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Evaluation of the role of genes encoding for dehydrin proteins (LEA D-11) during drought stress in arbuscular mycorrhizal *Glycine max* and *Lactuca sativa* plants

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Abstract

In this study, it has been determined whether the arbuscular mycorrhizal (AM) symbiosis is able to alter the pattern of dehydrin (LEA D-11 group) transcript accumulation under drought stress, and whether such a possible alteration functions in the protection of the host plants against drought. Two dehydrin-encoding genes have been cloned from *Glycine max* (*gmlea 8* and *gmlea 10*) and one from *Lactuca sativa* (*lslea 1*) and they have been analysed for their contribution to the response against drought in mycorrhizal soybean and lettuce plants. Results with soybean plants showed that most of the treatments did not show LEA gene expression under well-watered conditions. The higher gene expression was found in non-inoculated plants subjected to drought. Only plants singly inoculated with *Bradyrhizobium japonicum* showed an important level of LEA gene expression under well-watered conditions and a reduced level under drought-stress conditions. The same results were confirmed in subsequent experiments and at the latest stage of a time-course experiment. In lettuce, the *lslea 1* gene was also induced by drought stress in all treatments. However, the level of induction was clearly higher in roots from non-inoculated plants than in roots from the two AM treatments assayed. The overall results demonstrated that the levels of *lea* transcript accumulation in mycorrhizal treatments subjected to drought were considerably lower than in the corresponding non-mycorrhizal plants, indicating that the accumulation of LEA proteins is not a mechanism by which the AM symbiosis protects their host plant.

Introduction

Water deficit is one of the most common environmental stress factors experienced by soil plants. It interferes with both normal development and growth and has a major adverse effect on plant survival and productivity (Colmenero-Flores *et al.*, 1997; Kramer and Boyer, 1997). However, plants can respond to drought stress at morphological, anatomical and cellular levels with modifications that allow the plant to avoid the stress or to increase its tolerance (Bray, 1997). The morphological and anatomical adaptations can be of vital importance for some plant species, but they are not a general response of all plant species. By contrast, the cellular responses to water deficit seem to be conserved in the plant kingdom. In addition, most terrestrial plants can establish a symbiotic association with a group of soil fungi called arbuscular mycorrhizal (AM) fungi (Smith and Read, 1997). The AM symbiosis is present in all natural ecosystems, even in those affected by adverse environmental conditions (Barea and Jeffries, 1995). A number of studies have demonstrated that the AM symbiosis can protect the host plants against the detrimental effects of drought stress (for reviews see Augé, 2001; Ruiz-Lozano, 2003). It is accepted that the contribution of the AM symbiosis to plant drought tolerance results from a combination of physical, nutritional, and cellular effects (Ruiz-Lozano, 2003). Studies carried out so far have suggested several

mechanisms by which the AM symbiosis can alleviate drought stress in host plants. The most important are: direct uptake and transfer of water through the fungal hyphae to the host plant (Hardie, 1985; Allen, 1991; Ruiz-Lozano and Azcón, 1995; Marulanda *et al.*, 2003), better osmotic adjustment of AM plants (Augé *et al.*, 1992; Ruiz-Lozano *et al.*, 1995a; Kubikova *et al.*, 2001), enhancement of plant gas exchange (Augé *et al.*, 1987, 1992; Ruiz-Lozano *et al.*, 1995a, b; Duan *et al.*, 1996; Goicoechea *et al.*, 1997; Green *et al.*, 1998), changes in soil water retention properties (Augé *et al.*, 2001, 2004), and protection against the oxidative damage generated by drought (Ruiz-Lozano *et al.*, 1996, 2001a, b; Porcel *et al.*, 2003; Porcel and Ruiz-Lozano, 2004).

Among a diversity of responses, plants can adapt to water deficit by the induction of specific genes (Zhu *et al.*, 1997) such as the gene family encoding for a group of proteins called late embryogenesis abundant (LEA) proteins. These proteins accumulate in seeds during their maturation phase, when tolerance to desiccation is required (Close, 1996). A variety of studies have demonstrated that LEA proteins also accumulate in vegetative plant tissues during periods of water deficit, which reinforced a role for these proteins as desiccation protectants (Moons *et al.*, 1997). It has been proposed that, during cellular dehydration, LEA proteins play an important role in maintenance of the structure of other proteins, vesicles, or endomembrane structures, in the sequestration of ions such as calcium, in binding or replacement of water, and functioning as molecular chaperones (Close, 1996; Heyen *et al.*, 2002; Koag *et al.*, 2003). Currently, it is known that over-expression of LEA proteins in plants and yeast confers tolerance to osmotic stresses (Xu *et al.*, 1996; Imai, 1996). Dehydrins are an important group of LEA proteins (LEA D-11 family). They represent the most conspicuous soluble proteins induced by a dehydration stress and have been observed in over 100 independent studies of drought stress, cold acclimation, salinity stress, embryo development, and responses to ABA. Among the water-stress-induced proteins so far identified, dehydrins are the most frequently observed (Close, 1997; Cellier *et al.*, 1998). It seems that dehydrins play a fundamental role in the dehydration response of plants to a range of environmental and developmental stimuli (Close, 1996). The multiple targets of dehydrins (euchromatin, cytosol, cytoskeleton) suggest that the direct consequences of dehydrin activity are biochemically diverse.

