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Key words: C_4 photosynthesis, crop yield, food security, genetic engineering, *Oryza sativa*, rice.

Does the enhanced tolerance of arbuscular mycorrhizal plants to water deficit involve modulation of drought-induced plant genes?

Water deficit is one of the most common environmental stress factors experienced by land plants, having a major adverse effect on plant survival and productivity (Kramer & Boyer, 1997; Bray, 2004). Plants can respond to drought stress with modifications that allow the plant to avoid the stress or to increase its tolerance. Tolerance to drought stress in plants is a complex phenomenon and involves many changes at both biochemical and physiological levels. The cellular responses of plants to water deficit appear to be conserved in the plant kingdom. Among a diversity of responses, plants can adapt to water deficit by the induction of specific genes such as genes encoding late embryogenesis-abundant (LEA) proteins, or genes encoding proteins involved in the biosynthesis of osmoregulatory compounds, as well as by modulating the expression of genes encoding aquaporins (Zhu *et al.*, 1997; Bray, 2004; Luu & Maurel, 2005). Most terrestrial plants can also establish a symbiotic association with arbuscular mycorrhizal (AM) fungi. A number of studies have demonstrated that the AM symbiosis can protect 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).

Although in recent years there has been an increase in understanding of the water relations of AM plants and the physiological processes involved in the enhanced tolerance of mycorrhizal plants to water limitation, the molecular basis for the tolerance to water stress in AM plants remains far from being understood (Ruiz-Lozano, 2003). Thus our research group has initiated an investigation aimed at evaluating, at a molecular level, the possible participation of drought-induced genes in the enhanced tolerance of AM plants to drought stress. The most important results are discussed in the following sections.

Late embryogenesis-abundant proteins

The LEA proteins accumulate in plant seeds during their maturation phase, when they are developing tolerance to desiccation (Close, 1996). Nevertheless, a variety of studies have demonstrated that LEA proteins also accumulate in vegetative plant tissues during periods of water deficit, reinforcing a role for these proteins as desiccation protectant. 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; Koag *et al.*, 2003). The overexpression of LEA proteins in plants and yeast confers tolerance to osmotic stresses (Imai, 1996; Babu *et al.*, 2004).

Dehydrins are an important group of LEA proteins (LEA group 2). They represent the most conspicuous soluble proteins induced by a dehydration stress (Close, 1996). It appears 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.

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 possible alteration functions

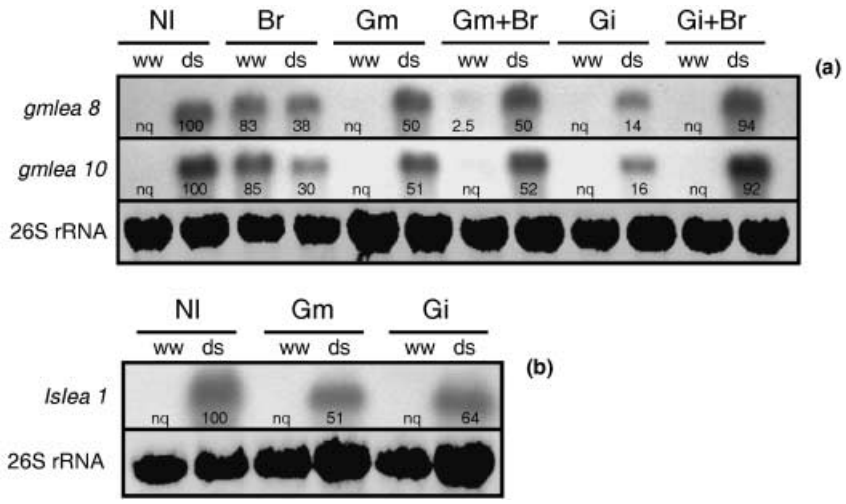

Fig. 1 Northern blot of total RNA (15 μg) from (a) soybean and (b) lettuce roots using *gmlea 8*, *gmlea 10* and *lslea 1* gene probes. Treatments: NI, noninoculated control; Br, *Bradyrhizobium japonicum*; Gm, *Glomus mosseae*; Gm + Br, *G. mosseae* plus *B. japonicum*; Gi, *Glomus intraradices*; Gi + Br, *G. intraradices* plus *B. japonicum*. Plants were either well watered (ww) or drought-stressed (ds) for 10 d. The percentage of gene expression is indicated by numbers close to each Northern (nq, not quantifiable). Lower panel, a representative example of the amount of 26S rRNA loaded for each treatment (methylene blue staining). Reproduced from Porcel *et al*. (2005a) with permission from Oxford University Press.

in protection of the host plants against drought. We cloned two dehydrin-encoding genes from *Glycine max* (*gmlea 8*; *gmlea 10*) and one from *Lactuca sativa* (*lslea 1*) and analysed their contribution to the response against drought in mycorrhizal soybean and lettuce plants.

