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A gene from the arbuscular mycorrhizal fungus *Glomus intraradices* encoding a binding protein is up-regulated by drought stress in some mycorrhizal plants

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Abstract

The contribution of the arbuscular mycorrhizal (AM) symbiosis to plant drought tolerance results from a combination of physical, nutritional and cellular effects. However, the exact mechanisms involved in such enhanced tolerance are still a matter of debate. In this study a BiP-encoding gene from the AM fungus *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. Results show that its expression was up-regulated by drought stress not only during *in vitro* conditions (AM monoxenic cultures) but also *ex vitro*, when forming natural symbioses with plants. The identification of *GiBiP* gene provides new evidences that the contribution of AM fungi to the enhanced drought tolerance of the host plant can be mediated by proteins with chaperone-like activity, such as that of BiP.

Keywords: Arbuscular mycorrhizal symbiosis; BiP; Drought stress

1. Introduction

Water deficit is one of the most common environmental stress factors experienced by terrestrial plants. It interferes both with normal plant development and growth, and has major adverse effects on plant survival and productivity (Kramer and Boyer, 1997).

The arbuscular mycorrhizal (AM) symbiosis can increase the plant survival and production under water deficit conditions (for reviews see Augé, 2001; Ruiz-Lozano, 2003). It is currently accepted that the contribution of the AM symbiosis to plant drought tolerance results from a combination of physical, nutritional and cellular effects, although the exact mechanisms involved are still a matter of debate (Ruiz-Lozano, 2003).

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 (Bray, 1997). Among a diversity of responses, living organisms can adapt to water deficit by the induction of specific genes (Zhu et al., 1997). One of these genes encodes for an important component of endoplasmic reticulum (ER): the luminal binding protein (BiP). The protein BiP is a molecular chaperone present in all kingdoms. The role of BiP in the ER is to transiently bind to unfolded proteins and to prevent intramolecular and intermolecular interactions that can result in permanent misfolding or aggregation, with the subsequent lost of their function (Gething and Sambrook, 1992; Hendershot et al., 1996). Thus, both the increase of secretory activity and accumulation of unfolded proteins within the ER, as usually happens under abiotic stresses, result in the induction of BiP (Boston et al., 1996; Galili et al., 1998).

Some studies have demonstrated that overexpression of BiP genes in cultured mammalian cells and tobacco leaf protoplast attenuates ER stress caused by ionophore or tunicamycin (Laitusis et al., 1999; Leborgne-Castel et al., 1999). It is also well known that overexpression of BiP in mammalian cultured cells (Laitusis et al., 1999) prevents the induction of unfolded protein response (UPR)-induced genes and increases cell tolerance to stress, suggesting that BiP directly alleviates the ER

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stress. Plant BiP expression has been shown to respond to a variety of abiotic and biotic stress conditions, such as water stress, fungus infestation, insect attack, nutritional stress, cold acclimation and elicitors of the plant–pathogenesis response (Anderson et al., 1994; Denecke and Vitale, 1995; Kalinski et al., 1995; Figueiredo et al., 1997; Fontes et al., 1999). Furthermore, it has been demonstrated that constitutive overexpression of BiP in tobacco is enough to confer tolerance to water stress (Alvim et al., 2001).

Many studies on BiP have been carried out in plants and animals, while little information is available on fungi. The little information available on fungal BiP focussed on yeasts. In *Saccharomyces cereviseae*, BiP is encoded by the *Kar2* gene (Normington et al., 1989). Expression of the *Kar2* gene is induced by stress, heat shock and tunicamycin treatment (Normington et al., 1989; Gething, 1992). It has been shown that the *S. cerevisiase* KAR2 (BiP) protein is essential for protein folding in the ER lumen, for translocation of newly synthesized secretory precursors across the ER membrane and for the transport back across the membrane of aberrant polypeptides destined for degradation by the proteasome. KAR2 is also required for the stage of karyogamy that involves fusion of the nuclear membranes, the outer of which is contiguous with the ER membrane (Gething, 1992). Based on these findings it has been proposed that, the function of BiP in fungi must be similar to that of animals or plants (Kasuya et al., 1999).

