Microbial Ecology

# Identification of a Gene from the Arbuscular Mycorrhizal Fungus *Glomus intraradices* Encoding for a 14-3-3 Protein that Is Up-Regulated by Drought Stress During the AM Symbiosis

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### Abstract

In the present study, a 14-3-3 protein-encoding gene from *Glomus intraradices* has been identified after differential hybridization of a cDNA library constructed from the fungus growing *in vitro* and subjected to drought stress by addition of 25% PEG 6000. Subsequently, we have studied its expression pattern under drought stress *in vitro* and also when forming natural symbioses with different host plants. The results obtained suggest that *Gi14-3-3* gene may be involved in the protection that the arbuscular mycorrhizal (AM) symbiosis confers to the host plant against drought stress. Our findings provide new evidences that the contribution of AM fungi to the enhanced drought tolerance of the host plant can be mediated by a group of proteins (the 14-3-3) that regulate both signaling pathways and also effector proteins involved in the final plant responses.

### Introduction

Drought stress is considered one of the most important abiotic factors limiting plant growth and yield [26]. However, it has been shown that the arbuscular mycorrhizal (AM) symbiosis can increase the plant survival and production under drought stress conditions [1, 44]. Investigations on that topic have demonstrated that the contribution of the AM symbiosis to plant drought tolerance results from a combination of physical, nutritional, physiological, and cellular effects [44]. This appears to be due, in many instances, to differences in tissue hydration between AM and non-AM plants: one treatment group manages to either absorb more water or lose less water as the soil dries [4]. However, this seems not to be the only mechanism by which AM symbiosis enhances drought tolerance of plants. Additional mechanisms have been proposed such as direct uptake and transfer of water through the fungal hyphae to the host plant [21, 30, 45], better osmotic adjustment of AM plants [2, 27, 46], enhancement of plant gas exchange [2, 3, 12, 18, 20, 46], changes in soil water retention properties [4, 5], and protection against the oxidative damage generated by drought [37, 39, 47, 48, 49].

Living organisms can respond to drought stress at morphological, anatomical, and cellular levels, with modifications that allow these organisms to avoid the stress or to increase its tolerance [7]. 14-3-3 proteins are ubiquitous eukaryotic proteins that have wide-ranging regulatory functions by acting as phosphoserine/phosphothreonine-binging proteins [42]. It is clear that these proteins function in the regulation of signal transduction pathways, generally functioning as adapters, chaperones, activators, or repressors [34], and that they regulate the activities of a wide array of targets via direct protein–protein interactions. Binding of 14-3-3 proteins to a target serves either to directly regulate the activity of that protein, to affect its interactions with other protein, or to modify the intracellular localization of the target [41]. Targets for 14-3-3 include proteins involved in metabolism, signal transduction, chromatin function, ion transport, and vesicle trafficking [41].

14-3-3 protein family plays a central role in stress resistance, disease, and growth control during the cell life cycle [10]. Plant 14-3-3 proteins bind a range of transcription factors and other signaling proteins and have roles regulating plant development and stress responses. In the case of stress responses, support for such roles comes from the observation of changes in 14-3-3 gene expression during stress responses and from the detec-

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tion of interactions between 14-3-3's and proteins with signaling or protective functions [32, 43, 58].

Many studies on 14-3-3 proteins have been carried out in plants, animals, and yeast. In contrast, there is no information about 14-3-3 proteins in AM fungi or in the AM symbiosis. Although in recent years there has been an increase in the understanding of the physiological processes involved in the enhanced tolerance of mycorrhizal plants to water limitation, the exact mechanisms involved are still a matter of debate [44]. In the present study, a 14-3-3 protein-encoding gene from *Glomus intraradices* has been identified following a nontargeted approach (differential hybridization of a cDNA library constructed from the fungus growing *in vitro* and subjected to drought stress by addition of 25% PEG 6000). Subsequently, we have studied its expression pattern under drought stress *in vitro* and also when forming natural symbioses with different host plants. The identification of this 14-3-3 protein-encoding gene in *G. intraradices* provides new insights into the complex mechanisms by which AM fungi can protect the host plants against water deficit.

