



# The arbuscular mycorrhizal fungal protein glomalin: Limitations, progress, and a new hypothesis for its function

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## Summary

Most research on glomalin-related soil proteins (GRSP) has focused on the role of arbuscular mycorrhizal fungi in soil aggregation and environmental engineering. Recently, with the description of the glomalin gene sequence and other results, work on this protein has shifted to include fungal physiology. Based on recent findings, we develop a new model for the role of glomalin; we postulate a primary role in fungal physiology and secondarily arising effects in the soil environment that lead to observed correlations of GRSP with soil aggregate stability. Concurrent with advances in molecular biology, several recent studies have highlighted problems with quantifying GRSP from soil. We summarize and discuss limitations of the methods currently used for GRSP extraction and quantification in soils. We finish with a set of recommendations for research directions that involve testing the model proposed here.

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## Introduction

Arbuscular mycorrhizal fungi (AMF) are widespread plant symbionts with multiple and pervasive influences on terrestrial plant communities and

ecosystem processes (Rillig, 2004a). Among these, their effects on soil aggregation have recently received increased attention (Rillig and Mummey, 2006), mostly precipitated by the discovery of glomalin-related soil protein (GRSP) (Wright et al., 1996; Rillig, 2004b) whose soil concentration was typically highly correlated with soil aggregate water stability (Wright and Upadhyaya, 1998). While GRSP constitutes only one of many interacting biochemical, physio-chemical and biological

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AMF-mediated mechanisms contributing to soil aggregation (Rillig and Mummey, 2006), it is an appealing factor to measure, since it is an operationally defined compound which is correlated to an ecosystem parameter of interest, soil structure. AMF ecology is a field with a relatively limited range of response variables suitable for measuring abundance and activity, like identification of field spores, measuring hyphal length, root colonization and inoculum potential. All these variables have limitations and entail fairly labor-intensive processes. This adds to the appeal of including GRSP in the suite of variables used in field studies, since protein quantification is fast, objective, cheap and relatively easily conducted.

GRSP is so termed because the correspondence with the AMF protein proper, glomalin, has not been clearly established (Rillig, 2004b). Although the subject of numerous phenomenological studies, GRSP research has remained a controversial area in soil microbiology since fundamental knowledge of the actual AMF protein is lacking, and because other components of soil organic matter not of AMF origin may be co-extracted and cross-reactive in the detection methods (Bradford protein assay, or ELISA based on the monoclonal antibody MAb32B11, raised against crushed spores of the AMF *Glomus intraradices* Wright et al., 1996).

In this paper, we discuss the current limitations of GRSP extraction and quantification in soils, taking into account recent progress. We also present recent findings that support the re-examination of previous hypotheses about the identity of the glomalin protein proper, and suggest new hypotheses for glomalin function (with fungal physiology as the centerpiece). We finish with a set of recommendations for research directions, and for the use of GRSP in soil ecology research.

## GRSP: the soil pool and its relationship with glomalin

GRSP quantification is reliant on reactivity in Bradford and MAb32B11-ELISA assays following extraction. It was initially hypothesized that the harsh extraction procedure (autoclaving at 121 °C in citrate buffer) destroys all protein except glomalin, which would then react with the coomassie brilliant blue (CBB) reagent in the Bradford assay. However, Rosier et al. (2006) showed that both bovine serum albumin (a protein of similar size to glomalin) and mixtures of proteins contained in leaf litter from three different plant sources were not eliminated through the extraction process, and

were detected in the reaction with CBB; additionally, also some interference with the ELISA assay was found.

These results point to the conclusion that the current soil extraction method can lead to the co-extraction of proteins of non-AMF origin. Moreover, the Bradford assay is unable to distinguish the origin of different proteins, justifying the proposed change in nomenclature from "Total glomalin" to "Bradford-reactive soil protein (BRSP)" (Rillig, 2004b).

Proteins are chemically versatile molecules in soil for which a large variety of biochemical modifications have been documented, or hypothesized to occur (Rillig et al., 2007), that can lead to their stabilization in the environment. Hence, it is not surprising that, in addition to proteins of non-AMF origin, the GRSP extract may also contain other compounds. Two recent studies have shown the co-extraction of tannic and humic acids (Whiffen et al., 2006; Schindler et al., 2006), supporting the hypothesis that GRSP extract may actually represent complex associations of polyphenolic substances with an AM associated protein (or proteins). In fact, in another recent study soil treatments with tannins resulted in darker extracts compared to control soil, which may have influenced the photometric measurement following the Bradford reaction and consequently caused incorrect estimations of soil protein (Halvorson and Gonzalez, 2006).

