition of a reduced S source such as met is mediated by controlling the activity of the positive regulatory protein through transcriptional regulation (Marzluf, 1997) or ubiquitination (Kuras *et al.*, 2002). Additionally, SAT is strongly inhibited by the product of the second enzymatic step, 3'-phosphoadenosine-5'-phosphosulfate (PAPS), in *A. nidulans*, *Penicillium chrysogenum*, *Penicillium duponti*, and *N. crassa*

(Renosto *et al.*, 1990), and sulfate uptake is completely suppressed by an order of magnitude lower concentration of met in *S. cerevisiae* (Breton & Surdin-Kerjan, 1977).

The results presented here show that germinating spores of *Glomus intraradices* can assimilate S from sulfate, cysteine (cys) and methionine (met). The addition of cys reduced sulfate assimilation, but less than it does in other fungi, and with diminishing potency after its addition. Met addition had little effect on the utilization of S from sulfate or cys, but increased the expression of putative genes of the sulfate assimilatory pathway. In contrast to labeling data, putative genes encoding CT pathway enzymes were up-regulated by more than four fold in response to met, suggesting the induction of the pathway from met to cys. In contrast to other fungi, assimilation of reduced and oxidized forms of S was simultaneous and non-preferential. Exposure to reduced glutathione (GSH) resulted in a reduction in sulfate uptake similar to cys addition and changes in gene expression similar to met addition, whereas buthionine sulfoximine, a specific inhibitor of GSH synthesis, increased sulfate utilization and had no effect on gene expression. The results demonstrate that presymbiotically growing *G. intraradices* assimilates S and possesses a complex regulatory system for S metabolism with distinctive features compared to all other fungi studied to date.

Experimental Procedures

Tissue culturing

Standardized germination cultures were prepared under sterile conditions by blending spores (Shenk & Smith; DAOM 181602) with solidified Gel-gro agar (MP Biomedicals, Solon, OH) and transferring 2 mL into each well of a sterile 12-well polystyrene plate. *Glomus intraradices* spores purchased from Premier Tech Biotechnologies (Revière-du-Loup, Québec, Canada) were used throughout. Prior to labeling, spore cultures contained only Gel-gro, spores, 2 mM $CaCl_2$, and 2 mM MES buffer (pH 6.0). This process resulted in consistent sample weights and a uniform spore density of approximately 2500 spores mL^{-1}. MES contains S, however its addition did not alter radio-labeled sulfate uptake (not shown). Germination plates were stored at 4°C for a minimum of one week before use. All experiments were conducted using germination plates unless, as in the 3 hr time point tested (Fig. 3-1) and the germination of tissue for gene expression analysis (Fig. 3-5), the timing of tissue collection was critical for the success of the experiment. For experiments where germination plates were impractical, spores were germinated in liquid media.

Radio-labeled sulfate ($Na_2[^{35}SO_4]$; MP Biomedicals) was supplied to spores with 0.1 mM Na_2SO_4 with two exceptions. The examination of ^{35}S-labeling from amino acids and sulfate combined (Fig. 3-3) was conducted with 0.1 mM total S, each source comprising 0.05 mM S. Also, the measurement of uptake at three hours from various sulfate concentrations (Fig. 3-1) utilized 0.01, 0.014, 0.022, 0.04, 0.1 or 0.2 mM Na_2SO_4.

Labeled cys and met (L-[^{35}S]cysteine and L-[^{35}S]methionine; MP Biomedicals) were supplied with either 0.1 mM (Fig. 3-3) or 0.05 mM (Fig. 3-4) of the corresponding non-labeled amino acid.

In all experiments conducted filter-sterilized stock solutions were added as a 1 mL solution to each well and spores germinated in a 30°C incubator. Samples in blended Gel-gro were collected by first transferring the contents of a well to a plastic bottle with 100 mL of 10 mM sodium citrate buffer (pH 6.0) to disintegrate the gellan. Surface radioactivity was removed by a sieving procedure described previously (Allen & Shachar-Hill, 2009). Briefly, 30 mL of cold 10 mM Na_2SO_4 was allowed to flow through the sample. This was repeated three times followed by 30 mL of cold deionized water. Samples were immediately frozen in liquid nitrogen, lyophilized, and weighed. In the case of ^{35}S-labeled samples not germinated in Gel-gro (Fig. 3-1), the sodium citrate buffer was replaced by 100mL of cold MiliQ water. Spores germinated for the measurement of gene expression were only washed with approximately 10 mL of MiliQ water over a sieve followed by immediate collection and freezing in liquid nitrogen. The collection of ^{35}S-labeled spores and mycelia lasted approximately five minutes while the collection of material for gene expression measurements took less than one minute. Samples for gene expression analysis were stored in a -80°C freezer until extracted.

Extraction and fractionation procedure

The relative allocation of ^{35}S was determined by the crude separation of the cellular components according to chemical solubility (Allen & Shachar-Hill, 2009). In

this way, fractions containing, but not limited to, sulfate, the free amino acids cysteine and methionine, proteins, and sulfonated lipids were isolated from one another. Two 3 mm stainless steel beads were added to each dried sample in 1.5mL screw-top centrifuge vials along with 0.1 mL of methanol:water solution (70:30). Fungal spore and mycelial structures were disintegrated using a bead mill (Retsch MM301) set at 30Hz for four minutes at 4°C. A representative sample was taken from each milling and examined under a dissecting microscope to ensure complete breakage. Another 0.9 mL of methanol:water solution (70:30) was added and the samples vortexed for five minutes to isolate ionic and small organic compounds including sulfate, sulfite, free amino acids, and glutathione. The samples were centrifuged and supernatants collected. A portion of the alcohol extract (0.5 mL) was added to a solution containing Na_2SO_4 and slightly acidified with 0.05 mL of 1 N HCl, followed by the addition of $BaCl_2$ solution. The final concentrations of sulfate and barium were 10 mM and 11 mM, respectively. Samples were placed in a 100°C heat block for 30min resulting in the precipitation of $BaSO_4$. In control experiments (not shown), this procedure was determined to be more than 99% effective in removing a known amount of radio-labeled sulfate from solution. The comparison between radioactivity levels in the pre- and post-barium precipitation solutions determined the amount of ^{35}S in the sulfate and amino acid pools. Proteins were extracted from the remaining pellets using Urea/SDS buffer consisting of 9 M urea, 1% SDS, 0.7 M β-mercaptoethanol, 25 mM Tris-HCl (pH 6.8), and 1 mM EDTA (Osherov and May, 1998). To completely solubilize the proteins, samples were heated to 100°C on a heat block for 5 min, vortexed for 1 min, and heated again for 1 min. After

centrifugation at 14,000 g for 5 min, 0.5 mL of the supernatant was analyzed by scintillation counting. The effect on counting efficiency of adding 1 M urea to scintillation fluid (BioSafe II, MP Biomedicals) was analyzed and found to be negligable (not shown). Pellets were transferred to glass ampules containing 0.5 mL TS-2 tissue solubilizer (MP Biomedicals), sealed over a gas flame, and incubated at 70°C for one week. Scintillation fluid (Bio-Safe II, MP biomedicals) was added directly to the tissue solubilizing solution followed by scintillation counter analysis, and was titrated with 1 N HCl solution until neutral before scintillation counting.

Sample preparation for gene expression analysis

Samples were pulverized using a bead mill (Retsch MM301) set to 30 Hz with 100 μL of RNA extraction buffer and two 3 mM stainless steel beads. Care was taken not to allow thawing by milling the samples for 2 min intervals followed by re-freezing in liquid nitrogen for three to four cycles. A small aliquot of two samples were checked under a dissecting microscope for tissue disruption. Total RNA was then extracted using the RNeasy Plant Mini Kit (Qiagen, Valencia, CA) immediately followed by DNA removal using RNase-free DNase (Turbo DNA-free; Ambion, Austin, TX). Following quantification of RNA by spectroscopy, an equal concentration of cDNA for each sample was synthesized with SuperScript II reverse transcriptase (Invitrogen corp., Carlsbad, CA) from each sample. Simultaneously, a set of reverse transcriptase free control samples were created.

Sequences of gene fragments with high homology to known sulfur assimilatory genes from filamentous fungi and yeast were obtained through a combination of high

throughput sequencing data (PJ Lammers, Y Shachar-Hill and coworkers unpublished results) and a published EST database (Jun *et al.*, 2002). These sequence contigs were verified by standard PCR using cDNA (obtained as already described) derived from spores germinated in water for one week followed by sequencing using an ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems, CA, USA). A QIAquick Gel Extraction Kit (QIAGEN GmbH, Germany) was used to isolate the DNA fragments after separation by electrophoresis. The primer sequences (IDT, Syntegen, Skokie, IL) used and fragment sizes are listed here: putative high affinity sulfate permease (482 bp), forward 5'-CGGGATTCCAGATGCACTT-3', reverse 5'-GGGAAGTTCATTCCATGGTC-3'; putative sulfate adenylyl-transferase (902 bp), forward 5'-ATATCGACCATTATACAAGAGTAAGAGTC-3', reverse 5'-CGATATATCCGTCCTTTTCGA-3'; putative phosphoadenylyl sulfate reductase (764 bp), forward 5'-ACCCGCGAATCAGTATATCG-3', reverse 5'-CCCACCTTCCATCTCTTTCA-3'; putative cysteine synthase (1404 bp), forward 5'-AAACCAACAAAGGGACAACG-3', reverse 5'-TTTATCCAGTTCCGGCAGTC-3'; putative cystathionine β-synthase (605 bp), forward 5'-ATGGAGTCTAATGACAAAGAAAATAATTG-3', reverse 5'-GAGAGATTTTGCAACTTGAATTGCTGCC-3'; putative cystathionine γ-lyase (922 bp), forward 5'-CCAAATCGAAGTGCATTTGAAGAGTC-3', reverse 5'-CCTAGCAAATCATCAACATCTTCGATACC-3'.

Real time quantitative PCR measurements

Gene expression was analyzed using an ABI Prism 7900 HT Sequence Detection System (Applied Biosystems, Austin, TX) using the following cycling program: 50°C for 2 min, 95°C for 10 min followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. Power SYBR Green 2-Step Master Mix (Applied Biosystems, Austin, TX) was used to produce 0.015 mL samples containing 1 ng of cDNA and 300 nM primers per sample. From these, 0.01 mL was transferred to a 384 well plate for analysis.

Gene expression of putative S assimilation genes was measured relative to the expression of an S4 ribosomal protein as described by Govindarajalu *et al.* (2005) using the $\Delta\Delta C_T$ (Livak & Schmittgen, 2001) and comparative C_T methods. Primer Express software (Applied Biosystems, Austin, TX) was used to create qRT-PCR specific primer sets for all sequences studied. Primer sequences for qRT-PCR are listed here: putative high affinity sulfate permease forward 5'-CAATTCTGGGATTTTATGGTCTTTTT-3', reverse 5'-GATTCCATATTCGACGGTAGTGAA-3'; putative sulfate adenylyl-transferase forward 5'-AGCTTTTGTAGCAGAGCTAAC-3', reverse 5'-GGCAAACGGAGCAATTGG-3'; putative phosphoadenylyl sulfate reductase forward 5'-AAAATCTTGGAATGGGCTCTT-3' and reverse 5'-GTGAGGCCGAAAGCAGTTG-3'; putative cystathionine β-synthase forward 5'-CAATTATTGAGCCAACTTCTGGAA-3', reverse 5'-TCCTTTGATAGCAGCCGCTAA-3'; putative cystathionine γ-lyase , forward 5'-AGCAAAGAAACAGGCAAGAGGAT-3', reverse 5'-TGCTTCCTTAAAACCACCATTAAT-3'; S4 ribosomal protein forward 5'-

AACAGGTGGTAGAAATATGGGAAGA-3' and reverse 5'-

AAGCCGCCTACGTGTCGTT-3'.

Results

Import and assimilation of sulfate by germinating spores and its regulation by S metabolites

The uptake and assimilation of exogenous sulfate during presymbiotic growth was measured by supplying radio-labeled sulfate to spores germinated 7 days prior. The rate of import increased linearly ($R^2 = 0.983$) when sulfate was supplied at concentrations ranging from 10-100 μM sulfate (Fig. 3-1). In this range there was also a linear relationship between external sulfate concentration and incorporation into different biochemical fractions: sulfate ($R^2 = 0.978$), amino acids ($R^2 = 0.964$), extractable proteins ($R^2 = 0.989$), and tissue solubilized cell material ($R^2 = 0.901$). In proportion to the total S uptake, incorporation into the amino acid fraction decreased by 5 fold, while intracellular sulfate doubled between 10 and 100 μM external sulfate. There was no further increase in uptake or assimilation of S into any fraction with concentrations exceeding 100 μM (Fig. 3-1; data not shown).

Import, reduction, and incorporation of ^{35}S from sulfate was continuous for at least 7 days and was affected by some, but not all, sulfur metabolites tested (Fig. 3-2). The total incorporation of ^{35}S over 2, 4, and 7 days increases linearly ($R^2 = 0.977$) with analogous increases in pools containing proteins and aqueous-alcohol soluble S-containing molecules. The uptake of sulfate was reduced by the addition of cysteine (cys) or reduced glutathione (GSH), but not methionine (met). Cysteine initially reduced total

^{35}S uptake by 35 ± 4% by day 2, however, the effect of cys diminished to a 21 ± 4% reduction by day 4 and finally a 13 ± 4% reduction by day 7. Less than 10% of the cys available in the media was consumed during the experiment. GSH addition and the inhibition of GSH synthesis had the most dramatic effects on sulfate uptake by the spores (Fig. 3-2). Uptake was reduced by 63 ± 1% and 64 ± 3% at days 2 and 4, respectively, when 1 mM GSH was added. At day 7, however, this effect was diminished to the point that a difference in uptake was not statistically significant between the control and GSH samples, similar to the diminished effect of cys addition. Buthionine sulfoximine (BSO) irreversibly inhibits GSH synthesis at the point of glutamate-cysteine ligase (Griffith & Meister, 1979). The effect of adding 10 mM BSO was to increase sulfate uptake, compared to control samples by 30 +/- 2% (day 2), 47 +/- 9% (day 4), and 82 +/- 10% (day 7). ^{35}S incorporation into the sulfate and reduced sulfur-containing small molecule pools increased most (Fig 3-2). Taken together the effects of GSH and BSO strongly suggest that intracellular GSH concentrations regulate sulfate assimilation. The uptake of sulfate in germinating spores exposed to exogenous met was not statistically significantly different from the uptake by spores germinated in sulfate alone at any time point.