Although in recent years there has been an increase in the understanding of the water relations of AM plants and the physiological processes involved in the enhanced tolerance of mycorrhizal plants to water limitation, there are still many unknown aspects which must be elucidated (Ruiz-Lozano, 2003). Moreover, a molecular basis for the tolerance to water stress in AM plants remains far from being understood. No studies have been published so far on dehydrins (LEA D-11 group) in mycorrhizal plants. As

these proteins seems to be part of the plant's universal responses against dehydration, it is of interest to determine whether the AM symbiosis is able to alter the pattern of dehydrin accumulation under drought stress and whether such a possible alteration functions in the protection of the host plants against drought. As a first approach, in the present study two dehydrin-encoding genes from *Glycine max* (*gmlea 8* and *gmlea 10*) and one from *Lactuca sativa* (*lslea 1*) have been cloned and their contribution to the response against drought in mycorrhizal soybean and lettuce plants has been analysed.

Materials and methods

Experimental designs and statistical analysis

First experiment (with Glycine max*)*: The experiment consisted of a randomized complete block design with six inoculation treatments: (i) plants inoculated with the nitrogen-fixing bacteria *Bradyrhizobium japonicum*, strain USDA 110 (Br), (ii) plants inoculated with the arbuscular mycorrhizal (AM) fungus *Glomus mosseae* (Nicol. and Gerd.) Gerd. and Trappe and *B. japonicum* (Gm+Br), (iii) plants inoculated with the AM fungus *Glomus intraradices* (Schenck and Smith) and *B. japonicum* (Gi+Br), (iv) plants inoculated with *G. mosseae* (Gm), (v) plants inoculated with *G. intraradices* (Gi), and (vi) non-inoculated control plants (NI). These six inoculation treatments included the different combinations of micro-organisms able to establish a symbiotic association with the soybean root. There were 12 replicates of each treatment, totalling 72 pots (one plant per pot), so that half was cultivated under well-watered conditions throughout the entire experiment while the other half was drought-stressed for 10 d before harvest (35 d after inoculation).

Second experiment (with Glycine max*)*: The experiment consisted of a randomized complete block design with three inoculation treatments: (i) plants inoculated with the nitrogen-fixing bacteria *Brady-rhizobium japonicum*, strain USDA 110 (Br), (ii) plants inoculated with the AM fungus *Glomus mosseae* (Nicol. and Gerd.) Gerd. and Trappe and *B. japonicum* (Gm+Br), and (iii) non-inoculated control plants (NI). For each treatment plants were cultivated at four time intervals: 5, 12, 20, or 35 d after inoculation (dai). There was a variable number of replicates of each treatment, ranging from 12 replicates for plants harvested after only 5 d, to six replicates for plants harvested after 35 d, totalling 108 pots (one plant per pot). Half of the plants was cultivated under well-watered conditions through-out the entire experiment, while the other half was drought-stressed for 5 d (for plants harvested 5 dai) or for 10 d (for the rest of treatments) before harvest.

Third experiment (with Lactuca sativa*)*: The experiment consisted of a randomized complete block design with three inoculation treat-ments: (i) plants inoculated with the AM fungus *Glomus mosseae* (Nicol. and Gerd.) Gerd. and Trappe (Gm), (ii) plants inoculated with the AM fungus *Glomus intraradices* (Schenck and Smith) (Gi), and (iii) non-inoculated control plants (NI). Ten replicates of each treatment were done, totalling 30 pots (one plant per pot), so that half of them was cultivated under well-watered conditions throughout the entire experiment, while the other half was drought-stressed for 10 d before harvest.

Data were subjected to analysis of variance (ANOVA) with microbial treatment, water supply, and microbial treatment–water supply interaction as sources of variation, and followed by Duncan's multiple range test (Duncan, 1955). Percentage values were arcsin transformed before statistical analysis.

Soil and biological materials

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 for 3 d). The soil had a pH of 8.1 (water); 1.81% organic matter, 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.

Soybean (*Glycine max* L. cv. Williams) seeds were sterilized in a 15% H$_2$O$_2$ solution for 8 min, then washed several times with sterile water to remove any trace of chemical that could interfere with seed germination, and placed on sterile vermiculite at 25 °C to germinate. Three-day-old seedlings were transferred to plastic pots containing 600 g of sterilized soil/sand mixture (1:1, v/v). When appropriate, a suspension (2 ml seed) of the diazotrophic bacterium *Bradyrhizobium japonicum*, strain USDA 110 (10^8 cell ml^{-1}) was sprinkled over the seedling at the time of planting.

Three seeds of lettuce (*Lactuca sativa* L. cv. Romana) were sown in pots containing 500 g of the same soil/sand mixture as described above and thinned to one seedling per pot after emergence.

Mycorrhizal inoculum for each endophyte was bulked in an open-pot culture of *Allium cepa* L. and consisted of soil, spores, mycelia, and infected root fragments. The AM species were *Glomus mosseae* (Nicol. and Gerd.) Gerd. and Trappe, isolate BEG 122 and *Glomus intraradices (*Schenck and Smith) isolate BEG 121. Ten grams of inoculum of the two *Glomus* isolates, possessing similar infective characteristics (about 115 infective propagules per gram, according to the MPN test), were added to appropriate pots at sowing time just below soybean seedlings or lettuce seeds.