In contrast to yeast, there is no information about BiP expression in AM fungi or during the AM symbiosis. It is true that in the last years there has been an increase in the understanding of the physiological processes involved in the enhanced tolerance of mycorrhizal plants to water limitation. However, there are still many unknown aspects which must be elucidated (Ruiz-Lozano, 2003). In the present study a BiP-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 both under *in vitro* conditions and also *ex vitro*, when forming natural symbioses with different host plants. The identification of this BiP-encoding gene in *G. intraradices* may provide new insights into the complex mechanisms by which AM fungi can protect the host plants against water deficit.

2. Materials and methods

2.1. In vitro mycorrhizal cultures

G. intraradices was established in monoxenic culture as described by St-Arnaud et al. (1996). 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 (Bécard and Piché, 1992). 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 of new liquid minimal medium supplemented with 25% PEG 6000 (Sigma–Aldrich Co., Madrid, Spain) in order 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 a 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 RT-PCR experiments.

2.2. Soil and biological material

Properties of soil and plants used in this study were as previously described (Porcel and Ruiz-Lozano, 2004; Porcel et al., 2005a,b).

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 wildtype) from *N. tabaccum* were used (see Siefritz et al., 2002; Porcel et al., 2005a). All plants were inoculated with the AM fungus *G. intraradices* (Schenck and Smith) isolate BEG 121. One plant per pot and 10 pots per treatment were used. In all cases, half of the plants was cultivated under well-watered conditions throughout the entire experiment, while the other half was drought-stressed for 10 days before harvest as previously described (Porcel and Ruiz-Lozano, 2004; Porcel et al., 2005a,b).

2.3. 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 (PPFD) 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), while the other half was maintained at field capacity. Plants were maintained under such conditions for additional 8 days. In order 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 in order 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.

2.4. Symbiotic development

The extent of mycorrhizal root colonization was calculated by the gridline intersect method (Giovannetti and Mosse, 1980) after root staining according to Phillips and Hayman (1970).

2.5. RNA isolation, construction of the cDNA library and screening

Total RNA was extracted from *G. intraradices* mycelia grown in monoxenic culture under non-stress conditions or subjected to drought stress by addition of 25% PEG. 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 as described by Ruiz-Lozano et al. (2002). The library was screened by differential hybridization as previously described (Ruiz-Lozano et al., 2002).

Differentially detected phage clones were used as template for a PCR-based screening with universal M13 forward and reverse primers in order 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 (Martin-Laurent et al., 1995). Southern blot analysis was used to confirm differentially expressed clones by probing the membranes with ^{32}P-labelled cDNAs from *G. intraradices* mycelia grown in monoxenic culture added or not of 25% PEG 6000.

2.6. Sequencing the cloned cDNA and analyses

Sequencing was performed by the dideoxy-sequencing method (Sanger et al., 1977) using fluorescent dye-linked universal M13 primers and a Perkin-Elmer ABI Prism model 373 DNA sequencer. Similarity searches were carried out in the EMBL databank, using the BLAST software or the FASTA program available on-line from the National Center for Biotechnology Information (NCBI).

2.7. Northern blot analysis

Northern blot analysis of *GiBiP* gene expression were carried out as described previously (Ruiz-Lozano et al., 2002; Porcel et al., 2005b). After hybridization to the *GiBiP* probe, blots were stripped and rehybridized with the β-tubulin gene probe from *G. intraradices* (accession no. BE603903). *GiBiP* and β-tubulin signals on autoradiograms were analyzed and quantified using Quantity One software (BioRad, Hemel Hempstead, UK). Transcript accumulation levels were normalized according to β-tubulin signals on autoradiograms (Ruiz-Lozano et al., 2002). Each quantification was repeated three times and the average value was used for normalization. Northern blot analyses were repeated twice with different set of plants.