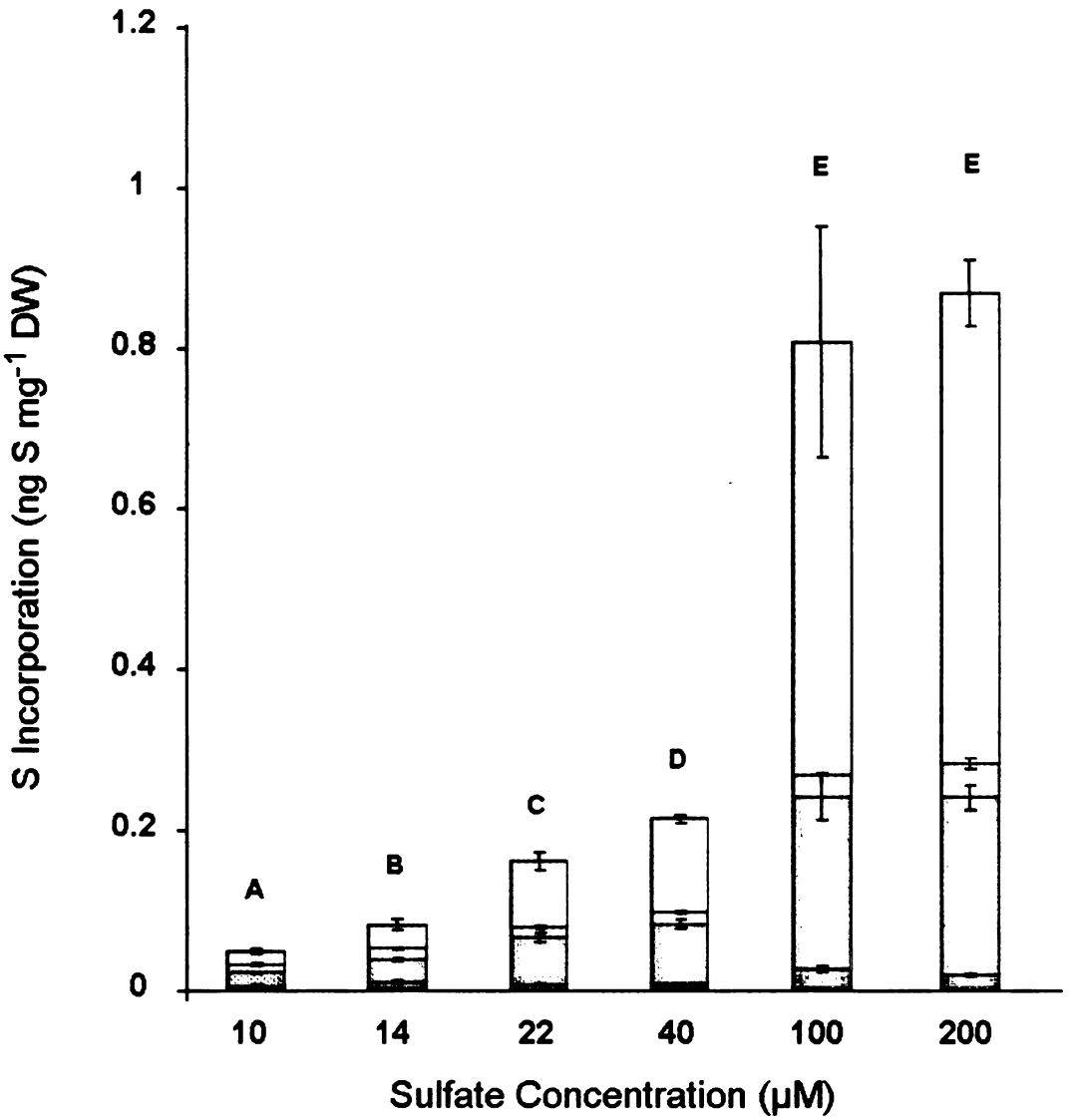


Figure 3-1 Three hour uptake and incorporation of radio-labeled sulfate by germinating spores. Spores were germinated in an S-free media for five days prior to the addition of 10, 14, 22, 40, 100, 120, 150, 200, or 300 µM sulfate labeled with $^{35}SO_4^{2-}$ at a specific activity of 7.2 µCi μg^{-1} S. Tissue was collected and counts fractionated into pools containing but not limited to sulfate (white bars), amino acids (light gray bars), proteins (gray bars), and tissue solubilized cell debris (dark gray bars). Error bars depict standard error of the mean (n = 5). Statistical analyses done by ANOVA single factor analysis, alpha = 0.05.

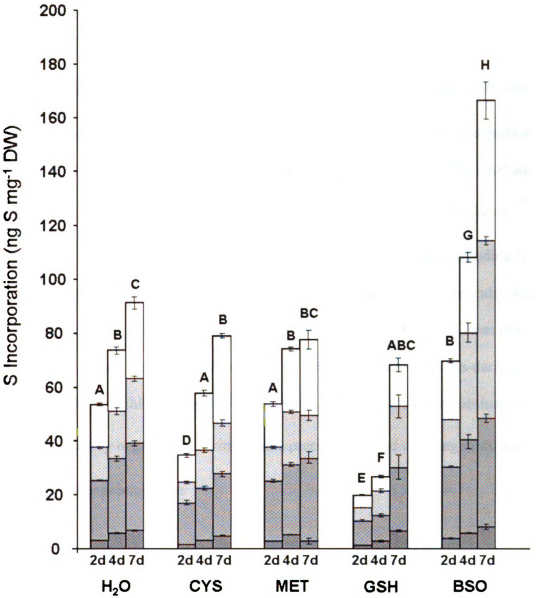


Figure 3-2 Radio-labeled sulfate uptake over time with the addition of sulfur metabolites. Spores were germinated for 2 (2d), 4 (4d), or 7 (7d) days with 7.85 µCi of $^{35}SO_4^{2-}$ in the presence or absence of 1 mM L-cysteine hydrochloride (CYS), 1 mM L-methionine (MET), 1 mM reduced glutathione (GSH), or 10 mM buthionine sulfoximine (BSO), an inhibitor of GSH synthesis. Tissue was collected and counts fractionated into pools containing but not limited to sulfate (white bars), amino acids (light gray bars), proteins (gray bars), and tissue solubilized cell debris (dark gray bars). Error bars depict standard error of the mean (n = 5). Statistical analyses done by ANOVA single factor analysis, alpha = 0.1.

Uptake and assimilation of S from cysteine

^{35}S incorporation from labeled cys and the effect of sulfate, met, GSH, BSO or *O*-acetylserine (OAS) addition was examined (Fig. 3-3) and the statistical significance of the results determined by ANOVA single factor analysis (p-value < 0.05). GSH addition increased (44 ± 11 %) while BSO addition decreased (11 ± 6 %) total uptake of [^{35}S]cys compared to controls. No other treatments significantly altered uptake including OAS, a precursor of cys, either with or without added sulfate. None of the compounds added significantly altered the amino acid or protein fractional content derived from labeled cys. The data also shows no significant synthesis of sulfate or sulfite from cys-derived S (Fig. 3-3). Any counts in this fraction are within the range of errors from contamination of the sulfate fraction by organic ^{35}S during sulfate precipitation and counting inaccuracies before and after precipitation.

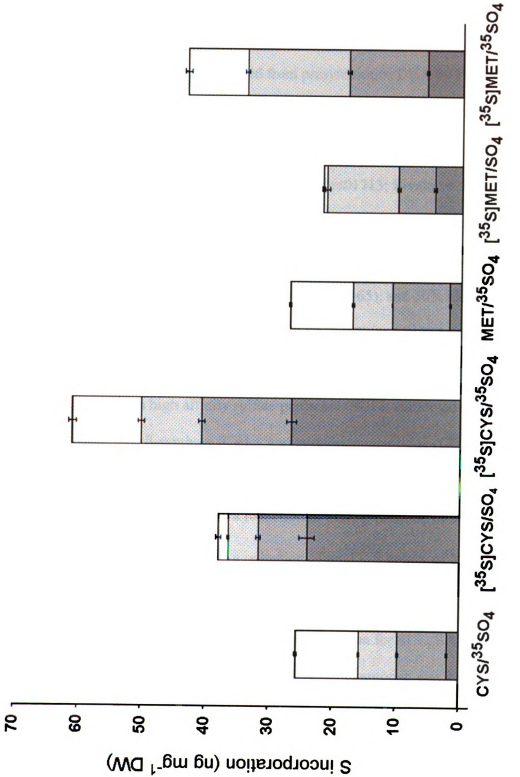
Simultaneous uptake of sulfate and reduced S species

An experiment was conducted to determine the degree of preference for the importation and assimilation of organic S sources (cys and met) compared to sulfate (Fig. 3-4). The incorporation of ^{35}S was measured when added as ^{35}S-sulfate or ^{35}S-amino acids in the presence of unlabeled amino acids and sulfate respectively, or when both sulfate and an amino acid were labeled. This was compared to the incorporation of ^{35}S added as either sulfate or an amino acid alone. When [^{35}S]cys and $^{35}SO_4^{2-}$ were

introduced to germinating spores together, the resulting labeling was equivalent to the sum of the labeling from either source individually to within the experimental accuracy. Labeled S from [^{35}S]cys was more readily taken up than from sulfate by 48% (Fig. 3-4), while the opposite was true for met, labeling with which resulted in an 18.7% decrease in total ^{35}S uptake compared to $^{35}SO_4^{-2}$ labeled samples. Overall labeling from $^{35}SO_4^{2-}$ was roughly equivalent whether unlabeled cys or met was added, only changing by 5 ± 2 %. This finding was similar to $^{35}SO_4^{2-}$ uptake with cys and met addition after 7 days from figure 2, which did not result in a statistically significant difference (2 ± 12%). While the overall uptake was similar, when the label was supplied as [^{35}S]cys compared to $^{35}SO_4^{2-}$, there was a 13 fold increase in the total counts discovered in the solubilized fraction (Fig. 3-4), and an increase in this fraction was also seen in the [^{35}S]CYS/$^{35}SO_4$ samples. Labeled met increased the solubilized fraction by 2.4 fold compared to labeled sulfate. The reason for this fractional increase is not clear, however it shows that inter-conversion of cys and met is limited and suggests that the insoluble fraction contains cys or a derivative thereof.

Figure 3-3 Combined uptake of sulfate and reduced S species cys and met. Equal amounts (0.05 mM) of sodium sulfate and either cys or met were added to germinating spores with labeling supplied as 8.8 μCi $Na_2[^{35}SO_4]$ (CYS/$^{35}SO_4$; MET/$^{35}SO_4$) , 8.2 μCi [^{35}S]cys ([^{35}S]CYS/SO_4), or 8.5 μCi [^{35}S]met ([^{35}S]MET/SO_4). The uptake and incorporation of ^{35}S was compared between additions of single labeling and double labeling with both $Na_2[^{35}SO_4]$ and [^{35}S]cys ([^{35}S]CYS/$^{35}SO_4$), or $Na_2[^{35}SO_4]$ and [^{35}S]met ([^{35}S]MET/$^{35}SO_4$). The resulting labeling was chemically separated after 7 days of germination into fractions containing but not limited to sulfate (white bars), amino acids (light gray bars), proteins (medium gray bars), and tissue solubilized residue (dark gray bars) and analyzed by scintillation counting. Error is expressed as standard error of the mean (n = 4). The total uptake was significantly different for all conditions by ANOVA single factor analysis (alpha = 0.05).

Figure 3-3


S incorporation (ng mg-1 DW)
70
60
50
40
30
20
10
0
CYS/35SO4
[35S]CYS/SO4
[35S]CYS/35SO4
MET/35SO4
[35S]MET/SO4
[35S]MET/35SO4

The expression of putative S metabolism and transport genes

An expressed gene sequence was identified by 454 sequencing of cDNA from extraradical mycelium that has close similarity to 3'-phosphoadenylsulfate reductase (PAPSR). This sequence was amplified from presymbiotic cDNA by PCR and sequenced to confirm its identity. PAPSR catalyzes the first reduction step converting sulfate to sulfite (see introduction). A BLAST search using the nonredundant database at NCBI revealed one conserved PAPSR-specific domain (cd01713; E-value = 5e-33). The protein sequence shares 54% identity with PAPSR from a fungal plant pathogen, *Pyrenophora tritici-repentis* (XP_001937780; E-value = 9e-70), 51% identity with MET16 (PAPSR) from *Aspergillus nidulans* (XP_662374; E-value = 4e-65), and 50% identity with PAPSR from *Neurospora crassa* (XP_964870; E-value = 2e-60), and MET16 from *Sacchaomyces cerevisiae* (NP_015493; E-value = 4e-59). The analyses of putative sequences encoding a high affinity sulfate permease (SUL), sulfate-adenylyl transferase (SAT), cystathionine β-synthase (CBL), and cystathionine γ-lyase (CGL) were previously reported (Allen & Shachar-Hill, 2009).

Exposure to cys led to both a reduction in SUL expression of 63 +/- 9% after a 6hr induction period (Fig. 3-5) and a reduction of total ^{35}S uptake of 35 ± 4% after two days of presymbiotic labeling (Fig. 3-2). In contrast, addition of either met or GSH to germinating spores resulted in increased gene expression for SUL, SAT, and PAPSR, and greatly increased expression of CBS and CGL. CBS expression was increased 3.5 ± 0.8 fold by met and 4.4 ± 0.8 fold by GSH addition, whereas CGL expression was increased 4.4 ± 0.8 fold by met and 4.9 ± 0.2 fold by GSH addition. Although BSO greatly increased uptake of $^{35}SO_4^{2-}$ (Fig. 3-2), it had little effect on the expression of any gene

analyzed. Changes in the expression of putative gene sequences encoding a high affinity sulfate permease (SUL) and the first and third steps of sulfate assimilation, sulfate-adenylyltransferase (SAT), and 3'-phosphoadenylsulfate reductase (PAPSR), were not biologically significant (between 0.5 and 2 fold) for cys, met, or GSH treated samples (Fig. 3-5).

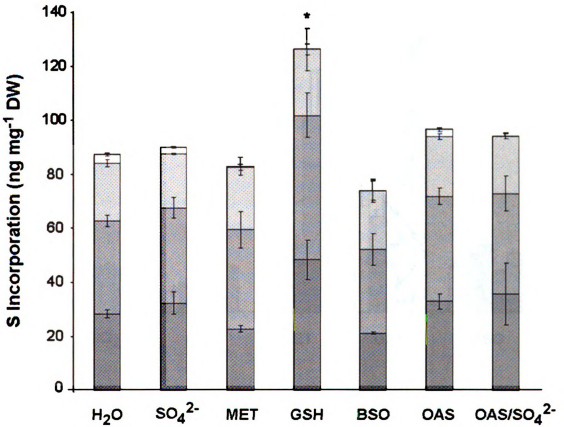


Figure 3-4 [^{35}S]Cysteine uptake and utilization in the presence of common sulfur metabolites. Spores were germinated in a 30°C incubator for 7 days in the presence of 5.25 µCi of [^{35}S]cys and 0.1 mM sulfate (SO_4), 1 mM methionine (MET), 1 mM reduced glutathione (GSH), 10 mM buthionine sulfoximine (BSO), 1 mM *O*-acetyl serine (OAS), 1 mM OAS with 0.1 mM sulfate (OAS/S), or nothing else (H_2O). Samples were collected and the S species separated by chemical fractionation yielding fractions containing but not limited to sulfate (white bars), amino acids (light gray bars), and proteins (dark gray bars), analyzed by scintillation counting. Error is expressed as standard error (n=3). Stars signify statistical differences (total uptake) from all other treatments by ANOVA single factor analysis (alpha = 0.1).

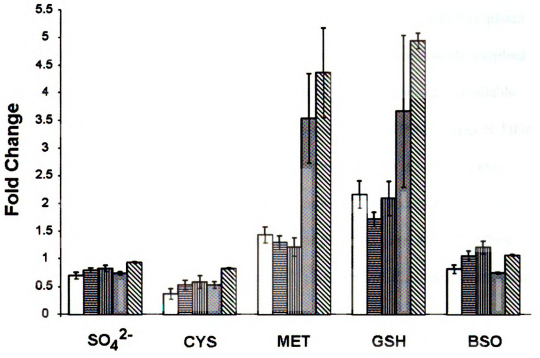


Figure 3-5 Real-time PCR analysis of changes in sulfur assimilation pathway gene expression in response to sulfur metabolites. Spores of *G. intraradices* were germinated for five days in 2 mM MES (pH 6.0) prior to induction for six hours with 0.1 mM sodium sulfate (SO_4^{2-}), 1 mM L-cysteine hydrochloride (CYS), 1 mM L-methionine (MET), 1 mM reduced glutathione (GSH), or 10 mM buthionine sulfoximine (BSO). Expression as fold change was calculated relative to control samples labeled with only deionized water. Putative sequences of the S assimilation pathway including a high affinity sulfate permease (SUL; white), S-adenosyl transferase (SAT; horizontal lines), phosphoadenylsulfate reductase (PAPSR; vertical lines), cytathionine β-synthase (CBS; gray), and cystathionine γ-lyase (CGL; down-slashed lines) were analyzed. Values are reported as mean ± SEM (n = 3 biological replicates).