It is clear that the current extraction protocol coupled with Bradford-assay detection could yield a potentially misleading picture regarding the AMF-origin of the material, particularly in circumstances where extraneous protein additions occur. The ELISA assay remains as a detection option of potentially greater reliability (Rosier et al., 2006), but has not been subjected to rigorous tests thus far.

## Glomalin: the protein

As a consequence of potentially contaminating compounds present in soil-extracted materials, characterizing glomalin in the soil-extracted phase has proven a difficult undertaking. Also the relatively harsh soil extraction conditions (autoclaving in citrate buffer) have presented problems with obtaining clear protein banding patterns (V. Gadkar, J. Driver, and M. Rillig, unpublished observation). Hence, it was an important step to test if glomalin is also produced in sterile culture conditions. Such a system exists in the form of dual

plant root organ/ fungus *in vitro* cultures, which are free of other microbes and soil organic matter components. In these culture containers, root and hyphae can be spatially separated, permitting the study of just the extraradical hyphae (St-Arnaud et al., 1996). Immuno-reactivity with the glomalin-defining MAb32B11 was indeed found using such *in vitro* AMF cultures, (Rillig and Steinberg, 2002; Driver et al., 2005; Gadkar et al., 2006). Based on this culture system, Gadkar and Rillig (2006) were able to isolate an MAb32B11-immunoreactive band from an *in vitro* culture of *G. intraradices*. This led to the isolation, sequencing and initial expression of the putative gene for glomalin. The expressed protein was cross-reactive with MAb32B11, and the corresponding gene was expressed in proliferating mycelium. Evidence was found, based on high amino-acid sequence identity and an initial phylogenetic analyses, that glomalin is a putative homolog of Heat Shock Protein 60 (Hsp60). This was the first time that a member of this ubiquitous protein group had been described from the eumycotan phylum Glomeromycota, containing the AMF.

At the same time this is a surprising result, because rather than a hypothesized “specialized” protein, with perhaps a function unique to AMF (fueled by the observation that protein similarity is often low – Gadkar and Rillig, 2006), or a hydrophobin-homolog (Rillig, 2005), it turns out that glomalin is a putative member of a virtually ubiquitous family of proteins.

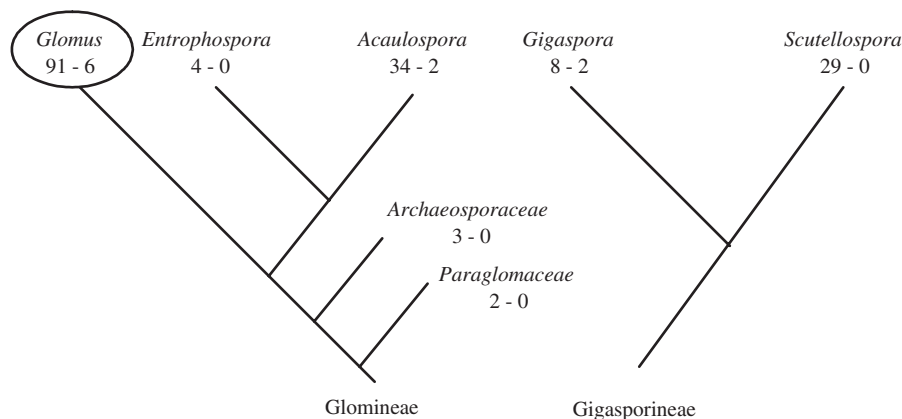
This raises issues regarding the extent to which glomalin genes or proteins are conserved within the

Glomeromycota; is it sufficient to explain the MAb32B11-immuno-reactivity of the AMF species examined thus far (Fig. 1)? Additionally important, are these proteins of non-AMF origin also reactive with MAb32B11? These new questions warrant a discussion of heat shock proteins.

