The arbuscular mycorrhizal fungal protein glomalin is a putative homolog of heat shock protein 60

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arbuscular mycorrhizal fungi; *Glomus intraradices*; soil aggregation; glomalin.

Abstract

Work on glomalin-related soil protein produced by arbuscular mycorrhizal (AM) fungi (AMF) has been limited because of the unknown identity of the protein. A protein band cross-reactive with the glomalin-specific antibody MAb32B11 from the AM fungus *Glomus intraradices* was partially sequenced using tandem liquid chromatography-mass spectrometry. A 17 amino acid sequence showing similarity to heat shock protein 60 (hsp 60) was obtained. Based on degenerate PCR, a full-length cDNA of 1773 bp length encoding the hsp 60 gene was isolated from a *G. intraradices* cDNA library. The ORF was predicted to encode a protein of 590 amino acids. The protein sequence had three N-terminal glycosylation sites and a string of GGM motifs at the C-terminal end. The *GiHsp* 60 ORF had three introns of 67, 76 and 131 bp length. The GiHsp 60 was expressed using an *in vitro* translation system, and the protein was purified using the 6xHis-tag system. A dot-blot assay on the purified protein showed that it was highly cross-reactive with the glomalin-specific antibody MAb32B11. The present work provides the first evidence for the identity of the glomalin protein in the model AMF *G. intraradices*, thus facilitating further characterization of this protein, which is of great interest in soil ecology.

Introduction

Arbuscular mycorrhizal fungi (AMF) are ubiquitous symbionts in terrestrial ecosystems with important contributions to plant performance, plant community composition, and ecosystem processes (Smith & Read, 1997; Rillig *et al*., 2003; Harner *et al*., 2004; Rillig, 2004a). Among these functions is their role in soil aggregation, hypothesized to be partly mediated by a proteinaceous compound released by an actively growing AMF mycelium in the soil: glomalin (Wright & Upadhyaya, 1998; Rillig & Mummey, 2006). Operationally defined and extracted from soil as glomalin-related soil protein (GRSP; Rillig, 2004b), this proteinaceous compound was highly correlated with an important soil parameter, aggregate water stability (Wright & Upadhyaya, 1998), spurring an active interest among soil ecologists (Rillig, 2004a, b). In the absence of biochemical and molecular biology knowledge of glomalin, numerous research projects were carried out aimed at a phenomenological description of GRSP responses to a variety of environmental and management factors (reviewed in Rillig, 2004b); however, it is clear that further progress in understanding the role of this compound in soil or in the biology of AMF depends on knowledge of the protein proper and the gene that codes for it.

Various GRSP fractions are obtained using harsh extraction methods (autoclaving in citrated buffer; Wright & Upadhyaya, 1996); also, the potential complexity associated with the protein-material extracted from soil may explain why attempts to define glomalin biochemically thus far have been foiled. As the glomalin protein has not been identified, the next logical steps of applying molecular biological tools to isolate the putative gene responsible for its synthesis have also not taken place. Hence, the first necessary step toward characterizing the glomalin gene was to demonstrate that the protein could also be detected and produced in soil-free sterile production systems (Rillig & Steinberg, 2002; Driver *et al*., 2005). This has opened up another avenue to isolating glomalin protein from AMF, which we pursued in the present work, avoiding the drawbacks of working with soil-derived material.

Based on earlier work on the putative identity and characteristics of the AMF glomalin protein, we attempted to characterize the immunoreactive protein isolated from an *in vitro* grown mycelium of AMF *Glomus intraradices*. Our goal was to identify and isolate the gene responsible for

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glomalin synthesis, and to synthesize the protein *in vitro* to determine its cross-reactivity with the glomalin-specific (and glomalin-defining) monoclonal antibody MAb32B11.

Materials and methods

Fungal material and culture conditions

Ri-T DNA-transformed roots of carrot (*Daucus carota*) colonized by *G. intraradices* (DAOM 197198 Biosystematics Research Center, Ottawa) were maintained as described before (St Arnaud *et al*., 1996). Cultures with liquid M-medium (Bécard & Piché, 1992) were added to the split plates in the 'fungus-only' compartment. Mycelial proliferation was observed in plates anytime after 2–3 weeks post-initiation. Glomalin contained in the growth medium was obtained, without autoclaving, as described before (Driver *et al*., 2005).

Glomalin reactive band isolation and protein sequencing using nano LC-MS/MS

To obtain a discrete protein cross-reacting with the glomalin-defining monoclonal antibody MAb32B11 (Wright & Upadhyaya, 1996), the components of the liquid fungal exudate were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), using the same running conditions as described before (Wright & Upadhyaya, 1996). Immunoblot analysis using the glomalin-specific monoclonal antibody MAb32B11 revealed a single protein band of *c*. 65 kDa. A duplicate SDS-PAGE gel was prepared and stained with colloidal Coomassie Blue G250 using the Gel Code Blue^{TM} stain (Pierce, Rockford, IL). The ~65 kDa cross-reactive band was cut out and submitted for partial sequencing using nano LC-MS; spectra were used to search the most recent nonredundant protein database from GenBank using the PROTQUEST software (Norristown, PA) suite.