Discussion

Germinating spores of *Glomus intraradices* remain metabolically active for approximately two weeks in the absence of host roots before germ tube cytoplasm retreats and spores regain dormancy (Smith & Read, 2008). Exogenously supplied phosphorus, nitrogen, acetate, amino acids, and glucose are imported if available (Bücking *et al.*, 2008; Lammers *et al.*, 2001; Thomson *et al.*, 1990; Hepper & Jakobsen, 1983; Bago *et al.*, 1999; Gachomo *et al.*, 2009), however little is currently known of the function of this uptake capacity. Imported nutrients and root secreted signaling compounds can affect extension and branching of the germ tube, as well as their metabolic capability (Hepper & Jakobsen, 1983; Besserer *et al.*, 2008; Tamasloukht *et al.*, 2003; 2007; Nagahashi *et al.*, 1996).

Sulfur is a macronutrient required in quantities similar to phosphorus and its assimilation is regulated similarly among other fungal species (Marzluf, 1997). Exogenously supplied sulfate is readily imported, reduced, and incorporated by germinating spores of *G. intraradices*, producing stable proportions of S incorporation into proteins and amino acids. This proportional relationship was unaffected by sulfate concentration (Fig. 3-1), the application of S amino acids, GSH or an inhibitor of GSH synthesis (Fig. 3-2), or total sulfate uptake. These data show that the relative rates of sulfate reduction and incorporation into proteins are constant, and but that sulfate uptake is less closely regulated relative to assimilation.

Sulfate assimilation is an energy intensive process and other fungi control the assimilation of different S forms through strict transcriptional and allosteric regulation of

sulfate uptake and reduction so as to preferentially use reduced forms of S when available (Foster *et al.*, 1994; Renosto *et al.*, 1990; Lowe, 1991). In *S. cerevisiae* for instance, exposure to 0.75 mM met results in a complete absence of sulfate permease activity (Breton & Surdin-Kerjan, 1977). In contrast both organic and inorganic S are imported and assimilated by spores of *Glomus intraradices* throughout germination and germ tube extension with no detectable preference for reduced forms of S (Fig. 3-4). The addition of met and its uptake by presymbiotic *G. intraradices* had no effect on sulfate uptake. In *S. pombe*, which lacks the enzymes needed to convert met to cys (Brzywczy *et al.*, 2002), met addition also fails to regulate sulfate uptake, presumably because the demand for cys cannot be met by met. Cysteine initially suppressed uptake by *G. intraradices* during spore germination although much less than in other fungi, and even this effect diminished during development. One week of exposure to cys as the sole S source led to S incorporation comparable to that from sulfate (Fig. 3-3), so the diminishing effect of cys on sulfate uptake is likely not due to a reduction in the import of cys. The simultaneous uptake and assimilation of sulfate and S amino acids was confirmed for cys and met (Fig. 3-4), and to our knowledge represents the only such case among fungi. Interestingly, the relative rates of incorporation of S from sulfate into amino acid and protein pools are not altered by the simultaneous uptake and incorporation of S from reduced sources (Figs. 3-2, 3-4), suggesting that assimilation of reduced and oxidized forms of S are regulated separately.

Regulation of sulfate permease expression controls the flux through the S assimilation pathway in fungi (For a review, see Marzluf 1997). The transcription of genes encoding a high affinity sulfate permease in *N. crassa*, and high and low affinity

sulfate permeases from *A. nidulans*, *P. chrysogenum*, and *S. cerevisiae* is completely suppressed by the availability of reduced S or high concentrations of sulfate (Cherest *et al.*, 1997; Pilsyk *et al.*, 2007; Van de Kamp *et al.*, 2000; Ketter and Marzluf, 1988; Natorff *et al.*, 2003). In *A. nidulans*, a mutant lacking a functional S-adenosyl transferase (SAT) accumulate up to 40 mM sulfate demonstrating that the reduction of sulfate is required for permease regulation (Bradfield *et al.*, 1970), and sulfate permeases in this species as well as *N. crassa* are known to be transcriptionally regulated by sulfur metabolite repression mediated through cys (Pilsyk *et al.*, 2007; Ketter *et al.*, 1991). In *G. intraradices*, the expression of a putative sulfate permease (SUL) was suppressed by the presence of cys. The reduction in SUL expression by cys agreed with the reduction in uptake of sulfate. This is consistent with data on this sequence previously published in which addition of 1 mM cys decreased both uptake of sulfate and expression of SUL by roughly half in the extraradical mycelium during the symbiotic phase of the life cycle (Allen & Shachar-Hill, 2009). However, met addition, while having no effect on sulfate uptake, doubled the expression of SUL. Sulfate uptake and SUL expression were also differently affected when spores were exposed to GSH or BSO (Figs. 3-2, 3-5), suggesting that changes in SUL transcript levels are not the primary level at which SO_4^{2-} uptake is regulated in this fungus.

There was no increase in the uptake of sulfate when it was supplied in concentrations higher than 0.1 mM, which agrees with the presence of a single high affinity transport system (Fig. 3-1). Single high and low affinity sulfate permease genes have been identified in the genomes of *S. cerevisiae, N. crassa*, and *P. chrysogenum* (Cherest *et al.*, 1997; Ketter *et al.*, 1991; Van de Kamp *et al*, 2000), and only in *A.*

nidulans has a third sulfate transporter been characterized (Pilsyk *et al.*, 2007). This third transporter is distinct from other known sulfate permeases, belonging to a separate family of ion permeases than the highly conserved SulP family in which other sulfate permeases, and SUL, are part of (Pilsyk *et al.*, 2007). The genome of *G. intraradices* is smaller than that of other filamentous fungi and from the large amount of unpublished genomic and EST sequence available, there is no evidence that *G. intraradices* possesses more isoforms of other genes than do other fungi. It is therefore unlikely that there is another high affinity sulfate transporter in the *G. intraradices* genome, and it seems more probable that the changes in uptake observed are due to post-translational modifications or alloteric controls on permease activity.

The S assimilation pathway enzymes sulfate adenosyltransferase (SAT) and phosphoadenosyl phosphosulfate reductase (PAPSR), catalyzing the first and third steps of sulfate reduction, are transcriptionally regulated in *A. nidulans* (Natorff *et al.*, 2003), and *S. cerevisiae* (Thomas *et al.*, 1990; 1992) by sulfur metabolite repression. In *S. cerevisiae*, the addition of 2 mM met to the media lowers transcription of the SAT gene by ten fold (Cherest *et al.*, 1985), and 1 mM met completely suppresses the expression of the gene encoding PAPSR (Thomas *et al.*, 1990). In *A. nidulans,* expression of the gene encoding SAT is strongly repressed by 5 mM met addition (Borges-Walmsley *et al.*, 1995). The expression of putative sequences encoding SAT and PAPSR from *G. intraradices* germinating spores was different than that of other fungal SAT's. Spore SAT and PAPSR shared the expression profile of SUL (Fig. 3-5), and it is likely that these three sequences are regulated coordinately as SAT and PAPSR genes are in *S. cerevisiae* and *A. nidulans* (Marzluf, 1997). The putative SAT sequence shared the

greatest homology with SAT from *P. chrysogenum*, including a cys residue implicated in the allosteric control of the *P. chrysogenum* protein (Renosto *et al.*, 1990). Additionally, the *G. intraradices* putative SAT sequence has homology with a PAPS-binding region in SAT genes from *P. chrysogenum*, *A. nidulans*, and *A. terreus* (Clarke *et al.*, 1997; Schierova *et al.*, 2000). In *Aspergillus terreus*, the activity of SAT is reduced by 90% after the addition of 1 mM met (Schierova *et al.*, 2000). The addition of met to germinating spores of *G. intraradices* doubled the expression of SUL, SAT, and PAPSR without changing the uptake or assimilation of sulfate (Figs. 3-2, 3-5).

The conversion of met to cys requires the enzymes cystathionine beta synthase (CBS) and cystathionine gamma lyase (CGL), constituting the CT pathway. This capacity varies among fungal species and its absence is associated with a lack of effect of met on transcriptional regulation. In contrast to cys, met addition had no effect on sulfate assimilation in *G. intraradices*, suggesting an absence of the CT pathway (Fig. 3-2). The presence of putative sequences with homology to CBS and CGL and their expression profiles, however, contradicted this hypothesis, and the expression of these putative genes differs from other fungi. In *A. nidulans* CBS is not transcriptionally regulated by cys or sulfate (Sienko & Paszewski, 1999) whereas cys suppressed transcript levels in *G. intraradices* twofold. In *S. cerevisiae*, CGL transcript levels are reduced 15 fold by met addition (Ono *et al.*, 1993) while the *G. intraradices* transcript level was unaffected by sulfate or cys addition, and exposure to met increased it by more than 4 fold (Fig. 3-5). Glutathione addition yielded similar results to met exposure, although the effects of GSH and met on sulfate and cys uptake were dissimilar (Figs. 3-2, 3-3). Changes in expression of CBS and CGL were, like SUL, not reflected by the ^{35}S uptake data. In *S. cerevisiae*,

CGL transcription is coordinately regulated with a high affinity GSH transporter (GSH11) (Hiraishi *et al.*, 2008) through a *cis*-element linked to the TOR pathway (Miyake *et al.*, 2003), which coordinates cell growth with growth factors and nutrient availability (reviewed by Dann & Thomas, 2006). It is possible that in *G. intraradices* the CT pathway enzymes are also regulated by more than one signal.

While the assimilation of sulfate in relation to reduced S has been well studied, reduced S assimilation itself has not. The addition of precursors of cys synthesis in other fungi, met and *O*-acetyl serine (OAS), failed to affect incorporation of cys-S by *G. intraradices* (Fig. 3-3). At least one strain of *S. cerevisiae* is unable to import OAS (Ono *et al.*, 1996). The failure of OAS addition, with or without sulfate, to alter assimilation of S from cys may also be due to an inability of *G. intraradices* to import the substance, as OAS also failed to alter sulfate assimilation (unpublished data). Although met addition up-regulated putative CT pathway genes, it also had no effect on cys-S acquisition, suggesting that either met uptake is reduced in the presence of cys, or that met conversion through the CT pathway is low. Consistent with this possibility, [^{35}S]cys increased the proportion of radioactivity in the insoluble S fractions much more than [^{35}S]met (Fig. 3-4). In a reverse of their effects on sulfate uptake (Fig. 3-2), GSH increased while BSO decreased cys uptake (Fig. 3-3), again suggesting that the intracellular concentration of GSH is an important regulator of S uptake and assimilation. Cys-S incorporation was otherwise essentially constitutive.

Glutathione is present in millimolar quantities in fungal cells (Penninckx and Jaspers, 1982; Pocsi *et al.*, 2004) and is considered a reservoir of reduced S as its catabolism produces free cys (Miyake *et al.*, 1999). In *S. cerevisiae* and *S. pombe*,

exogenous GSH produces changes in gene expression and enzymatic activity similar to the addition of cys (Kumar *et al.*, 2003; Wheeler *et al.*, 2002; Brzywczy *et al.*, 2002). Both GSH and cys addition to germinating spores of *G. intraradices* had a suppressive effect on sulfate uptake which diminished over time (Fig. 3-2), yet had opposite effects on gene regulation (Fig. 3-5). Additionally, glutathione increased the assimilation of cys-S (Fig. 3-3), which, together with the gene expression results, is indicative that GSH effects are not the result of the catabolic breakdown to cys. In support of this, an inhibitor of GSH synthesis, buthionine sulfoximine (BSO), nearly doubled sulfate assimilation while having no effect on the transcript levels of putative S assimilation genes (Fig. 3-5). Thus it seems that the assimilation of sulfate is negatively correlated with intracellular GSH levels. This should be tested by measuring the intracellular concentration of GSH. In fungi, GSH has a broad range of effects on cellular processes (Pocsi *et al.*, 2004), one example of which is the regulation of vacuolar H+-ATPase by redox state in barley leaves and the bacterium *Dictyostelium discoideum* (Tavakoli *et al.*, 2001; Jeong *et al.*, 2006). Vacuolar and plasma membrane H+-ATPases are also coordinately regulated in *S. cerevisiae* in response to cytoplasmic pH (Fernandez & Sa-Correia, 2001), which has the potential to impact the activity of sulfate permease, a proton symporter.

Summary

The addition of cys led to decreased uptake of radio-labeled sulfate and decreased transcription of putative genes encoding a high affinity sulfate permease, CT pathway, sulfate assimilatory pathway enzymes (Fig. 3-5). Unlike other fungi studied with the exception of *S. pombe*, met addition failed to change sulfate uptake or the transcription of

related genes (Fig. 3-2). In *S. pombe*, the failure of met addition to elicit a regulatory response is due to the lack of a CT pathway converting met to cys, and the observation agrees with the hypothesis that cys is the principal regulatory molecule in fungi (Brzywczy *et al.*, 2002; Schierova *et al.*, 2000). *G. intraradices* germinating spores, however, possess putative transcripts homologous to the CT pathway genes, CBS and CGL, and these transcripts are up-regulated by met addition suggesting functionality (Fig. 3-5). Cysteine addition had moderate effects in *G. intraradices*, reducing transcription by two fold compared to similar studies in other fungi demonstrating much more extreme reductions of five to ten fold (Thomas *et al.*, 1990; Cherest *et al.*, 1985; Borges-Walmsley *et al.*, 1995). Aside from cys effects, sulfate assimilation in *G. intraradices* germinating spores was independent of transcriptional regulation (Fig. 3-5). These observations, along with the apparent up-regulation of putative S assimilation genes by GSH (Fig. 3-5), demonstrate that *G. intrardices* germinating spores are unique in the regulation of S assimilation among fungi studied.

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Chapter 4

The Relationship between N and S Assimilation in Germinating Spores of *Glomus intraradices*

Abstract

The relationship between the assimilation of two macronutrients, N and S, was analyzed in germinating spores of the arbuscular mycorrhizal fungi *Glomus intraradices* by measuring the effect of various N sources on $^{35}SO_4^{2-}$ uptake, sequestration, and assimilation into amino acid and protein pools. Additionally, ^{15}N-labeling of free amino acids by GC-MS and HPLC based amino acid concentration analyses were used to determine the effect of various S sources on N assimilation. Changes in putative S uptake and assimilation genes associated with exogenous ammonia availability were measured using quantitative real-time PCR and the results analyzed in relation to ^{35}S-uptake data. All N sources tested increased sulfate uptake by a minimum of two fold. Ammonia and urea led to the greatest increases in SO_4^{2-} uptake, and were also preferred N sources as reflected by increased free amino acid concentrations and ^{15}N-labeling compared to supplementation with nitrate, Gln, and Arg. Ammonia addition failed to increase sulfate uptake in the presence of reduced glutathione (GSH), which also halved the concentrations of amino acids related to N assimilation and doubled that of Leu, which is a regulator of S assimilation in other organisms. Cysteine, methionone, and *O*-acetylserine addition failed to counteract the effect of ammonia addition. When S was supplied to germinating spores as cysteine, ammonia addition had no effect on assimilation in the presence of absence of GSH, whereas GSH increased assimilation by 30%. The transcription of putative high affinity sulfate permease, sulfate adenosyl transferase, phosphoadenosylephosphosulfate reductase, cystathionine beta-synthase, and

cystathionine gamma-lyase was analyzed in relation to labeling data and found to be unrelated. While N assimilation is not altered by S addition, S assimilation is increased with N addition in proportion to general increases in amino acid concentrations from *de novo* synthesis.