## Materials and Methods

*In Vitro Mycorrhizal Cultures.* *G. intraradices* was established in monoxenic culture as described by St-Arnaud *et al.* [55]. Briefly, clone DC2 of carrot *(Daucus carota* L.) Ri-T DNA transformed roots were cultured with the AM fungus *G. intraradices* Smith and Schenck (DAOM 197198, Biosystematic Research Center, Ottawa, Canada) in two-compartment petri dishes. Cultures were initiated in one compartment ("root compartment") of each plate, which contained minimal medium. Fungal hyphae, but not roots, were allowed to grow over to the second compartment ("hyphal compartment"), which contained liquid minimal medium without sucrose (M-C medium). The plates were incubated in the dark at 24°C for 3 months. The hyphal compartment medium was then carefully removed with a Pasteur pipette and added with new liquid minimal medium supplemented with 25% PEG 6000 (Sigma-Aldrich Co., Madrid, Spain) to subject the extraradical hyphae to water stress. The water potential of the minimal medium after PEG addition was −1.5 MPa, as measured with a C-52 thermocouple psychrometer chamber coupled to an HR-33T dew point microvoltmeter (Wescor Inc., Logan, UT, USA). The extraradical mycelium was allowed to grow under such conditions for 5 days and then harvested and stored in liquid nitrogen for subsequent RNA extraction, library construction, and reverse transcriptase–polymerase chain reaction (RT-PCR) experiments.

*Soil and Biological Material.* Properties of soil and plants used in this study were as previously described [36, 38, 39]. Loamy soil was collected from the Zaidin Experimental Station (Granada, Spain), sieved (2 mm), diluted with quartz sand (<1 mm; 1:1, soil/sand, v/v), and sterilized by steaming (100°C for 1 h on three consecutive days). The soil had a pH of 8.1 (water), 1.81% organic matter, and with the following nutrient concentrations (mg $kg^{-1}$): N, 2.5; P, 6.2 ($NaHCO_3$-extractable P); K, 132.0. The soil texture was made up of 35.8% sand, 43.6% silt, and 20.5% clay.

The plants used were soybean (*Glycine max* L. cv. Williams), lettuce (*Lactuca sativa* L. cv. Romana), maize (*Zea mays* L. cv. Prisma), and tobacco (*Nicotiana tabacum* L. cv. Samsun). Two plant lines (an aquaporin antisense mutant and the corresponding wild type) from *Nicotiana tabaccum* were used [38, 54]. All plants were inoculated with the AM fungus *G. intraradices* (Schenck and Smith) isolate BEG 121 (Gi). In all cases, half of the plants was cultivated under well-watered conditions throughout the entire experiment (ww), whereas the other half was drought-stressed for 10 days before harvest (ds) as previously described [36, 38, 39].

*Growth Conditions.* Plants were grown in a controlled environmental chamber with 70–80% RH, day/night temperatures of 25/15°C, and a photoperiod of 16 h at a photosynthetic photon flux density of 460 μmol $m^{-2}$ $s^{-1}$ (Licor, Lincoln, NE, USA, model LI-188B).

Soil moisture was measured with a ML2 ThetaProbe (AT Delta-T Devices Ltd., Cambridge, UK), which measures volumetric soil moisture content by responding to changes in the apparent dielectric constant of moist soil. Water was supplied daily to maintain constant soil water content close to field capacity (17% volumetric soil moisture, as determined experimentally) during the first 4 weeks of plant growth. At that time, half of the plants was allowed to dry until soil water content reached 70% field capacity (2 days needed), which corresponded to 10% volumetric soil moisture (also determined experimentally in a previous assay), whereas the other half was maintained at field capacity. Plants were maintained under such conditions for additional 8 days. To control the level of drought stress, the soil water content was daily measured with the ThetaProbe ML2 (at the end of the afternoon), and the amount of water lost was added to each pot to return soil water content at the desired 10% of volumetric soil moisture (70% of field capacity). However, during the 24-h period comprised between each rewatering, the soil water content was progressively decreasing until a minimum value of 60% of field capacity.

*Symbiotic Development.* The extent of mycorrhizal root colonization was calculated by the gridline intersect method [17] after root staining according to Phillips and Hayman [35].