2.8. Quantitative real time RT-PCR

GiBiP gene expression was studied by real-time PCR by using iCycler (Bio-Rad, Hercules, CA, USA), as described by Gonzalez-Guerrero et al. (2005) 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, California, USA) and 200 U of Superscript II Reverse Transcriptase (Invitrogen). The primer set used to amplify *GiBiP* and *Gi 18S rRNA* genes in the synthesized cDNAs were the following (5′–3′): GiBiPForw agatgctggcgtaattgctgg; GiBiPRev tggcggcaccatatgcaactg; 18SGiForw tgttaataaaaatcggtgcgttgc; 18SGiRev aaaacgcaaatgatcaaccggac. 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 $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen, 2001). Negative controls without cDNA were used in all PCR reactions.

3. Results

3.1. Symbiotic development

The degree of mycorrhizal root colonization varied with the plant considered (Table 1), ranging from 41% for soybean plants to 96% for tobacco plants. Drought stress for 10 days did not affect the mycorrhizal root colonization of any of the host plants assayed.

3.2. Identification of GiBiP gene

The differential screening of the library allowed detection of a cDNA clone hybridizing with the cDNA probe obtained from the AM fungus *G. intraradices* growing in monoxenic culture in presence of 25% PEG 6000, but not with the probe from the fungus growing in minimal medium without PEG. The clone was sequenced and the sequence obtained gave a 100 of identity with accession no. AJ319773, corresponding to the *BiP* gene from *G. intraradices*.

Table 1
Percentage of mycorrhizal root colonization by *Glomus intraradices* in the different plants used in this study

Treatment	Maize	Soybean	Lettuce	Tobacco	
				Wildtype	Antisense
Well-watered	89 a	45 a	81 a	95 a	94 a
Droughted	92 a	41 a	78 a	96 a	91 a

Within each plant means followed by the same letter are not significantly different ($P < 0.05$) as determined by Duncan's multiple-range test ($n = 4$).

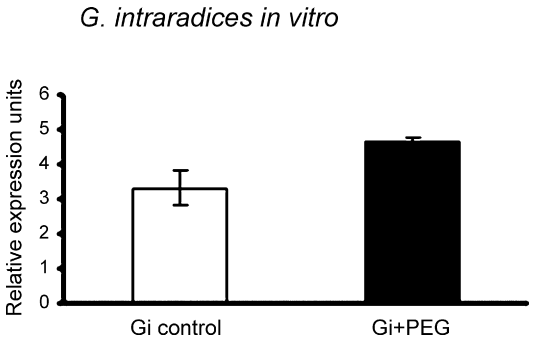

Fig. 1. Analysis of *GiBiP* gene expression by real time quantitative RT-PCR in the AM fungus *Glomus intraradices* grown *in vitro*. The fungus was subjected to drought by addition of PEG 6000 (25%) to the growing medium or maintained under control conditions without PEG.

3.3. Expression of GiBiP gene in vitro and ex vitro, during symbiosis with different plants

The expression of *GiBiP* 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 *GiBiP* gene when the fungus was growing *in vitro* with carrot root organ cultures and subjected to an osmotic stress by adding 25% PEG 6000 to the growing medium (Fig. 1). The addition of PEG to the medium increased *GiBiP* gene expression by 41%.

We then analyzed the expression of that fungal gene while forming natural symbiosis with whole plants cultivated under well-watered or under drought stress conditions. The results obtained differed according to the host plant assayed. The expression of *GiBiP* gene increased by over 600% as a consequence of drought when *G. intraradices* colonized maize plants (Fig. 2). A lower increase (52%) in gene expression was observed in soybean plants subjected to drought stress (Fig. 3) and also in wildtype tobacco plants (Fig. 4), that increased by 106% *GiBiP* gene expression. In contrast, when the host plant was the antisense tobacco line or lettuce, *GiBiP* gene expression was not significantly affected by drought stress (Figs. 4 and 5).

4. Discussion

Protein folding in the ER is facilitated by molecular chaperones, which prevent nonproductive intermolecular interactions of folding intermediates and subsequent misaggregation of proteins within the lumen of the ER (Hammond and Helenius, 1995). The induction of *BiP* genes is achieved through a signalling pathway named the UPR pathway, which coordinately up-regulates the transcription of a set of ER-resident proteins, including BiPs (Alvim et al., 2001).