Isolation of the full-length *Hsp* 60 gene

PCR-assisted screening of *G. intraradices* cDNA library

A previously described procedure for isolation of the full-length cDNA from λ libraries using PCR was used to isolate the full-length *Hsp* 60 gene (Griffin *et al*., 1993; Gonzalez & Chan, 1993; Preston, 1996). A cDNA library prepared earlier from 11-day-old germinating *G. intraradices* spores (Lammers *et al*., 2001) in the λTriplEx2 lambda vector system (Clontech, Palo Alto, CA) was used for this purpose. Before the screening effort, the λ cDNA library was amplified to a titer $> 10^8$ PFU, the phage lysate pooled and stored at $-80\,^{\circ}C$ using dimethyl sulfoxide, as per the manufacturer's protocol. A working stock of the phage lysate was stored at 4 °C.

The two PCR primers specific for amplifying the conserved region (*c*. 800 bp) of the *Hsp* 60 genes (Rusanganwa & Gupta, 1993) were used in conjunction with the λTriplEx2 lambda vector primers, LD-F/LD-R (Fig. S1). All primers were custom synthesized from Sigma-Genosys (Woodlands, TX), and are listed in Table 1. They were used in the following combinations to obtain the full-length sequence: (1) LD-F and Hsp 60-R, (2) LD-F and Hsp 60-F1, (3) LD-R and Hsp 60-F and (4) LD-R and Hsp 60-R1. The components of the PCR reaction (20 μL volume) were the same as described earlier (Gadkar & Rillig, 2005), with the primer

Table 1. List of PCR primers used in the present study for screening the λTriplEx2 cDNA library from *Glomus intraradices* and *in vitro* expression using 6xHis-tag system

Name	Sequence (5′–3′)	Note	Reference
LD-F	CTCGGGAAGCGCGCCATTGTGTTGGT	λTriplEx2 vector primer (right flank)	GenBank Acc # U39779
LD-R	ATACGACTCACTATAGGGCGAATTGGCC	λTriplEx2 vector primer (left flank)	GenBank Acc # U39779
Hsp 60 -F	GGNGAYGGNACNACNACNGCNACNGT	Degenerate forward primer for Hsp 60	Rusanganwa & Gupta (1993)
Hsp 60 -R	TCNCCRAANCCNGGNGCYTTNACNGC	Degenerate reverse primer for Hsp 60	Rusanganwa & Gupta (1993)
Hsp 60 -F1	ACNGTNGCNGTNGTNGTNCCRTCNCC	Reverse of Hsp 60-F	Present work
Hsp 60 -R1	GCNGTNAARGCNCCNGGNTTYGGNGA	Reverse of Hsp 60-R	Present work
NHis6x	ATCACCATCACCACGGT**ATGCAGCGCGTCTCACAA**	*GiHsp* 60 gene specific forward primer (The underline denotes the N-terminal 6xHis-tag)	Present work
CHis6x	CTTGGTTAGTTAGTTA**TTACATCATACCCATATCACCCATTCC**	*GiHsp* 60 gene-specific reverse primer (The underline denotes the additional sequence to which the second round PCR primer will hybridize)	Present work

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concentration maintained at 1 μM. A 1 μL aliquot of the phage lysate from the high-titer λ library ($\sim 1.8 \times 10^8$) was added in each reaction. A touch-down PCR (Don *et al.*, 1991) protocol was used with the following cycling conditions: 94 °C for 80 s, 10 cycles of 94 °C for 45 s, 65 °C (− 1 °C per cycle) for 80 s and 67 °C for 2 min, and then 20–25 cycles of 94 °C for 45 s, 55 °C for 80 s and 67 °C for 2 min and a final extension at 67 °C for 10 min. Amplicons of expected size were cleaned for primer dimers using the QIAquick PCR purification kit (Qiagen, Valencia, CA) and cloned into a pGEM-T plasmid vector (Promega, Madison, WI) as per the manufacturer's instructions. Colony screening of white colonies for the presence of inserts was performed using the SP6/T7 primer combination using the following cycling conditions: 94 °C for 90 s, followed by 30 cycles of 94 °C for 30 s, 55 °C for 90 s and 67 °C for 2 min and 30 s and a final extension of 67 °C for 7 min. The plasmids were then purified using the QIAprep mini prep kit (Qiagen) and sequenced using the ABI Prism Big Dye™ chemistry (Applied Biosystems, Foster City, CA) at the Murdock core sequencing facility located at University of Montana, Missoula. The raw sequence data were processed using the Chromas™ software (version 1.45; Griffith University, Queensland, Australia) and used for further analysis.

Sequencing of *Hsp* 60 gene from *G. intraradices* genomic DNA

PCR primers flanking the whole Hsp 60 ORF were designed using the sequence information obtained from the cDNA library (Table 1). Genomic DNA of *G. intraradices* was isolated from *in vitro* grown spores and mycelia as described earlier (Gadkar & Rillig, 2005). The same touch-down PCR cycling conditions described above were used here. As the amplicon was > 2 kb and hence difficult to sequence completely in a single run, this amplicon was amplified as two separate fragments using the following primer combinations: (1) NHis6x and Hsp 60 R and (2) Hsp 60 R1 and CHis6x (see Table 1 for sequence information). Individual amplicons from each combination were cloned in the pGEM-T vector as described above, and sequenced from both directions using the SP6/T7 primer combination. The obtained sequences were then aligned to yield the whole *Hsp* 60 genomic sequence.

Sequence analyses

The vector sequences were identified using the VecScreen utility available on the NCBI server. Sequence identity searches were carried out using the BLASTX (Altschul *et al.*, 1990) and FASTA (Pearson & Lipman, 1988) programs on the NCBI server. Multiple sequence alignments and analyses were conducted using the program CLUSTAL X (Thompson *et al.*, 1997). For constructing the phylogenetic trees, the relative support for groups was determined based upon 1000 bootstrap trees. Searches for signal sequences and N-linked glycosylation sites were carried out using the PROSITE database on the ExPASy server (http://ca.expasy.org/).