The analysis of *gmlea* and *lslea* gene expression showed that, in general, these genes responded to drought and were expressed only in drought-stressed treatments (Fig. 1a,b), suggesting that these dehydrins are important for the plant response against drought stress (Giordani *et al*., 1999). In any case, a consistent effect observed both for soybean and lettuce plants is that the expression of *gmlea* and *lslea* genes was lower in drought-stressed AM plants than in noninoculated plants. To understand this effect, it must be considered that abscisic acid (ABA) induces the expression of water deficit-responsive genes such as *lea* (Giordani *et al*., 1999). It has been proposed that mycorrhization can alter the levels of ABA in the host plant, and that under drought stress levels of ABA are lower in AM than in nonAM plants (Goicoechea *et al*., 1997; Estrada-Luna & Davies, 2003); thus the level of *lea* gene expression may be lower in these plants. Additionally, AM plants can be less affected than nonAM plants by drought stress, and for that reason the expression of the *lea* genes studied is lower. It has been proposed that primary drought-avoidance mechanisms (direct water uptake by hyphae) or increased water uptake related to mycorrhizal changes in root morphology or soil structure (Augé, 2001) might have contributed to the AM protection of host plants against drought. This hypothesis was supported by data on relative water content and leaf water potential (Ψ), which were significantly higher in AM plants than in nonAM plants subjected to a similar level of drought stress (Porcel & Ruiz-Lozano, 2004; Porcel *et al*., 2005a).

In conclusion, our results demonstrate that the levels of *lea* transcript accumulation in soybean and lettuce plants colonized by either *Glomus mosseae* or *Glomus intraradices* were considerably lower than those of the corresponding nonmycorrhizal plants, suggesting that the accumulation of LEA proteins is not a mechanism by which the AM symbiosis protects the host plant.

Δ^1-pyrroline-5-carboxylate synthetase (P5CS)

Maintenance of a favourable water flow gradient from soil into roots is a fundamental process for plants under conditions of water deficit, when soil water potential becomes more negative. The most important mechanism of plants to decrease their water potential is to decrease the osmotic potential in their tissues by active accumulation of organic ions or solutes, a phenomenon known as osmotic adjustment or osmoregulation (Morgan, 1984). Of these metabolites, proline is probably the most widespread in plants, although it is not the only one, and it has been shown that proline accumulates under conditions of water shortage, high salinity, chilling, heat and heavy metal exposure. It plays a major role in osmoregulation and osmotolerance (Yoshiba *et al*., 1995; Armengaud *et al*., 2004).

Accumulation of proline is caused primarily by *de novo* synthesis, although a reduced rate of catabolism has also been observed. The first two steps of proline biosynthesis are catalysed by Δ^1-pyrroline-5-carboxylate synthetase (P5CS) by means of its γ-glutamyl kinase and glutamic-γ-semialdehyde dehydrogenase activities. Subsequently, the Δ^1-pyrroline-5-carboxylate (P5C) formed is reduced to proline by P5C reductase (P5CR) (Hu *et al*., 1992). The rate-limiting step in this pathway is represented by the γ-glutamyl kinase activity of P5CS. The overexpression of the P5CS-encoding gene in transgenic tobacco plants has been shown to increase proline production and to confer to such plants tolerance to osmotic stress. Hence the P5CS-encoding gene is of key importance for the biosynthesis of proline in plants (Ábrahám *et al*., 2003).