Introduction

In fungi, N assimilation is tightly controlled on a transcriptional level and involves a global positive acting transcriptional factor and a host of associated pathway specific regulatory proteins (Marzluf, 1993). Orthologs of the global transcriptional factor responsible for the amino acid control circuit (gC) in yeasts and cross pathway control circuits (CPC) in filamentous fungi have been characterized in *Neurospora crassa* (Nit2; Chiang & Marzluf, 1995), *Aspergillus nidulans* (AreA/NirA; Okamoto *et al.*, 1993) and *Saccharomyces cerevisiae* (Gcn4/Gln3; Sosa *et al.*, 2003; Roussou *et al.*, 1988). Additionally, orthologs have been identified in the basidiomycete biotrophic plant pathogen *Ustilago maydis* (McCann & Snetselaar, 2008), the ascomycete ectomycorrhizal truffle *Tuber borchii* (TbNre1; Guescini *et al.*, 2009), the yeast *Hansula polymorpha* (Siverio *et al.*, 2002), as well as several other *Aspergilli* (Braus *et al.*, 2006), which show a high degree of conservation. Amino acid control in *S. cerevisiae* is initiated by the binding of a sensor kinase, Gcn2p, to uncharged tRNAs, resulting in a phosphorylation cascade, the general reduction of the translation initiation rate, and the increase in the transcription of Gcn4-controlled sequences (Dong *et al.*, 2000). Both AreA and Nit2 contain putative glutamine (gln) binding sites, disruption of which results in a loss of sensitivity to N repression (Marzluf, 1993). Intracellular gln concentrations in *A. nidulans* are inversely related to the activity of AreAp, suggesting that the cross pathway control regulatory system senses gln (Berger *et al.*, 2008).

There are direct and indirect associations between N and S assimilation on both the enzymatic and transcriptional level. The Gcn4p/Nit2 proteins directly or indirectly

regulate the expression of hundreds of genes (Tian *et al*, 2007; Natarajan *et al.*, 2001), including genes involved with S amino acid synthesis and sulfate reduction pathways. In *S. cerevisiae*, several of these genes have known Gcn4 promoter sequences including methionine synthase, sulfite reductase, 3'-phospho-5'-adenylylsulfate (PAPS) reductase, and the positive transcriptional activator Met4 (Thomas & Surdin-Kerjan, 1997). Interestingly, the only known transcriptional regulator of Met4, which is not regulated ny S source, is Gcn4p (Mountain *et al.*, 1993). Additionally, an analysis of conserved direct targets of Gcn4 and orthologs in *S. cerevisiae*, *C. albicans*, and *N. crassa* using genomic and transcriptomic data by Tian and co-workers (2007) revealed ten directly regulated genes, one of which is part of the transsulfuration pathway converting cysteine to methionine, cystathionine β-synthase. On the level of metabolism, the synthesis of met is directly related to threonine (thr) and indirectly related to aspartate (asp) and branched chain amino acid synthesis (Thomas & Surdin-Kerjan, 1997).

General amino acid control over transcription is known to be required for life cycle completion and survival in several fungi. The expression of AreA/Nit2/Gcn4 are required for complete virulence of *A. funigatus* in immuno-suppressed mice, morphogenesis in *C. albicans*, and cell-cell or cell-surface adhesion in *S. cerevisiae* (Braus *et al.*, 2006; Yin *et al.*, 2004; Braus *et al.*, 2003; Kleinschmidt *et al.*, 2005). The TbNre1 gene is also specifically upregulated in the *Tuber borchii* symbiotic state, demonstrating a possible role for cross pathway control in a mycorrhizal symbiosis (Guescini *et al.*, 2009). The widespread use of the endomycorrhizal symbiosis in agriculture is currently restricted by difficulties obtaining large scale inoculum, a consequence of their obligatory symbiotic nature. The study of nutrient regulation may

uncover the limitations on the transition from the pre-symbiotic to symbiotic state in arbuscular mycorrhizal fungi, enabling their large scale agricultural use.

In *Glomus intraradices* pre-germinating spores both N and S are imported and utilized, though exogenous sources are unnecessary for germination (Gachomo *et al*, 2009; Smith & Read, 2008). The present study examined the inter-relationship between N and S metabolism in arbuscular mycorrhizal fungi using germinating spores of *Glomus intraradices* as a model system. Labeling with ^{35}S revealed a dramatic response of increased sulfate uptake and assimilation when N sources, especially ammonia, were supplied. The addition of glutathione (GSH) counteracted the effects of ammonia on sulfate uptake whereas cys and met had no suppressive effects. The addition of a specific inhibitor of GSH synthesis, buthionine sulfoximine increased sulfate uptake with and without available ammonia. The concentrations of free amino acids related to ammonia assimilation were halved when GSH was supplied exogenously. The data suggests that intracellular GSH concentrations supercede the affects of N source availability on sulfate acquisition by altering the assimilation of ammonia. Gene expression analysis of putative sulfate assimilation genes failed to correlate with sulfate uptake data, and transcriptional regulation therefore does not explain the control of sulfate assimilation by ammonia. Finally, a model is presented in interpretation of the results of the study and discussed.

Experimental Procedures

Amino acid quantification

Approximately 25,000 spores (Premier Tech Biotechnologies, Québec, Canada) were germinated per sample at 30°C in sterile 12 well petri plates containing 1mL of M medium (St.Arnaud *et al.*, 1996) modified to remove N (Govindarajalu *et al.*, 2005) and sucrose. Four samples were analyzed for each condition for each experiment (Figures 4-1, 4-2, and 4-8). Additional to the modified M medium, MilliQ water, 2 mM urea, 4 mM NH_4Cl, 4 mM KNO_3, 2 mM L-glutamine, or 2 mM L-arginine hydrochloride (Sigma-Aldrich, St. Louis, MO) were added as 0.05 mL of filtered stock solutions to study the effect of exogenous nitrogen sources (Figure 4-2). Germinating spores were collected at 0, 7, or 14 days (Figure 4-1) by sieving and immediate freezen in liquid nitrogen, lyophilized and weighed. Measurements of the effects of GSH addition on amino acid concentration were made by adding 2 mM MES (pH 6.0) with or without 1 mM GSH.

Free amino acids were extracted by first disintegrating spores and germ tubes in 0.1 mL of a 0.01 N HCl solution using a bead mill (Retsch MM301) set to 30 Hz and two 3 mM stainless steel beads for 4 min. The complete disintegration was confirmed by analyzing small aliquots of five randomly chosen samples under a dissecting microscope. An additional 0.4 mL of 0.01 N HCl was added followed by 10 min of vortexing. Approximately 2500 pmols of norleucine was added as an internal standard prior to extraction. Chloroform (0.5 mL) was added followed by 5 min of further vortexing, after which the chloroform, aqueous solution, and cell debris were separated by centrifugation. The supernatants were collected and lyophilized. Dried samples were re-dissolved in 0.02

mL of 0.02 N HCl , 0.06 ml of 0.1 M sodium borate buffer (pH 10.0), and 0.02 mL of AccQ-Fluor™ reagent (6-aminoquinolyl-*N*-hydroxysuccinimidylcarbamate; Waters, Milford, MA, USA). Free amino acids were analyzed from aliquots of 0.01 or 0.02 mL of each sample by HPLC-fluorescence using a Waters 2695 Alliance HPLC (Waters, Milford, MA) equipped with a 37°C column oven, a Waters 2474 Fluorescence Detector, and Empower software (Waters, Milford, MA). A four buffer system was used for HPLC: A and B, sodium acetate buffer (Waters, Milford, MA) adjusted to pH 5.7 and pH 6.8 with phosphoric acid; C, acetonitrile; and D, methanol:water (1:9).

^{35}S-labeling experiments

Approximately $5x10^3$ sterile spores of *Glomus intraradices* (Premier Tech Biotechnologies, Revière-du-Loup, Québec, Canada) per sample were washed on a 300 mesh sieve, collected, and blended together with cold solidified sterile Gel-gro agar (MP Biomedicals, Solon, OH) using a Waring blender to form a slurry of uniform spore density, and aliquots of 2 mL were transferred to the wells of sterile 12 well polystyrene plates. The germination slurry contained only 2 mM MES buffer (pH 6.0), 2 mM $CaCl_2$, and spores, and were maintained at 4°C for at least one week prior to labeling with either 16.5 μCi of $Na_2[^{35}SO_4]$ or 9.3 μCi of L-[^{35}S]cysteine (MP Biomedicals, Solon, OH). Labeled sulfate was supplied with 0.1 mM Na_2SO_4, and ^{35}S-cysteine with 0.1 mM unlabeled cysteine as carrier molecules. Additionally, 1 mM *O*-acetylserine, 1 mM reduced glutathione, 1 mM cysteine, 1 mM methionine, or 10 mM buthionine sulfoximine were added with or without 4 mM NH_4Cl (Figures 4-5 and 4-6). For the

analysis of N source effects on sulfate uptake (Figure 4-3), N was supplied as 4 mM KNO_3, 2 mM L-glutamine, 2 mM L-arginine, 2 mM urea, or 4 mM NH_4Cl. Spores were germinated for seven days after labeling in a 30°C incubator. Samples were collected and S-containing compounds extracted and fractionated into different biochemical pools exactly as described in Chapter 3.

^{15}N-labeling experiments

Experiments examining percent ^{15}N-labeling used 4 mM $^{15}NH_4Cl$ or 4 mM $K^{15}NO_3$ as N sources in Gel-gro gellan (MP Biomedicals, Solon, OH), 2 mM $CaCl_2$, and 2 mM MES (pH 6.0) as previously described (Chapter 3). All sources of ^{15}N were 98% labeled. Approximately $1x10^4$ sterile spores of *Glomus intraradices* (Premier Tech Biotechnologies, Revière-du-Loup, Québec, Canada) were germinated per sample in a 30°C incubator and in the presence of ^{15}N label prior to collection after one week. Additionally, 0.1 mM Na_2SO_4, 1 mM L-cysteine hydrochloride, 1 mM reduced glutathione, or 10 mM buthionine sulfoximine were added at the time of labeling. Samples were collected by adding them to 100 mL of cold 10 mM sodium citrate buffer (pH 6.0) and sieving the solution two minutes later on a 300 mesh wire sieve, followed immediately by liquid N_2 freezing and lyophilization. Amino acids were extracted by pulverizing each sample in 0.1 mL of 0.01 M HCl solution with two 3 mm stainless steel beads using a bead mill (Retsch MM301) oscilating at 30Hz for four minutes (Allen and Shachar-Hill, 2009). The amino acid pool was extracted by the addition of 0.4 mL of 0.01

M HCl followed by moderate vortexing for 10 min. Proteins and non-polar compounds were removed by the addition of 0.5 mL of $CHCl_3$ followed by 2 min of moderate vortexing. Samples were centrifuged at 14,000 g for 10 min and the supernatants collected. The final solutions were dried under gaseous N_2 streams, heated to 60°C on a heat block. The dried amino acid extracts were reconstituted in 0.1 mL of MilliQ water, transferred to GC-MS vial inserts, and lyophilized in preparation of derivatization.

Amino acid samples were derivatized in pyridine: N-tert-butyldimethylsilyl-trifluoroacetamide (MTBSTFA) (1:1) for 1 hr at 55°C prior and 1 μL injected at 280°C using an Agilent 7683 series injector (Santa Clara, CA, USA) into an Agilent GC-MS system (6890N Netwrok GC System and 5973 *inert* Mass Selective Detector) equipped with a J&W Scientific 30m DB5-ms column (Agilent Technologies, Santa Clara, CA, USA). The oven temperature was maintained at 100°C for 4 min, ramped to 200°C at 5°C/min, then to 300°C at 10°C/min where it was held for 2min. Amino acid standards were used to identify GC peaks and mass fragments.

RNA extraction and gene expression measurements

RNA was extracted from frozen tissue as described in previous chapters. Briefly, tissue for extraction was oscillated in 1.5 mL screw-cap microcentrifuge tubes at 30 Hz in the presence of 100uL extraction buffer (RNeasy Plant Mini Kit, Qiagen, Valencia, CA) and two 3 mm stainless steel beads using a Retsch MM301 bead mill three times for two minutes each time. Five 2 min homogenation steps were done in total and the samples were refrozen in liquid nitrogen between steps. Aliquots of 2 μL were examined microscopically for complete spore breakage. Extractions using an RNeasy Plant Mini

Kit (Qiagen, Valencia, CA) were followed by treatment with RNase-free DNase (Turbo DNA-free; Ambion, Austin, TX), after which aliquots were measured by UV absorbance to determine RNA concentration and approximate purity. Equal quantities of RNA were used to synthesize cDNA with SuperScript II reverse transcriptase (Invitrogen corp., Carlsbad, CA), and negative controls were generated with parallel sample sets which did not contain the enzyme. Sequences of putative sulfate assimilation-related genes were obtained by BLAST batch analyses using homologous sequences of conserved regions of each gene of interest as previously described (chapters 2 & 3), and these sequences were verified by PCR amplification and sequencing using an ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems, CA, USA). Primer sequences used (Primer Express software, Applied Biosystems, Austin, TX; IDT, Syntegen, Skokie, IL) are identical to those listed in chapter 3. Gene expression measurements were analyzed as previously reported in chapter 3.

Results

Free amino acid concentrations during germination

Free amino acid (FAA) concentrations were measured at three time points, 0, 7, and 14 days (Figure 4-1). The total N content in the FAA pool decreased by 27 ± 4% between 0 and 7 days of germination, 72 ± 14% of that amount was due to a fall in asp content. The total N content of the FAA pool did not significantly change significantly between days 7 and 14 by ANOVA single factor analysis (alpha = 0.05). Aspartate represented almost half (41 ± 0.7%) of the total N in this pool before germination, dropping to 20 ± 2% by day 14. On day 14 the levels of five FAA's, asn, gly, leu, pro, and gln, had increased and three FAA's, ala, asp, and ile, had decreased more than two fold compared to the levels at day 0. Fewer than half of the FAA concentrations changed significantly between days 7 and 14 (ANOVA; alpha = 0.05), and only two out of nineteen, glycine and leucine, showed greater than two fold differences. With few exceptions FAA concentrations were stable after 7 days of germination, and experiments were therefore conducted over one week time periods.

Figure 4-1. Time course of free amino acid concentration in germinating spores of *Glomus intraradices*. Spores were germinated in deionized water for 0 (white bars), 7 (down slashed bars), and 14 (gray bars) days prior to the extraction of free amino acids and quantification by HPLC analysis, n = 4 ± SEM.