*RNA Isolation, Construction of the cDNA Library, and Screening.* Total RNA was extracted from *G. intraradices* mycelia grown in monoxenic culture and subjected to drought stress by addition of 25% PEG 6000. The RNA was extracted using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). cDNA was synthesized from 2.5 μg of total RNA using the Smart PCR cDNA Synthesis Kit (Clontech, Palo Alto, CA) and cloned into the bacteriophage lambda ExCell vector (Amersham, Little Chalfont, UK) [50]. The library was screened by differential hybridization of identical plaque lifts. Approximately $10^4$ $\lambda$ bacteriophage particles were plated at low density (1500–2000 pfu per plate) on the host *Escherichia coli* (strain NM522). After incubation for 7–8 h at 37°C, the colonies were produced and the petri plates placed at 4°C for 1 h to harden the top agarose. They were then overlaid with a Hybond-N + nylon membrane (Amersham) and left for 2 min at room temperature or for 4 min for duplicate filters. DNA was denatured and fixed by autoclaving the filter for 5 min at 120°C. Each of the duplicate filters was then hybridized at 65°C with a total cDNA radioactive probe from mycorrhizal roots or from uninoculated control roots, under standard conditions [51]. After washing twice for 5 min at room temperature in 2× standard saline citrate (SSC) and 0.1% sodium dodecyl sulfate (SDS), and once for 15 min at 65°C in 0.5× SSC and 0.1% SDS, membranes were exposed overnight to Kodak X-RAY-OMAT at −70°C.

Differentially detected phage clones were used as template for a PCR-based screening with universal M13 forward and reverse primers to verify the purity of the clones as well as the size of the inserts. The PCR products obtained were divided into two equal fractions, separated in agarose gels, and transferred onto nylon membranes [29]. Southern blot analysis was used to confirm differentially expressed clones by probing the membranes with 32 P-labeled cDNAs from *G. intraradices* mycelia grown *in vitro* and added or not added with 25% PEG 6000.

*Sequencing the Cloned cDNA and Analyses.* Sequencing was performed by the dideoxy-sequencing method [52] using fluorescent dye-linked universal M13 primers and a PerkinElmer ABI Prism model 373 DNA sequencer. Similarity searches were carried out in the European Molecular Biology Laboratory (EMBL) databank, using the BLAST software or the FASTA program available on-line from the National Center for Biotechnology Information (NCBI).

Sequence alignments were performed using the program package ClustalW, also available through the NCBI. After sequence alignments, a phylogenetic tree was constructed using the Neighbor-joining method from the program package Phylip.

*Northern Blot Analysis.* Total RNA (15 μg) extracted from roots of the different plant treatments was separated by electrophoresis on 1.2% agarose gel containing 2.2 M formaldehyde and blotted onto Hybond-$N^+$ nylon membranes (Amersham) by capillarity [51]. Equal RNA loading and transfer were verified by methylene blue staining of nylon membranes before hybridization [22]. Blots were prehybridized 2–3 h at 65°C in 5× Denhardt's solution, 5× SSC, 0.5% SDS, and hybridized with *Gi14-3-3* specific probe obtained by radioactive PCR labeling of plasmid insert. Unincorporated $^{32}P$ was removed using Mini Quick Spin TM columns (Boehringer Manheim, Indianapolis, IN). A total of $10^7$ cpm probe was heat-denatured and used to hybridize the blots overnight at 65°C under standard conditions [51]. After washing twice for 5 min at room temperature in 2× SSC and 0.1% SDS, and twice for 15 min at 65°C with 0.5× SSC and 0.1% SDS, membranes were exposed overnight to Kodak X-RAY-OMAT at −70°C. Signals on autoradiograms were analyzed and quantified using Quantity One software (Bio-Rad, Hemel Hempstead, UK). Transcript accumulation levels were normalized according to the amount of rRNA in the corresponding membrane, which had been also quantified with Quantity One software. Each quantification of signals on autoradiograms and of rRNA was repeated three times, and the average value was used for normalization. Northern blot analyses were repeated twice with different set of plants.