Expression of *GiHsp* 60 gene

Extraradical *G. intraradices* mycelia

Extraradical mycelium (comprised of spores and actively growing hyphae) was harvested by liquefying the gelling agent (Doner & Bécard, 1991), from the 'fungus-only' compartment of a *c.* 5-month-old dual *G. intraradices*-carrot *in vitro* culture. The total amount of fungal tissue obtained from the plate was *c.* 200 mg (fresh weight), which was ground in liquid nitrogen before total RNA extraction using the TRIzol™ reagent (Invitrogen, Carlsbad, CA). The total RNA after quantification was treated with DNase-I before removing any genomic DNA contamination. The cDNA synthesis was carried out using the cMaster RTplusPCR system (Eppendorf, Westbury, NY) using oligo$(dT)_{18}$VN primer (V = A/G/C and N = A/T/G/C). The final cDNA reaction was diluted either 1/5 to 1/20 in TE buffer before PCR amplification. An 870 bp fragment of the *GiHsp* 60 gene was amplified using the Hsp 60-R1/CHis6x (Table 1) gene-specific primer combination, with the same touch-down cycling protocol described in the earlier section. To rule out the possibility of genomic DNA contamination as the source of the amplified product, total RNA (10 ng) was used as the sole template in a separate PCR reaction.

In vitro expression of *GiHsp 60* cDNA

In vitro expression of the *GiHsp 60* cDNA was performed using the EasyXpress™ Protein synthesis kit (Qiagen) as per the manufacturer's instruction. To facilitate the downstream purification of the GiHsp 60 protein, an N-terminal 6xHis-tag was added to the gene using PCR with the following affinity primers: NHis6x and CHis6x (Table 1). The *in vitro* synthesized protein was purified using a His-Spin Protein Miniprep™ kit (Zymo Research, Orange, CA), after running a small aliquot to verify the synthesis on an SDS-PAGE gel. The column wash solutions were also run in parallel during the SDS-PAGE analysis.

ELISA-dot-blot assay of GiHsp 60 protein

An ELISA-dot-blot assay was carried out on the purified protein using the procedure described earlier (Wright & Morton, 1989). Briefly, a nitrocellulose membrane (0.45 mm pore size; Bio-Rad Laboratories, Richmond, CA) was soaked

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in phosphate-buffered saline (PBS) for 5 min and allowed to dry between filters. The eluate of the final wash (i.e. the GiHsp 60 protein) was spotted using a dot-blotting apparatus. The glomalin soil standard (Wright & Upadhyaya, 1996) was also spotted similarly. For negative controls, the following were used: water, PBS buffer, elution buffer from the 6xHis-tag purification kit (50 mM sodium phosphate buffer, pH 7.7, 300 mM sodium chloride 250 mM imidazole) and EasyXpress^{TM} translation lysate. After the membranes were allowed to dry, they were immersed in blocking solution (2% nonfat milk in PBS) and washed three times in PBS-Tween 20 (PBS-T) solution. The membrane was then soaked in the diluted (1 : 2 in PBS) MAb32B11antibody solution and incubated on a shaker for 1 h. The antibody solution was removed and the membrane was washed in PBS-T buffer, three times, each for 5 min. The membrane was incubated in diluted (5 μL/6 mL 1% BSA) biotinylated anti-mouse IgM with the membrane for 1 h on a shaker. The solution was removed and the membrane was washed three times, each for 5 min with PBS-T solution. The membrane was then incubated in diluted ExtrAvidin peroxidase solution (3.0 μL per 6 mL 1% BSA) and incubated for 1 h on a shaker. The membrane was finally washed three times in PBS-T buffer and finally color development was performed using 4-chlor-1-naphthol prepared in methanol. After color development, the membrane was dried and stored at room temperature.

Results

Sequence of the immunoreactive protein

The immunoreactive band (Fig. 1) yielded the following partial amino acid sequence: TALLDAAGVASLLTTAE. Comparison of this sequence with other accessions in GenBank showed it to be > 80% identical to the mitochondrial Hsp 60 class of proteins. Using this sequence information, we proceeded to isolate the complete *Hsp* 60 gene from the AMF *G. intraradices*, hence termed *GiHsp* 60, using a fungus-only cDNA library.

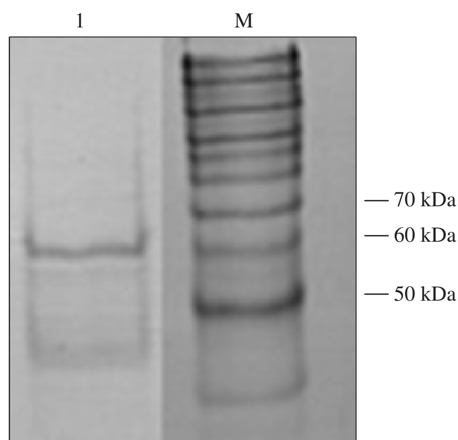

Fig. 1. Immunoblot of the MAb32B11 cross-reactive protein isolated from liquid exudate of the AMF *Glomus intraradices* produced *in vitro* (lane 1). M: Benchmark^{TM} Protein ladder (Invitrogen).