Figure 4-1

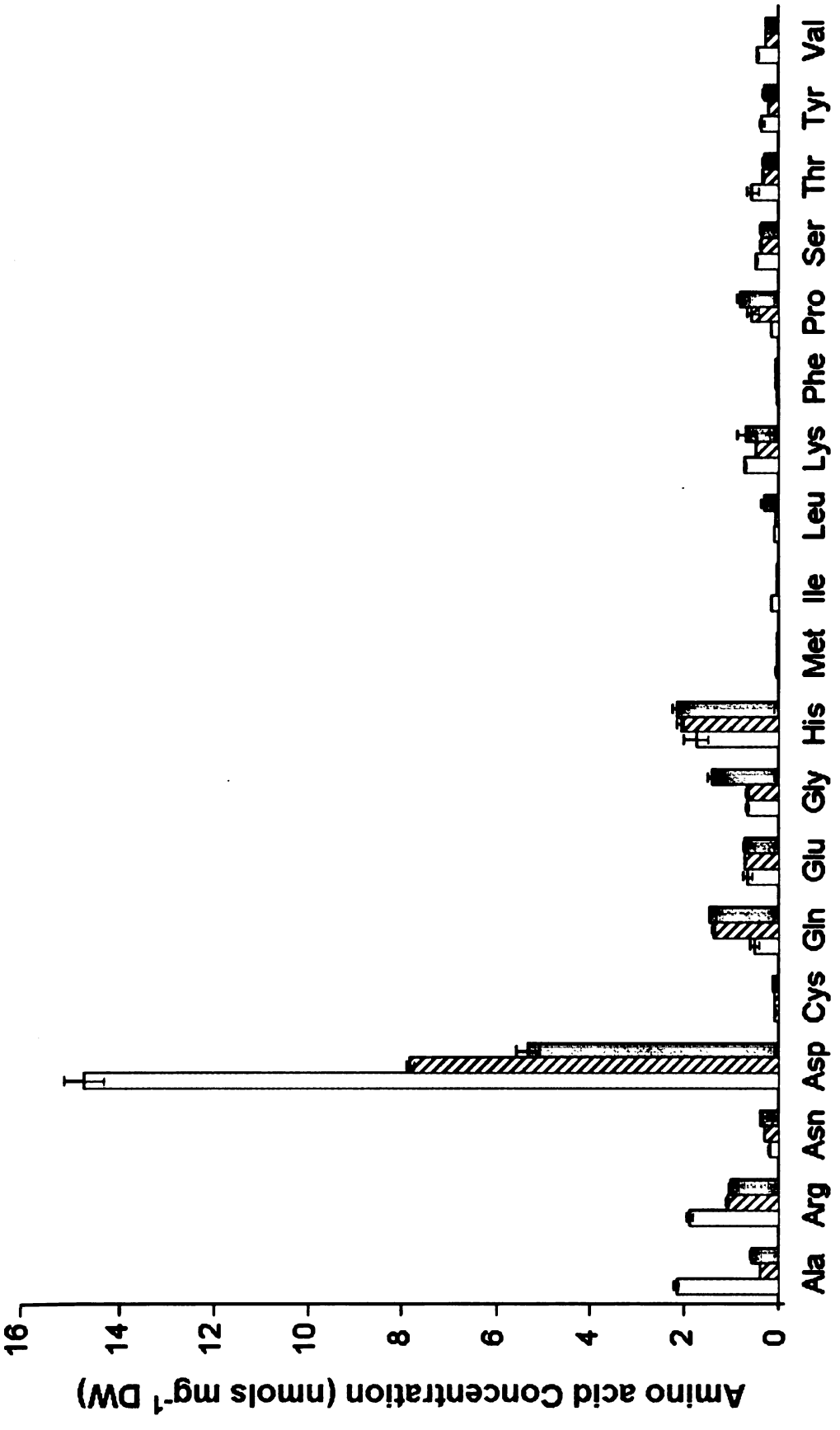

Amino acid Concentration (nmols mg-1 DW)
0
2
4
6
8
10
12
14
16
Ala
Arg
Asn
Asp
Cys
Gln
Glu
Gly
His
Met
Ile
Leu
Lys
Phe
Pro
Ser
Thr
Tyr
Val

Figure 4-2 Free amino acid concentrations in the presence of exogenous N sources. The free amino acid composition of extracts of *Glomus intraradices* spores germinated for 7 days with no nitrogen source (white bars), 4mM KNO_3 (horizontally striped bars), 2mM Gln (light gray bars), 2mM Arg (up slashed bars), 2mM urea (dark gray bars), or 4mM NH_4Cl (down slashed bars). The charts represent concentrations (A) above or (B) below 2 nmols mg^{-1} DW, and (C) the total N concentration under each condition derived from the sum of amino acid concentrations adjusted for the number of N atoms per molecule, n = 4 ± SEM.

Figure 4-2

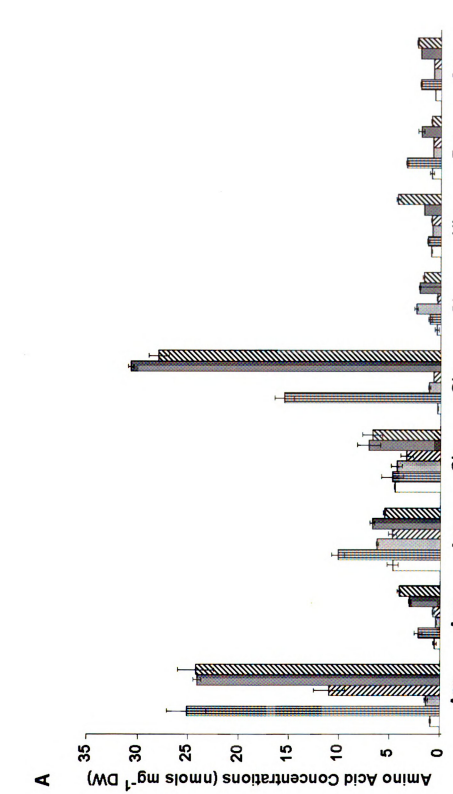
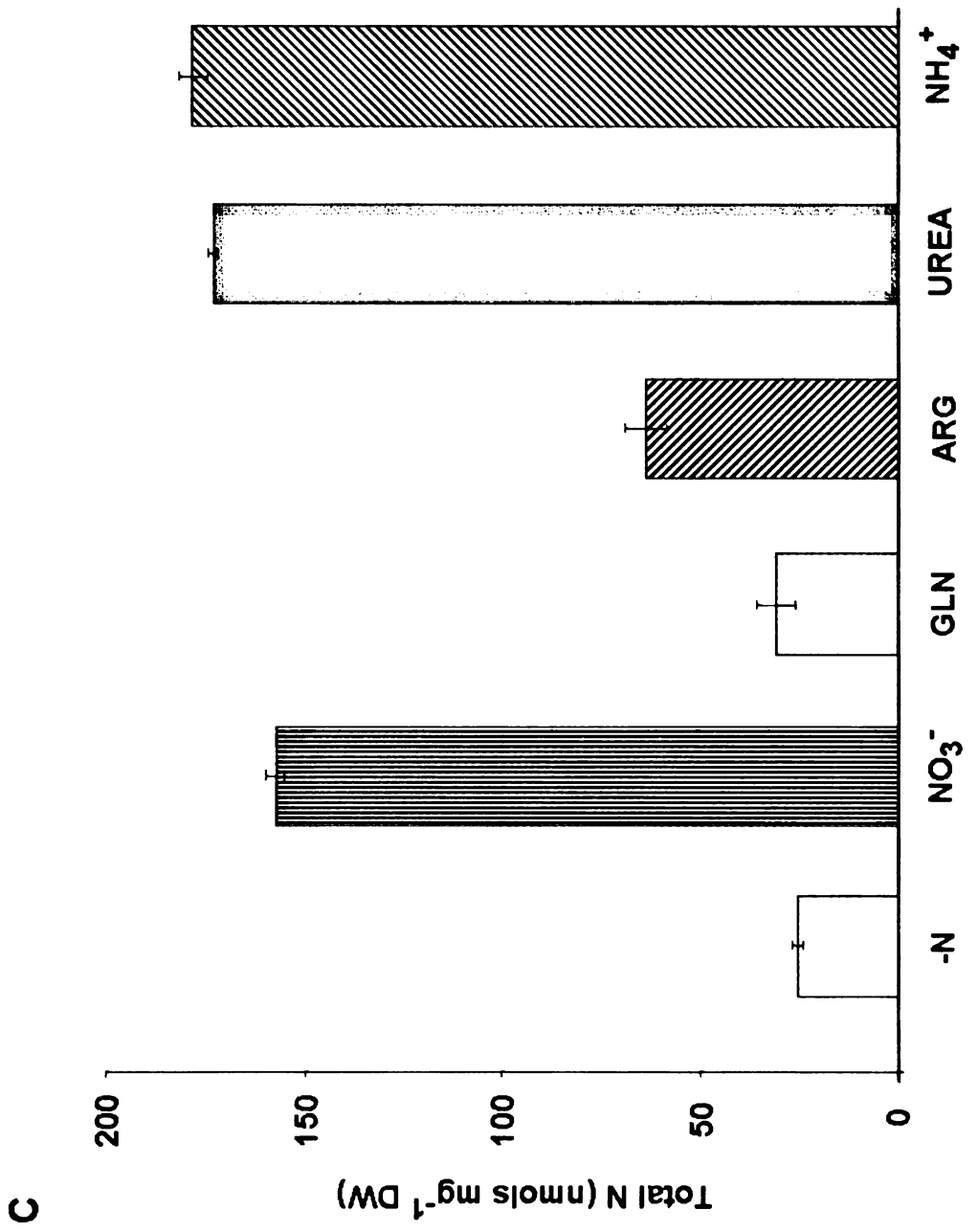

A
Amino Acid Concentrations (nmols mg-1 DW)
35
30
25
20
15
10
5
0
Arg
Asn
Asp
Gln
Glu
Gly
His
Pro
Ser

Figure 4-2 (cont'd)

Figure 4-2 (cont'd)



The effects of N sources on free amino acid concentrations

The reduction of sulfate and nitrate are energy intensive processes and the oxidative state of either nutrient may have an effect on the assimilation of the other through the consumption of reducing equivalents. Control of the S assimilation pathway has also been directly linked to general amino acid control in *S. cerevisiae* (Mountain *et al.*, 1993). Organic (arg and gln), reduced inorganic (NH_4^+ and urea), or oxidized inorganic (NO_3^-) N were therefore supplied to germinating spores to determine the plasticity of the FAA content (Figure 4-2). Inorganic N sources had equivalent effects on the FAA pool overall, although there were differences in responses of glu, tyr, his, and pro. The total N content in the FAA pool increased by more than 6 fold compared to control samples with ammonia, urea, or nitrate addition; the response to these N sources was not statistically distinct by ANOVA single factor analysis (Figure 4-2). In fungi, gln concentrations modulate the amino acid control circuit (gC) in yeasts and cross pathway control circuits (CPC) in filamentous fungi by directly binding to a global transcriptional regulatory protein (Marzluf, 1993; Berger *et al.*, 2008), and arg is a putative form of N transfer to the hosts of AM fungal symbioses (Govindarajale *et al.*, 2005). In contrast to the inorganic N sources, supplying arg or gln led to only 2.5 ± 0.5 fold or 1.2 ± 0.2 fold increases in total N. However, an analysis of percent labeling by 15N revealed that $^{15}NO_3^-$ labeled amino acids 43 ± 5% less on average than comparable amounts of $^{15}NH_4^+$ (Figure 4-3), revealing that the utilization of external nitrate is different than that of reduced inorganic N. The internal source of N which makes up the difference in the FAA total N content was not identified.

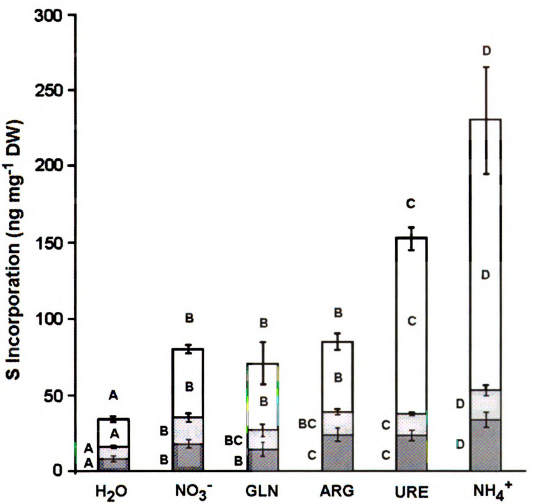


Figure 4-3 Influence of N sources on $^{35}SO_4^{2-}$ uptake, reduction, and allocation. Spores were germinated in the presence of 0.1 mM Na_2SO_4 and 16.5 µCi of $Na_2[^{35}SO_4]$ for seven days. Prior to germination at 30°C, 4 mM KNO_3 (NO_3), and NH_4Cl (NH_4), 2 mM urea (URE), glutamine (GLN), and arginine (ARG) were added. Samples were collected and ^{35}S separated by chemical fractionation yielding fractions containing but not limited to sulfate (white bars), amino acids (light gray bars), and proteins (dark gray bars), analyzed by scintillation counting. Error is expressed as standard error of the mean from the analysis of five samples per condition, and letters ANOVA single factor analyses (alpha = 0.05) between fractions and total uptake (above white bars).

The effects of N sources on sulfate uptake and assimilation

The addition of different N sources revealed a general stimulation of S incorporation into all fractions tested, disproportionately increasing sulfate sequestration in some cases. While gln and arg addition did not change the amount of S incorporation into the protein pool compared to nitrate addition, the introduction of urea and ammonia produced significant increases in this S pool (Figure 4-3). Urea increased protein S by 37% more than nitrate, which increased protein S by 2.1 +/- 0.24 fold compared to controls germinated without N. Ammonia addition doubled protein S incorporation compared to nitrate, which caused an increase of the protein fraction of 4.1 +/- 0.24 fold over controls, and also led to a disproportionately large amount of sequestered sulfate (Figure 4-3). The type of N added had the greatest effect on the sulfate fractions. Urea addition led to an increase in the sulfate fraction of 6.1 +/- 0.08 fold compared to controls, ^{35}% less than ammonia addition. Sulfate fractions from urea and gly additions were not statistically different by ANOVA analysis. Other treatments led to a doubling of the sulfate fraction compared to controls and 2-3 fold increases in S incorporation into the protein and amino acid (Figure 4-3). The addition of ammonia, however, led to a disproportionate 9.4 +/- 0.2 fold increase in the sulfate fraction, the largest change measured (Figure 4-3). Compared to the absence of N, nitrate, gln, arg, gly, urea, or ammonia addition led to total increases in S incorporation of 2.31 +/- 0.08, 2.03 +/- 0.21, 2.46 +/- 0.10, 4.61 +/- 0.26, 4.41 +/- 0.08, and 6.66 +/- 0.16 fold, respectively.