Uninoculated control plants for each microbial treatment received the same amount of autoclaved rhizobial and/or mycorrhizal inoculum.

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} (Li-Cor, 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 using a pressure plate apparatus and applying a pressure of one-third atmosphere for 48 h, and then determining the volumetric soil moisture) during the first 4 weeks of plant growth (first and third experiments). At that time, half of the plants was allowed to dry until soil water content reached 70% field capacity (two days were 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 an additional 10 d. In order to control the level of drought stress, the soil water content was measured daily 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 between each rewatering, the soil water content progressively decreased to a minimum value of 60% of field capacity. For the second experiment, half of the plants was maintained at field capacity during the entire experiment, while the other half was drought-stressed as indicated above for 5 d (plants harvested 5 dai) or for 10 d for the rest of the harvests.

Each week throughout the experiment, soybean plants received 10 ml of Hewitt's nutrient solution lacking N and P (Hewitt, 1952). Three weeks after planting, plants received nutrient solution amended with N and/or P as follows (Goicoechea *et al.*, 1997): 0.18 mM K$_2$HPO$_4$ and 2 mM NH$_4$NO$_3$ (NI plants), 0.35 mM K$_2$HPO$_4$ (Br plants), 3 mM NH$_4$NO$_3$ (Gm and Gi plants). Nutrient concentrations were chosen in an attempt to obtain well-watered plants of similar size and nutrient contents in all the microbial treatments.

Each week throughout the experiment, uninoculated control lettuce plants received 10 ml of Hewitt's nutrient solution (Hewitt, 1952), modified to contain 4 mM N+1 mM P. Mycorrhizal plants did not receive nutrient solution. The use of such a fertilization level for non-mycorrhizal plants was devised to obtain well-watered control plants of similar size and nutrient contents to the AM plants tested in this assay.

Symbiotic development

The percentage of mycorrhizal root infection in soybean and lettuce plants was estimated by visual observation of fungal colonization after clearing washed roots in 10% KOH and staining with 0.05% trypan blue in lactic acid (v/v), according to Phillips and Hayman (1970). The extent of the mycorrhizal colonization was calculated according to the gridline intersect method (Giovannetti and Mosse, 1980). Nodule number in soybean roots was determined using a binocular microscope.

Relative water content

The relative water content (*RWC*) in plant shoots was determined as previously described by Ruiz-Lozano and Azcón (1997).

RNA isolation and synthesis of first strand cDNA

Total RNA was isolated from soybean or lettuce roots by phenol/chloroform extraction (Kay *et al.*, 1987). DNase treatment of total RNA was performed according to Promega's recommendations. Total RNAs (2.5 µg) from soybean and from lettuce roots subjected to drought stress were reverse transcribed to first strand cDNA using AMV-RT enzyme (Finnzymes, Espoo, Finland) and oligo(dT)$_{15}$ primer (Promega, Madison, WI), in a final volume of 25 µl with the buffer and temperature recommended by the enzyme supplier.

Total RNA was also isolated from soybean nodules (0.3 g FW) that had been previously separated from the roots at the harvest time and kept at −80 °C in order to be used for molecular and biochemical determinations. The RNA from nodules was extracted using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany).

Cloning the gmlea and lslea genes

Dehydrins (LEA D-11 family) have specific conserved domains such as the S, Y, and K segments (Dure, 1993). While the S segment is a tract of Ser residues, the Y (VDYGNP) and the K (EKKGIMDKI-KEKLPG) segments are useful for degenerate primer design. These two stretches were used to design degenerate oligonucleotide primers as described by Numberg *et al.* (1989): forward primer 5′-GTC GAC GA(GA) TAC GG(CT) AAC CC-3′ and reverse primer 5′-CC (AG)GG (CA)AG (CT)TT CTC (CT)TT (AG) CT-3′. Using cDNA from either soybean or from lettuce roots subjected to drought stress as the template, a cDNA fragment of about 610 bp was amplified for soybean with these primers and the polymerase chain reaction (PCR), while for lettuce the cDNA fragment that was amplified was about 340 bp. PCR was carried out in a final volume of 50 µl containing 10 mM TRIS–HCl pH 8.3, 50 mM KCl, 2.5 mM MgCl$_2$, 0.25 mM dNTPs, 100 pmol of each primer and 2 units of *Taq* DNA polymerase (Sigma, St Louis, MO, USA). A Perkin/Elmer thermocycler (model 2400) was used with the following values: initial denaturation at 95 °C for 5 min, followed by 35 denaturation cycles at 95 °C for 45 s, annealing at 52 °C for 45 s, elongation at 72 °C for 75 s, and a final elongation at 72 °C for 5 min. The amplified cDNA was purified following electrophoresis through a 1.2% agarose gel with the

QIAEX II Gel Extraction Kit (Qiagen, Hilden, Germany) and cloned into pGME plasmid (Promega). Recombinant plasmids were used to transform competent *E. coli* DH-5α cells. Positive clones screened by PCR were subcultured and plasmid DNA isolated using QIAprep^R Spin Miniprep kit (Qiagen).

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 using the BLAST software or the FASTA program, available on-line from the National Center for Biotechnology Information (NCBI).