## Homology and functionality of heat shock proteins

Heat shock proteins are conserved proteins found in eukaryotes and prokaryotes (Lindquist and Craig, 1988), grouped into five families: Hsp100, Hsp90, Hsp70, Hsp60 and small Hsps. The synthesis of these proteins was discovered by a heat shock effect, but other stress factors, like pH change and starvation, are known to stimulate their production (Tereshina, 2005). Functionally, Hsps includes proteins with catalytic activity (Hsp100) and chaperones, which assist protein folding and protect them against denaturation (Hsps 70 and 60). It is known that some chaperones also have the ability to act as signals resulting in increased thermotolerance, control of spore viability, and long-term viability of starving vegetative cells (Parsell and Lindquist, 1993; Burnie et al., 2006).

Despite their ubiquitous occurrence and their role in maintenance of cell activity and integrity, heat shock proteins remain poorly known in many groups of organisms, including the fungi. The synthesis of Hsps has been studied in some groups in response to stressing factors like heat shock, low



**Fig. 1.** Approximate phylogenetic relationships within the Glomeromycota (based on data provided by INVAM – <http://invam.caf.wvu.edu>) showing the number of described species (first number) and the number of species already shown to produce glomalin-related soil proteins (second number). Note that *Glomus intraradices*, the species from which the MAb32B11 antibody was obtained, is in a genus distantly related to some other groups, raising the question to what degree results obtained for some species can be extrapolated to that of other taxa in the Glomeromycota phylum. Data from GRSP production were obtained from Wright et al. (1996), Wright and Upadhyaya (1999), Wright (2000), Rillig and Steinberg (2002), Augé (2003), Lovelock et al. (2004), González-Chávez et al. (2004), Driver et al. (2005), Caravaca et al. (2005) and Gadkar et al. (2006).

pH or oxidizing conditions (Kamei et al., 1992; Goldani et al., 1994; Chen and Chen, 2004). Evidently, the cellular roles of Hsps can be varied and complex, and we have relatively limited information on functionality in eumycotan fungi (see Table 1). There are few reports of Hsp occurrence in the Glomeromycota in addition to glomalin. Using the AMF species *G. intraradices*, Porcel et al. (2006) demonstrated the expression of a small Hsp (30 kDa), which improved plant tolerance to drought stress.

However, the identity in amino-acid sequence may not necessarily result in a similar function, as appreciated generally, and shown specifically for Hsps by Yoshida et al. (2001). These authors studied an Hsp60 that is homologous to GroEl in *Escherichia coli*. This protein is produced by a bacterial symbiont (*Enterobacter aerogenes*) that occurs in the saliva of *Myrmeleon bore*, and could paralyze cockroaches. However, this insecticidal effect was not produced by GroEl isolated from *E. coli*, which illustrates specifically for this group of proteins that homology does not necessarily imply functional similarity.

Given these results, there are many questions to be elucidated for the putative Hsp60 homolog from *G. intraradices*. Does this AMF protein have any functional similarity to other well-known Hsps? How different are glomalins among AMF species, given the wide range of fungal life histories in this group (Hart and Reader, 2002), and the differences in isolate symbiotic efficiency (Van der Heijden et al., 1998). Could differences in glomalin genes among clades of AMF contribute to differences in functioning? Do all AMF species secrete/ release this protein (Fig. 1), and what environmental stress factors

would induce or inhibit glomalin production? Does the Hsp60 homolog have any additional functions, such as illustrated in the example above, that manifest themselves either in the cell or in the environment? This leads us to a new model of glomalin function.

### A new hypothesis for glomalin function: cellular function, palatability and secondary environmental effects

The original hypothesis, formulated by Wright et al. (1996), stated that glomalin is secreted (or otherwise released) into the soil from AMF where it would aid in soil aggregation. This model was directly based on the observed correlation of GRSP concentrations with soil aggregate water stability. Increased soil aggregation would benefit both the host plant and associated AMF justifying the energetic "cost" of glomalin production. Experimental evidence, albeit from a very artificial model system, suggests that feedbacks between glomalin production, soil aggregation and improved extraradical AMF hyphal growth may indeed exist (Rillig and Steinberg, 2002), supporting a habitat engineering functionality (*sensu* Jones et al., 1997). However, AMF also appear to produce GRSP in soils in which organic matter is not the primary soil binding agent, and where GRSP and soil aggregation are not correlated (Rillig et al., 2003). This suggests that promotion of soil aggregation may not be the primary function of glomalin. Also, AMF occur as communities, and many other soil biota groups other than host roots stand to