Figure 4-4 Effect of SO_4^{2-} **(A)** and S metabolites **(B)** on ^{15}N-labeling of free amino acids. **(A)** Spores were germinated at 30°C for 7 days in the presence of 4 mM K[$^{15}NO_3$] either alone (white bars) or with 0.1 mM Na_2SO_4 (down slashed bars); or with 4 mM [$^{15}NH_4$]Cl either alone (light gray bars) or with 0.1 mM Na_2SO_4 (up slashed bars). **(B)** Spores were germinated at 30°C for 7 days in the presence of 4 mM [$^{15}NH_4$]Cl and either no S source (white bars), or 0.1 mM Na_2SO_4 (down slashed bars), 1 mM L-cysteine (light gray bars), 1 mM reduced glutathione (up slashed bars), or 10 mM buthionine

Figure 4-4

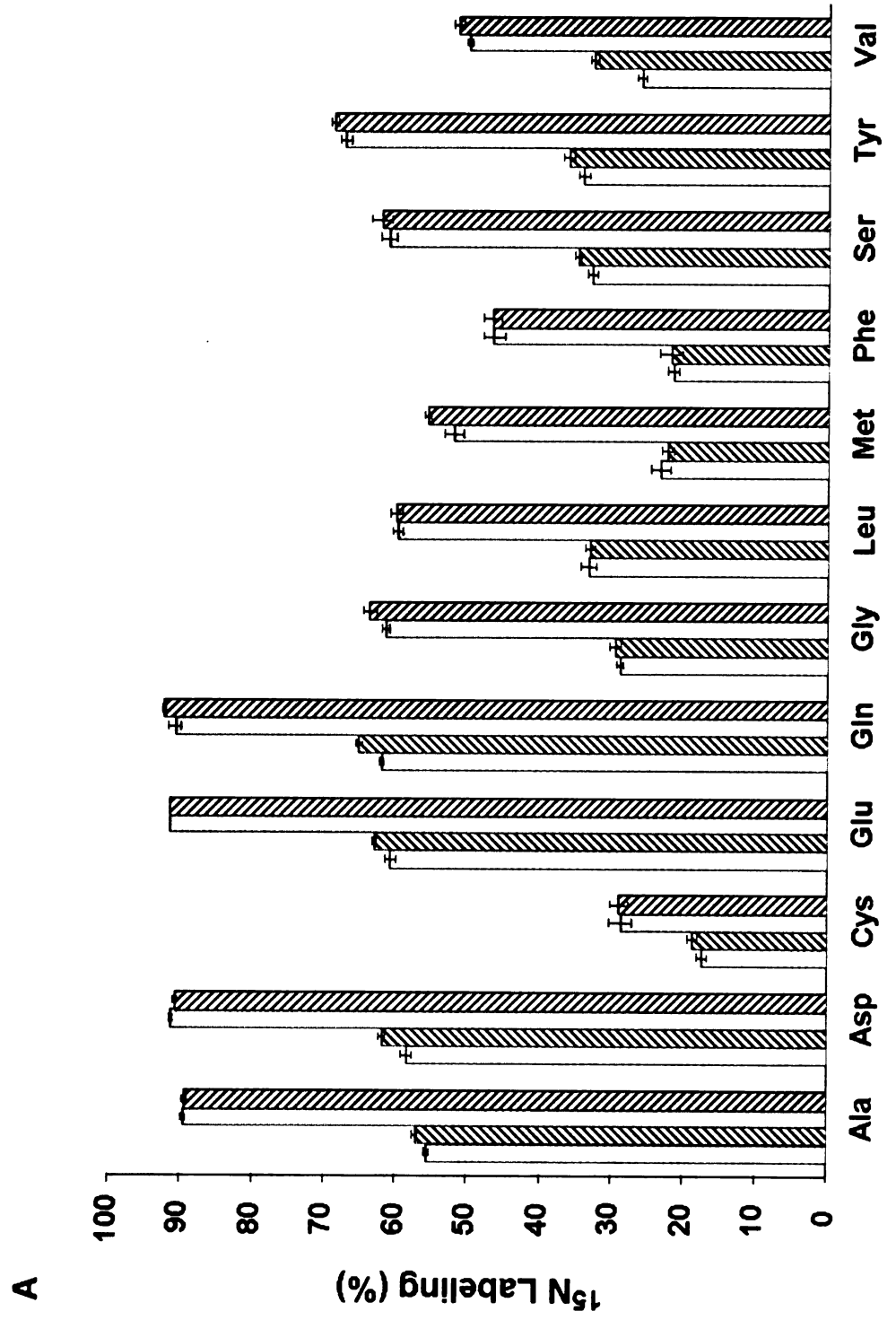

A
^{15}N Labelling (%)
100
90
80
70
60
50
40
30
20
10
0
Ala
Asp
Cys
Glu
Gln
Gly
Leu
Met
Phe
Ser
Tyr
Val

Figure 4-4 (cont'd)

Interactions between sulfate and nitrogen assimilation pathways

The interaction of N and S metabolism was examined in two experiments. In the first, the effects of S on N metabolism was examined by supplying 15N nitrate or 15N ammonium with or without SO_4^{2-} or S metabolites and analyzing the ^{15}N-labeling of free amino acids (Figure 4-4). In the second experiment the effect of N on the regulation of S assimilation was examined through an analysis of $^{35}SO_4^{2-}$ labeling in different S fractions when S metabolites were added in the presence or absence of ammonia (Figure 4-5). Reduced glutathione altered both the labeling of FAA's from ^{15}N-ammonia and also the effect of ammonia on sulfate uptake. When supplied with ^{15}N-ammonia, the average labeling after one week of germination was 74.5 ± 12.6% for all amino acids measured (Figure 4-5). Among the compounds examined, only reduced glutathione (GSH) significantly altered percent labeling, reducing it modestly in all amino acids examined. The largest reductions were in val, leu, met, and tyr which were 31.5 ± 5.1%, 27.2 ± 10.3%, 22.7 ± 9.5%, and 12.9 ± 4.1% less labeled than the average of other treatments, respectively. The experiments revealed little effect of sulfate, cys, or a specific inhibitor of GSH synthesis, buthionine sulfoximine (BSO).

The effect of ammonia addition (which stimulates S assimilation, Figure 4-3) was further analyzed by the introduction of 1 mM *O*-acetyl serine (oas), the immediate precursor of cysteine biosynthesis in *A. nidulans* and *N. crassa* (Marzluf, 1997). S uptake and assimilation was not significantly different when oas was added (Figure 4-4A). The simultaneous addition of oas and 2 mM ammonia reduced S incorporation into sulfate (34.4 +/- 3.3%), amino acids (16.5 +/- 0.5%), and the solubilized fraction (31.6 +/- 4.4%).

There was no significant effect of ammonia on S incorporation into the protein fractions (ANOVA; alpha = 0.05). In contrast, the addition of 1 mM GSH sharply reduced total and fractional S incorporation in the presence or absence of ammonia (Figure 4-4B). GSH addition reduced S incorporation by 88.5 +/- 4.5%. Introducing 2 mM ammonia with gsh failed to significantly alter either fractional or total S incorporation compared to GSH alone.

Glutathione addition in relation to sulfate assimilation and free amino acid concentrations

An inhibitor of GSH synthesis, buthionine sulfoximine (BSO) was used to determine if the effects of GSH addition were mediated via the intracellular GSH concentration (Figure 4-4B). Inhibiting GSH synthesis with 10 mM BSO resulted in significant changes in S incorporation only in the amino acid fraction, which was increased by 3.1 +/- 0.5 fold. The addition of 2 mM ammonia (Figures 4-4A and B) led to less S incorporation than 4 mM (Figure 4-3). Both figures 4A and 4B show a more than 3 fold increase in total S incorporation (3.3 +/- 0.2 fold in both cases) compared to the 6.7 +/- 0.2 fold enhancement shown in Figure 4-3.

To examine the effects of N and GSH on the uptake and incorporation of organic S, [^{35}S]cys was supplied in the presence or absence of ammonia and/or GSH. Sulfate was not added to the germination solution and the 0.1 mM cys added was the only S source available. Ammonia (2 mM) had no effect on incorporation alone or with GSH addition (1 mM). The application of GSH resulted in three changes: a 71 +/- 11% reduction in amino acid fraction radioactive counts, a 2.1 +/- 0.21 fold increase in protein fraction

counts, and an increase of 56 +/- 17% in tissue solubilized fraction counts (Figure 4-5). The possibility of conversion of cys to sulfate was also analyzed by measuring the sulfate fractions, which contained less than 1.5% of the total radioactivity under all conditions. This low level is within the error intrinsic to the barium sulfate precipitation procedure.

Since glutathione was the only compound tested that affected both ammonia (Figure 4-5) and sulfate (Figure 4-6) assimilation, we analyzed its effects on N metabolism further. Exposure to GSH in the absence of an N source led to decreased concentrations of glu (2.3 ± 0.1 fold), gln (1.9 ± 0.2 fold), asp (2 ± 0.1 fold), asn (1.8 ± 0.2 fold), and arg (1.5 ± 0.1 fold) compared to germination in pure water, while most of the FAA concentrations measured did not change (Figure 4-7). Aside from ala, which showed a slight increase, the only FAA to increase in concentration was leu, which more than doubled (2.1 ± 0.2 fold) in the presence of GSH.

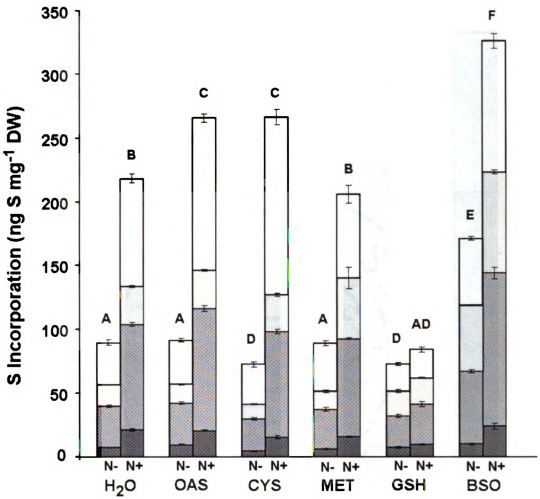


Figure 4-5 ^{35}S-sulfate uptake, reduction, and allocation in the presence of ammonia and S metabolites. Spores were germinated for 7 days at 30°C with 0.1 mM Na_2SO_4 labeled with 16.53 µCi of $^{35}SO_4^{2-}$ per sample and 1 mM *O*-acetylserine (OAS), 1 mM reduced glutathione (GSH), 1 mM L-cysteine hydrochloride (CYS), 1 mM L-methionine (MET), or 10 mM buthionine sulfoximine (BSO) in the absence (N-) or presence (N+) of 4 mM ammonium chloride. Tissue was collected and S fractionated into pools containing sulfate (white bars), amino acids (light gray bars), proteins (gray bars), and solubilized cell debris (dark gray bars), n = 4 ± SEM.

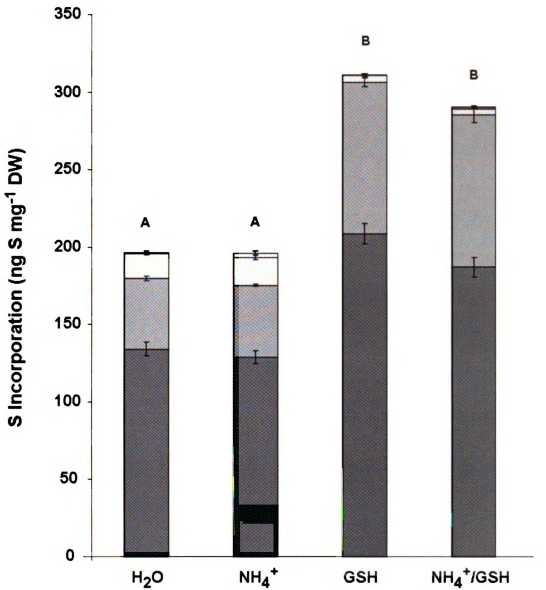


Figure 4-6 Effects of ammonia and glutathione (GSH) on [^{35}S]Cysteine uptake and utilization. Spores were germinated for 7 days in a 30°C incubator with 0.1 mM L-cysteine labeled with 9.3 μCi of [^{35}S]Cys in the presence of water alone (H_2O), 4 mM ammonia (NH_4^+), 1 mM reduced glutathione (GSH), or 1 mM GSH with 4 mM NH_4^+ (GSH/NH_4^+). Samples were collected and the S species separated by chemical fractionation yielding fractions containing but not limited to sulfate (white bars), amino acids (light gray bars), proteins (medium gray bars), and tissue solubilized cellular debris (dark gray bars), analyzed by scintillation counting, n = 6 ± SEM.

Figure 4-7 Change in amino acid concentrations from glutathione addition. The free amino acid composition of spores germinated for 7 days in water (gray bars) or with 1 mM reduced glutathione (up slashed bars) were evaluated by HPLC, n = 4 ± SEM.

Figure 4-7

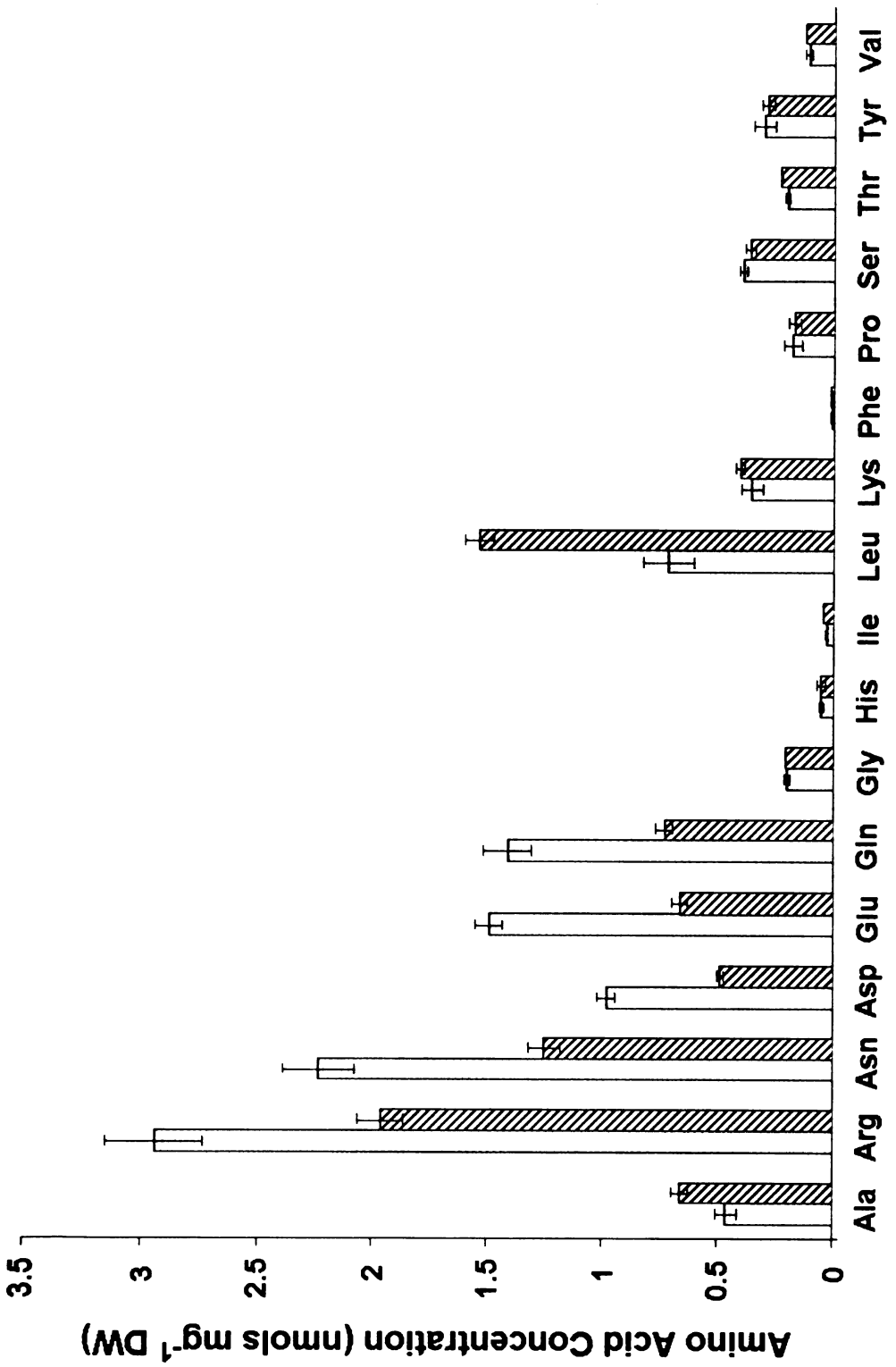
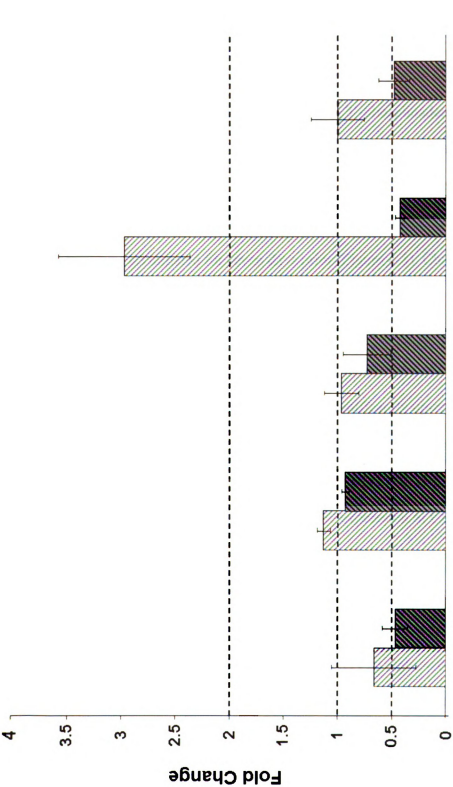

Amino Acid Concentration (nmols mg-1 DW)
3.5
3
2.5
2
1.5
1
0.5
0
Ala
Arg
Asn
Asp
Glu
Gln
Gly
His
Ile
Leu
Lys
Phe
Pro
Ser
Thr
Tyr
Val

Figure 4-8 Transcriptional changes of putative sulfur assimilation pathway genes after NH_4^+ addition. Spores of *G. intraradices* were germinated for five days in 2 mM MES (pH 6.0) prior to induction for six hours with 4 mM NH_4Cl (up slashed bars) or a combination of 4 mM NH_4Cl and 0.1 mM Na_2SO_4 (down slashed bars). Expression was calculated relative to control samples given only deionized water and reported as fold change. Putative sequences of the S assimilation pathway including a high affinity sulfate permease (SUL), S-adenosyl transferase (SAT), phosphoadenylsulfate reductase (PAPSR), cytathionine β-synthase (CBS), and cystathionine γ-lyase (CGL) were analyzed, n = 3 ± SEM.