Northern blot analysis

Total RNA (15 µg) was separated by electrophoresis on 1.2% agarose gel containing 2.2 M formaldehyde and blotted onto Hybond-N$^+$ nylon membranes (Amersham, Little Chalfont, UK) by capillarity (Sambrook *et al.*, 1989). Equal RNA loading and transfer were verified by methylene blue staining of nylon membranes before hydridization (Herrin and Schmidt, 1988). Blots were prehybridized for 2–3 h at 42 °C in 5× Denhardt's solution, 5× SSC and 0.5% SDS, and hybridized with *gmlea 8*, *gmlea 10*, or *lslea 1* specific probes obtained by radioactive PCR labelling of plasmid inserts. Unincorporated ^{32}P was removed using Mini Quick Spin™ 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 (Sambrook *et al.*, 1989). 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 analysed and quantified using Quantity One software (Bio-Rad, Hemel Hempstead, UK). Transcript accumulation levels for each gene probe 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.

Nucleotide sequence accession number

The nucleotide sequences corresponding to *gmlea 8*, *gmlea 10*, and *lslea 1* cDNAs have been deposited in the EMBL database under accession numbers AJ704825, AJ704824, and AJ704826, respectively.

Results

Symbiotic development in soybean and lettuce plants

Non-inoculated soybean plants did not show any mycorrhizal infection or nodules from *Bradyrhizobium* (Table 1). Plants inoculated with *G. mosseae* or *G. intraradices* showed an important level of colonization, but no differences were observed among the different AM treatments. The number of nodules varied with the microbial treatment. The highest nodule number was found in plants singly inoculated with *B. japonicum* and cultivated under well-watered conditions. No significant differences were found for this parameter between the two AM treatments. Drought stress decreased the number of nodules only in plants singly inoculated with *B. japonicum*.

Table 1. *Relative water content* (RWC, %) *in soybean shoots and percentage of mycorrhizal infection and nodule number in soybean roots*

Treatments are designed as NI, non-inoculated controls; Br, *Bradyrhizobium japonicum*; Gm, *Glomus mosseae*; Gi, *Glomus intraradices*; Gm+Br, *G. mosseae* plus *B. japonicum*; Gi+Br, *G. intraradices* plus *B. japonicum*. Plants were either well-watered or drought-stressed for 10 d.

Treatment	RWC^a	AM $(\%)^a$	Nodule number^a
Well-watered			
NI	84 a	0 b	0 c
Br	83 a	0 b	52 a
Gm	82 ab	92 a	0 c
Gm+Br	83 a	96 a	43 ab
Gi	85 a	88 a	0 c
Gi+Br	84 a	96 a	30 b
Droughted			
NI	71 e	0 b	0 c
Br	74 de	0 b	37 b
Gm	77 cd	89 a	0 c
Gm+Br	78 bc	95 a	35 b
Gi	79 bc	87 a	0 c
Gi+Br	78 bc	86 a	29 b

^{a} Within each column, means followed by the same latter are not significantly different ($P <0.05$) as determined by Duncan's multiple range test ($n=4$).

In the second experiment, plants harvested 5 dai did not show intraradical mycorrhizal structures, only some appresoria on the roots were observed, while no nodules were detected (Table 2). At 12 dai the level of AM infection was about 15% in *G. mosseae*-inoculated plants under both well-watered and drought-stressed conditions. The number of nodules ranged from 7 to 10 for Br or Gm+Br plants, with no significant differences between both treatments. At 20 dai the level of AM infection was over 25% in *G. mosseae*-inoculated plants and the number of nodules averaged 14 in all *B. japonicum*-inoculated treatments. Finally, at 35 dai, the AM infection was close to 50% in *G. mosseae*-inoculated plants and the number of nodules ranged from 20 to 30 in *B. japonicum*-inoculated treatments.

Lettuce plants exhibited a good percentage of mycorrhizal infection for both AM fungi and there were no significant differences between well-watered or drought-stressed treatments (Table 3).

Relative water content

No significant differences in the *RWC* of soybean and lettuce plants were found when cultivated under well-watered conditions, regardless of the microbial treatment (Tables 1, 2, 3). By contrast, when plants were cultivated under drought-stress conditions, mycorrhizal plants maintained higher *RWC* values compared with non-mycorrhizal ones (Tables 1, 3). The only exception to this was found in the time-course experiment (Table 2), since AM plants showed similar values of *RWC* than non-AM plants (5, 12, and 20 dai). Only at 35 dai, did plants colonized by *G. mosseae* have a significantly higher *RWC* than non-inoculated plants.

Table 2. *Relative water content (RWC, %) in soybean shoots, percentage of mycorrhizal infection and nodule number in soybean roots*

Plants were harvested at 5, 12, 20, or 35 d after inoculation (dai). Treatments are designed as NI, non-inoculated controls; Br, *Bradyrhizobium japonicum* and Gm+Br, *G. mosseae* plus *B. japonicum*; Plants were either well-watered (ww) or drought-stressed (ds) before harvest.