Hsp 60 gene from *G. intraradices*

A cDNA fragment whose translation displayed high identity (66–73%) to the *Hsp 60* genes previously identified from other fungi (Fig. 2) was identified from a *G. intraradices* cDNA library. Using CLUSTAL X (Thompson *et al.*, 1997), an unrooted phylogenetic tree was constructed based on the full-length amino acid sequence of *Hsp* 60 ORF from various organisms (Fig. 3). The resulting tree shows that the *GiHsp* 60 is in a clade with *Saccharomyces pombe*, *Ustilago maydis* and *Cryptococcus neoformans*. Sequence analysis of the full-length *GiHsp* 60 cDNA revealed that it contains an ORF encoding a protein of 590 amino acid length. Use of PCR primers flanking the *GiHsp* 60 ORF on genomic DNA resulted in the isolation of a genomic fragment, 2047 bp in length, suggesting the presence of intron(s). Three introns, each with its conserved 5′ GT, 3′ AG and lariat sequences characteristic of fungal introns (Balance, 1986), were found to be present in the Hsp 60 ORF. The lengths of these three introns were as follows: intron I (131 bp), intron II (76 bp) and intron III (67 bp). The *GiHsp* 60 gene contained a 5′ untranslated region (UTR) (100 bp) and 3′ UTR (174 bp) at the upstream and downstream region of the coding region, respectively. The GC content of the 5′ and 3′ UTR sequence was 28.2% and 14.4%, respectively, while the ORF had a GC content of 38%. The low GC content of *GiHsp* 60 is characteristic of genes isolated from AMF (Hosny *et al.*, 1999; Ferrol *et al.*, 2000; Breuninger *et al.*, 2004). The nucleotide sequence of GiHsp 60 cDNA and its corresponding genomic encoding region has been submitted to GenBank under the accession number DQ383980 and DQ383981, respectively.

Analysis of the deduced protein sequence of the GiHsp 60 protein (Fig. 4) revealed the presence of the sequences that are characteristics of Hsp 60 chaperone peptides, e.g. AAVEEGTVPGGG at position 442–453, which is characteristic of the family [A-(AS)-X-(DEQ)-E-(X_4)-G-G-(GA)], and the GGM amino acid residues at the C terminus (Hemmingsen *et al.*, 1988). Based upon the protein sequence, the molecular weight of the GiHsp 60 protein was predicted to be 63.1 kDa with an isoelectric focusing point (*p*I) of 5.91. Three putative N-terminal glycosylation sites were found, namely, NKTN (position: 115), NATR (position: 438) and NLSP (position: 465). The GiHsp 60 protein had three predicted transmembrane domains with an average length between 17 and 33 amino acids.