*Quantitative Real-Time RT-PCR.* *Gi14-3-3* gene expression was studied by real-time PCR by using iCycler (Bio-Rad, Hercules, CA, USA), as described by Gonzalez-Guerrero *et al.* [19]. cDNAs were obtained from 2.5 μg of total DNase-treated RNA in a 20-μL reaction containing 500 ng random hexamer primer, 0.5 mM each dNTP, 10 mM DTT, 40 U of RNase inhibitor, 1× first strand buffer (Invitrogen, Carlsbad, CA, USA), and 200 U of Superscript II Reverse Transcriptase (Invitrogen).

The primer set used to amplify *Gi14-3-3* and *Gi18S rRNA* genes in the synthesized cDNAs were the following (5′ to 3′): Gi14-3-3Forw cgcaatctcctctcagtcgc; Gi14-3-3Rev gcaatagcatcatcaaatgc; Gi18SForw tgttaataaaaatcgg tgcgttgc; and Gi18SRev aaaacgcaaatgatcaaccggac. Each 25-μL reaction contained 1 μL of a dilution 1:10 of the cDNA, 200 nM dNTPs, 400 nM each primer, 3 mM $MgCl_2$, 2.5 μL of 1× SyBR Green (Molecular Probes, Eugene, Oregon, USA), and 0.5-U Platinum *Taq* DNA polymerase (Invitrogen) in 1× PCR buffer (20 mM Tris–HCl, pH 8.4, 50 mM KCl).

The PCR program consisted of a 4-min incubation at 95°C to activate the hot-start recombinant *Taq* DNA polymerase, followed by 30 cycles of 45 s at 95°C, 45 s at 60°C, and 45 s at 72°C, where the fluorescence signal was measured. The specificity of the PCR amplification

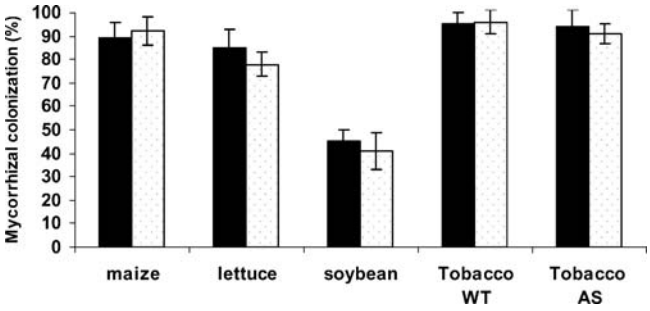


**Figure 1.** Percentage of mycorrhizal root infection by *G. intraradices* in the different plants used in this study. Plants were either well-watered (*dark columns*) or drought stressed for 10 days (*white columns*). Data are means of four replicates, and *lines* over the columns represent the standard error.

procedure was checked with a heat dissociation protocol (from 70 to 100°C) after the final cycle of the PCR. The efficiency of the primer set was evaluated by performing real-time PCR on several dilutions of plasmid DNA. The results obtained on the different treatments were standardized to the 18S rRNA levels, which were amplified with the primers Gi18S previously shown.

Real-time PCR experiments were carried out at least five times, with the threshold cycle ($C_T$) determined in triplicate. The relative levels of transcription were calculated by using the $2^{-\Delta\Delta Ct}$ method [28]. Negative controls without cDNA were used in all PCR reactions.

*Nucleotide Sequence Accession Number.* The nucleotide sequence corresponding to *Gi14-3-3* gene has been deposited in the EMBL database under accession number AM049264.

## Results

*Symbiotic Development.* The percentage of mycorrhizal root colonization varied with the host plant considered (Fig. 1), ranging from 41% for soybean plants to 96% for tobacco plants. The exposition of plants to drought stress for 10 days did not affect the mycorrhizal root infection of any of the host plants assayed.

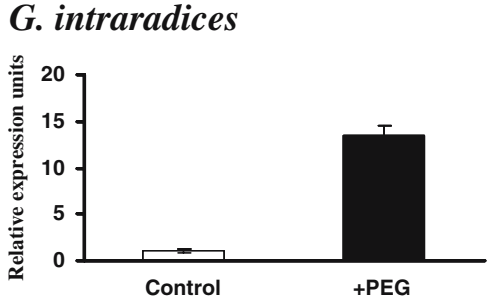


**Figure 2.** Analysis of *Gi14-3-3* gene expression by real-time quantitative RT-PCR in the AM fungus *G. intraradices* grown *in vitro*. The fungus was subjected to drought by addition of PEG (25%) to the growing medium or maintained under control conditions without PEG.