Treatment	RWC[a]		AM (%)[a]		Nodule number[a]	
	ww	ds	ww	ds	ww	ds
5 dai						
NI	85 a	79 c	0 d	0 d	0 e	0 e
Br	84 a	80 bc	0 d	0 d	0 e	0 e
Gm+Br	83 ab	79 c	0 d	0 d	0 e	0 e
12 dai						
NI	84 a	77 cd	0 d	0 d	0 e	0 e
Br	84 a	78 c	0 d	0 d	10 d	7 d
Gm+Br	85 a	77 cd	17 c	15 c	10 d	8 d
20 dai						
NI	84 a	72 ef	0 d	0 d	0 e	0 e
Br	86 a	73 ef	0 d	0 d	14 c	14 c
Gm+Br	83 a	72 ef	30 b	25 b	15 c	14 c
35 dai						
NI	83 a	71 f	0 d	0 d	0 e	0 e
Br	85 a	72 ef	0 d	0 d	23 b	20 b
Gm+Br	83 a	75 de	55 a	47 a	30 a	21 b

[a] Within each column, means followed by the same latter are not significantly different (*P* <0.05) as determined by Duncan's multiple range test (*n*=4).

Cloning gmlea and lslea genes

The use of the degenerate primers designed on the Y and K segments of dehydrins allowed several clones to be obtained containing inserts of the expected size using cDNA from soybean and from lettuce roots. The sequencing of five of the clones obtained from soybean cDNA showed that two of them contained a cDNA insert putatively encoding for LEA proteins. These clones were named *gmlea 8* and *gmlea 10*. The first clone (*gmlea 8*) contained a cDNA fragment of 591 bp encoding for a putative protein of 77% identity with a dehydrin protein from *Glycine max* (accession Q39805, $e=1e^{-85}$). The second clone (*gmlea 10*) contained a cDNA fragment of 641 bp encoding for a putative protein of 65% identity with another dehydrin protein from *Glycine max* (accession CAE47771, $e=2e^{-73}$). The homology between *gmlea 8* and *gmlea 10* nucleotide sequences was 83%.

In the case of lettuce, three clones were sequenced, of which only one contained a cDNA insert putatively encoding for a LEA protein. This clone was named *lslea 1*. It contained a cDNA fragment of 338 bp that showed 92% identity at the nucleotide level with a dehydrin gene from *Aster tripolium* (accession AB090885, $e=6e^{-20}$). The putative protein encoded by this cDNA gave 35% identity with a dehydrin from *Aster tripolium* (accession BAC57962, $e=2e^{-11}$).

Northern blot analysis with soybean RNAs

Both cDNA inserts from soybean (*gmlea 8* and *gmlea 10*) were used as probes in northern blot analyses with soybean

Table 3. *Relative water content (RWC, %) in lettuce shoots and percentage of mycorrhizal infection in lettuce roots*

Treatments are designed as NI, non-inoculated controls; Gm, *Glomus mosseae* and Gi, *Glomus intraradices*. Plants were either well-watered or drought stressed for 10 d.

Treatment	RWC[a]	AM (%)[a]
Well-watered		
NI	88 a	0 b
Gm	86 a	75 a
Gi	85 a	82 a
Droughted		
NI	70 c	0 b
Gm	75 b	77 a
Gi	74 b	87 a

[a] Means followed by the same latter are not significantly different (*P* <0.05) as determined by Duncan's multiple range test (*n*=4).

roots RNA from a variety of microbial treatments (see experimental design). The results obtained were very similar for both *gmlea* probes (Fig. 1). Most of the treatments did not show LEA gene expression under well-watered conditions. The highest gene expression was found in non-inoculated plants subjected to drought, that was set as 100% in arbitrary units after normalization according to the amount of ribosomal RNA loaded in the blots. Only plants singly inoculated with *B. japonicum* showed a significant level of LEA gene expression under well-watered conditions (83% compared with non-inoculated plants subjected to drought) and reduced LEA gene expression under drought-stress conditions (only 38% of non-inoculated plants, being more visible with the *gmlea 10* probe). Plants singly inoculated with *G. mosseae* only showed detectable LEA gene expression under drought stress (50% of the level found in non-inoculated plants), while those dually inoculated with *B. japonicum* plus *G. mosseae* exhibited very little LEA gene expression under well-watered conditions (more visible with the *gmlea 8* probe). In any case, the level of expression in that treatment was less than 3% of the expression found in non-inoculated plants. These plants showed LEA gene expression under drought-stress conditions (50% of non-inoculated plants). Plants singly inoculated with *G. intraradices* only showed detectable LEA gene expression under drought-stress conditions, but this expression was considerably lower than with the other fungal treatment (14% of non-inoculated plants). Finally, plants dually inoculated with *B. japonicum* plus *G. intraradices* only exhibited LEA gene expression under drought-stress conditions (94% of non-inoculated plants).