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Gi MQRVSQFFNKAPHIT----ISPSLSMVSRNSKRPFLSRFYA-THKDLKFGVEGRASLLKGVDILAKAVAVTLGPKGRNVLIEQPYGSPKITKDGVTVAKSISLKDKFENLGARLVQDVAN
Um MSLLRNAA------------TPARKAAS-NQGRSFSTSLVANAHKEVKFSNDGRAAMLNGVNLLANAVSVTLGPKGRNVIIEQPFGGPKITKDGVTVAKSITLKDKFENLGARLVQDVAN
Yl MQR--VIARSS--------------RLRPQQIRGFA-------HKELKFGVEGRAALLKGVDTLAKAVSVTLGPKGRNVLIEQSFGSPKITKDGVTVARSITLEDKFENMGARLLQEVAS
Sc MLRSSVVRSRA--------------TLRPLLRRAYSS------HKELKFGVEGRASLLKGVETLAEAVAATLGPKGRNVLIEQPFGPPKITKDGVTVAKSIVLKDKFENMGAKLLQEVAS
Ci MHRALSASSRASVLSSAASTRGQLSHFRPALSSGLNLQQQRYAHKELKFGVEGRAALLKGVDTLAKAVTTTLGPKGRNVLIESSYGSPKITKDGVTVAKAISLQDKFENLGARLLQDVAS
Af MQRALSS--RTSVLS-AASKRAAFTKP-----AGLNLQQQRFAHKELKFGVEARAQLLKGVDTLAKAVTSTLGPKGRNVLIESPYGSPKITKDGVSVAKAITLQDKFENLGARLLQDVAS
Nc MQRALTR---ASVGK--AATR-------------LPAQQLRFAHKELKFGVEGRAALLAGVETLAKAVATTLGPKGRNVLIESSFGSPKITKDGVTVAKSISLKDKFENLGARLIQEVAG
Sp MVSFLSSSVSRLPLR----------IAGRRIPGRFAVPQVRTYAKDLKFGVDARASLLTGVDTLARAVSVTLGPKGRNVLIDQPFGSPKITKDGVTVARSVSLKDKFENLGARLVQDVAS
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Gi KTNEMAGDGTTTATILTRAIFVEGVKNVAAGCNPMDLRRGVQMAVDSIVKFLREKSRVITTSEEIAQVATISANGDTHVGKLIANAMEKVGKEGVITVKEGKTIEDELEITEGMRFDRGY
Um KTNEIAGDGTTTATVLARAIYAEGVKNVAAGCNPMDLRRGVQAGVDAVIKFLETNKRAVTTSAEIAQVATISANGDQHVGQLIATAMEKVGKEGVITVKEGKTLEDEIEITEGMRFDRGY
Yl KTNETAGDGTTSATVLGRSIFTESVKNVAAGCNPMDLRRGSQAAVDAVVEFLQKNKREITTSEEIAQVATISANGDTHIGQLIANAMEKVGKEGVITVKEGKTIEDELEITEGMRFDRGY
Sc KTNEAAGDGTTSATVLGRAIFTESVKNVAAGCNPMDLRRGSQVAVEKVIEFLSANKKEITTSEEIAQVATISANGDSHVGKLLASAMEKVGKEGVITIREGRTLEDELEVTEGMRFDRGF
Ci KTNEIAGDGTTTATVLARAIFSETVKNVAAGCNPMDLRRGIQAAVDSVVEYLQANKREITTSEEIAQVATISANGDTHIGKLISNAMERVGKEGVITVKDGKTIEDELEVTEGMRFDRGY
Af KTNEIAGDGTTTATVLARAIFSETVKNVAAGCNPMDLRRGIQAAVDAVVDYLQKNKRDITTGEEIAQVATISANGDTHIGKLISTAMERVGKEGVITVKEGKTIEDELEVTEGMRFDRGY
Nc KTNEVAGDGTTSATVLARAIFSETVKNVAAGCNPMDLRRGIQAAVEAVVEYLQANKRDVTTSEEVAQVATISANGDKHIGELIASAMEKVGKEGVITCKEGKTLYDELEVTEGMRFDRGY
Sp KTNEVAGDGTTTATVLTRAIFSETVRNVAAGCNPMDLRRGIQLAVDNVVEFLQANKRDITTSEEISQVATISANGDTHIGELLAKAMERVGKEGVITVKEGRTISDELEVTEGMKFDRGY
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Gi ISPYFITEAKTQKVEFEKPLILLSEKKISVLQDILPALETSSTQRRPLLIISEDIDGEALAACILNKLRGNIQVAAVKAPGFGDNRKSILGDLAILTGGTVFSDELDIKLERATPDLFGS
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Sc ISPYFITDPKSSKVEFEKPLLLLSEKKISSIQDILPALEISNQSRRPLLIIAEDVDGEALAACILNKLRGQVKVCAVKAPGFGDNRKNTIGDIAVLTGGTVFTEELDLKPEQCTIENLGS
Ci VSPYFITDTKTQKVEFEKPLILLSEKKISAVQDIIPALEASTTLRRPLVIIAEDIEGEALAVCILNKLRGQLQVAAVKAPGFGDNRKSILGDIGILTNSTVFTDELDMKLDKATPDMLGS
Af TSPYFITDTKSQKVEFEKPLILLSEKKISAVQDIIPALEASTTLRRPLVIIAEDIEGEALAVCILNKLRGQLQVAAVKAPGFGDNRKSILGDLAVLTNGTVFTDELDIKLEKLTPDMLGS
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Sp ISPYFITDVKSQKVEFENPLILLSEKKVSAVQDILPSLELAAQQRRPLVIIAEDVDGEALAACILNKLRGQLQVVAIKAPGFGDNRRNMLGDLAVLTDSAVFNDEIDVSIEKAQPHHLGS
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Gi TGSVTITKEDTILLNGDGSKDFINQRCEQIRAAINDASVSDYEKEKLQERLAKLSGGVAVIKVGGSSELEVGEKKDRFVDALNATRAAVEEGTVPGGGVALLKSIK-CLDNLSPANFDQK
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Sc CDSITVTKEDTVILNGSGPKEAIQERIEQIKGSIDITTTNSYEKEKLQERLAKLSGGVAVIRVGGASEVEVGEKKDRYDDALNATRAAVEEGILPGGGTALVKASR-VLDEVVVDNFDQK
Ci TGSITITKEDTIILNGEGSKDAIAQRCEQIRSIIADPATSEYEKEKLQERLAKLSGGVAVIKVGGASEVEVGEKKDRVVDALNATRAAVEEGILPGGGTALLKASANGLKDVKPANFDQQ
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Gi LGIDIVKSALQKPAKTIVDNAGEEGAVIVGKILDNHVDDFNYGYDAAKGEYGDLVSRGIVDPLKVVRTALVDASGVASLLTTTECMITEAPEENKGAAGGMGRMGGMGGMGDMGMM--
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Sc LGVDIIRKAITRPAKQIIENAGEEGSVIIGKLIDEYGDDFAKGYDASKSEYTDMLATGIIDPFKVVRSGLVDASGVASLLATTEVAIVDAPEP--PAAAGAGGMP--GGMPGMPGMM-
Ci LGVSIVKSAIQRPARTIVENAGLEGSVIVGKLTDEFAGDFNRGFDSAKGEYVDMIGAGIVDPLKVVRTALVDASGVASLLGTTEVAIVEAPEEKAPAAAGMGGMGGMGGMGG-MY---
Af LGVSIIKNAITRPARTIVENAGLEGSVIVGKLTDEFAKDFNRGFDSSKGEYVDMISSGILDPLKVVRTALLDASGVASLLGTTEVAIVEAPEEKGPAAPGMGGMGGMGGMGGGMF---
Nc LGVTIVRNAITRPAKTIIENAGLEGSVVVGKLTDEFANDFNKGFDSAKAEYVDMIQAGILDPLKVVRTGLVDASGVASLLGTTEVAIVEAPEEKGPAP--MGGMGGMGGMGGMM----
Sp LGVEIVRKAITRPAQTILENAGLEGNLIVGKLKELYGKEFNIGYDIAKDRFVDLNEIGVLDPLKVVRTGLVDASGVASLMGTTECAIVDAPEE-SKAPAGPPGMGGMGGMPGMM----
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Fig. 2. Alignment of the deduced amino acid sequence of the *GiHsp* 60 gene against similar sequences from other fungal species. The values in bracket represent the identity score (%). *Gi*, *Glomus intraradices*; *Um*, *Ustilago maydis* (73); *Yl*, *Yarrowia lipolytica* (73); *Sc*, *Saccharomyces cerevisiae* (69); *Ci*, *Coccidioides immitis* (73); *Af*, *Aspergillus fumigatus* (72); *Nc*, *Neurospora crassa* (73); *Sp*, *Schizosaccharomyces pombe* (66). The asterisks and periods below the sequence alignment indicate residues that are identical or similar between species.