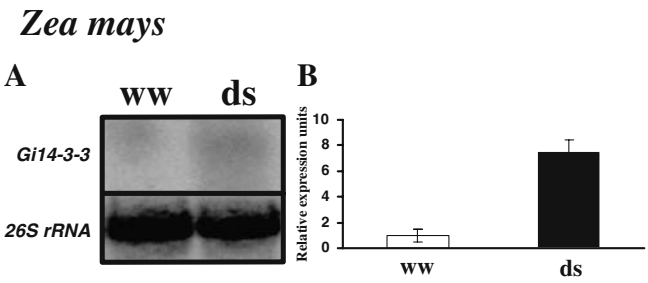


**Figure 3.** Analysis of *Gi14-3-3* gene expression in maize plants colonized by the AM fungus *G. intraradices*. Plants were either well-watered (ww) or drought stressed (ds). (A) Northern blot of total RNA (15 μg). The *lower panels* show the amount of 26S rRNA loaded for each treatment (methylene blue staining). (B) Real-time quantitative RT-PCR analysis of *Gi14-3-3* gene expression.

*Isolation of Gi14-3-3 Gene.* The differential screening of the library allowed detection of a cDNA clone hybridizing with the cDNA probe obtained from the fungus growing in monoxenic culture in the presence of 25% PEG, but not with the probe from the fungus growing in minimal medium without PEG. The clone was sequenced, and the sequence obtained had 884 bp, including the 3′ nontranslated region and the polyA. The GC content of the coding sequence was 41%. The putative protein encoded by such sequence had 260 amino acids and exhibited 80% of identity with the 14-3-3 protein from the fungus *Lentinula edodes* (accession Q9UR29, $E = e^{-105}$), published by Zhou *et al.* [59].

*Expression of Gi14-3-3 Gene in Vitro and ex Vitro During Symbiosis with Different Plants.* The expression of *Gi14-3-3* gene was studied by Northern blot when enough RNA was available and/or by quantitative real-time PCR when the amount of RNA limited Northern blot analysis.

We first analyzed the expression of *Gi14-3-3* gene when the fungus was growing *in vitro* and subjected to an

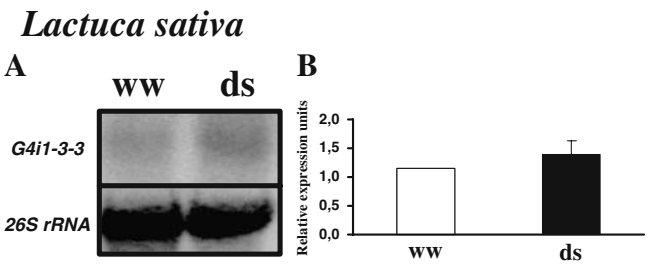


**Figure 4.** Analysis of *Gi14-3-3* gene expression in lettuce plants colonized by the AM fungus *G. intraradices*. See legend for Fig. 3.

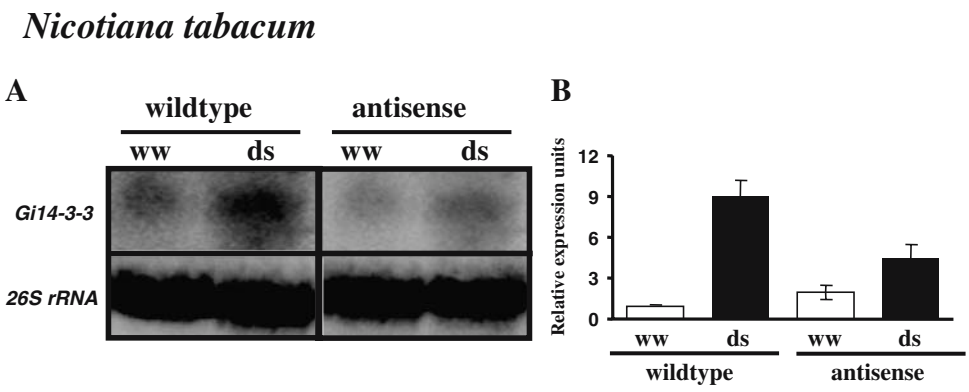


**Figure 5.** Analysis of *Gi14-3-3* gene expression in two tobacco plant lines (wild type or an aquaporin antisense mutant) colonized by the AM fungus *G. intraradices*. See legend for Fig. 3.