Since plants inoculated only with *B. japonicum* showed a different and unexpected pattern of LEA transcript accumulation compared with the other treatments, the northern blot analysis was repeated with a different set of plants (data not shown). The results obtained agreed with the previous findings, since plants inoculated with
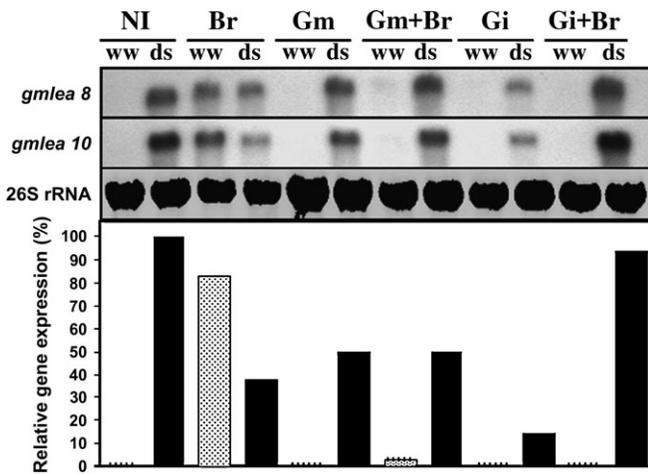
Fig. 1. Northern blot of total RNA (15 µg) from soybean roots. Treatments are designed as NI, non-inoculated controls; Br, *Bradyrhizobium japonicum*; Gm, *Glomus mosseae*; Gm+Br, *G. mosseae* plus *B. japonicum*; Gi, *Glomus intraradices*; Gi+Br, *G. intraradices* plus *B. japonicum*. Plants were either well-watered (ww) or drought-stressed (ds) for 10 d. The lower panel shows a representative example of the amount of 26S rRNA loaded for each treatment (methylene blue staining). Histogram shows the relative gene expression (after normalization to rRNA) presented as a percentage of the value for droughted non-inoculated plants.

B. japonicum only showed significant LEA transcript accumulation under well-watered conditions; and plants inoculated with *G. intraradices* only also had a low level of LEA transcript accumulation.

Following the unexpected results obtained for the expression of LEA genes in roots of plants singly inoculated with *B. japonicum*, a time-course experiment was planned in order to study the expression level of such genes at different time intervals, both in non-inoculated soybean plants and after inoculation with either *B. japonicum* or with *B. japonicum* plus *G. mosseae*. Plants harvested 5 dai did not show *gmlea 10* transcript accumulation under any condition (Fig. 2). At 12 dai, *gmlea 10* transcript accumulation was visible in the three treatments, but only when subjected to drought. After normalization, the level of gene expression in arbitrary units was 100% for non-inoculated plants, 115% for *B. japonicum*-inoculated plants and 102% for Gm+Br plants. At 20 dai, again the LEA transcript only accumulated in the three treatments subjected to drought, although the level of expression was considerably lower in the non-inoculated plants (set as 100%) compared with the other two treatments (about 210% compared with non-inoculated plants). Finally at 35 dai, the pattern of LEA transcript accumulation returned to similar levels to those observed in plants from the first experiment (Fig. 1) since plants inoculated only with *B. japonicum* showed an important expression level under well-watered conditions (about 78% compared with non-inoculated plants) and little expression under drought conditions (9% compared with droughted non-inoculated plants). Plants dually inoculated with *B. japonicum* plus *G. mosseae* also showed little
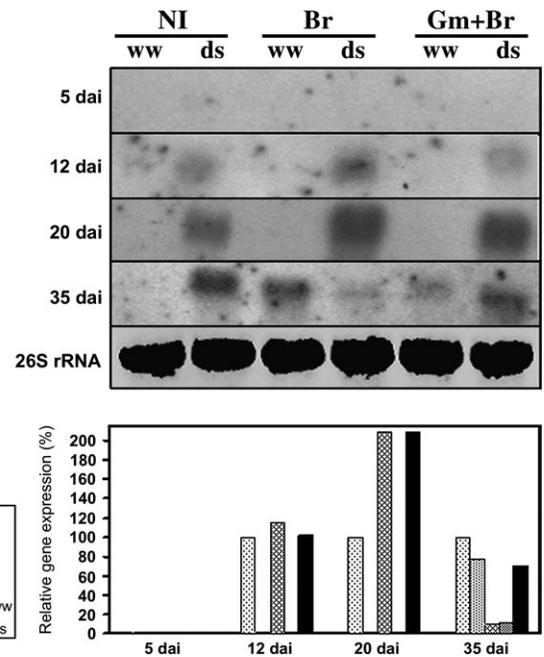

Fig. 2. Northern blot of total RNA (15 µg) from soybean roots. Plants were harvested 5, 12, 20, or 35 d after inoculation (dai). Treatments are designed as NI, non-inoculated controls; Br, *Bradyrhizobium japonicum*; Gm+Br, *G. mosseae* plus *B. japonicum*. Plants were either well-watered (ww) or drought-stressed (ds) for 10 d. The lower panel shows a representative example of the amount of 26S rRNA loaded for each treatment (methylene blue staining). Histogram shows the relative gene expression (after normalization to rRNA) presented as a percentage of the value for droughted non-inoculated plants.

expression under well-watered conditions (11% of non-inoculated plants), but enhanced expression under drought stress (70% of non-inoculated plants).

RNA from soybean root nodules was also used for northern blot analysis of LEA transcript accumulation. Figure 3 shows results obtained with the *gmlea 10* probe in nodules from plants inoculated only with *B. japonicum* or dually inoculated with *B. japonicum* plus *G. mosseae*. In contrast to what happened in roots, it is clearly visible that there was no expression of that LEA gene in nodules of *B. japonicum*-inoculated plants under well-watered conditions, while the expression notably increased in nodules of these plants when subjected to drought stress (which was set as 100%). In nodules from plants dually inoculated with both symbiotic micro-organisms, the level of LEA gene expression under drought-stress conditions was lower than in the non-AM plants (45% of Br plants).