Mycelium expression, *in vitro* expression of *GiHsp* 60 cDNA and dot-blot assay

The *GiHsp* 60 gene was found to be expressed in the extraradically growing *G. intraradices* mycelium that included spores (Fig. S3). No amplicon was found in the PCR reaction that contained RNA as the template. This indicated that the amplicon was from the synthesized cDNA and not from any genomic DNA contamination in the RNA sample.

In vitro expression of the *GiHsp* 60 gene resulted in the synthesis of a protein of ~60 kDa (Fig. S2). As this protein was tagged at the N-terminal end with a 6xHis-tag, it was affinity purified and used for the dot-blot assay to determine its cross-reactivity with the glomalin-specific monoclonal antibody MAb32B11 (Wright & Upadhyaya, 1996). The GiHsp 60 protein was highly cross-reactive with the MAb32B11 antibody (Fig. 5) as seen by the intensity of the color development. The negative control was non-reactive and no detectable immunoreactivity was observed.

Discussion

Considerable indirect evidence has accumulated on the putative characteristics of the glomalin protein based on work with soil-derived material (Table 2). Glomalin has been hypothesized to be a glycoprotein of *c.* > 60 kDa released by the fungal mycelium. The putative functional roles of this protein include aggregating substrate (Wright & Upadhyaya, 1998) and binding iron (Rillig *et al.*, 2001). Given that these studies were carried out using material isolated from soil by autoclaving, it has been difficult to unequivocally isolate and define the properties of this protein (Rillig, 2004b). However, our ability to detect the presence of the glomalin signal in the extracts of *in vitro* root organ culture systems of *G. intraradices* has alleviated this technical problem (Rillig & Steinberg, 2002). Using a similar system, this laboratory has previously shown that the protein is present predominantly in the fungal mycelium (80%) rather than secreted into the culture medium (Driver

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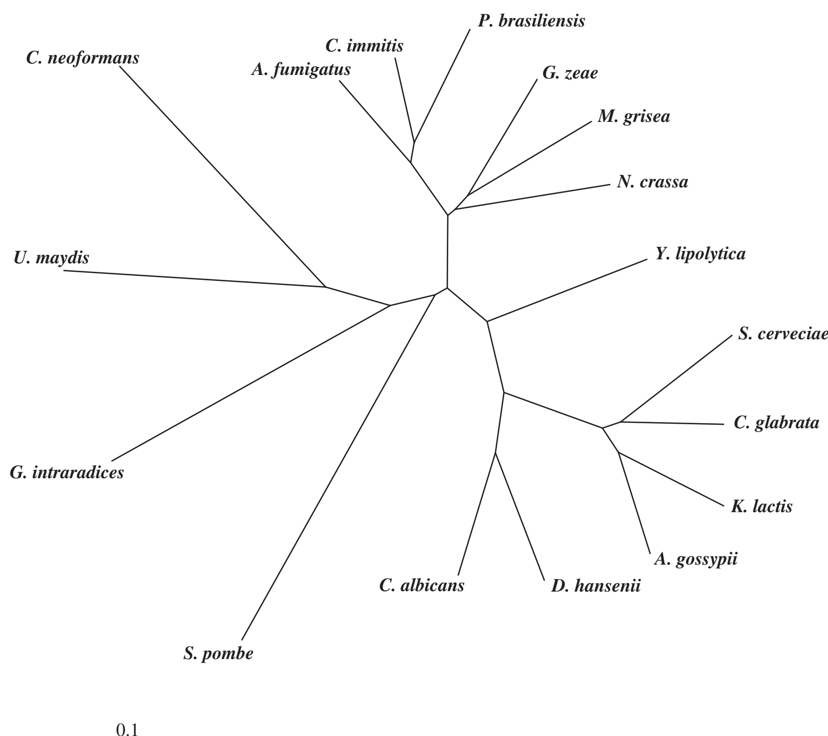

Fig. 3. Unrooted phylogenetic tree showing the relationship between the *GiHsp* 60 gene and other fungi based on the predicted protein sequence. For constructing the tree, the relative support for groups was determined based upon 1000 bootstrap trees. The bar indicates the number of amino acid substitutions per site (0.1). The following are the fungal sequences used to construct the unrooted tree, with the corresponding GenBank accession number (in parentheses): *C. immitis*, *Coccidioides immitis* (AAD00521); *Y. lipolytica*, *Yarrowia lipolytica* (XP_504920); *P. brasiliensis*, *Paracoccidioides brasiliensis* (AAC14712); *A. fumigatus*, *Aspergillus fumigatus* (XP_755263); *U. maydis*, *Ustilago maydis* (XP_761978); *N. crassa*, *Neurospora crassa* (XP_956500); *G. zeae*, *Gibberella zeae* (XP_386422); *M. grisea*, *Magnaporthe grisea* (XP_360622); *S. cerevisiae*, *Saccharomyces cerevisiae* (NP_013360); *D. hansenii*, *Debaryomyces hansenii* (XP_459575); *C. glabrata*, *Candida glabrata* (XP_448482); *C. albicans*, *Candida albicans* (XP_713100); *K. lactis*, *Kluyveromyces lactis* (XP_455510); *C. neoformans*, *Cryptococcus neoformans* (XP_569211), *A. gossypii*, *Ashbya gossypii* (AAS53526), and *S. pombe*, *Schizosaccharomyces pombe* (CAA91499).