**Table 1.** Examples of roles of Hsps described in some eumycotan fungi

Protein family	Species	Function	Reference
Small Hsps (30 kDa)	<i>Saccharomyces cerevisiae</i>	Inhibition the activity of the proton ATPase under heat shock conditions, conserving energy in the form of ATP	Thevelein and Hohmann (1995)
	<i>Glomus intraradices</i>	Protection of the host plant against drought stress	Porcel et al. (2006)
Hsp 70	<i>Histoplasma capsulatum</i>	Conversion of mycelial to yeast phase	Caruso et al. (1987)
	<i>Neurospora crassa</i>	Expression and transport of sporulation-specific proteins, stabilization of macromolecules in the spores and their preservation for germination	Rensing et al. (1998)
Hsp 90	<i>Saccharomyces cerevisiae</i>	Formation of macromolecular complexes with Hsps 70 and 60	Mager and De Kruijff (1995)
Hsp 100	<i>Saccharomyces cerevisiae</i>	Protease with ATPase activity	Sanchez et al. (1992)

profit from an improved soil structure (e.g. nematodes, Niklaus et al., 2003); this makes it perhaps unlikely that promoting soil aggregation is the primary function of glomalin.

Driver et al. (2005), using a sterile *in vitro* culture system, found that most (ca. 80%) of the glomalin found was contained in the fungal mycelium rather than in the liquid growth medium. It is unclear if this result translates from the artificial aqueous culture system to the soil environment, or if it applies to fungi across the spectrum of AMF species (Fig. 1). However, if it does, it suggests that a primary function of glomalin may be in the living fungus. This notion is of course supported by the finding that glomalin is a putative heat shock protein homolog (with the caveats regarding homology discussed in the preceding section).

Based on the above observations, we propose a new model of glomalin function as a new working hypothesis. This model has the following key components: (a) glomalin has (or has had) a primary cellular function, such as chaperonins; (b) another function has been acquired relating to wall-location of the protein and effects on palatability of the mycelium; (3) the environmental function in the soil in the context of soil aggregation has arisen secondarily as a by-product of the primary physiological function.

Glomalin may have (or may have had) a primary cellular function, related to the documented properties of other Hsp, such as chaperonins. We hypothesize that glomalin may then have acquired a secondary function, much like that observed by Yoshida et al. (2001). An essentially toxic effect of glomalin to potential fungal grazers (like an insecticide) could have presented a very powerful trait for selection. In contrast to many earlier findings, it has been shown that AM fungal hyphae are far less palatable to microarthropods than other, saprobic soil fungi (Klironomos and Kendrick, 1996). The mechanisms for this reduced palatability are not known, and we suggest that it may be related to glomalin production. Preliminary studies in our lab indicated that a fraction of AMF *in vitro* culture supernatant enriched in MAb32B11-reactive glomalin strongly decreased palatability of fungi to *Folsomia candida* (Collembola), compared to a control (Rillig, unpublished observation). Why would AMF soil mycelium be so well defended? AMF function by maintaining continuous transport of carbon from the host plant cell in the root cortex to the tips of the growing mycelium (like all fungi, they only grow at the hyphal apex); simultaneously they transport other material (e.g. forms of P and N) from the mycelium in the soil to the host plant. AMF as obligate biotrophs are dependent upon an

uninterrupted flow of C from the host, a dependence not occurring in saprobic fungi. The latter have the enzymatic capabilities to access C resources directly from organic matter in the soil. It hence seems plausible that particularly effective defenses might have evolved in the ancient phylum (460 Million years old; Redecker et al., 2002) comprising the AMF (Klironomos and Kendrick, 1996). These defenses may also extend to hyphal and spore parasites, not only to consumers of spores and hyphae. In this model, physiology of the mycelium is central; effects that glomalin has once in the soil environment are incidental. This certainly does not contradict feedback effects under conditions of unfavorable hyphal growth, such as observed previously (e.g. Rillig and Steinberg, 2002). It merely shifts the focus from effects on the soil to reactions within the fungal mycelium. So, how does this secondary effect of glomalin in the soil environment arise?

First, it is important to realize that evidence regarding glomalin involvement in soil aggregation is correlational in nature (Rillig and Mummey, 2006); causality remains yet to be established. Hence the null hypothesis that glomalin has no mechanistic influence on soil aggregation needs to first be convincingly rejected. AMF certainly influence soil aggregation in a variety of ways, including physical, biological, and biochemical factors other than glomalin (Rillig and Mummey, 2006). If glomalin is in fact mechanistically involved in soil aggregation, for example, by serving as a biochemical binding agent like many proteins (Rillig et al., 2007), we suggest that this function has arisen because of the persistence of this protein in the environment and the location in the hyphal wall. The persistence may be related to its toxic nature postulated above, or simply caused by the protein not being primarily secreted, but deposited into the soil covalently bound in the hyphal wall matrix. This could also lead to the protein being "delivered" into micro-pores, simply as a function of invasive hyphal growth, potentially making the protein less vulnerable to enzymatic attack.