The up-regulation of *GiBiP* gene under conditions of water deficit (induced by PEG or by withholding plant irrigation) indicates that this fungal protein has a role in the answer of the fungus against drought. The GiBiP protein is probably involved in the protection of the fungus itself (as indicated by the induction of the gene within the extraradical hyphae under *in vitro* conditions) and may be also involved in the protection of the host plant when forming a natural symbiosis (induction of gene expression during the symbiosis with maize, soybean and wildtype tobacco). The induction of BiP mRNA by osmotic stress may represent a primary response to water stress that is activated as soon as the stress is sensed and may accommodate a regulatory function (Cascardo et al., 2000). The protective role of BiP against water stress may be associated with preservation of protein structure and of high secretory activity mediated by the water stress adaptive cellular response (Ingram and Bartels, 1996).

Curiously, we obtained quite different responses when the fungus was associated to the two plants that are more sensitive to drought. In previous studies we have demonstrated the sensitivity to drought stress of lettuce and tobacco antisense plants (Ruiz-Lozano et al., 1995; Porcel et al., 2005a). This is especially clear for the antisense tobacco plants that show an 80% of inhibition of an aquaporin and this result in a higher sensitivity to drought (Siefritz et al., 2002). In contrast, the pattern of AM colonization of these antisense mutants is the same that the corresponding wildtype plants (Porcel et al., 2005a). Some studies have shown that the BiP stress response may differ significantly in plants (Cascardo et al., 2000). Hence, the results obtained in lettuce and tobacco antisense plants suggest that the protection the AM symbiosis confers to these plants against drought

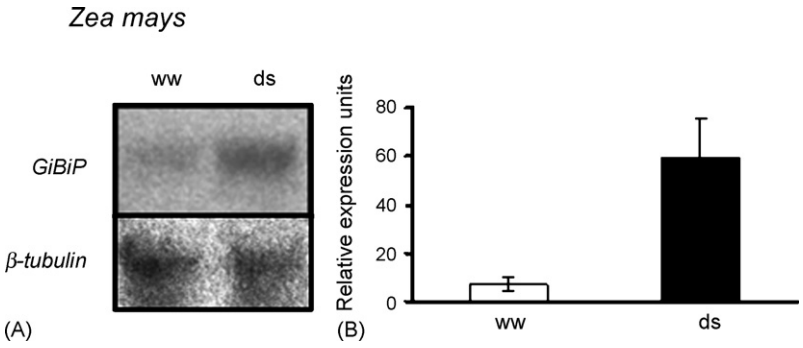

Fig. 2. Analysis of *GiBiP* gene expression in maize plants colonized by the AM fungus *G. intraradices*. Plants were either well-watered (ww) or drought stressed (ds). (A) Northern blot of total RNA (15 μg). Blots were hybridized with *GiBiP* probe and with the *G. intraradices* β-tubulin probe (accession no. BE603903) for calibration of *GiBiP* gene expression. (B) Real-time quantitative RT-PCR analysis of *GiBiP* gene expression.

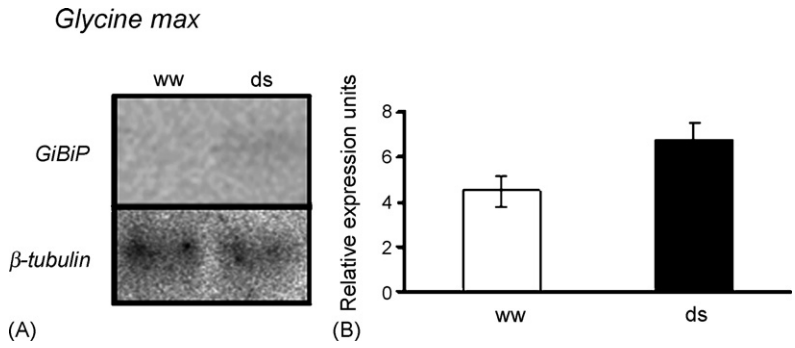

Fig. 3. Analysis of *GiBiP* gene expression in soybean plants colonized by the AM fungus *G. intraradices*. See legend in Fig. 2.