osmotic stress by adding 25% PEG to the growing medium (Fig. 2). The addition of PEG to the medium increased *Gi14-3-3* gene expression by 1200%.

We then analyzed the expression of that fungal gene during natural symbiosis with different plants cultivated under well-watered or under drought stress conditions. The results obtained differed according to the host plant assayed. The expression of *Gi14-3-3* gene increased by over 640% as a consequence of drought when *G. intraradices* colonized maize plants (Fig. 3). A lower increase (38%) in gene expression was observed in lettuce plants subjected to drought stress (Fig. 4), whereas in tobacco (Fig. 5), the increase of *Gi14-3-3* gene expression was by 823 and 127% for wild-type and antisense plants, respectively. When the host plant was the soybean, *Gi14-3-3* gene expression could not be detected by Northern blot, and only real-time quantitative PCR allowed quantification of gene expression that, curiously, was not significantly affected by drought stress (Fig. 6).

*Phylogenetic Tree for Gi14-3-3 Nucleotide Sequence.* We constructed a phylogram showing the relationship between *Gi14-3-3* nucleotide sequence and that of other fungi (Fig. 7). As can be seen, the closest relative to *G. intraradices* was *Pneumocystis carinii*, but the bootstrap values are low, indicating that their degree of relationship is also low. Other fungi that showed a certain degree of relationship with *G. intraradices* were *Schizosaccharomyces pombe*, *Debaryomyces hansenii*, or *Candida albicans*. Curiously, *Oidiodendron maius* (an ericoid mycorrhizal fungus) or *Paxillus involutus* (an ectomycorrhizal fungus) are located far from *G. intraradices* in the phylogram.

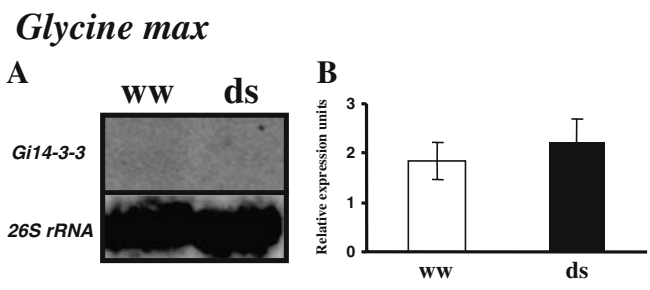


**Figure 6.** Analysis of *Gi14-3-3* gene expression in soybean plants colonized by the AM fungus *G. intraradices*. See legend for Fig. 3.

## Discussion

Abiotic stresses, such as drought, salinity, or extreme temperatures, share a common osmotic component because of the generation of a water deficit for plants and are serious threats to agriculture, resulting in the deterioration of the environment. Osmotic stress is the primary cause of crop loss worldwide, reducing average yields for most major crop plants by more than 50% [57]. Osmotic stress evokes multiple responses that involve a series of physiological, biochemical, and molecular events to prevent cellular damage and to reestablish cellular homeostasis [57].

Among the diverse roles of 14-3-3 proteins, a common theme emerges of adaptation to a changing environment. 14-3-3 proteins are found in association with key control enzymes of primary metabolism, regulation of which could rapidly alter metabolic flux in response to signals such as water, osmotic, or salt stress [15]. They could also regulate the expression of stress-inducible genes by regulating the activity and or localization of transcription factors [32]. The up-regulation of *Gi14-3-3* gene under conditions of water deficit (induced *in vitro* by PEG addition or *in vivo* by withholding plant irrigation) indicates that this fungal gene has a role in the answer of the fungus against drought. This gene is probably involved in the protection of the fungus itself (induction of the gene *in vitro*) and may be also involved in the protection of the host plant (induction of gene expression when forming natural symbiosis with maize, lettuce, and tobacco plants). In this study, PEG was used as osmoticum to induce osmotic stress-responsive genes in *G. intraradices* and subsequent construction of a cDNA library aimed at identifying such induced genes.