MQRVSQFFNKAPHITISPSLSMVSRNSKRPFLSRFYATHKDLKFGVEGRASLLKGVDIL

AKAVAVTLGPKGRNVLIEQPYGSPKITKDGVTVAKSISLKDKFENLGARLVQDVA**NKTN**

EMAGDGTTTATILTRAIFVEGVKNVAAGCNPMDLRRGVQMAVDSIVKFLREKSRVITTS

EEIAQVATISANGDTHVGKLIANAMEKVGKEGVITVKEGKTIEDELEITEGMRFDRGYI

SPYFITEAKTQKVEFEKPLILLSEKKISVLQDILPALETSSTQRRPLLIISEDIDGEAL

AACILNKLRGNIQVAAVKAPGFGDNRKSILGDLAILTGGTVFSDELDIKLERATPDLFG

STGSVTITKEDTILLNGDGSKDFINQRCEQIRAAINDASVSDYEKEKLQERLAKLSGGV

AVIKVGGSSELEVGEKKDRFVDAL**NATR**AAVEEGTVPGGGVALLKSIKCLD**NLSP**ANFD

QKLGIDIVKSALQKPAKTIVDNAGEEGAVIVGKILDNHVDDFNYGYDAAKGEYGDLVSR

GIVDPLKVVRTALVDASGVASLLTTTECMITEAPEENKGAAGGMGRMGGMGGMGDMGMM

Fig. 4. Analysis of the deduced protein sequence of the GiHsp 60 protein. The AAVEEGTVPGGG (position 442–453) sequence is similar to the characteristic A-(AS)-X-(DEQ)-E-(X_4)-G-G-(GA) sequence, present in all Hsp 60 family of proteins. The three predicted N-terminal glycosylation sites and the C terminal located GGM amino acid residues are in bold and underlined, respectively.

et al., 2005). This indicated that glomalin serves a primary function in the living hypha, and that effects arising in the soil are likely secondary consequences. Therefore, using the *in vitro* cultured mycelia from the AMF *G. intraradices*, the *c.* 65 kDa band cross-reacting with the glomalin-specific monoclonal antibody MAb32B11 was isolated and partially sequenced.

GiHsp 60 showed the highest amino acid identity to the *CiHsp* 60 gene from the fungus *Coccidioides immitis* (Thomas *et al.*, 1997). The immunocytological analysis of the CiHsp 60 protein in *C. immitis* showed that it was present exclusively in the fungal cytoplasm and cell walls, even though a mitochondrial targeting signal was detected (Thomas *et al.*, 1997). Interestingly, the GiHsp 60 protein also has a signal sequence for mitochondrial targeting; when immunolocalization using the glomalin-specific antibody MAb32B11 was performed in the AMF *G. intraradices* to map the cellular binding sites, signals in the fungal

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cytoplasm and cell walls were obtained (J.D. Driver & M.C. Rillig, unpublished data). No satisfactory explanation is available for the observation that a protein predicted to be present at a particular cellular location (here mitochondria) is empirically found elsewhere within the cell. This atypical cellular localization has led to classifying Hsp 60 proteins as 'moonlighting' proteins (Maguire *et al*., 2002), a hypothesized class of proteins that apparently do not require a signal peptide to determine their cellular location(s).

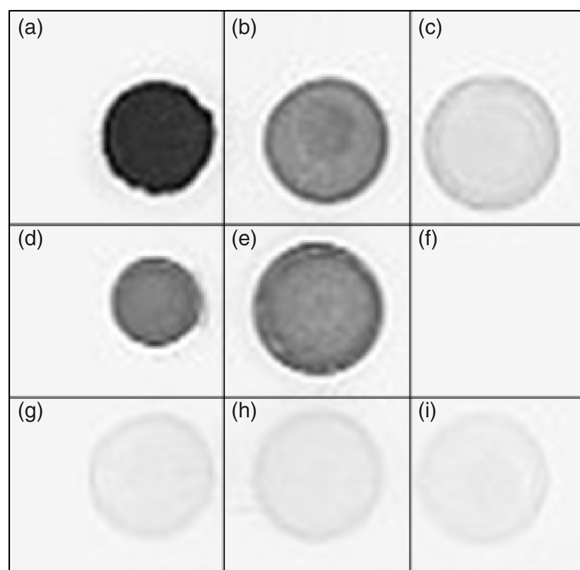

Fig. 5. ELISA-dot blot-analysis of the *in vitro* synthesized GiHsp 60 protein. Deep-colored dots show a positive reaction. GiHsp 60 protein: (a) 2.0 μg, (b) 1.0 μg (c) 0.5 μg. Glomalin standard protein: (d) 0.08 μg, (e) 0.04 μg. Negative controls: (f) water, (g) PBS buffer, (h) elution buffer from the 6x His-tag purification kit (i) EasyXpress™ translation lysate.