Northern blot analysis with lettuce RNA

In order to test the behaviour of LEA genes in non-legume plants and to avoid the interference of AM symbiosis with that of *Bradyrhizobium* symbiosis, the cDNA cloned from lettuce (*lslea 1*) was also used for northern blot analysis with RNA from non-inoculated AM lettuce roots that were cultivated either under well-watered or drought-stressed
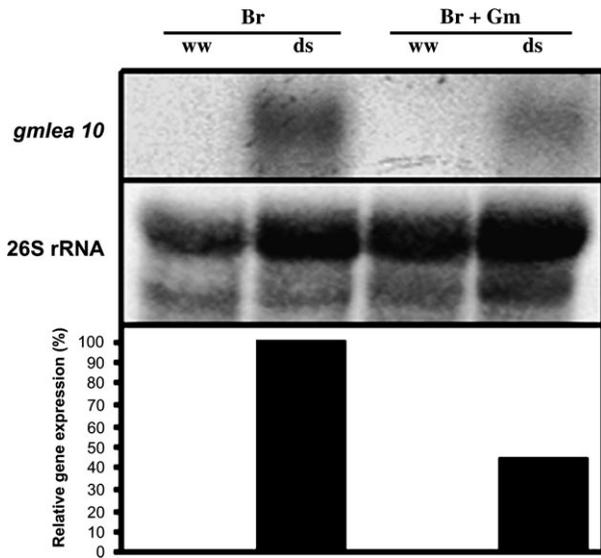
Fig. 3. Northern blot of total RNA (10 μg) from soybean nodules. Treatments are designed as Br, *Bradyrhizobium japonicum* and Gm+Br, *G. mosseae* plus *B. japonicum*. Plants were either well-watered (ww) or drought-stressed (ds) for 10 d. The lower panel shows the amount of 26S rRNA loaded for each treatment (methylene blue staining). Histogram shows the relative gene expression (after normalization to rRNA) presented as a percentage of the value for droughted non-inoculated plants.

Fig. 4. Northern blot of total RNA (15 μg) from lettuce roots. Treatments are designed as NI, non-inoculated controls; Gm, *Glomus mosseae*; Gi, *Glomus intraradices*; Plants were either well-watered (ww) or drought-stressed (ds) for 10 d. The lower panel shows the amount of 26S rRNA loaded for each treatment (methylene blue staining). Histogram shows the relative gene expression (after normalization to rRNA) presented as a percentage of the value for droughted non-inoculated plants.

conditions. Results showed that the *lslea 1* gene was also induced by drought stress in the three treatments assayed (Fig. 4). However, the level of induction was clearly higher in roots from non-inoculated plants than in roots from both AM treatments. After normalization to the corresponding RNA loaded in the blots, the level of *lslea 1* gene expression in non-inoculated plants was set as 100%. The level of *lslea 1* gene expression in plants colonized by *G. mosseae* was 51% compared with non-inoculated plants, and the level of expression in plants colonized by *G. intraradices* was 64% compared with the non-inoculated plants.

Discussion

The changes induced in the host plant by the AM symbiosis in order to enhance its tolerance to drought seem to be varied (Augé, 2001; Ruiz-Lozano, 2003). In this work previous physiological observations have been extended by studying the possible participation of LEA proteins in the enhanced tolerance to drought stress in mycorrhizal soybean and lettuce plants at the molecular level. As Harrison (1999) stated in a review on molecular aspects of this symbiosis, little is known about how AM fungi influence plant gene expression.

The analysis of *gmlea* gene expression showed that, in general, these genes responded to drought and were only expressed in drought-stressed treatments, suggesting that these dehydrins are important for the plant response against
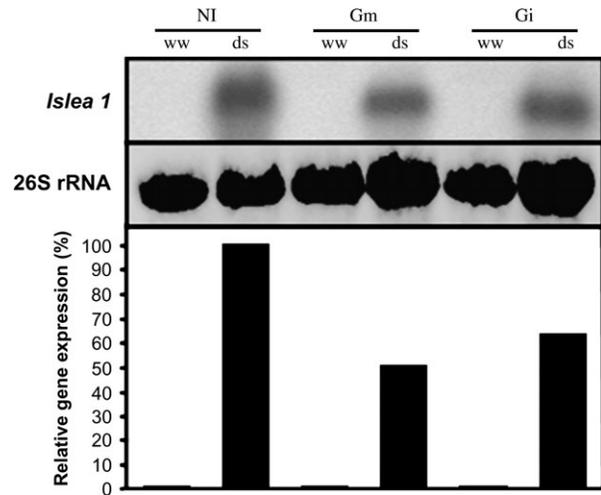
drought stress (Robertson and Chandler, 1992; Colmenero-Flores *et al.*, 1997; Cellier *et al.*, 1998; Giordani *et al.*, 1999). However, contrasting results were obtained in plants singly inoculated with *B. japonicum*, where *gmlea* genes were up-regulated in roots under well-watered conditions. By contrast, no up-regulation of *gmlea 10* was observed in nodules from well-watered plants inoculated with *B. japonicum* only. In nodules, the expression of the *gmlea 10* gene was induced only under drought-stress conditions.