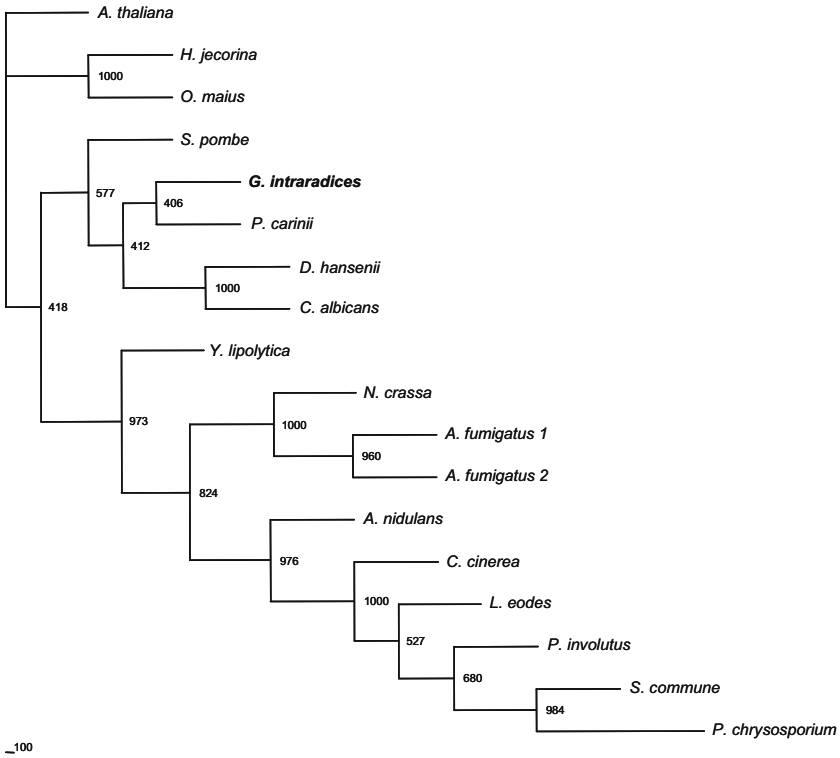


**Figure 7.** Phylogenetic tree constructed after alignment of *Gi14-3-3* sequence from *G. intraradices* and other fungi. The tree was constructed using the neighbor-joining method from the program package Phylip. *Scale* represents the estimated number of nucleotide substitutions per sequence position. Accession numbers of sequences used to construct the tree are as follows: *Arabidopsis thaliana* NM202155, which was set up as the outgroup in the tree; *Aspergillus fumigatus* 1 NW876235; *A. fumigatus* 2 XM744371; *Aspergillus nidulans* XM658256; *Candida albicans* AF038154; *Coprinopsis cinerea* DR775106; *Debaryomyces hansenii* NC006049; *Glomus intraradices* AM049264; *Hypocrea jecorina* CF932611; *Lentinula eodes* AB029308; *Neurospora crassa* XM330735; *Oidiodendron maius* CN200257; *Paxillus involutus* AY857493; *Phanerochaete chrysosporium* AY971673; *Pneumocystis carinii* AF461162; *Schizophyllum commune* AY029473; *Schizosaccharomyces pombe* Z99292.1; *Yarrowia lipolytica* XM501483.

The use of PEG as osmoticum in nutrient solutions implies that the PEG is of high molecular weight, to avoid its uptake, that the time of exposure do not exceed 6 days, and that its degree of purity is high to avoid contaminants [23, 40, 56]. All these precautions have been taken into account in this study.