Investigations carried out so far on proline in the AM symbiosis are scarce and somewhat contradictory. While some

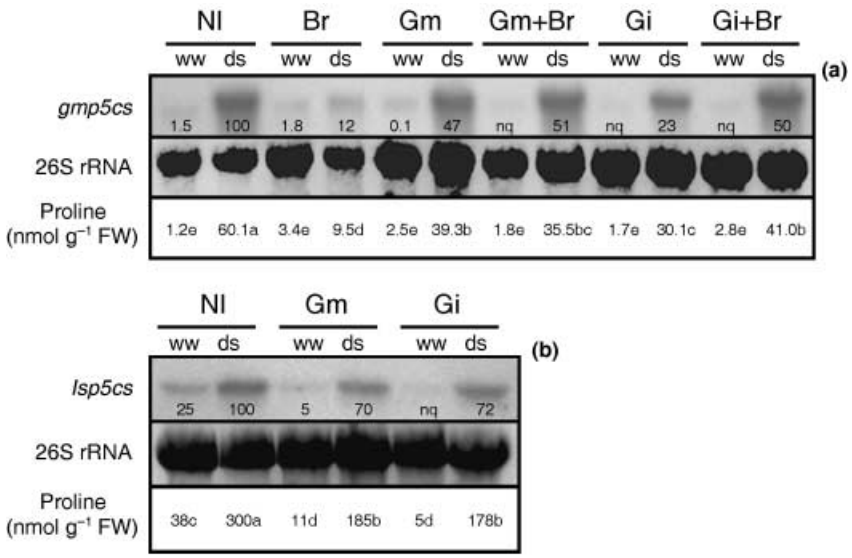

Fig. 2 Northern blot of total RNA (15 μg) from (a) soybean roots and (b) lettuce roots using *gmp5cs* and *lsp5cs* gene probes. Treatments as in Fig. 1. The bottom panel shows the proline content in roots. Reproduced from Porcel *et al*. (2004) with permission from Elsevier.

studies have shown an increase in proline accumulation in mycorrhizal plants subjected to drought (Ruiz-Lozano *et al*., 1995; Azcón *et al*., 1996; Goicoechea *et al*., 1998), the same studies also demonstrated that the increase in proline accumulation was quite variable depending on the AM fungus involved (Ruiz-Lozano *et al*., 1995). By contrast, other studies on drought (Ramakrishnan *et al*., 1988) or salt stress (Ruiz-Lozano *et al*., 1996) have shown a lower proline accumulation in AM plants than in nonAM plants.

The establishment of the expression pattern of genes such as *p5cs* in AM plants under osmotic stress conditions should provide new insights into the role of the AM symbiosis in the process of osmotic adjustment during drought stress. We cloned a P5CS-encoding gene from *G. max* (*gmp5cs*) and another from *L. sativa* (*lsp5cs*), and analysed their contribution to the response against drought in mycorrhizal soybean and lettuce plants.

The expression of *gmp5cs* and *lsp5cs* (Fig. 2a,b) genes responded to drought and was upregulated in drought-stressed treatments, suggesting that these genes are important for the plant response against water deficit (Parvanova *et al*., 2004). Results on proline accumulation paralleled those on *p5cs* gene expression in both soybean and lettuce (Fig. 2a,b). A contrasting result was obtained in soybean plants singly inoculated with *Bradyrhizobium japonicum*, where the *gmp5cs* gene showed little upregulation in roots under drought-stressed conditions, and there was also low proline accumulation (Fig. 2a). To explain this result, it must be considered that the expression of *p5cs* genes has two regulatory pathways, an ABA-dependent and an ABA-independent pathway, and that both can act simultaneously and with cumulative effects (Ábrahám *et al*., 2003). Hence it may be possible that nodulation itself may affect one of these regulatory pathways, avoiding the accumulation of *p5cs* transcripts. By contrast, the mycorrhization of nodulated plants restores the normal *p5cs* transcripts accumulation pattern, at least in part, by compensating such ABA-dependent and ABA-independent pathways in some way.

In any case, as also happened with *lea* genes, the expression of *gmp5cs* and *lslp5cs* genes decreased in drought-stressed AM plants compared with noninoculated plants (Fig. 2a,b). This was probably caused by a decrease in the ABA level in AM plants, and by the fact that AM plants were less affected by drought stress than nonAM plants because of primary drought-avoidance mechanisms. The results suggest that the induction of *p5cs* genes does not appear to be a mechanism by which the AM symbiosis protects the host plant (Porcel *et al*., 2004).