Figure 4-8



Changes in gene expression of putative sulfate assimilation genes in relation to nitrogen addition

Genes from *Glomus intraradices* germinating spores were identified from 454 sequencing data based on homology to fungal sequences encoding a high affinity sulfate transporter (SUL), sulfate adenylytransferase (SAT), 3'-phosphoadenylsulfate reductase (PAPSR), and the enzymes making up the reverse transsulfuration pathway (CT pathway) cystathionine β-synthase (CBS) and cystathionine γ-lyase (CGL) (see chapters 2 & 3). The expression of these genes was analyzed after a six hour induction by the addition of ammonia to spores germinated for seven days prior (Figure 4-8). Changes in expression more than two fold greater or lower than two fold lesser than control samples were considered biologically significant. None of the sequences were biologically significantly down-regulated, and only CBS, which controls the condensation of homocysteine and serine to form the intermediate cystathionine, was significantly up-regulated (3 ± 0.58 fold). The second step of the pathway converting cystathionine to cysteine and α-ketobutyric acid governed by CGL was not up-regulated (1 ± 0.23 fold). The addition of sulfate with ammonia had the opposite effect on the expression of CBS, down-regulating it by 60 ± 4% (0.41 ± 0.04 fold; Figure 4-8), and also halved expression of CGL (0.45 ± 0.13 fold). The high affinity sulfate permease putative sequence tested (SUL) was generally down-regulated by ammonia addition, and the fold change from sulfate addition with the ammonia was not statistically different (ANOVA; alpha = 0.05) from the addition of ammonia alone (Figure 4-8). Expression of SAT and PAPSR was not statistically significantly different from 1 as measured by ANOVA single factor analysis (alpha = 0.05), with or without sulfate addition. In general there were few changes in

gene expression of the sequences tested due to ammonia addition, and the reduction of expression from ammonia/sulfate addition does not clearly correlate to the increase in sulfate uptake and assimilation under these conditions (Figure 4-3).

Discussion

During spore germination exogenous nitrogen sources were readily taken up and used to synthesize amino acids; they also increased the uptake, reduction and incorporation of exogenous sulfate. Stable isotope labeling and amino acid concentration data demonstrated a preference for ammonia and urea, and both of these reduced N sources also led to larger increases sulfate uptake than the organic and oxidized inorganic N sources (Figure 4-3). Urea is catabolized to, and nitrate is reduced to, ammonia before assimilation, and their effects are likely reflective of the efficiency of their uptake and conversion to ammonia. The results of these experiments suggest a correlation between the amount of N assimilated and the uptake of sulfate. The addition of organic N as gln or arg, however, seems to be in opposition to a direct correlation between N and S assimilation, as neither increased the total N content of the amino acid pool as much as nitrate addition (Figure 4-2A), and both led to similar increases in sulfate assimilation compared to nitrate addition (Figure 4-3). Additionally, gln is imported by germinating spores of *G. intraradices* at ten times the rate of arg (Gachomo *et al.*, 2009). Glutamine is known to have a regulatory role in fungal gene expression. The global positive acting transcriptional factors controlling cross pathway control circuits (CPC) in *A. nidulans* (AreA) and *N. crassa* (Nit2) have gln binding sites, and in *A. nidulans* the concentration of gln negatively correlates with the activity of AreA (Marzluf, 1993; Berger *et al.*, 2008). The concentration of gln in the FAA pool, however, does not correlate with the amount of sulfate uptake, although there is a slight increase from urea or ammonia assimilation (Figure 4-2A). The data is therefore suggestive, but inconclusive, that gln

and arg addition led to a regulatory effect on sulfate assimilation unrelated to both the total N in the FAA pool and the concentration of specific amino acids.

In *Aspergillus nidulans*, a result of biotin deficiency is an increase in ammonia assimilation, which leads to a 70% increase in protein content as well as higher concentrations of glu, asp, leu, and met (Desai, 1979). Data presented in this study are consistent with the hypothesis that ammonia availability influences protein synthesis in *Glomus intraradices* as well. As the largest N pool, the rate of protein synthesis or degradation likely determines the intracellular concentration of FAA's. If these rates remain static, an increase in $\%^{15}N$ labeling of an amino acid from an exogenous N source will lead to an equivalent relative increase in its concentration. The addition of $^{15}NH_4^+$, however, resulted in 30% more labeling in the free amino acid (FAA) pool than did adding $^{15}NO_3^-$ (Figure 4-4), while ammonia addition led to an increase of only 12% in the total N in the FAA pool relative to nitrate addition (Figure 4-2C). Since ^{35}S labeling results indicate a nearly twofold increase in the protein-S pool when ammonia is added compared to nitrate, it is likely that ammonia increases protein synthesis (Figure 4-9), leading to less total N in the FAA pool than expected from the ^{15}N-labeling data.

The addition of reduced glutathione (GSH) counteracted the increased sulfate uptake measured when spores were germinated with ammonia whereas the addition of buthionine sulfoximine (BSO), a specific inhibitor of the first enzyme of GSH synthesis, γ-glutamylcysteine synthetase, induced sulfate uptake and assimilation (Figure 4-3). These data repeat the finding of the previous chapter on GSH and BSO addition at seven days in the absence of ammonia, as well as the data on cys and met addition (Chapter 3).

In contrast to GSH, addition of cys slightly increased ammonia stimulation of sulfate uptake (Figure 4-5). Since cys is readily imported by germinating spores (Figure 4-6), GSH does not appear to affect sulfate uptake by contributing to the cys pool through its catabolism. In *S. cerevisiae*, GSH is readily imported and utilized as a source of cys (Elskens *et al.*, 1991; Kumar *et al.*, 2003; Miyake *et al.*, 1999). Addition of 10 mM BSO has been shown to reduce the intracellular GSH concentration by 76% in the yeast *Kluyveromyces lactis* (Coulon *et al.*, 2007) and 70% in *S. cerevisiae* (Rossi *et al.*, 1997). The intracellular concentration of GSH in *G. intraradices* was not measured directly, and therefore the effects of GSH and BSO addition on GSH concentration in the germinating spores is uncertain, although it seems plausible that both have their effects either through changes in the free GSH levels or indirectly through their effects on FAA levels.

The concentrations of the amino acids that are synthesized from glu, gln and arg either directly or through transamination reactions involving glu, asp, and asn, were reduced by roughly half with GSH addition (Figure 4-7). Glutamate and asp concentrations have been shown to specifically increase in *A. nidulans* in response to increased assimilation of ammonia (Desai, 1979), as expected from N assimilation through the GS/GoGAT cycle. Both gln and arg addition increased the concentration of glu by more than two fold (Figure 4-2A), however, preliminary evidence suggests that the addition of glu has no effect on sulfate uptake with or without N addition (data not shown). Based on these data, the concentration of glu is likely to be symptomatic of the efficiency of N assimilation into the FAA pool rather than being directly involved in sulfate uptake. Glutathione may therefore effect sulfate assimilation indirectly by reducing ammonia assimilation in this fungus.

In contrast to other amino acids measured under GSH exposure, leu accumulated to more than double the concentration found in control samples (Figure 4-5). In *S. cerevisiae*, genes encoding two enzymes in the leu biosynthetic pathway, isopropylmalate isomerase (*LEU1*) and beta-isopropylmalate dehydrogenase (*LEU2*) are transcriptionally suppressed by the regulatory protein Leu3p or activated by Leu3p complexed with the pathway intermediate alpha-isopropylmalate (Kohlhaw, 2003). The influence of Leu3p extends to seven genes including *GDH1* (Kohlhaw, 2003; Hu *et al.*, 1995), which encodes an NADP+-dependent glutamate dehydrogenase, an integral enzyme in the assimilation of ammonia. All of the amino acid concentrations reduced when GSH is present are either synthesized from or transaminated by glu (Figure 4-7). Leucine accumulation is possibly symptomatic of how GSH exposure negates the hyper-accumulation of sulfate when ammonia is added to germinating spores, by affecting leu pathway intermediate concentrations, and is suggestive that a similar regulatory system exists in *G. intraradices* as *S. cerevisiae* and *N. crassa* with regard to Leu3p (Baichwal *et al.*, 1983; Gross, 1965). Additionally in *S. cerevisiae*, S-adenosylmethionine transport to the vacuole is greatly reduced by leu (Murphy & Spence, 1972), which may increase the concentration of the cytosolic pool and affect S assimilation.

Exposure to BSO leads to a reduction in GSH (Rossi *et al.*, 1997; Coulon *et al.*, 2007), which may leave the germinating spores vulnerable to oxidative damage, as has been shown in *S. cerevisiae* (Izawa *et al.*, 1995; Grant *et al.*, 1996). Also in *S. cerevisiae*, transcriptional suppression of the S assimilatory pathway is known to be superseded by oxidative stress. Under Cd^{++} stress, the ubiquination controlling the activity of the positive regulatory protein met4p is removed, up-regulating S assimilatory pathway

enzymes and increasing GSH synthesis (Barbey *et al.*, 2005). This further illustrates the possibility that S assimilation is regulated independently by the oxidative state of the germ tube. However, not enough data is presented here to draw any definite conclusions about the mechanism by which BSO exerts its regulatory effects, or about its relationship to N-mediated effects on sulfate assimilation.

By contrast with sulfate assimilation, uptake of reduced S in the form of cys was unaffected by ammonia and was increased by GSH addition, suggesting that the assimilation of exogenous cys is not regulated by the same mechanism as sulfate utilization. The increase in cys utilization with GSH addition has been previously demonstrated (Chapter 3), and was repeated here. A single cys transporter was recently shown to be the primary route of entry into *S. cerevisiae* cells (Kaur & Bachhawat, 2007), and its regulation has not been studied. To date, little other evidence exists concerning cys transport in fungi.

Although ammonia addition increased sulfate uptake and assimilation, it failed to alter the transcription of putative sulfate permease or primary S assimilatory pathway genes after six hours of induction (Figure 4-8). Ammonia added without sulfate led to a three fold increase of a putative sequence encoding cystathionine β-synthase (CBS), the first step of the reverse trans-sulfuration pathway (CT pathway), however sulfate added in equal quantity as in uptake experiments reduced the expression of this gene by roughly six fold in comparison (chapter 3). The data for gene expression in the presence of both sulfate and ammonia is similar to data obtained from cys addition (Chapter 3), and may indicate the results of a short term increase of cys concentration. The data is clear that six hours of incubation with ammonia and sulfate does not result in transcriptional changes

that would account for the stimulation of sulfate uptake. Further analyses are necessary to determine the relationship between N source availability and transcriptional regulation of the S assimilatory pathway at later time points to form meaningful conclusions of the interaction between N and S assimilation pathway regulation.

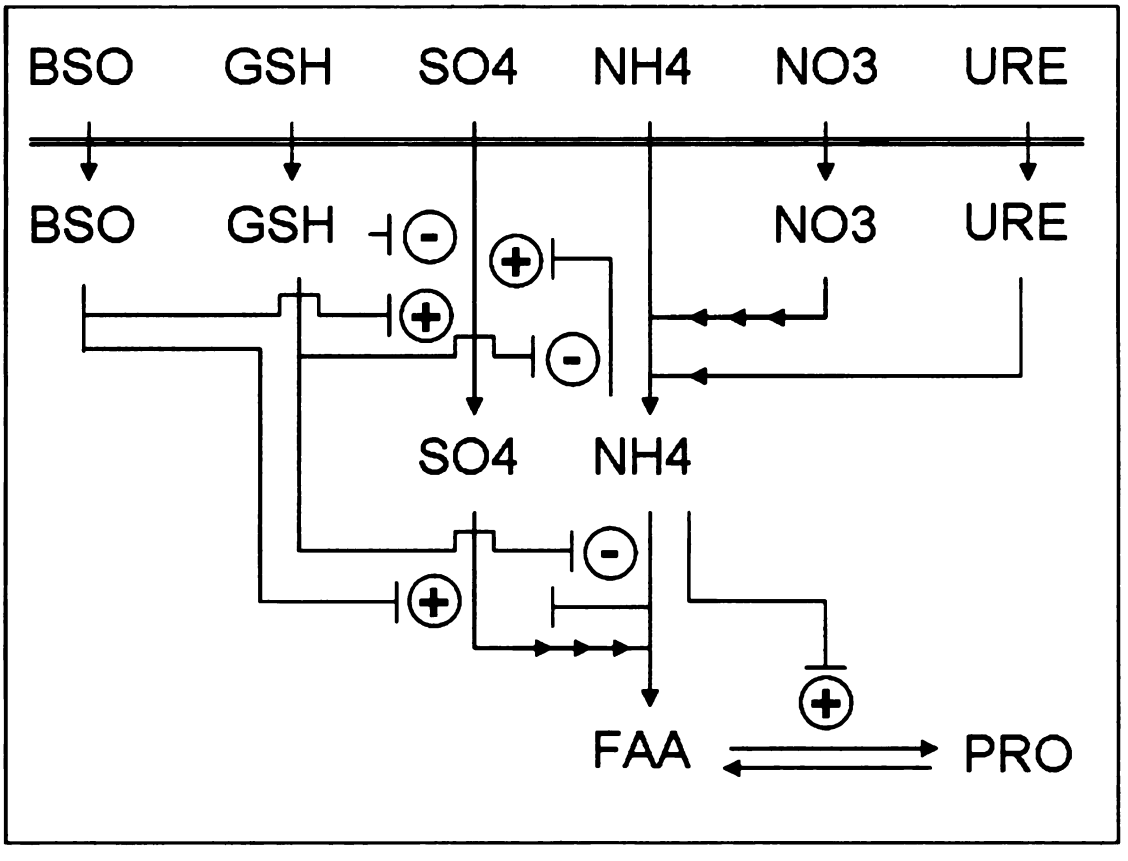


Figure 4-9 Working model of the regulation of sulfate assimilation in germinating spores of *Glomus intraradices*. Sulfate uptake was increased by exogenous ammonia (NH4) and buthionine sulfoximine (BSO). Nitrate (NO3) and urea (URE) likely affect S metabolism through their conversion to ammonia. Reduced glutathione (GSH) antagonized the stimulatory effect of ammonia on sulfate uptake, reduced ammonia assimilation through glu, and has been previously shown to decrease importation of sulfate. Ammonia also increased both the assimilation of sulfate into free amino acids (FAA) and their incorporation into protein (PRO). There is evidence that ammonia generally increases the flux from the free amino acid to protein pool as well.