It has been demonstrated that one of the effects of 14-3-3 proteins against osmotic stresses is carried out through the activation of the plasma membrane proton ATPase [6, 9, 24]. The activity of plasma membrane $H^+$-ATPase is highly regulated by factors that affect the cell physiology, including stress conditions [33], and enhanced ATPase activity is crucial for the protective system that different organisms have developed against external adverse influences [53]. In the case of plants, the effects of 14-3-3 proteins are important for tolerance to water limitation because it has been shown that the plasma membrane H-ATPase plays an essential role in the regulation of plant cell turgor. In fact, it exports protons to create an electrochemical gradient across the plasma membrane, which is then used by cell as the driving force for nutrient uptake, phloem loading, water movement, stomatal closure, and opening [11]. There is clear evidence for 14-3-3-mediated activation of the $H^+$-ATPase in response to osmotic stresses. It has been demonstrated that osmotic stress induces a redistribution of 14-3-3 proteins between the cytoplasm and the plasma membrane of sugar beet cells. This effect is accompanied by an increase in H-pump activity [6, 9, 24]. Increased $H^+$ transport through phosphorylation and 14-3-3 binding to the proton ATPase are part of the early responses of cells to perturbation in growth conditions such as osmotic stress [14, 24].

It is noteworthy that the expression of $H^+$-ATPases is also induced in barley, transgenic tobacco, and tomato roots infected with AM fungi [13, 16, 31]. However, the possible participation of 14-3-3 proteins in the corresponding pumping activity of AM symbiosis-induced ATPases has never been investigated.

In addition to $H^+$-ATPases, other factors with potential roles in abiotic stress responses have also been identified as targets of 14-3-3 proteins. A calcium-

dependent protein kinase (CDPK) isoform from *Arabidopsis* has been shown to be activated by 14-3-3 proteins [8]. CDPKs constitute a unique family of plant kinases that are defined by a C-terminal calmodulin-like regulatory domain and have been shown to be involved in drought and salinity responses [15, 25]. Although physiological processes regulated by CDPKs have remained largely elusive, a number of proteins phosphorylated by these kinases have been identified [8].

Curiously, a no significant effect of drought stress on *Gi14-3-3* gene expression has been observed in this study when the fungus is associated to soybean plants. This result may be related to the fact that the AM colonization in these plants was considerably lower than in the rest of treatments. In any case, the induction of *Gi14-3-3* gene was more or less intense depending on the host plant. This is quite evident with the two tobacco plant lines that exhibited a higher induction of *Gi14-3-3* gene expression in wild-type plants and a lower induction in the plant line that is more sensitive to drought (antisense). Antisense tobacco plants show an 80% inhibition of a plasma membrane intrinsic aquaporin [54]. In previous studies, we and other authors have demonstrated the high sensitivity to drought stress of antisense tobacco plants [38, 54]. The varying results obtained with the different plants here assayed suggest that the importance of *Gi14-3-3* when coping with drought stress may depend on the intrinsic physiological characteristics of the host plant. Hence, it may be speculated that 14-3-3 proteins take part of the mechanisms by which the AM symbiosis enhances the tolerance of the host plant against drought, but that the contribution and predominance of other mechanisms, as those already reported in previous revisions on the subject [1, 44], when alleviating drought stress, may depend on the physiological characteristic of the host plant.

In conclusion, the results obtained here suggest that Gi14-3-3 protein is involved in the protection that the AM symbiosis confers to the host plant against drought stress, and that the real implication of these proteins depends on the physiological characteristics of the host plant. In any case, as 14-3-3 proteins regulate the activity of many proteins involved in signal transduction, there are multiple levels at which 14-3-3 proteins may play roles in stress responses [43], and the precise mechanism of Gi14-3-3-mediated drought stress tolerance remains unknown. It is likely, however, that Gi14-3-3 protein can regulate the activity of plasma membrane H+-ATPases of either the fungus or the host plant, to activate its pumping activity, which is essential to cope with osmotic stress [11, 33, 53]. Additionally, Gi14-3-3 protein can activate a fungal or plant CDPK to modulate the activity of final effector proteins involved in the response to the osmotic stress [8]. Our findings provide new evidences that the contribution of AM fungi to the enhanced drought tolerance of the host plant can be mediated by a group of proteins (the 14-3-3) that regulate both signaling pathways and also proteins involved in the final responses.

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