This model depends on glomalin being contained in the hyphal wall. We have preliminary evidence from EM-immunocytochemistry that glomalin may in fact be located in the spore and hyphal walls (Driver and Rillig, unpublished). Previous studies using light microscopy fluorescence techniques indicated the presence of GRSP not only on hyphal and spore surfaces, but also surfaces of roots and soil aggregates (Wright et al., 1996; Wright, 2000). What evidence is there for other Hsp to be located in cell/ hyphal walls? Frisk et al. (1998) reported that a GroEL homolog produced by the bacteria



*Haemophilus ducreyi* was also associated with the cell surface. The extracellular localization of some Hsps is also known in some fungi. Most of 70 and 47-kDa Hsps produced by *Candida albicans*, *Paracoccidioides brasiliensis* and *Saccharomyces cerevisiae* are located in the cell wall and sometimes appear to reach the cell surface through channels, as observed when using immuno-electron microscopy (Matthews et al., 1988; López-Ribot and Chaffin, 1996; López-Ribot et al., 1996; Burnie et al., 2006; Batista et al., 2006).

## Conclusions: research priorities

The recent description of the glomalin gene represents a springboard for investigations into glomalin functionality. Further clues regarding the role of the putative Hsp60 homolog, particularly in response to a variety of environmental conditions, must come from studies of gene expression and/or protein production. Another important approach for studying the function of glomalin is heterologous expression of this protein; owing to the suspected multi-genomic nature of AMF simple mutation/knockout studies are not possible. Availability of the protein from such sources would in turn facilitate our understanding of the fate, persistence, and role in aggregation of this protein in the soil environment, and permit the execution of studies that causally relate glomalin to soil aggregation.

In order to test our conceptual model of glomalin function, studies relating glomalin production to hyphal palatability and/or parasitism need to be conducted. Another key point is the localization of glomalin protein to the hyphal and spore walls. Also, we have postulated this "dual" functionality of one protein primarily in the physiology of the mycelium and secondarily in the soil environment. Further analysis of the molecular biology of glomalin/Hsp 60 may indicate if this dual role of the same molecule necessarily needs to be invoked. An alternative is that AMF may carry several genes that encode glomalins or other Hsps. *S. cerevisiae* and *C. albicans*, for example, are two fungi that have multiple genes encoding heat shock proteins (reviewed by Chaffin et al., 1998). This would not fundamentally change our working hypothesis, but this "division of labor" could be most interesting evolutionarily and also practically (e.g., which one is the glomalin-antibody MAb32B11 preferentially detecting?). Such specialization is certainly known for another important group of fungal proteins, the hydrophobins (Wösten 2001; Linder et al., 2005).

In hydrophobins, different functions can be carried out by different hydrophobin proteins produced by the same organism, for example, at different developmental stages or in response to different environmental conditions.

The molecular and physiological work underway needs to eventually be applied to the soil environment. Our confidence in glomalin environmental detection tools is currently low, because it is uncertain to what extent the Bradford-reactive protein pool is of AMF origin, and this operationally defined soil extract (GRSP) may also contain other compounds linked with proteins. Hence this assay must be used with caution, especially under conditions of extraneous (proteinaceous) organic matter additions. There is currently no evidence what level of extraneous organic matter would interfere unacceptably with the Bradford-based assay. Until this is known, the Bradford assay used in isolation (easily extractable or other fractions) as the sole quantification method for GRSP is not recommended if the goal is a link to AMF. We currently have only limited evidence for the specificity of the monoclonal antibody; further testing this tool for potential interference in soil (Rosier et al., 2006) or cross-reactivity with other soil constituents must be a priority. However, it is clear that one cannot test all potentially MAb32B11 cross-reacting biomolecules or biota in soil; hence, ultimately the usefulness of this assay will hinge on developing additional antibodies. It is precisely this goal to which the molecular work currently being done can contribute decisively. Availability of the protein will permit raising antibodies against it directly, rather than against fungal spore walls.

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