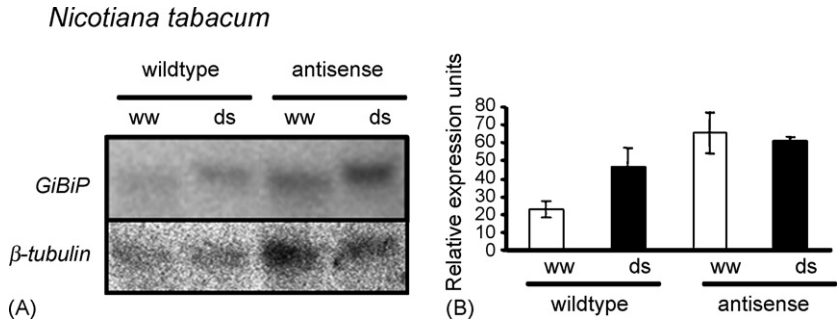

Fig. 4. Analysis of *GiBiP* gene expression in two tobacco plant lines (wildtype or an aquaporin antisense mutant) colonized by the AM fungus *G. intraradices*. See legend in Fig. 2.

stress, evidenced in several former studies (Ruiz-Lozano, 2003; Porcel et al., 2005a), is not mediated by BiP activity. For these plants, the protection seems to be mediated by other mechanisms, as those already reported in previous revisions on the subject (Augé, 2001; Ruiz-Lozano, 2003). However, it should be also taken into account the intensity of the stress applied. In this study we subjected the plants to a moderate stress. It is known that plant response depends on drought intensity (Siemens and Zwiazek, 2003, 2004). Therefore, the induction of *GiBiP* gene should be expected to vary during the initial, mild or later drought stages, when the plant is severely stressed. For instance, in trembling aspen an increase in root hydraulic conductivity was measured during mild stress and a decrease during severe stress (Siemens and Zwiazek, 2004). Additional studies, using a more severe drought stress level should help to understand whether or not *GiBiP* is also induced in plants such as lettuce or the antisense tobacco line.

In conclusion, the results obtained here suggest that BiP proteins could be 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 sensitivity of the host plant against water deficit or on the severity of the stress. In any case, as there are multiple targets proteins for BiP activity, pleiotropic physiological effects of BiP under water stress have been proposed (Alvim et al., 2001), and the precise mechanisms of BiP-mediated water stress tolerance remain unknown. It is likely, however, that GiBiP protein can facilitate the proper folding and maturation of water stress-induced secretory proteins involved in the osmotic response mechanism. In fact, drought-induced proteins, which are targeted to the secretory pathway have been identified in a wide range of species (Ingram and Bartels, 1996; Riccardi et al., 1998) and BiP has been shown to associate with water-stress induced proteins (Cascardo et al., 2000). Our findings provide new evidence that the contribution

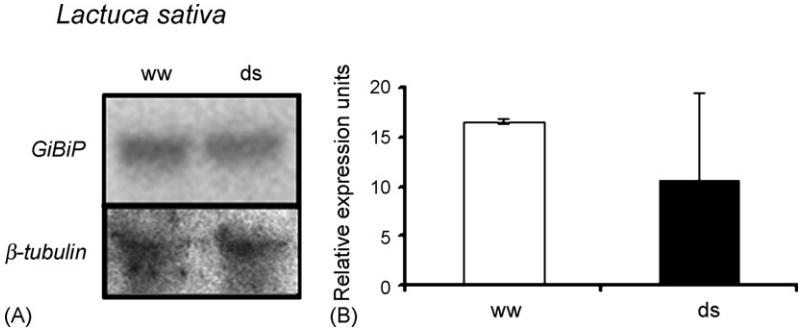

Fig. 5. Analysis of *GiBiP* gene expression in lettuce plants colonized by the AM fungus *G. intraradices*. See legend in Fig. 2.

of AM fungi to the enhanced drought tolerance of the host plant can be mediated by proteins with chaperone-like activity, such as that of BiP.

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