Sequence identity to *Hsp* 60 gene, and likely homology (based on our initial phylogenetic analysis), does not necessarily imply an Hsp-like functionality; for example, Yoshida *et al*. (2001) observed that the Hsp 60 protein isolated from the endosymbiotic bacterium *Enterobacter aerogenes* functioned as a nerve toxin, all attributed to a single residue substitution. The results from the present study make possible further examination of the functionality and properties of glomalin. For example, future studies should compare glomalin genes from a variety of different representatives of the Glomeromycota phylum containing the AMF. Previous studies have shown that the MAb32B11 antibody cross-reacts with numerous members of the AMF (Wright *et al*., 1996; M.C. Rillig, unpublished). Also, larger scale production of the GiHsp 60 protein in a heterologous expression system, currently being attempted in this laboratory, could yield protein material suitable for testing specific hypotheses relating to the function of glomalin in the life of AMF. More detailed knowledge of GiHsp 60 protein production rates and the expression changes in response to environmental factors (stress) could inform the development of management approaches aimed at maximizing its production in soils, which could maximize soil aggregation (Wright & Upadhyaya, 1998; Rillig, 2004b).

The presence of three N-terminal glycosylation sites within the GiHsp 60 protein sequence suggests that the mature protein is glycosylated. Earlier observations on

Table 2. Comparison of previous hypotheses and observations with the results obtained through present study

Hypotheses and observations based on previous studies (mostly conducted in soil)	Support from present results
Involvement in soil aggregation/surface interactions (attachment); may be a hydrophobin-like protein (Wright & Upadhyaya, 1998)	No direct support from this study, but Hsp play an important role in cellular adhesion (Hennequin *et al*., 2001; Maguire *et al*., 2002)
Proteinaceous substance about > 60 kDa in size (Wright & Upadhyaya, 1996)	Predicted size of protein: 63.1 kDa
Presence of glycosylation (capillary electrophoresis); lectin-binding (Wright *et al*., 1996, 1998)	Three N-glycosylation sites predicted from the sequence
Iron-containing/binding (Rillig *et al*., 2001)	No direct support from this study for a Fe binding domain. However, Hsp 60 proteins are known to indirectly modulate free Fe pools to prevent cell damage (Cabiscol *et al*., 2002)
Production increased with limited hyphal growth (Rillig & Steinberg, 2002; Lovelock *et al*., 2004)	Stress-protein homology
Conserved/ cross-reactive among glomeromycotan fungi (Wright *et al*., 1996)	Hsp 60 genes are highly conserved and are used for molecular taxonomy of microorganisms(Hill *et al*., 2005; Zhu & Dong, 2001)
Tightly mycelium or hyphal wall-bound (Driver *et al*., 2005)	Mitochondrial target sequence, but 'moonlighting' commonly observed resulting in cell wall localization of mature protein
Heat stable (extraction by autoclaving) (Wright & Upadhyaya, 1996)	Can survive boiling (Lewthwaite *et al*., 2001)

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GRSP, including lectin-binding and capillary electrophoresis, arrived at the conclusion that N-terminal glycosylation may be present (Wright *et al*., 1996); this is corroborated by our findings here. Other researchers have also found that the mature Hsp 60 protein can be both glycolsylated and present in the cell wall (Gomez *et al*., 1991, 1995; Thomas *et al*., 1997). It is currently not known whether glycosylation is essential to the functionality of glomalin. It was previously hypothesized that the MAb32B11-reaction is at least partially dependent on the presence of glycosylation; however, here we showed that the *in vitro* expressed protein was immunoreactive in the absence of glycosylation.

The *GiHsp* 60 gene was found to be expressed in the extraradical mycelium of *G. intraradices* (Fig. S3), strongly indicating the extraradical mycelium as the principal site for the glomalin gene expression. As the RNA extraction was performed on the whole extraradical mycelia that included spores, it is difficult to say at this juncture whether the *GiHsp* 60 expression is confined to the spores, mycelia or any specific part of the *G. intraradices* fungal structure(s).

There is already evidence, corroborated by our present finding of glomalin having high amino acid sequence identity to stress-related proteins, that glomalin production increases sharply under conditions of limited growth of the mycelium, i.e. stressed conditions (Rillig & Steinberg, 2002; Lovelock *et al*., 2004). In fact, Lovelock and co-workers showed a negative curvilinear relationship between glomalin-related soil protein concentration in the growth substrate and fungal mycelium growth. Keeping this observation in mind, we are at present studying the expression of *GiHsp 60* gene under various stress conditions, with particular emphasis on nutritional stress and other factors that may govern its expression.

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Supplementary material

The following supplementary material is available for this article online:

Fig. S1. Relative location of the PCR primers used for screening the *G. intraradices* λTriplEx2 cDNA library. Open box indicates the open reading frame flanked by the lambda vector.

Fig. S2. SDS-PAGE analysis of the *in vitro* synthesized 6xHis-tagged GiHsp 60 protein. Lane 1: translation lysate only; Lane 2: Translation lysate with template; Lane 3: Translation lysate first column wash; Lane 4: column wash 2; Lane 5: column wash 3; Lane 6: column wash 5; Lane 6: elution wash.

Fig. S3. PCR amplification of the *GiHsp* 60 gene (870 bp fragment) from cDNA prepared using *G. intraradices* extraradical mycelium. Replicate dilution of the cDNA preparation was done prior to performing the PCR analysis. Lane 1 & 2: 1/10 dilution; Lane 3 & 4: 1/20 dilution and Lane 5 & 6: 1/5 dilution. Lane 7: minus reverse transcriptase (RT) sample. M: 100 bp DNA molecular weight marker (Bioline, Boston, MA)

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