Regulation of aquaporin abundance

Aquaporins are water channel proteins that facilitate and regulate the passive movement of water molecules down a water potential gradient. The discovery of aquaporins in plants has caused a significant change in the understanding of plant water relations. In recent years, much effort has been concentrated on investigating the function and regulation of plasma membrane aquaporins (plasma membrane intrinsic proteins, PIPs). These aquaporins appear to play a specifically important role in controlling transcellular water transport. For instance, they are abundantly expressed in roots, where they mediate most soil water uptake (Javot & Maurel, 2002); transgenic plants downregulating one or more PIP genes had lower root water-uptake capacity (Siefritz *et al*., 2002). However, the relationship between aquaporins and plant responses to drought remains elusive and shows contradictory results (Aharon *et al*., 2003). Moreover, the contribution of aquaporin genes to the enhanced tolerance to drought in

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AM plants had never been investigated. However, it has been shown that the impairment of a PIP gene in an antisense tobacco mutant reduced the symbiotic efficiency of two AM fungi under drought stress conditions (Porcel *et al.*, 2005b).

Mechanisms of osmotic adjustment and modulation of tissue hydraulic conductivity are required to maintain tissue water potential. Such mechanisms, which regulate water flux, are likely to be mediated, in part, by aquaporins (Maurel, 1997). It is of interest to study whether the expression of aquaporin-encoding genes in roots is altered by AM symbiosis as a mechanism to enhance host-plant tolerance to water deficit. To achieve this, genes encoding PIPs from soybean and lettuce were cloned and their expression pattern studied, in AM and nonAM plants cultivated under well watered or drought-stress conditions. If AM fungi can transfer water to the roots of host plants, it is expected that the plant must increase its permeability for water and that aquaporin genes should be upregulated in order to allow a higher rate of transcellular water flow (Javot & Maurel, 2002).

In contrast to the above hypothesis, our results showed that the PIP genes studied were downregulated under drought stress in both soybean (Fig. 3a, harvest time, 35 d after inoculation (dai)) and lettuce plants (Fig. 3b), and that such downregulation was even more severe in plants colonized by *G. mosseae* than in nonAM plants. A similar result was obtained recently by Ouziad *et al.* (2006) regarding the expression of PIP and tonoplast intrinsic protein (TIP) genes in roots of AM tomato plants subjected to salt stress. When the expression of *gmPIP2* was analysed in a time course (Fig. 3a), it was clearly visible that AM plants already downregulated that gene significantly at 5 and 12 dai, while both nonAM control plants still maintained *gmPIP2* gene expression almost unaltered. At 20 dai, the more intense downregulation of that gene in AM plants than in both nonAM plants was still clearly visible. Finally, at 35 dai all treatments had the same level of *gmPIP2* gene expression. This effect of the AM symbiosis anticipating the downregulation of the *gmPIP2* gene may have a physiological importance in helping AM plants cope with drought stress (Aharon *et al.*, 2003). The decreased expression of plasma membrane aquaporin genes during drought stress in AM plants may be a regulatory mechanism to limit the water lost from cells (Smart *et al.*, 2001). In support of this hypothesis, data on leaf Ψ and relative water content show that AM plants (soybean and lettuce) had higher leaf Ψ and water content than nonAM plants.

Data obtained with lettuce plants also colonized by *G. mosseae* point in the same direction (Fig. 3b): under drought-stress conditions there is a higher downregulation of the PIP genes studied (and also at the protein level, as revealed by Western blot) in AM than in nonAM plants. In contrast to *G. mosseae*, plants colonized by *G. intraradices* do not exhibit such downregulation of PIP gene expression or protein accumulation. The expression of PIP genes under drought stress in these plants is similar to control nonAM plants.