Sufficient evidence was obtained from this study to make an initial model of the relationship between N assimilation and the regulation of the S assimilatory pathway (Figure 4-9). The addition of N sources increased sulfate uptake, assimilation, and sequestration. Additionally, an analysis of the ^{15}N labeling of the FAA pool in relation to the concentration of amino acids, coupled with ^{35}S-labeling evidence, indicates that ammonia increased the synthesis of proteins. Glutathione had opposing effects on the ability of the fungus to use exogenous sources of oxidized and organic S, slightly decreasing sulfate assimilation and increasing uptake of cys. It was the only compound tested which antagonized the stimulation of sulfate acquisition from ammonia addition. The addition of BSO increased uptake and assimilation of sulfate alone and in an ammonia background. The data overall reveals a close relationship between N and S assimilation, which may be mediated by FAA concentrations.

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Chapter 5

Conclusions and Future Research

Introduction

Global atmospheric sulfur emissions increased by more than 3000% between 1860 and 1990 (Lefohn *et al.*, 1999), representing approximately 0.0003% of terrestrial plant evolutionary time (Wellman *et al.*, 2003). In the absence of anthropogenic sources, plants and other organisms evolved complex regulatory mechanisms for the attainment and assimilation of sulfur from the environment. Additionally, terrestrial plant life evolved in conjunction with arbuscular mycorrhizal fungi (AMF) since the Devonian Period (400 MYA; Pirozynsky & Malloch, 1975). The symbiosis assists plant roots in obtaining otherwise unavailable soil nutrient resources (Smith & Read, 2008) and is today found in an estimated 80% of land plants (Wang & Qiu, 2006). Although the mycorrhizal symbiosis is widely considered to be a defining factor in plant phosphorus nutrition (Cress *et al.*, 1979; Murdock *et al.*, 1967), and several studies point towards an important role in plant nitrogen acquisition (Govindarajalu *et al.*, 2005; Hodge *et al.*, 2001), the role of mycorrhizal symbioses in sulfur acquisition has not been as well characterized, and the assimilation of sulfur by mycorrhizal fungi themselves not analyzed at all. The removal of sulfur from fossil fuel sources prior to combustion has reduced sulfur emissions in the United States by more than 50% between 1980 to 2000 (Baumgardner *et al.*, 2002), increasingly leading to sulfur deficiency symptoms in crop plants. Since mycorrhizae constitute the dominant soil symbiosis, information of their role in plant S nutrition is important for assessing the impact of the rise and fall of environmental sulfur concentrations. In this thesis, three major aspects of mycorrhizal S acquisition were analyzed using the AMF *Glomus intraradices*: their role in plant S

nutrition, their assimilation of S in the absence of the host, and the regulation of uptake in comparison to other known fungal systems.

AMF and plant S nutrition

The role of AMF in plant S nutrition has been little and inconclusively studied. A lack of basic measurements make it impossible to even assess whether or not AMF transfer S to plants in physiologically relevant amounts. Therefore, the research outlined in chapter 2 was to provide reliable, basic measurements of S transfer through an AMF. These measurements were achieved through ^{35}S tracer studies using a monoxenic, bi-compartmental petri dish system containing a mycorrhiza established between *Glomus intradarices* and transformed *Daucus carota* roots (StArnaud *et al.*, 1986). This system allows the fungal mycelium to separate itself from the colonized roots, and enables the isolated labeling of fungus and host. Measuring in parallel experiments the uptake of $^{35}SO_4^{2-}$ by mycorrhizal roots and the transfer of $^{35}SO_4^{2-}$ from the fungal sides, the transfer of S through the symbiosis was quantified in a non-growth limiting sulfate concentration in the context of direct root uptake. The amount of ^{35}S supplied to roots in this condition amounted to 25% of total root uptake during the labeling period. The percentage of a nutrient obtained from mycorrhizal transfer compared to direct uptake has only been measured in one other study. Mycorrhizal transfer of P has been measured for flax, medic, and tomato plants, and constitutes close to 100% of the total plant uptake during the labeling period (Smith *et al.*, 2003), leading the authors to speculate that AMF

directly affect direct root uptake of P. Smith an co-workers (2003) used pot-grown plants, which generally obtain higher percentages of mycorrhzial root length, whereas colonization in the monoxenic split plate system is normally much lower. Given this, whether or not AMF directly affect the ability of roots to import sulfate is inconclusive based on the data obtained as much of the direct ^{35}S uptake was likely by non-mycorrhizal root sections. Additionally, whether effects on direct root uptake mediated by AMF are systemic or localized to colonized root sections, or if photosynthetic tissue has a role, are all unknowns. However, the 25% of total uptake measured is clearly of physiological importance. Additional to this measurement, the functional importance of fungal S supply to the plant was also assessed by tacking ^{35}S transferred through the AMF to roots grown in limiting or non-limiting S concentrations. Transfer under non-limiting conditions was halved compared to low S concentrations, suggesting that the transfer amount is relative to root S status and not constitutive. This implies that AMF may play a functional role in the acquisition of S for plant hosts.

Having assessed the regulation of sulfate transfer through an AMF from the perspective of the plant S status, a further goal of the research outlined in chapter 2 was to assess the regulation of uptake by the fungus itself. To achieve this, S metabolites known to have regulatory effects on sulfate assimilation in other fungi were exogenously supplied to isolated mycorrhizal mycelium and $^{35}SO_4^{2-}$ transfer tracked. Methionine (met) and cysteine (cys) reduced transfer by 26% and 45%, respectively, over a one month labeling period. Also due to cys addition, quantitative real-time PCR measurements of *G. intarardices* extra-radical mycelium revealed a roughly four fold

decrease in the expression of a putative high affinity sulfate permease gene, suggesting this may be the cause of the lower transfer rate for labeled sulfate. Uptake of $^{35}SO_4^{2-}$ by the fungus was not significantly reduced by met and was halved by cys addition, in contrast to other fungi. Equivalent concentrations of cys or met completely suppress the expression of sulfate assimilation genes in *S. cerevisiae*, *A. nidulans*, and *N. crassa* (Kuras & Thomas, 1995; Breton & Surdin Kerjan, 1977; Ketter & Marzluf, 1988; Ketter *et al.*, 1991; Grynberg *et al.*, 2001).

Sulfate was shown to be transferred through an AMF symbiosis in physiologically significant quantities which are inversely related to sulfate availability to host roots. Additionally, the transfer of reduced S sources cysteine and methionine was demonstrated, which could provide plants an alternate option to their dependency on re-mineralized sulfate from organic material by microorganisms in the soil. Interestingly, cysteine, and to a lesser extent methionine, made available to the extra-radical fungal mycelium reduced transfer of sulfate to the roots. Since these amino acids are transferred in a reduced state to host roots, whether or not this is due to regulation by the fungus was investigated by measuring gene expression changes from exogenous cys application. There was a roughly four fold reduction in mRNA concentration for a putative high affinity sulfate permease gene corresponding to the reduction in uptake and transfer of sulfate. Other putative pathway genes were not down-regulated significantly, suggesting that control of sulfate transfer to the host may be on the level of extra-radical mycelial uptake.

Uptake and assimilation of S by AMF

The use of pre-symbiotic fungal mycelium for the study of the sulfate assimilation pathway reduces the time needed for an experiment from a minimum of two months to one week as well as the variability inherent to split plate studies due to differences in colonization time and extent within the roots. It is also necessary to remove the influence of the host roots on the regulation of fungal uptake and transfer to assess fungal-specific regulation. The study of S metabolism in pre-symbiotic AMF is therefore an important component in understanding the regulation of S transfer in the symbiotic phase. To this end, two lines of research were conducted using germinating spores: the regulation of S assimilation and the interaction between N and S regulation.

Regulation of sulfate assimilation and the synthesis of cysteine is well studied in *S. cerevisiae*, *A. nidulans*, and *N. crassa* (for a review see Marzluf, 1997). Symbiotic transfer of nutrients, however, requires uptake of those nutrients when uptake by other fungi would likely be suppressed. Since fungal regulatory systems for S assimilation and metabolism are well conserved in fungi, differences between AMF and other fungal systems are possibly specific to the symbiosis and may therefore provide valuable information concerning the transition from pre-symbiotic to symbiotic fungal states. The ability of germinating spores to import sulfate and the fractionation profiles of the incorporation of imported sulfate into various metabolic pools was analyzed and the results compared with data from other fungi. Exposure to unlabeled and labeled cys and met, glutathione (GSH) and buthionine sulfoximine (BSO) was analyzed. In contrast to other fungi, *Glomus intraradices* did not show a preference for reduced or oxidized S, but

imported an assimilated both sources simultaneously and to a relatively equal extent. The addition of met failed to reduce sulfate uptake at all, and cys reduces uptake by a third at the most, far less suppression than in other fungi (Grynberg *et al.*, 2001; Marzluf, 1997). The symbiotic nature of AMF may reduce the sensitivity of the regulatory system for sulfate assimilation. Additionally, based on the data it is likely that the intracellular concentration of GSH is negatively correlated with the importation of sulfate, as GSH addition initially reduced uptake by more than two thirds, while the addition of BSO, a specific inhibitor of GSH synthesis, more than doubled it. Although GSH is considered to be a reservoir for reduced S in fungi (Miyake *et al.*, 1999), increased incorporation from BSO addition suggests that the effect of GSH/BSO is not due to GSH catabolism. In support of this argument, the exogenous addition of GSH does not decrease $\%^{15}N$ labeling of cys from ammonia, and actually increases [^{35}S]cys uptake by about a third. Vacuolar H+-ATPase in *S. cerevisiae* is regulated by redox state (Fernandez et al., 2003), altering transport of S metabolites to and from the vacuole. This may provide an explanation for the effect of GSH/BSO, however the full determination of this aspect of S assimilation in AMF is beyond the scope of this thesis.

The assimilation of N and S are linked through a set of orthologous positive acting global transcriptional factors controlling the amino acid control circuit (gC) in yeasts, or the cross pathway control circuits (CPC) in filamentous fungi (Chiang & Marzluf, 1995; Sosa *et al.*, 2003; Okamoto *et al.*, 1993). In *S. cerevisiae*, expression of the S regulatory protein, Met4p, is not controlled by S metabolite concentrations, but through the gC (Mountain *et al.*, 1993). The addition of various sources of N uniformly led to a minimum two fold increase in sulfate uptake. Ammonia addition had the most

pronounced effect of the N sources, increasing sulfate uptake by six fold. This was also the most pronounced effect of any exogenously supplied substance, suggesting that the assimilation of N, and subsequent demand for S-amino acids, controls sulfate assimilation to a greater extent than S metabolite availability in this fungus. In support of this, the combinatory effect of adding S metabolites and ammonia was equivalent to the addition of ammonia alone with the exception of GSH, which completely negated the ammonia effect. An analysis of free amino acid (FAA) concentrations and $\%^{15}N$ labeling in relation to N addition demonstrated a likely positive correlation between ammonia availability and protein synthesis. This is in agreement with the hypothesis that sulfate assimilation is demand driven. Further analysis of the mechanism of the GSH mediated reduction in sulfate assimilation by FAA concentration measurements revealed that GSH addition roughly halved the concentrations of amino acids associated directly with ammonia assimilation. This supports a hypothesis that GSH reduces sulfate assimilation by countermanding ammonia assimilation by an unknown mechanism, although a speculative mechanism based on control at the level of gene expression was discussed in chapter 4.

The analysis of transcriptional regulation of putative genes involved in sulfate assimilation demonstrated extreme differences between the AMF studied and other fungi. Additionally, changes in gene expression failed to correlate in most cases with uptake data. The most apparent difference is the induction of pathway genes by met addition in AMF, compared to their complete suppression in other fungi under similar conditions (Natorff *et al.*, 2003; Cherest *et al.*, 1985; Borges-Walmsley *et al.*, 1995; Marzluf, 1997; Schierova et al., 2000). Exposure to met and GSH had very similar effects

of gene expression, increasing the transcript levels of the enzymes of the reverse-transsulfuration pathway, cytathionine β-synthase and cystathionine γ-lyase, by more than four fold. In comparison, met had no effect on sulfate uptake, and GSH reduced uptake of sulfate by two thirds. The expression of the putative high affinity sulfate permease gene did, however, coincide with uptake measurements. Exposure to cys led to a halving of transcript levels and a reduction of uptake of a third after two days of labeling. The addition of ammonia failed to affect gene expression of putative pathway enzymes in most cases, and certainly failed to account for the dramatic increases in sulfate uptake and assimilation under similar conditions. These data do not support the hypothesis that transcriptional regulation in AMF is controlled by an orthologous transcriptional regulator responding to amino acid starvation, as in *S. cerevisiae* (Mountain *et al.*, 1993), however uptake data does. Gene expression data obtained demonstrate that AMF may have a distinct transcriptional regulatory environment among the fungi with respect to sulfate uptake and assimilation. However, the incongruity with uptake data requires further examination of these findings, possibly through yeast complementation experiments where the comparison between AMF genes and those of *S. cerevisiae* are more direct.

Future research

The thesis presented was designed to provide key and conclusive data on S assimilation and transfer by AM fungi which can be used as a basis for further research. The transport and reduction of sulfate, as well as the synthesis of cysteine, are well

conserved and studied pathways in fungi. Therefore, much of the future research on this subject lies in the comparison between pathway elements from AMF, *S. cerevisiae*, *A. nidulans*, and *N. crassa*. In the short term, obtaining full length sequence information for all of the genes identified to this point would allow for complementation studied of known *S. cerevisiae* pathway mutants to determine if, and the extent to which, functional and regulatory homologies exist between the two systems. Unreported in this thesis, putative sequences obtained from *Glomus intraradices* germinating spore 454 sequencing data of both a positive and a negative transcriptional regulatory gene were discovered. Complementation studies of these genes would immediately reveal the correlations existing between AMF and other fungi in transcriptional regulation of the sulfate assimilation pathway. Additionally, the over-expression of these genes in a yeast background would make possible kinetic analyses of the pathway components, which could be compared to previously determined fungal enzyme kinetics.

The importance of S metabolic regulation to the AMF symbiosis is a topic of interest with respect to the root-less propagation of the fungus. Regulation of the S assimilation genes is connected to N assimilation and nutrient status in fungi, and therefore may provide an indicator of important steps in elucidating the genetic basis of the changes in life cycle stages. A study analyzing the expression patterns of sulfate assimilatory pathway genes in relation to life cycle stage would be a good first step in this regard, and may lead to a global regulatory cycle.

Finally, the incorporation of mutant *Medicago truncatula* plants impaired in sulfate assimilatory pathway genes would help determine more definitively to what extent AMF contribute to plant S metabolism. A mutant with impairment in sulfate

transport, for instance, could directly demonstrate the extent to which AMF can replace plant uptake by roots. A selective screening of S assimilation mutants for the ability to create a functional AMF symbiosis also has the potential for discovery of any S-related genes required for the establishment of a functional endomycorrhiza.

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