Giordani *et al.* (1999) have demonstrated the existence of two regulation pathways for dehydrin accumulation in sunflower, an ABA-dependent and an ABA-independent one, which may have cumulative effects. The influence of ABA on LEA gene expression could explain why the *gmlea* genes were up-regulated in roots from nodulated non-AM soybean plants cultivated under well-watered conditions. This could simply be an unspecific response mediated by ABA. In fact, there have been several studies showing that four phytohormones, including ABA, are present in nodulated roots at concentrations higher than in uninfected roots (Hirsch and Fang, 1994). By contrast, the levels of *gmlea* gene expression in nodulated, drought-stressed AM plants were similar to those from non-inoculated plants or to those from non-nodulated AM plants. It has been proposed that mycorrhization can also alter the levels of ABA in the host plant and that, under drought stress, the levels of ABA are lower in AM than in non-AM plants (Duan *et al.*, 1996; Goicoechea *et al.*, 1997; Ludwig-Müller, 2000; Estrada-Luna and Davies, 2003). This could explain the decrease in the expression of the *gmlea genes*

in these double inoculated plants compared with single nodulated soybean plants.

In the time-course study it was observed that at 12 dai and 20 dai, soybean plants inoculated with only *B. japonicum* or in combination with *G. mosseae* showed a higher level of dehydrin gene expression relative to non-inoculated plants. This effect must be linked to the time-dependent nature of the interaction between the root and both symbiotic micro-organisms. In fact, at 12 dai and 20 dai both symbioses should not be completely functional. This is even more evident in the case of the AM symbiosis that, at these stages, may still show a low number of arbuscules (Gianinazzi-Pearson and Dénarié, 1997). It is expected that the influence of both symbioses on the two regulatory pathways of *lea* gene expression is different at 12 dai and 20 dai than at 35 dai, when both symbioses must be completely functional. In support of that, it was found here that the *RWC* of AM plants was significantly different from non-inoculated plants, but only at 35 dai.

Another effect observed in this study in the case of soybean (but not of lettuce), was the lower *gmlea* gene expression in roots from plants colonized by *G. intra-radices* alone compared with that of plants colonized by *G. mosseae* alone (Fig. 1). However, functional diversity among different AM fungi has been widely observed in several aspects of the symbiosis. Burleigh *et al.* (2002) showed that the functional diversity between AM fungal species occurs not only at the level of mycorrhiza formation, plant nutrient uptake, or plant growth, but also at the molecular level. These authors studied seven AM fungal species and found that the seven species widely varied in their influence on the root expression of *MtPT2* and *Mt4* genes from *Medicago truncatula* and also of *LePT1* and *TPSI1* genes from *Lycopersicon esculentum* involved in plant P nutrition.

In any case, a consistent effect observed both for soybean and lettuce plants is that the expression of *gmlea* and *lslea* genes decreased in drought-stressed AM plants compared with non-inoculated plants, conversely to the initial hypothesis that expected a possible role of LEA proteins in the alleviation of drought stress by the AM symbiosis. This can be related to hormonal or any other developmental change induced in AM plants. It is also possible that AM plants were less strained by drought stress and thus the level of *lea* gene expression was lower in AM than in non-AM plants. In previous studies in which other authors and ourselves have found physiological or biochemical mechanisms involved in the enhanced tolerance to drought stress in AM plants, it has been proposed that primary drought-avoidance mechanisms (i.e. direct water uptake by hyphae) or increased water uptake related to mycorrhizal changes in root morphology (Kothari *et al.*, 1990) or soil structure (Augé *et al.*, 2001, 2004) might have contributed to the AM protection of host plants against drought (Porcel *et al.*, 2003). Indeed, a recent study has revealed that, apart from

direct hyphal water uptake, it seems that the AM symbiosis first enhances osmotic adjustment in roots, and this could contribute to maintaining a water potential gradient favourable to water entry from the soil into the roots. This would enable higher leaf water potential in AM plants during drought and would keep plants protected against oxidative stress. All these cumulative effects would increase the plant tolerance to drought (Porcel and Ruiz-Lozano, 2004). The hypothesis about AM plants being less strained by drought stress than non-AM plants and consequently having a lower *lea* gene induction under drought-stress conditions is supported by the *RWC* data, which are significantly higher in AM plants than in non-AM plants. In addition, previous studies with soybean plants subjected to a similar drought-stress level have shown that AM plants exhibit higher leaf water potential than non-AM plants (Porcel and Ruiz-Lozano, 2004).

In conclusion, this study's results demonstrate that the cloned *lea* genes clearly responded to drought stress and they are accumulated under drought conditions in roots and nodules of soybean plants and also in roots of lettuce plants, contributing to their protection against drought. Mycorrhization of these plants with either *G. mosseae* or *G. intraradices* did not induce the expression of the *lea* genes analysed. Moreover, the levels of *lea* transcript accumulation in mycorrhizal treatments subjected to drought were considerably lower than those of the corresponding non-mycorrhizal plants, indicating that the accumulation of LEA proteins is not a mechanism by which the AM symbiosis protects their host plant and suggesting that mycorrhizal plants were less strained by drought due to primary drought-avoidance mechanisms.

Acknowledgement

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