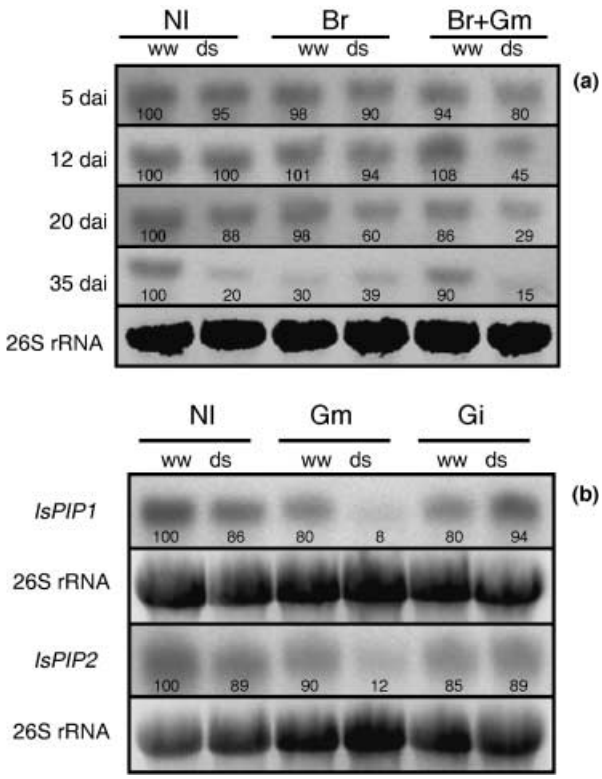

Fig. 3 (a) Northern blot of total RNA (15 μg) from soybean roots harvested 5, 12, 20 or 35 d after inoculation (dai) using the *gmPIP2* gene probe. Treatments as in Fig. 1. (b) Northern blot of total RNA (15 μg) from lettuce roots, using *lsPIP1* and *lsPIP2* gene probes. Treatments: NI, noninoculated controls; Gm, *Glomus mosseae*; Gi, *Glomus intraradices*. See Fig. 1 legend. Reproduced from Porcel *et al*. (2006a) with permission from Springer.

The reason for the differing influence of *G. mosseae* and *G. intraradices* on lettuce PIP gene expression is not known. However, in a previous study, also with lettuce, we evaluated the ability of six AM fungal species, including *G. mosseae* and *G. intraradices*, to enhance the amount of soil water uptake by these plants (Marulanda *et al.*, 2003). The study demonstrated that there were substantial differences among the six AM fungi used. One of the most efficient fungi stimulating water uptake by plants was *G. intraradices*, while *G. mosseae* showed a reduced ability to improve plant water uptake. This may suggest that the strategy of both fungi to protect the host plant against water deficit is different. *Glomus intraradices* appears to have an important capacity to enhance the rate of water uptake by lettuce roots. This means that water movement in these roots must be enhanced and thus root water permeability must also increase, maybe by maintaining high levels of PIP aquaporin gene expression, as observed in this study. By contrast, *G. mosseae* appears to direct its strategy for plant protection against water deficit toward the conservation of the water existing in the plant, and thus downregulates the expression of PIP genes. Such downregulation of PIP genes has been interpreted as a

mechanism to decrease membrane water permeability and to allow cellular water conservation (Smart *et al.*, 2001). In any case, both strategies appear to protect the host plant in a similar way, as lettuce plants had similar relative water content and leaf Ψ regardless of the fungus colonizing their roots (Porcel *et al.*, 2006).

In conclusion, the results suggest that AM plants respond to drought stress by downregulating the expression of the two PIP genes studied and anticipating downregulation, as compared with nonAM plants, rather than by maintaining high levels of expression of these PIP genes. This downregulation of PIP genes is likely to be a mechanism to decrease membrane water permeability and to allow cellular water conservation. It must be considered, however, that as PIP are members of a multigene family, other PIP isoforms in soybean and lettuce plants may be regulated differently and that, depending on the AM fungus implicated in the symbiosis, the pattern of aquaporin gene expression may also be different.

Perspectives for future investigation

Considering the overall results presented here, it is evident that some aspects are more promising than others for future research. It appears that there is no sense in investigating further the possible roles of *lea* and *p5cs* genes; however this must be considered with caution, as we analysed only a few of the *lea* genes belonging to the dehydrin group, while other *lea* genes in plants remain to be checked. The evidence obtained with these two genes suggests that it will be of interest to study the role of ABA in modulating host-plant responses to water deficit by AM symbiosis. Results obtained with aquaporin genes suggest that studies of the correlation between up- or downregulation of aquaporin gene expression, root hydraulic conductivity and plant water status may be of interest. The number of aquaporin genes analysed should also be enhanced as aquaporins constitute a multigenic family in plants, and it is likely that some genes not yet analysed may be regulated by AM symbiosis in relation to the alleviation of drought stress in the host plant.

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Key words: aquaporin, arbuscular mycorrhizal symbiosis, drought stress, late embryogenesis-abundant (LEA) protein, Δ^1-pyrroline-5-carboxylate synthetase (P5CS), tolerance.

